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Development and Characterization of Novel Bioluminescence Sources

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Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

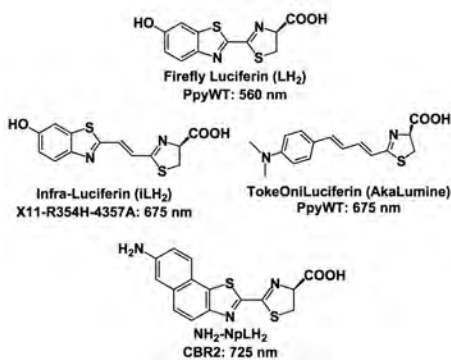


Figure 1. Luciferase-substrate emission maxima.

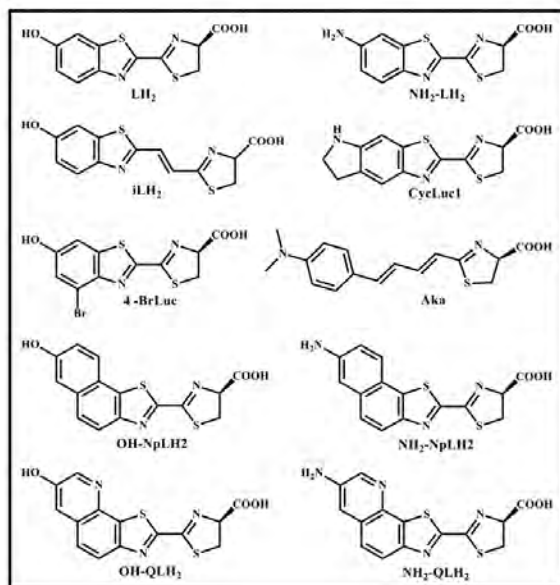
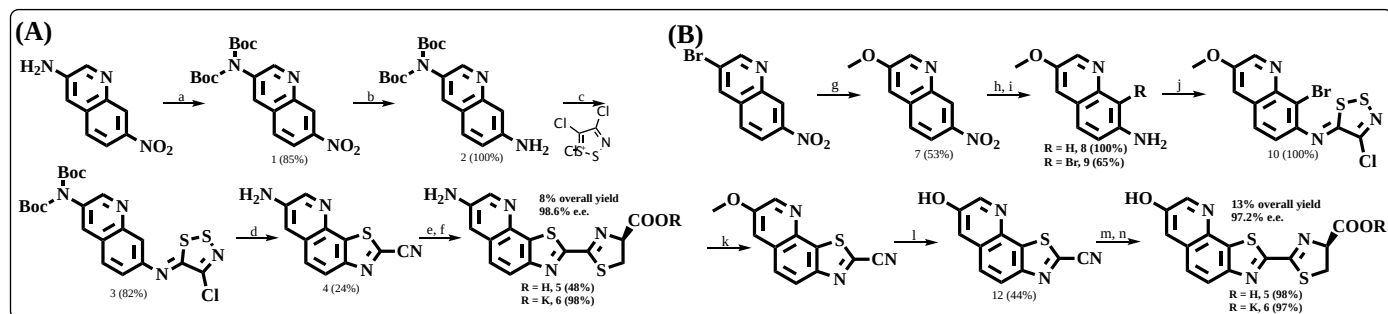


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

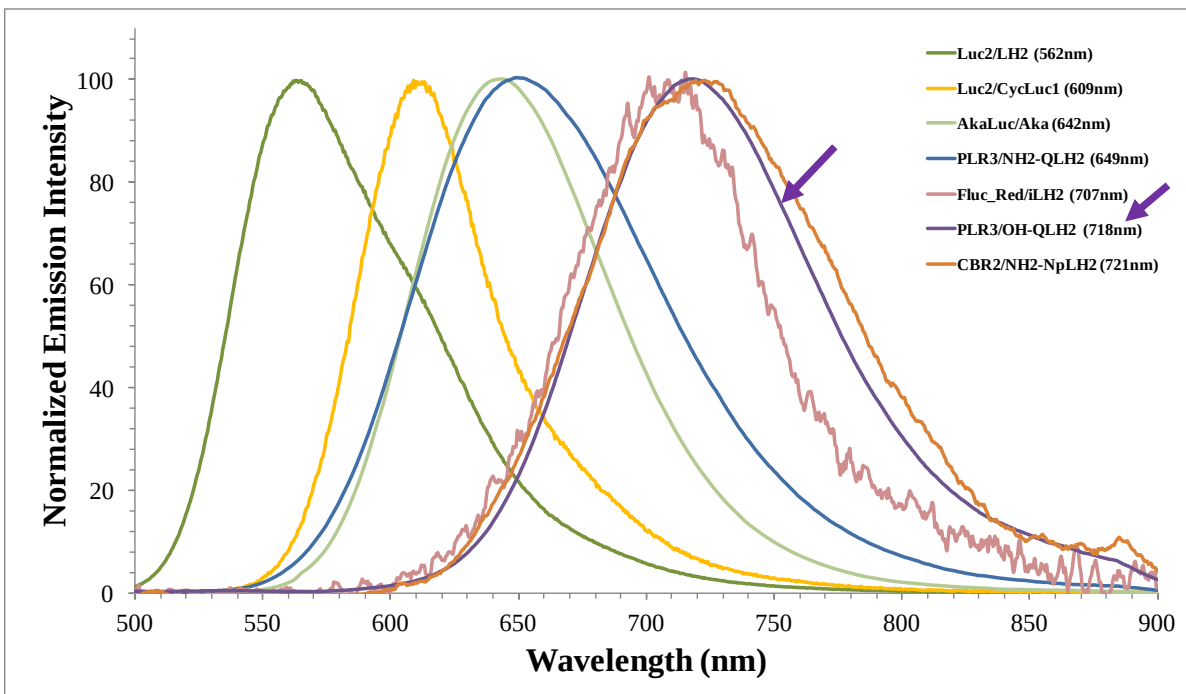


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

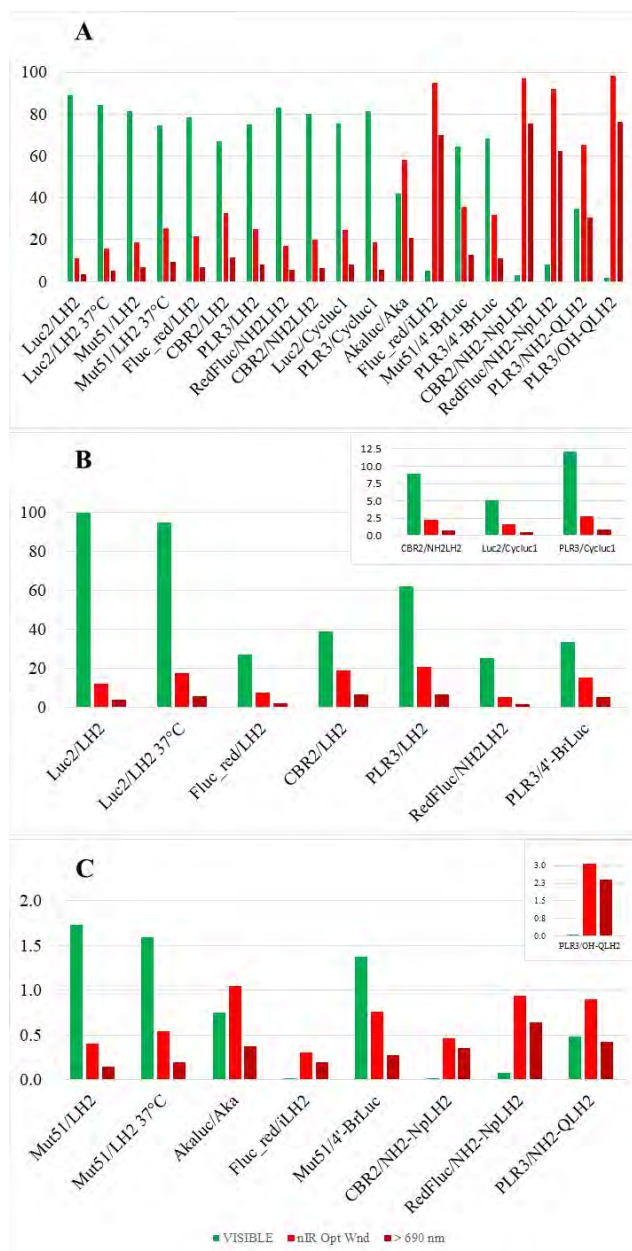


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

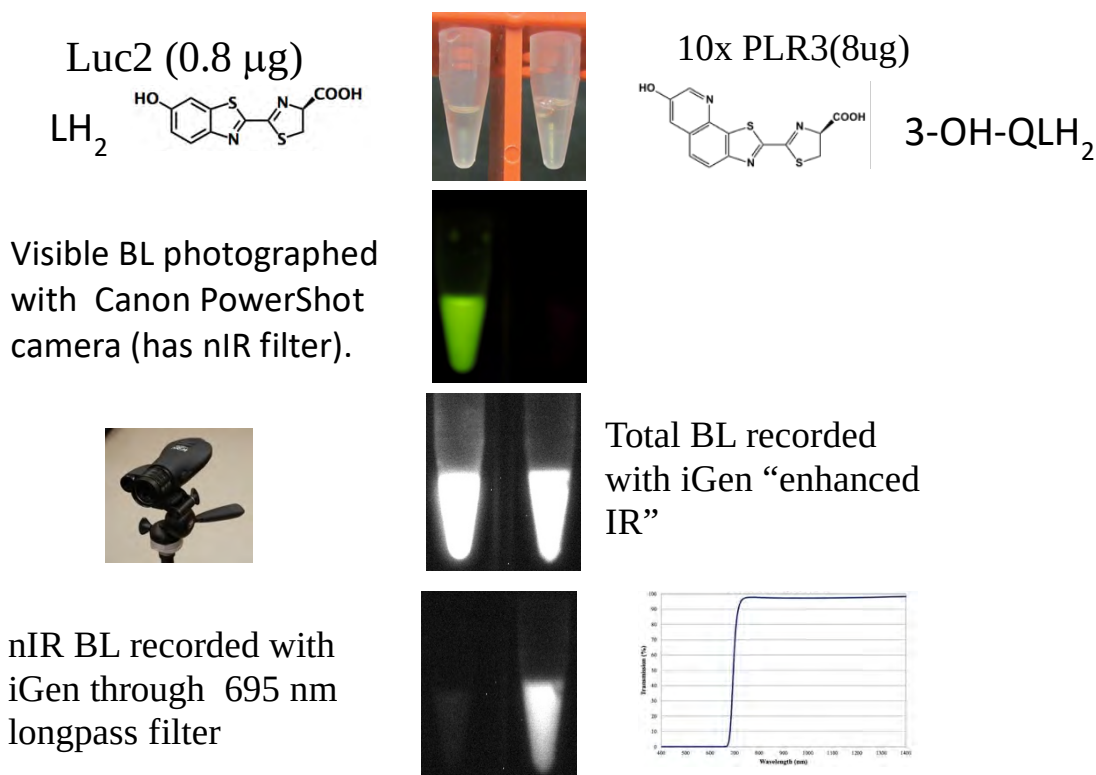
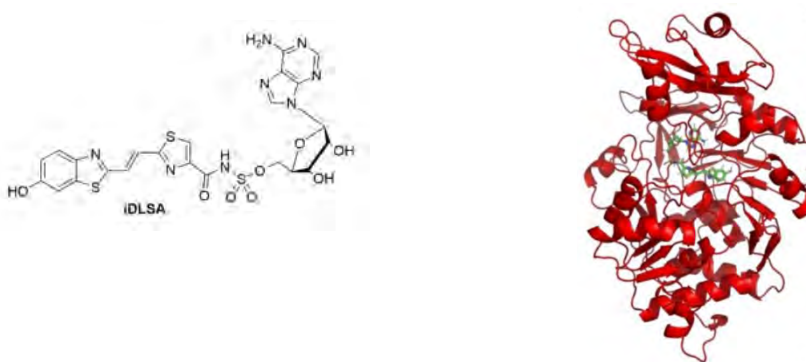


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T – OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

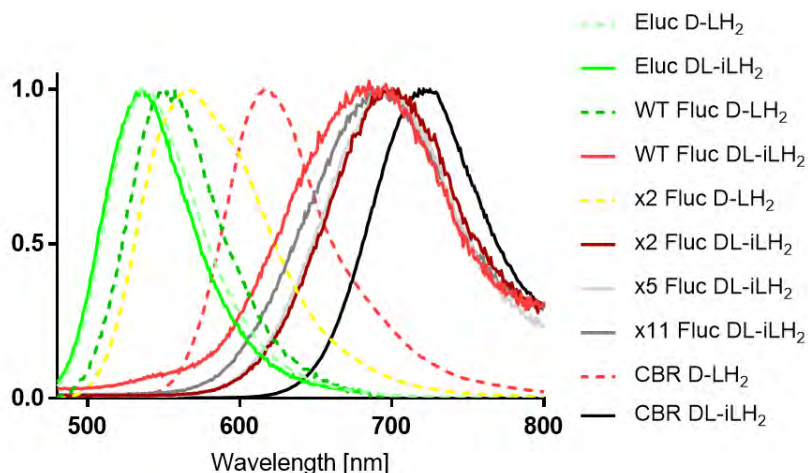


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

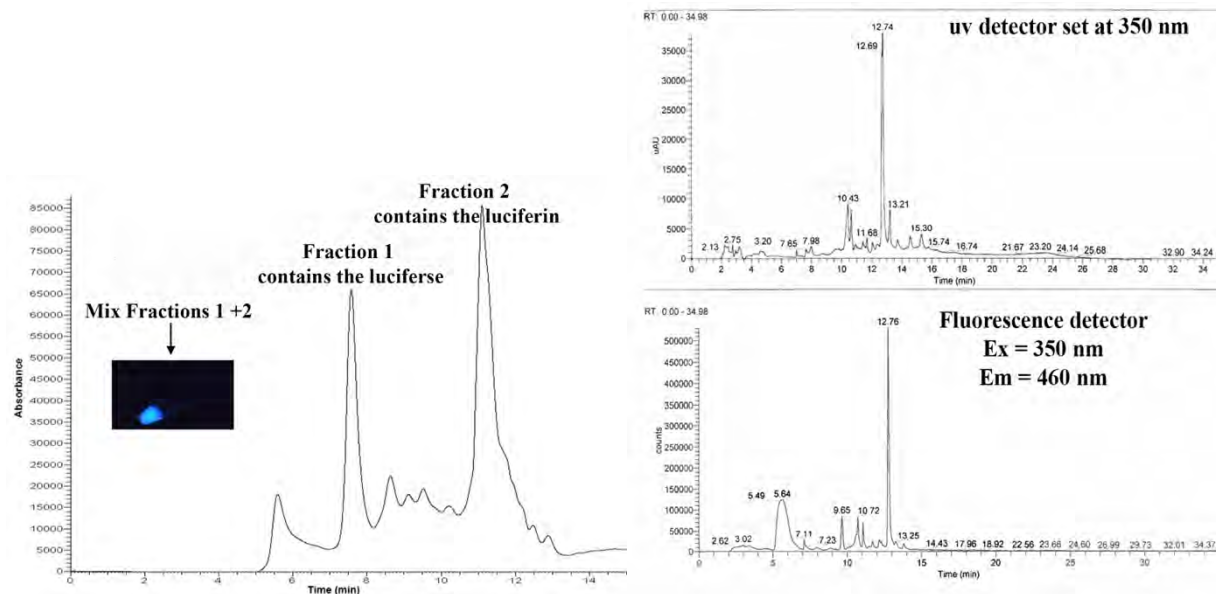
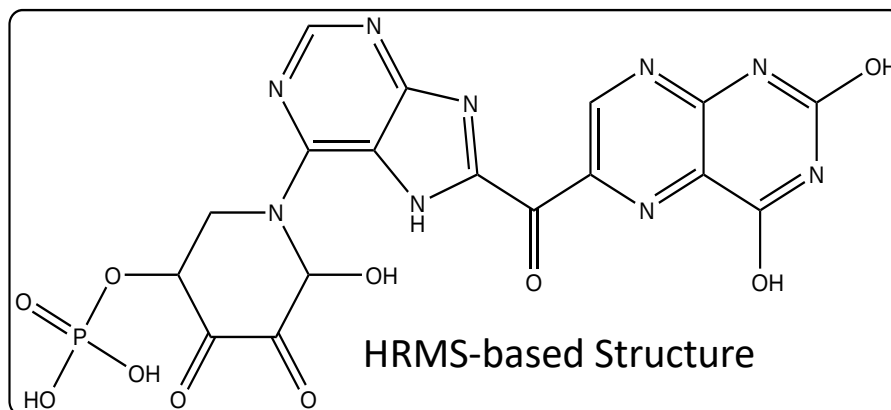


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

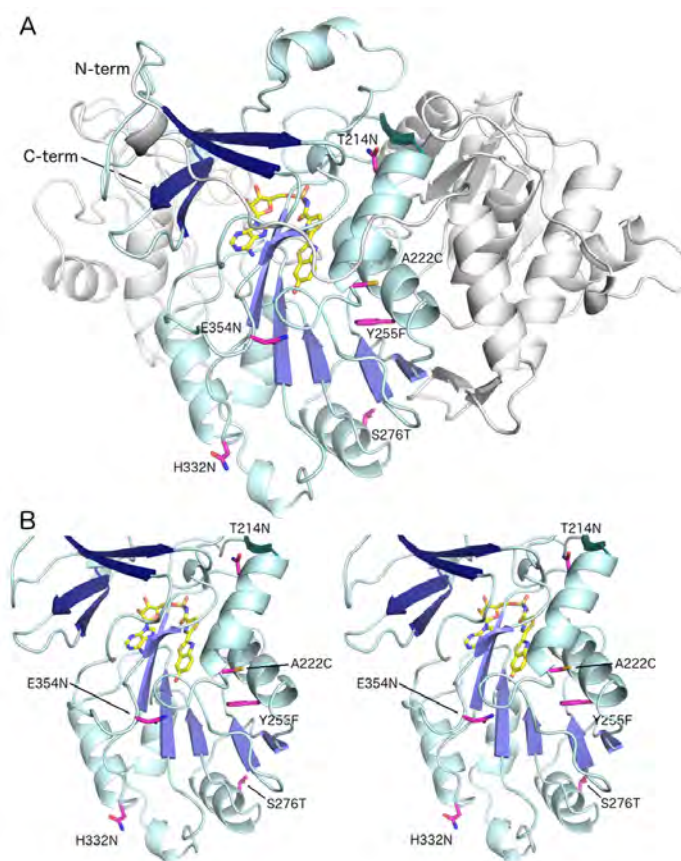


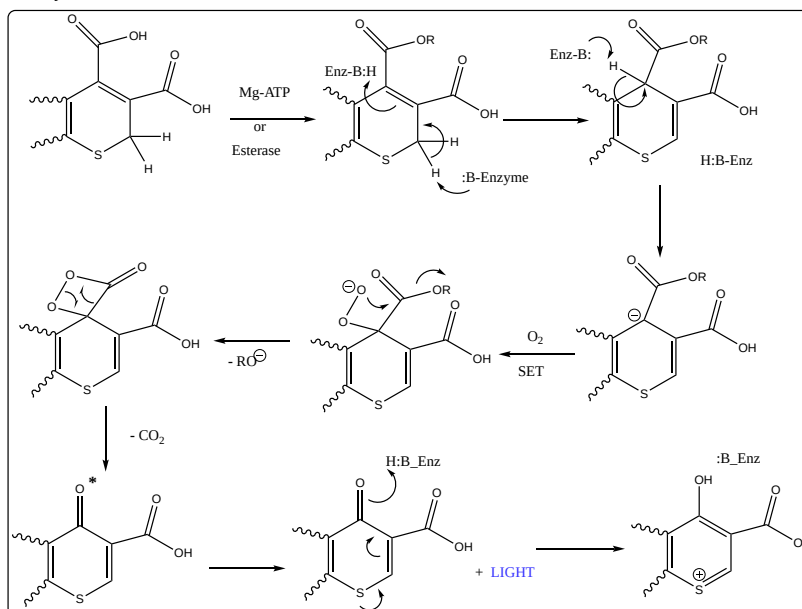
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaetae that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdonata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Universita degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

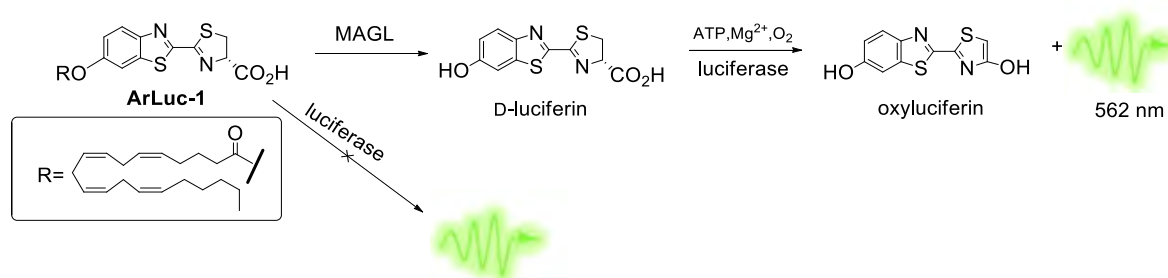


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 1. "Red-emitting chimeric firefly luciferase for in vivo imaging in low ATP cellular environments." Bruce R. Branchini, Tara L. Southworth, Danielle M. Fontaine, Dawn Kohrt, Franceine S. Welcome, Catherine M. Florentine, Emma R. Henricks, Demetria B. DeBartolo, Elisa Michelini, Luca Cevenini, Aldo Roda, Martha J. Grossel, *Analytical Biochemistry*, 2017;534:36–39. doi: 10.1016/j.ab.2017.07.001.**

- 2. "A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events." Branchini BR, Southworth TL, Fontaine DM, Kohrt D, Florentine CM, Grossel MJ. *Sci Rep.* 2018; 8(1):5990. doi: 10.1038/s41598-018-24278-2.
- 3. " Mutagenesis and structural studies reveal the basis for the activity and stability properties that distinguish the photinus luciferases scintillans and pyralis." Branchini, B. R., Fontaine, D. M., Southworth, T. L., Huta, B. P., Racela, A., Patel, K. D., and Gulick, A. M. (2019), *Biochemistry* 58, 4293-4303. doi/10.1021/acs.biochem.9b00719
- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
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- 6. " Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin." Stowe, C. L., Burley, T. A., Allan, H., Vinci, M., Kramer-Marek, G., Ciobota, D. M., Parkinson, G. N., Southworth, T. L., Agliardi, G., Hotblack, A., Lythgoe, M. F., Branchini, B. R., Kalber, T. L., Anderson, J. C., and Pule, M. A. (2019) *eLife* 8. doi/10.7554/eLife.45801
- 7. "A new brilliantly blue-emitting luciferin- luciferase system from *Orfelia fultoni* and *Keroplantinae* (Diptera)." V.R. Viviani, J. R. Silva, D. T. Amaral, V. R. Bevilaqua, F. C. Abdalla, B. R. Branchini, and C. H. Johnson (2020) *Sci. Rep.*, 10:9608 | <https://doi.org/10.1038/s41598-020-66286-1>
- 8. "A new ultrasensitive bioluminescence -based method for assaying monoacylglycerol lipase." M. Miceli, S. Casati, P. Allevi, S. Berra, R. Ottria, P. Rota, B.R. Branchini, and P. Ciuffreda, *Int. J. Mol. Sci.*, 22, 6148 (2021) <https://doi.org/10.3390/ijms22116148>

- 9. “Dendritic cells maintain anti-tumor immunity by positioning CDB skin-resident memory T cells.” J. L. Vella, A. Molodtskov, C. V. Angeles, B. R. Branchini, M. J. Turk, and Y. H. Huang, *Life Science Alliance* 4 (10), e202101056 (2021).
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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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*** Corresponding author inadvertently left out AFOSR acknowledgement.

COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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Distribution Statement: Approved for Public release

Program Officer: Bennett L. Ibey

Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaeta that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

I. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

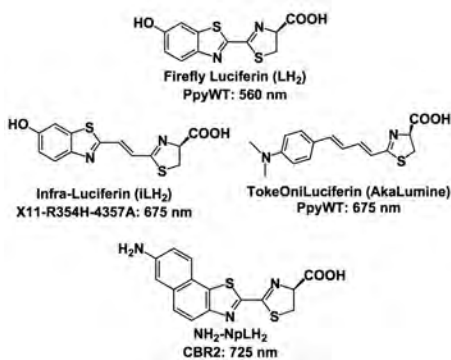


Figure 1. Luciferase-substrate emission maxima.

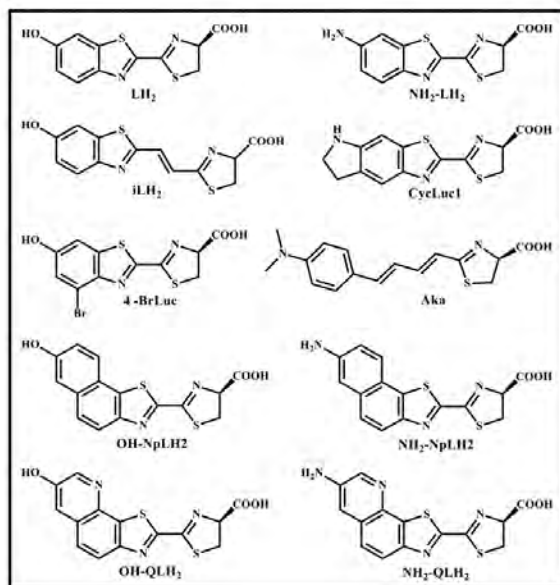
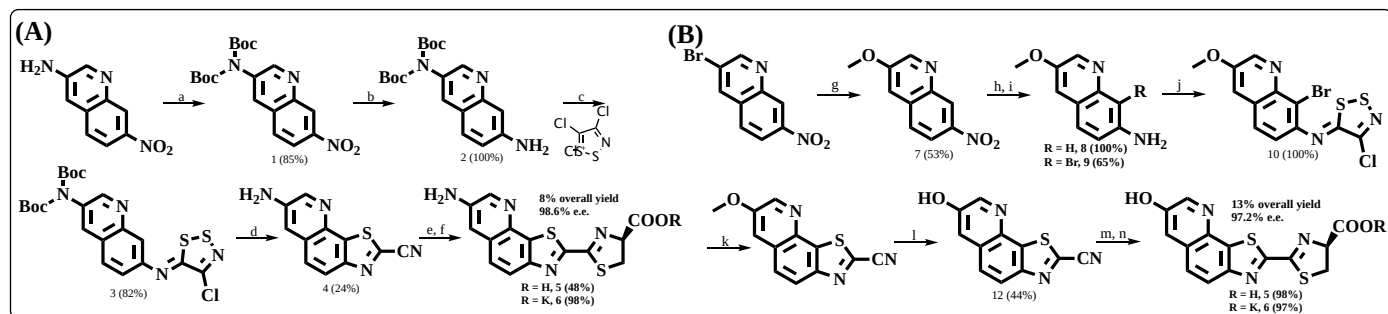


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

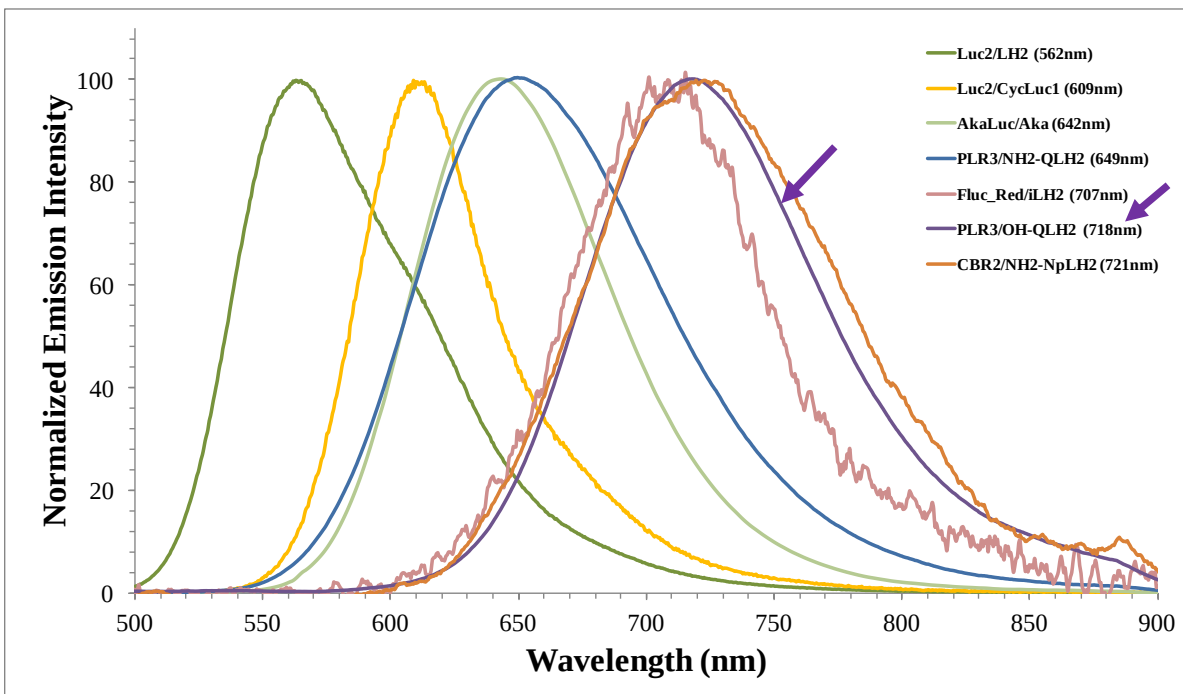


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

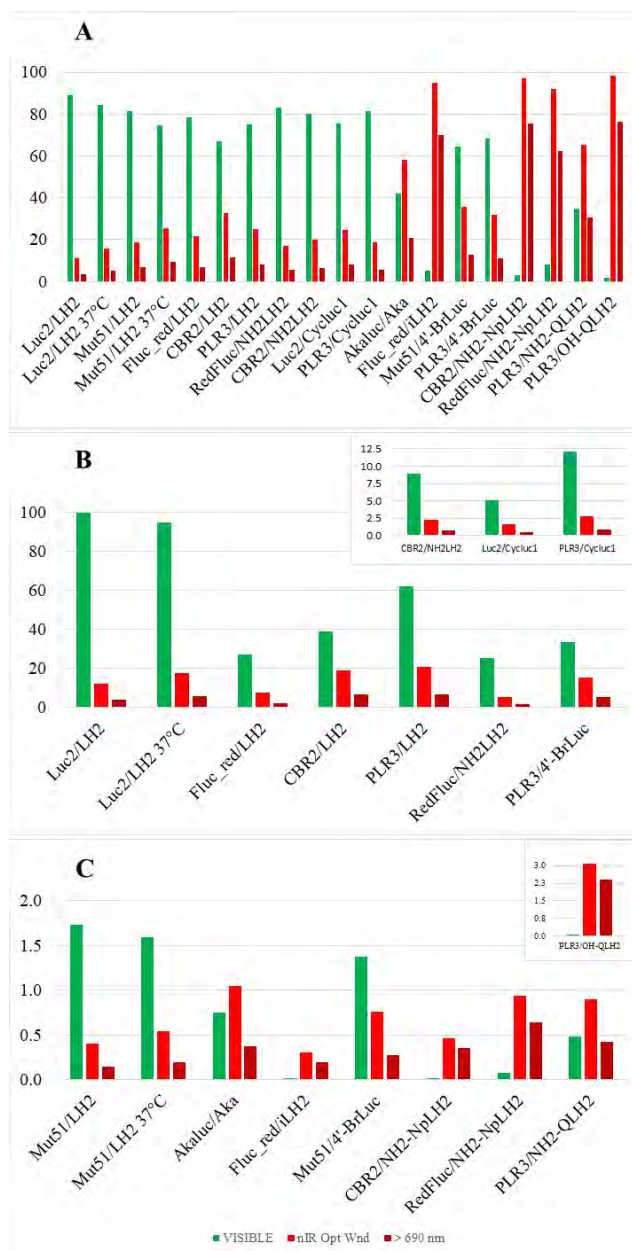


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

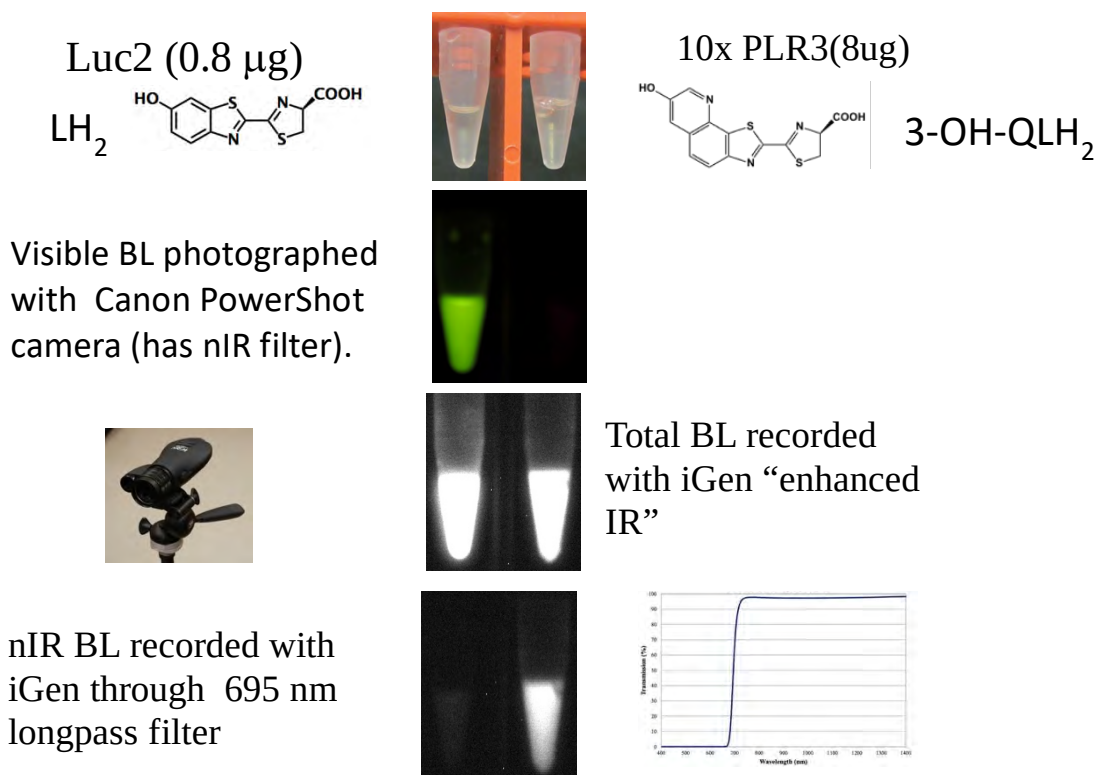
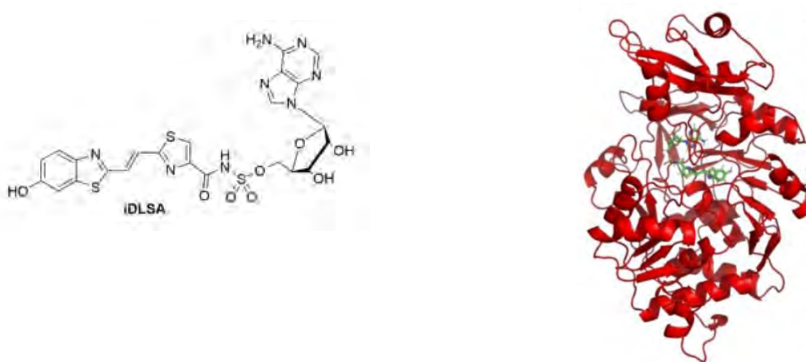


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T – OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

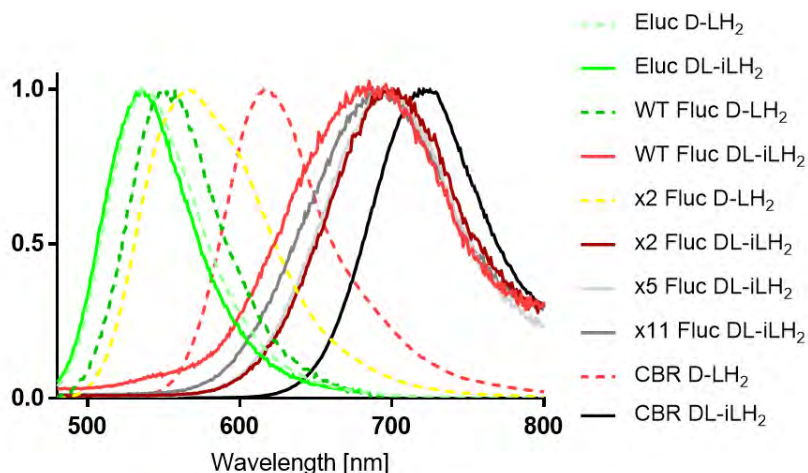


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultoni*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

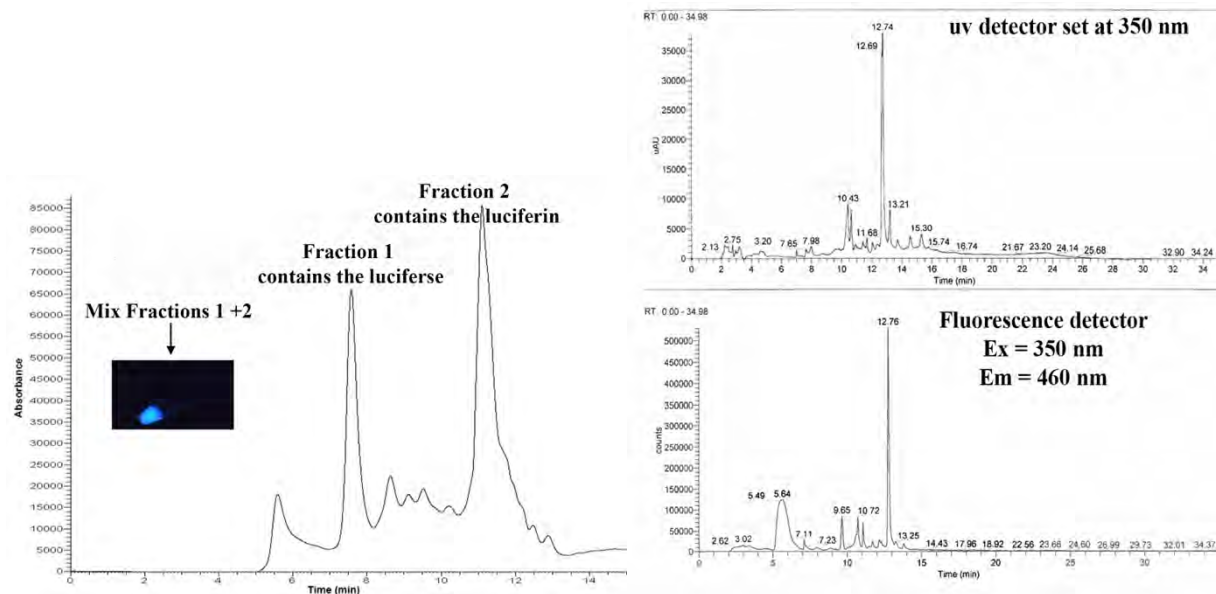
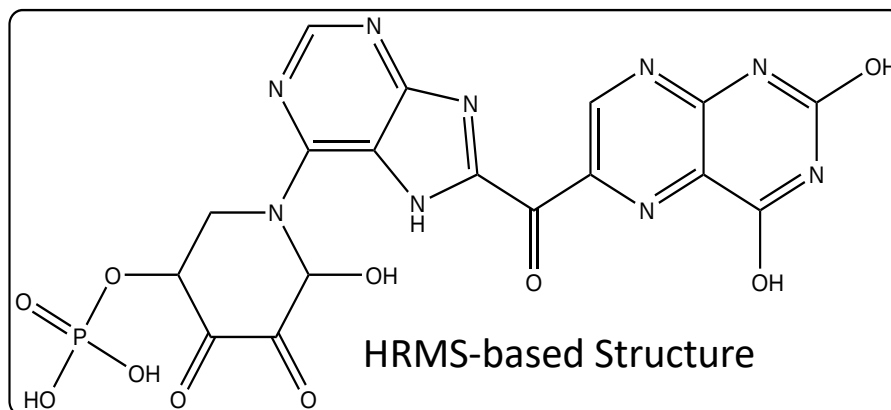


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



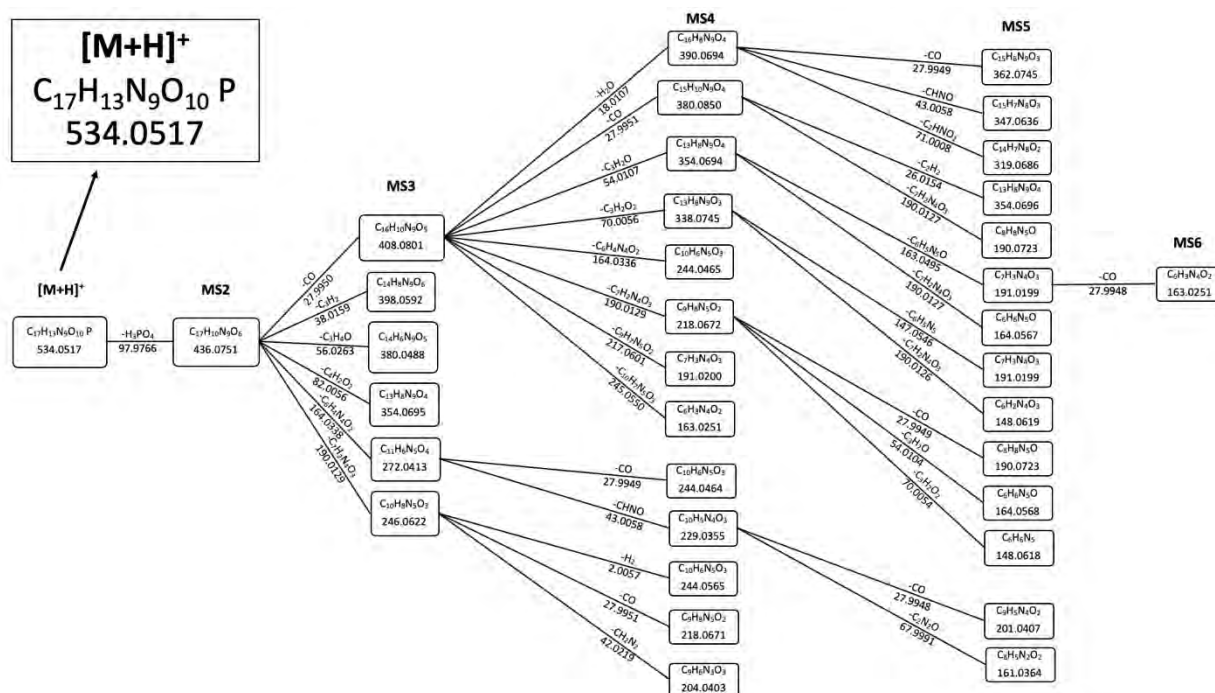


Figure 8. HRMS analysis of putative *Orfelia* oxyluciferin.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the *Orfelia* oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

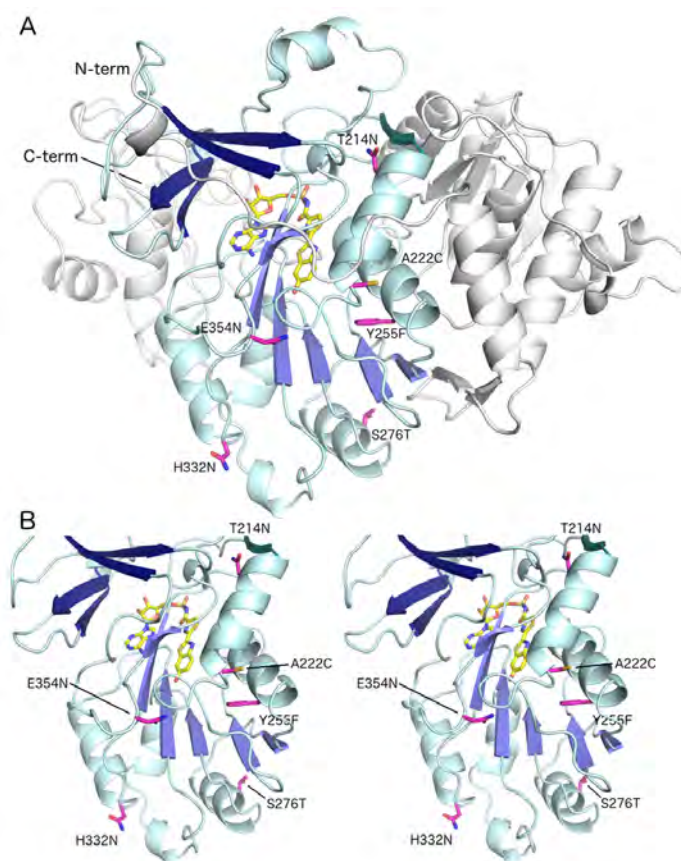


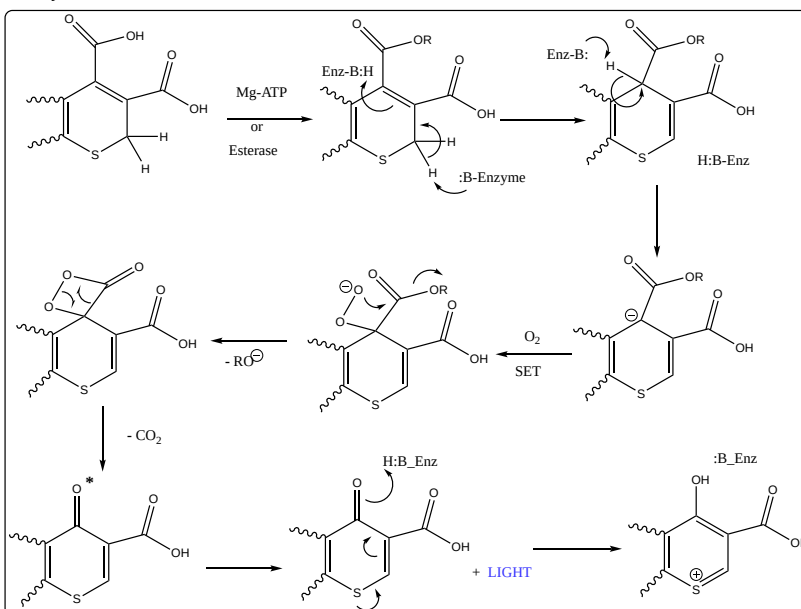
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

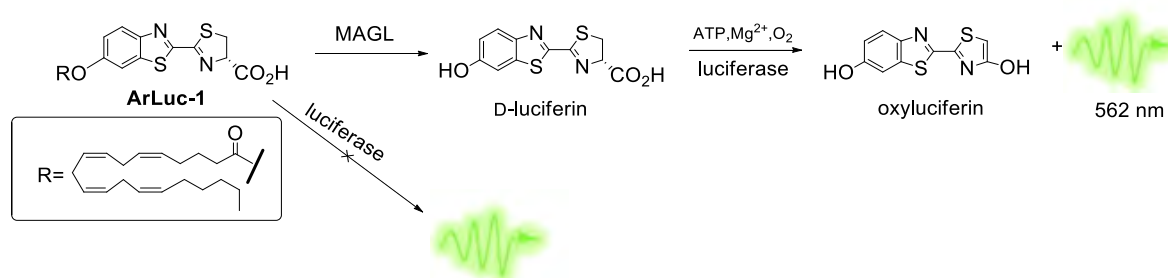


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 2. "A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events." Branchini BR, Southworth TL, Fontaine DM, Kohrt D, Florentine CM, Grossel MJ. *Sci Rep.* 2018; 8(1):5990. doi: 10.1038/s41598-018-24278-2.
- 3. " Mutagenesis and structural studies reveal the basis for the activity and stability properties that distinguish the photinus luciferases scintillans and pyralis." Branchini, B. R., Fontaine, D. M., Southworth, T. L., Huta, B. P., Racela, A., Patel, K. D., and Gulick, A. M. (2019), *Biochemistry* 58, 4293-4303. doi/10.1021/acs.biochem.9b00719
- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
- 5. "Prêt-à-porter nanoYESA and nanoYESB bioluminescent cell biosensors for ultrarapid and sensitive screening of endocrine-disrupting chemicals." Lopreside, A., Calabretta, M. M., Montali, L., Ferri, M., Tassoni, A., Branchini, B. R., Southworth, T., D'Elia, M., Roda, A., and Michelini, E. (2019) *Anal. Bioanal. Chem.* 411, 4937-4949. doi/10.1007/s00216-019-01805-2
- 6. " Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin." Stowe, C. L., Burley, T. A., Allan, H., Vinci, M., Kramer-Marek, G., Ciobota, D. M., Parkinson, G. N., Southworth, T. L., Agliardi, G., Hotblack, A., Lythgoe, M. F., Branchini, B. R., Kalber, T. L., Anderson, J. C., and Pule, M. A. (2019) *eLife* 8. doi/10.7554/eLife.45801
- 7. "A new brilliantly blue-emitting luciferin- luciferase system from *Orfelia fultoni* and *Keroplantinae* (Diptera)." V.R. Viviani, J. R. Silva, D. T. Amaral, V. R. Bevilaqua, F. C. Abdalla, B. R. Branchini, and C. H. Johnson (2020) *Sci. Rep.*, 10:9608 | <https://doi.org/10.1038/s41598-020-66286-1>
- 8. "A new ultrasensitive bioluminescence -based method for assaying monoacylglycerol lipase." M. Miceli, S. Casati, P. Allevi, S. Berra, R. Ottria, P. Rota, B.R. Branchini, and P. Ciuffreda, *Int. J. Mol. Sci.*, 22, 6148 (2021) <https://doi.org/10.3390/ijms22116148>

- 9. “Dendritic cells maintain anti-tumor immunity by positioning CDB skin-resident memory T cells.” J. L. Vella, A. Molodtskov, C. V. Angeles, B. R. Branchini, M. J. Turk, and Y. H. Huang, *Life Science Alliance* 4 (10), e202101056 (2021).
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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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*** Corresponding author inadvertently left out AFOSR acknowledgement.

COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

Award Number: FA9550-18-1-0017

Report Type: Final Report

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Program Officer: Bennett L. Ibey

Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

I. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferin that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

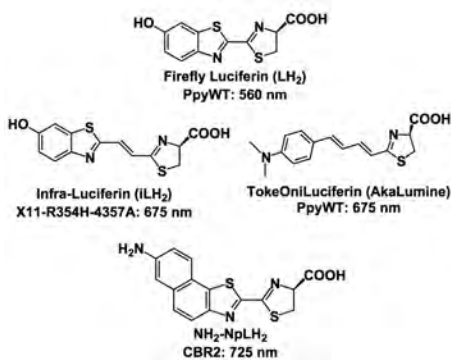


Figure 1. Luciferase-substrate emission maxima.

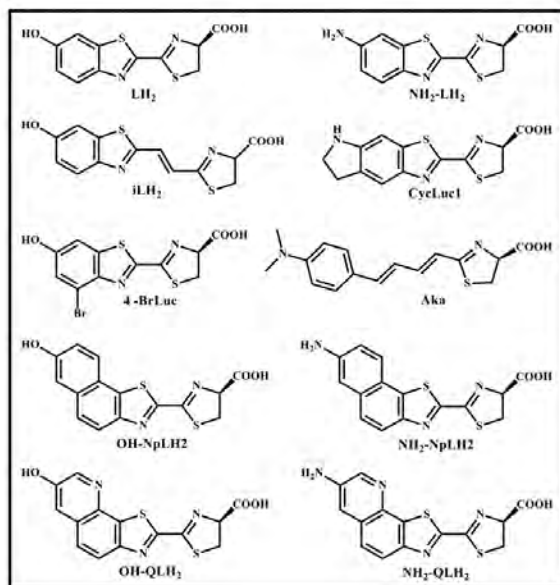
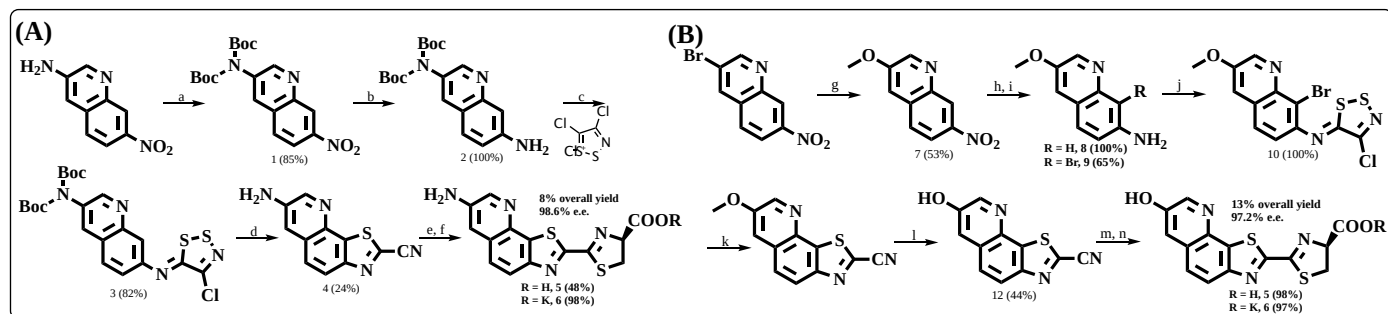


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

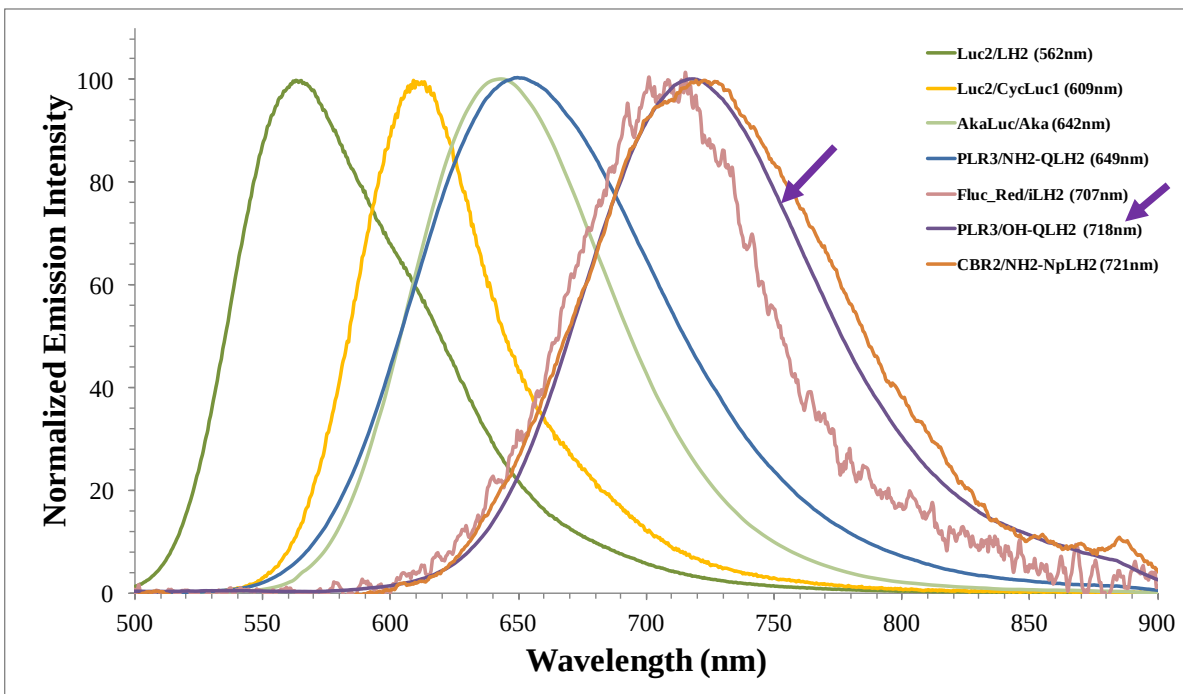


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

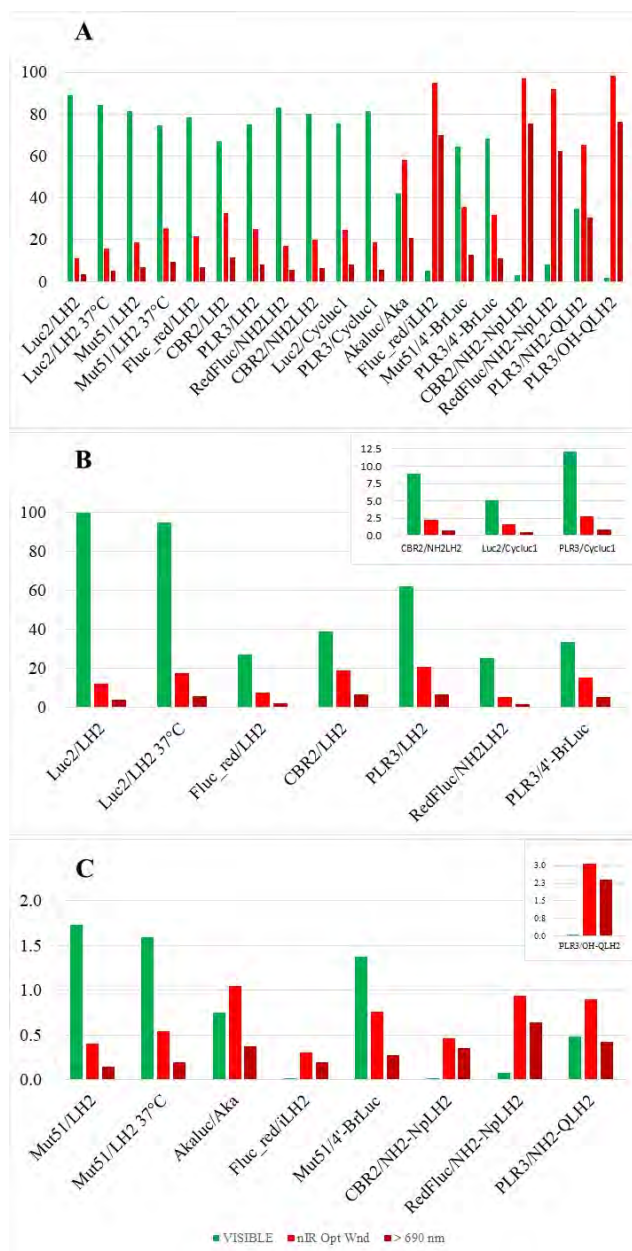


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

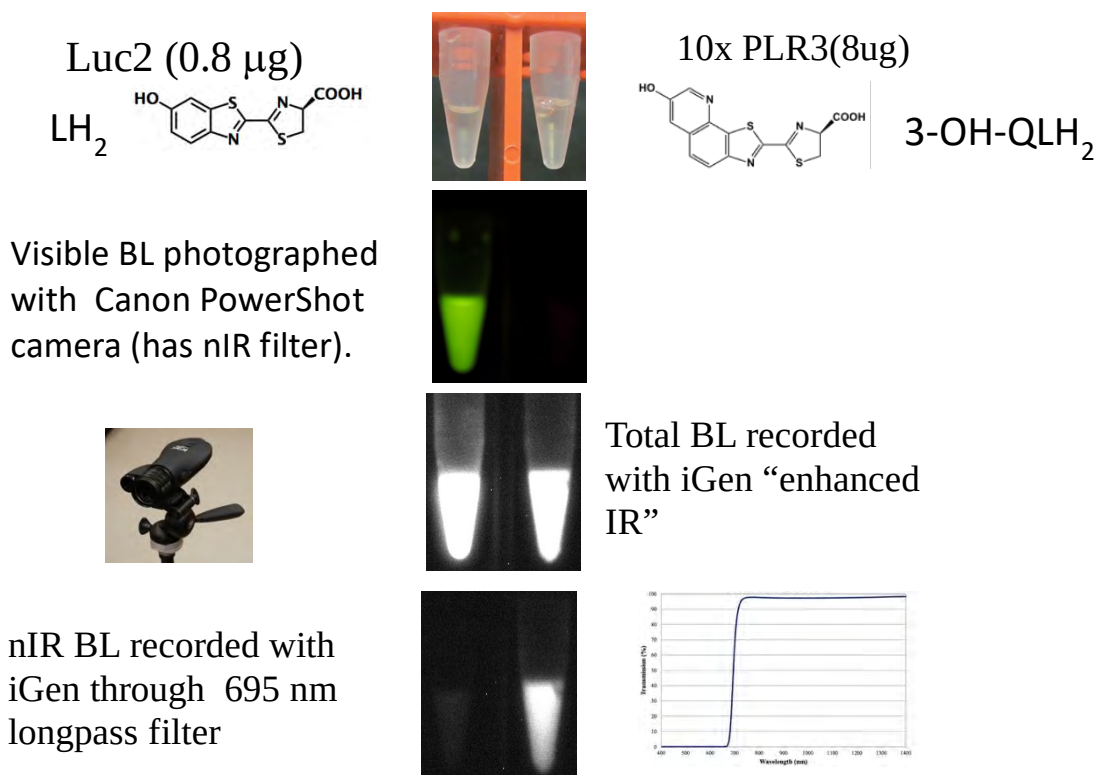
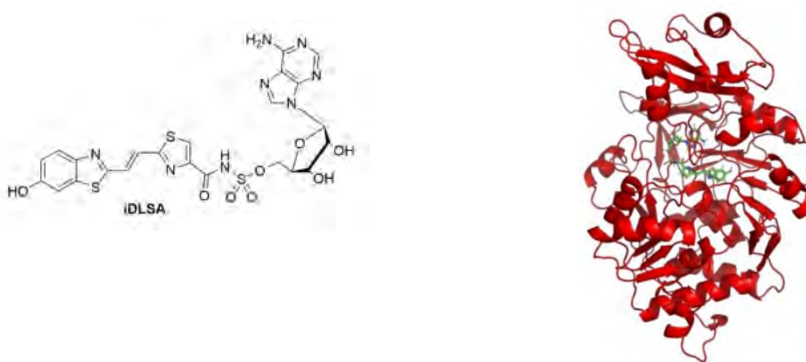


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T – OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

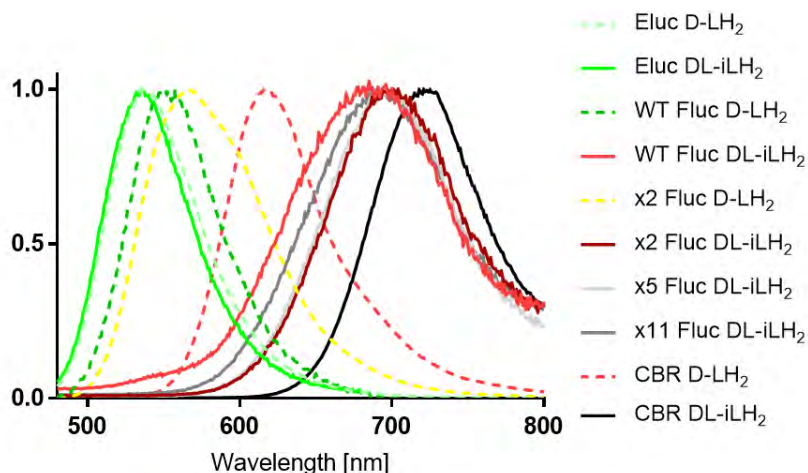


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

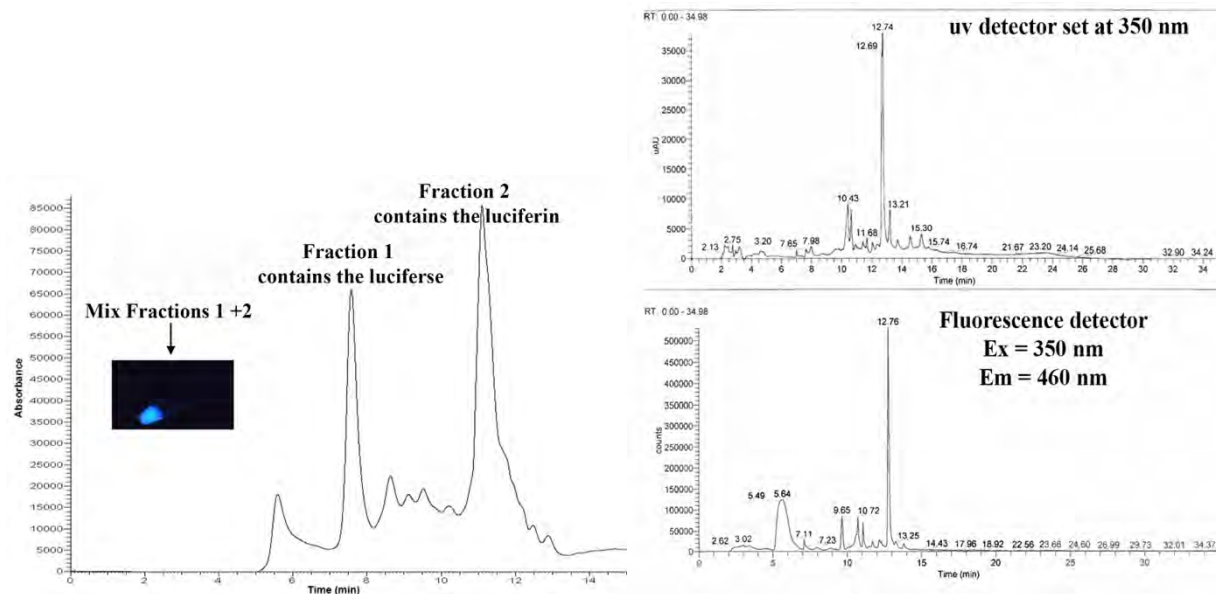
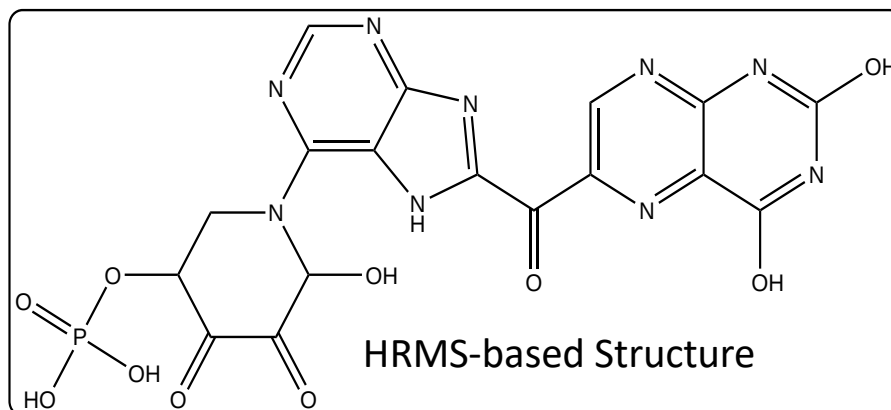


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

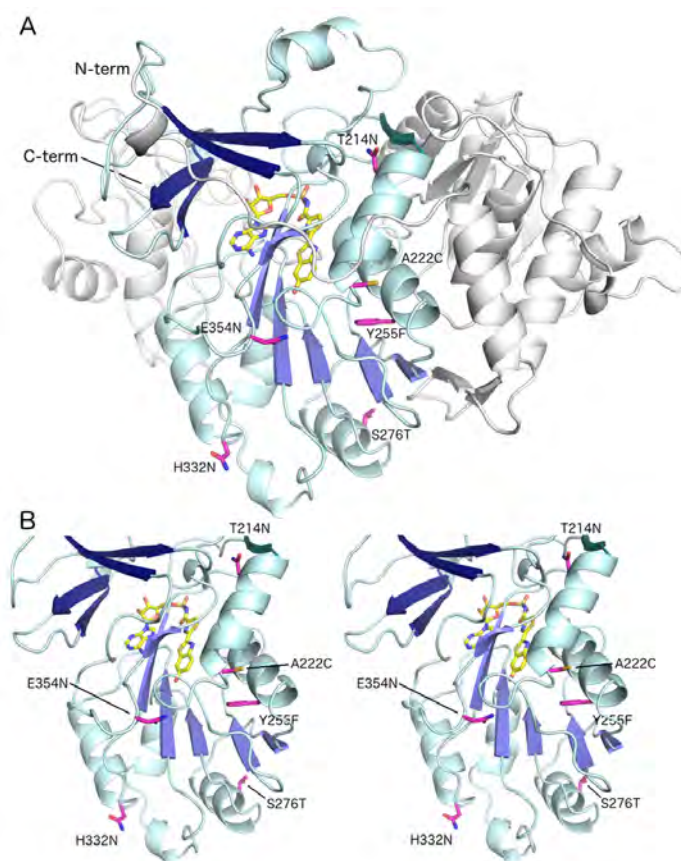


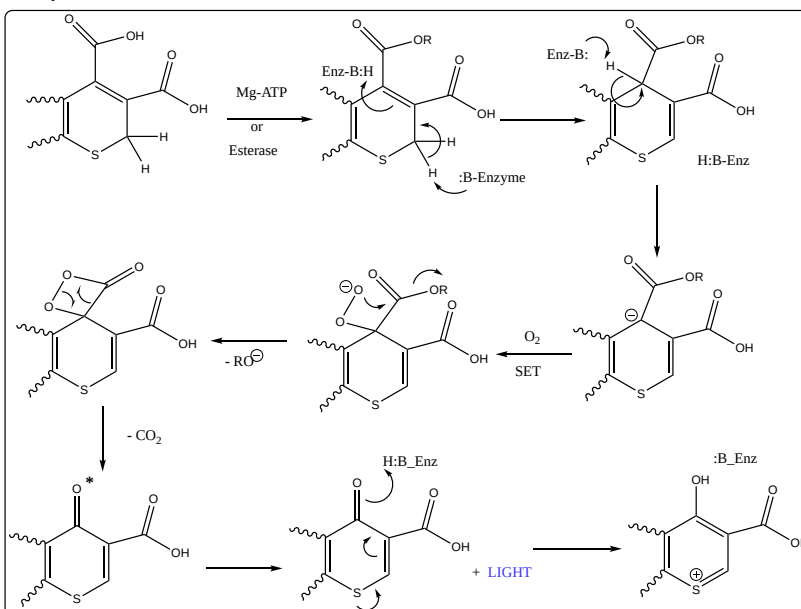
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

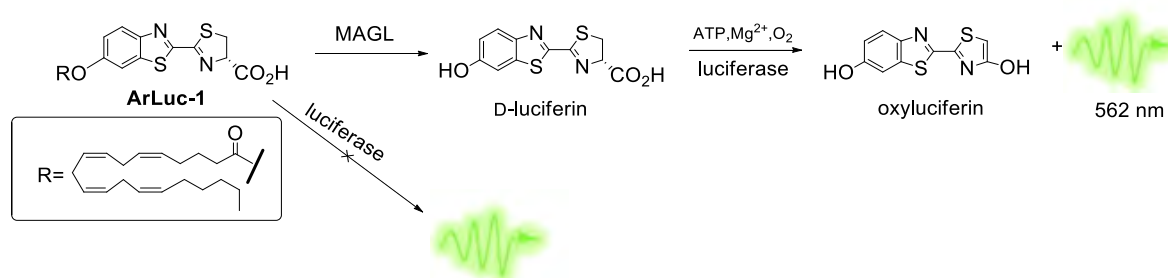


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
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- 6. " Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin." Stowe, C. L., Burley, T. A., Allan, H., Vinci, M., Kramer-Marek, G., Ciobota, D. M., Parkinson, G. N., Southworth, T. L., Agliardi, G., Hotblack, A., Lythgoe, M. F., Branchini, B. R., Kalber, T. L., Anderson, J. C., and Pule, M. A. (2019) *eLife* 8. doi/10.7554/eLife.45801
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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferin that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

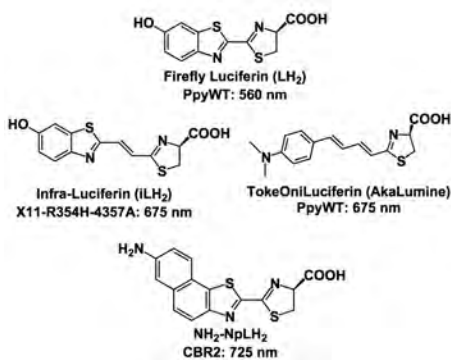


Figure 1. Luciferase-substrate emission maxima.

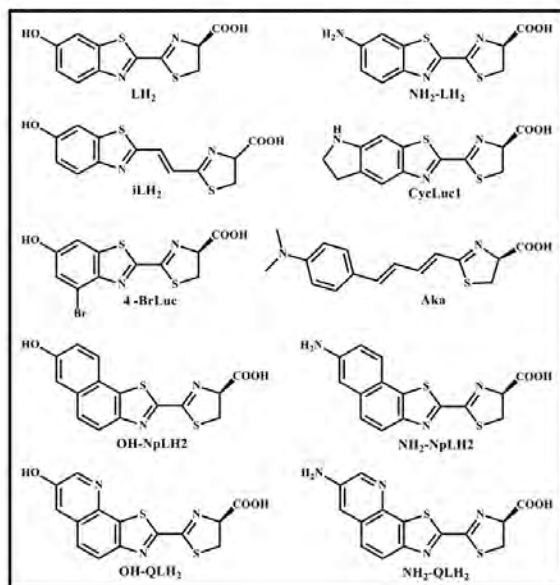
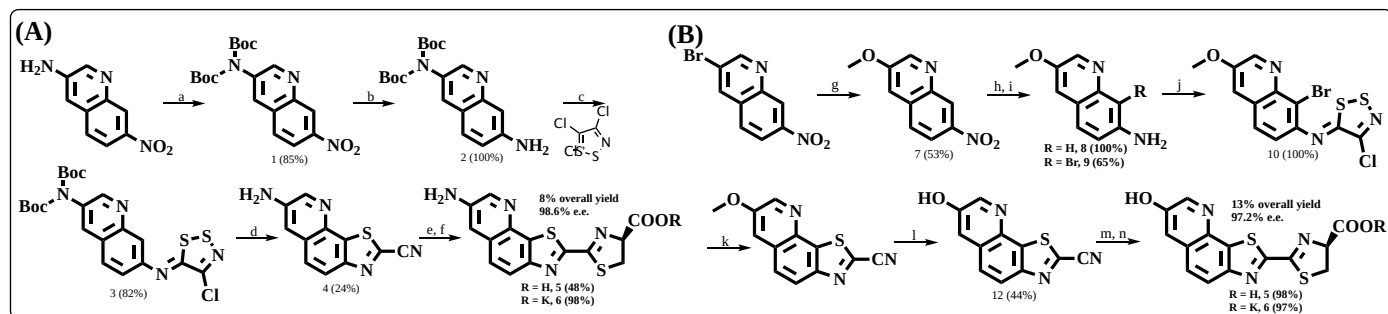


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

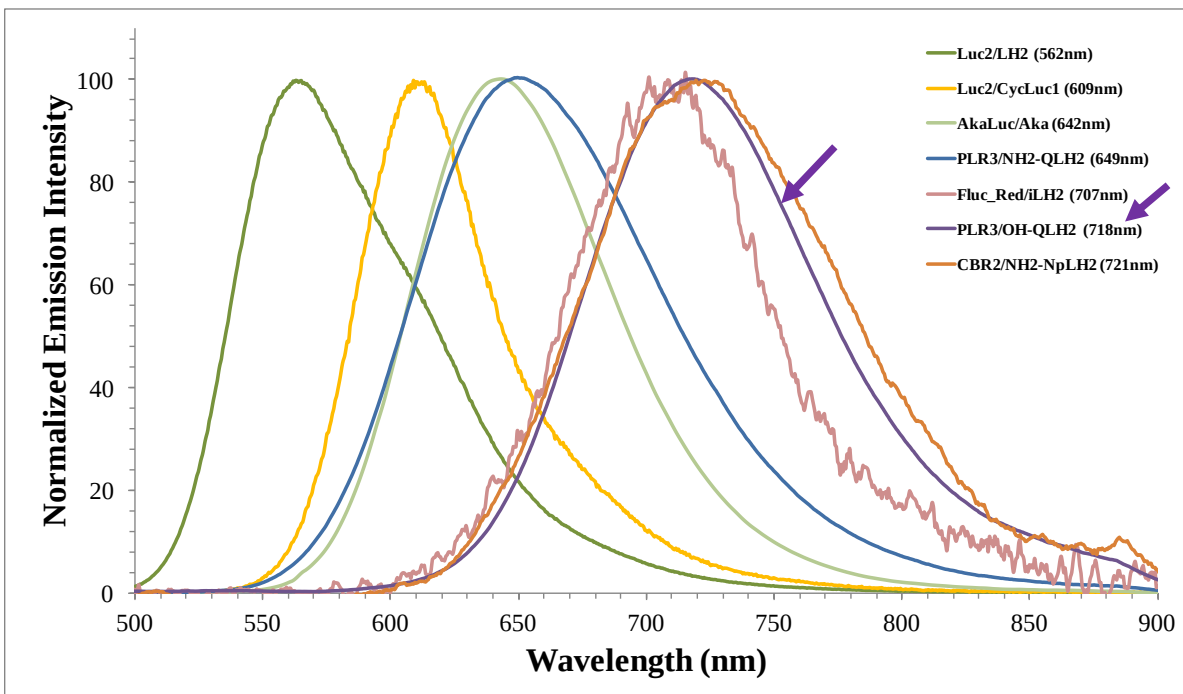


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

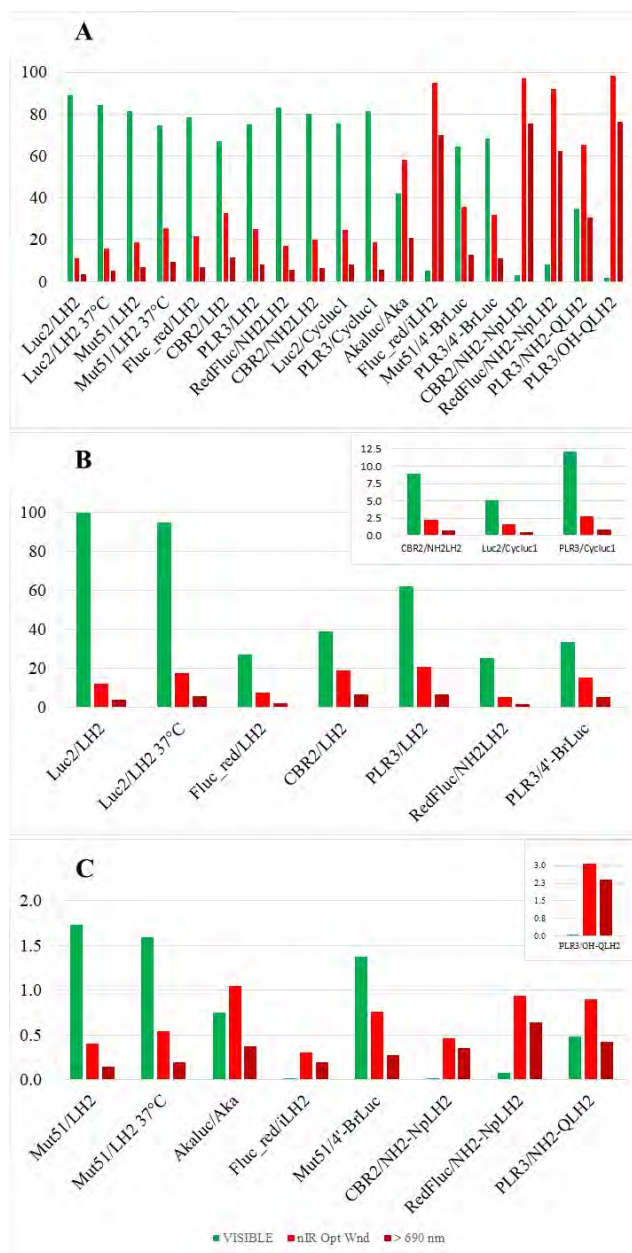


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

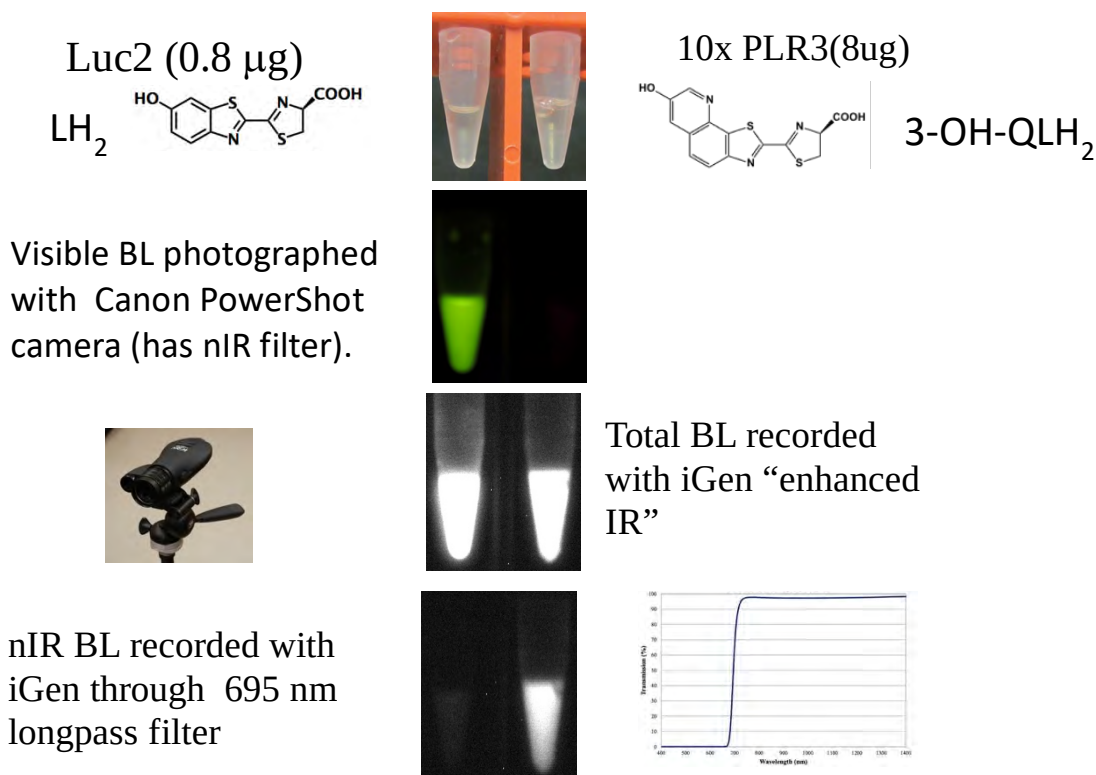
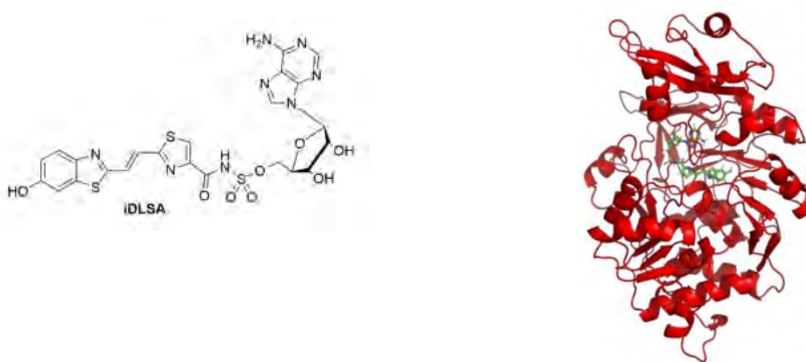


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T – OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

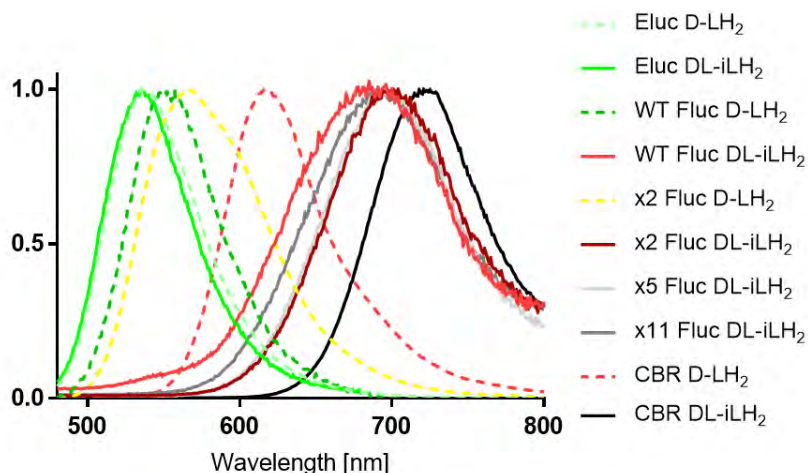


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

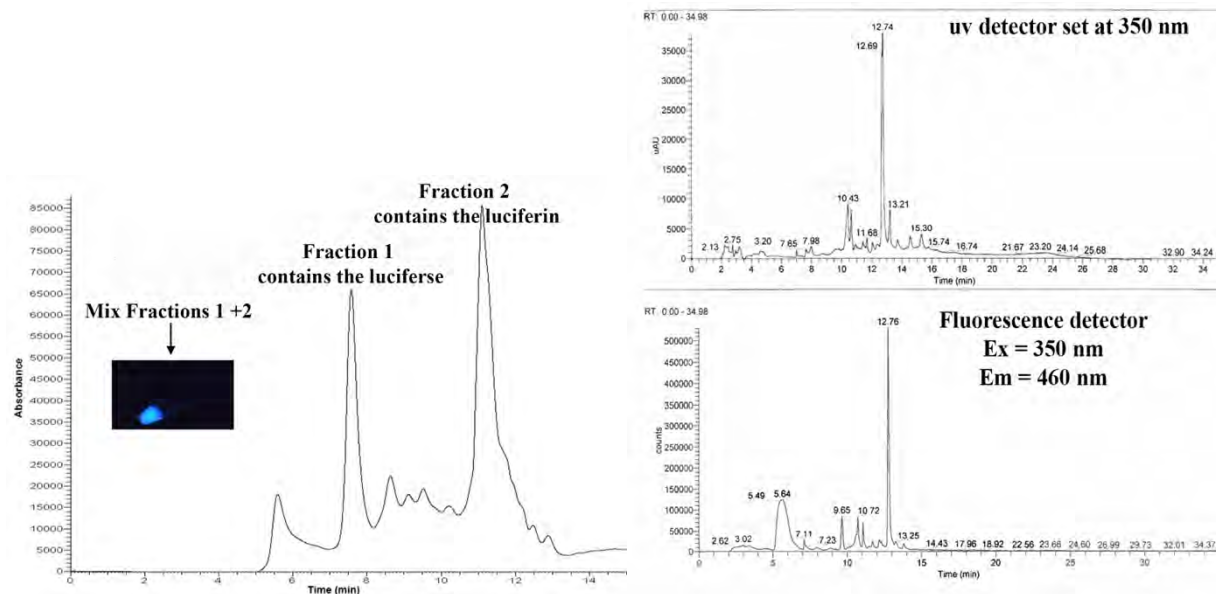
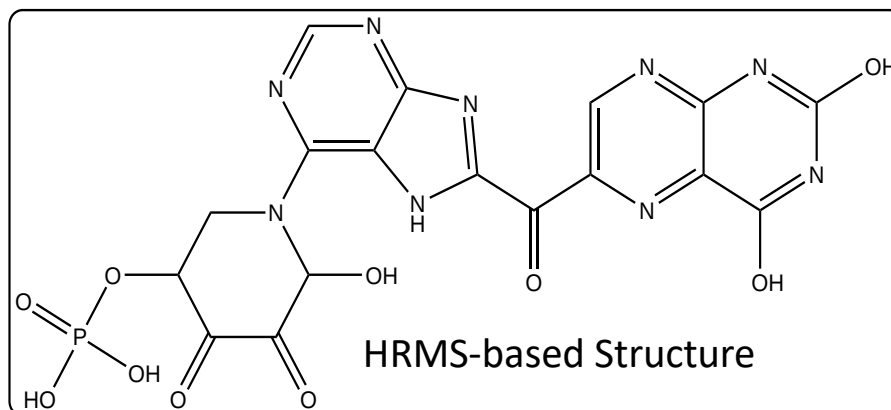


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



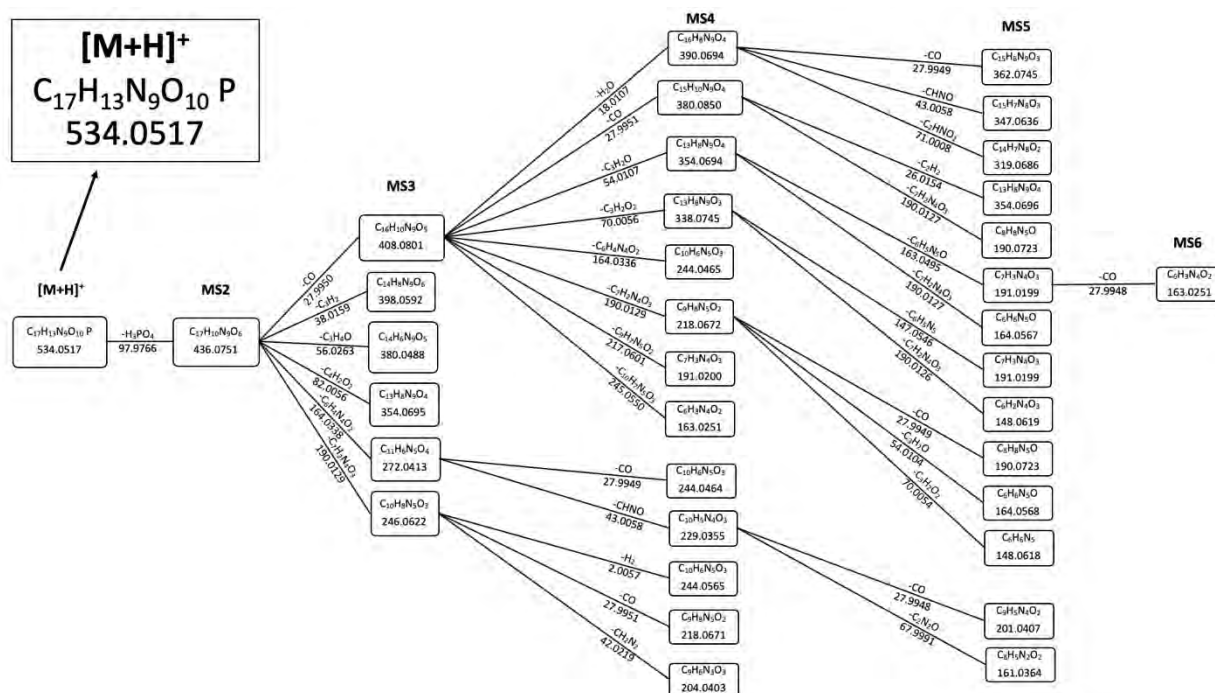


Figure 8. HRMS analysis of putative *Orfelia oxyluciferin*.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the Orfelia oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

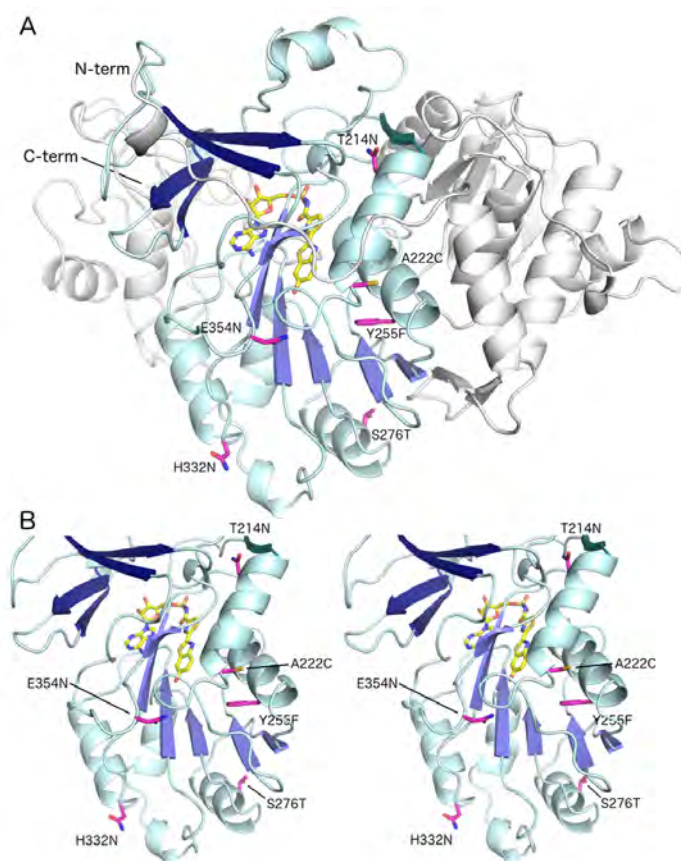


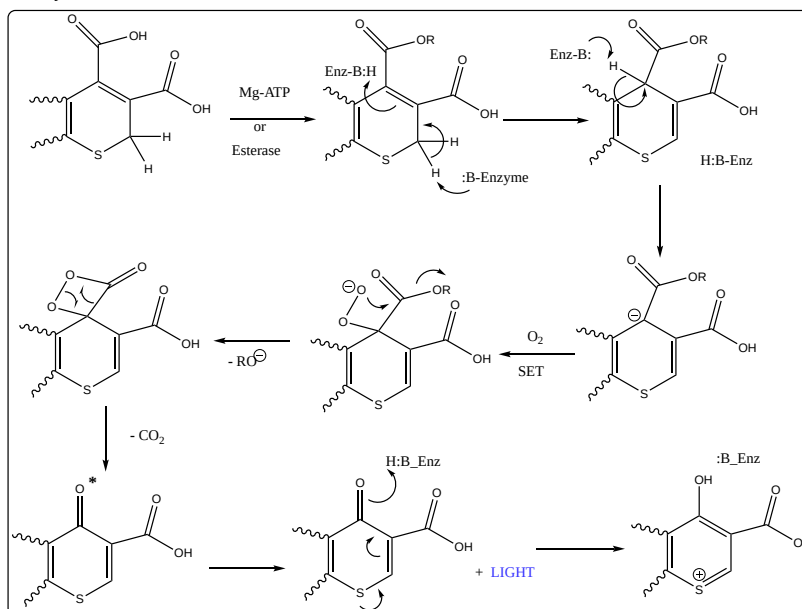
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaetae that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdonata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

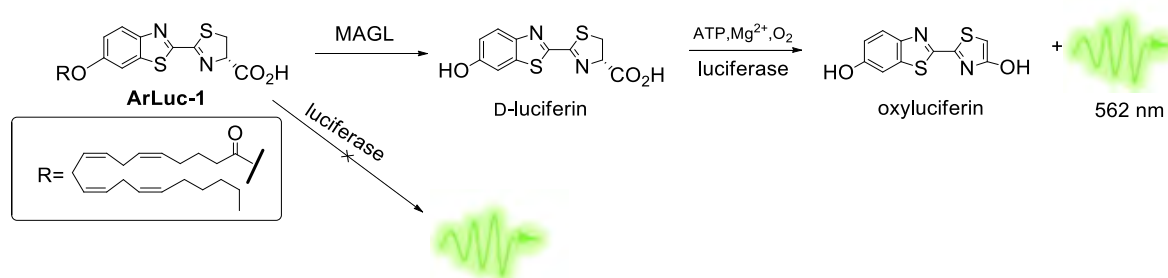


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

Publications

- 1. "Red-emitting chimeric firefly luciferase for in vivo imaging in low ATP cellular environments." Bruce R. Branchini, Tara L. Southworth, Danielle M. Fontaine, Dawn Kohrt, Franceine S. Welcome, Catherine M. Florentine, Emma R. Henricks, Demetria B. DeBartolo, Elisa Michelini, Luca Cevenini, Aldo Roda, Martha J. Grossel, *Analytical Biochemistry*, 2017;534:36–39. doi: 10.1016/j.ab.2017.07.001.**

- 2. "A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events." Branchini BR, Southworth TL, Fontaine DM, Kohrt D, Florentine CM, Grossel MJ. *Sci Rep.* 2018; 8(1):5990. doi: 10.1038/s41598-018-24278-2.
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COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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Principal Investigator: Bruce Branchini

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ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultoni* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

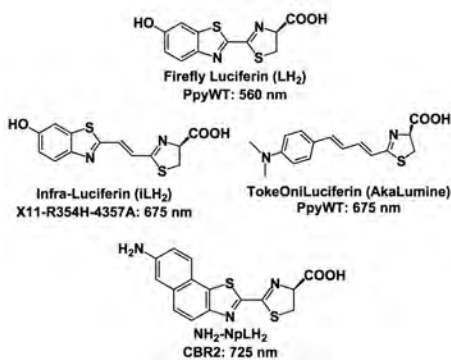


Figure 1. Luciferase-substrate emission maxima.

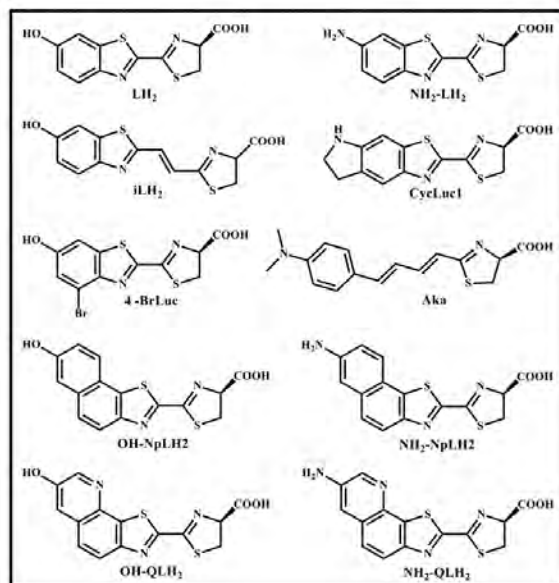
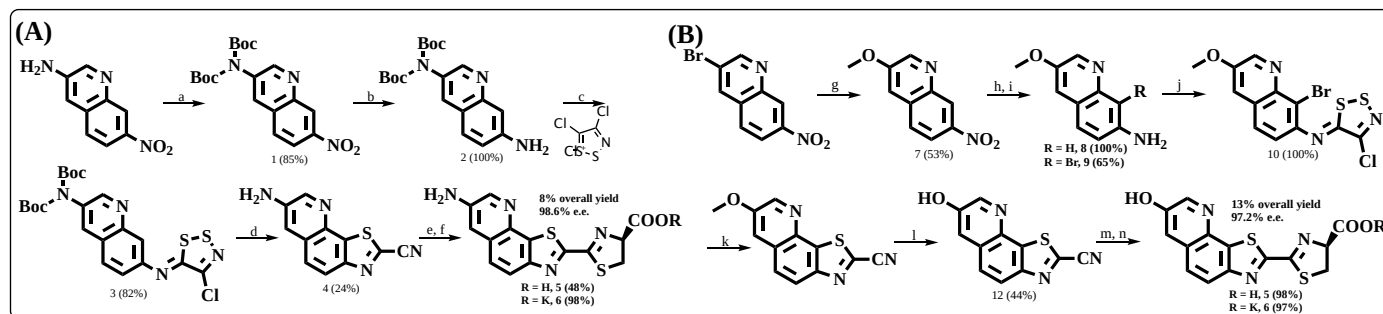


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

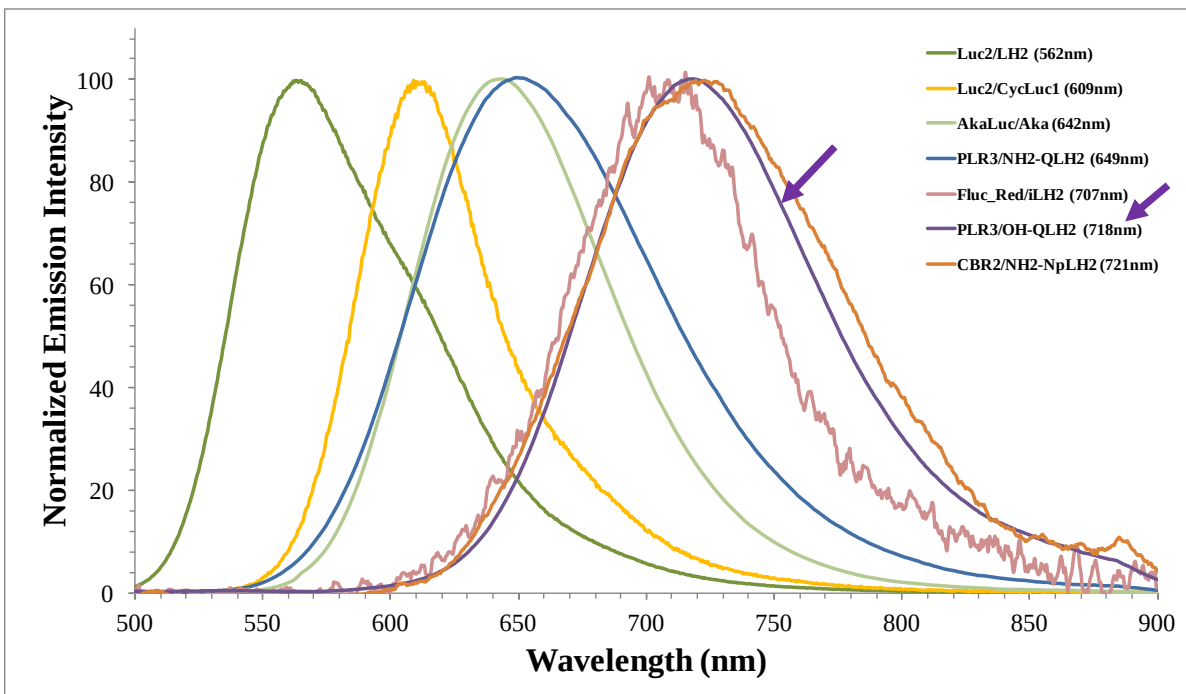


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

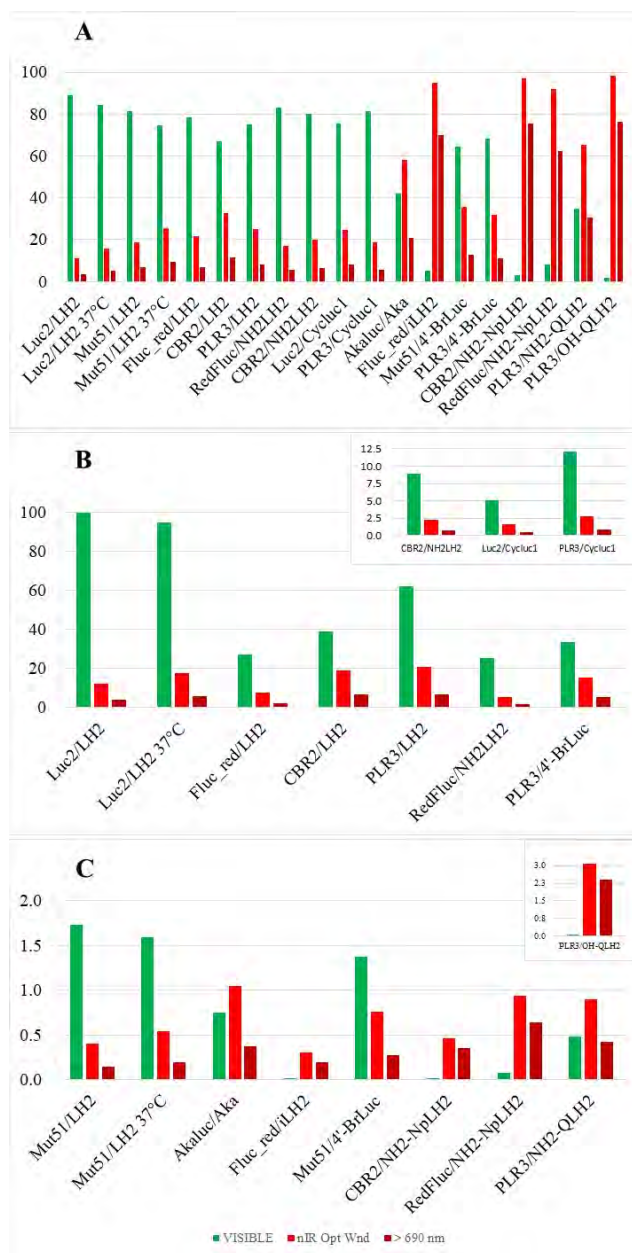


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

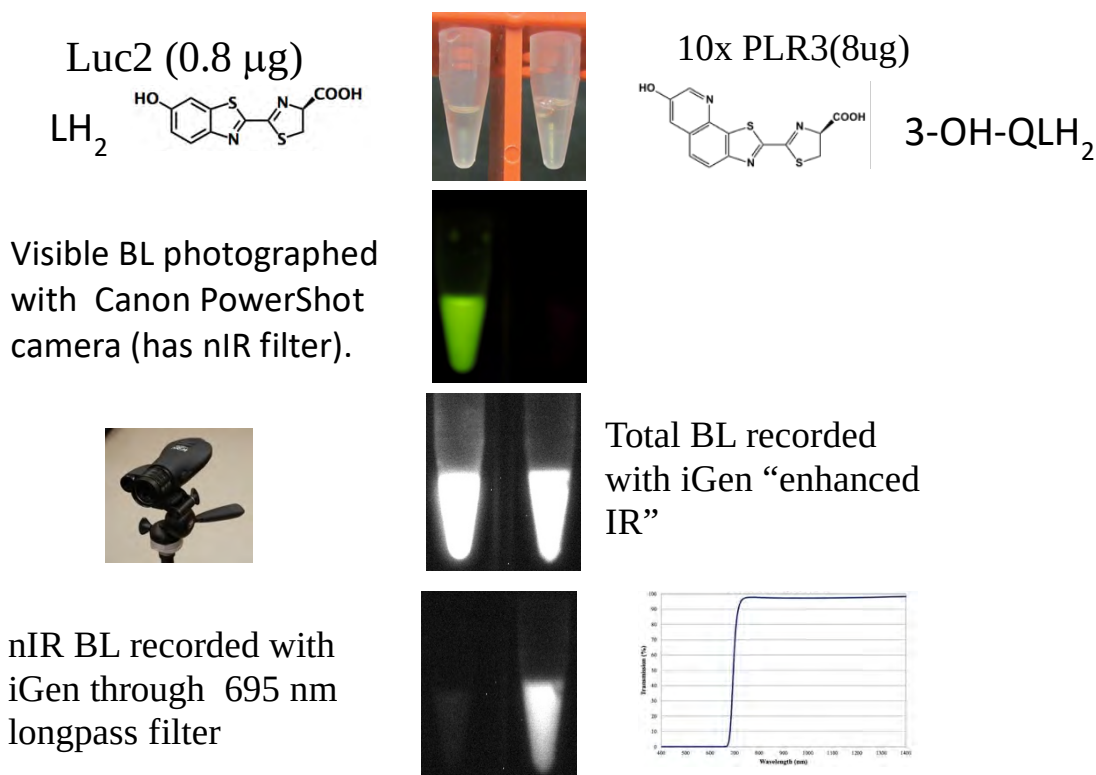
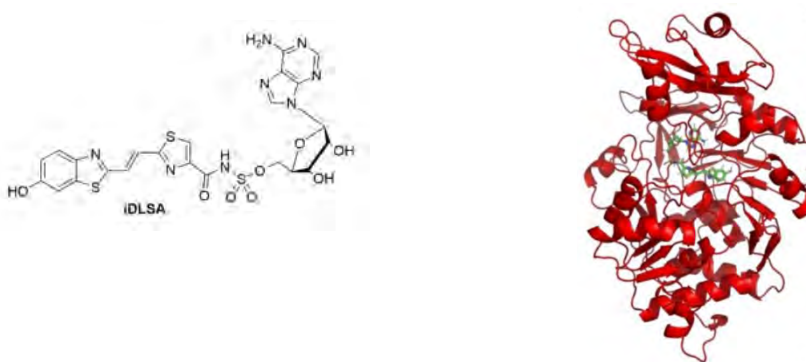


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T – OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

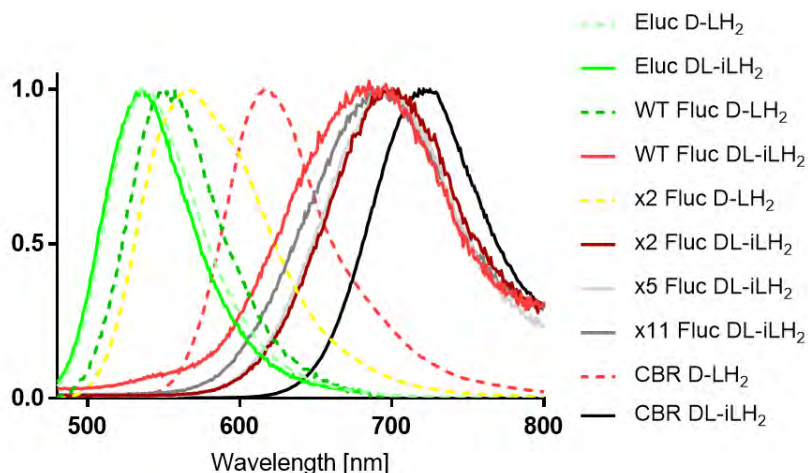


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultoni*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

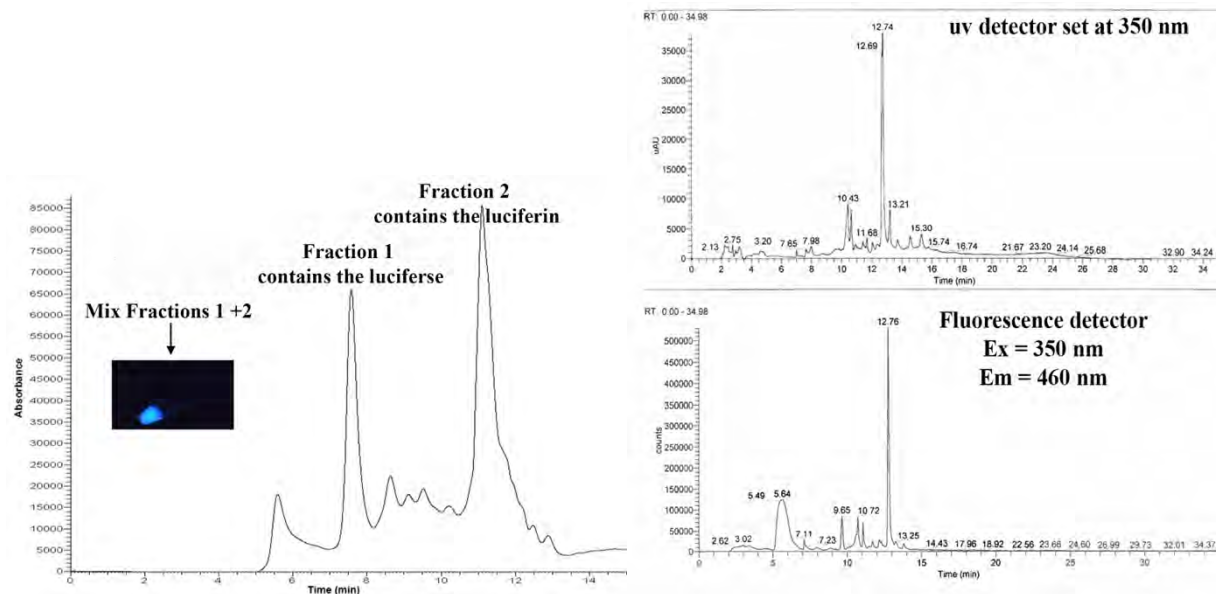
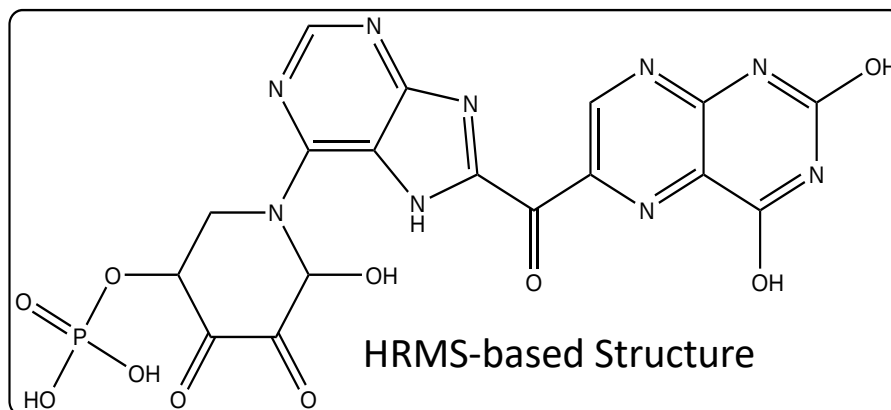


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

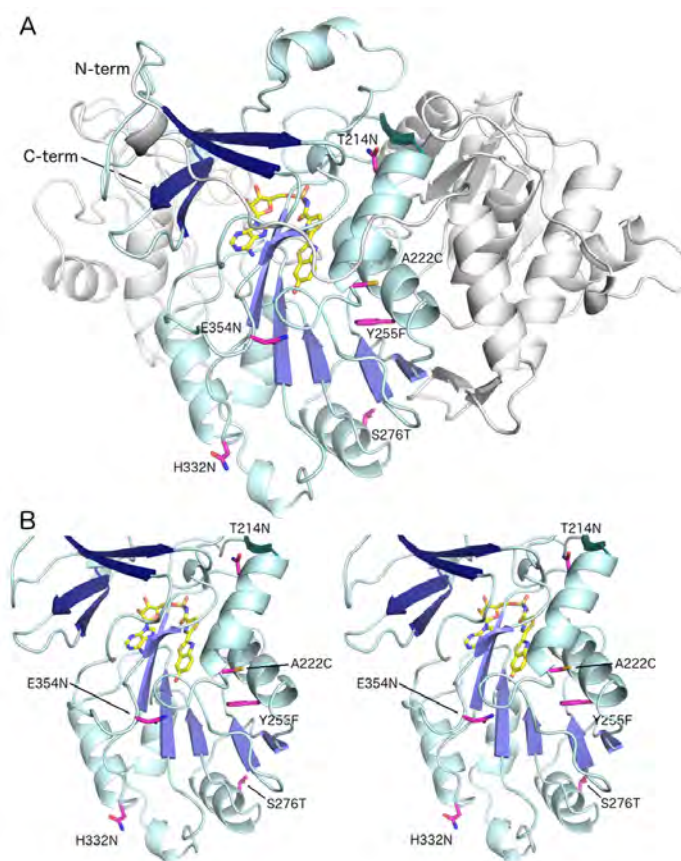


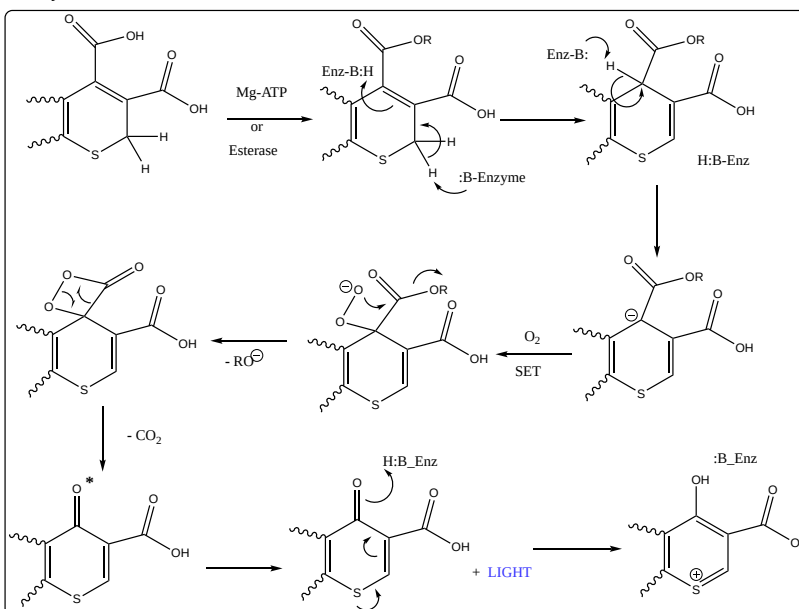
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

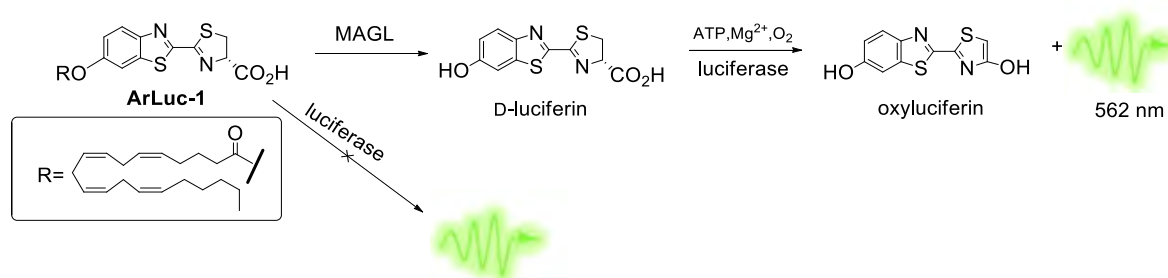


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 x g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18-20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at -80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 1. "Red-emitting chimeric firefly luciferase for in vivo imaging in low ATP cellular environments." Bruce R. Branchini, Tara L. Southworth, Danielle M. Fontaine, Dawn Kohrt, Franceine S. Welcome, Catherine M. Florentine, Emma R. Henricks, Demetria B. DeBartolo, Elisa Michelini, Luca Cevenini, Aldo Roda, Martha J. Grossel, *Analytical Biochemistry*, 2017;534:36-39. doi: 10.1016/j.ab.2017.07.001.**

- 2. "A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events." Branchini BR, Southworth TL, Fontaine DM, Kohrt D, Florentine CM, Grossel MJ. *Sci Rep*. 2018; 8(1):5990. doi: 10.1038/s41598-018-24278-2.
- 3. " Mutagenesis and structural studies reveal the basis for the activity and stability properties that distinguish the photinus luciferases scintillans and pyralis." Branchini, B. R., Fontaine, D. M., Southworth, T. L., Huta, B. P., Racela, A., Patel, K. D., and Gulick, A. M. (2019), *Biochemistry* 58, 4293-4303. doi/10.1021/acs.biochem.9b00719
- 4. " Bioluminescence chemistry of fireworm Odontosyllis." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
- 5. "Prêt-à-porter nanoYESA and nanoYESB bioluminescent cell biosensors for ultrarapid and sensitive screening of endocrine-disrupting chemicals." Lopreside, A., Calabretta, M. M., Montali, L., Ferri, M., Tassoni, A., Branchini, B. R., Southworth, T., D'Elia, M., Roda, A., and Michelini, E. (2019) *Anal. Bioanal. Chem.* 411, 4937-4949. doi/10.1007/s00216-019-01805-2
- 6. " Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin." Stowe, C. L., Burley, T. A., Allan, H., Vinci, M., Kramer-Marek, G., Ciobota, D. M., Parkinson, G. N., Southworth, T. L., Agliardi, G., Hotblack, A., Lythgoe, M. F., Branchini, B. R., Kalber, T. L., Anderson, J. C., and Pule, M. A. (2019) *eLife* 8. doi/10.7554/eLife.45801
- 7. "A new brilliantly blue-emitting luciferin- luciferase system from *Orfelia fultoni* and *Keroplantinae* (Diptera)." V.R. Viviani, J. R. Silva, D. T. Amaral, V. R. Bevilaqua, F. C. Abdalla, B. R. Branchini, and C. H. Johnson (2020) *Sci. Rep.*, 10:9608 | <https://doi.org/10.1038/s41598-020-66286-1>
- 8. "A new ultrasensitive bioluminescence -based method for assaying monoacylglycerol lipase." M. Miceli, S. Casati, P. Allevi, S. Berra, R. Ottria, P. Rota, B.R. Branchini, and P. Ciuffreda, *Int. J. Mol. Sci.*, 22, 6148 (2021) <https://doi.org/10.3390/ijms22116148>

- 9. “Dendritic cells maintain anti-tumor immunity by positioning CDB skin-resident memory T cells.” J. L. Vella, A. Molodtskov, C. V. Angeles, B. R. Branchini, M. J. Turk, and Y. H. Huang, *Life Science Alliance* 4 (10), e202101056 (2021).
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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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*** Corresponding author inadvertently left out AFOSR acknowledgement.

COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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Distribution Statement: Approved for Public release

Program Officer: Bennett L. Ibey

Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

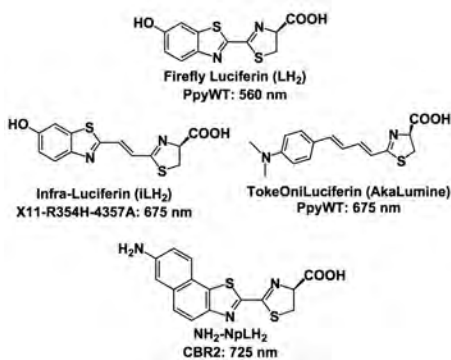


Figure 1. Luciferase-substrate emission maxima.

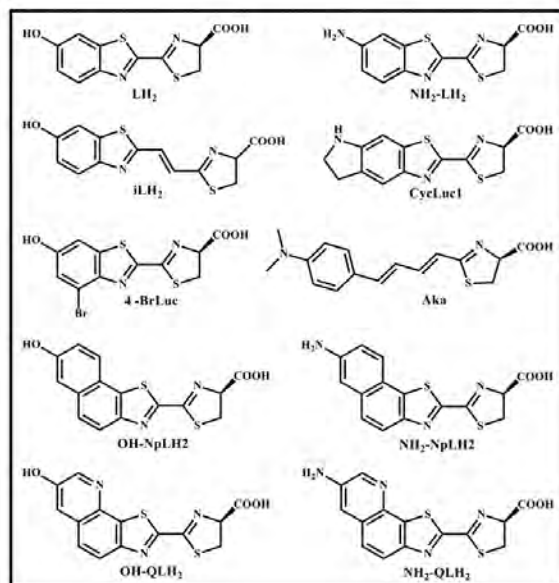
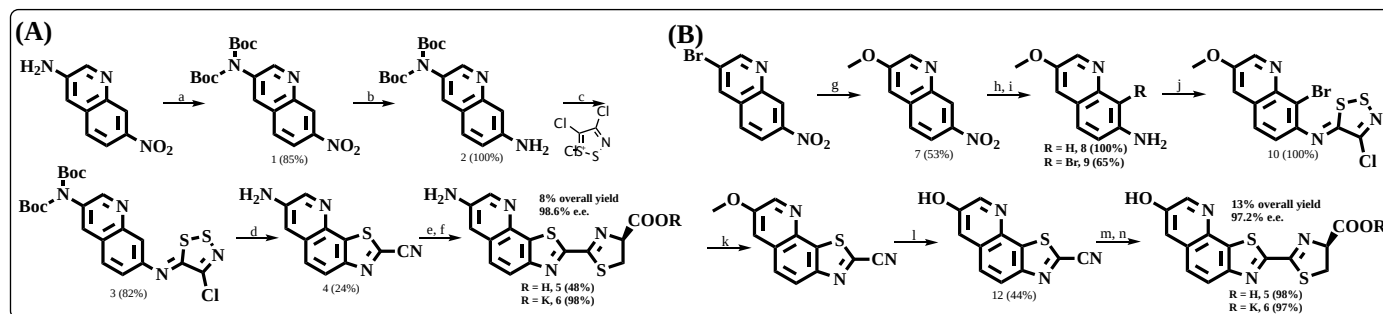


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

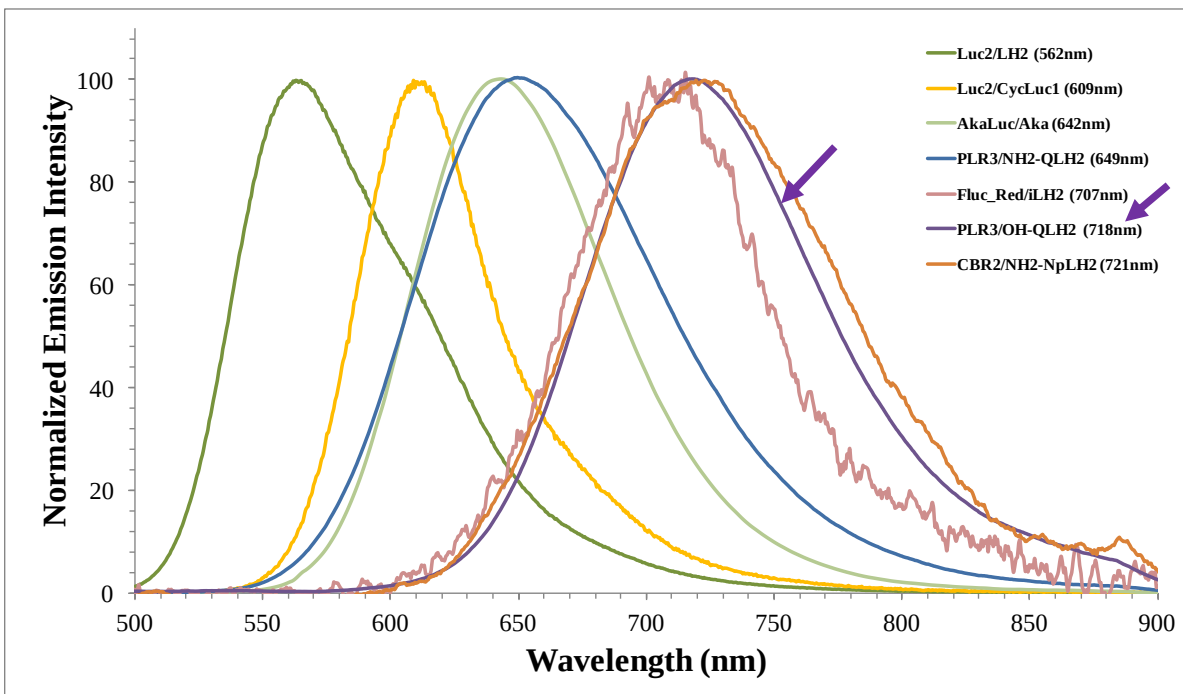


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

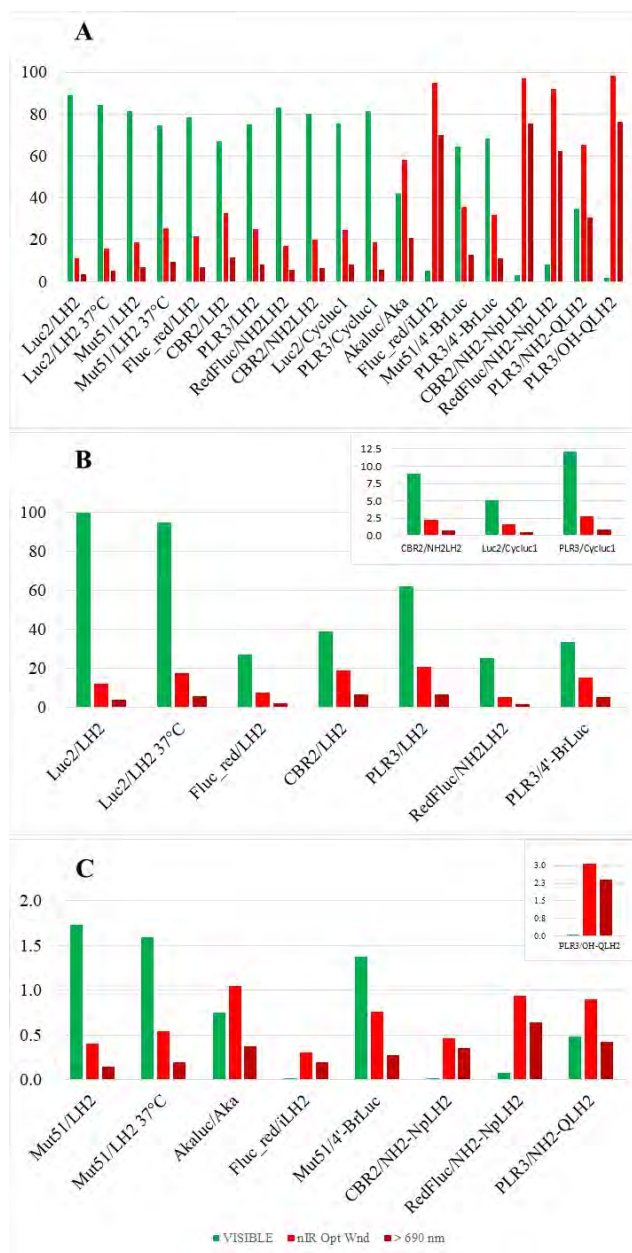


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

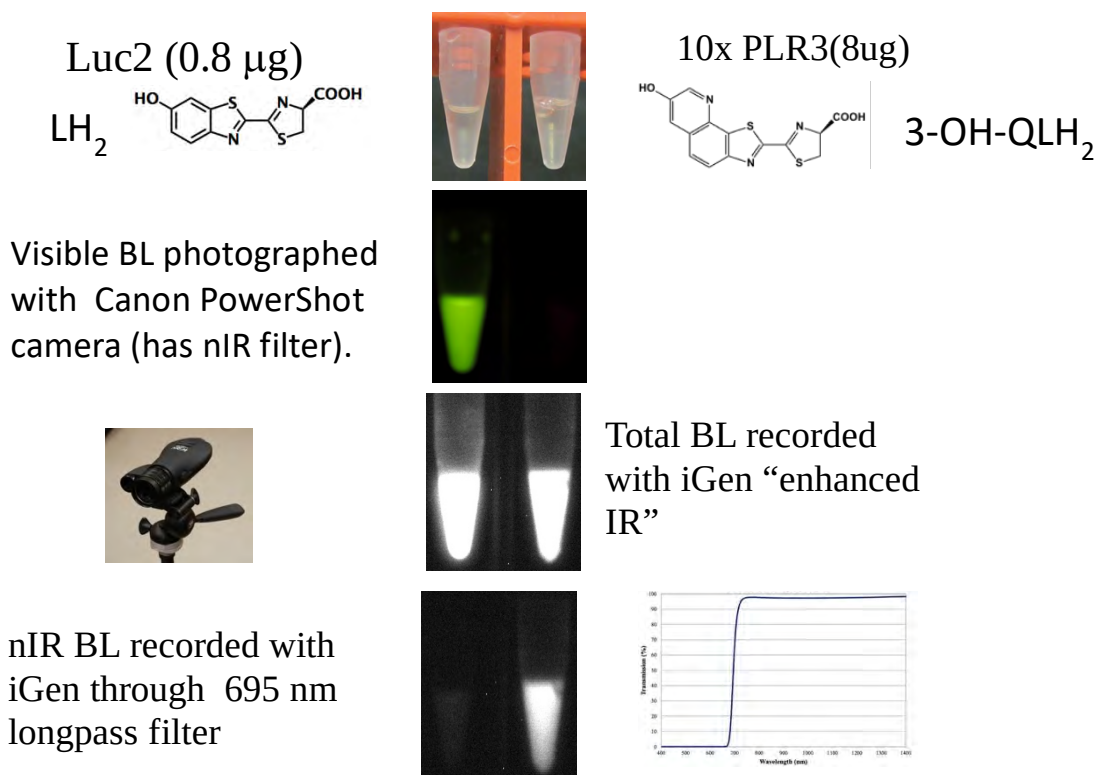
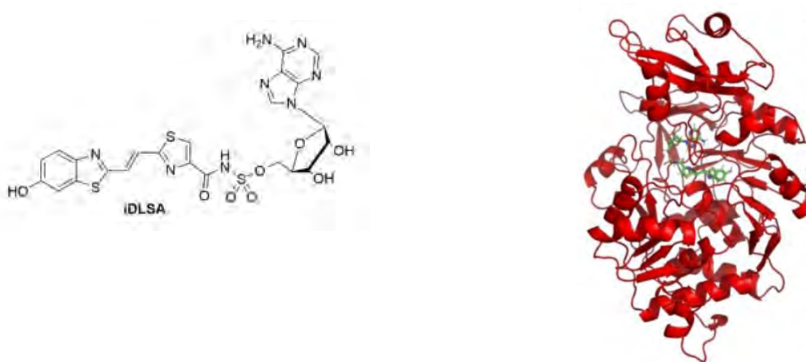


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

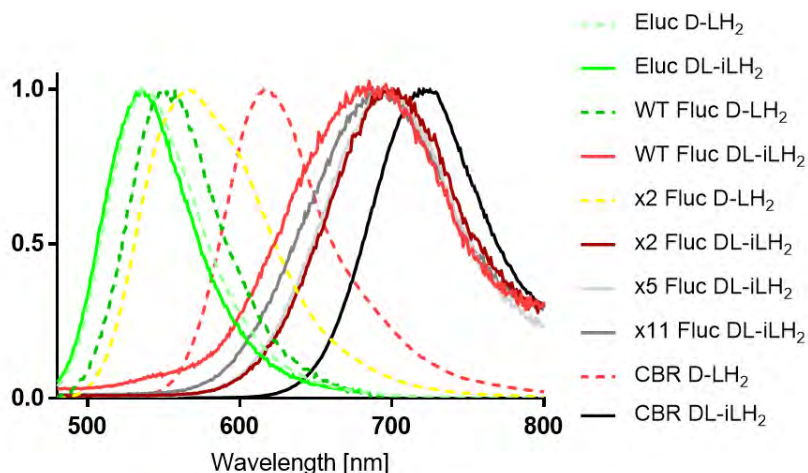


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultoni*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

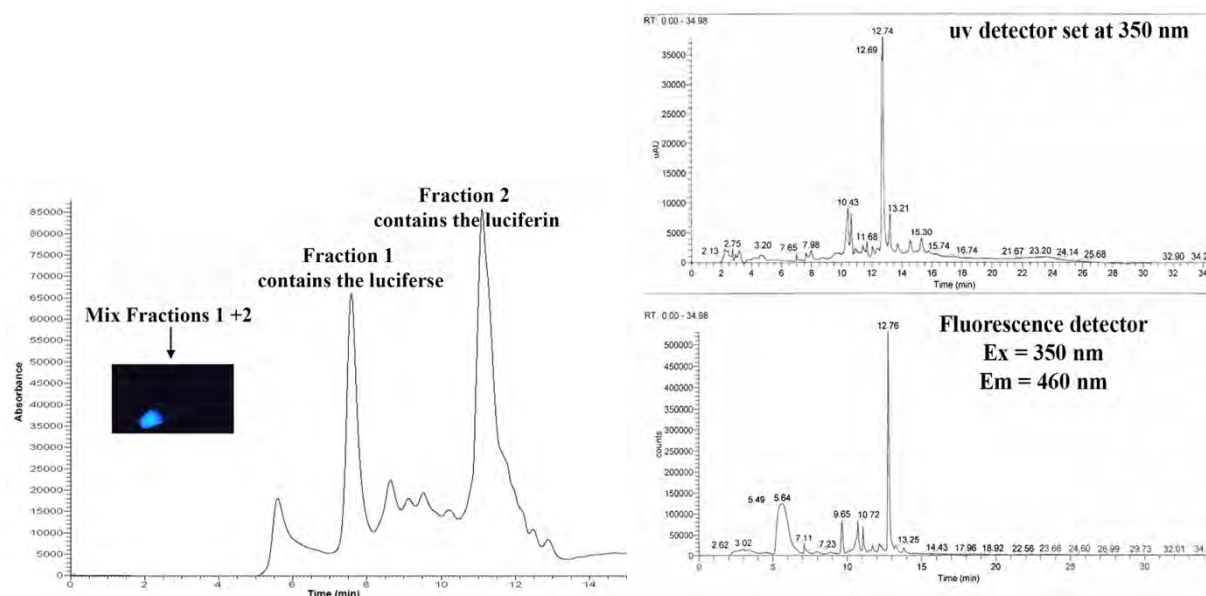
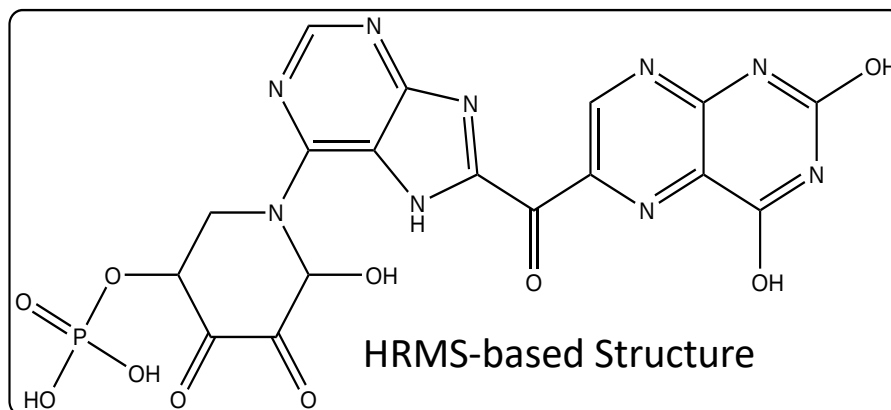


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of Orfelia bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

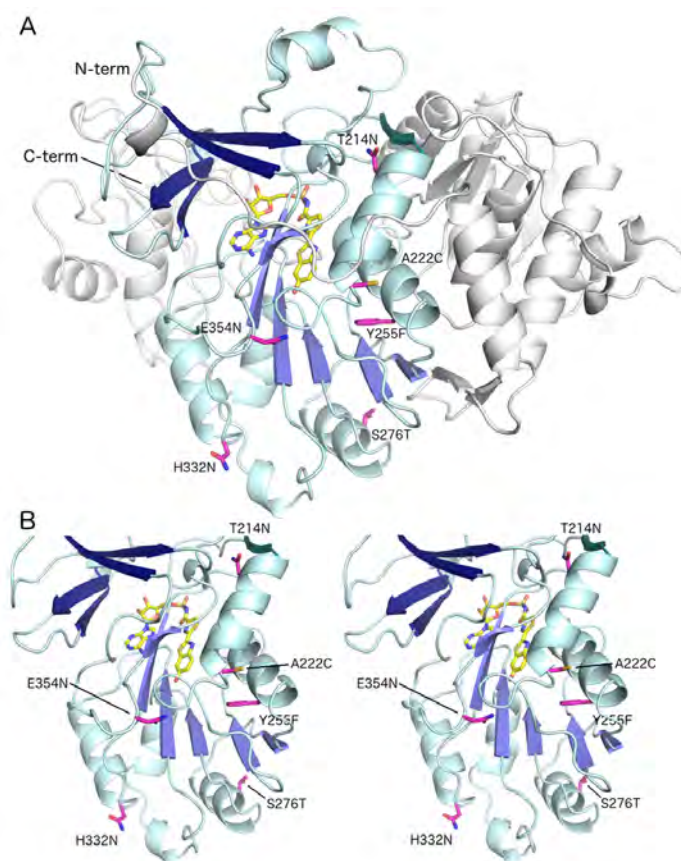


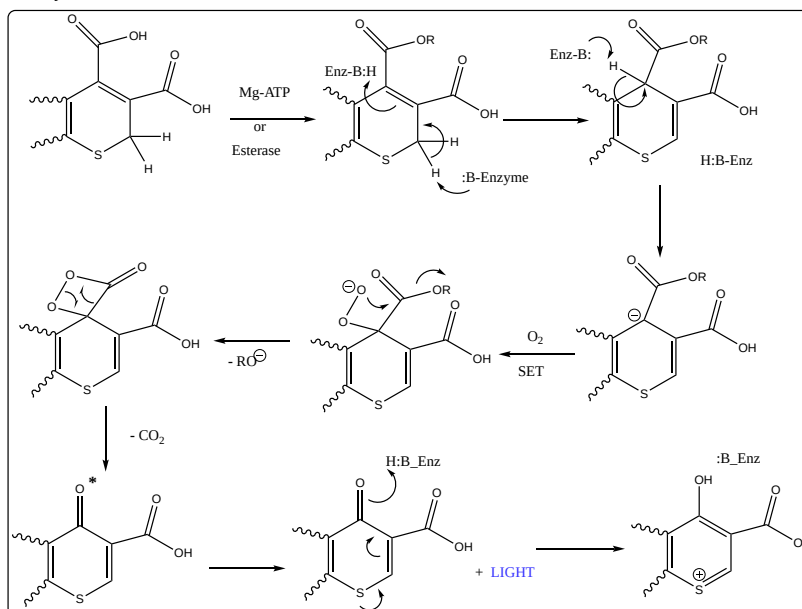
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaetae that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdonata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

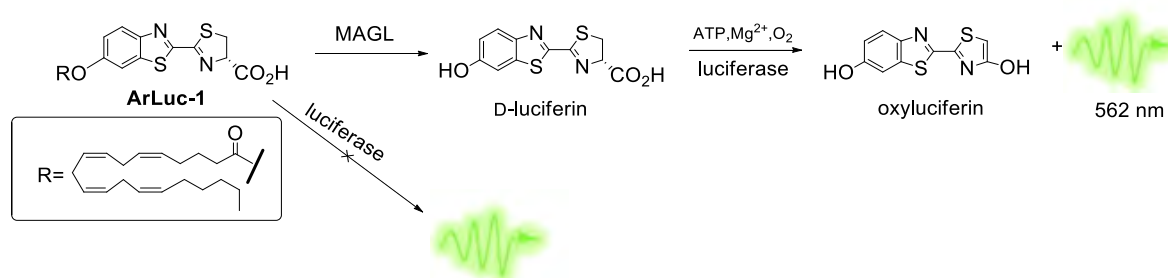


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 x g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18-20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at -80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of K_m for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). K_m values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 1. "Red-emitting chimeric firefly luciferase for in vivo imaging in low ATP cellular environments." Bruce R. Branchini, Tara L. Southworth, Danielle M. Fontaine, Dawn Kohrt, Franceine S. Welcome, Catherine M. Florentine, Emma R. Henricks, Demetria B. DeBartolo, Elisa Michelini, Luca Cevenini, Aldo Roda, Martha J. Grossel, *Analytical Biochemistry*, 2017;534:36-39. doi: 10.1016/j.ab.2017.07.001.**

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- 3. " Mutagenesis and structural studies reveal the basis for the activity and stability properties that distinguish the photinus luciferases scintillans and pyralis." Branchini, B. R., Fontaine, D. M., Southworth, T. L., Huta, B. P., Racela, A., Patel, K. D., and Gulick, A. M. (2019), *Biochemistry* 58, 4293-4303. doi/10.1021/acs.biochem.9b00719
- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
- 5. "Prêt-à-porter nanoYESA and nanoYESB bioluminescent cell biosensors for ultrarapid and sensitive screening of endocrine-disrupting chemicals." Lopreside, A., Calabretta, M. M., Montali, L., Ferri, M., Tassoni, A., Branchini, B. R., Southworth, T., D'Elia, M., Roda, A., and Michelini, E. (2019) *Anal. Bioanal. Chem.* 411, 4937-4949. doi/10.1007/s00216-019-01805-2
- 6. " Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin." Stowe, C. L., Burley, T. A., Allan, H., Vinci, M., Kramer-Marek, G., Ciobota, D. M., Parkinson, G. N., Southworth, T. L., Agliardi, G., Hotblack, A., Lythgoe, M. F., Branchini, B. R., Kalber, T. L., Anderson, J. C., and Pule, M. A. (2019) *eLife* 8. doi/10.7554/eLife.45801
- 7. "A new brilliantly blue-emitting luciferin- luciferase system from *Orfelia fultoni* and *Keroplantinae* (Diptera)." V.R. Viviani, J. R. Silva, D. T. Amaral, V. R. Bevilaqua, F. C. Abdalla, B. R. Branchini, and C. H. Johnson (2020) *Sci. Rep.*, 10:9608 | <https://doi.org/10.1038/s41598-020-66286-1>
- 8. "A new ultrasensitive bioluminescence -based method for assaying monoacylglycerol lipase." M. Miceli, S. Casati, P. Allevi, S. Berra, R. Ottria, P. Rota, B.R. Branchini, and P. Ciuffreda, *Int. J. Mol. Sci.*, 22, 6148 (2021) <https://doi.org/10.3390/ijms22116148>

- 9. “Dendritic cells maintain anti-tumor immunity by positioning CDB skin-resident memory T cells.” J. L. Vella, A. Molodtskov, C. V. Angeles, B. R. Branchini, M. J. Turk, and Y. H. Huang, *Life Science Alliance* 4 (10), e202101056 (2021).
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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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*** Corresponding author inadvertently left out AFOSR acknowledgement.

COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

Award Number: FA9550-18-1-0017

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Program Officer: Bennett L. Ibey

Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

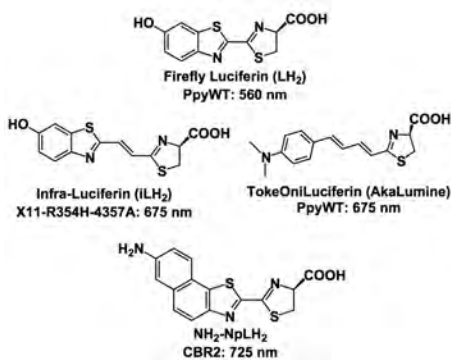


Figure 1. Luciferase-substrate emission maxima.

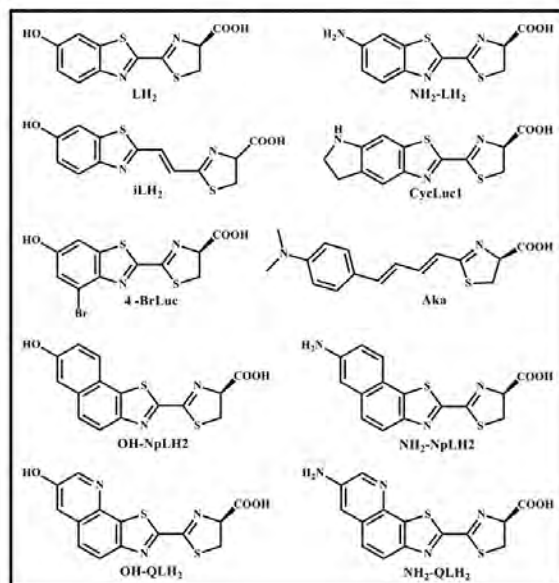
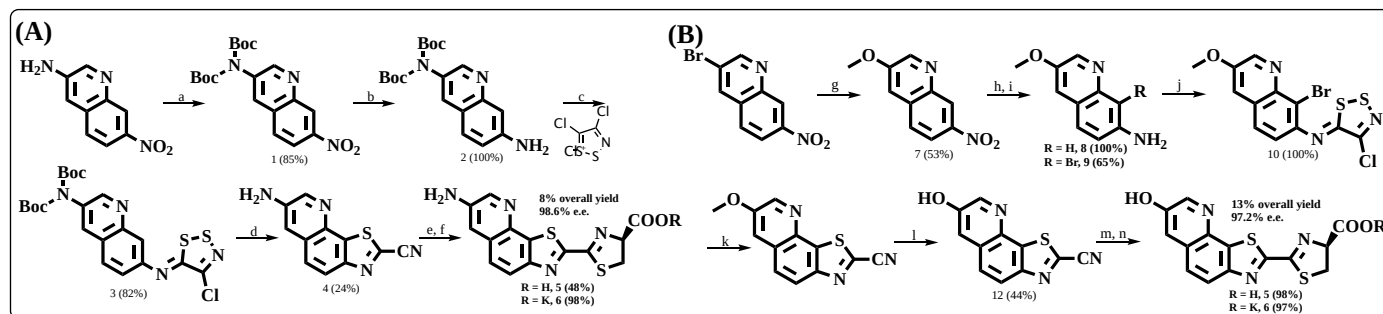


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

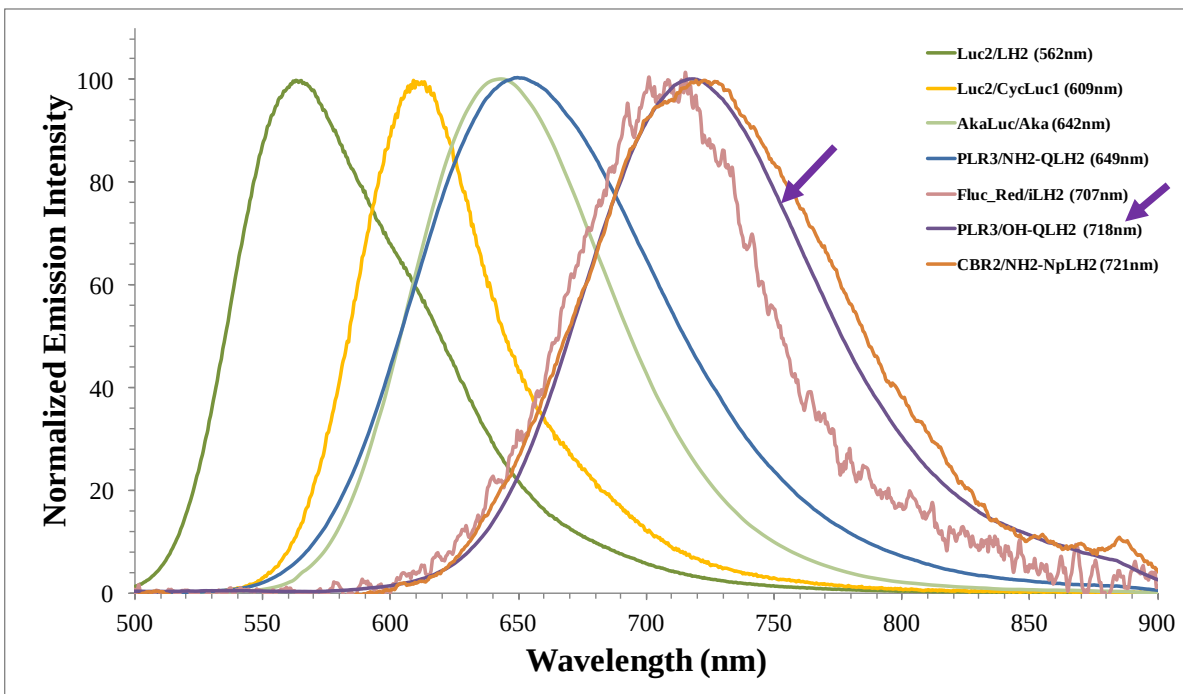


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

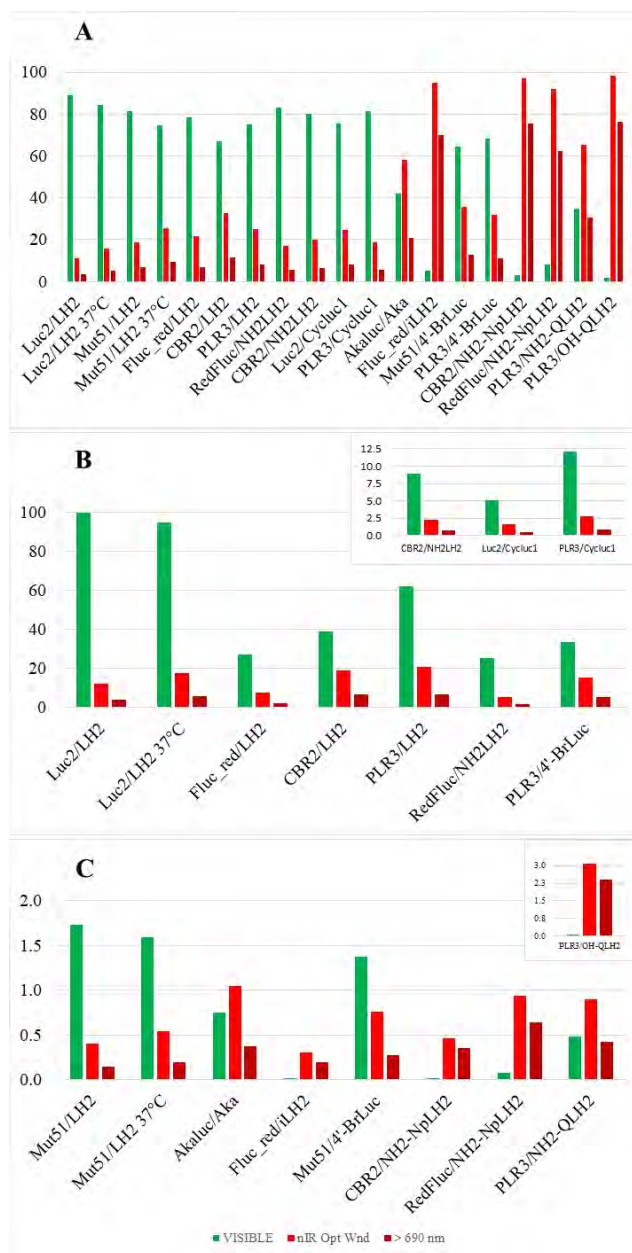


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

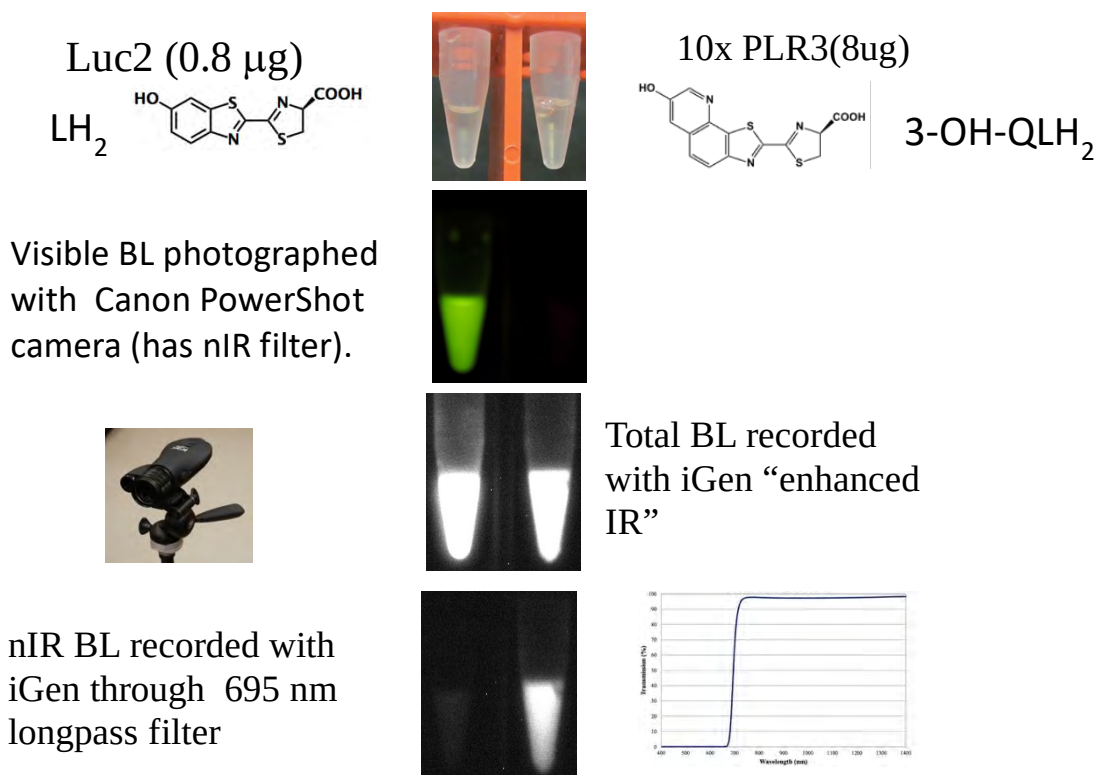
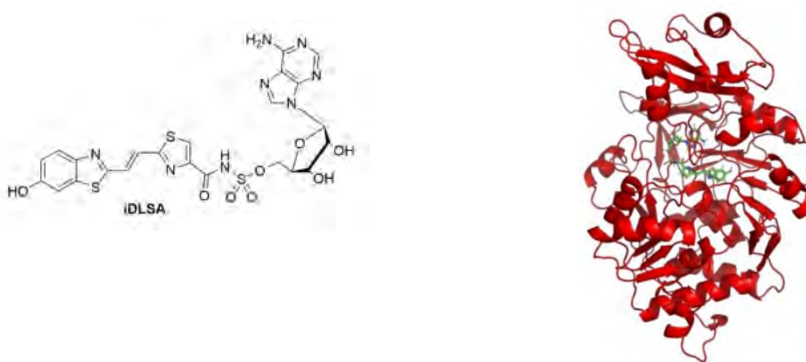


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

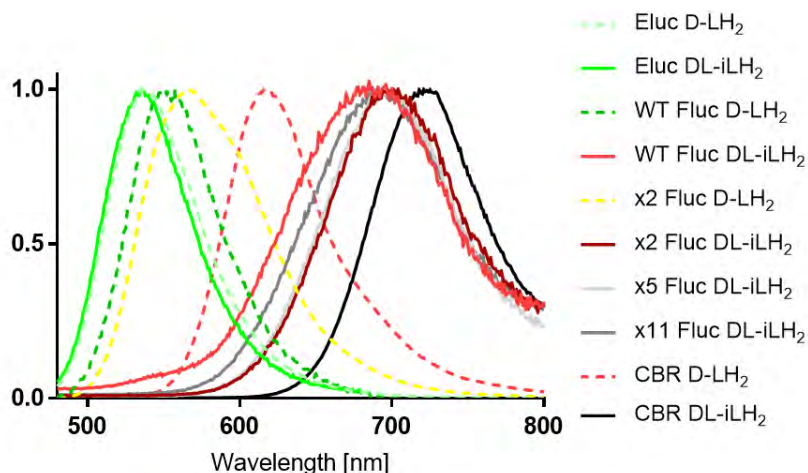


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

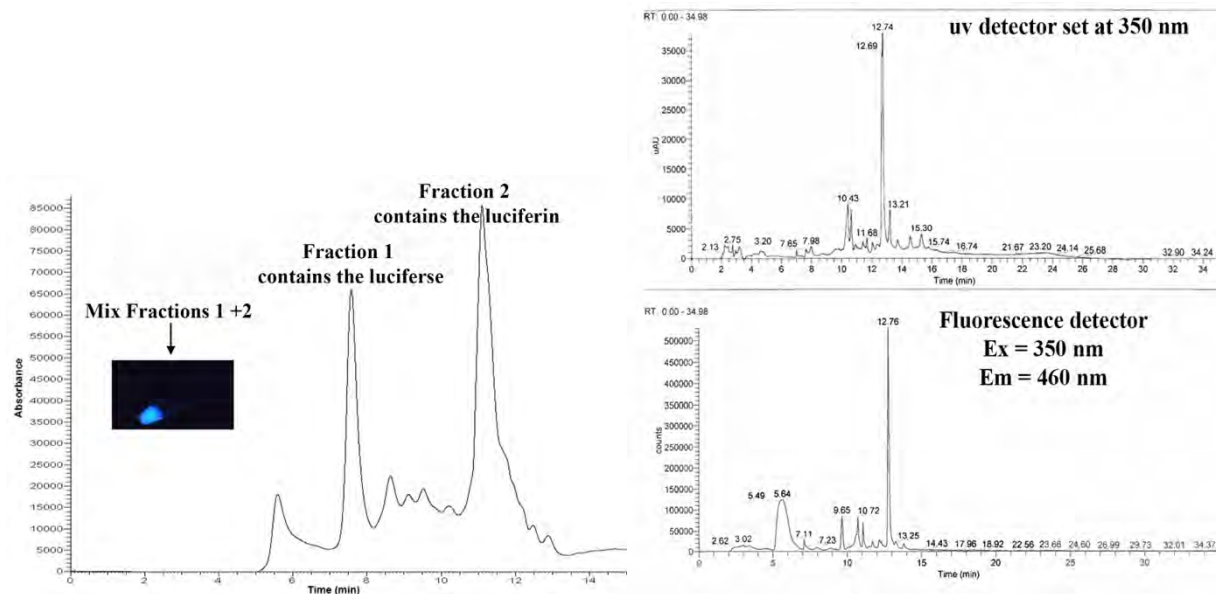
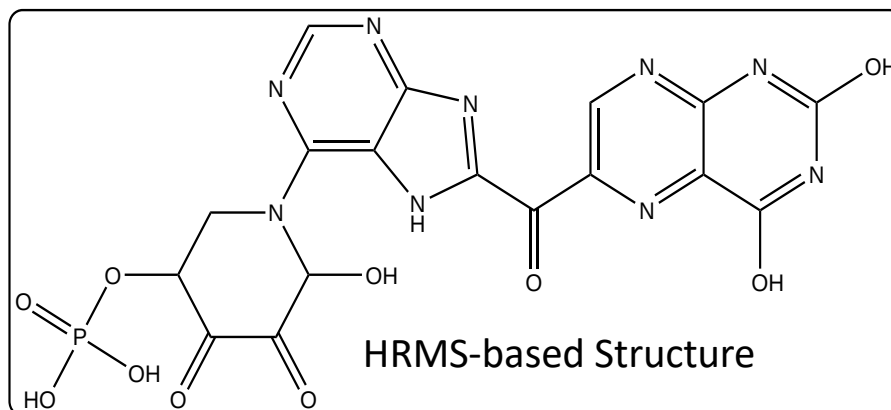


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



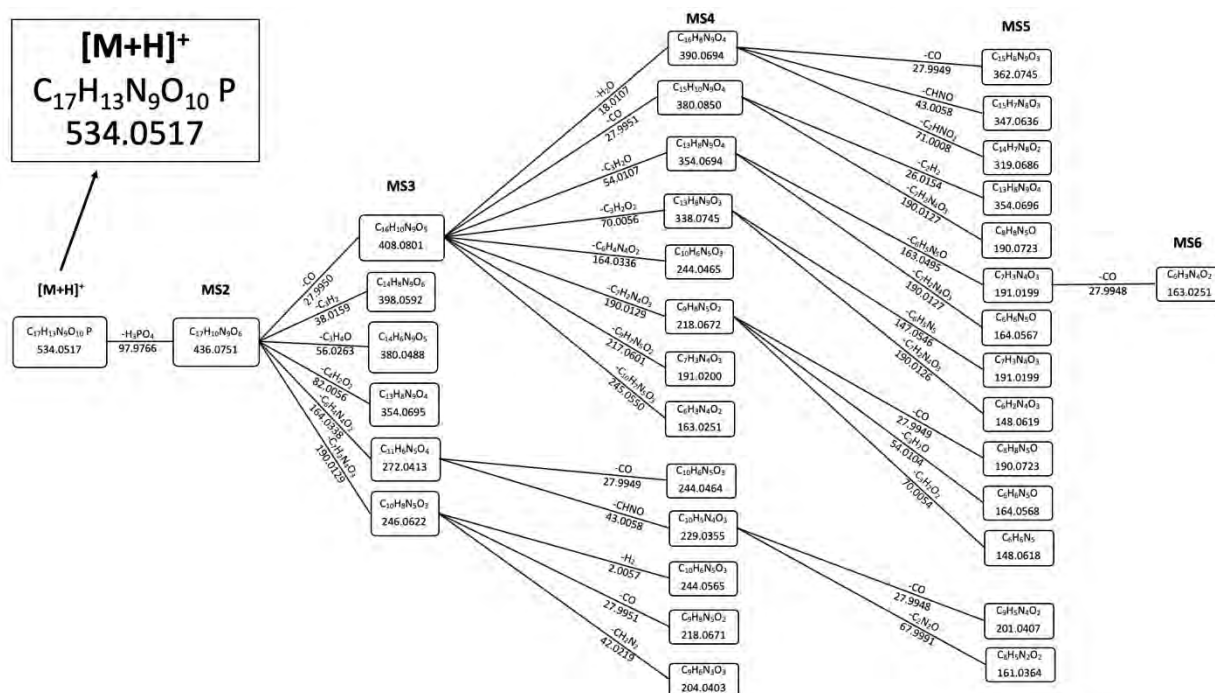


Figure 8. HRMS analysis of putative Orfelia oxyluciferin.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the Orfelia oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

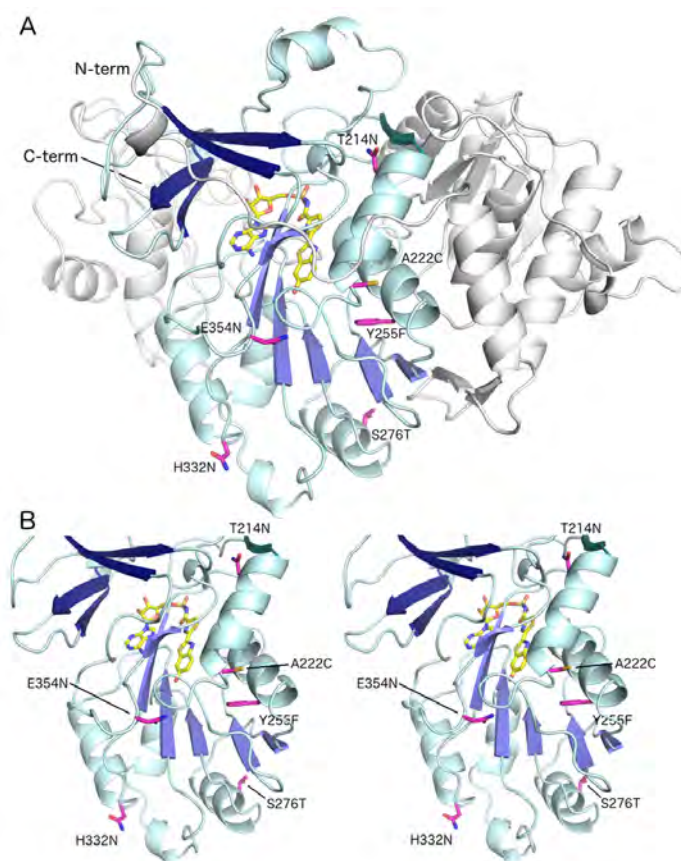


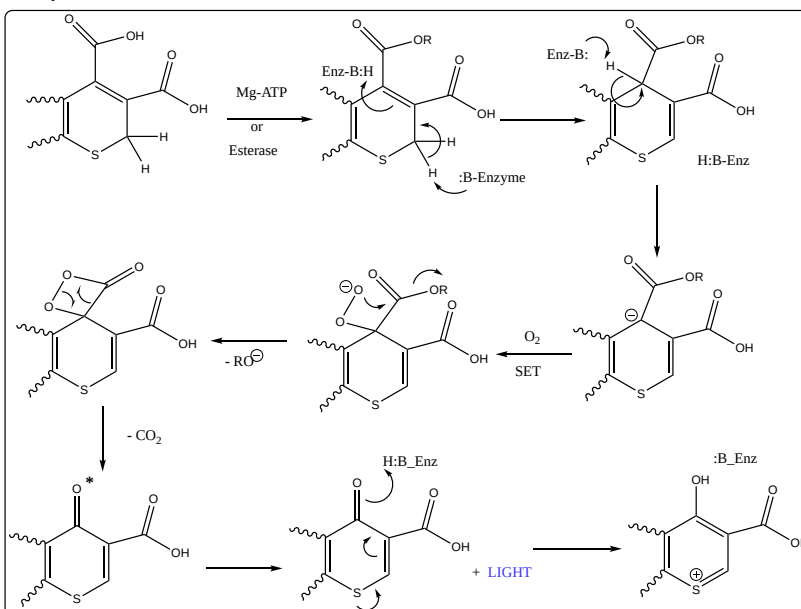
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

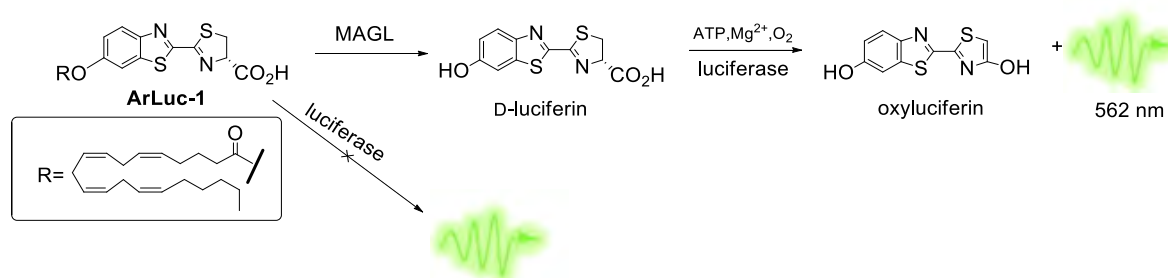


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 2. "A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events." Branchini BR, Southworth TL, Fontaine DM, Kohrt D, Florentine CM, Grossel MJ. *Sci Rep.* 2018; 8(1):5990. doi: 10.1038/s41598-018-24278-2.
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- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
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- 6. " Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin." Stowe, C. L., Burley, T. A., Allan, H., Vinci, M., Kramer-Marek, G., Ciobota, D. M., Parkinson, G. N., Southworth, T. L., Agliardi, G., Hotblack, A., Lythgoe, M. F., Branchini, B. R., Kalber, T. L., Anderson, J. C., and Pule, M. A. (2019) *eLife* 8. doi/10.7554/eLife.45801
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- 8. "A new ultrasensitive bioluminescence -based method for assaying monoacylglycerol lipase." M. Miceli, S. Casati, P. Allevi, S. Berra, R. Ottria, P. Rota, B.R. Branchini, and P. Ciuffreda, *Int. J. Mol. Sci.*, 22, 6148 (2021) <https://doi.org/10.3390/ijms22116148>

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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferin that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

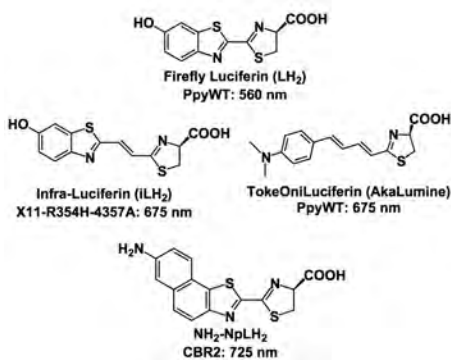


Figure 1. Luciferase-substrate emission maxima.

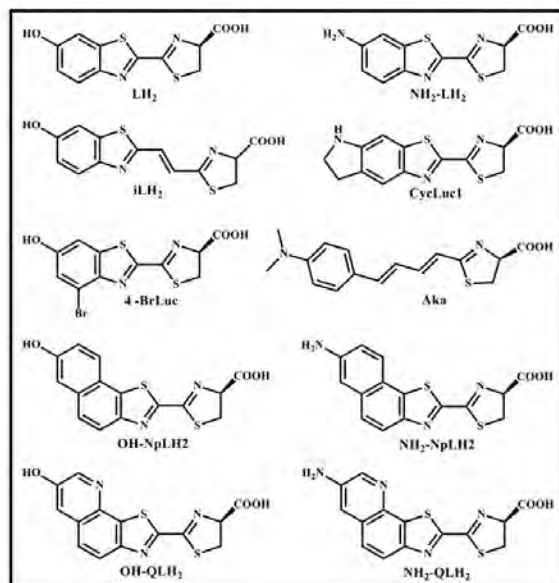
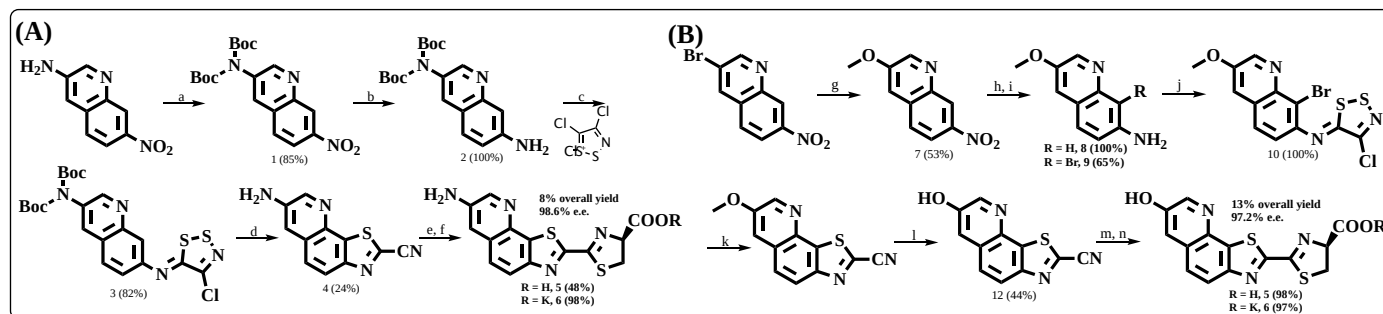


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

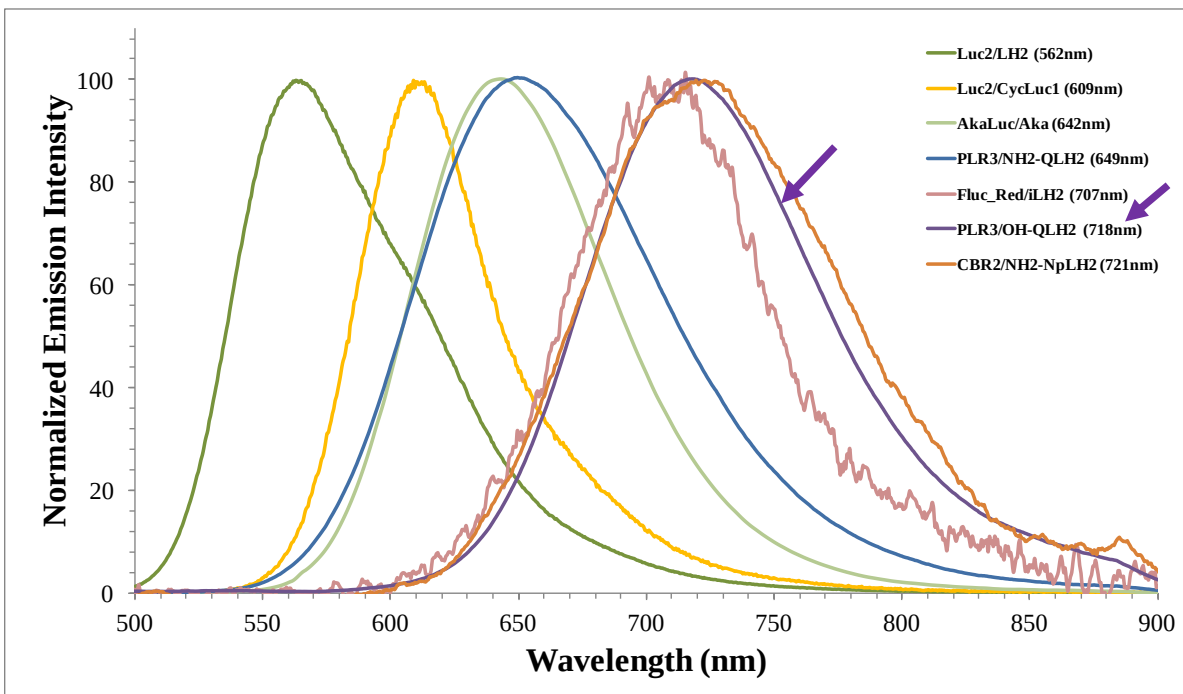


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

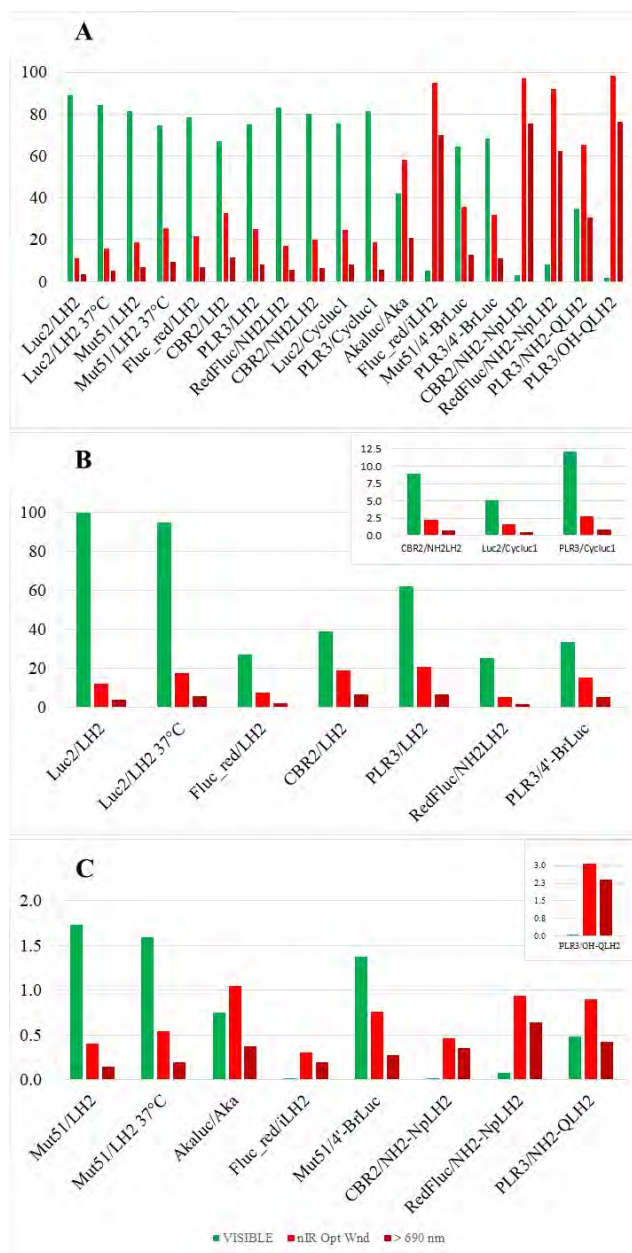


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

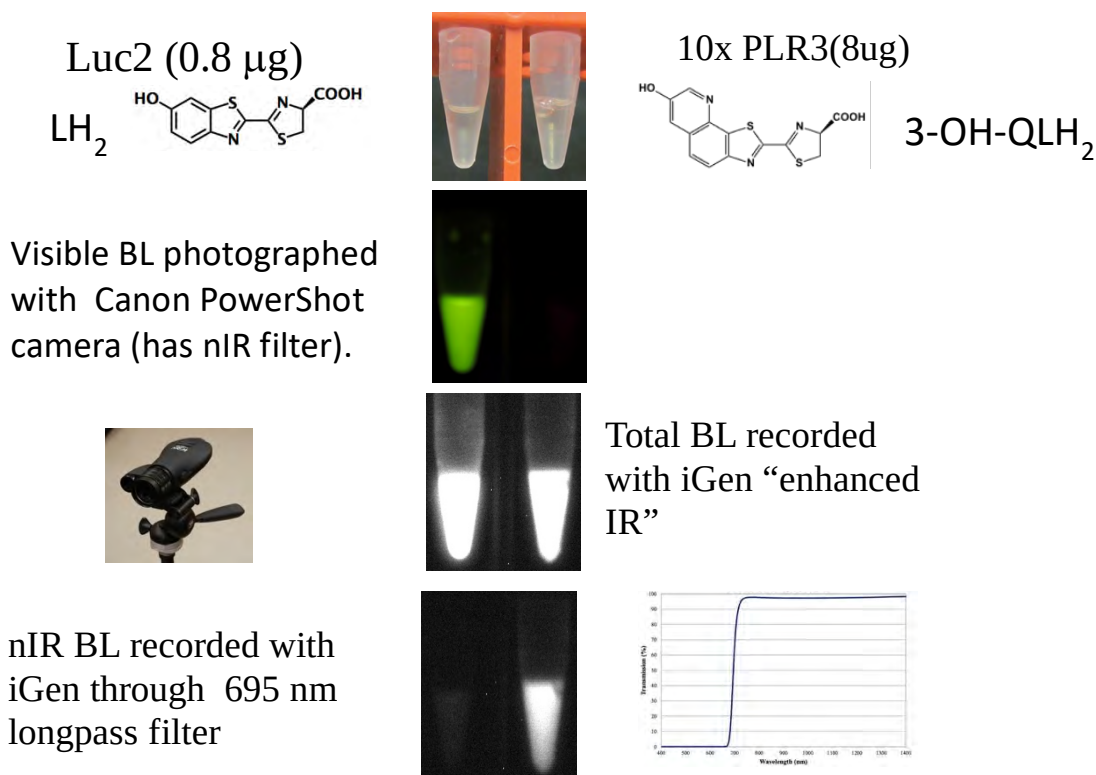
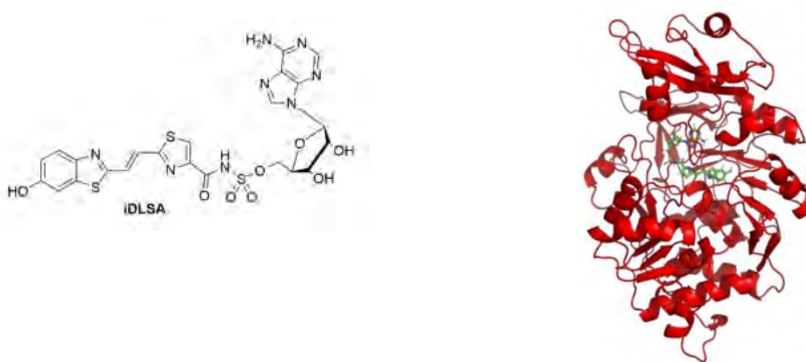


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T – OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

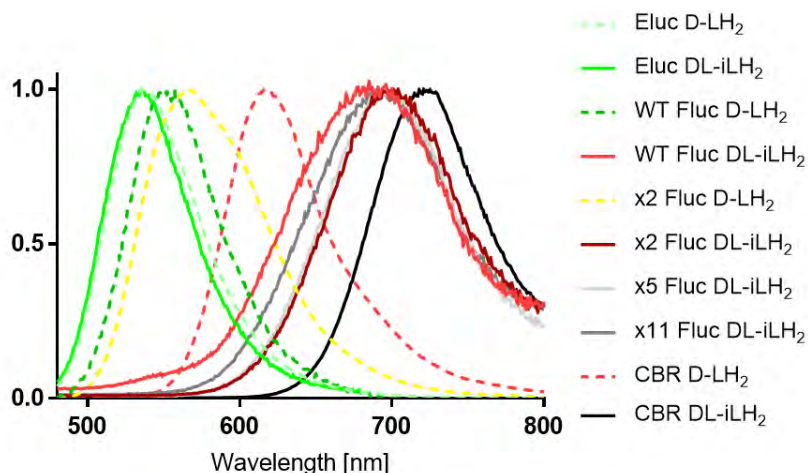


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultoni*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

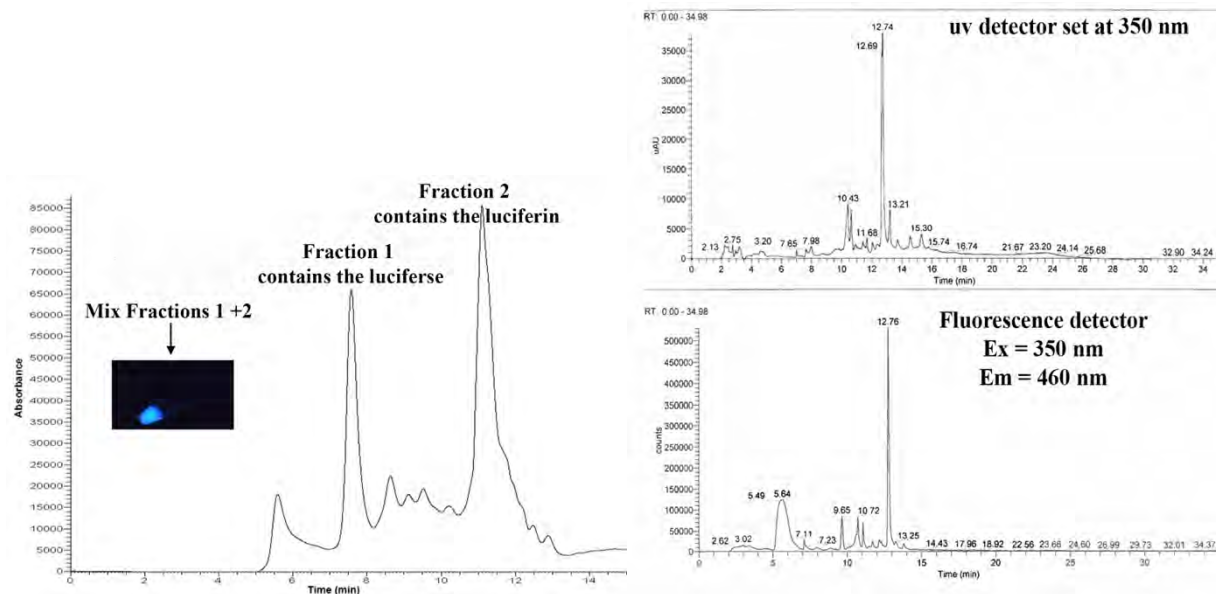
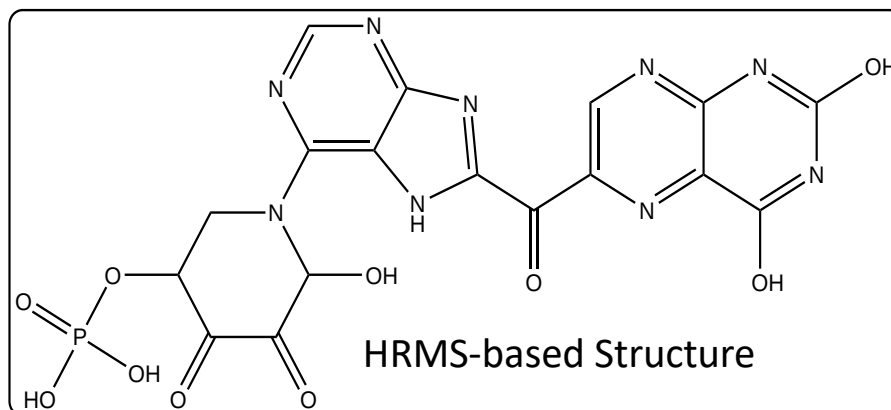


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



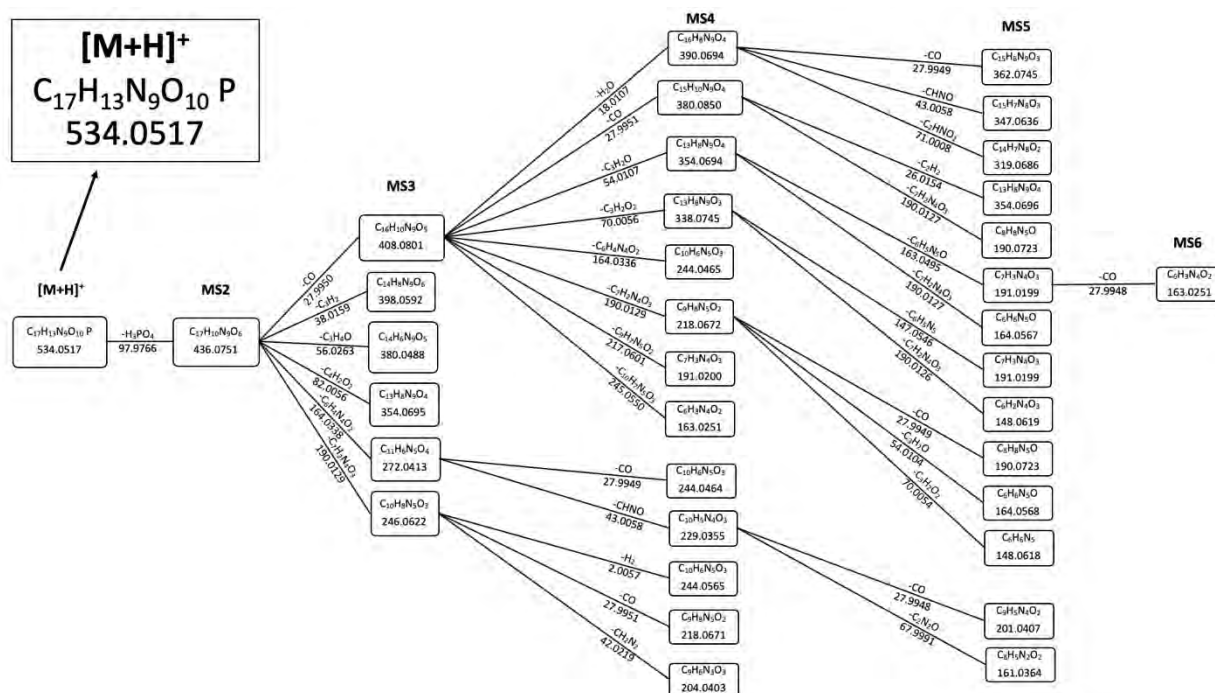


Figure 8. HRMS analysis of putative *Orfelia oxyluciferin*.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the Orfelia oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

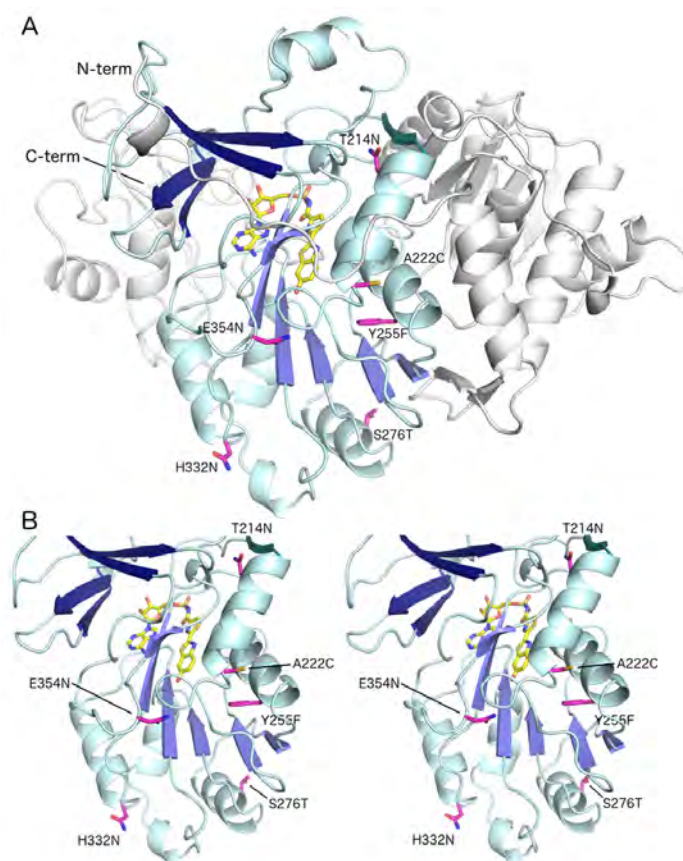


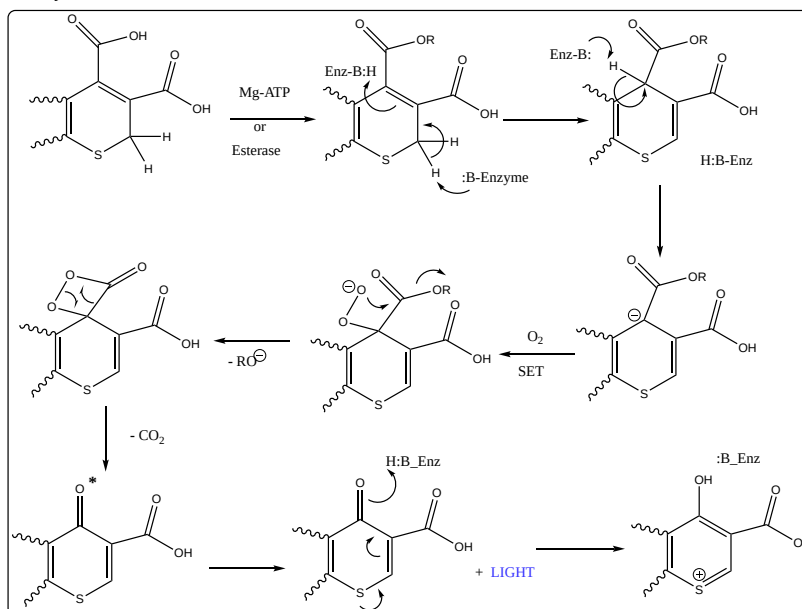
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaetae that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdonata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

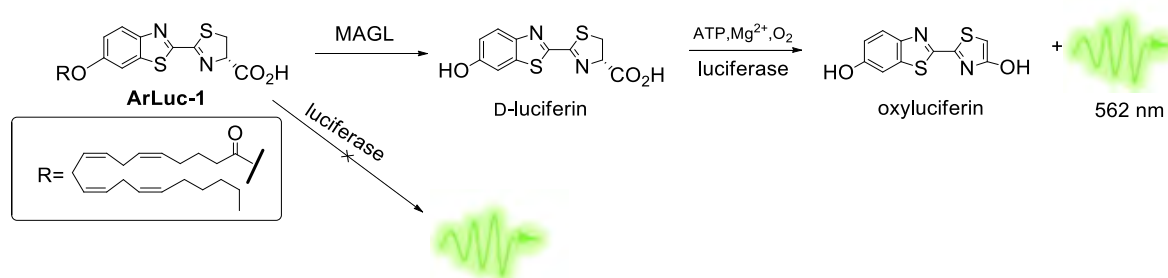


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 x g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18-20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at -80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of K_m for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). K_m values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

Publications

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- 2. "A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events." Branchini BR, Southworth TL, Fontaine DM, Kohrt D, Florentine CM, Grossel MJ. *Sci Rep.* 2018; 8(1):5990. doi: 10.1038/s41598-018-24278-2.
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COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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Principal Investigator: Bruce Branchini

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ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

I. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

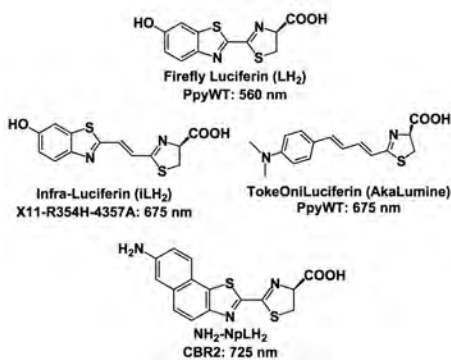


Figure 1. Luciferase-substrate emission maxima.

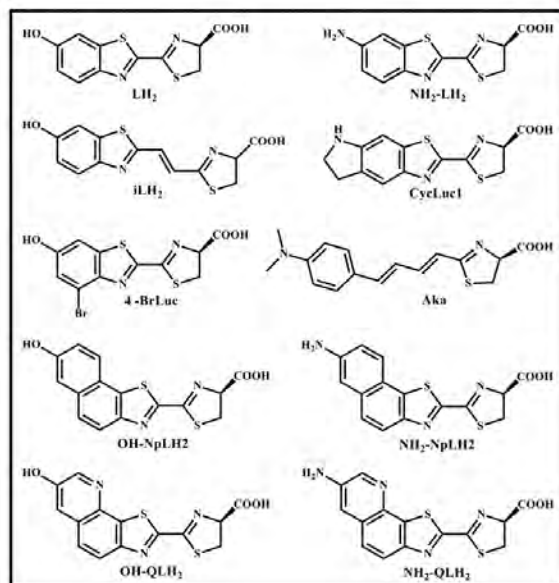
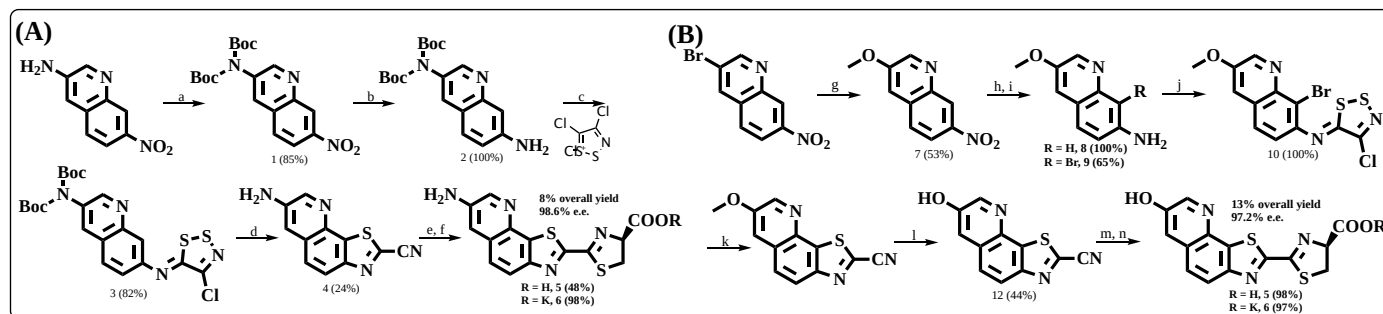


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

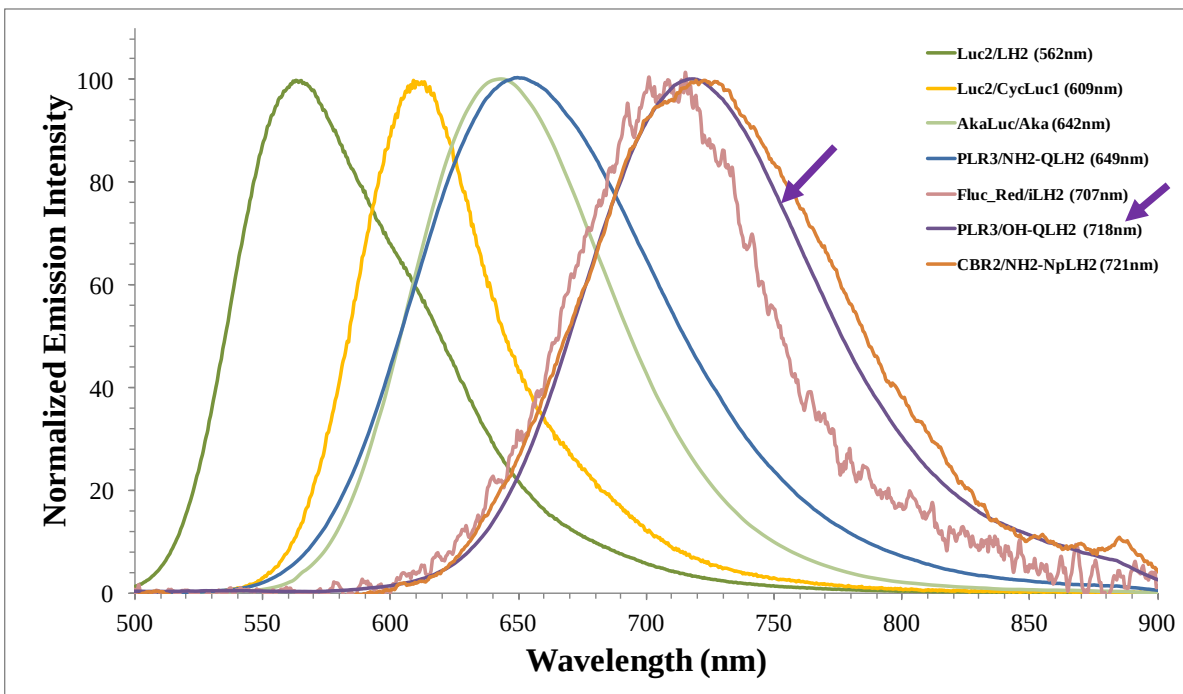


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

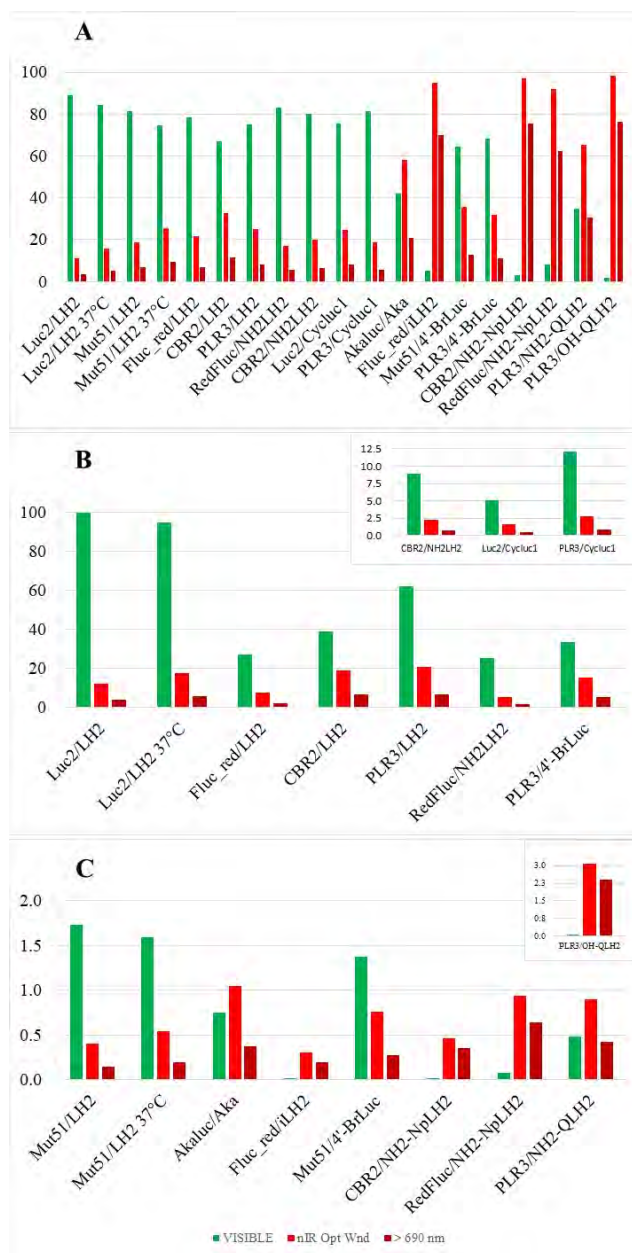


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

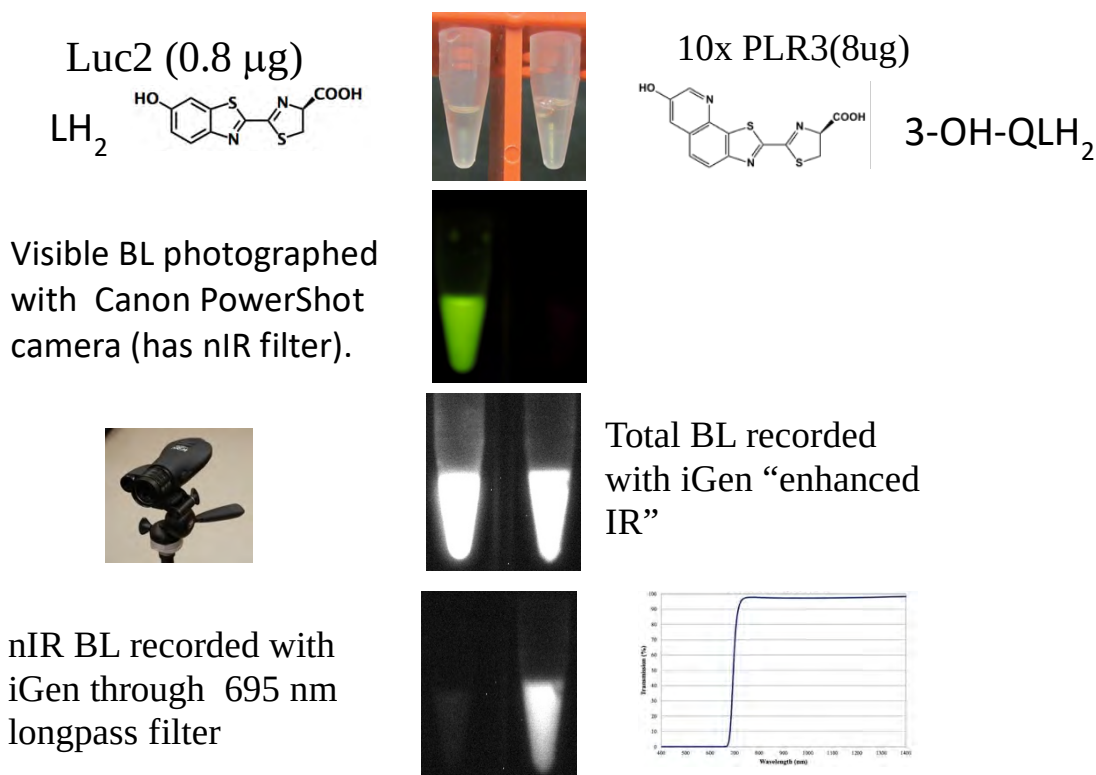
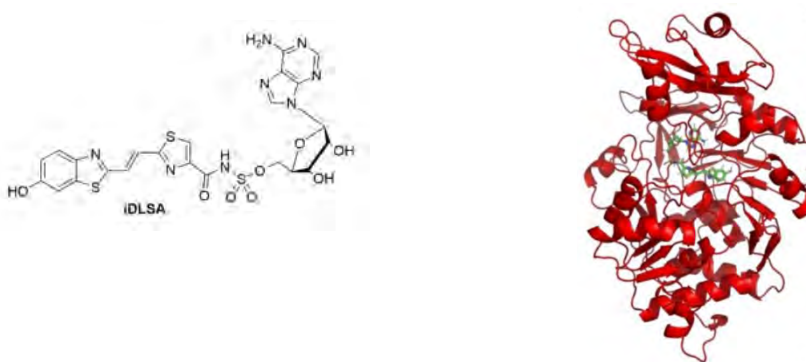


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T – OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

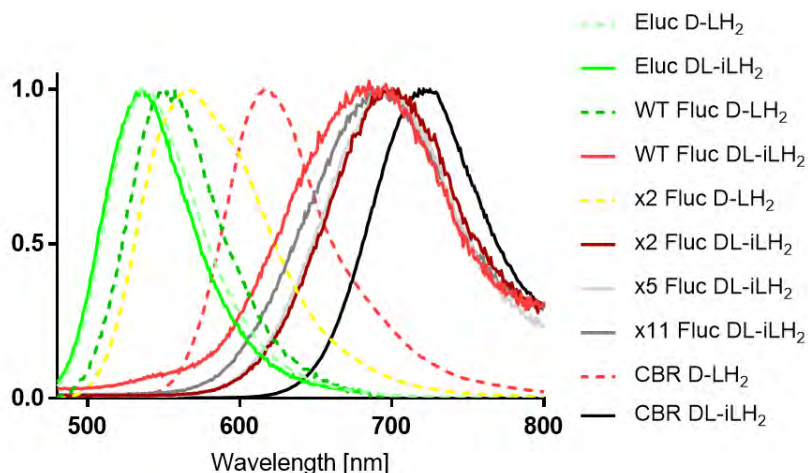


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultoni*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

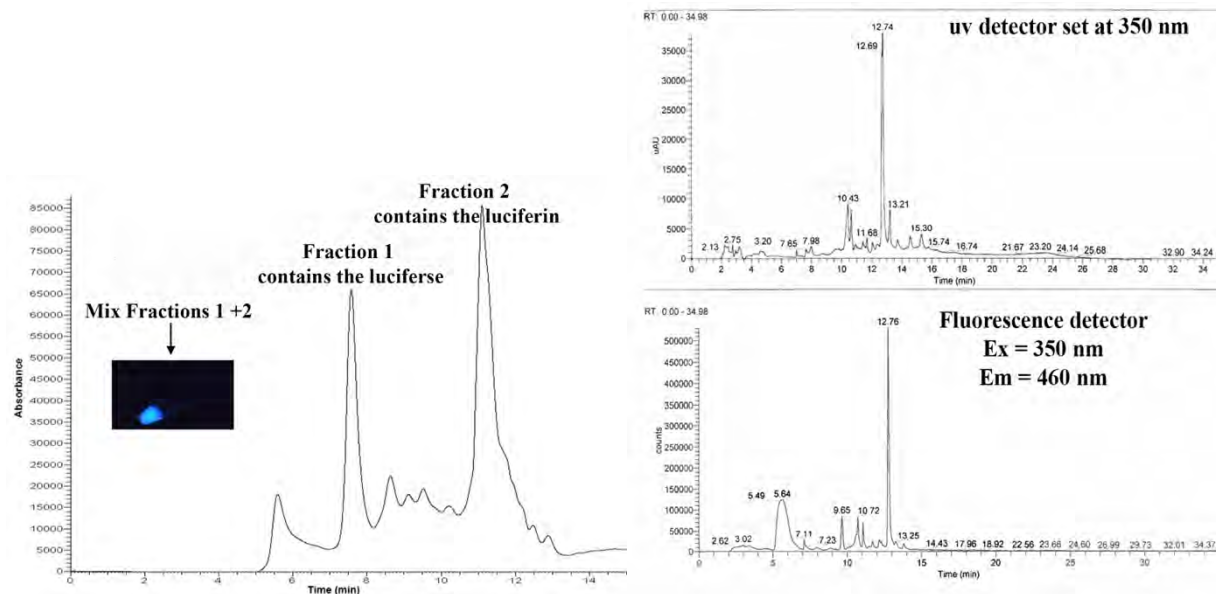
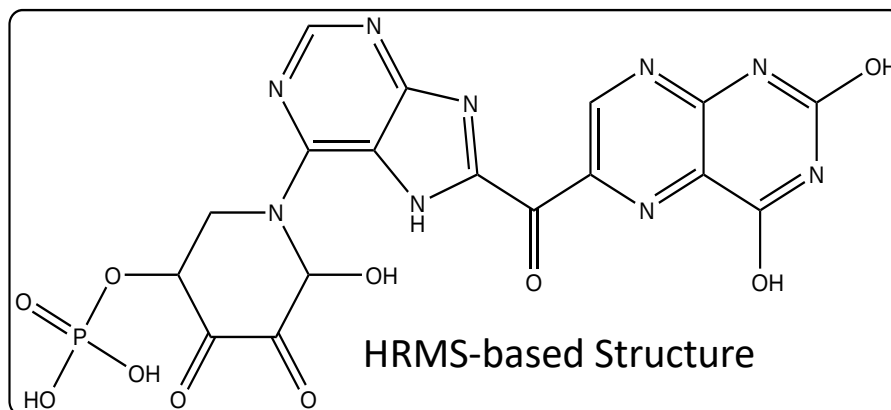


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of Orfelia bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



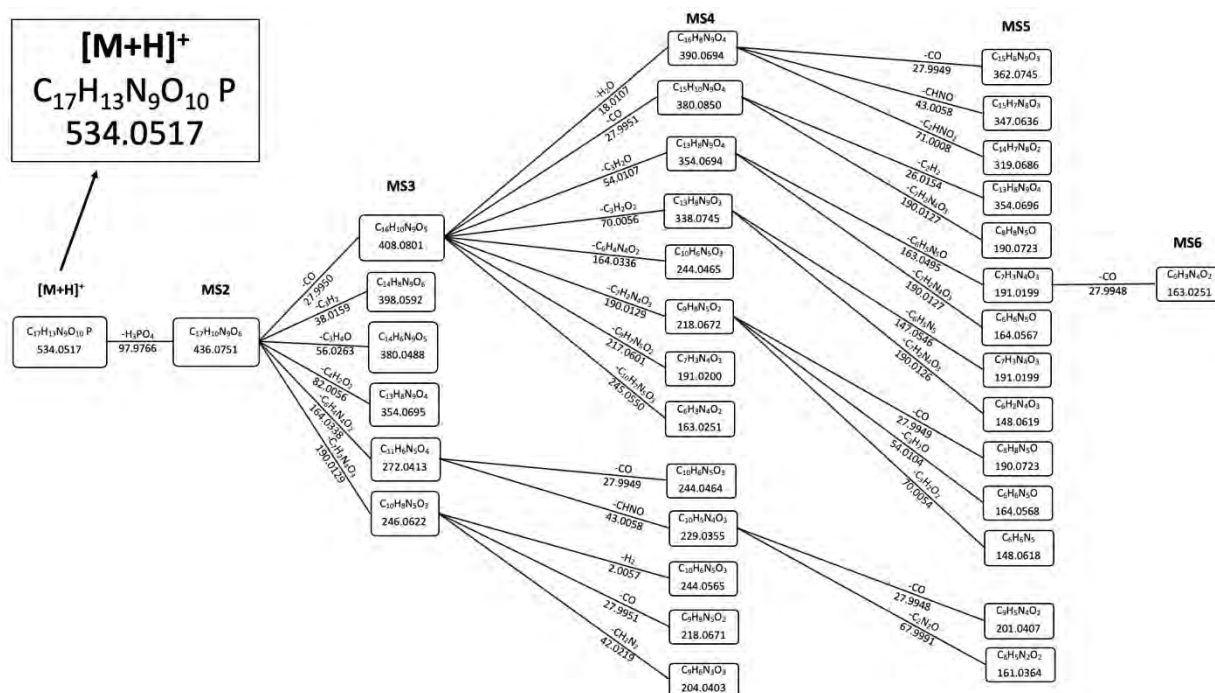


Figure 8. HRMS analysis of putative Orfelia oxyluciferin.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the Orfelia oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

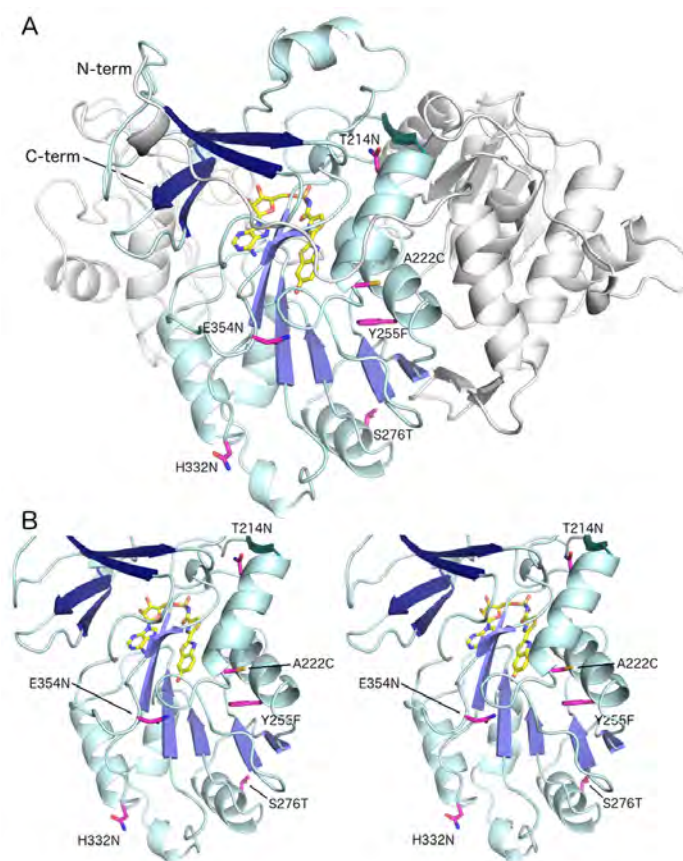


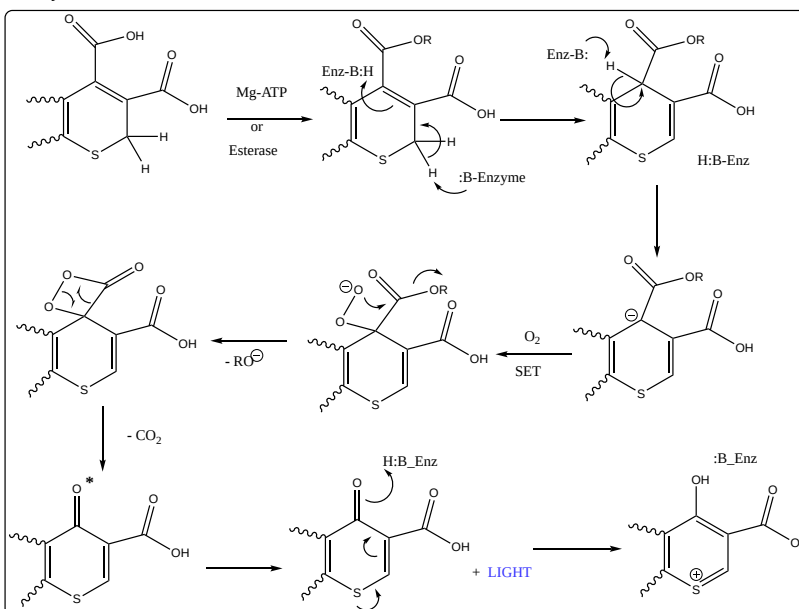
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Universita degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

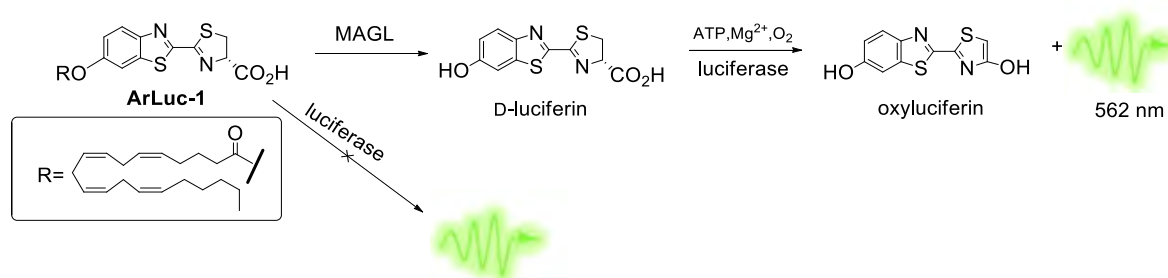


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 1. "Red-emitting chimeric firefly luciferase for in vivo imaging in low ATP cellular environments." Bruce R. Branchini, Tara L. Southworth, Danielle M. Fontaine, Dawn Kohrt, Franceine S. Welcome, Catherine M. Florentine, Emma R. Henricks, Demetria B. DeBartolo, Elisa Michelini, Luca Cevenini, Aldo Roda, Martha J. Grossel, *Analytical Biochemistry*, 2017;534:36–39. doi: 10.1016/j.ab.2017.07.001.**

- 2. "A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events." Branchini BR, Southworth TL, Fontaine DM, Kohrt D, Florentine CM, Grossel MJ. *Sci Rep*. 2018; 8(1):5990. doi: 10.1038/s41598-018-24278-2.
- 3. " Mutagenesis and structural studies reveal the basis for the activity and stability properties that distinguish the photinus luciferases scintillans and pyralis." Branchini, B. R., Fontaine, D. M., Southworth, T. L., Huta, B. P., Racela, A., Patel, K. D., and Gulick, A. M. (2019), *Biochemistry* 58, 4293-4303. doi/10.1021/acs.biochem.9b00719
- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
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- 9. “Dendritic cells maintain anti-tumor immunity by positioning CDB skin-resident memory T cells.” J. L. Vella, A. Molodtskov, C. V. Angeles, B. R. Branchini, M. J. Turk, and Y. H. Huang, *Life Science Alliance* 4 (10), e202101056 (2021).
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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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*** Corresponding author inadvertently left out AFOSR acknowledgement.

COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

Award Number: FA9550-18-1-0017

Report Type: Final Report

Reporting Period: Start 15 October 2017 - End 14 October 2022

Distribution Statement: Approved for Public release

Program Officer: Bennett L. Ibey

Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

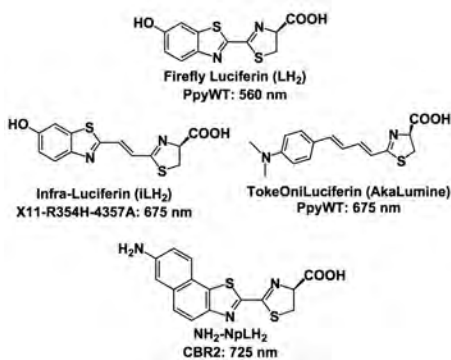


Figure 1. Luciferase-substrate emission maxima.

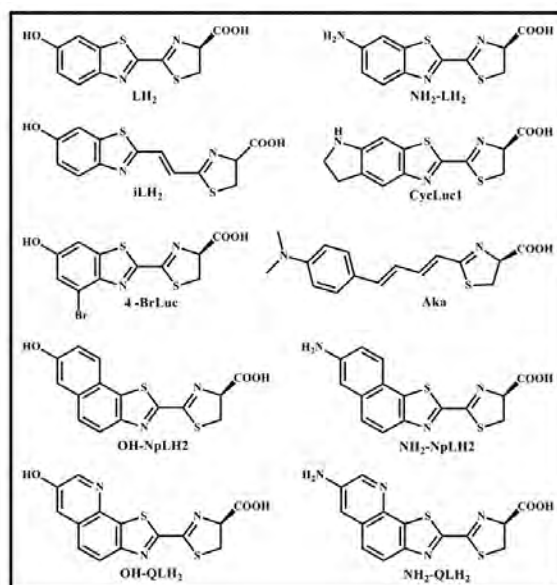
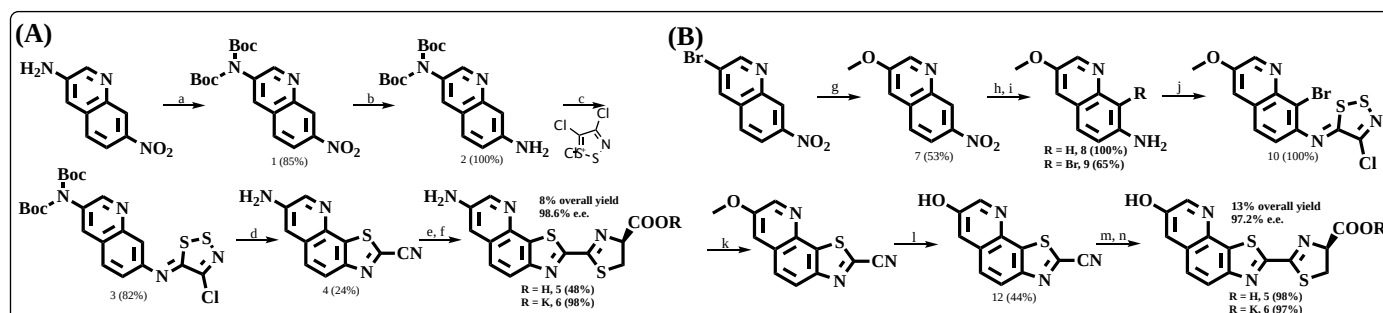


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

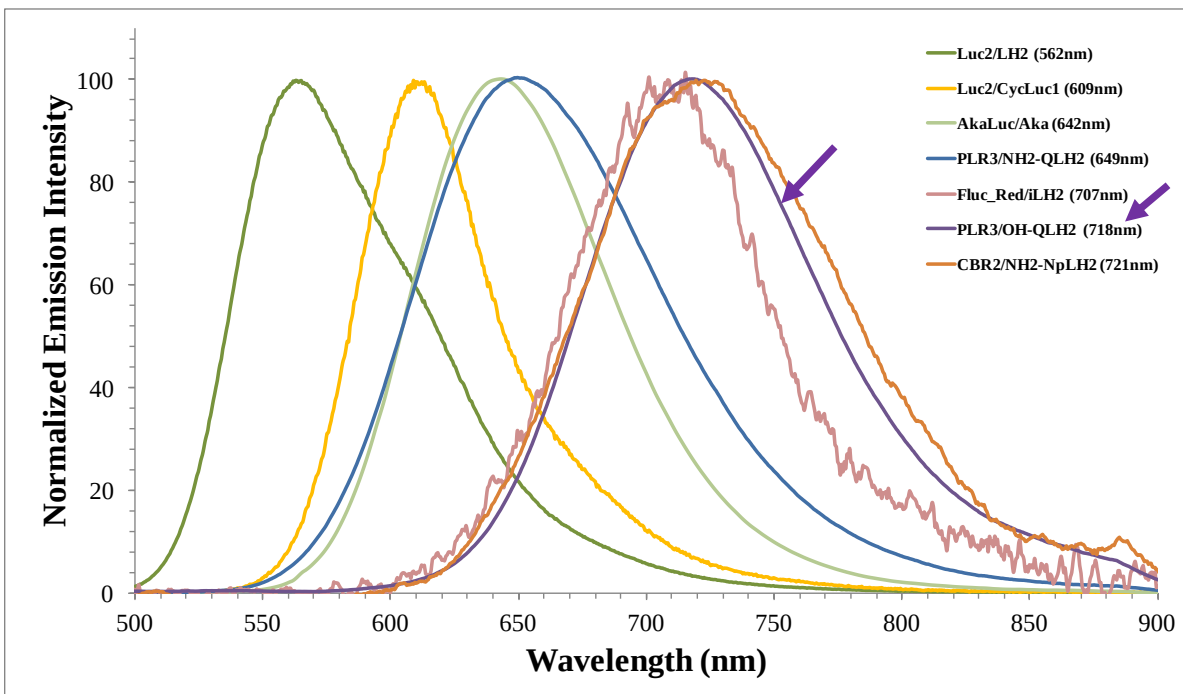


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

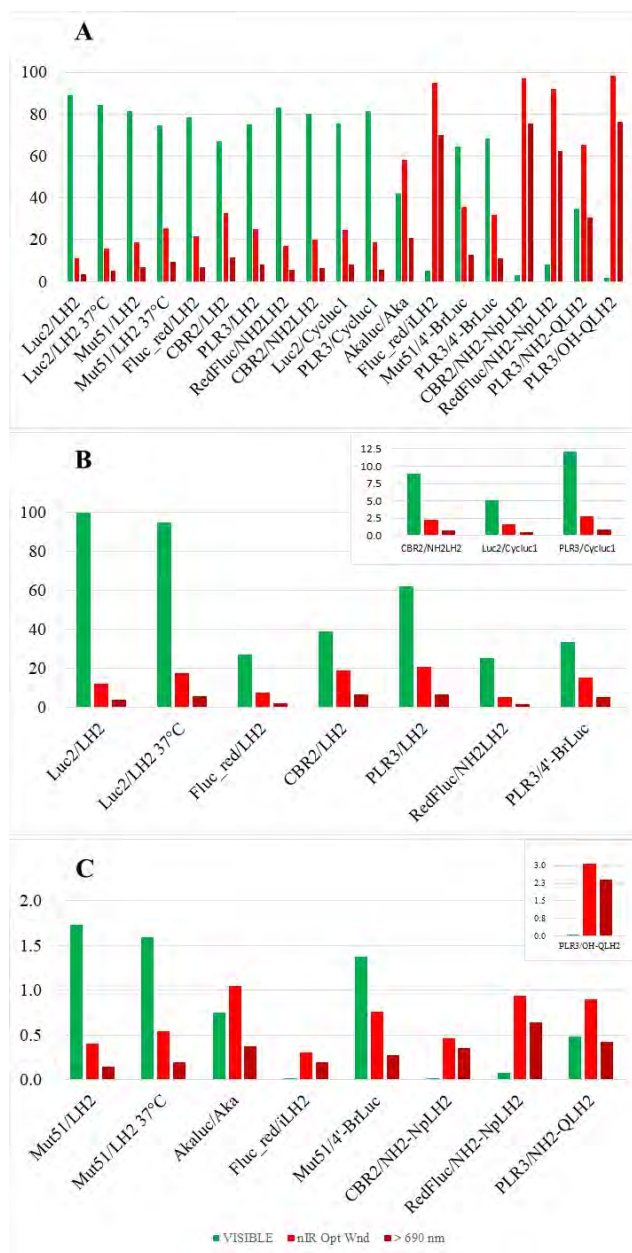


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

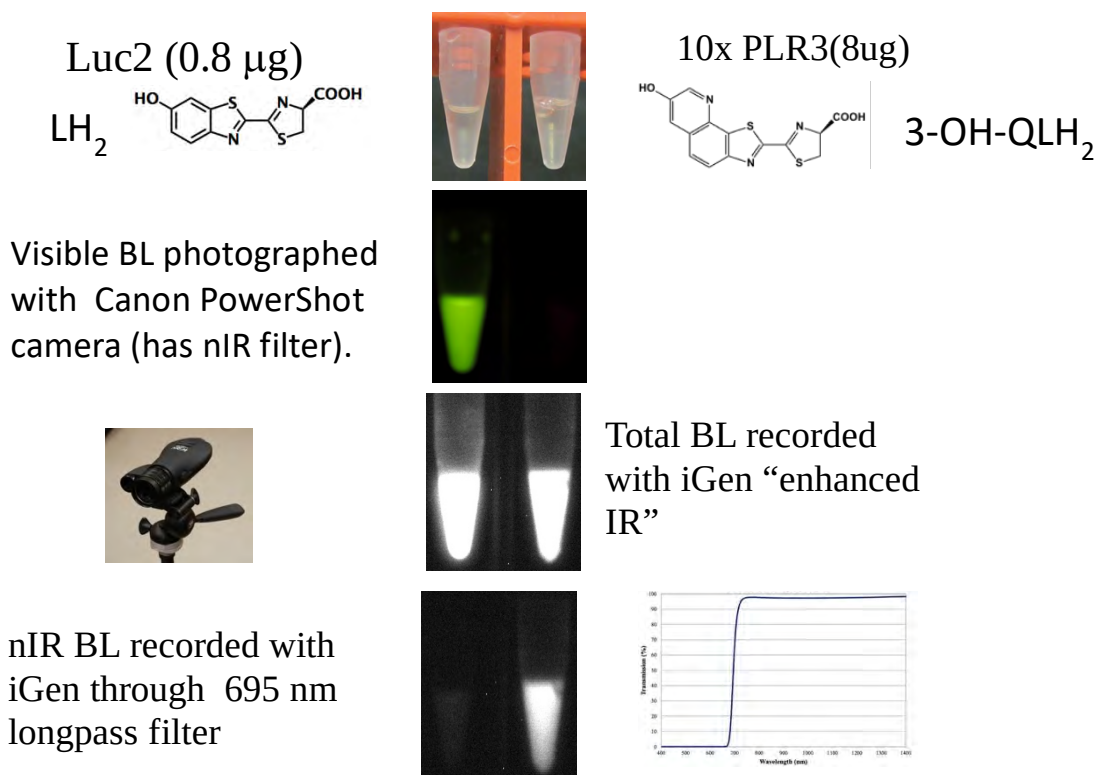
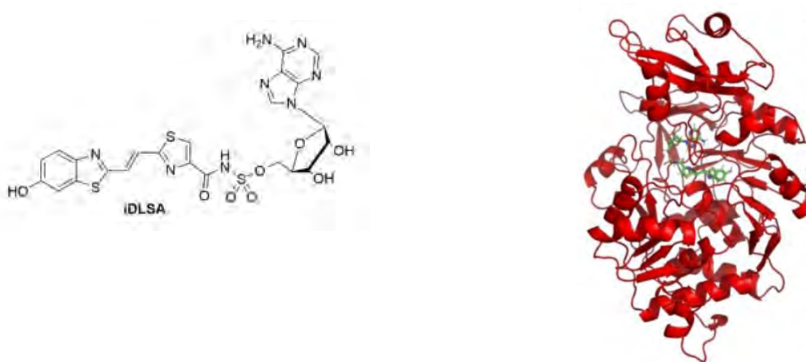


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

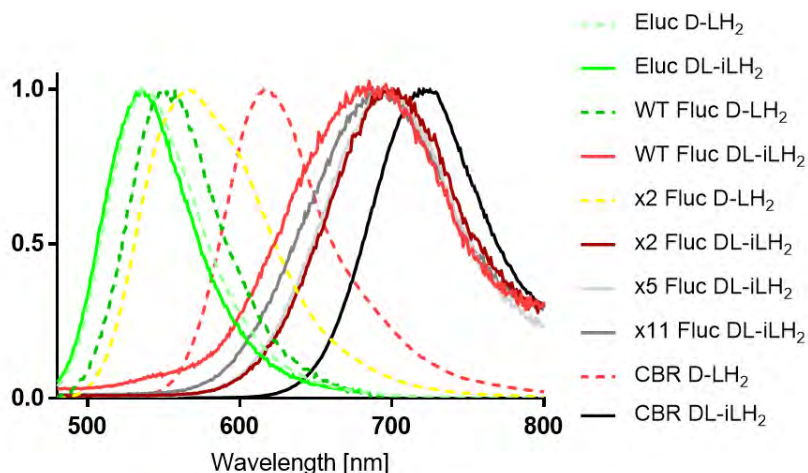


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultoni*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

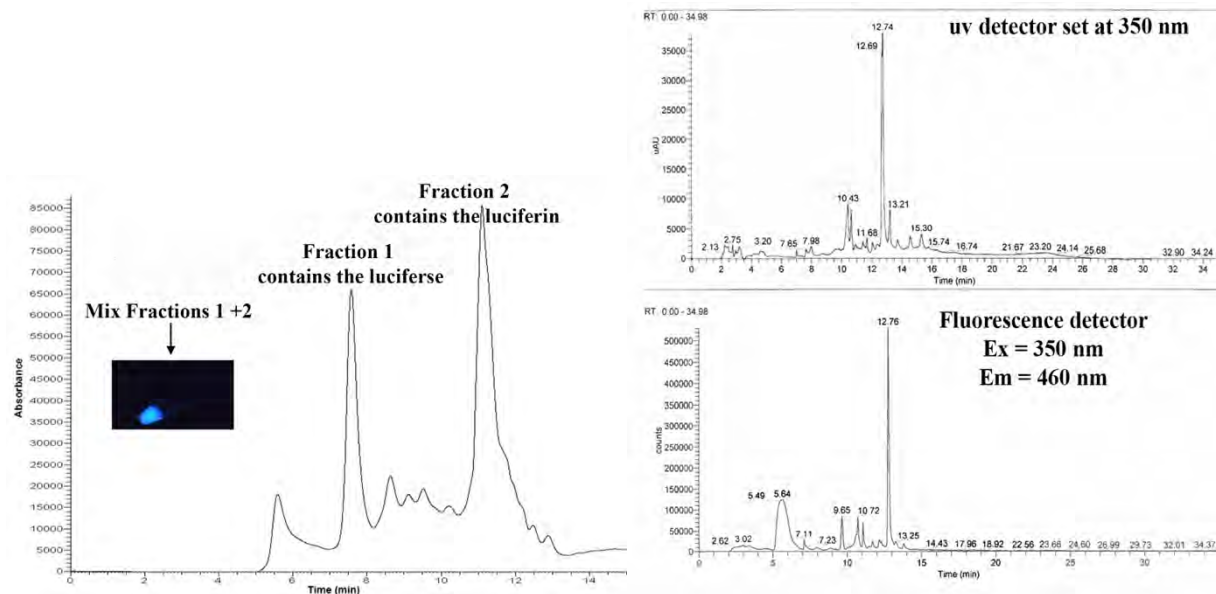
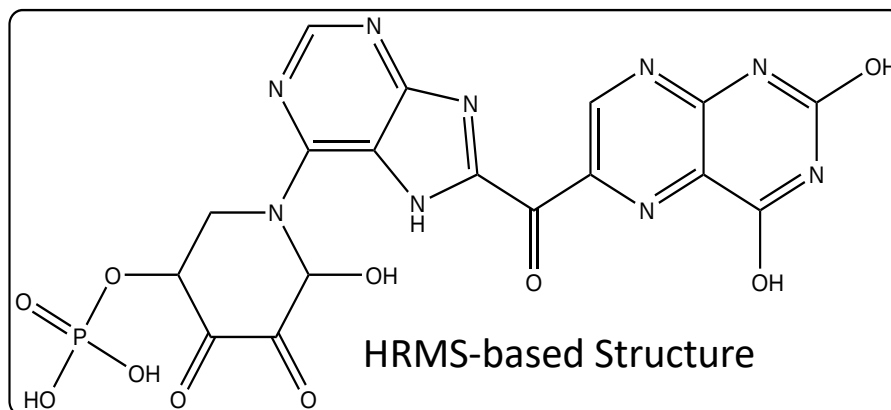
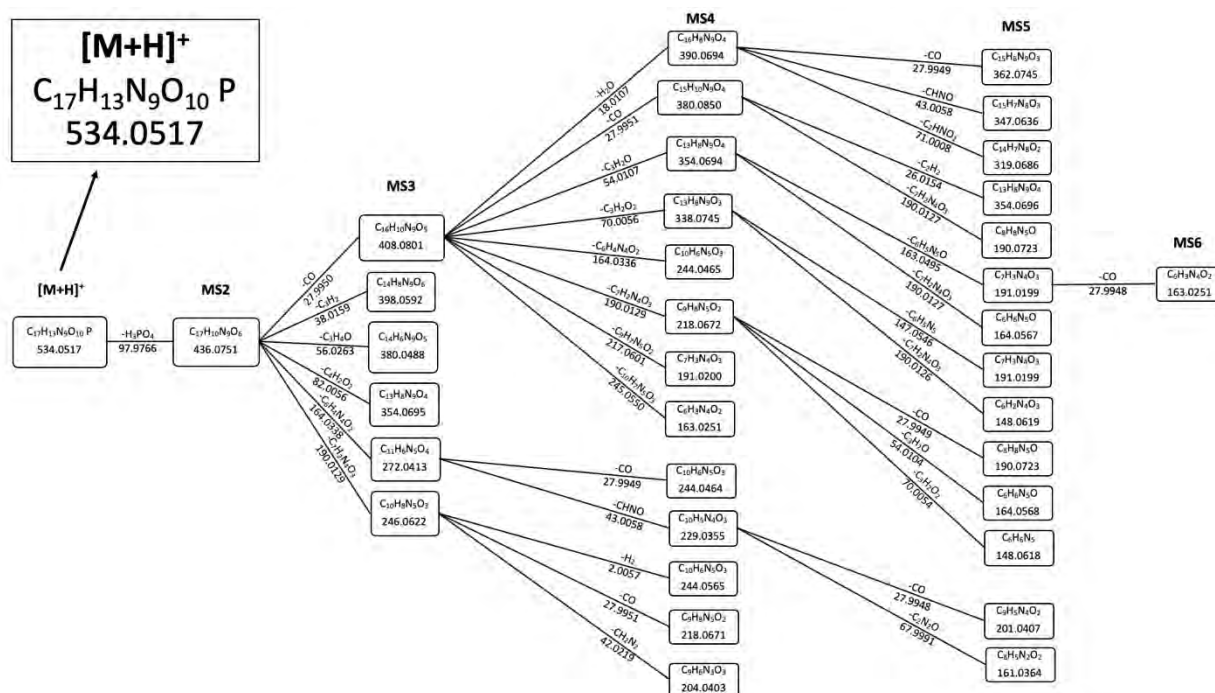


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.





sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

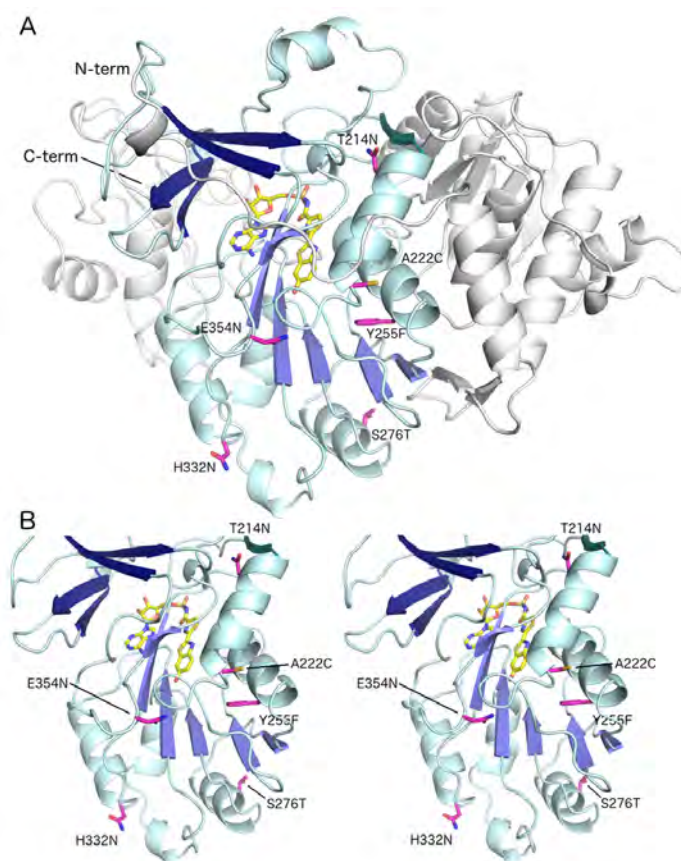


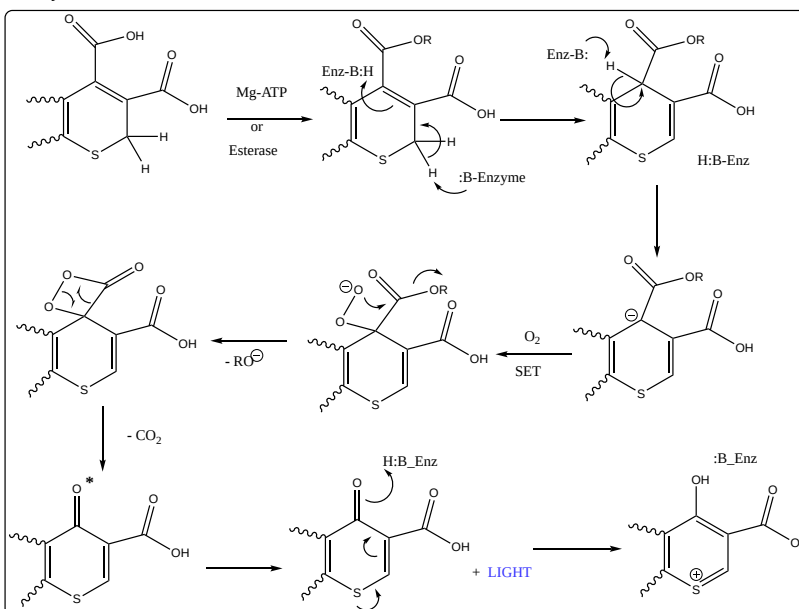
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Universita degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

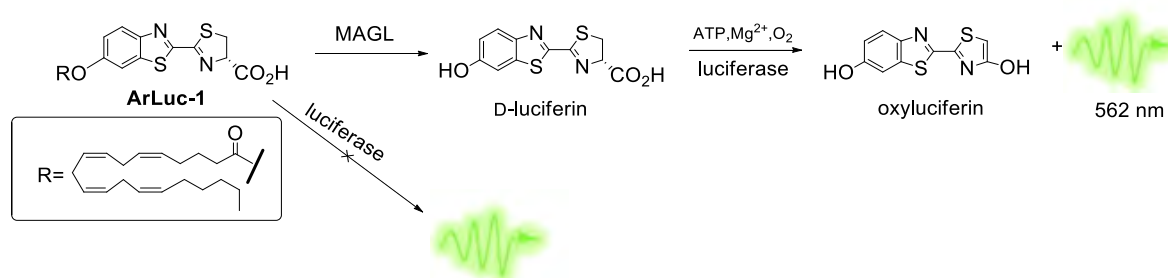


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18-20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at -80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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*** Corresponding author inadvertently left out AFOSR acknowledgement.

COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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Distribution Statement: Approved for Public release

Program Officer: Bennett L. Ibey

Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

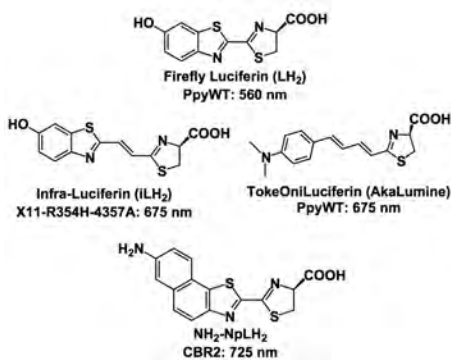


Figure 1. Luciferase-substrate emission maxima.

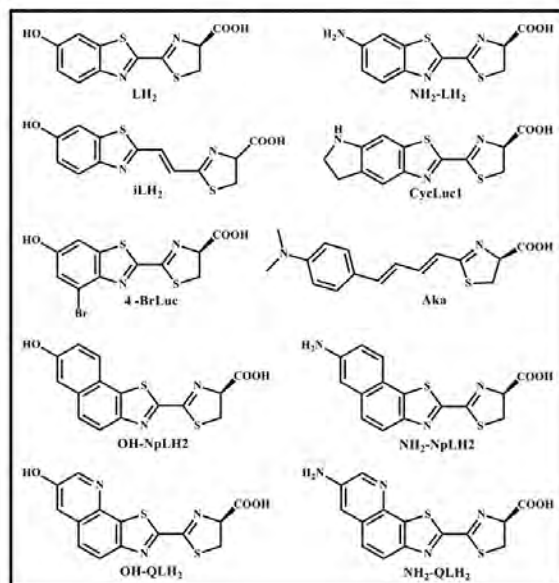
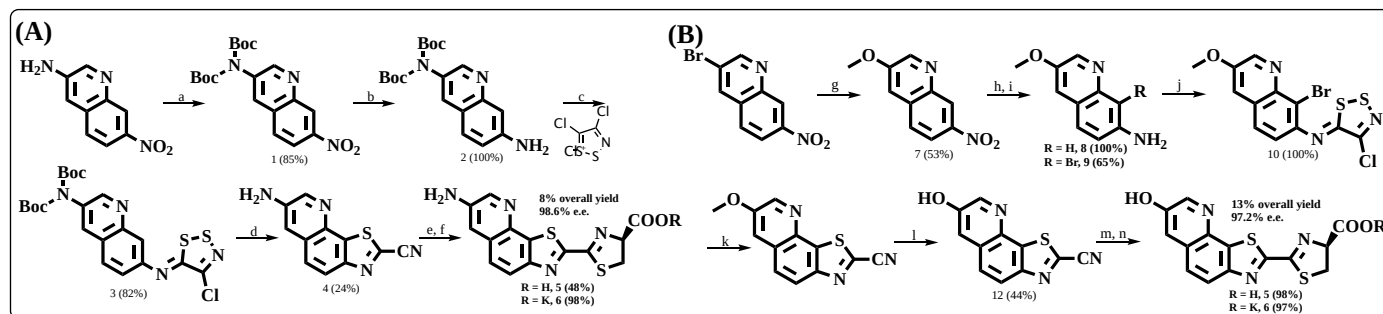


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

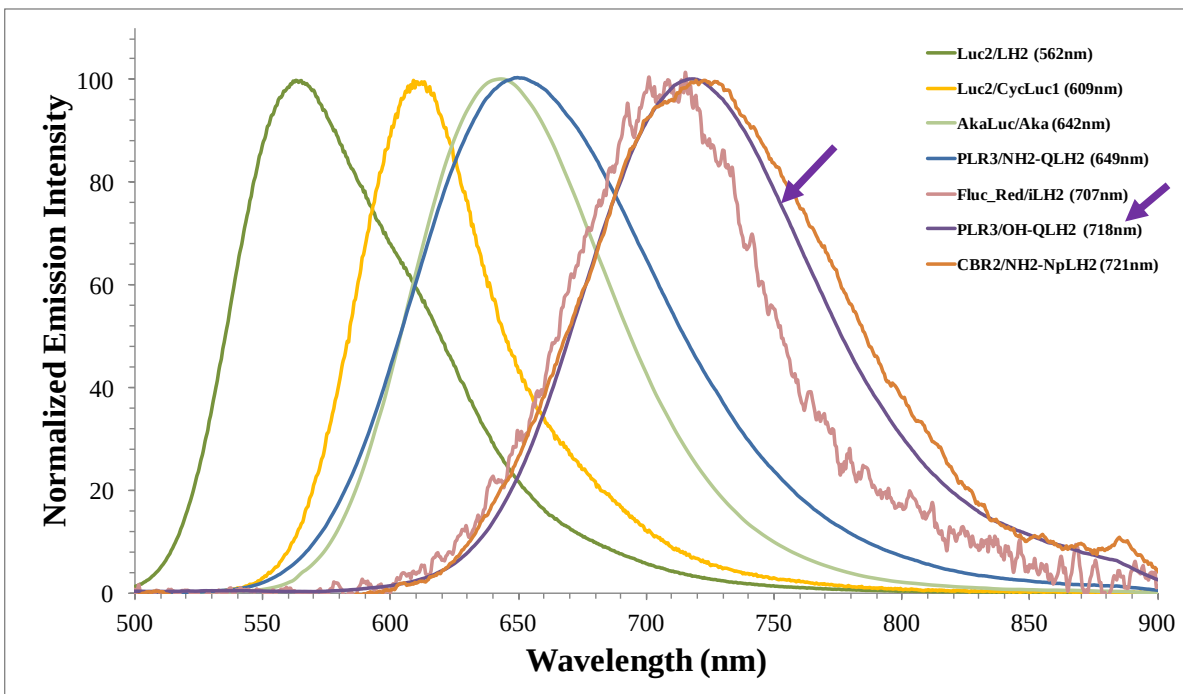


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

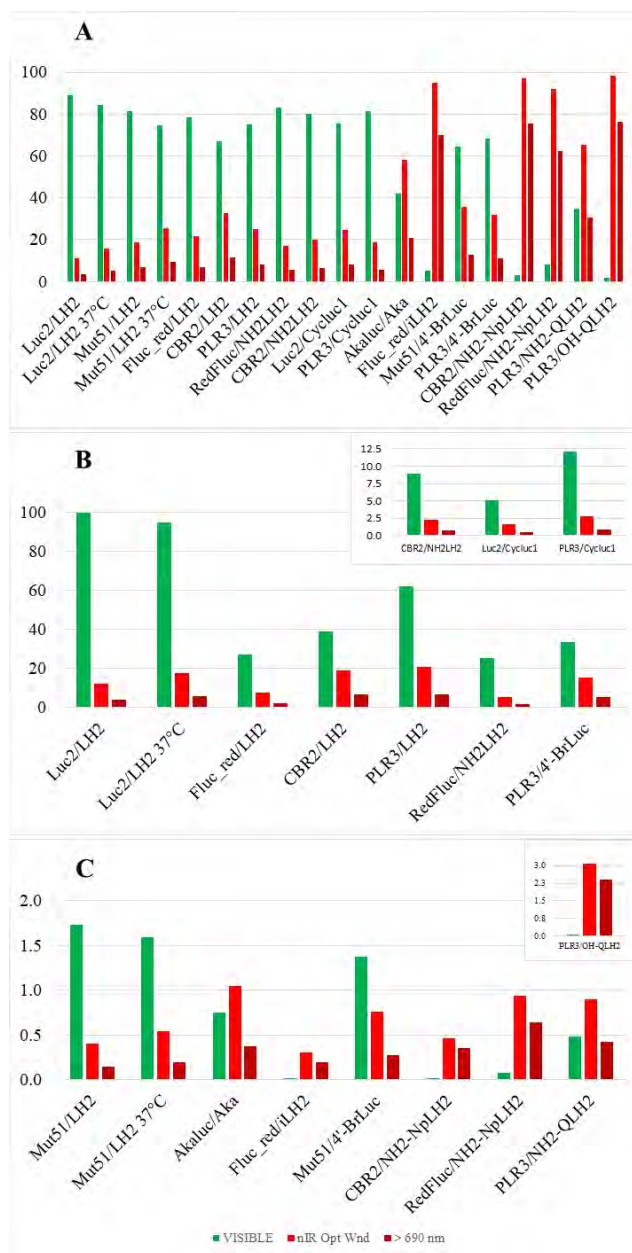


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

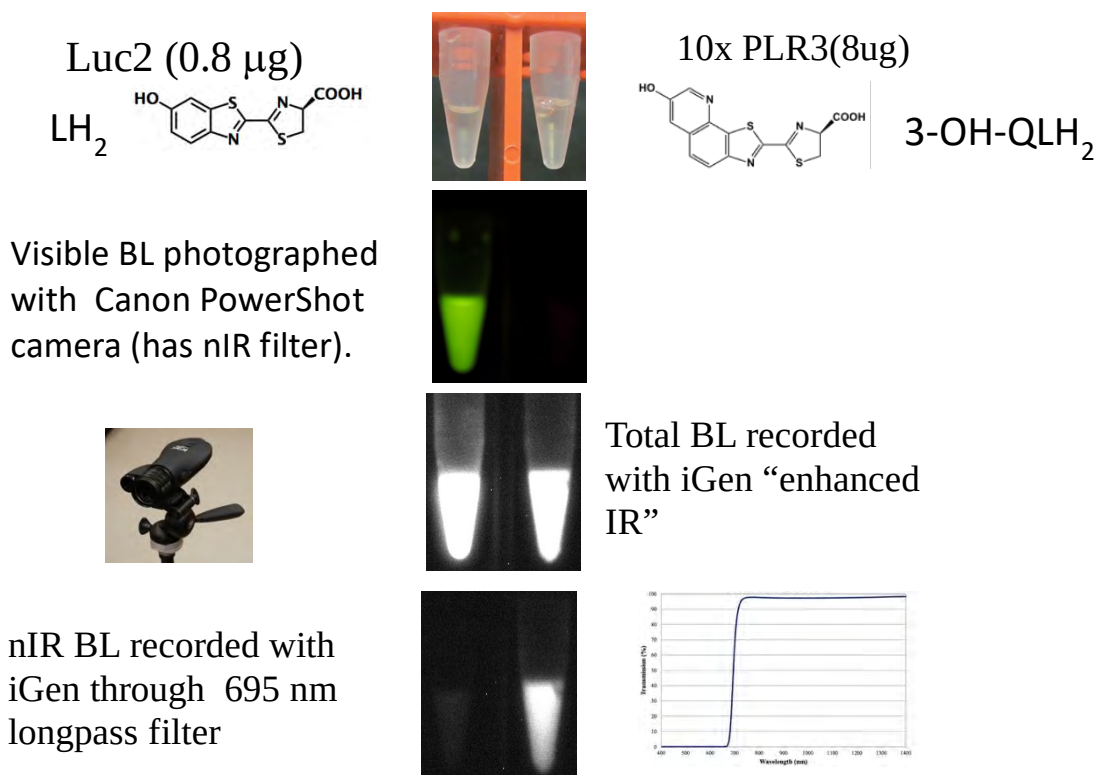
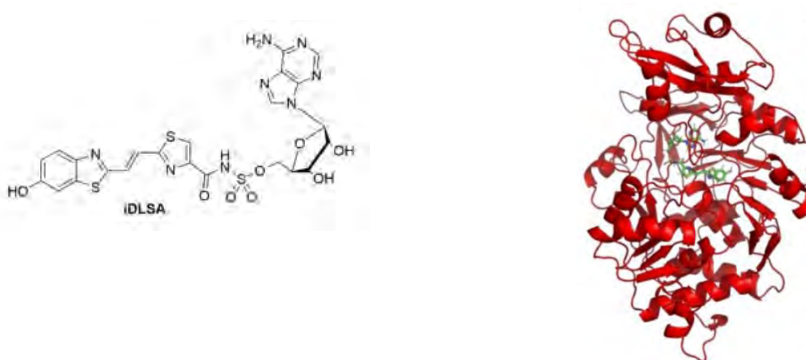


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

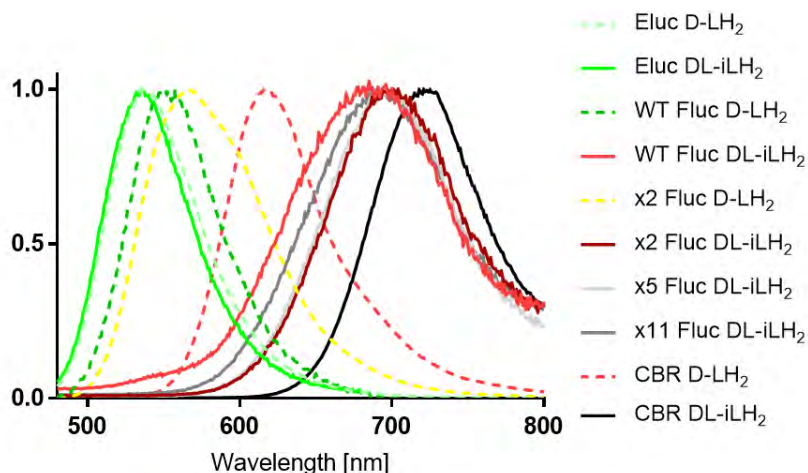


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

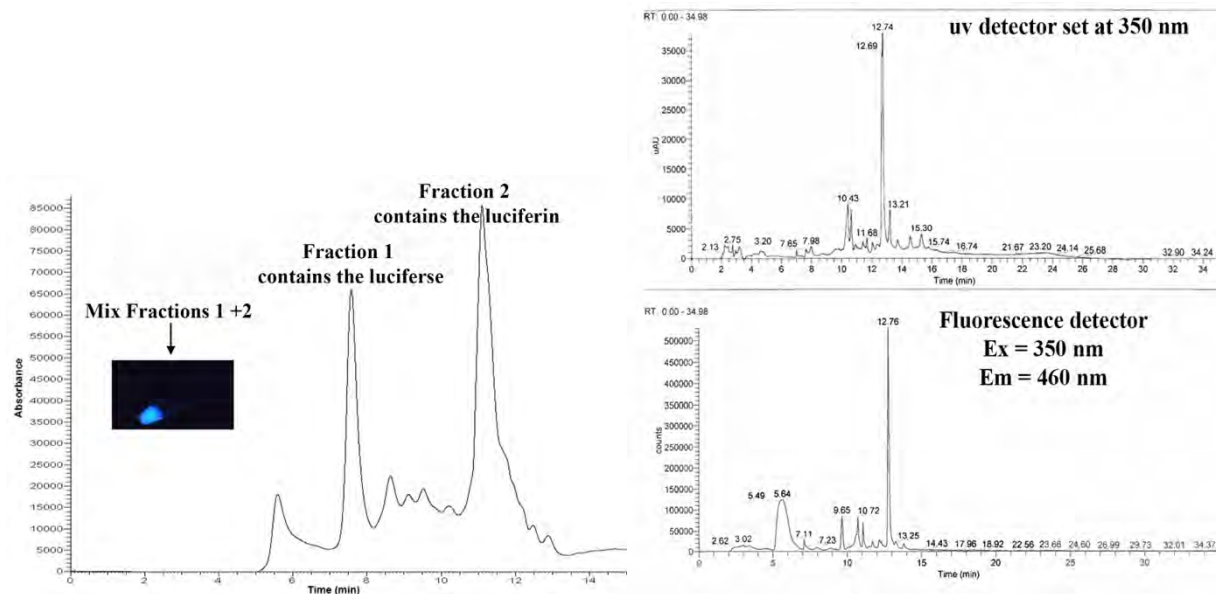
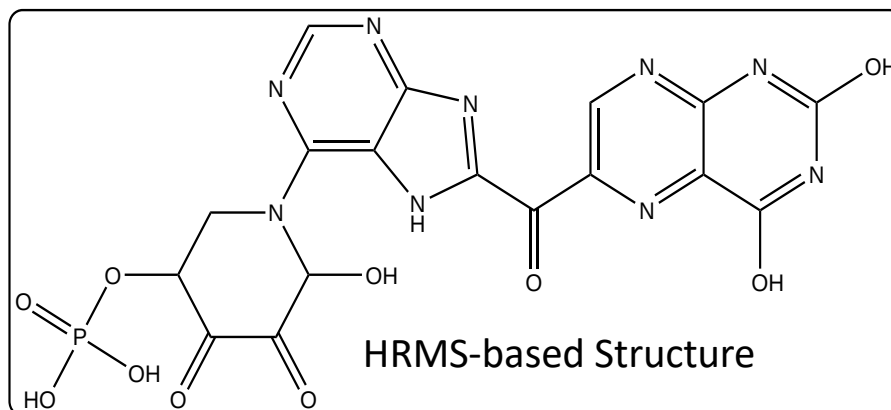


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



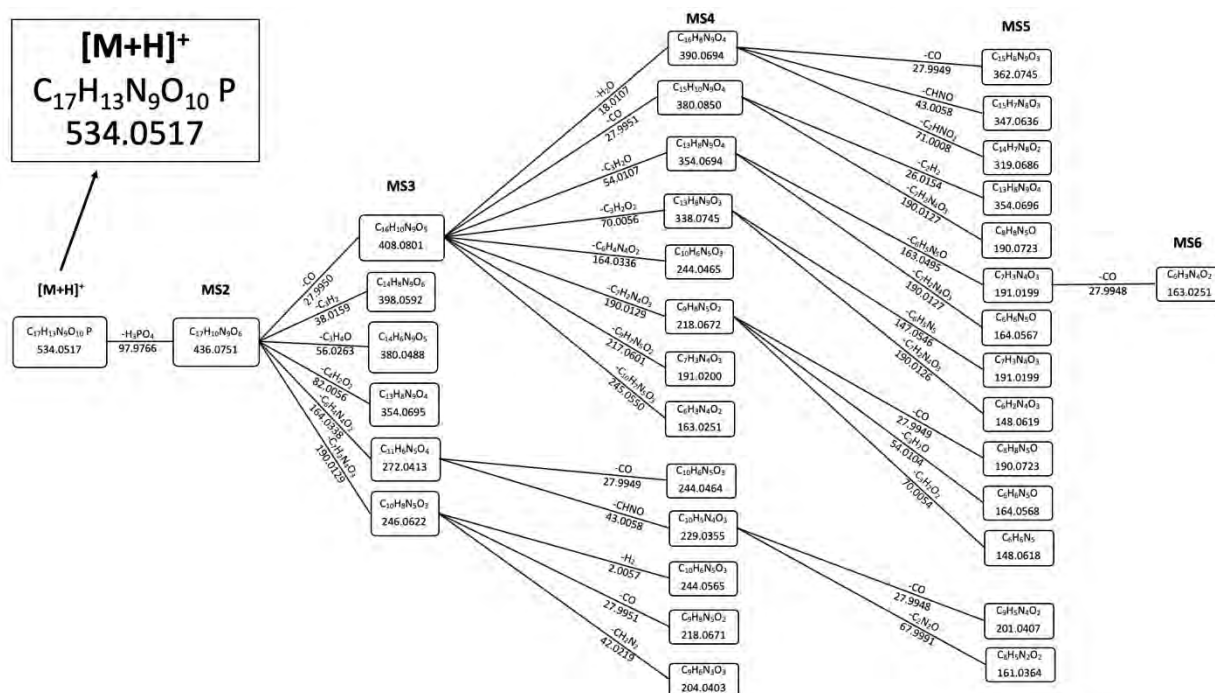


Figure 8. HRMS analysis of putative Orfelia oxyluciferin.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the Orfelia oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

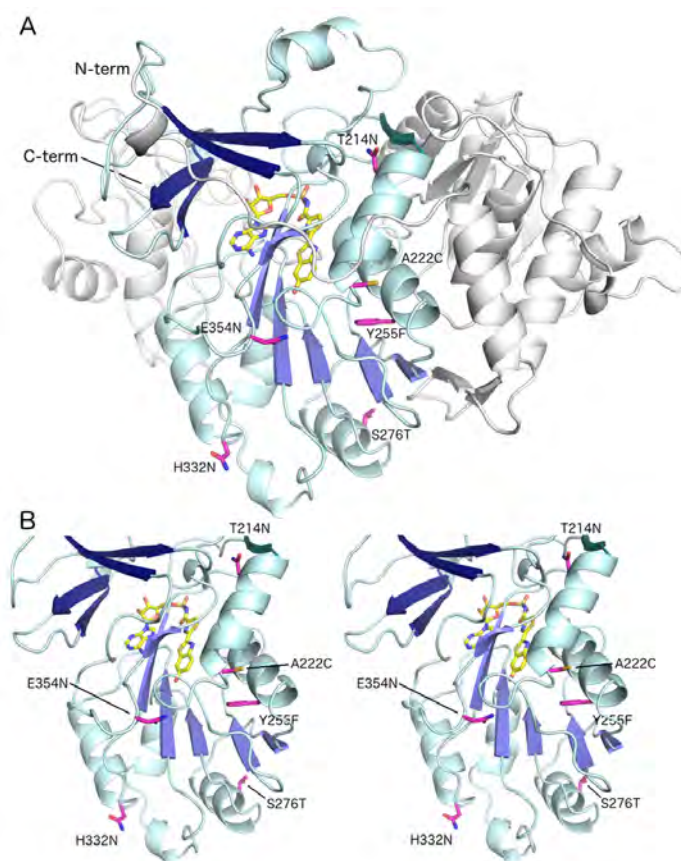


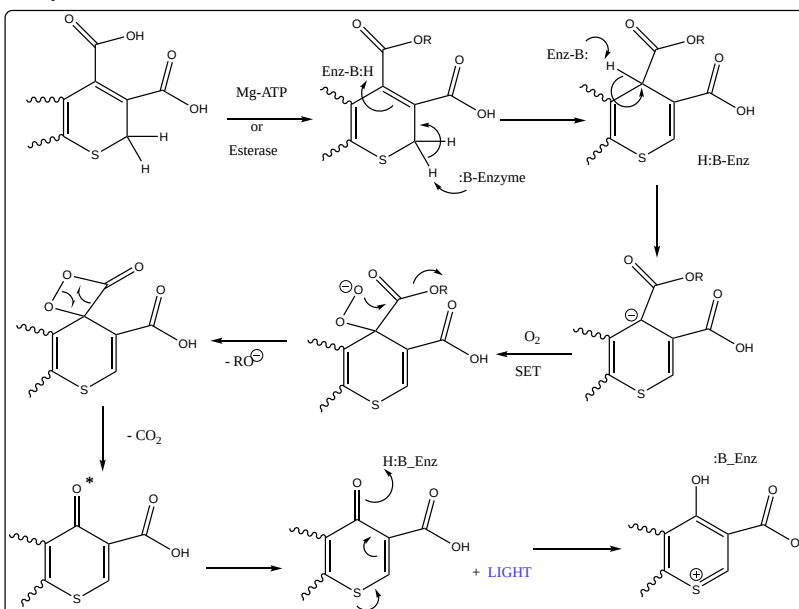
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Universita degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

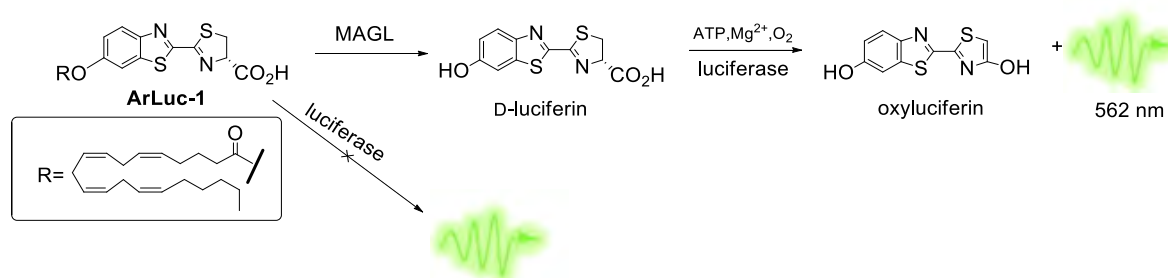


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
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- 8. "A new ultrasensitive bioluminescence -based method for assaying monoacylglycerol lipase." M. Miceli, S. Casati, P. Allevi, S. Berra, R. Ottria, P. Rota, B.R. Branchini, and P. Ciuffreda, *Int. J. Mol. Sci.*, 22, 6148 (2021) <https://doi.org/10.3390/ijms22116148>

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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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*** Corresponding author inadvertently left out AFOSR acknowledgement.

COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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Program Officer: Bennett L. Ibey

Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

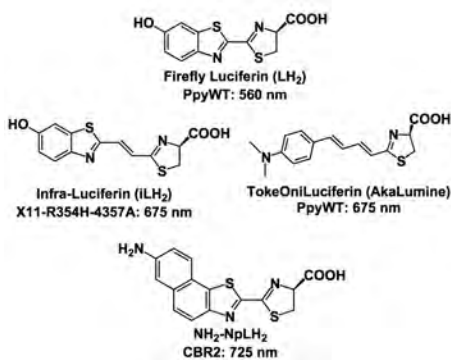


Figure 1. Luciferase-substrate emission maxima.

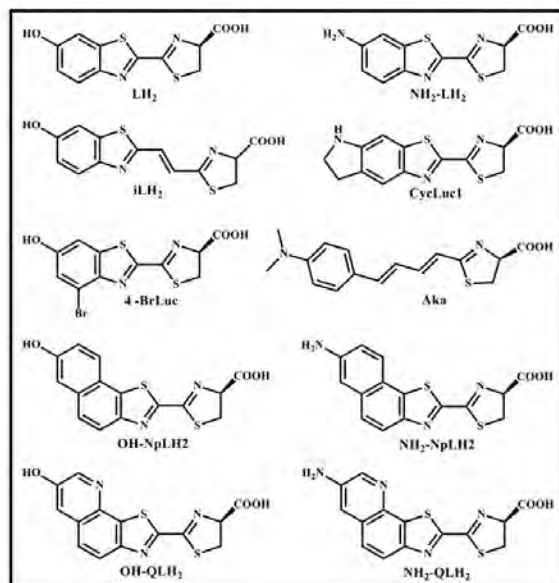
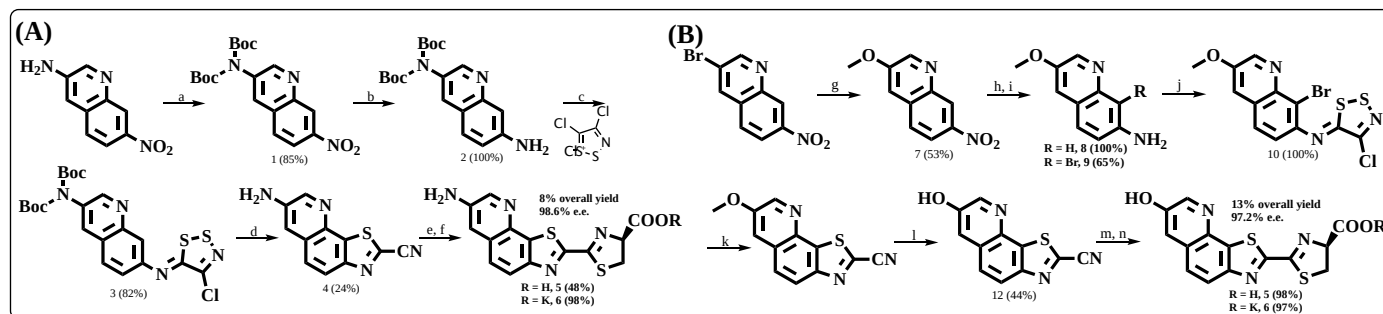


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

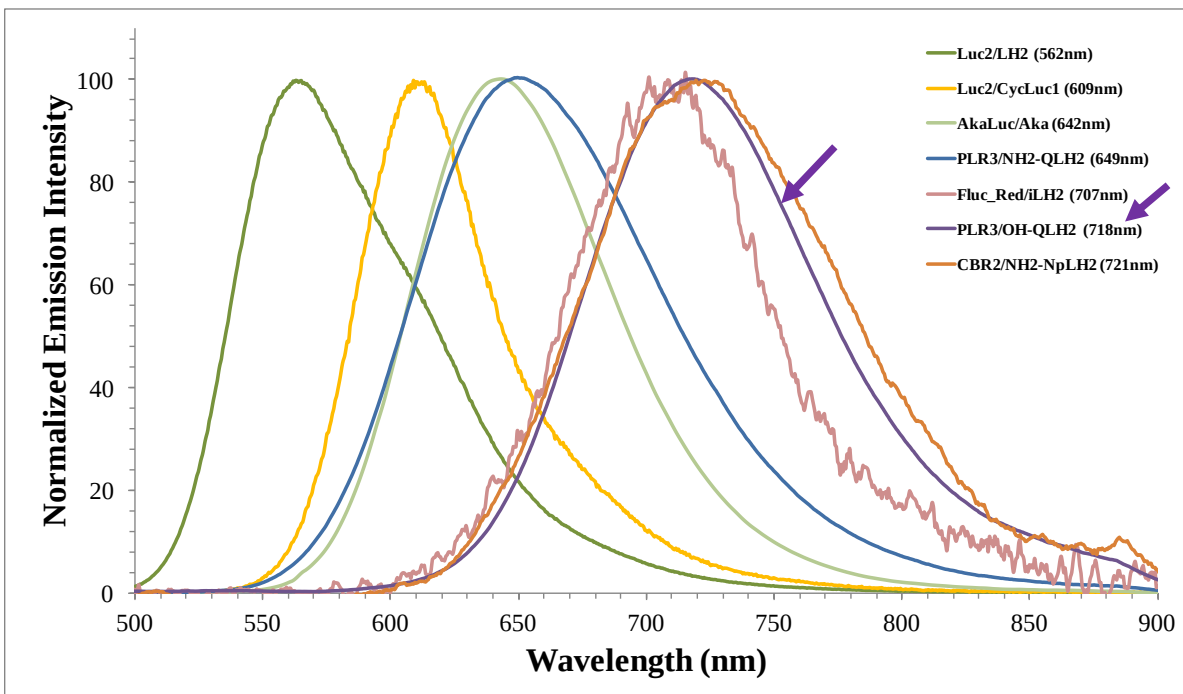


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

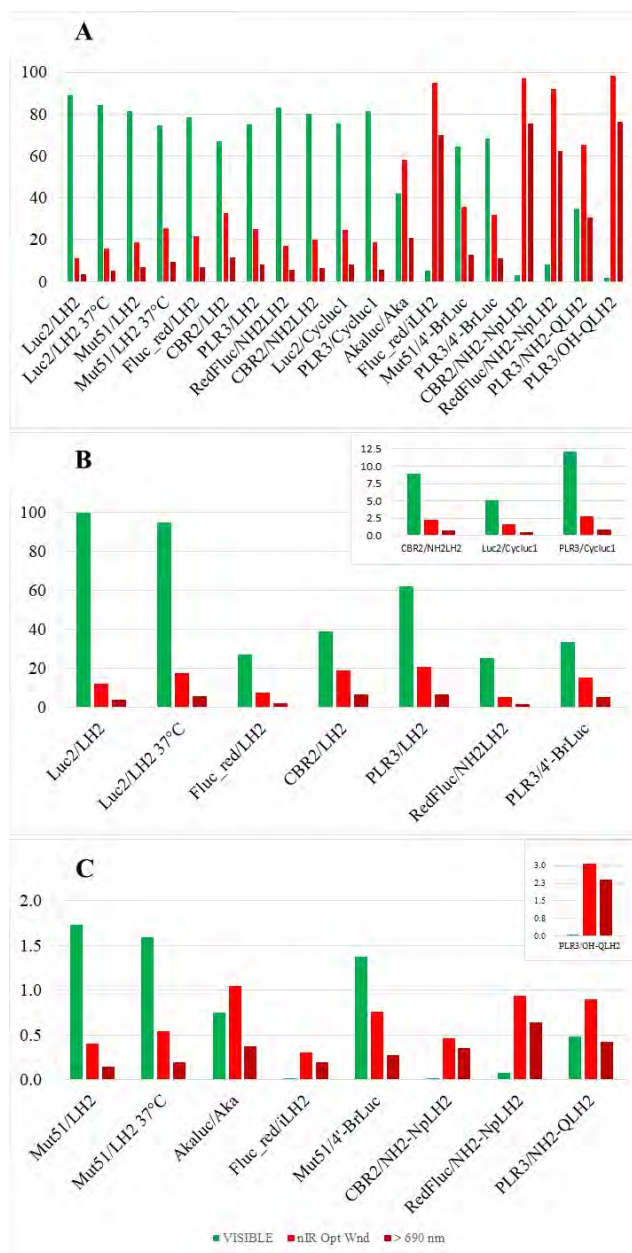


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

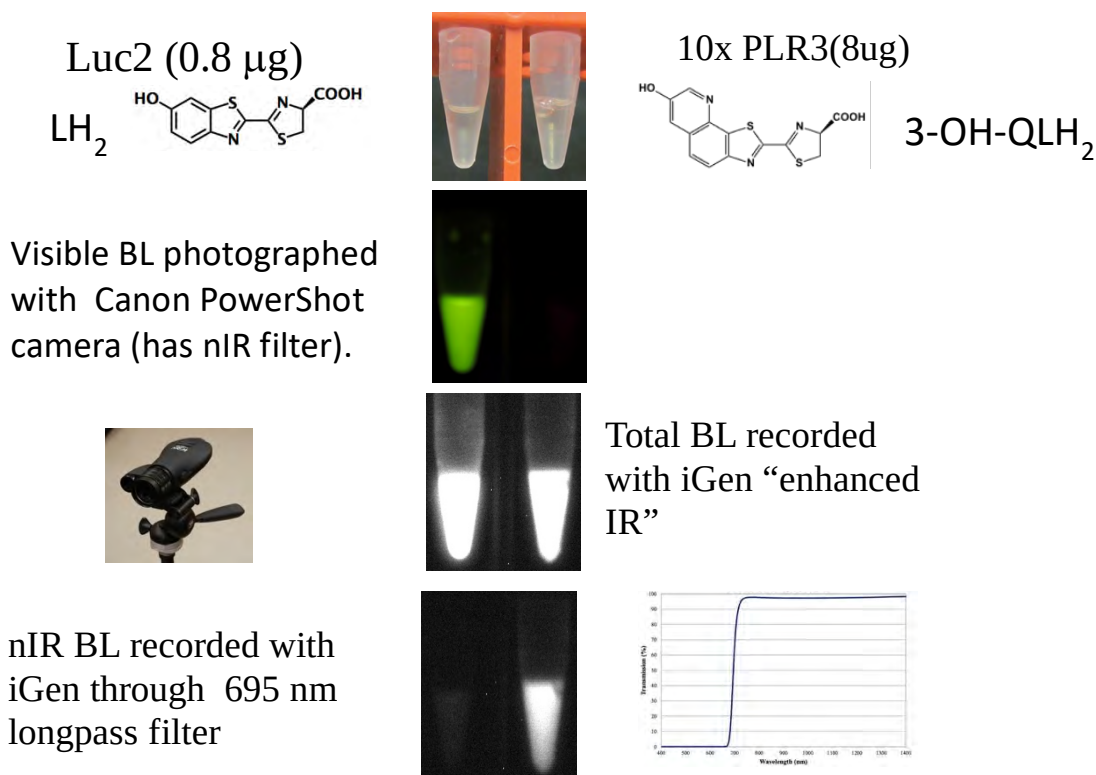
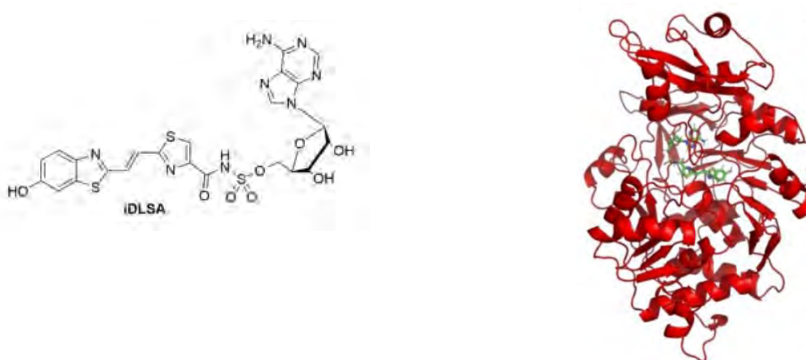


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

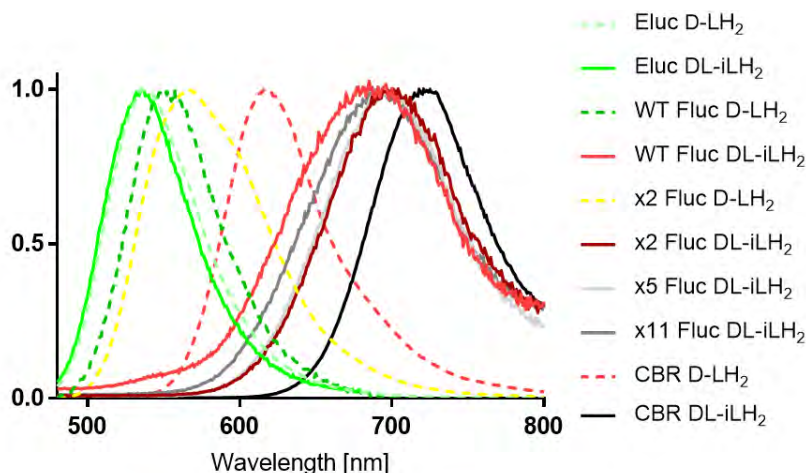


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

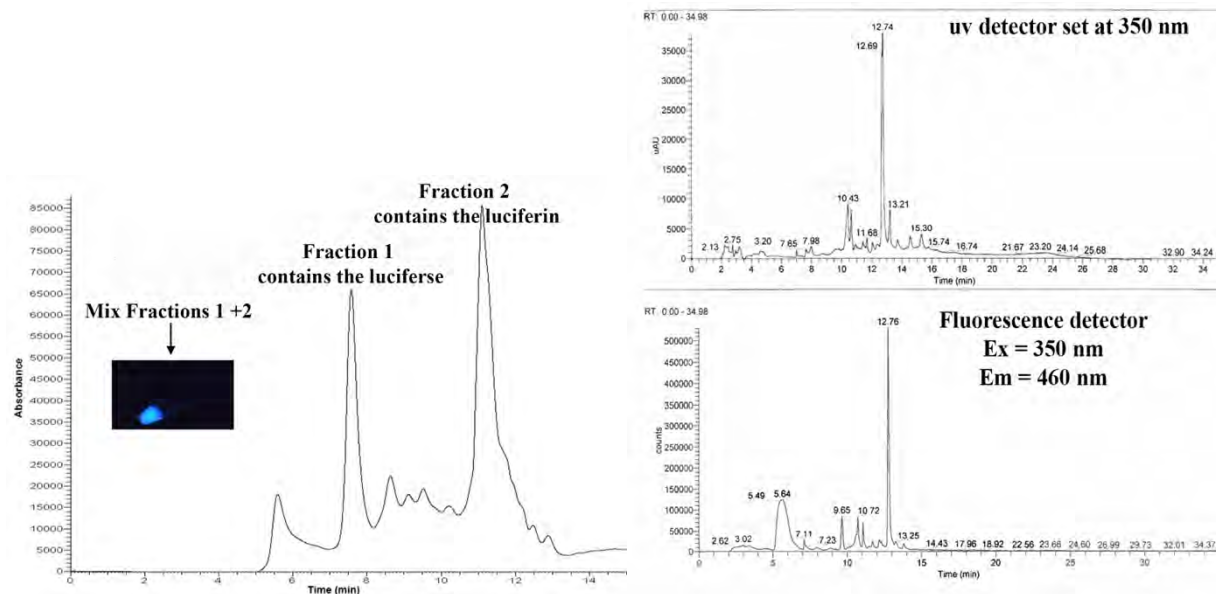
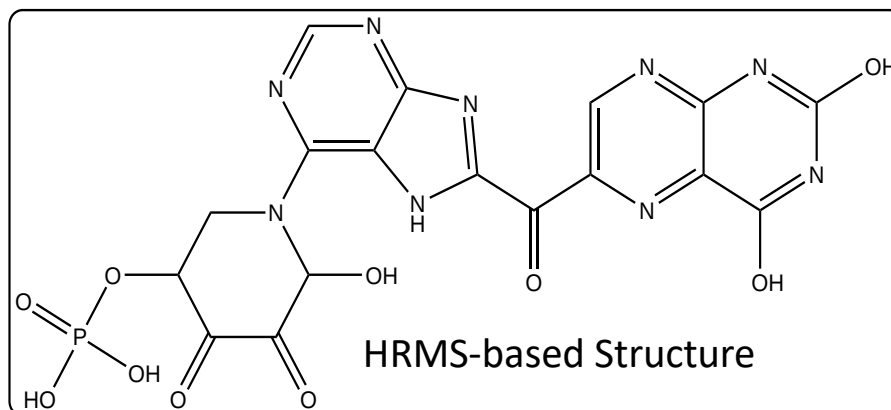


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of Orfelia bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

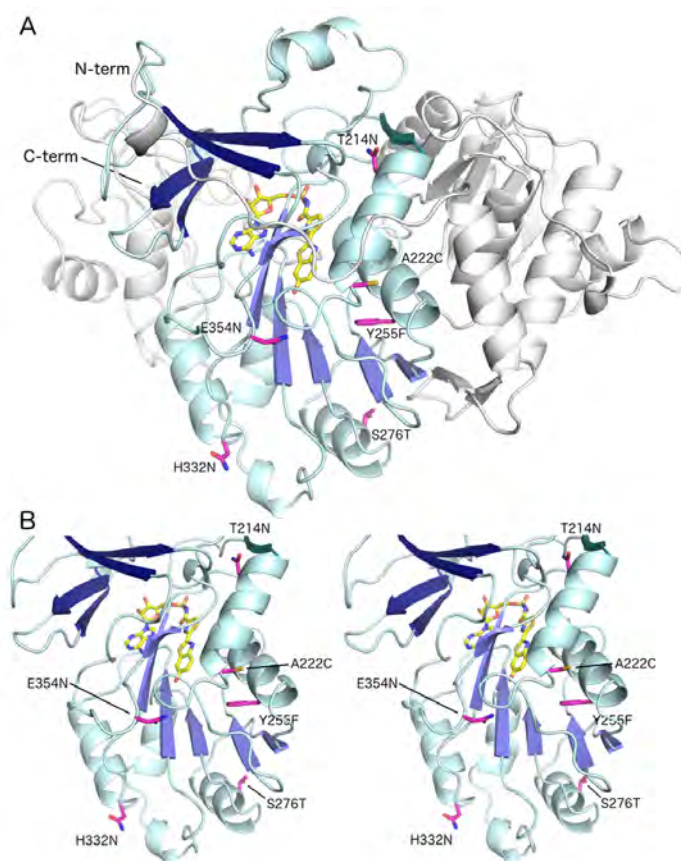


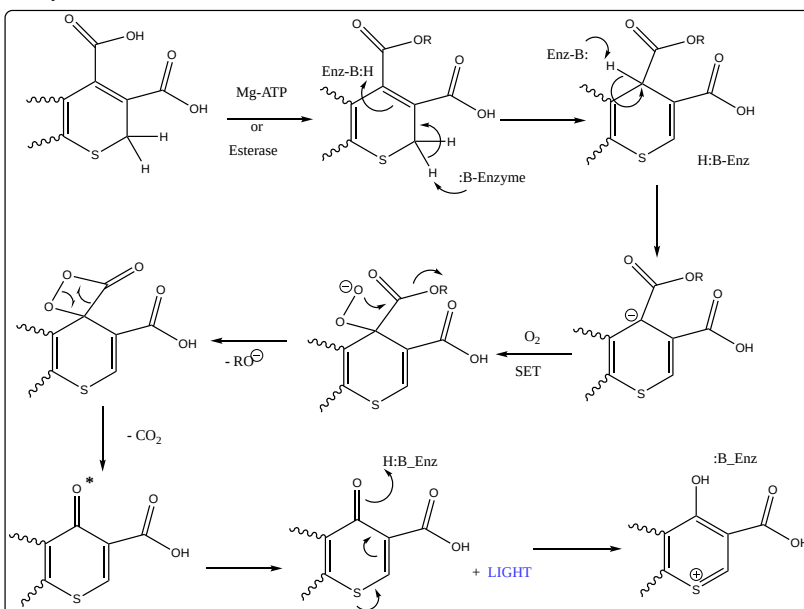
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

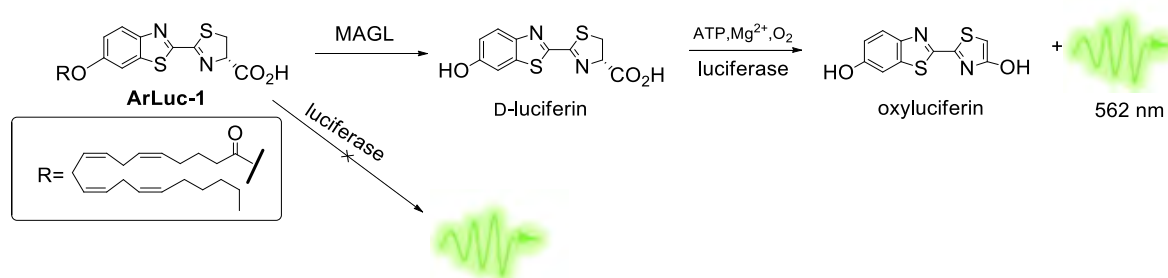


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

Publications

- 1. "Red-emitting chimeric firefly luciferase for in vivo imaging in low ATP cellular environments." Bruce R. Branchini, Tara L. Southworth, Danielle M. Fontaine, Dawn Kohrt, Franceine S. Welcome, Catherine M. Florentine, Emma R. Henricks, Demetria B. DeBartolo, Elisa Michelini, Luca Cevenini, Aldo Roda, Martha J. Grossel, *Analytical Biochemistry*, 2017;534:36–39. doi: 10.1016/j.ab.2017.07.001.**

- 2. "A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events." Branchini BR, Southworth TL, Fontaine DM, Kohrt D, Florentine CM, Grossel MJ. *Sci Rep*. 2018; 8(1):5990. doi: 10.1038/s41598-018-24278-2.
- 3. " Mutagenesis and structural studies reveal the basis for the activity and stability properties that distinguish the photinus luciferases scintillans and pyralis." Branchini, B. R., Fontaine, D. M., Southworth, T. L., Huta, B. P., Racela, A., Patel, K. D., and Gulick, A. M. (2019), *Biochemistry* 58, 4293-4303. doi/10.1021/acs.biochem.9b00719
- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
- 5. "Prêt-à-porter nanoYESA and nanoYESB bioluminescent cell biosensors for ultrarapid and sensitive screening of endocrine-disrupting chemicals." Lopreside, A., Calabretta, M. M., Montali, L., Ferri, M., Tassoni, A., Branchini, B. R., Southworth, T., D'Elia, M., Roda, A., and Michelini, E. (2019) *Anal. Bioanal. Chem.* 411, 4937-4949. doi/10.1007/s00216-019-01805-2
- 6. " Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin." Stowe, C. L., Burley, T. A., Allan, H., Vinci, M., Kramer-Marek, G., Ciobota, D. M., Parkinson, G. N., Southworth, T. L., Agliardi, G., Hotblack, A., Lythgoe, M. F., Branchini, B. R., Kalber, T. L., Anderson, J. C., and Pule, M. A. (2019) *eLife* 8. doi/10.7554/eLife.45801
- 7. "A new brilliantly blue-emitting luciferin- luciferase system from *Orfelia fultoni* and *Keroplata* (Diptera)." V.R. Viviani, J. R. Silva, D. T. Amaral, V. R. Bevilaqua, F. C. Abdalla, B. R. Branchini, and C. H. Johnson (2020) *Sci. Rep.*, 10:9608 | <https://doi.org/10.1038/s41598-020-66286-1>
- 8. "A new ultrasensitive bioluminescence -based method for assaying monoacylglycerol lipase." M. Miceli, S. Casati, P. Allevi, S. Berra, R. Ottria, P. Rota, B.R. Branchini, and P. Ciuffreda, *Int. J. Mol. Sci.*, 22, 6148 (2021) <https://doi.org/10.3390/ijms22116148>

- 9. “Dendritic cells maintain anti-tumor immunity by positioning CDB skin-resident memory T cells.” J. L. Vella, A. Molodtskov, C. V. Angeles, B. R. Branchini, M. J. Turk, and Y. H. Huang, *Life Science Alliance* 4 (10), e202101056 (2021).
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- 10. “A higher spectral range of beetle bioluminescence with infraluciferin.” A. P. Jathoul, B. R. Branchini, J. C. Anderson and J. A. H. Murray, *Front. Bioeng. Biotechnol.*, 10, 897272 (2022). <https://doi.org/10.3389/fbioe.2022.897272>
- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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Principal Investigator: Bruce Branchini

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ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

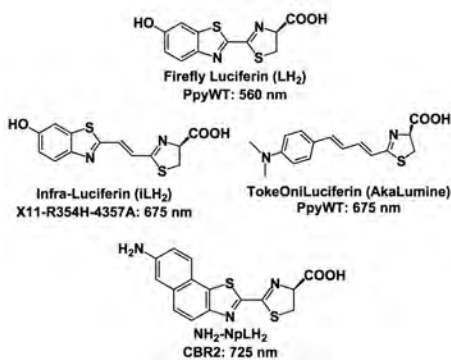


Figure 1. Luciferase-substrate emission maxima.

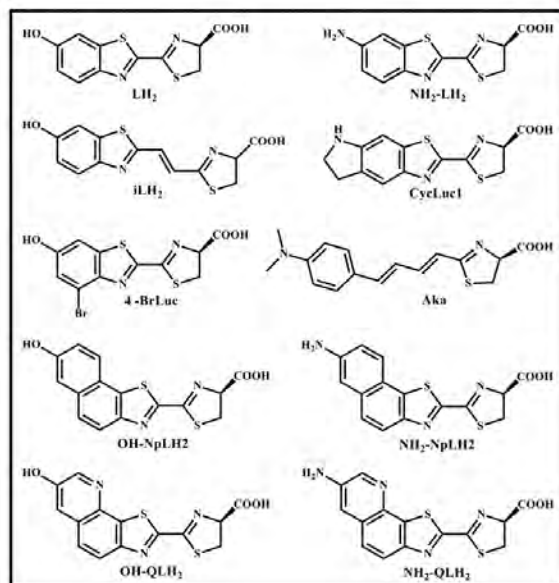
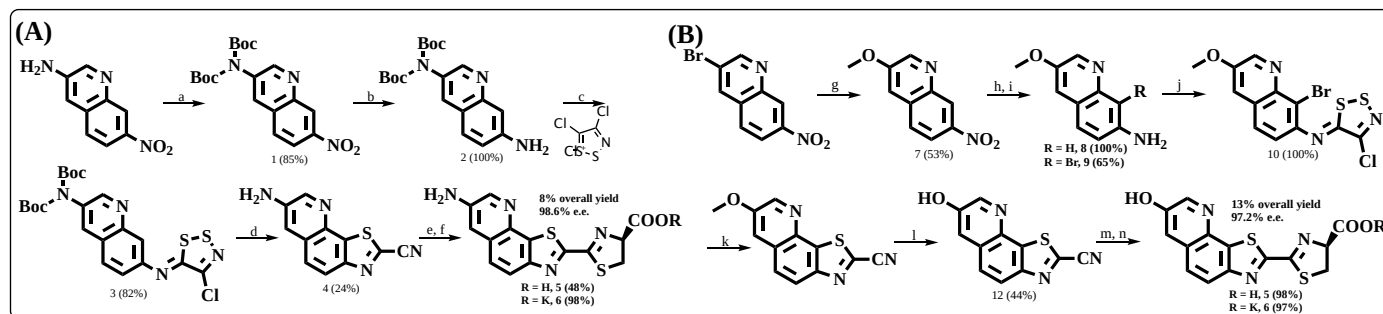


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

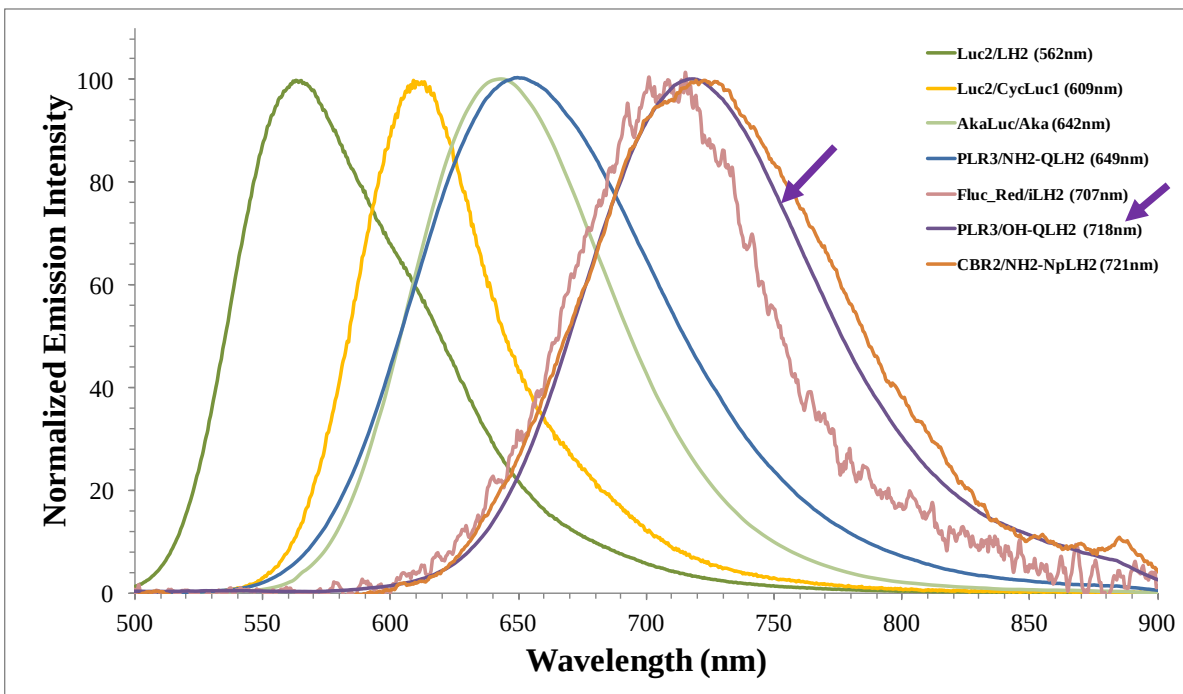


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

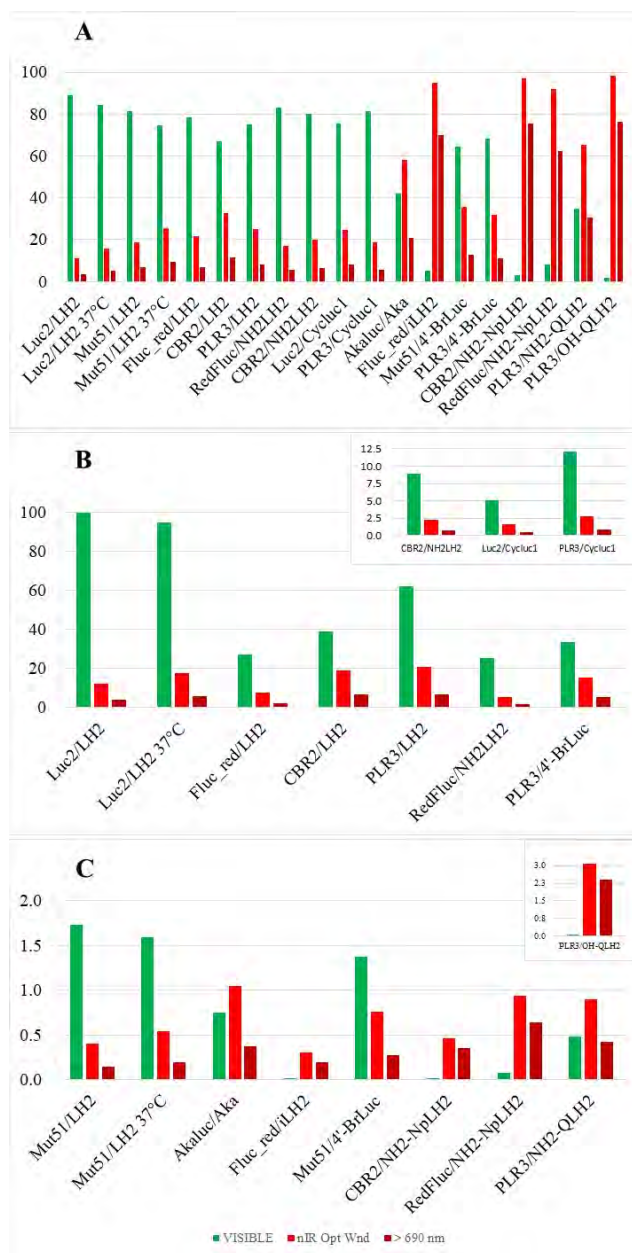


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

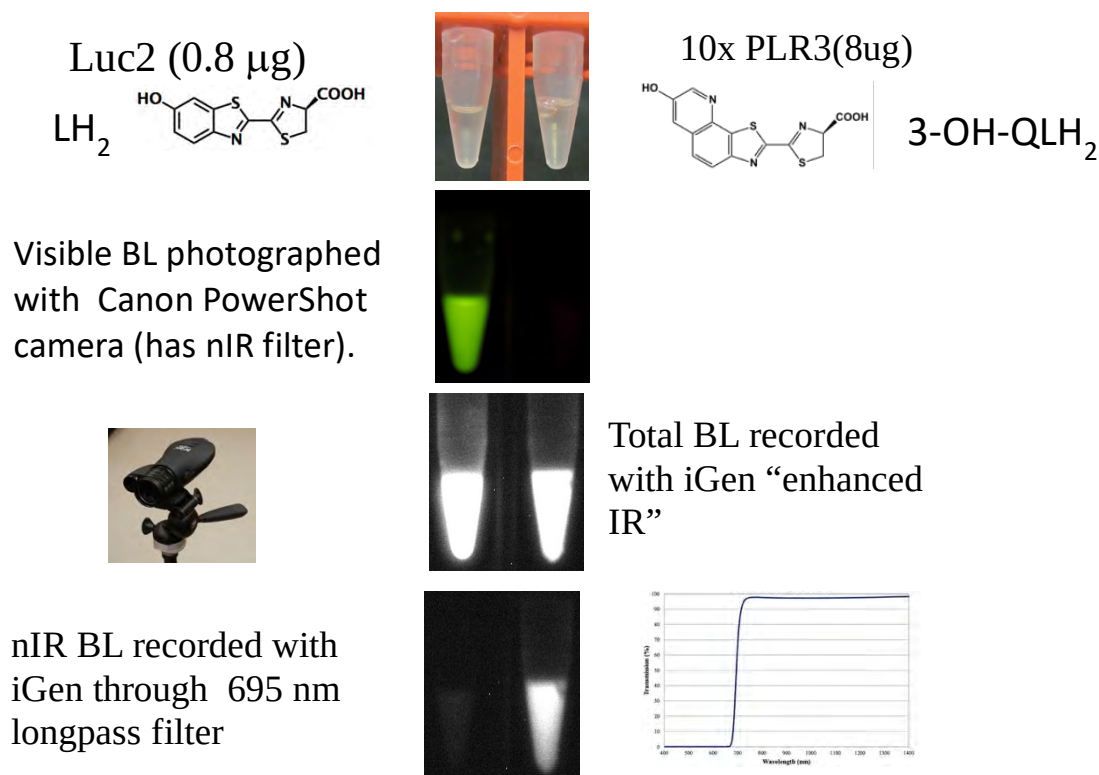
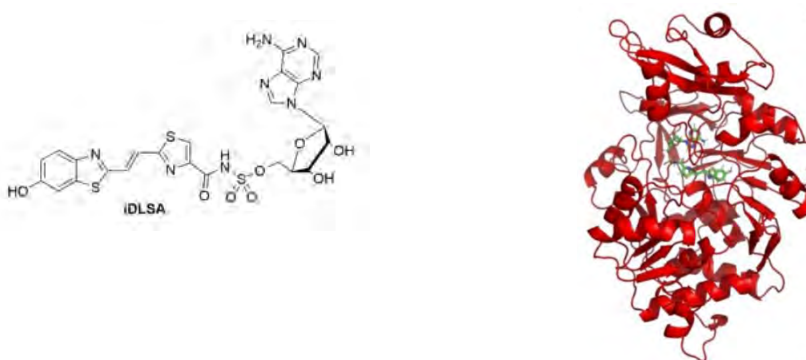


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

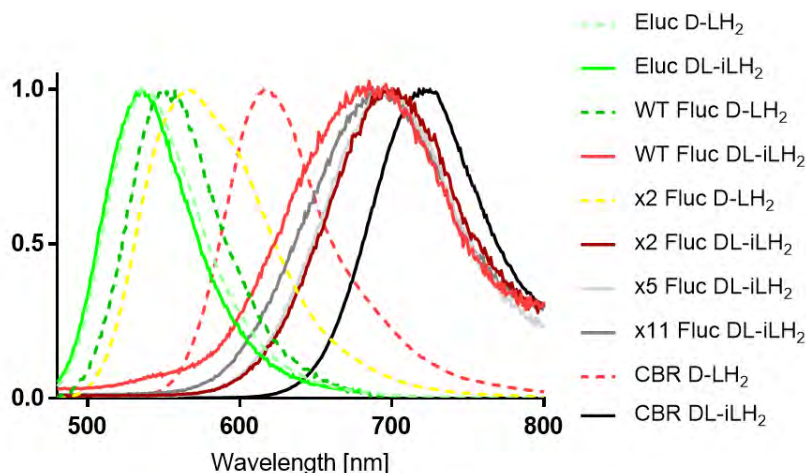


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultoni*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

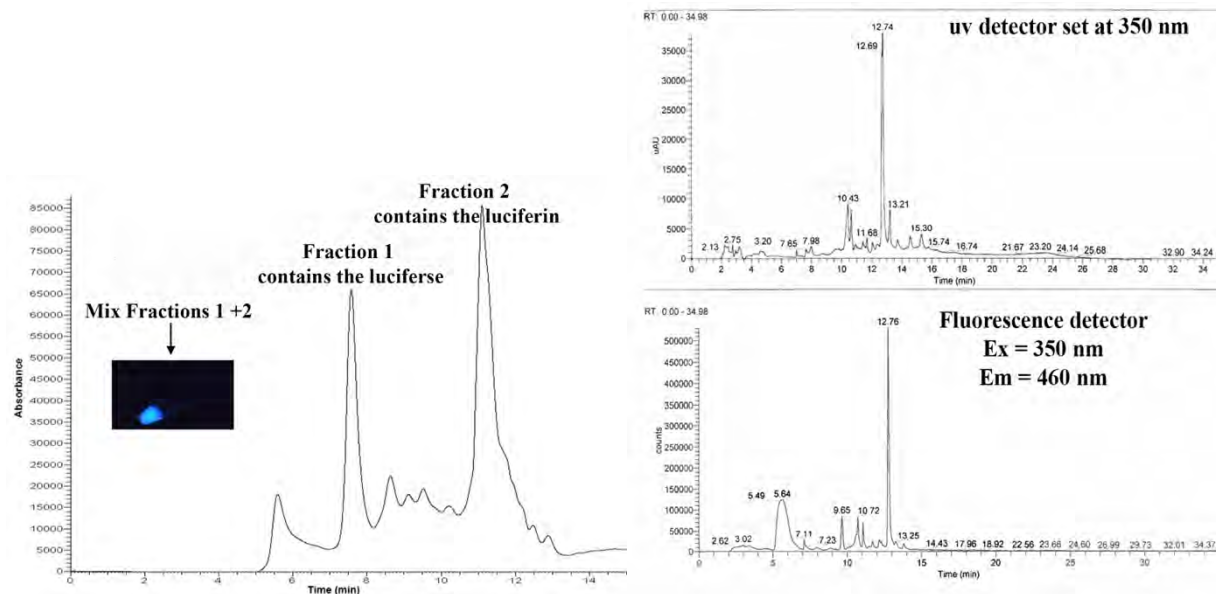
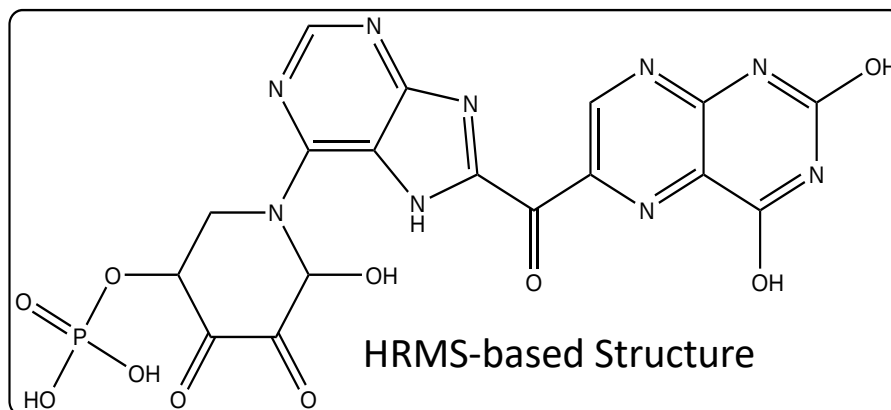


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of Orfelia bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



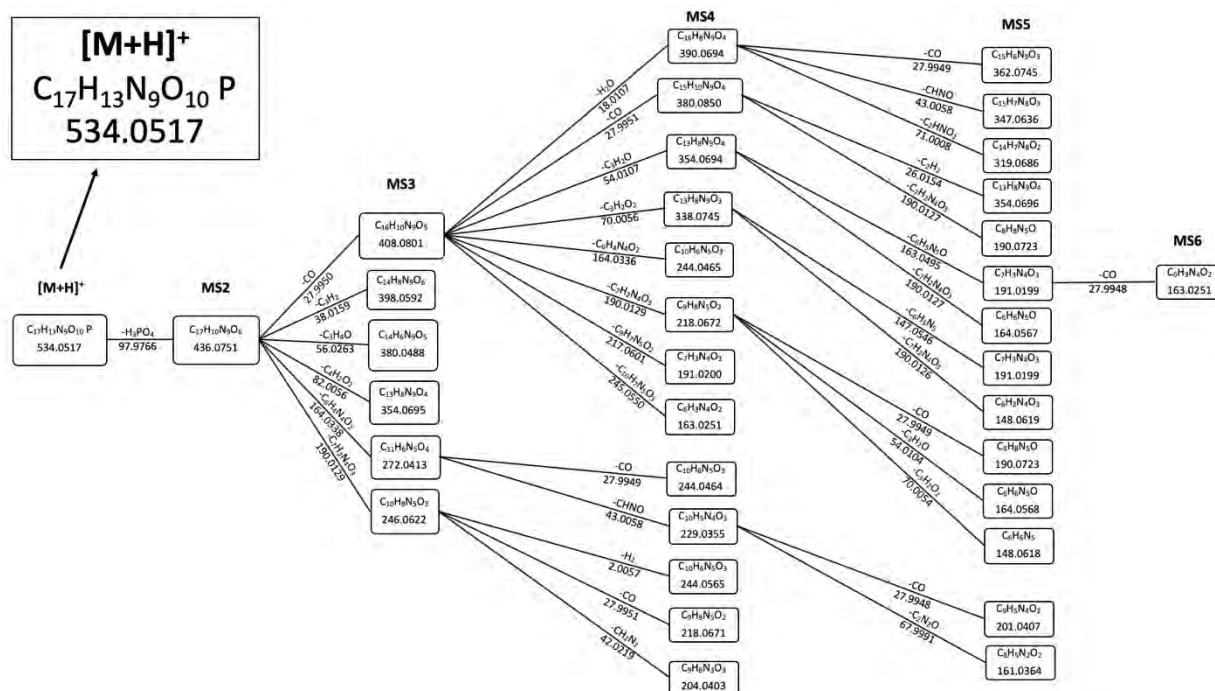


Figure 8. HRMS analysis of putative Orfelia oxyluciferin.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the Orfelia oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

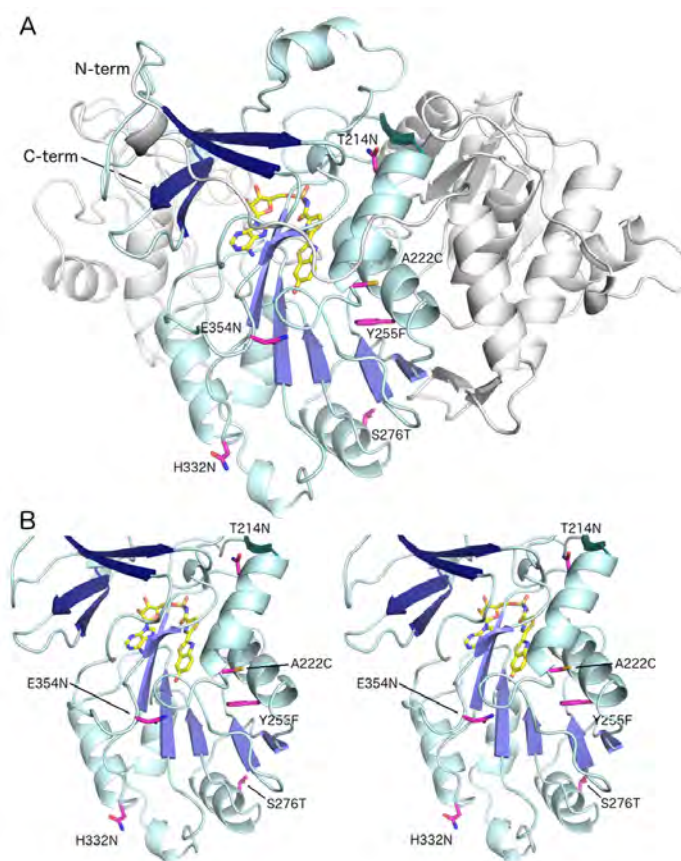


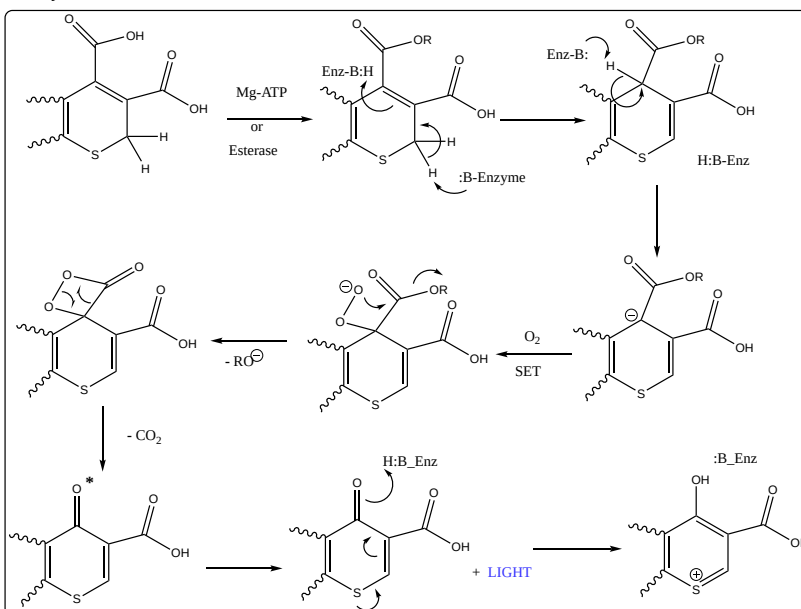
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

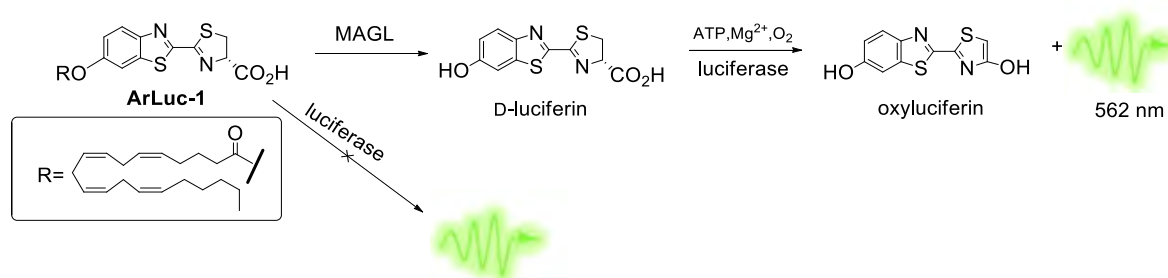


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 1. "Red-emitting chimeric firefly luciferase for in vivo imaging in low ATP cellular environments." Bruce R. Branchini, Tara L. Southworth, Danielle M. Fontaine, Dawn Kohrt, Franceine S. Welcome, Catherine M. Florentine, Emma R. Henricks, Demetria B. DeBartolo, Elisa Michelini, Luca Cevenini, Aldo Roda, Martha J. Grossel, *Analytical Biochemistry*, 2017;534:36–39. doi: 10.1016/j.ab.2017.07.001.**

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COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

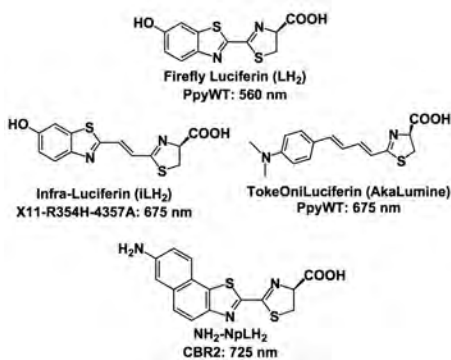


Figure 1. Luciferase-substrate emission maxima.

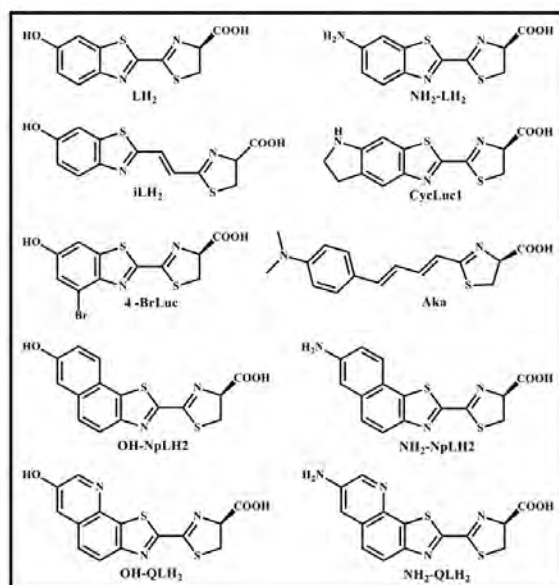
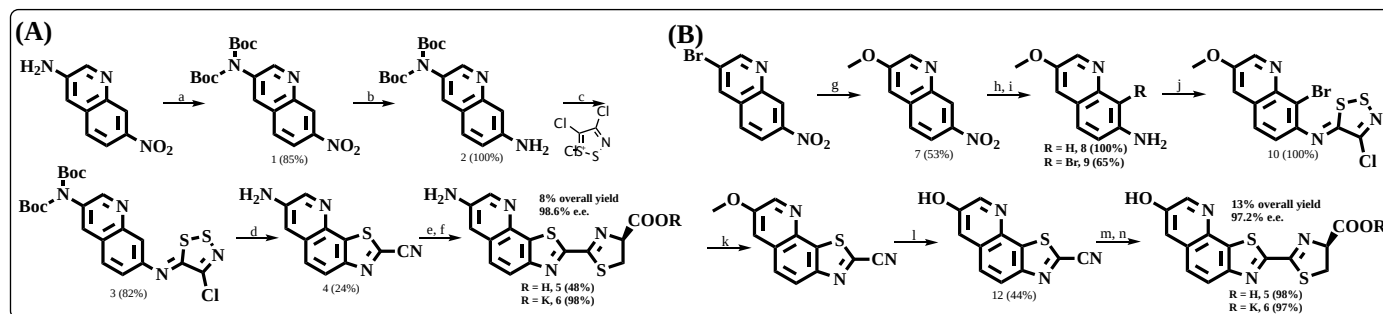


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

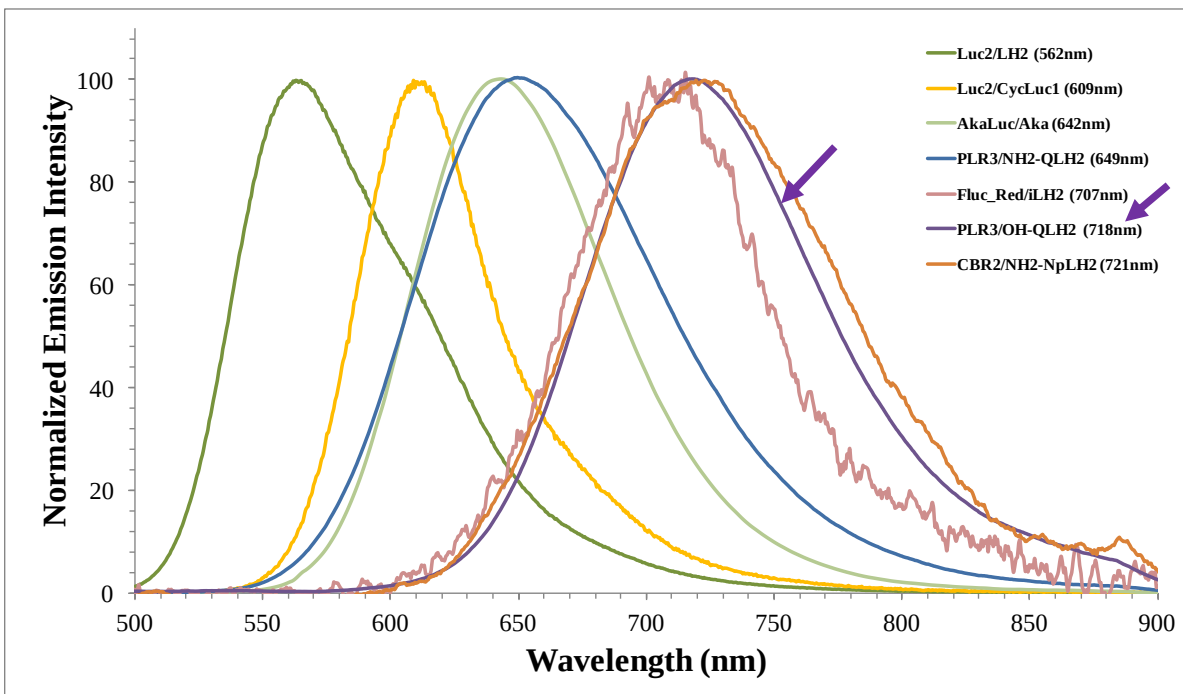


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

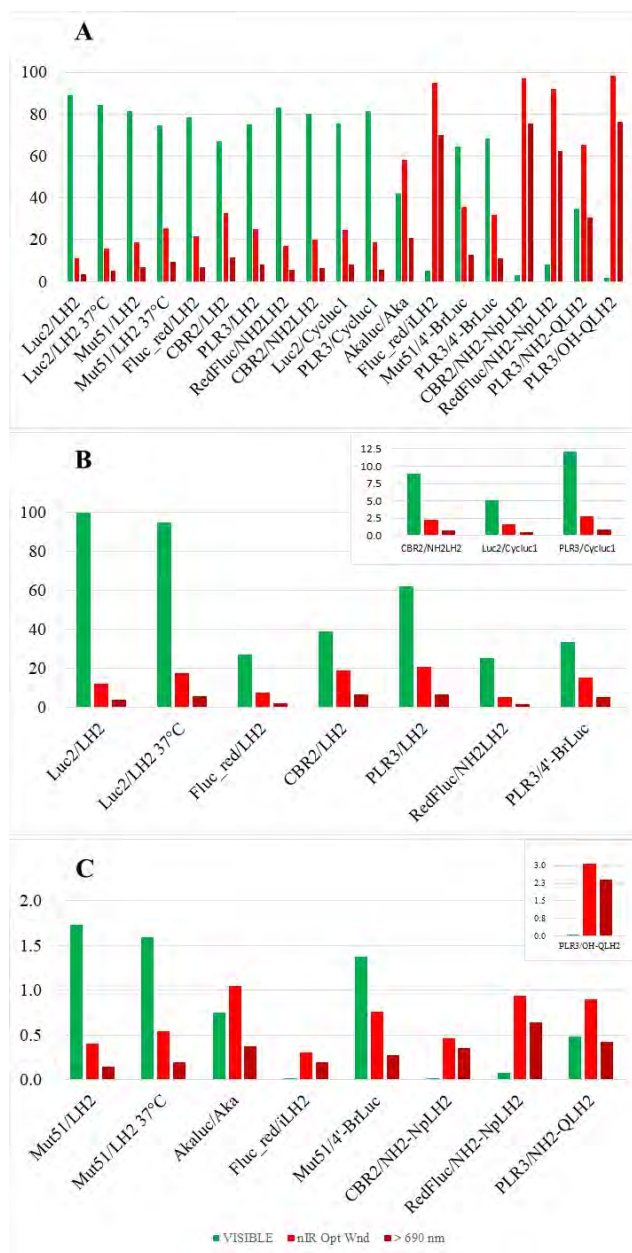


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

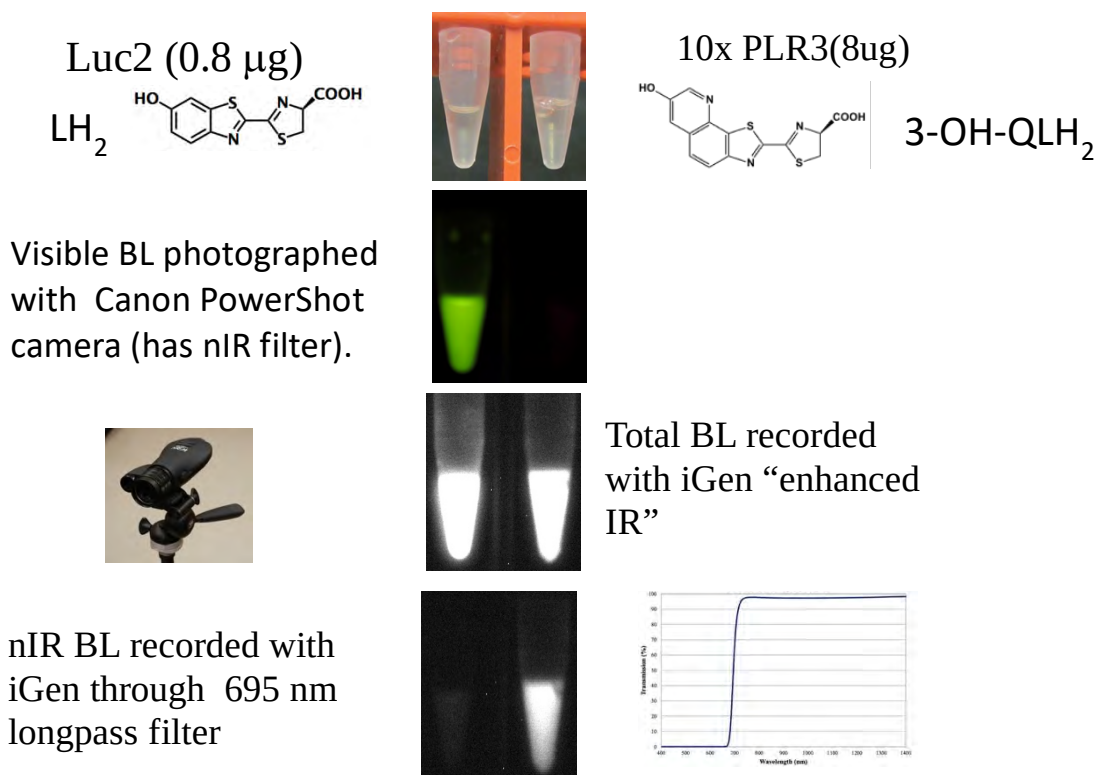
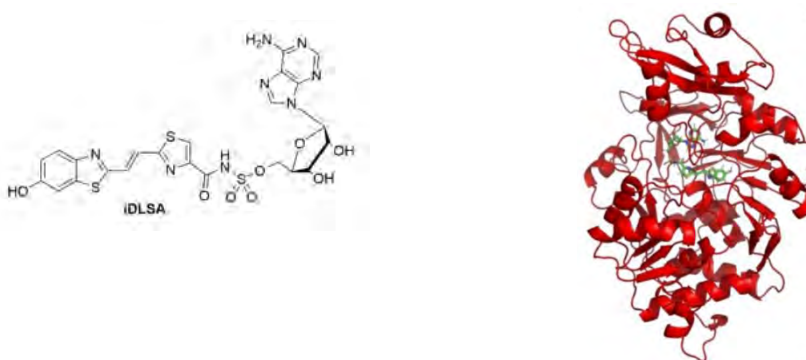


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

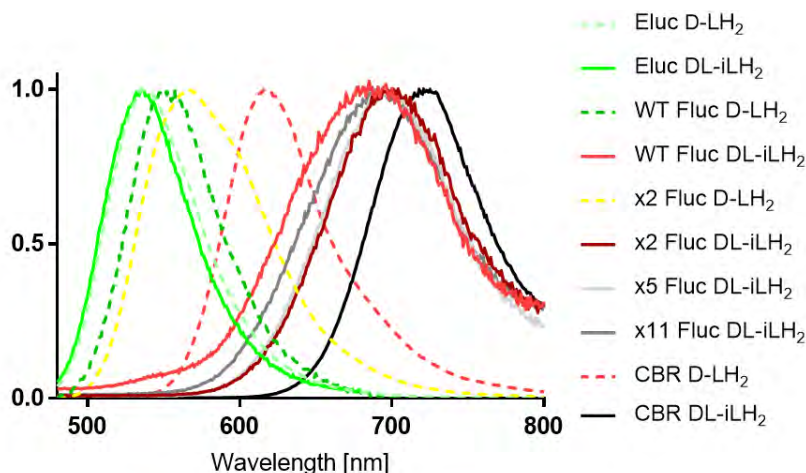


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

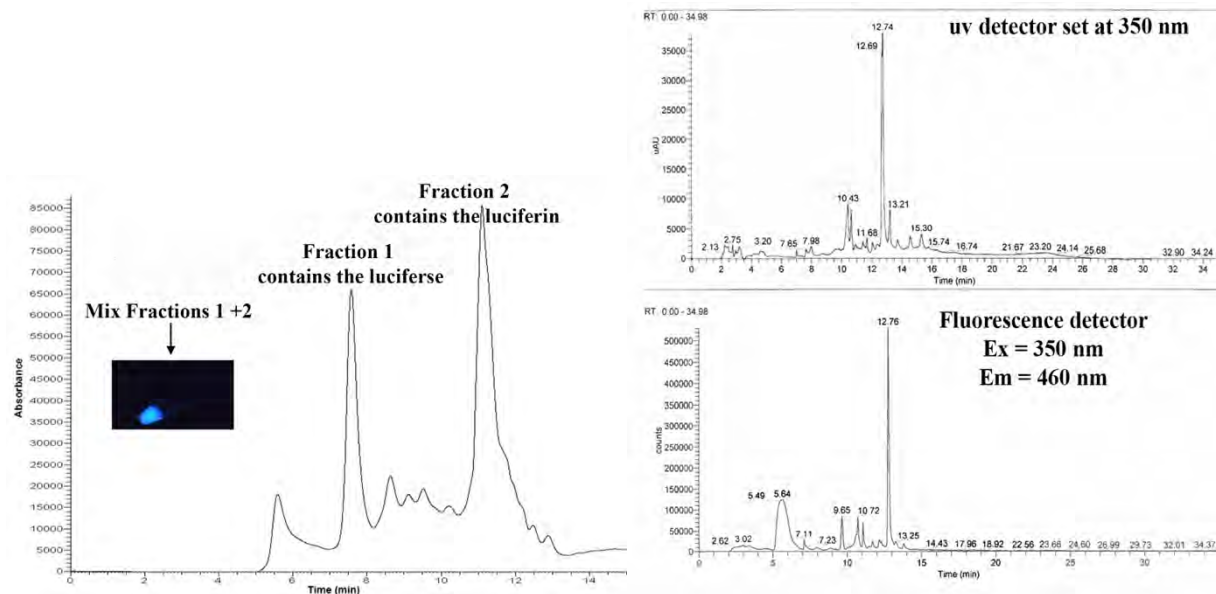
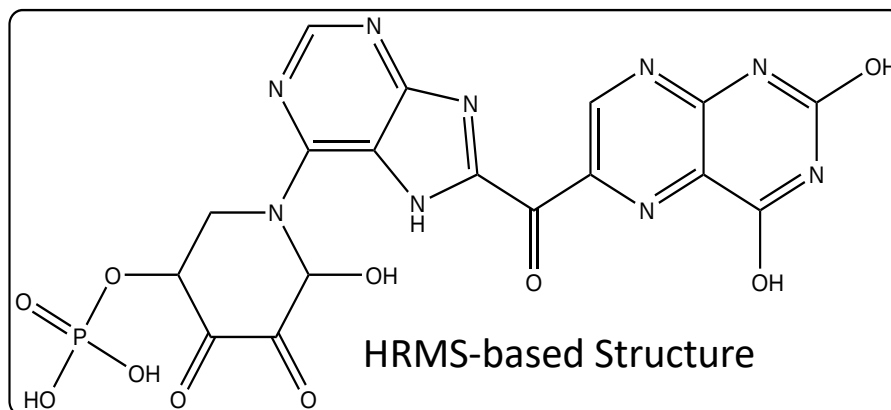


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



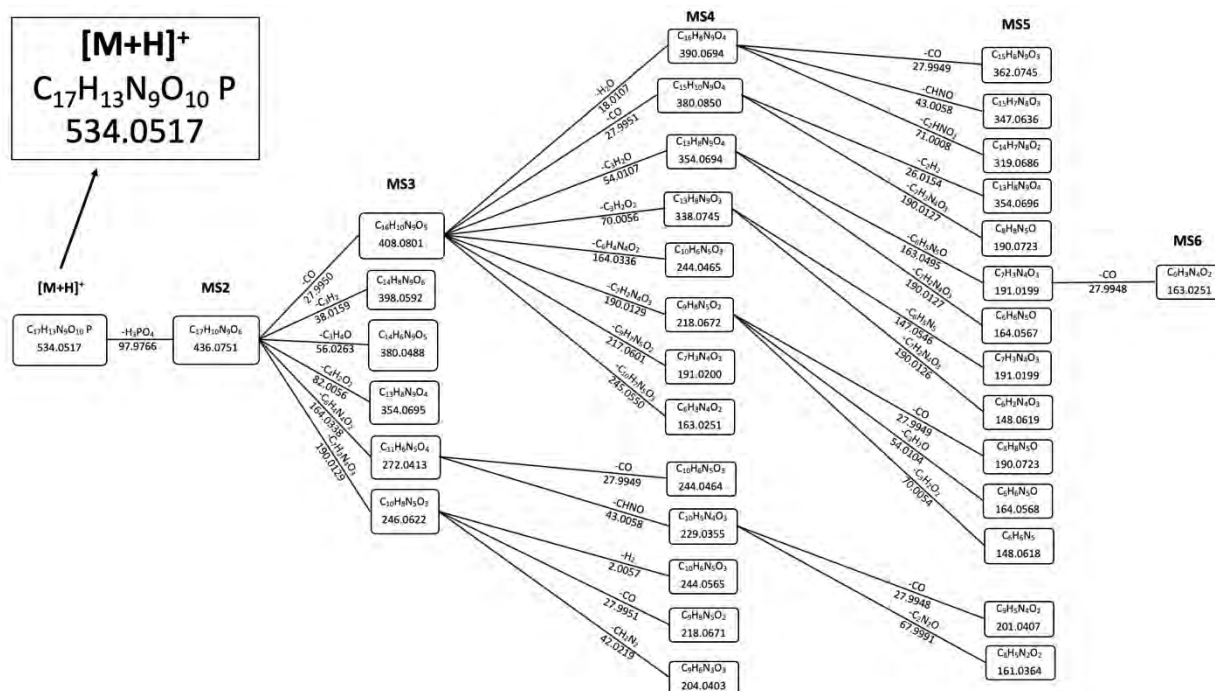


Figure 8. HRMS analysis of putative Orfelia oxyluciferin.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the Orfelia oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

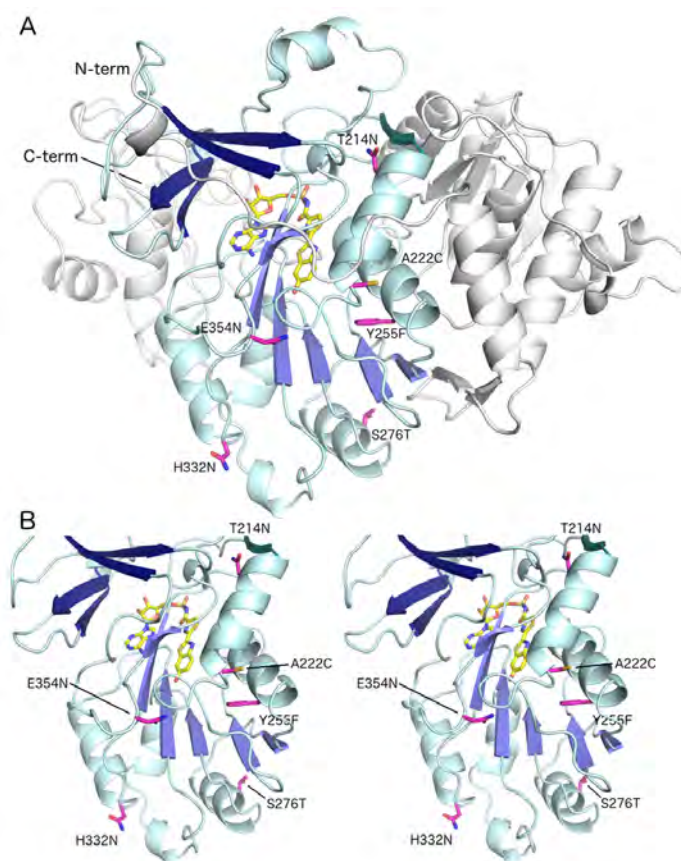


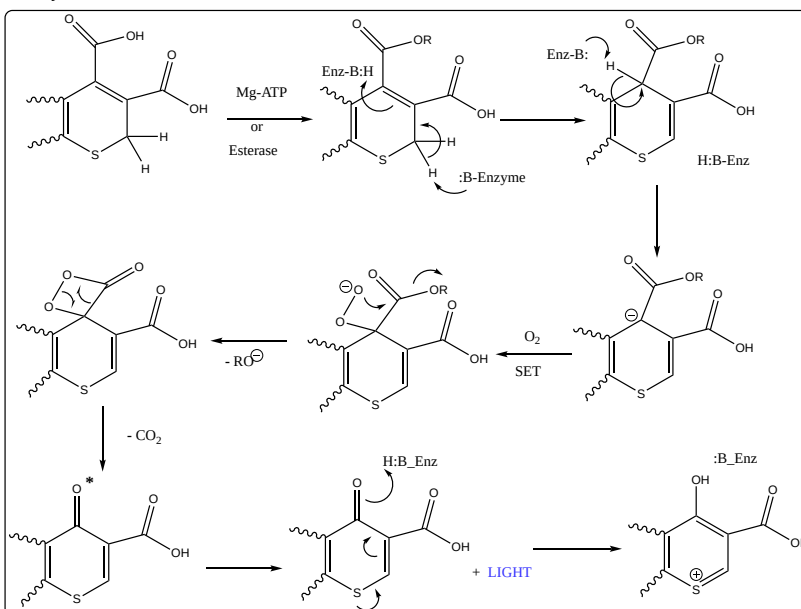
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Universita degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

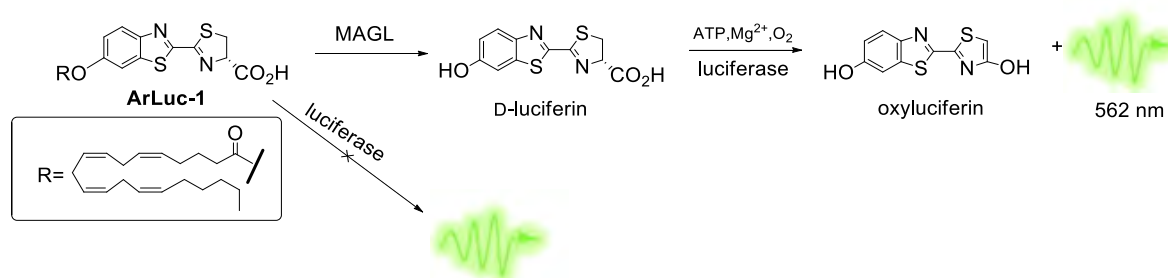


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 1. "Red-emitting chimeric firefly luciferase for in vivo imaging in low ATP cellular environments." Bruce R. Branchini, Tara L. Southworth, Danielle M. Fontaine, Dawn Kohrt, Franceine S. Welcome, Catherine M. Florentine, Emma R. Henricks, Demetria B. DeBartolo, Elisa Michelini, Luca Cevenini, Aldo Roda, Martha J. Grossel, *Analytical Biochemistry*, 2017;534:36–39. doi: 10.1016/j.ab.2017.07.001.**

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- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
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- 9. “Dendritic cells maintain anti-tumor immunity by positioning CDB skin-resident memory T cells.” J. L. Vella, A. Molodtskov, C. V. Angeles, B. R. Branchini, M. J. Turk, and Y. H. Huang, *Life Science Alliance* 4 (10), e202101056 (2021).
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*** Corresponding author inadvertently left out AFOSR acknowledgement.

COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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Program Officer: Bennett L. Ibey

Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

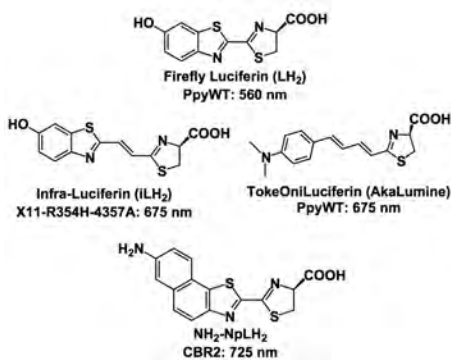


Figure 1. Luciferase-substrate emission maxima.

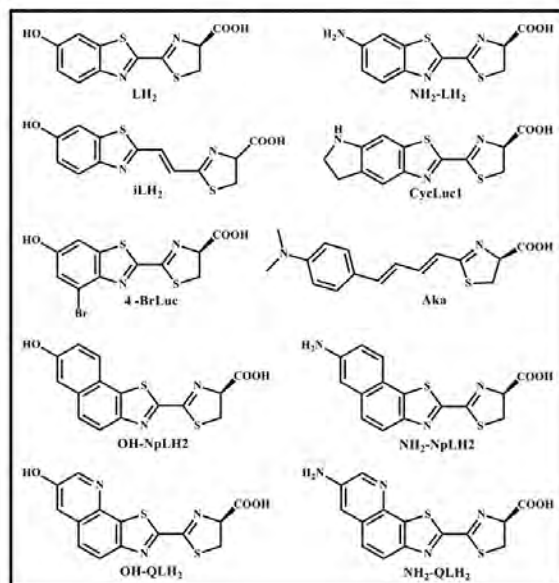
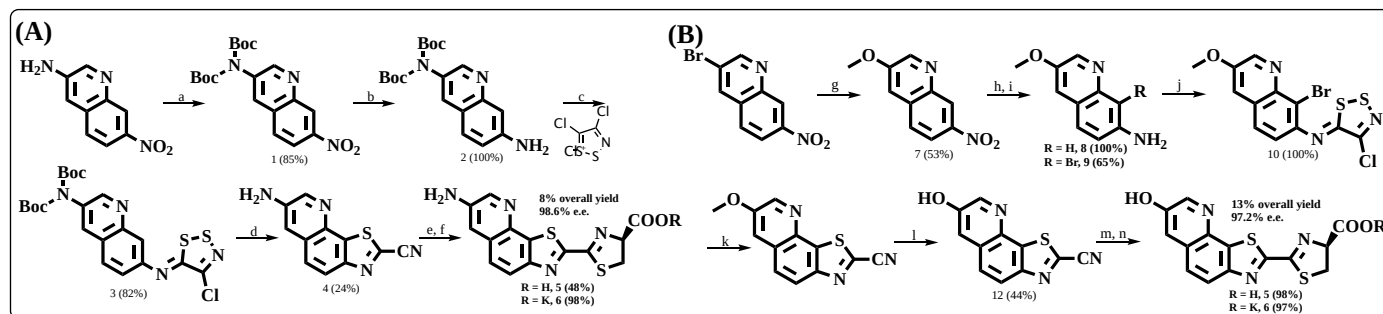


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

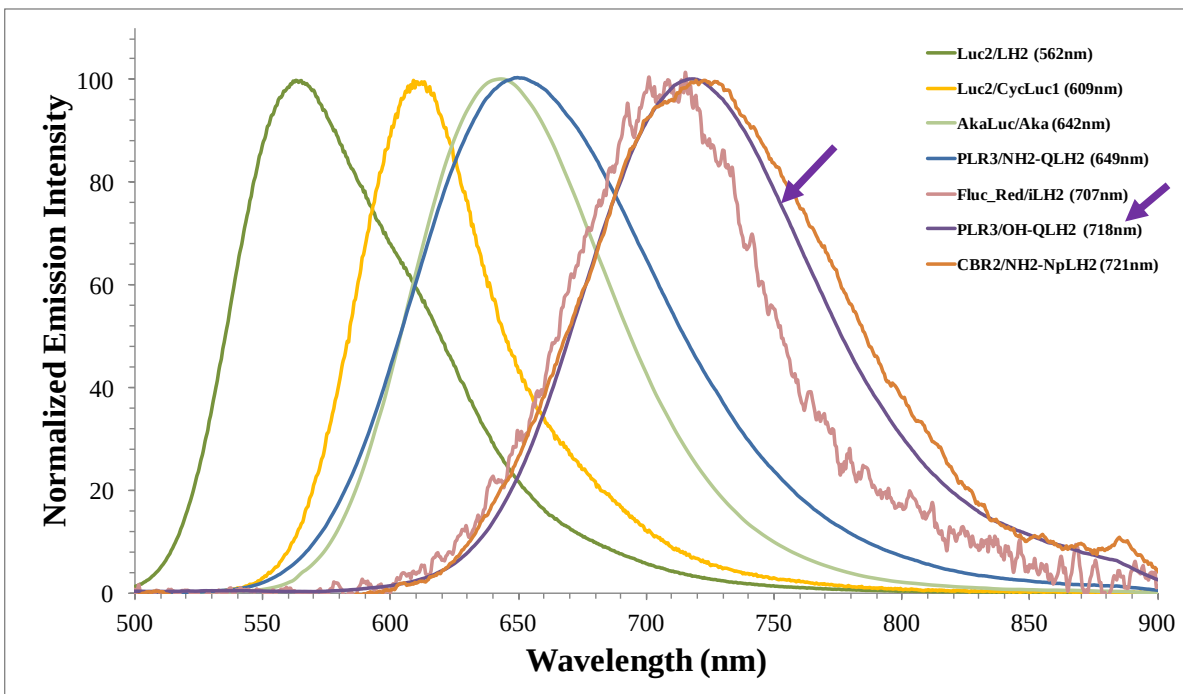


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

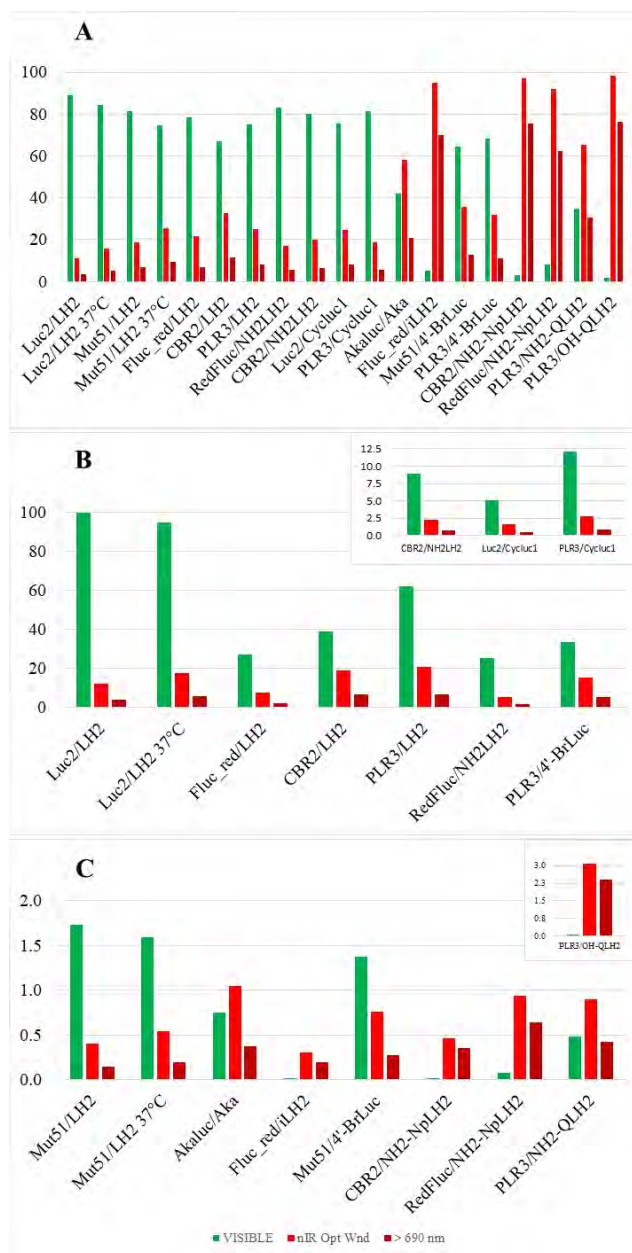


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

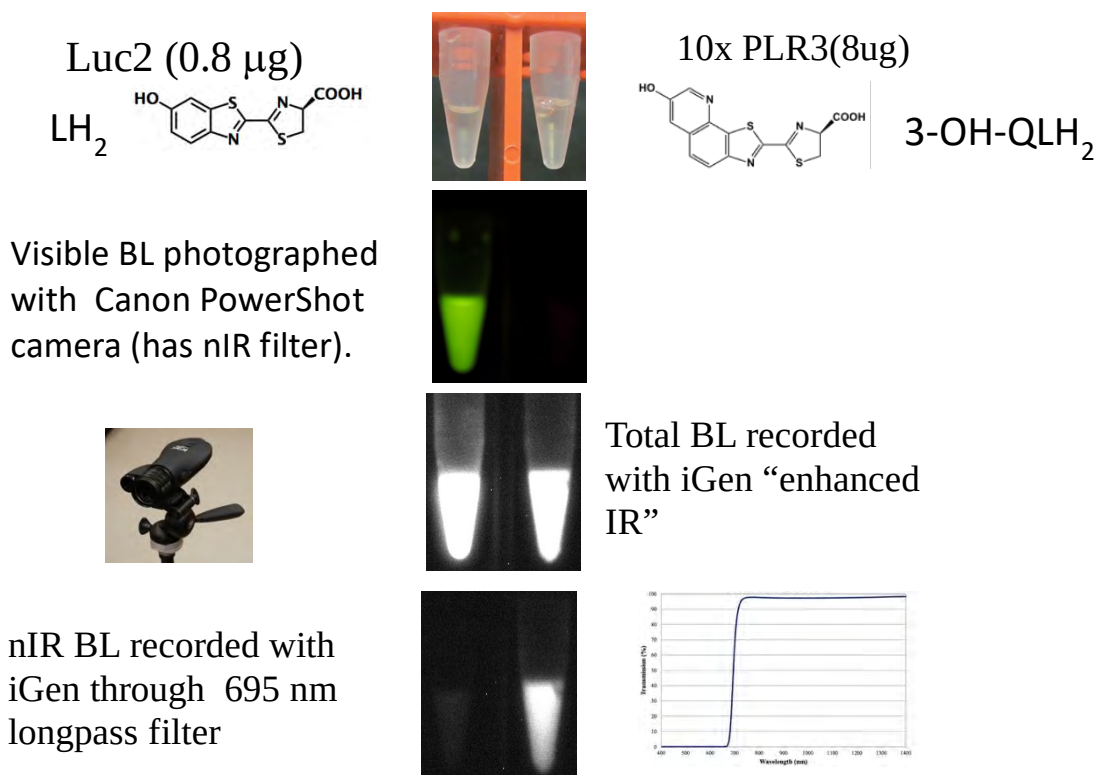
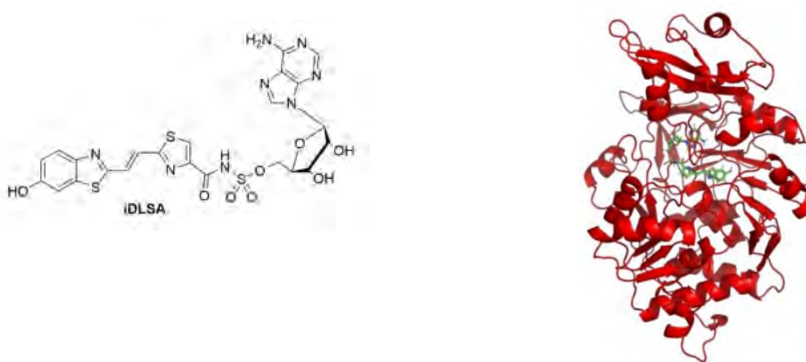


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

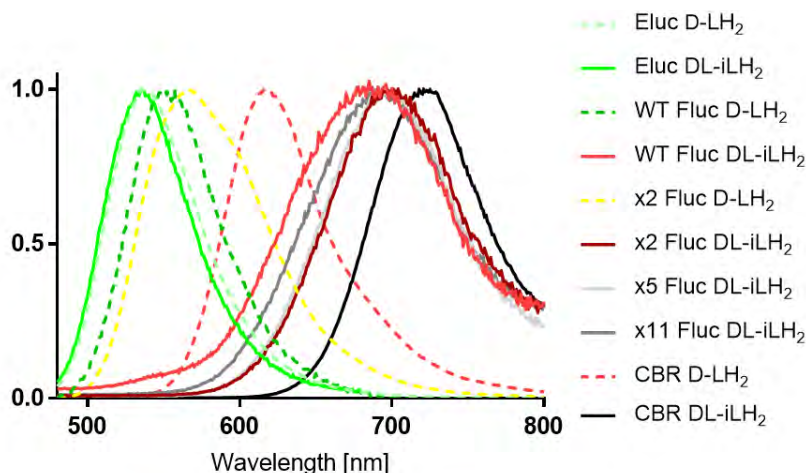


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultoni*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

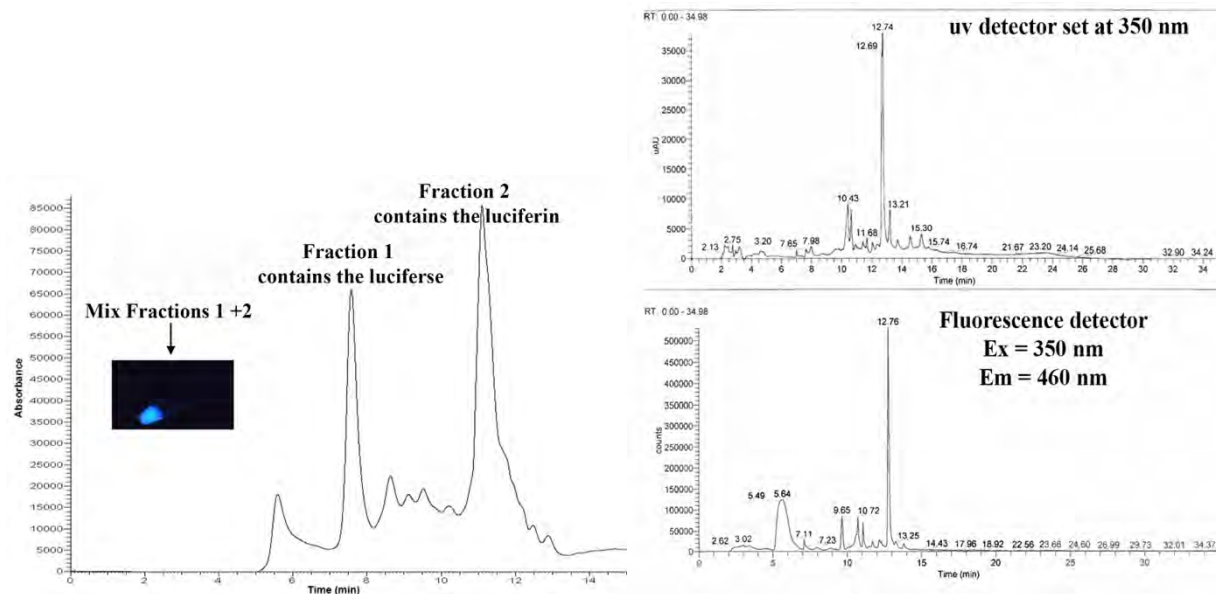
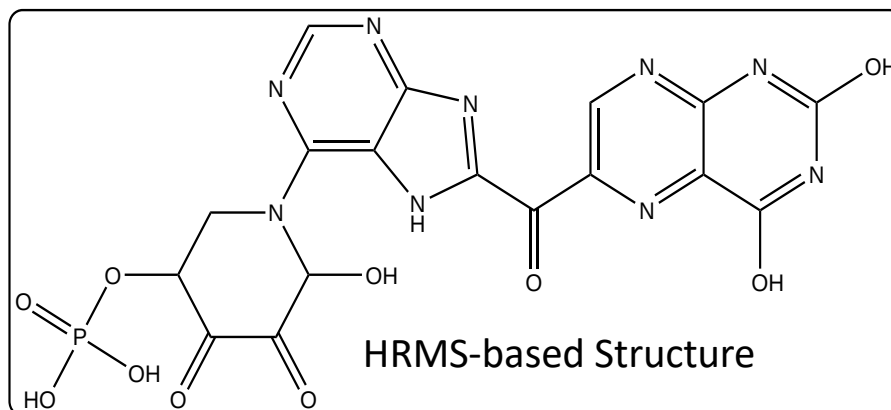


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

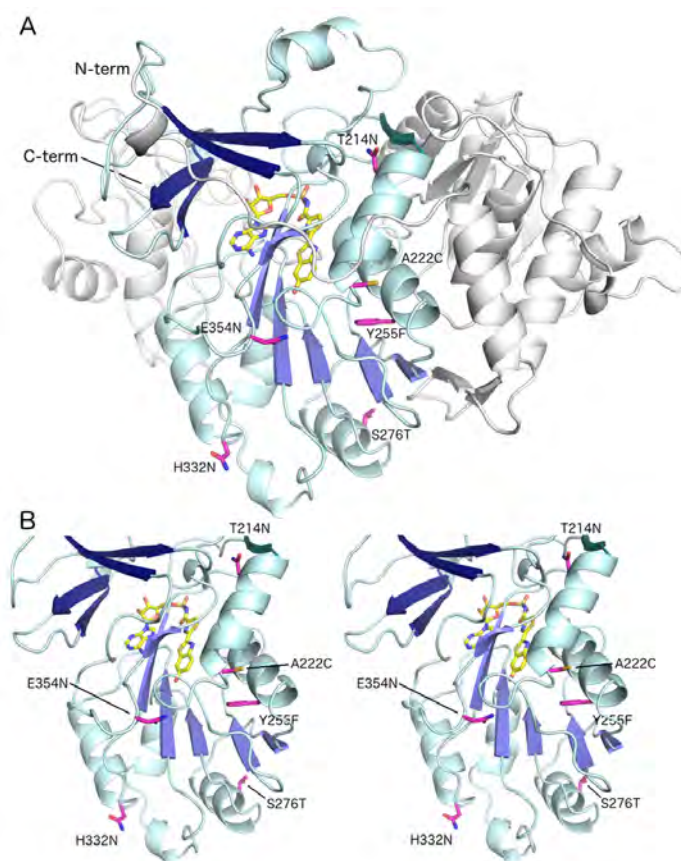


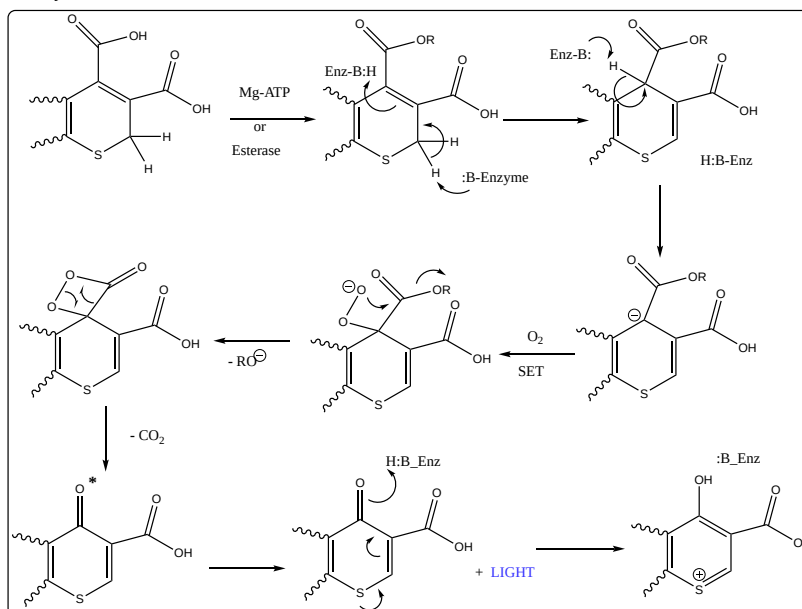
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

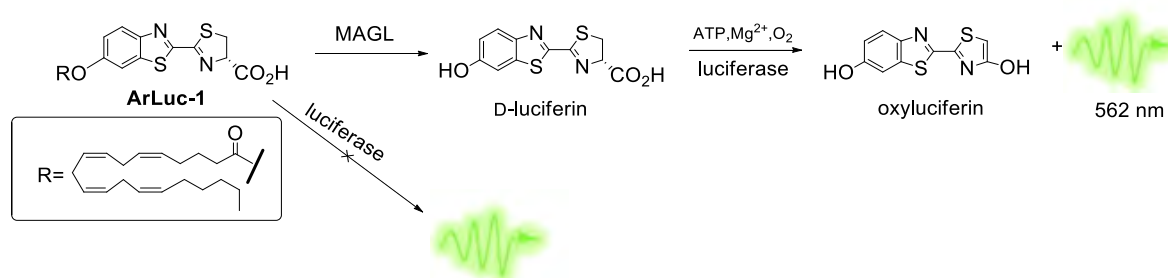


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 x g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18-20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at -80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of K_m for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). K_m values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
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- 6. " Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin." Stowe, C. L., Burley, T. A., Allan, H., Vinci, M., Kramer-Marek, G., Ciobota, D. M., Parkinson, G. N., Southworth, T. L., Agliardi, G., Hotblack, A., Lythgoe, M. F., Branchini, B. R., Kalber, T. L., Anderson, J. C., and Pule, M. A. (2019) *eLife* 8. doi/10.7554/eLife.45801
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- 8. "A new ultrasensitive bioluminescence -based method for assaying monoacylglycerol lipase." M. Miceli, S. Casati, P. Allevi, S. Berra, R. Ottria, P. Rota, B.R. Branchini, and P. Ciuffreda, *Int. J. Mol. Sci.*, 22, 6148 (2021) <https://doi.org/10.3390/ijms22116148>

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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

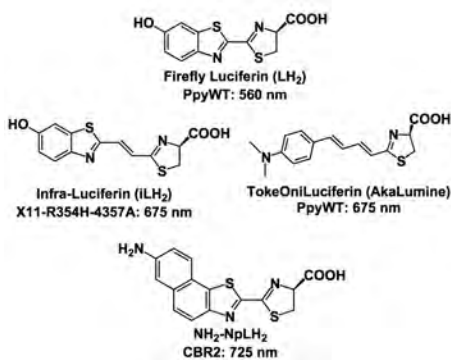


Figure 1. Luciferase-substrate emission maxima.

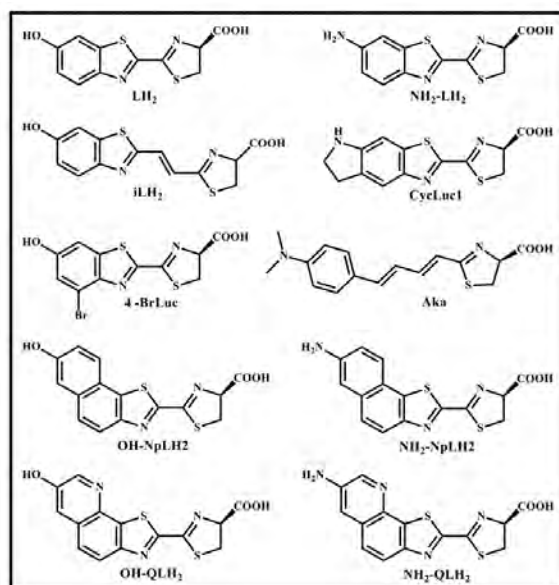
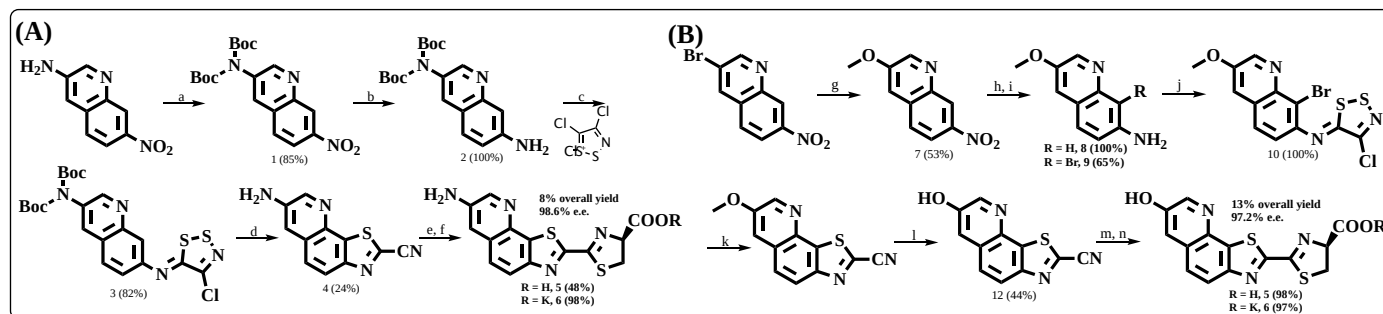


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

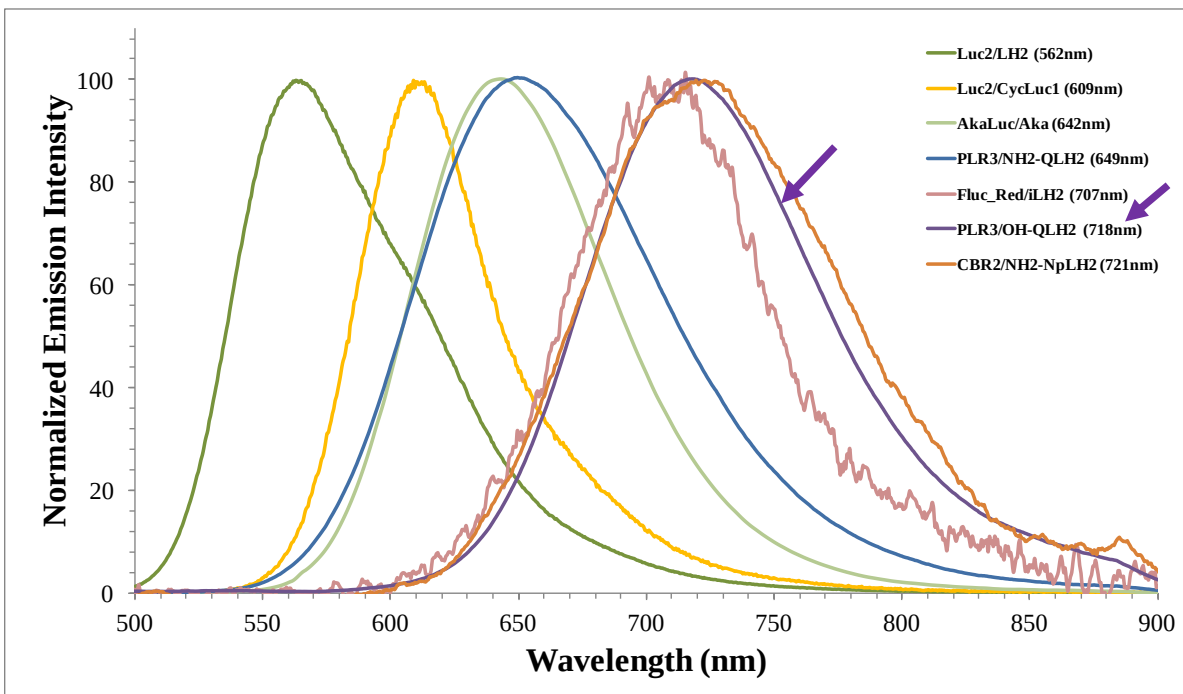


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

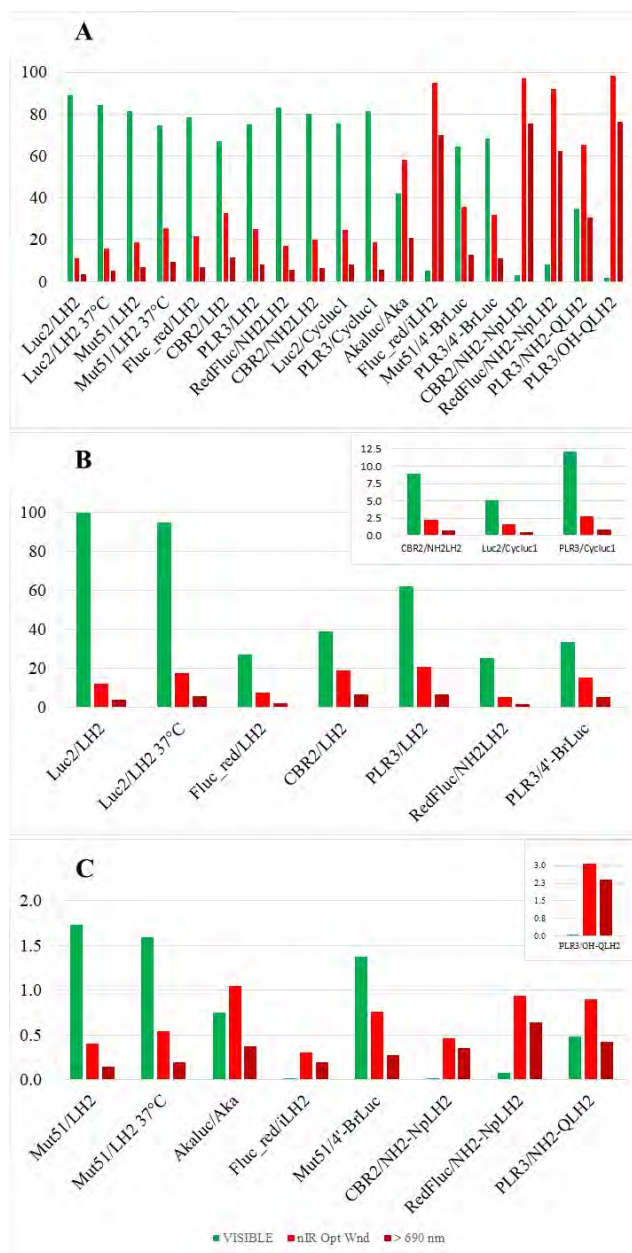


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

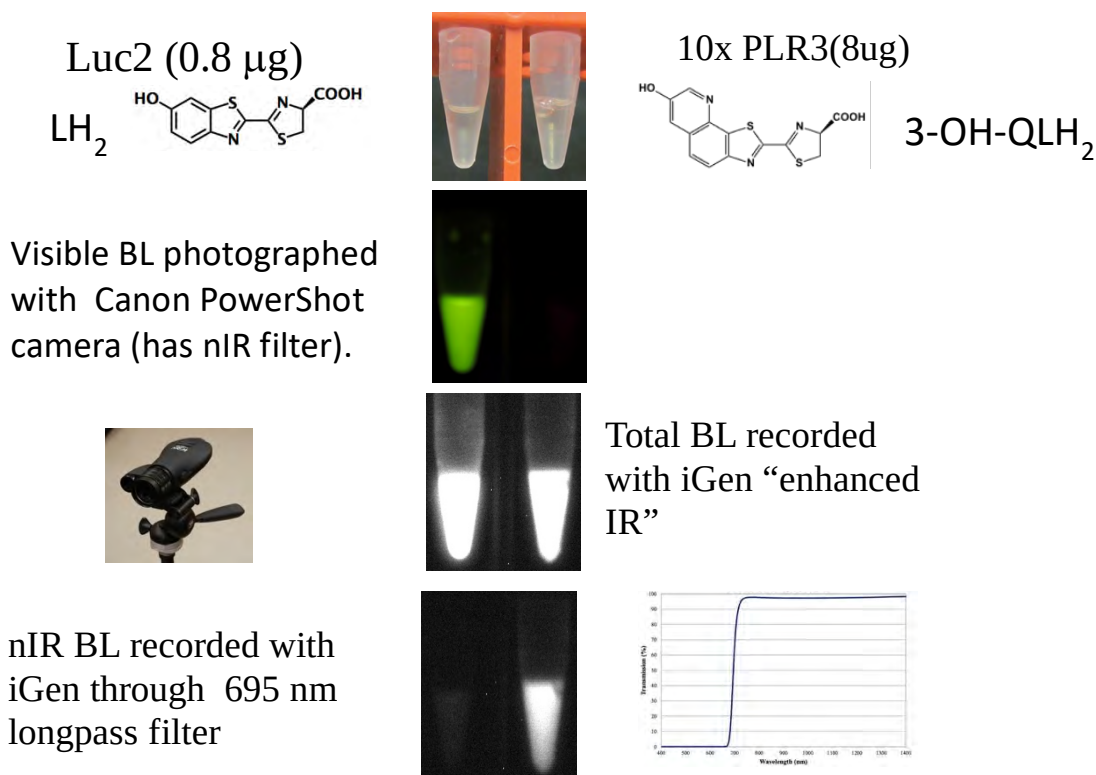
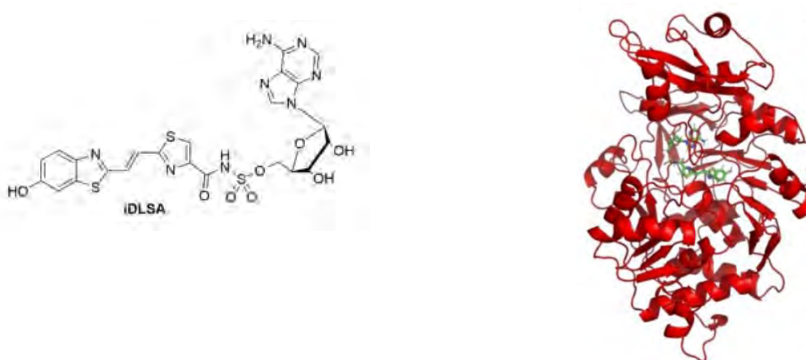


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

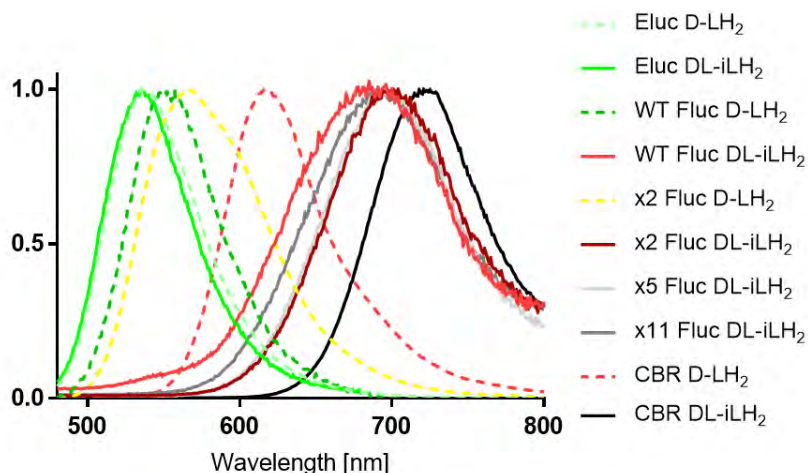


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

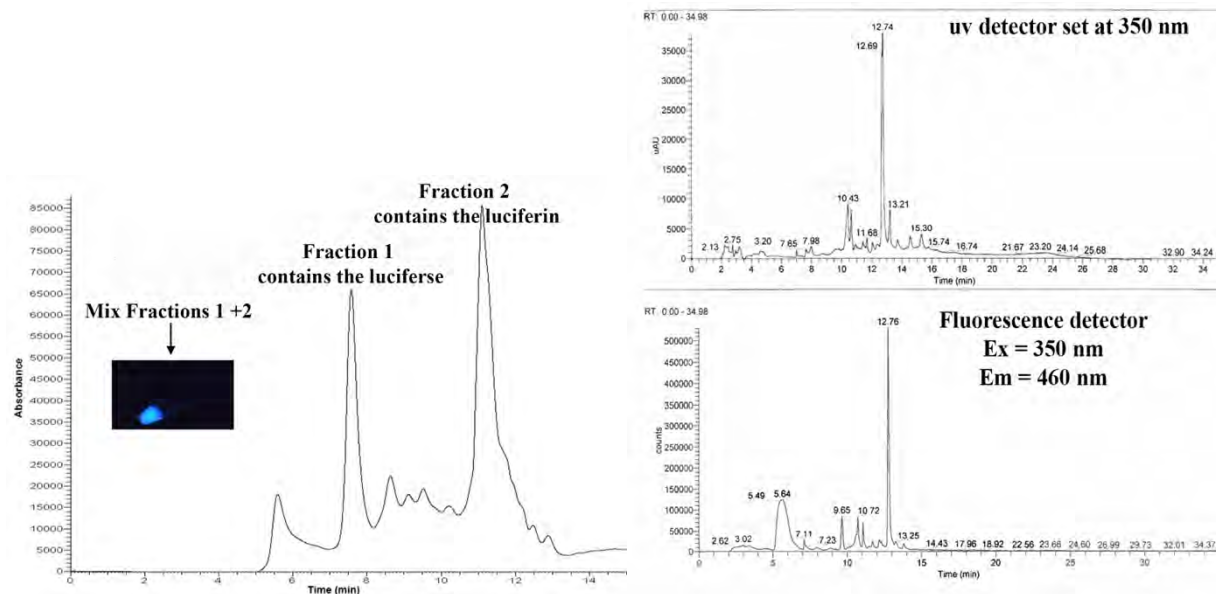
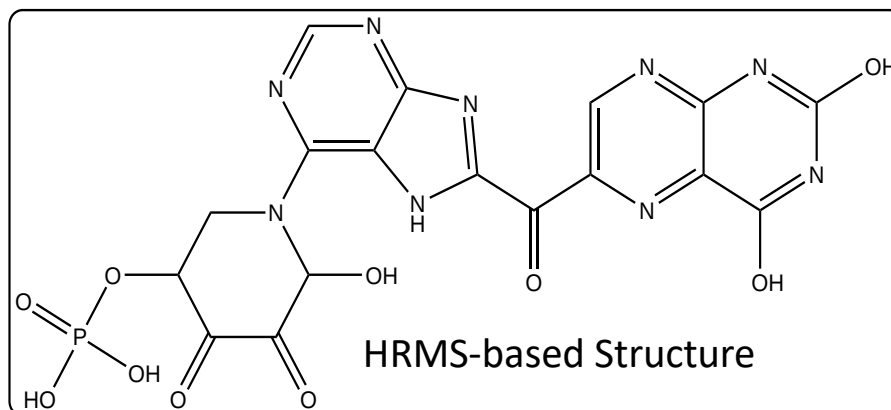


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

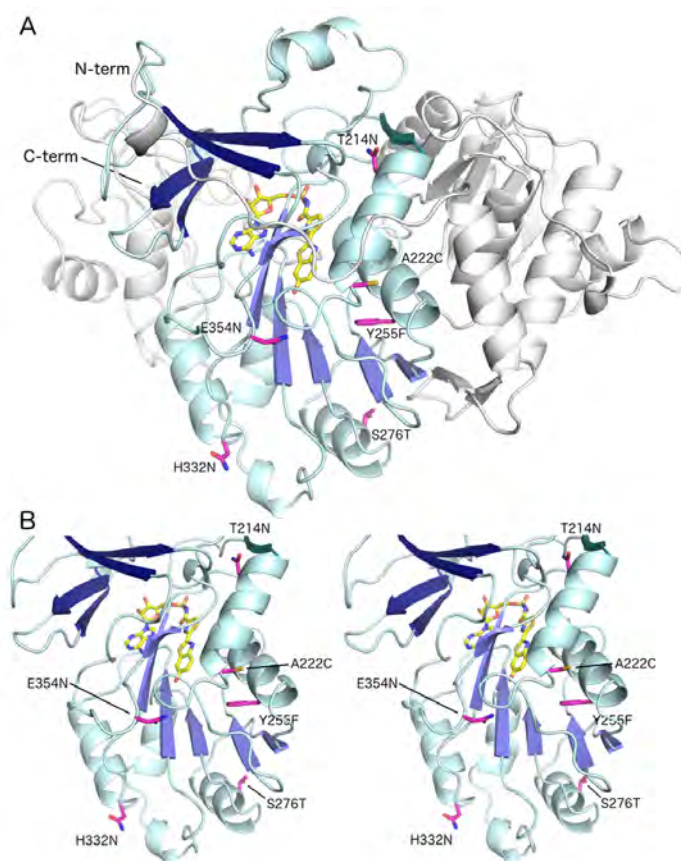


Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Universita degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

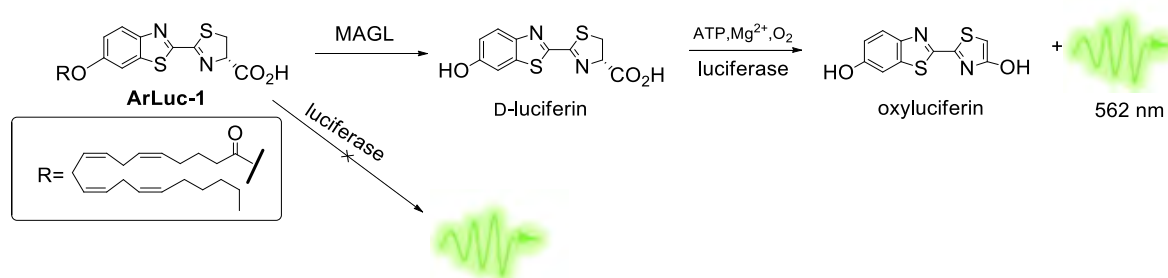


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

Publications

- 1. "Red-emitting chimeric firefly luciferase for in vivo imaging in low ATP cellular environments." Bruce R. Branchini, Tara L. Southworth, Danielle M. Fontaine, Dawn Kohrt, Franceine S. Welcome, Catherine M. Florentine, Emma R. Henricks, Demetria B. DeBartolo, Elisa Michelini, Luca Cevenini, Aldo Roda, Martha J. Grossel, *Analytical Biochemistry*, 2017;534:36–39. doi: 10.1016/j.ab.2017.07.001.**

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COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultoni* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

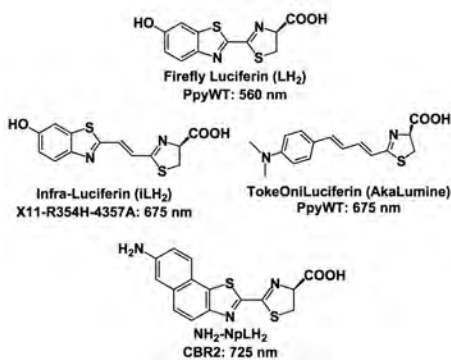


Figure 1. Luciferase-substrate emission maxima.

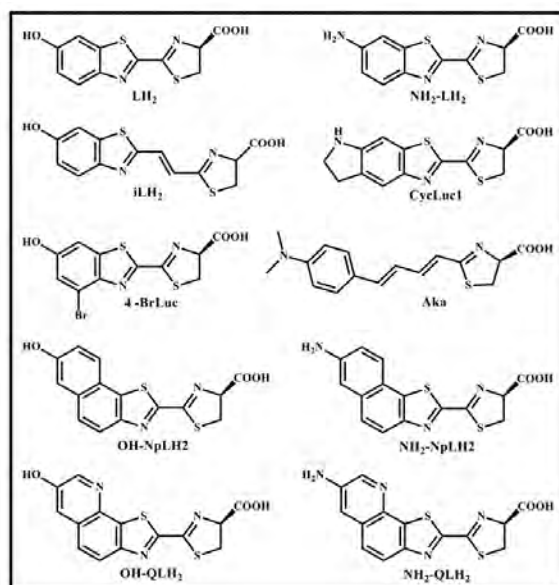
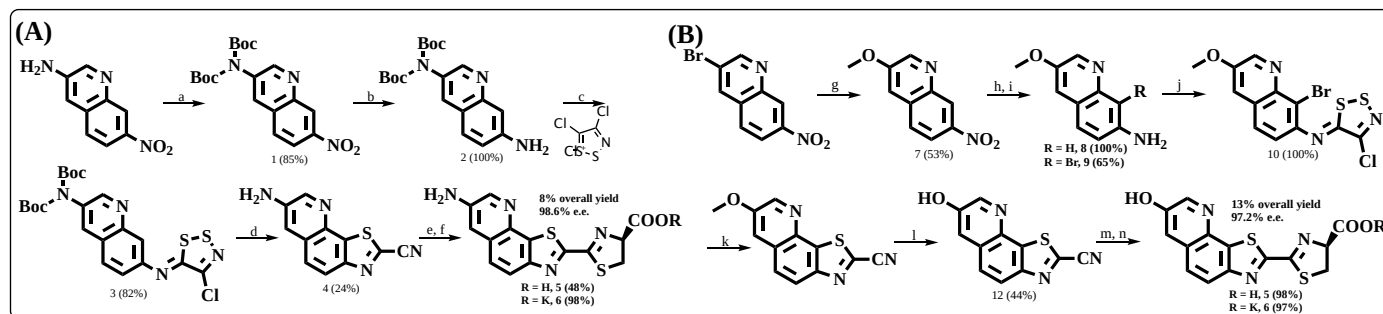


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

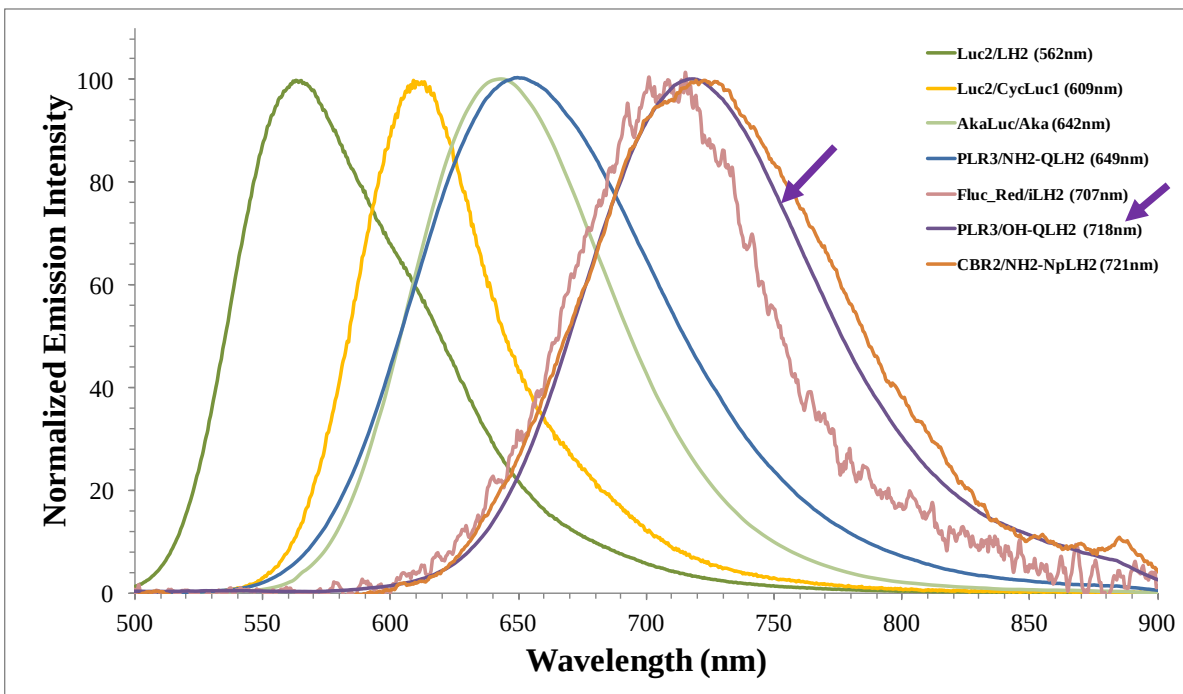


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

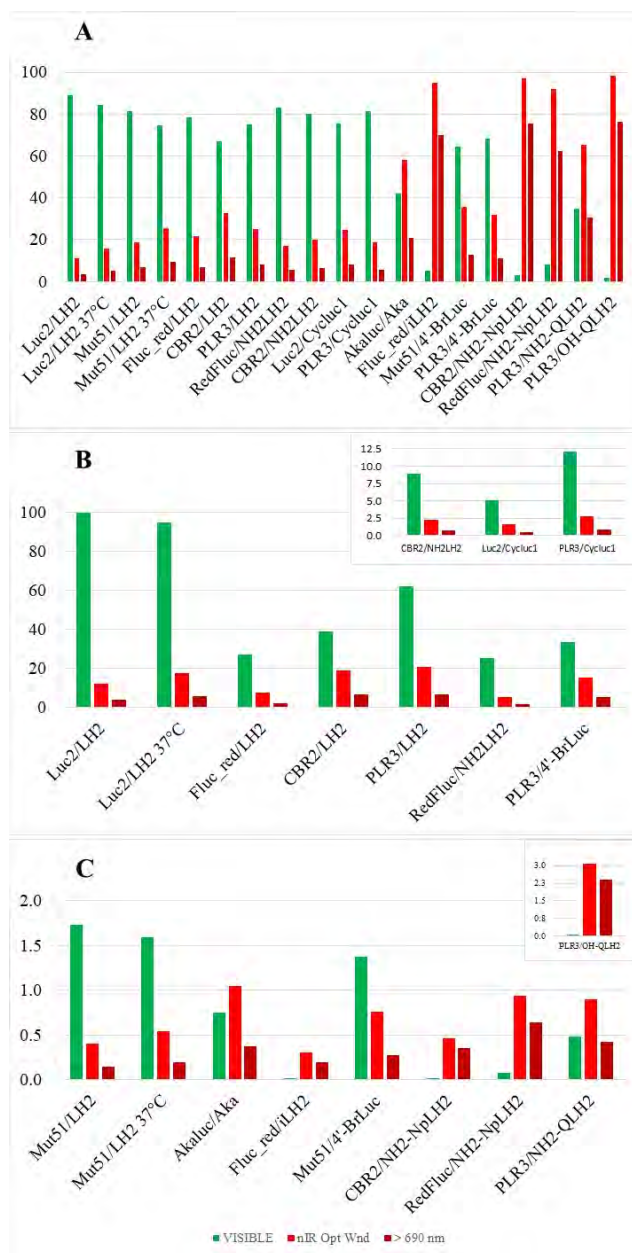


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

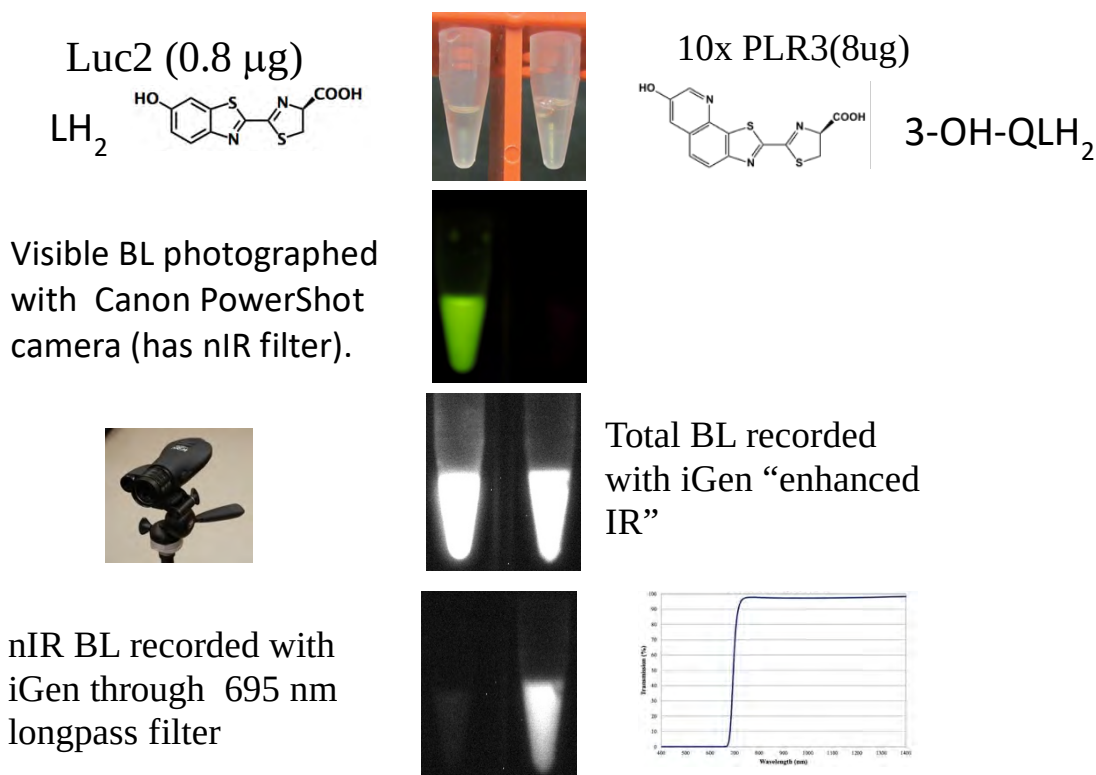
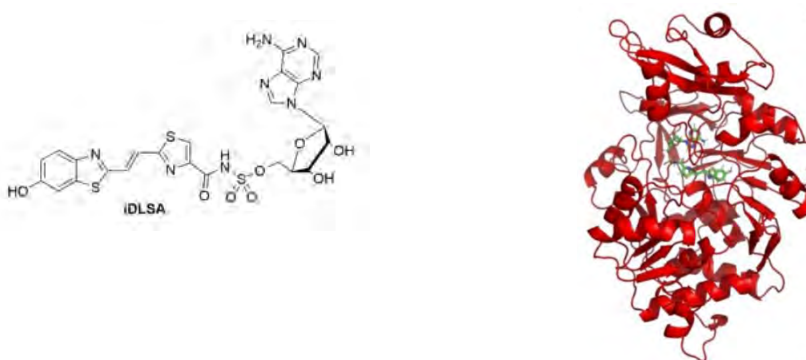


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

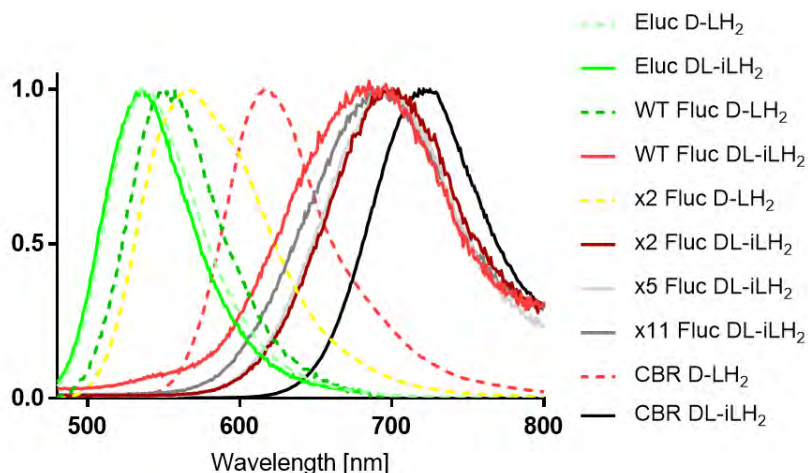


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

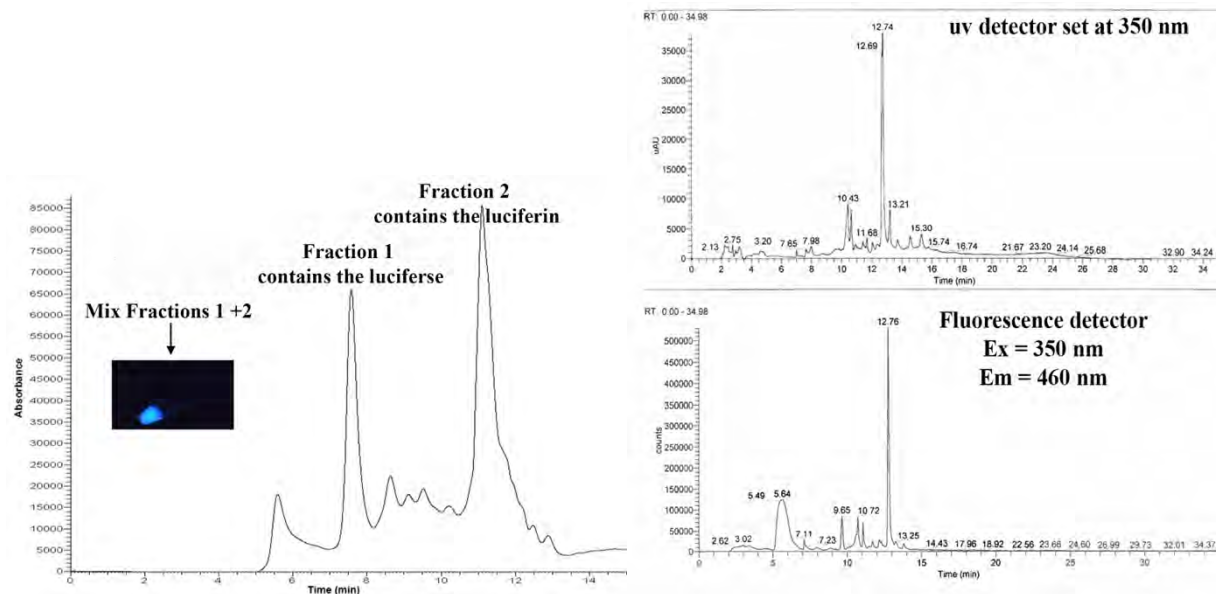
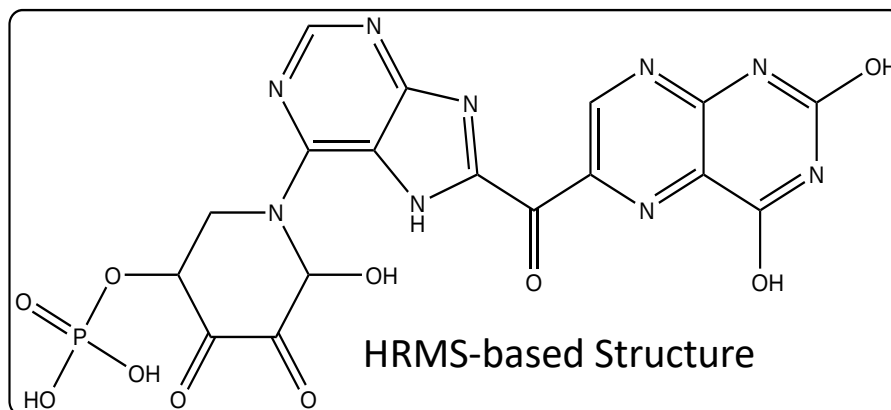


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of Orfelia bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



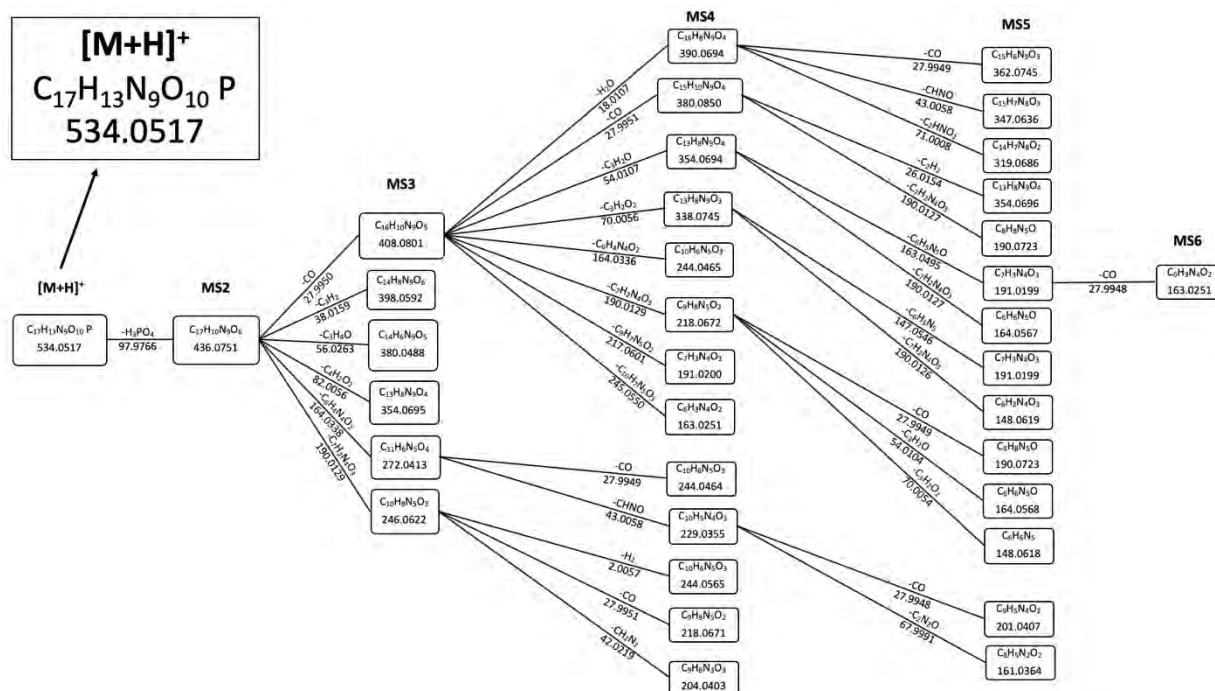


Figure 8. HRMS analysis of putative Orfelina oxyluciferin.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the Orfelina oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

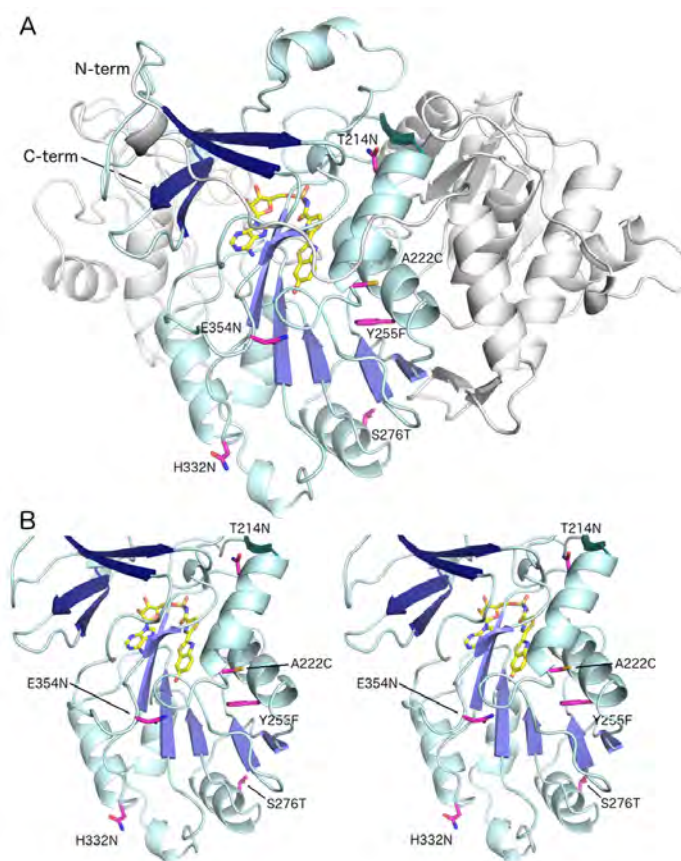


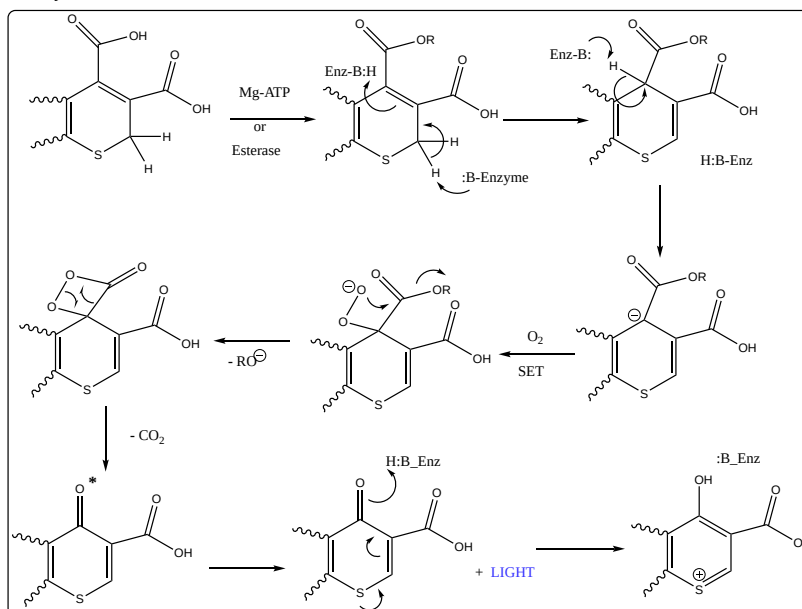
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaetae that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdonata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

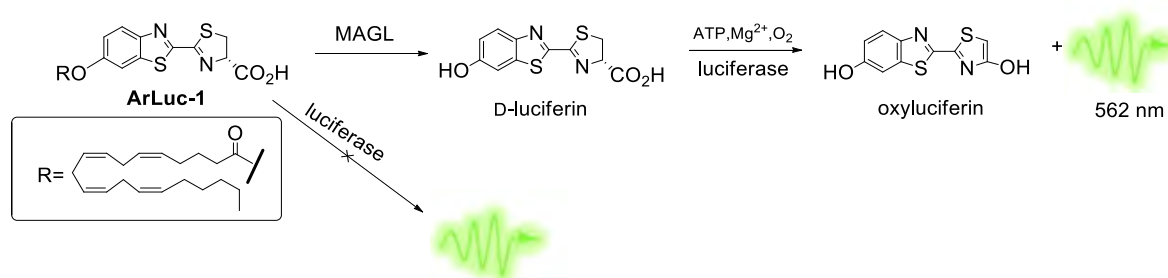


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the Orfelia bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 1. "Red-emitting chimeric firefly luciferase for in vivo imaging in low ATP cellular environments." Bruce R. Branchini, Tara L. Southworth, Danielle M. Fontaine, Dawn Kohrt, Franceine S. Welcome, Catherine M. Florentine, Emma R. Henricks, Demetria B. DeBartolo, Elisa Michelini, Luca Cevenini, Aldo Roda, Martha J. Grossel, *Analytical Biochemistry*, 2017;534:36–39. doi: 10.1016/j.ab.2017.07.001.**

- 2. "A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events." Branchini BR, Southworth TL, Fontaine DM, Kohrt D, Florentine CM, Grossel MJ. *Sci Rep.* 2018; 8(1):5990. doi: 10.1038/s41598-018-24278-2.
- 3. " Mutagenesis and structural studies reveal the basis for the activity and stability properties that distinguish the photinus luciferases scintillans and pyralis." Branchini, B. R., Fontaine, D. M., Southworth, T. L., Huta, B. P., Racela, A., Patel, K. D., and Gulick, A. M. (2019), *Biochemistry* 58, 4293-4303. doi/10.1021/acs.biochem.9b00719
- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
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- 9. “Dendritic cells maintain anti-tumor immunity by positioning CDB skin-resident memory T cells.” J. L. Vella, A. Molodtskov, C. V. Angeles, B. R. Branchini, M. J. Turk, and Y. H. Huang, *Life Science Alliance* 4 (10), e202101056 (2021).
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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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*** Corresponding author inadvertently left out AFOSR acknowledgement.

COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

Award Number: FA9550-18-1-0017

Report Type: Final Report

Reporting Period: Start 15 October 2017 - End 14 October 2022

Distribution Statement: Approved for Public release

Program Officer: Bennett L. Ibey

Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferin that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

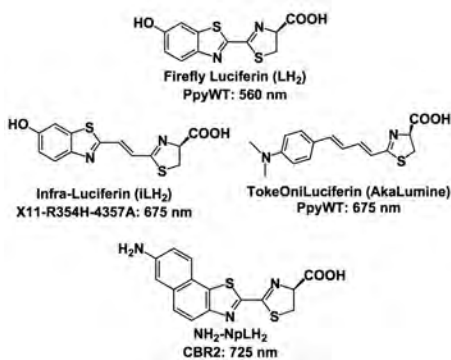


Figure 1. Luciferase-substrate emission maxima.

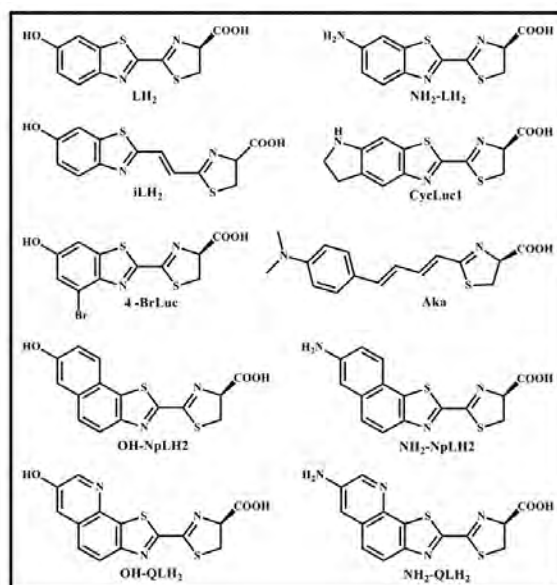
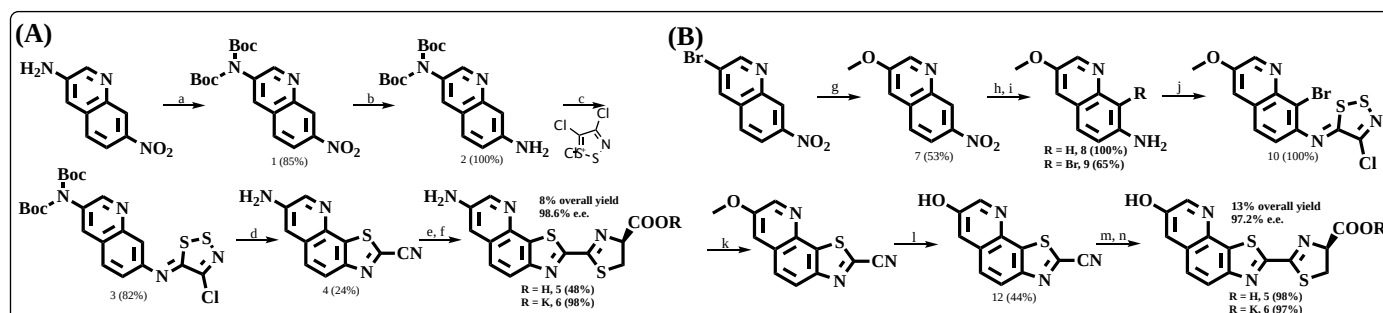


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

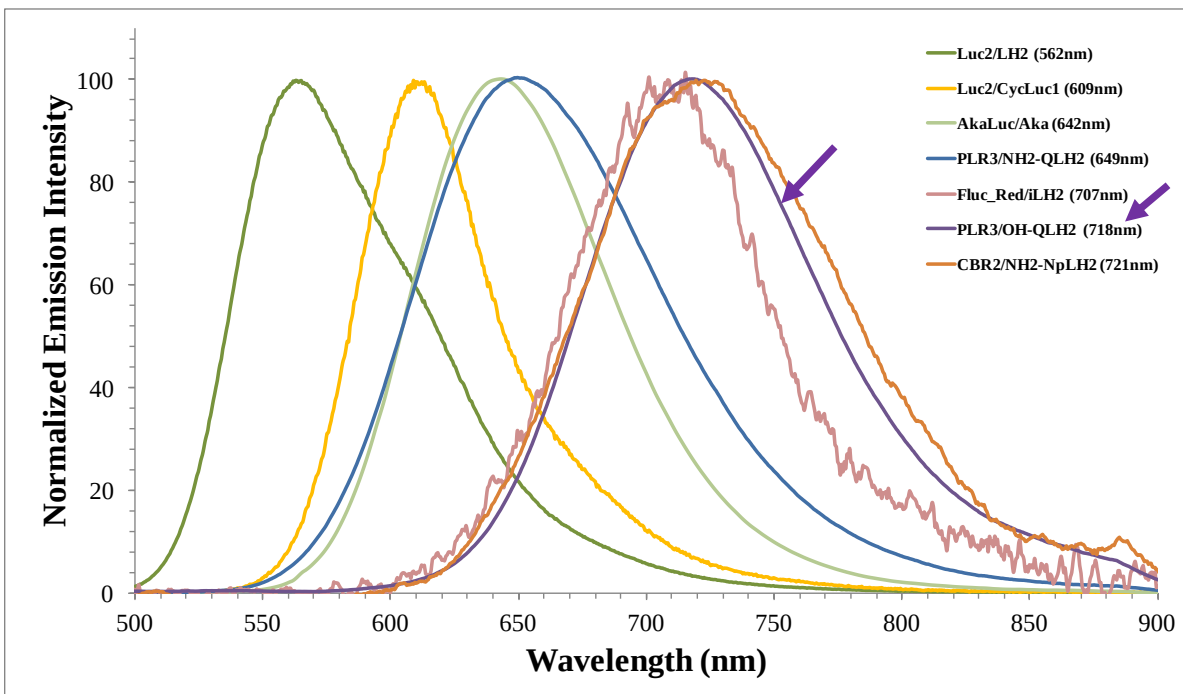


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

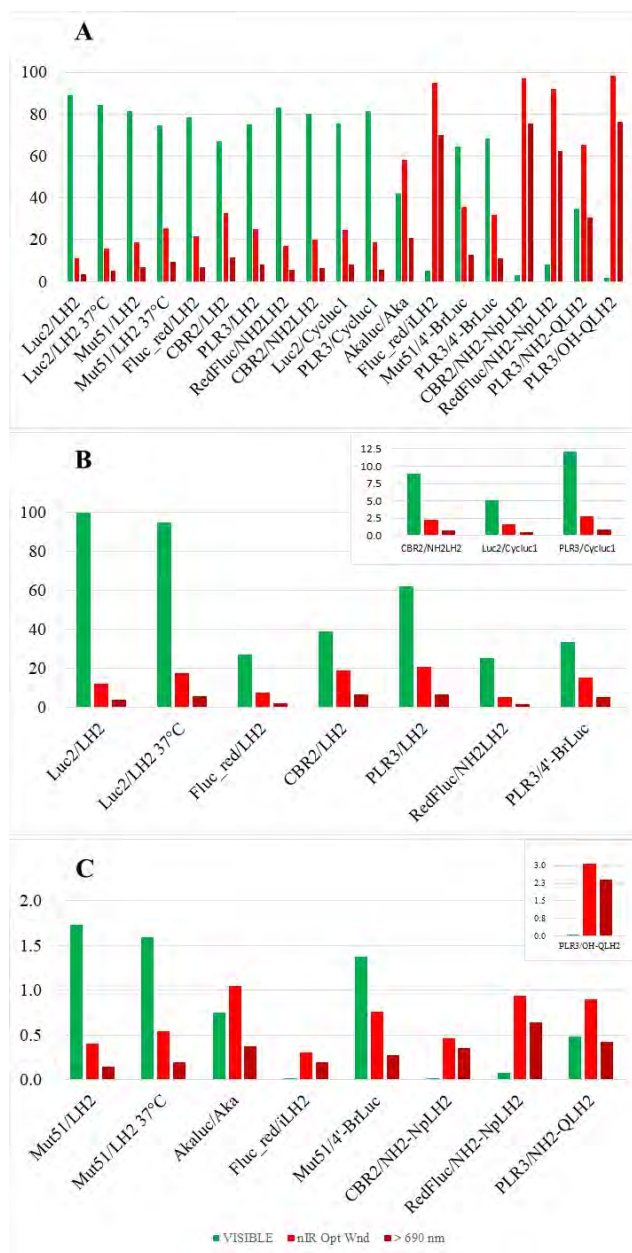


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

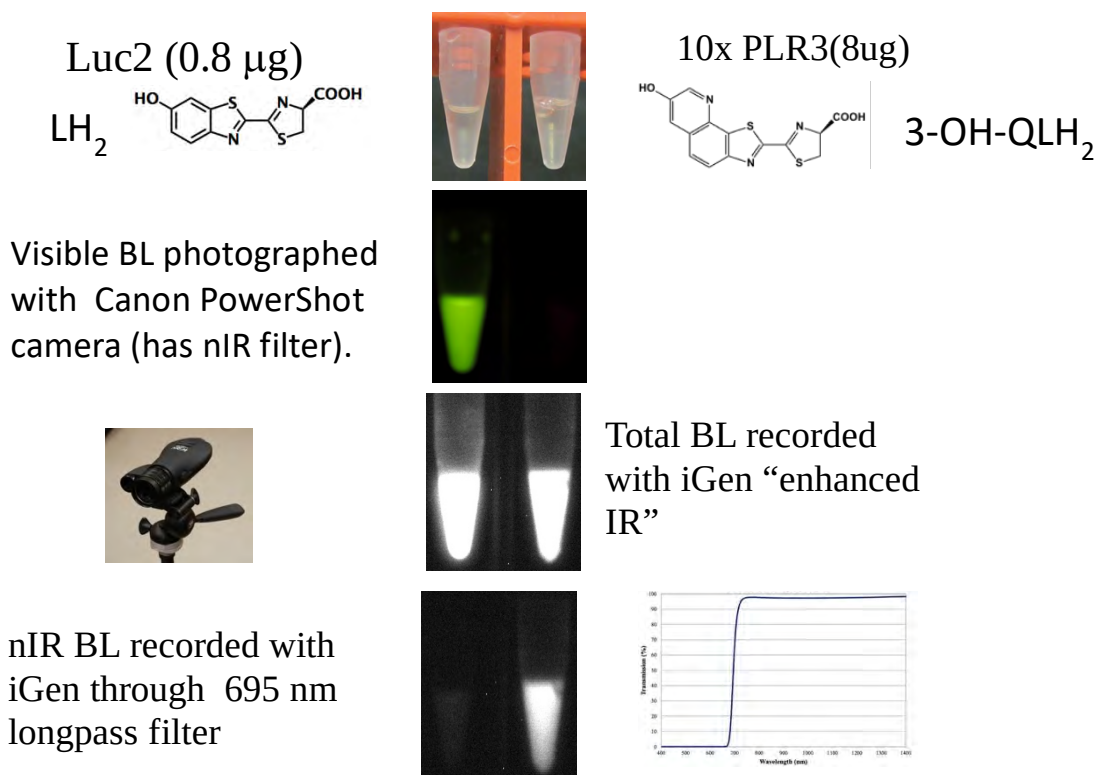
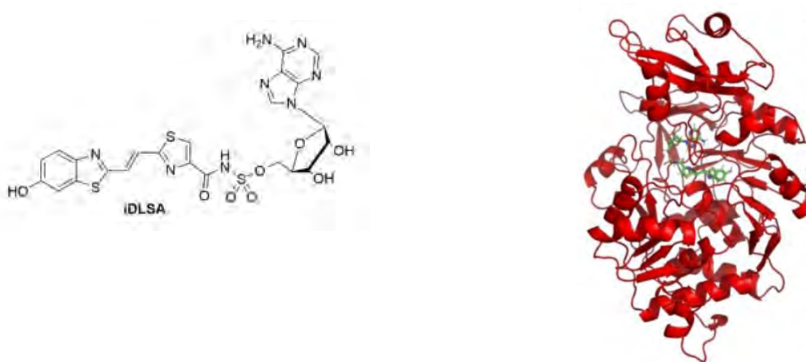


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

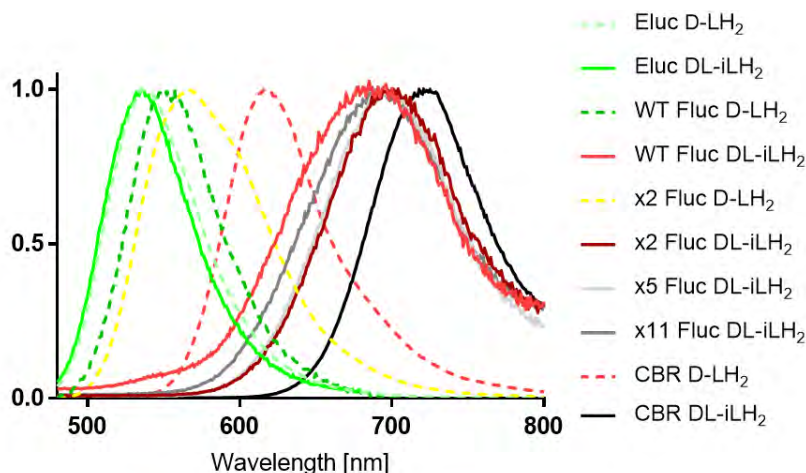


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

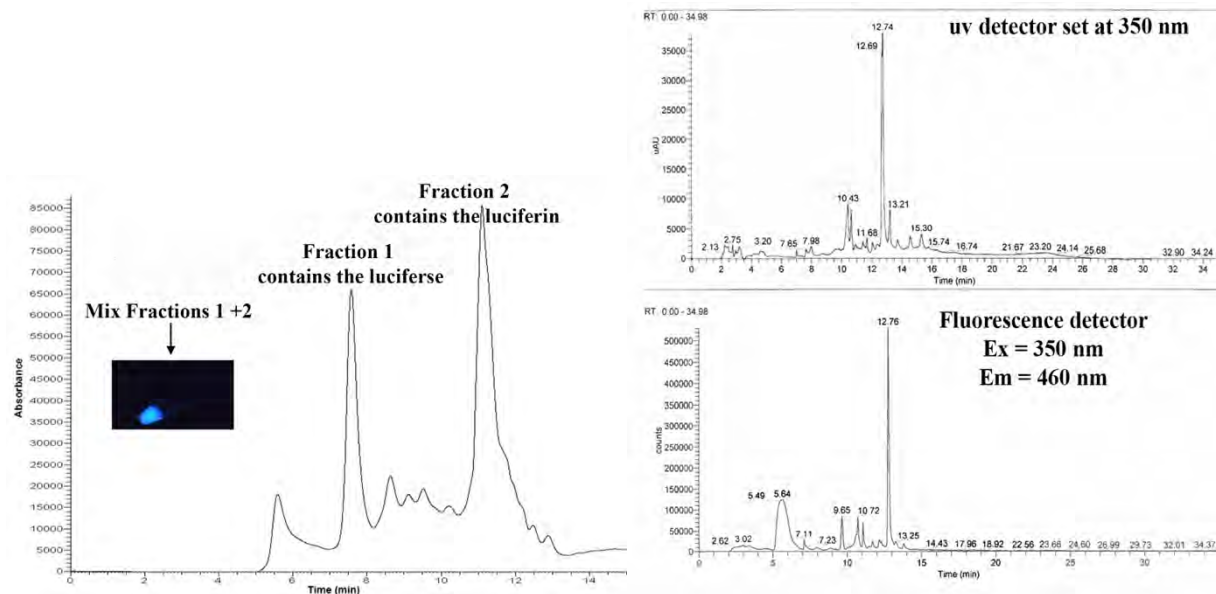
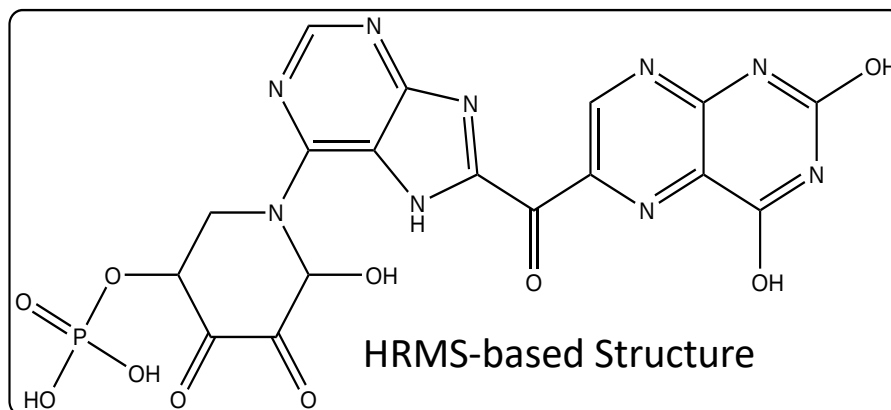


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

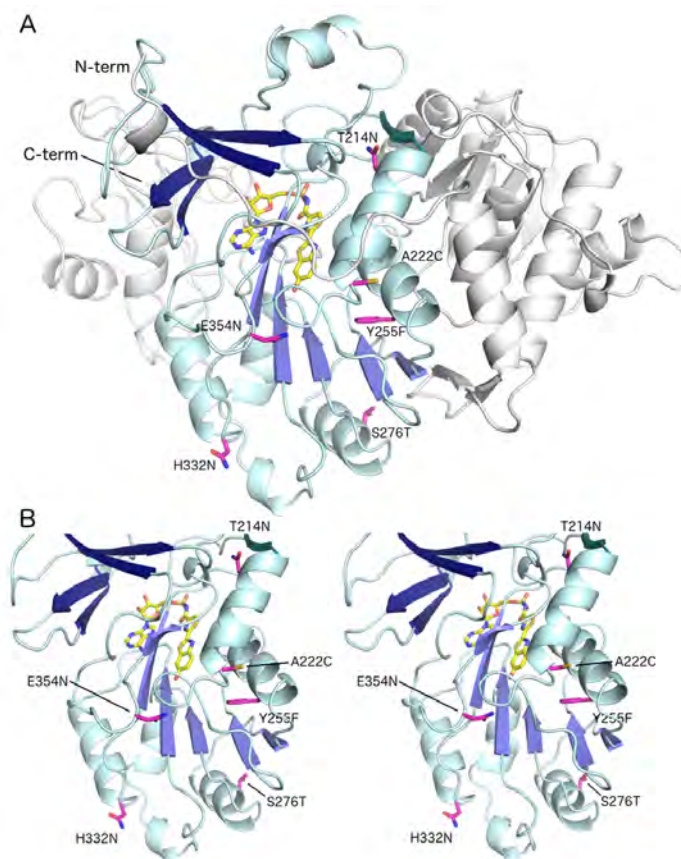


Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

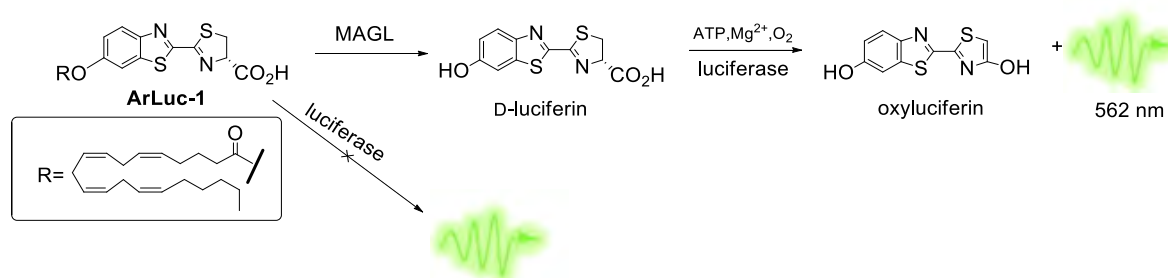


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the *Orfelia* bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 2. "A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events." Branchini BR, Southworth TL, Fontaine DM, Kohrt D, Florentine CM, Grossel MJ. *Sci Rep.* 2018; 8(1):5990. doi: 10.1038/s41598-018-24278-2.
- 3. " Mutagenesis and structural studies reveal the basis for the activity and stability properties that distinguish the photinus luciferases scintillans and pyralis." Branchini, B. R., Fontaine, D. M., Southworth, T. L., Huta, B. P., Racela, A., Patel, K. D., and Gulick, A. M. (2019), *Biochemistry* 58, 4293-4303. doi/10.1021/acs.biochem.9b00719
- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
- 5. "Prêt-à-porter nanoYESA and nanoYESB bioluminescent cell biosensors for ultrarapid and sensitive screening of endocrine-disrupting chemicals." Lopreside, A., Calabretta, M. M., Montali, L., Ferri, M., Tassoni, A., Branchini, B. R., Southworth, T., D'Elia, M., Roda, A., and Michelini, E. (2019) *Anal. Bioanal. Chem.* 411, 4937-4949. doi/10.1007/s00216-019-01805-2
- 6. " Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin." Stowe, C. L., Burley, T. A., Allan, H., Vinci, M., Kramer-Marek, G., Ciobota, D. M., Parkinson, G. N., Southworth, T. L., Agliardi, G., Hotblack, A., Lythgoe, M. F., Branchini, B. R., Kalber, T. L., Anderson, J. C., and Pule, M. A. (2019) *eLife* 8. doi/10.7554/eLife.45801
- 7. "A new brilliantly blue-emitting luciferin- luciferase system from *Orfelia fultoni* and *Keroplantinae* (Diptera)." V.R. Viviani, J. R. Silva, D. T. Amaral, V. R. Bevilaqua, F. C. Abdalla, B. R. Branchini, and C. H. Johnson (2020) *Sci. Rep.*, 10:9608 | <https://doi.org/10.1038/s41598-020-66286-1>
- 8. "A new ultrasensitive bioluminescence -based method for assaying monoacylglycerol lipase." M. Miceli, S. Casati, P. Allevi, S. Berra, R. Ottria, P. Rota, B.R. Branchini, and P. Ciuffreda, *Int. J. Mol. Sci.*, 22, 6148 (2021) <https://doi.org/10.3390/ijms22116148>

- 9. “Dendritic cells maintain anti-tumor immunity by positioning CDB skin-resident memory T cells.” J. L. Vella, A. Molodtskov, C. V. Angeles, B. R. Branchini, M. J. Turk, and Y. H. Huang, *Life Science Alliance* 4 (10), e202101056 (2021).
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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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*** Corresponding author inadvertently left out AFOSR acknowledgement.

COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

Award Number: FA9550-18-1-0017

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Program Officer: Bennett L. Ibey

Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferin that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

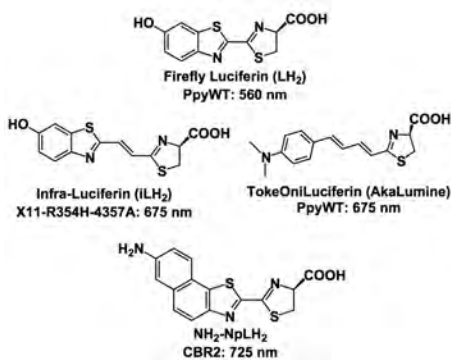


Figure 1. Luciferase-substrate emission maxima.

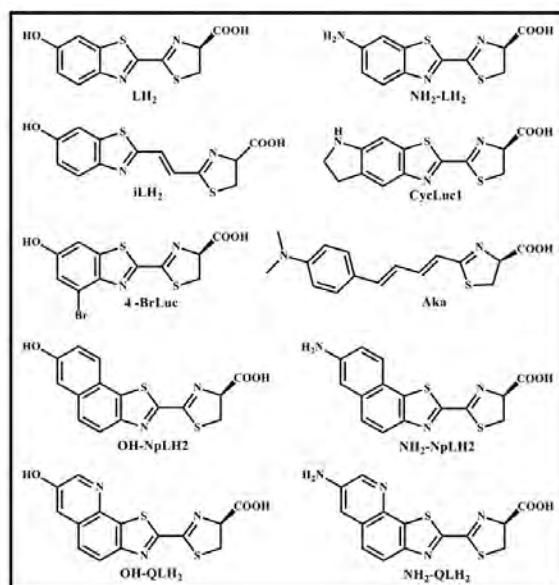
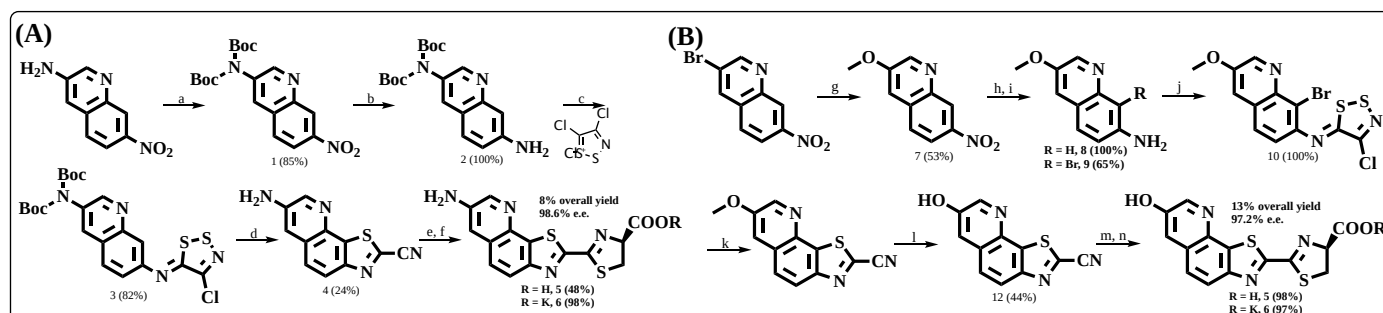


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

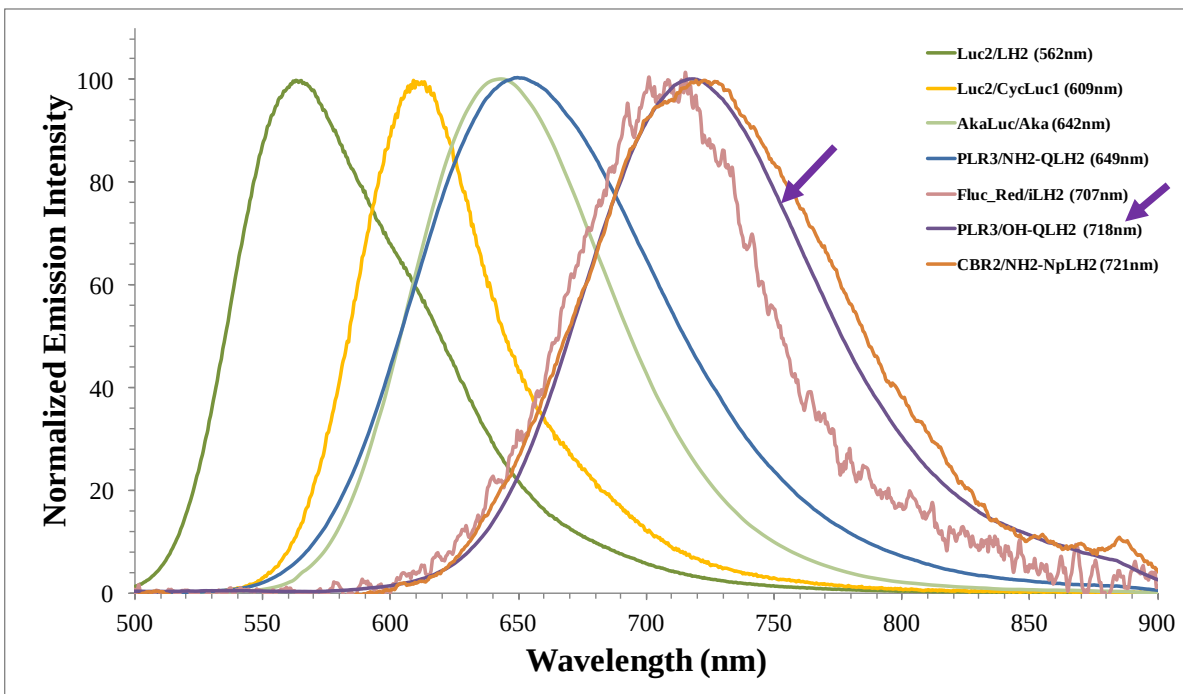


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

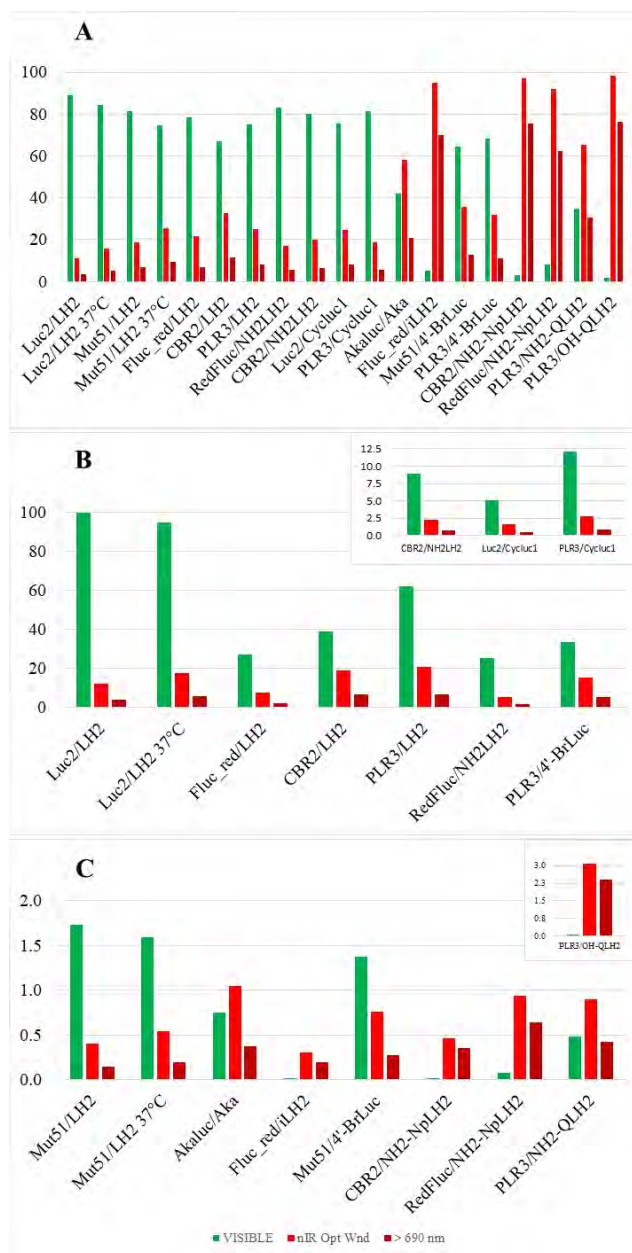


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

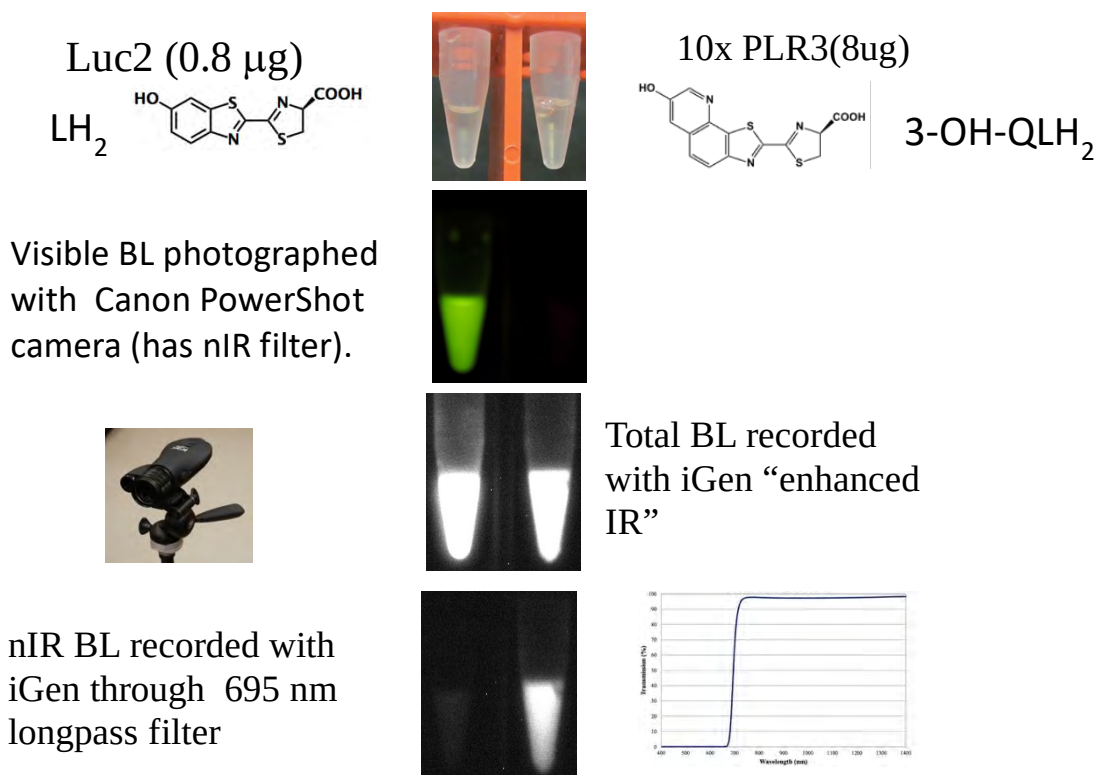
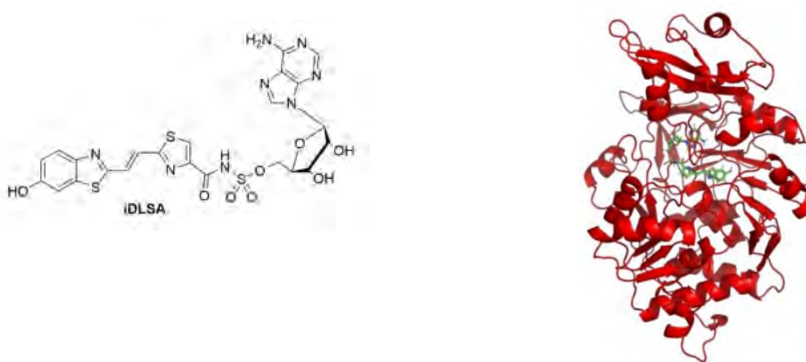


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

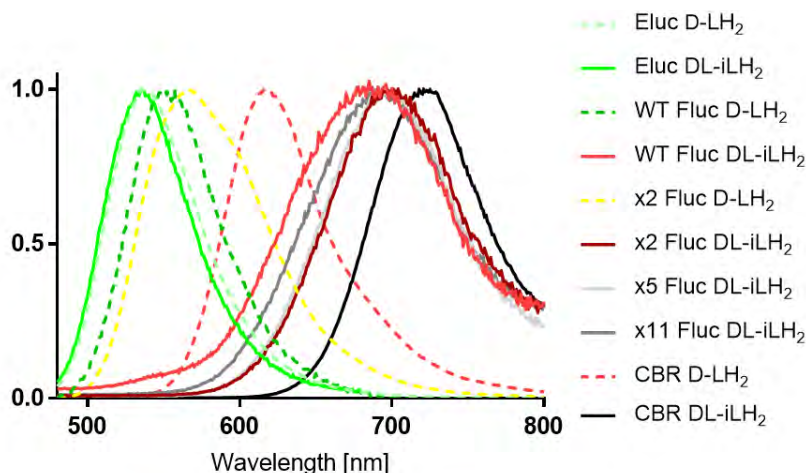


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

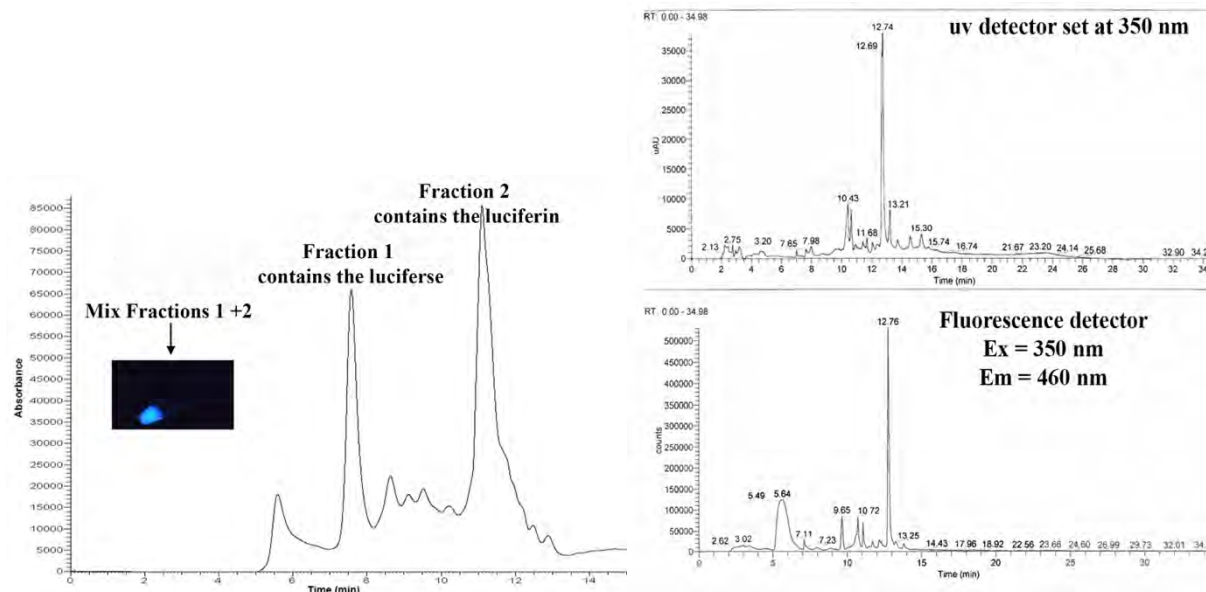
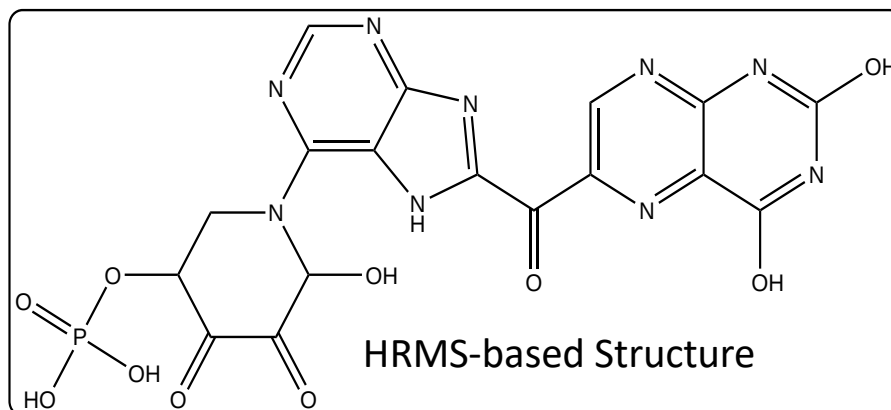


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of Orfelia bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



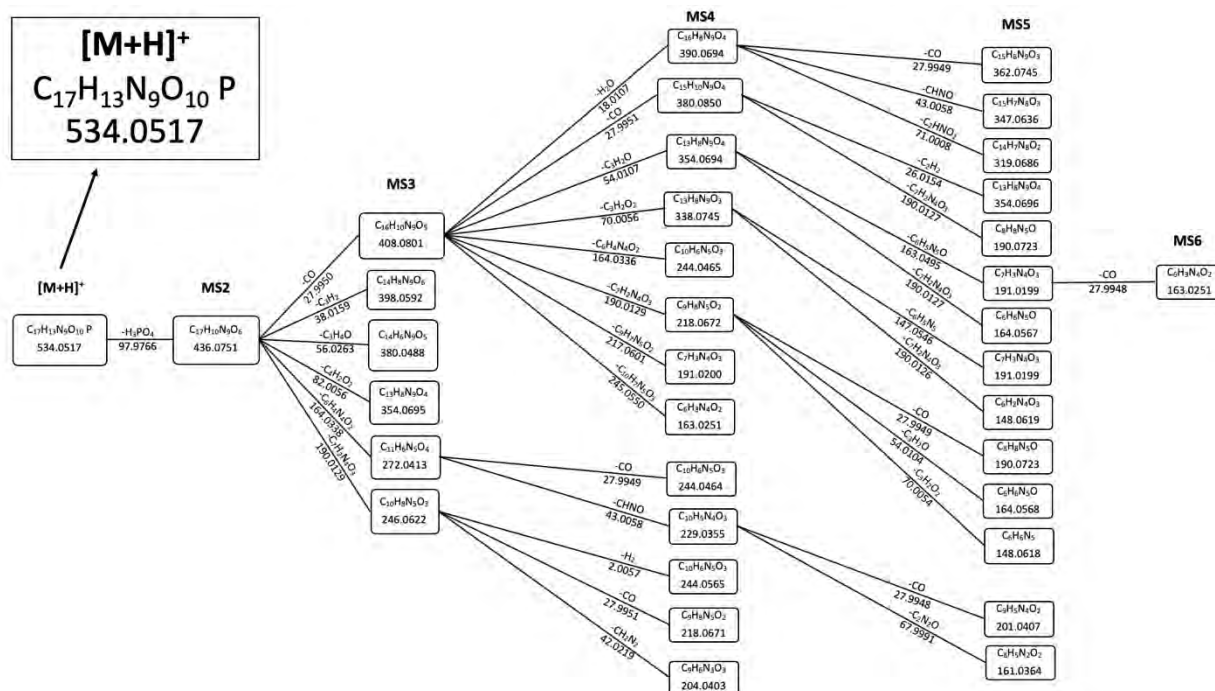


Figure 8. HRMS analysis of putative Orfelia oxyluciferin.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the Orfelia oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

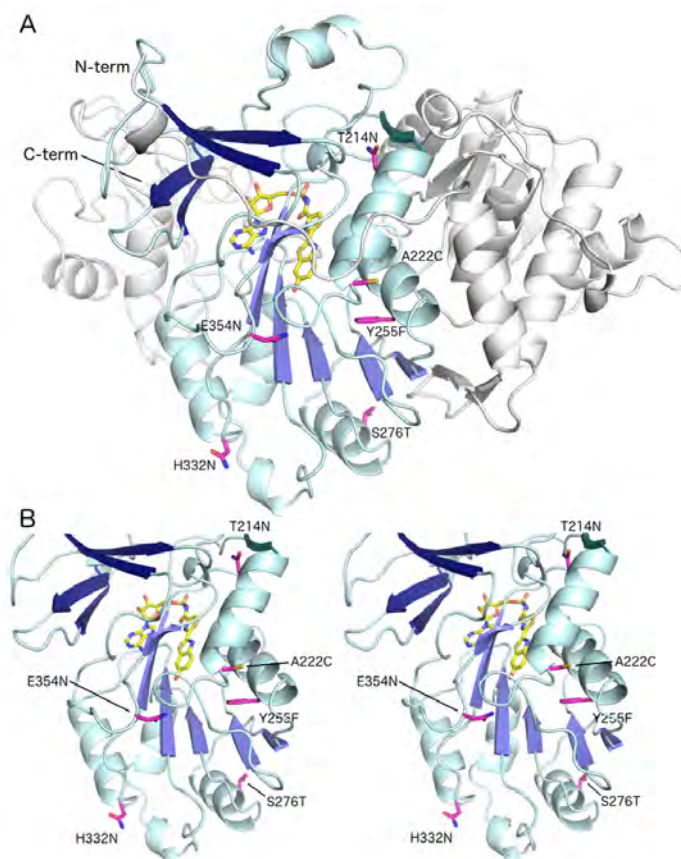


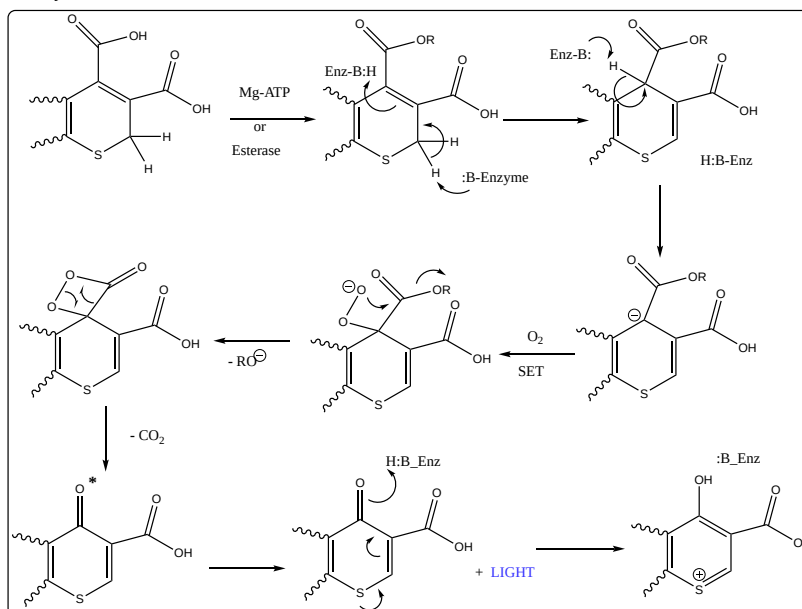
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Universita degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

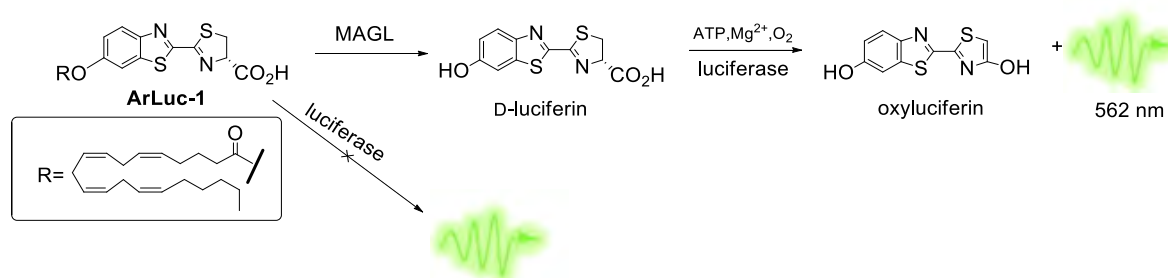


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the *Orfelia* bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 x g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18-20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at -80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
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- 6. " Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin." Stowe, C. L., Burley, T. A., Allan, H., Vinci, M., Kramer-Marek, G., Ciobota, D. M., Parkinson, G. N., Southworth, T. L., Agliardi, G., Hotblack, A., Lythgoe, M. F., Branchini, B. R., Kalber, T. L., Anderson, J. C., and Pule, M. A. (2019) *eLife* 8. doi/10.7554/eLife.45801
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- 8. "A new ultrasensitive bioluminescence -based method for assaying monoacylglycerol lipase." M. Miceli, S. Casati, P. Allevi, S. Berra, R. Ottria, P. Rota, B.R. Branchini, and P. Ciuffreda, *Int. J. Mol. Sci.*, 22, 6148 (2021) <https://doi.org/10.3390/ijms22116148>

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- 11. “Systematic comparison of beetle luciferase-luciferin pairs as sources of Near-Infrared Light for in vitro and in vivo applications.” B. R. Branchini, D. M. Fontaine, D. Kohrt, B. P. Huta, A. R. Racela, B. R. Fort, T. L. Southworth, and A. Roda, *Int. J. Mol. Sci.*, 23, 2451-2465 (2022) <https://doi.org/10.3390/ijms23052451>

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COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

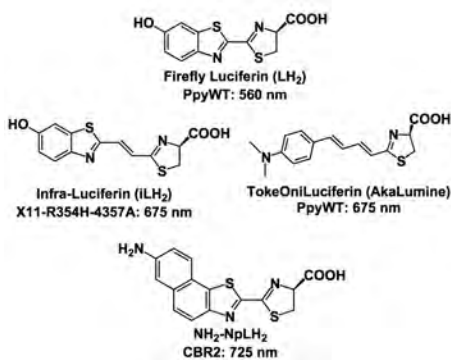


Figure 1. Luciferase-substrate emission maxima.

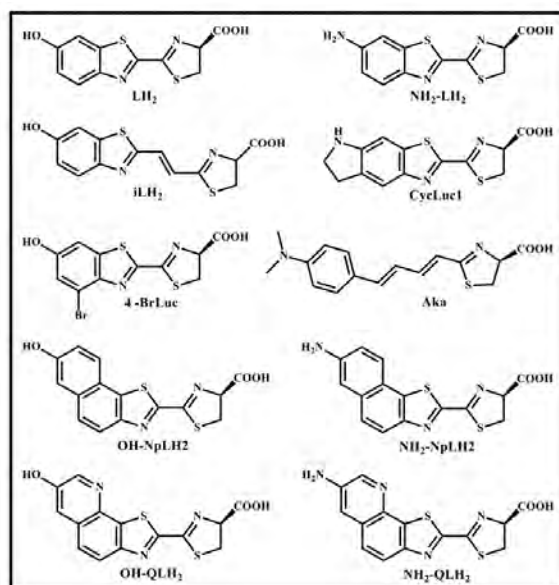
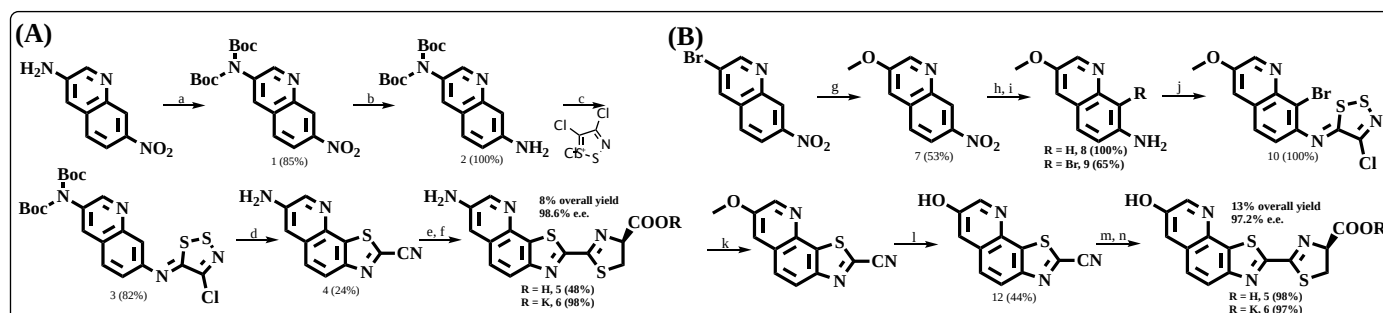


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

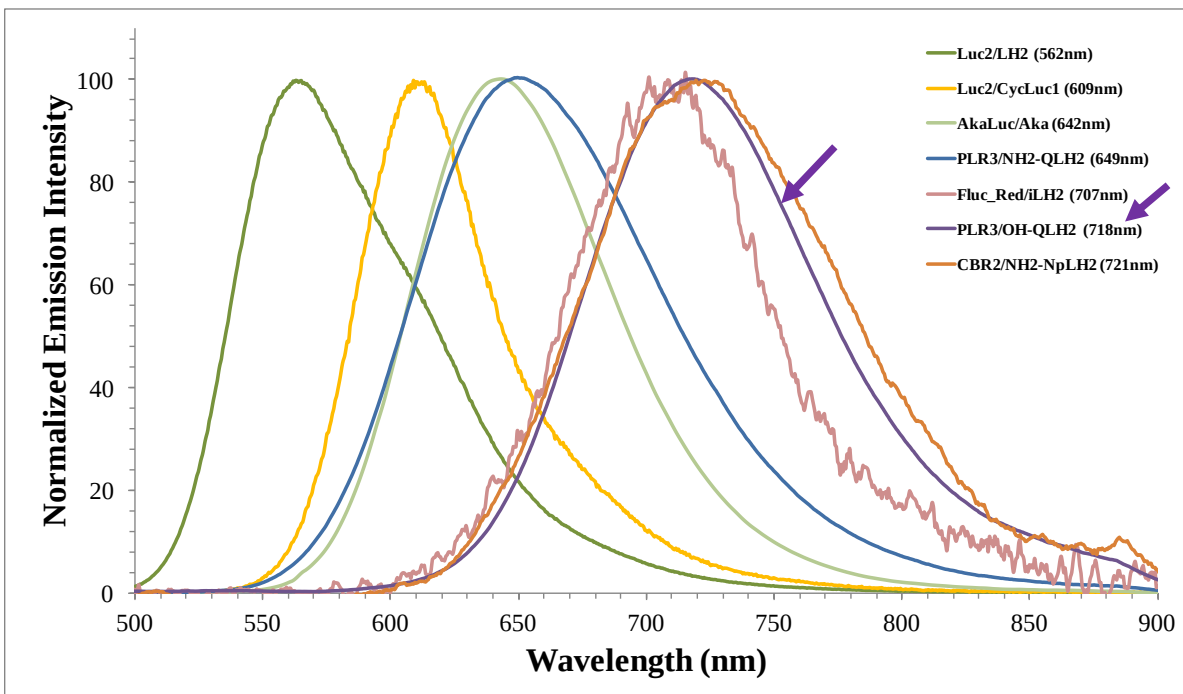


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

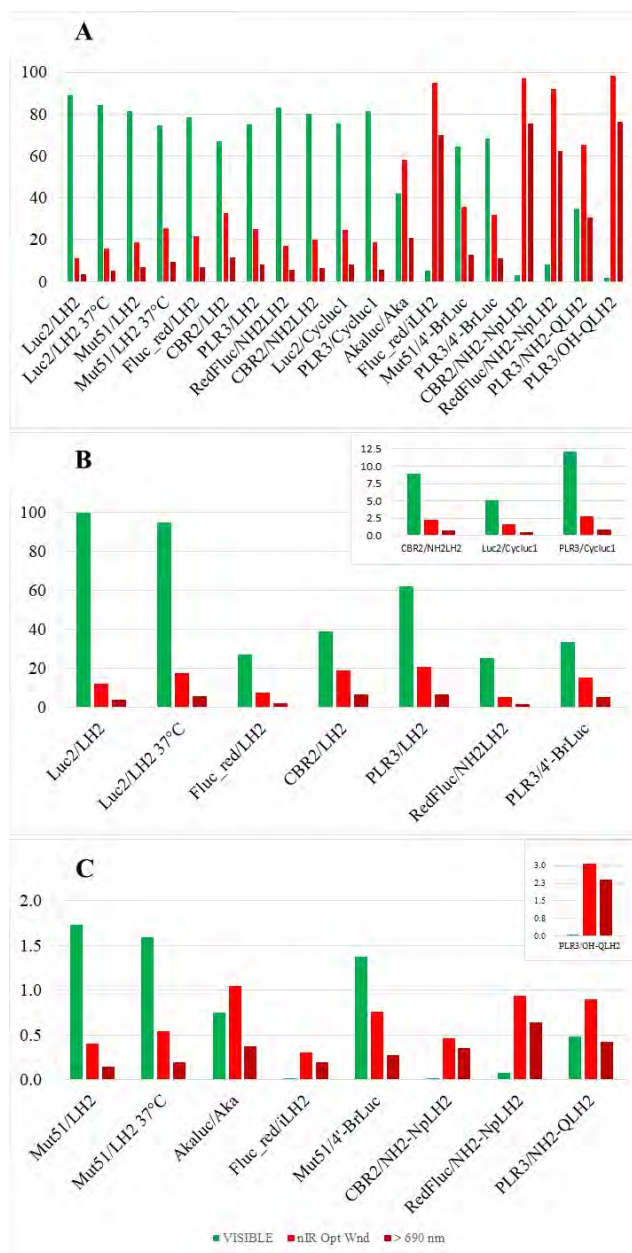


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

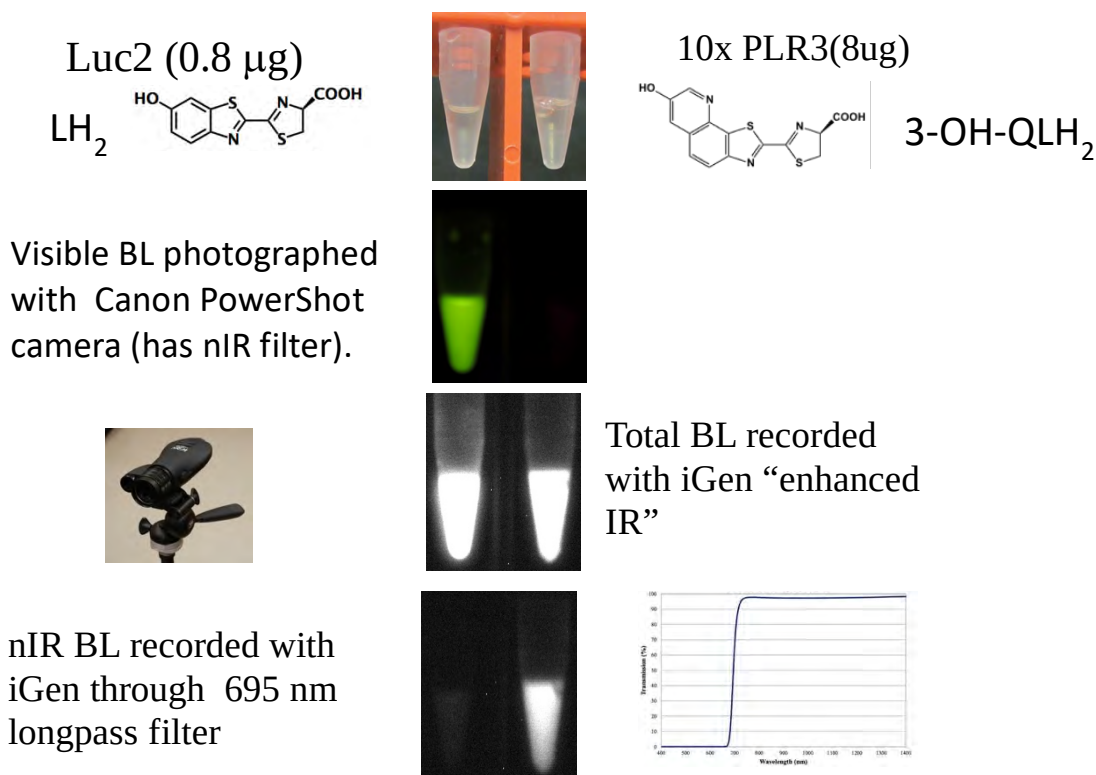
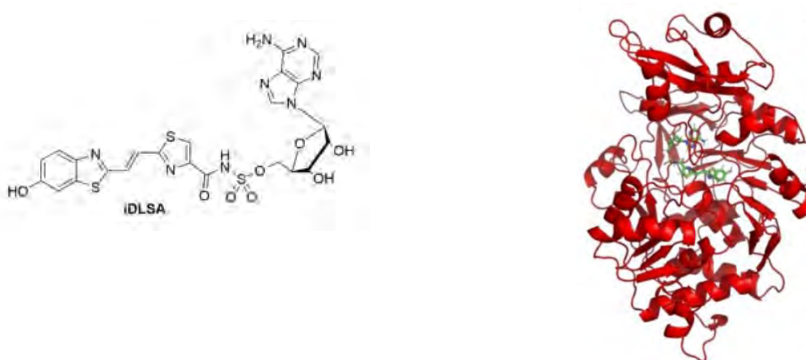


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

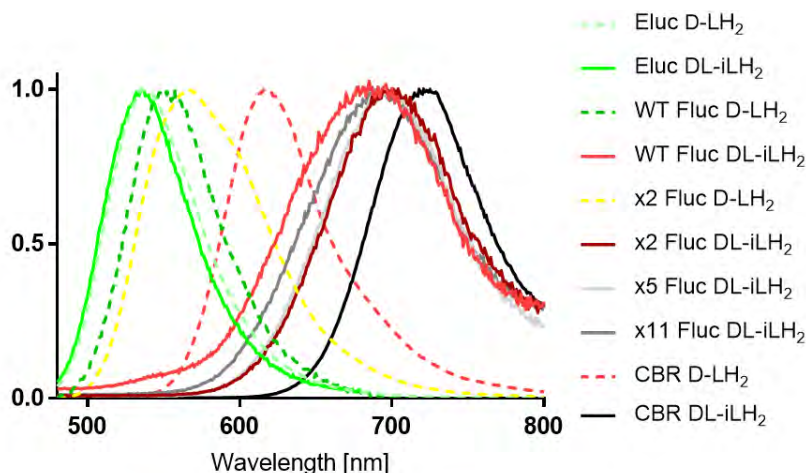


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

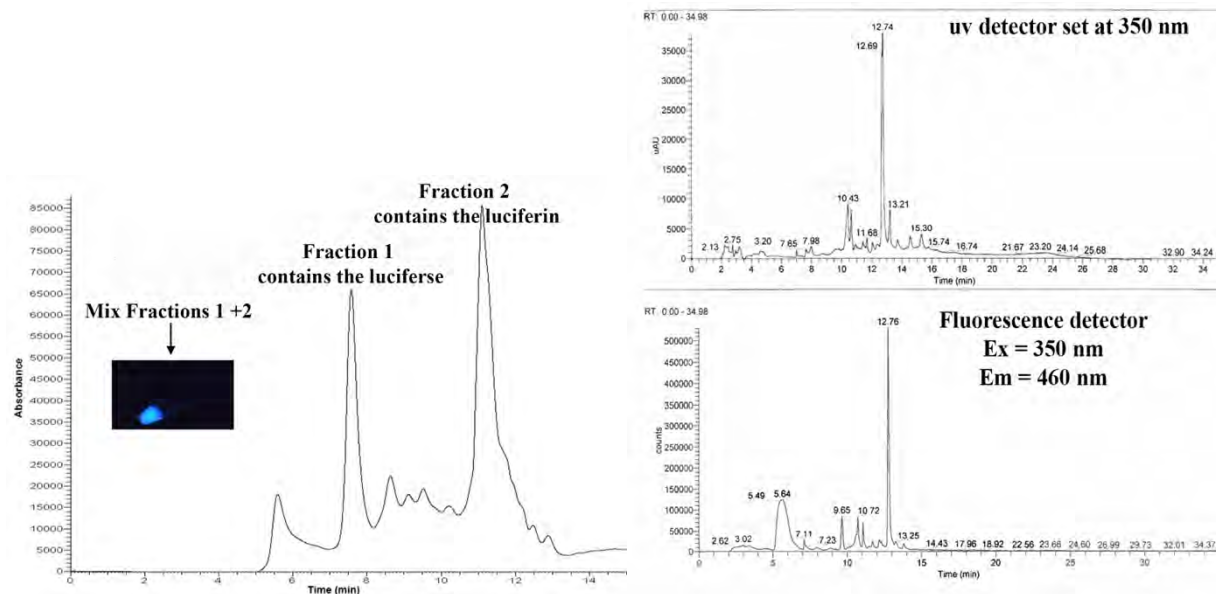
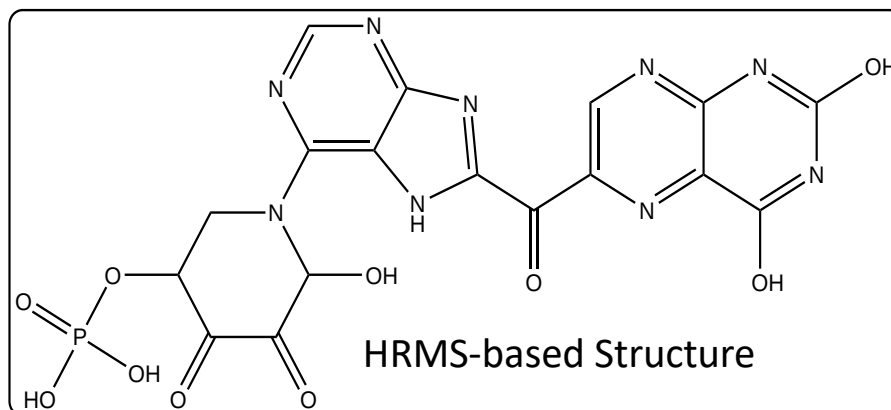


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



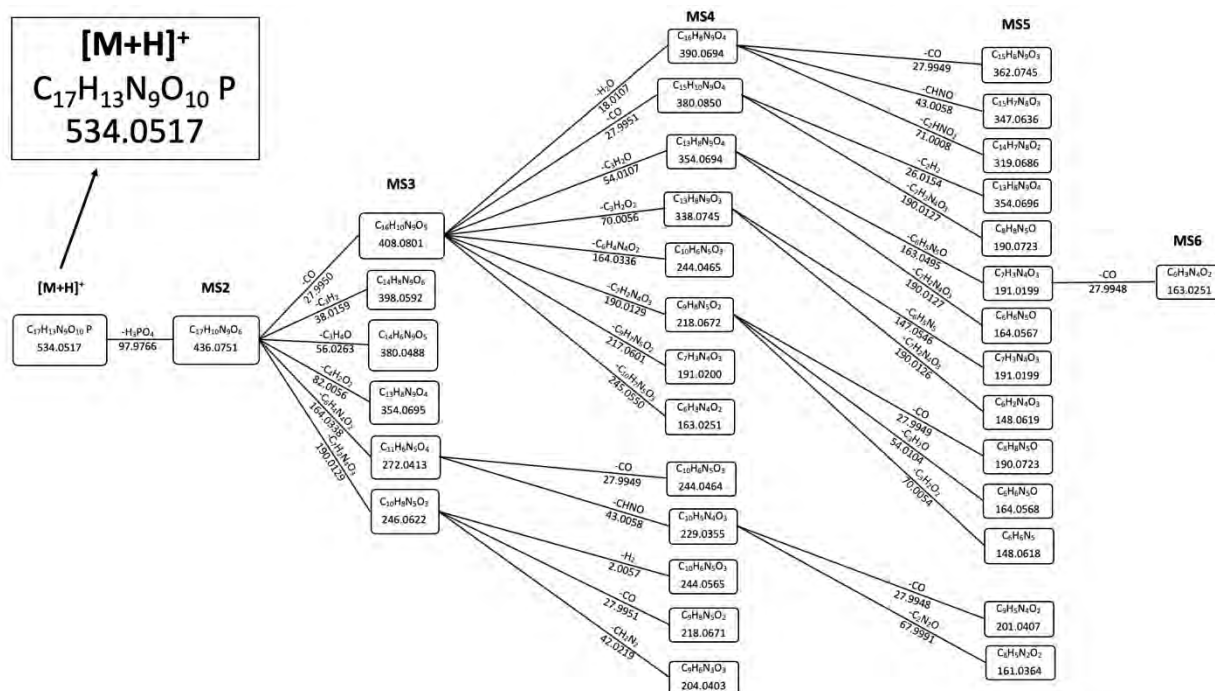


Figure 8. HRMS analysis of putative Orfelia oxyluciferin.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the Orfelia oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

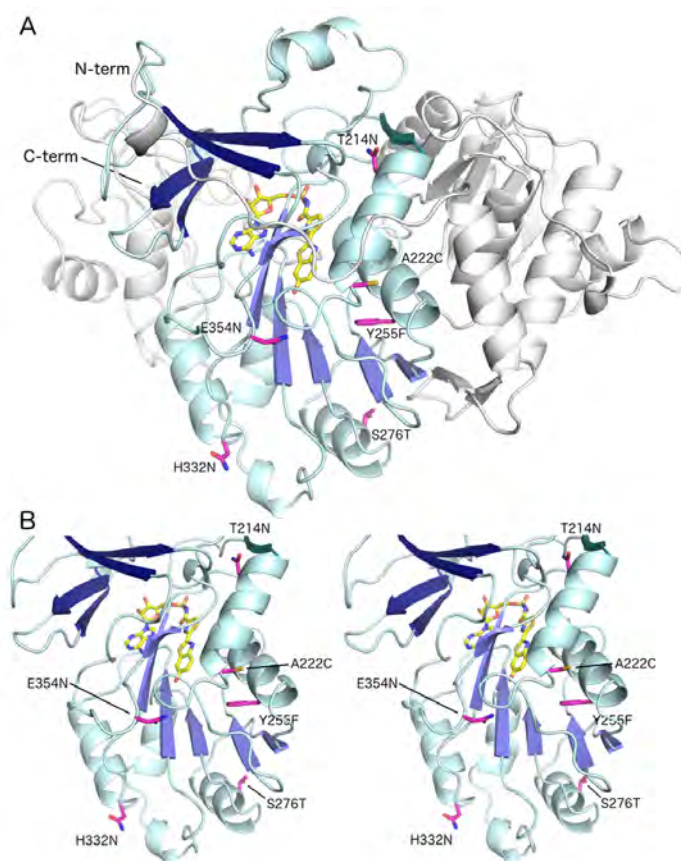


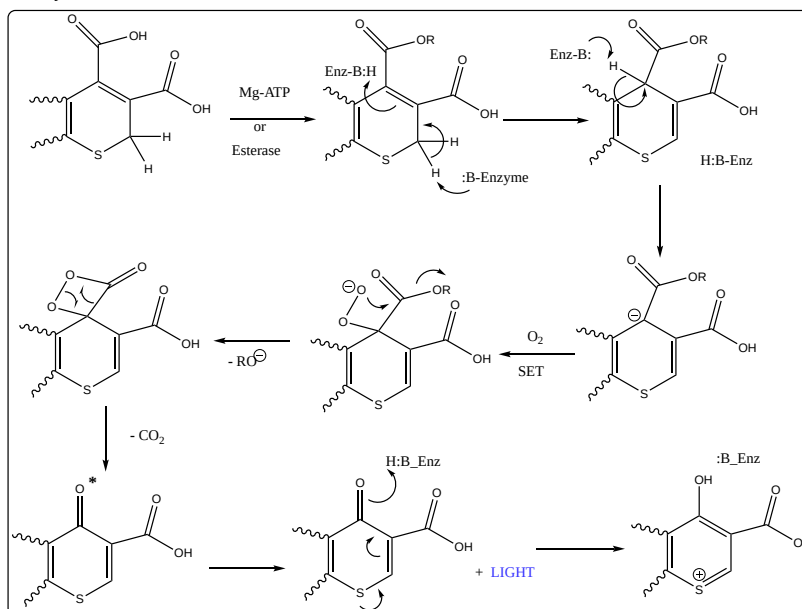
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

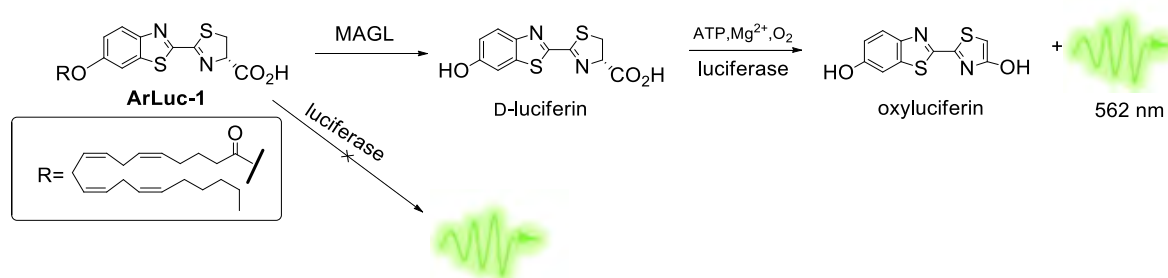


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the *Orfelia* bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All *Luc* gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) *Luc*–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase (*A*₆₀₀ = 0.5-0.7) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of K_m for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). K_m values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

Publications

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- 2. "A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events." Branchini BR, Southworth TL, Fontaine DM, Kohrt D, Florentine CM, Grossel MJ. *Sci Rep.* 2018; 8(1):5990. doi: 10.1038/s41598-018-24278-2.
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COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

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Principal Investigator: Bruce Branchini

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ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

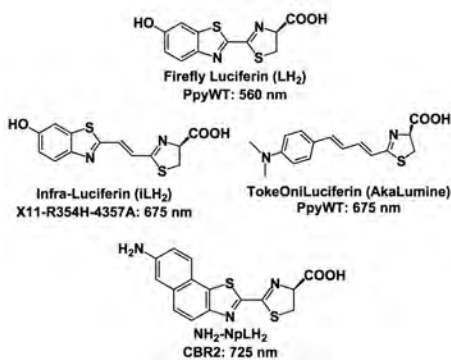


Figure 1. Luciferase-substrate emission maxima.

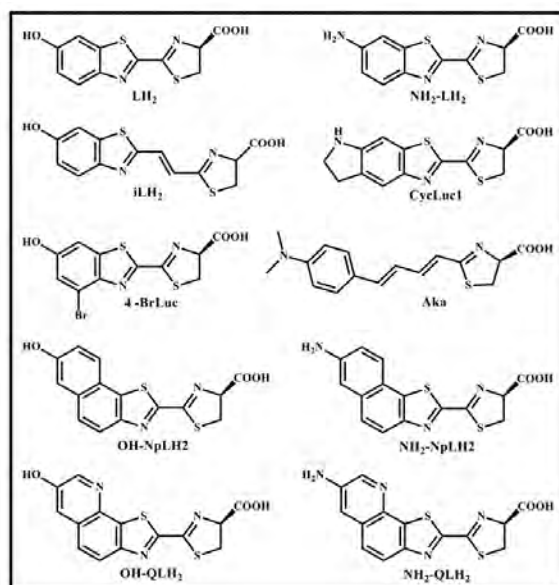
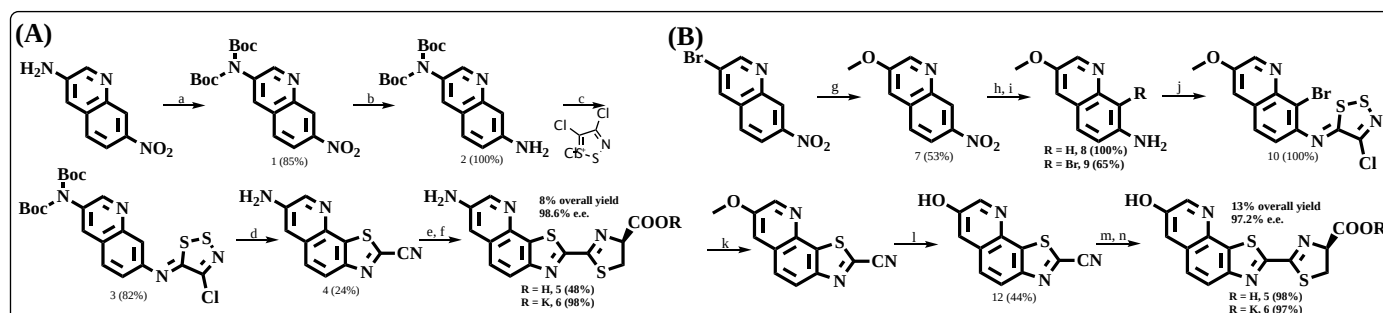


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

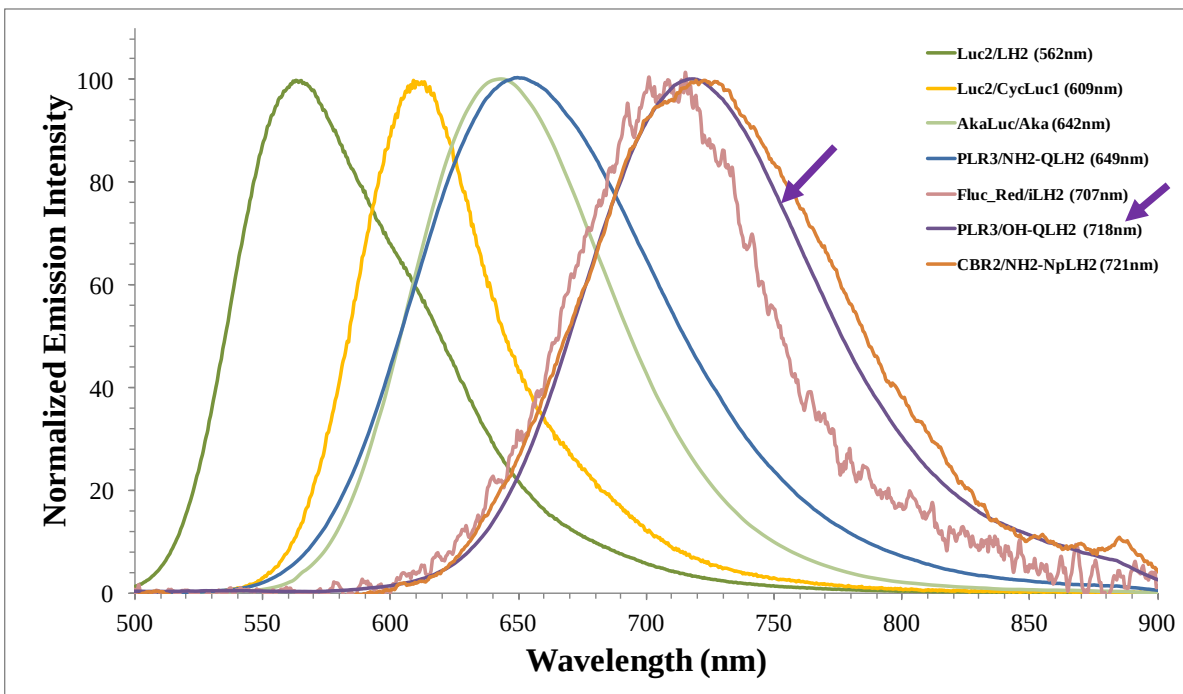


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

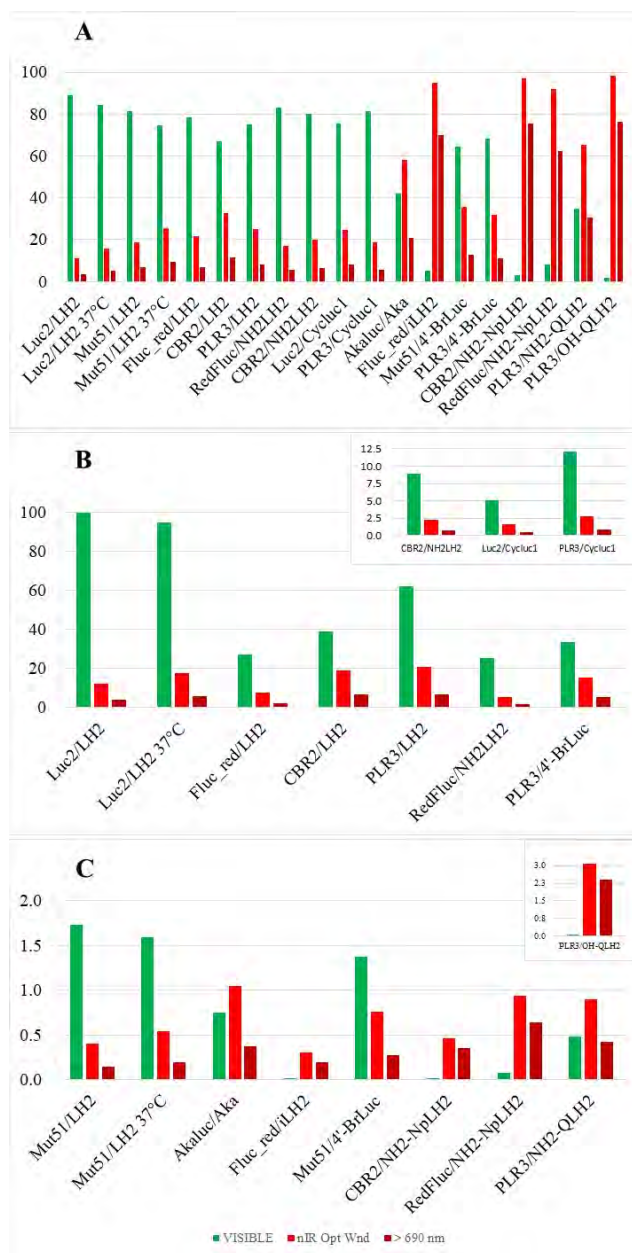


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

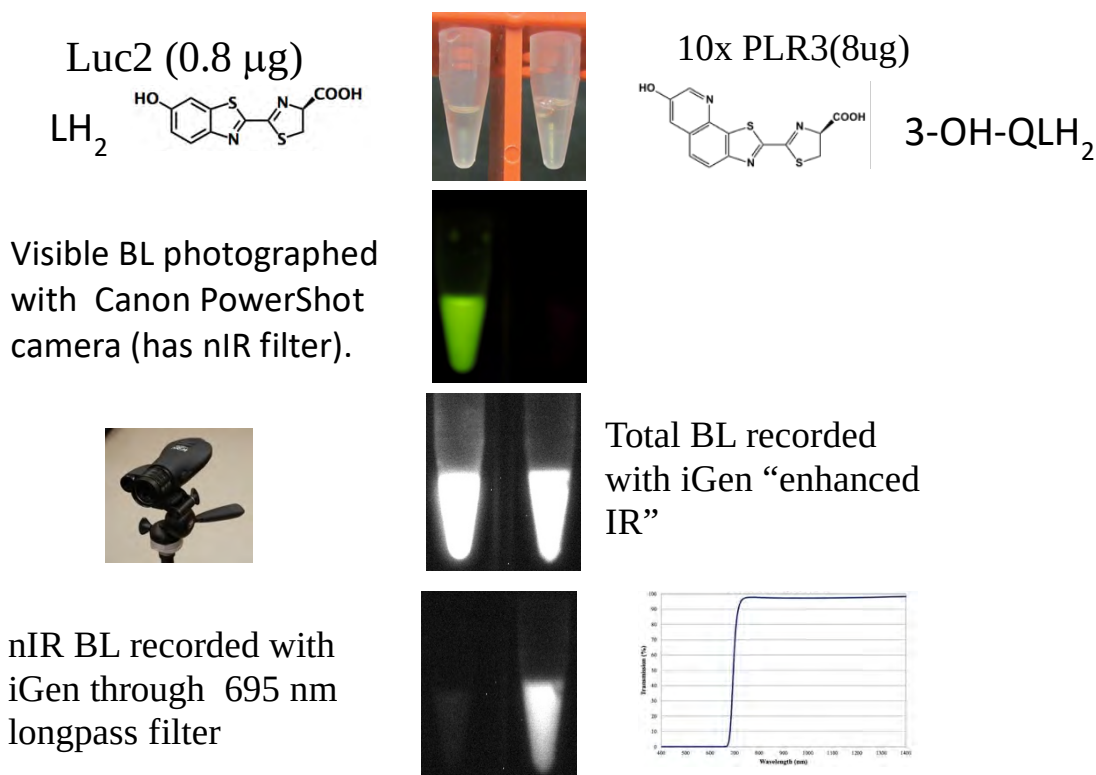
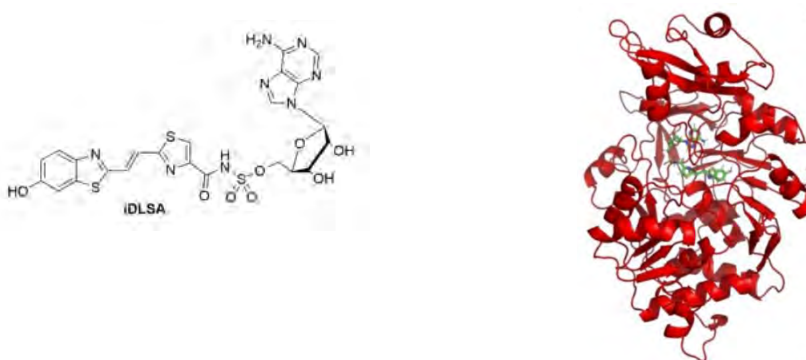


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

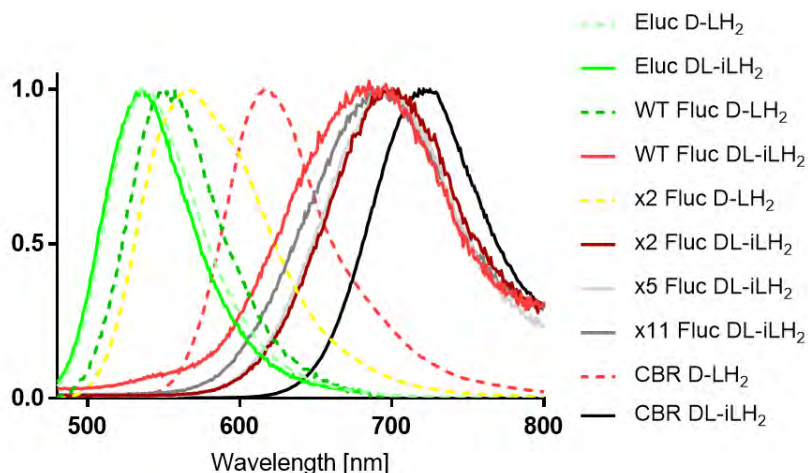


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultonii*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

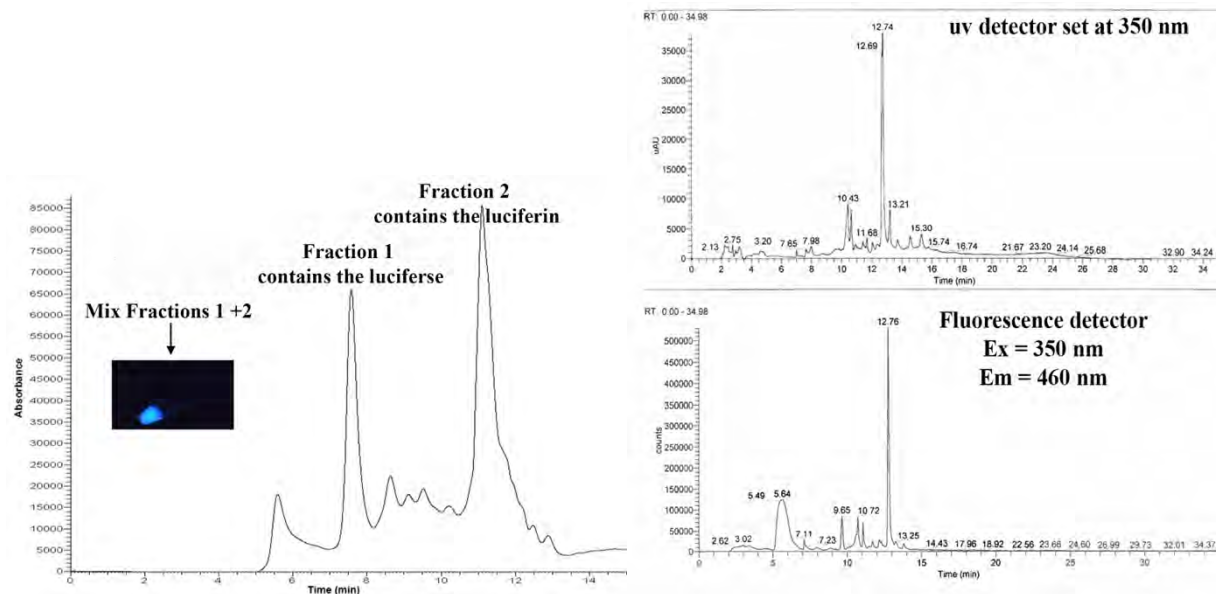
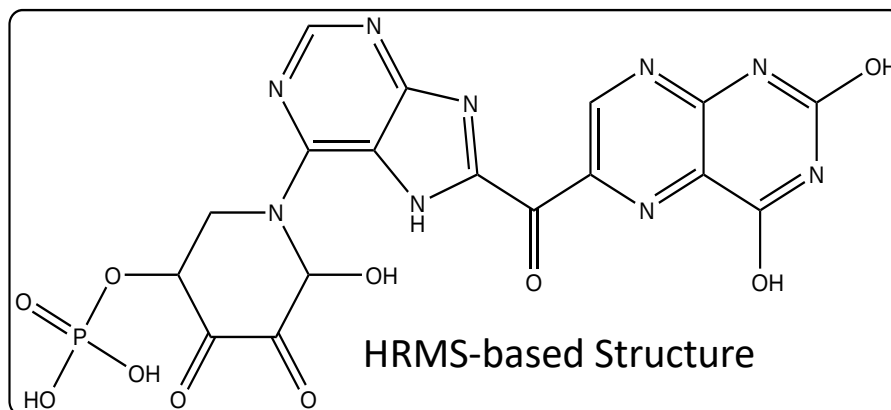


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



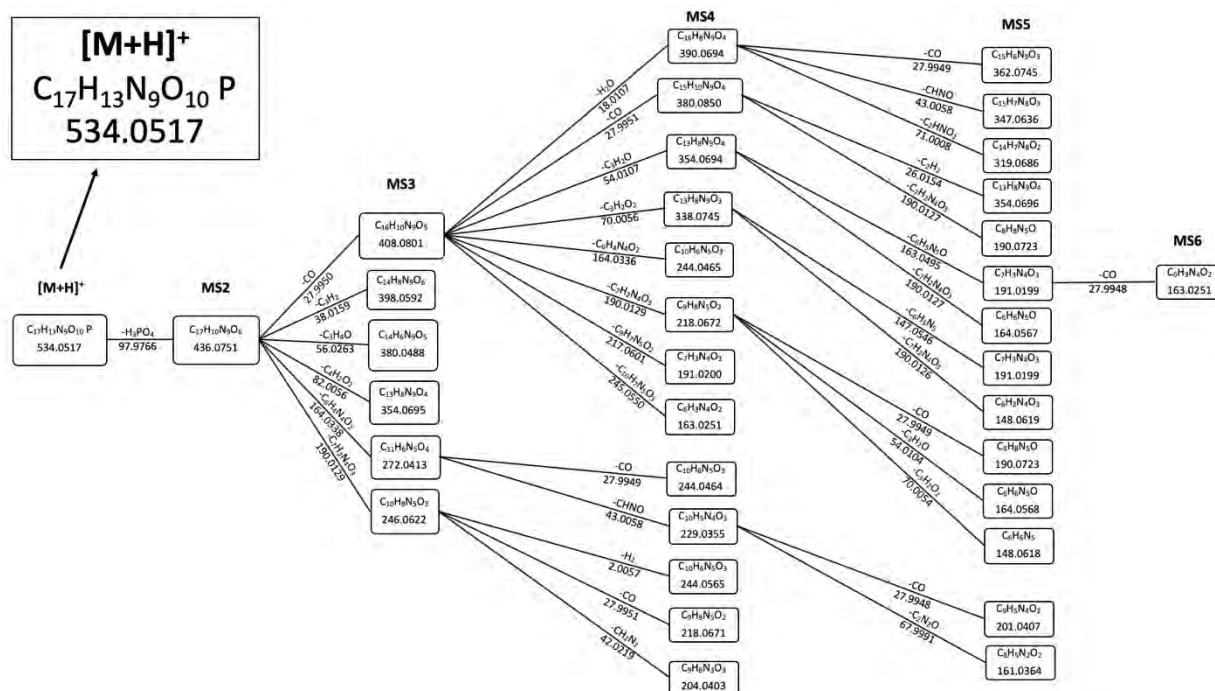


Figure 8. HRMS analysis of putative Orfelia oxyluciferin.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the Orfelia oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

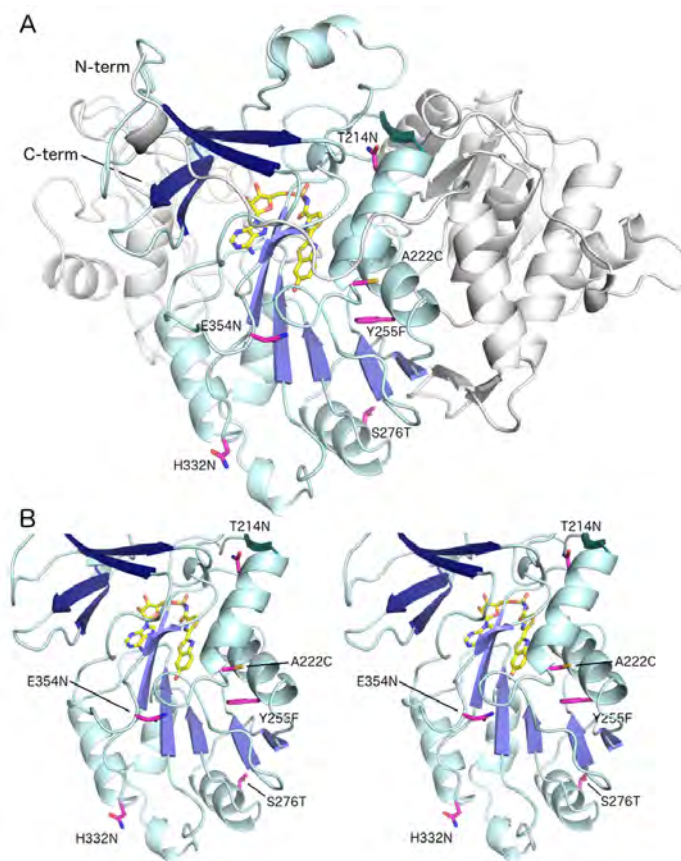


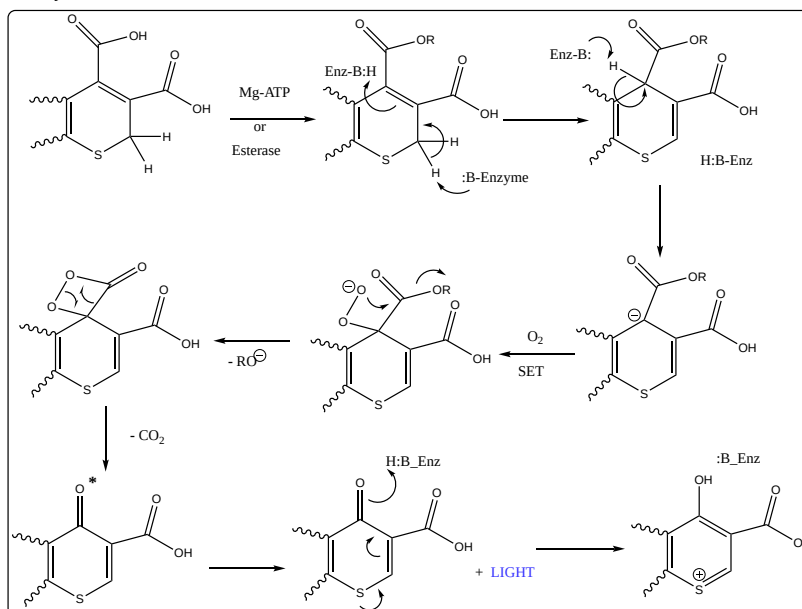
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaeta that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdongata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Universita degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

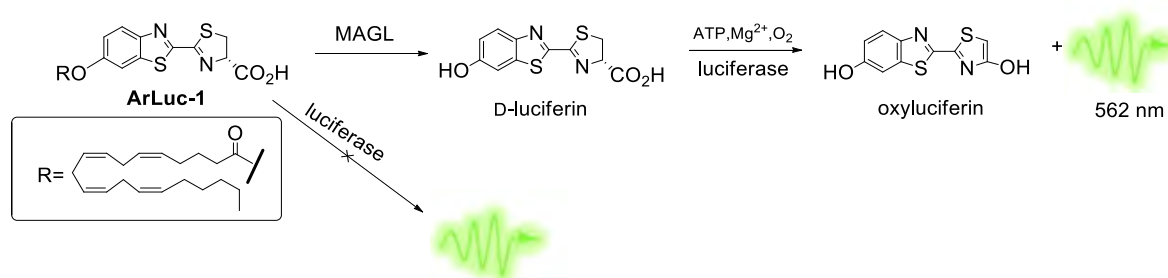


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the *Orfelia* bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase (*A*₆₀₀ = 0.5-0.7) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of *K_m* for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). *K_m* values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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- 1. "Red-emitting chimeric firefly luciferase for in vivo imaging in low ATP cellular environments." Bruce R. Branchini, Tara L. Southworth, Danielle M. Fontaine, Dawn Kohrt, Franceine S. Welcome, Catherine M. Florentine, Emma R. Henricks, Demetria B. DeBartolo, Elisa Michelini, Luca Cevenini, Aldo Roda, Martha J. Grossel, *Analytical Biochemistry*, 2017;534:36–39. doi: 10.1016/j.ab.2017.07.001.**

- 2. "A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events." Branchini BR, Southworth TL, Fontaine DM, Kohrt D, Florentine CM, Grossel MJ. *Sci Rep.* 2018; 8(1):5990. doi: 10.1038/s41598-018-24278-2.
- 3. " Mutagenesis and structural studies reveal the basis for the activity and stability properties that distinguish the photinus luciferases scintillans and pyralis." Branchini, B. R., Fontaine, D. M., Southworth, T. L., Huta, B. P., Racela, A., Patel, K. D., and Gulick, A. M. (2019), *Biochemistry* 58, 4293-4303. doi/10.1021/acs.biochem.9b00719
- 4. " Bioluminescence chemistry of fireworm *Odontosyllis*." Kotlobay, A. A., Dubinnyi, M. A., Purtov, K. V., Guglya, E. B., Rodionova, N. S., Petushkov, V. N., Bolt, Y. V., Kublitski, V. S., Kaskova, Z. M., Ziganshin, R. H., Nelyubina, Y. V., Dorovatovskii, P. V., Eliseev, I. E., Branchini, B. R., Bourenkov, G., Ivanov, I. A., Oba, Y., Yampolsky, I. V., and Tsarkova, A. S. (2019) *Proc Natl Acad Sci USA* 116, 18911-18916. doi/10.1073/pnas.1902095116
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- 8. "A new ultrasensitive bioluminescence -based method for assaying monoacylglycerol lipase." M. Miceli, S. Casati, P. Allevi, S. Berra, R. Ottria, P. Rota, B.R. Branchini, and P. Ciuffreda, *Int. J. Mol. Sci.*, 22, 6148 (2021) <https://doi.org/10.3390/ijms22116148>

- 9. “Dendritic cells maintain anti-tumor immunity by positioning CDB skin-resident memory T cells.” J. L. Vella, A. Molodtskov, C. V. Angeles, B. R. Branchini, M. J. Turk, and Y. H. Huang, *Life Science Alliance* 4 (10), e202101056 (2021).
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*** Corresponding author inadvertently left out AFOSR acknowledgement.

COVER SHEET

Development And Characterization of Novel Bioluminescent Sources

Award Number: FA9550-18-1-0017

Report Type: Final Report

Reporting Period: Start 15 October 2017 - End 14 October 2022

Distribution Statement: Approved for Public release

Program Officer: Bennett L. Ibey

Principal Investigator: Bruce Branchini

Project Title: Development and Characterization of Novel Bioluminescent Sources

ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultoni*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Section 2: Technical Report Submission

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Report Abstract: The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. The results of the project were disseminated in 10 publications in peer reviewed scientific journals that acknowledged the support of AFOSR. A total of 12 funded and non-funded participants including research technicians, undergraduates, a graduate student, and 9 collaborating laboratories contributed to the project. Two major objectives were pursued: (1) to develop efficient firefly luciferase-based sources of near-Infrared (nIR) light; and (2) to develop and characterize new bioluminescence systems. Among the significant accomplishments, the following are highlighted. (1) The development of an efficient biological source of nIR light (emission maximum = 718 nm) that produced minimal visible light. This result was achieved by engineering the bioluminescent system of firefly luciferase-firefly luciferin that normally emits yellow green light. It was necessary to synthesize ~12 substrate analogs and to screen them with luciferase variants produced in our lab. Additional directed evolution trials and a systematic study of other important luciferase-analog systems resulted in the identification of our PLR3/C47T - OH-QLH₂ system. (2) We investigated another nIR-producing substrate analog called iLH₂. In collaborative studies, we expanded the usefulness of this compound for multianalyte monitoring applications based on its ability of iLH₂ to also produce green light with different luciferases. (3) We made progress on the characterization of the bioluminescence system of the North American glow worm bioluminescence system of *Orfelia fultonii*. High resolution mass spectrometry and NMR results have provided a preliminary structure of the putative blue light emitter. (4) Working collaboratively, we characterized the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaetae that emits blue-green light and provided insights into the mechanism of light production. (5) We provided materials and assisted in the development of (i) methodology for the assay of endocrine-disrupting chemicals and (ii) the development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase.

Accomplishments

- **Research Objectives:**

(1) To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

The goal to produce a highly efficient biological source of nIR light (maximum intensity >700 nm with minimal emission in the visible) was pursued throughout this grant period. The general approach involved the organic synthesis of new substrate analogs and screening them for optimal nIR emission intensity and maximum wavelength with an in house developed library of *P. pyralis* luciferase variants. With the discovery of the PLR3-OH-QLH₂ pair, additional directed evolution studies of PLR3 were performed to improve the sensitivity of the enzyme-analog combination. OH-QLH₂ is a substrate analog that contains a quinoline ring that extends the conjugation of the light emitter beyond that of the natural substrate firefly luciferin. PLR3 is a chimeric enzyme consisting of the N-terminal domain of *P. pyralis* luciferase and the C-domain of *L. italica* luciferases. PLR3 has 14 amino acid changes that provide enhanced thermostability at 37 °C, red-shifted emission ($\lambda_{\text{max}} = 613 \text{ nm}$) with the natural substrate, and high specific activity compared to other red-shifted luciferases. The development of and properties of PLR3 and QLH₂ are described in *Publications 1, 2, 3 and 11*.

Under this major objective, we participated in 2 collaborative studies in which we performed testing of a substrate called infraLuciferin (iLH₂) developed by Anderson and Jathoul (*Publications 6 and 10*). Our lab also prepared a complex of *P. pyralis* luciferase and a tight binding active site bound inhibitor based on the structure of iLH₂ for crystallization by our collaborators. The resulting 3D structure provided insight into how larger substrate analogs could be accommodated at the LH₂ binding site offering a structural model for analog design.

(2) To develop and characterize new bioluminescence systems.

This major objective was investigated through collaborative work with several labs. With Yampolsky and Tsarkova, insight was provided into the biochemistry of the bioluminescence process of *Odontosyllis undecimdonata*, a marine polychaetae that emits blue-green light (*Publication 4*). With Vadim Viviani, we produced an overview of the processes that lead to light emission of the dipteran *Orfelia fultonii* (*Publication 7*). These tiny seasonal larvae are quite rare and were collected along the Appalachians in North Carolina. Preliminary studies to identify the putative light emitting compound by mass spectrometry and NMR were interrupted by Covid restrictions. Additionally, we worked collaboratively with 3 labs by providing luciferases and bioluminescence expertise for the development of: biosensors for endocrine-disrupting chemicals with the Roda lab

(*Publication 5*); an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase with the Ciuffreda lab (*Publication 8*); and a method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study by Yina Huang (*Publication 9*).

In addition to the publication of our results, oral presentations at AFOSR and International Society for Bio- and Chemiluminescence meetings disseminated our results.

- **Details Of Accomplishments**

1. To develop efficient firefly luciferase-based sources of near-Infrared (nIR) light.

1A. Synthesis of quinoline luciferin analogs OH-QLH₂ and NH₂-QLH₂. In our effort to produce an efficient luciferase-analog source of nIR light, our strategy was to produce an analog with extended conjugation compared to the natural substrate firefly luciferase that cannot produce maximal emission beyond ~ 630 nm. This strategy had already proven to be possible based on reports from other labs (Figure 1). We chose to focus on substituting the quinoline ring for the naphthyl ring of NH₂-NpLH₂ and to explore both hydroxy- and amino- electron donating substituents and regioisomers possible with the quinoline ring. We designed 10 quinoline substrates, and later added 2 other ring systems. We successfully prepared and chemically characterized 8 of the 10 analogs. Testing with an in house library of *P. pyralis* luciferase variants, the wild-type enzyme (Luc2), and 5 luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51), revealed that OH-QLH₂ and NH₂-QLH₂ (Figure 2) paired with our PLR3 variant were the most promising analogs for continued study. Interestingly, the analog containing a naphthyridine ring system extended the nIR emission maximum to 735 nm with a new Luc variant, its activity was only ~12% of that of the current best substrate/Luc combinations. Also, we observed the longest wavelength emission observed to date (760 nm maximum with the beetle enzyme CBR2), however, the emission intensity was too low to be useful. The bioluminescence (BL) intensities of the new substrates were disappointing, but the very long wavelength BL we achieved was impressive.

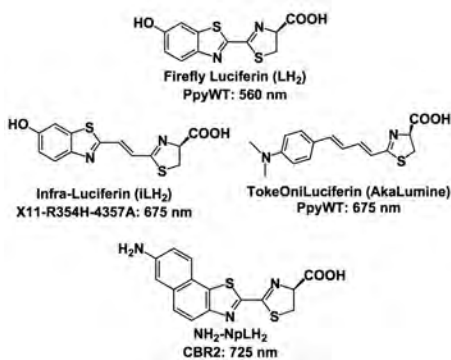


Figure 1. Luciferase-substrate emission maxima.

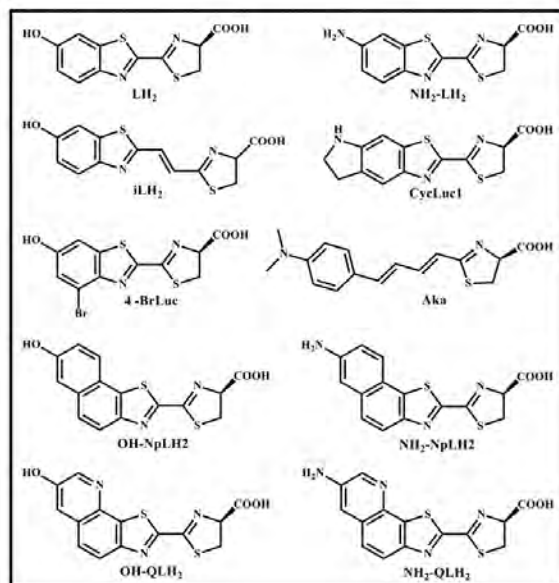
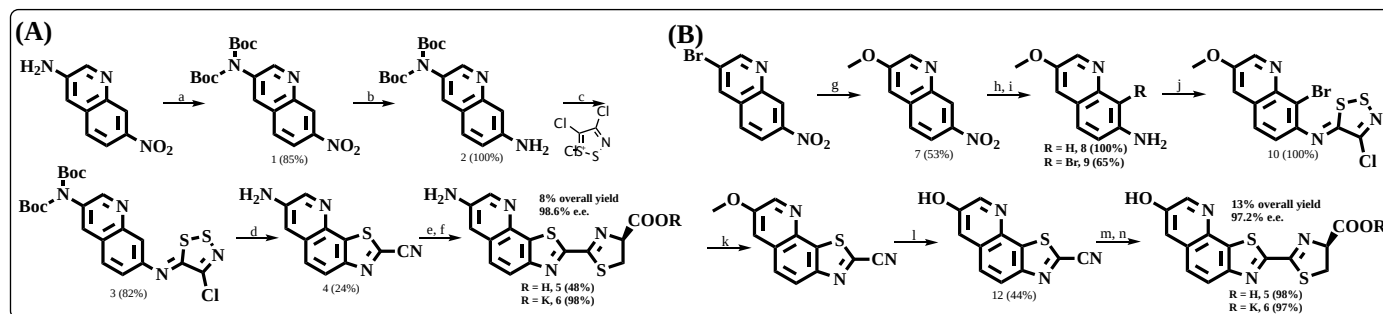


Figure 2. Natural substrate LH2 and Substrate Analogs.

The chemical syntheses of OH-QLH₂ and NH₂-QLH₂ are presented in Scheme 1 and the detailed synthetic procedures and spectral characterization of the analogs are presented in *Publication 11*.



Scheme 1. (A) a. THF, Boc₂O, DMAP (cat), 50 °C, 2.5 h; b. MeOH/THF, 10% Pd/C, H₂ 72 h; c. DCM, 1 h; d. sulfolane, microwave, 150 °C, 7 h; e. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 3 h; f. H₂O, KCl, KHCO₃. (B) g. MeOH/toluene, Pd(OAc)₂, Ad-BGPhos, Cs₂CO₃ 80 °C, 5 h; h. THF/MeOH, 10% Pd/C, H₂, 16 h; i. CH₃CN, NH₄OAc (cat), NBS, -5 °C, 2.25 h; j. DCM, Appel's salt, 4.5 h; k. pyridine, CuI, 130 °C, 1.5 h; l. pyridine-HCl, microwave, 200 °C, 20 min; m. MeOH/50 mM NaHPO₄ pH 8, D-cysteine, 30 min; n. H₂O, KCl, KHCO₃.

1B. Systematic comparison of luciferase-substrate pair nIR emission properties. The quinoline-based firefly luciferin (LH₂) substrate analogs were evaluated as potential near-IR (nIR) bioluminescence sources with our PLR3, the wild-type enzyme (Luc2), and 5 other luciferases reported to produce nIR light (commercial CBR2 and RedFluc, Akaluc, Fluc-red, and Mut51). Comparative *in vitro* testing with 8 other analogs and the natural substrate LH₂ with Lucs as purified proteins demonstrated that one of our synthetic substrates called OH-QLH₂, an extended conjugation LH₂ quinoline ring-containing analog, produced the most intense light emission (peak wavelength at 718 nm) with a Luc variant called PLR3 (*Table 1*).

The BL emission spectra for the 7 Lucs with natural substrate LH₂ and the optimal Luc-substrate analog pairs tested to date are presented in *Figure 3*. The data illustrate the limitation of

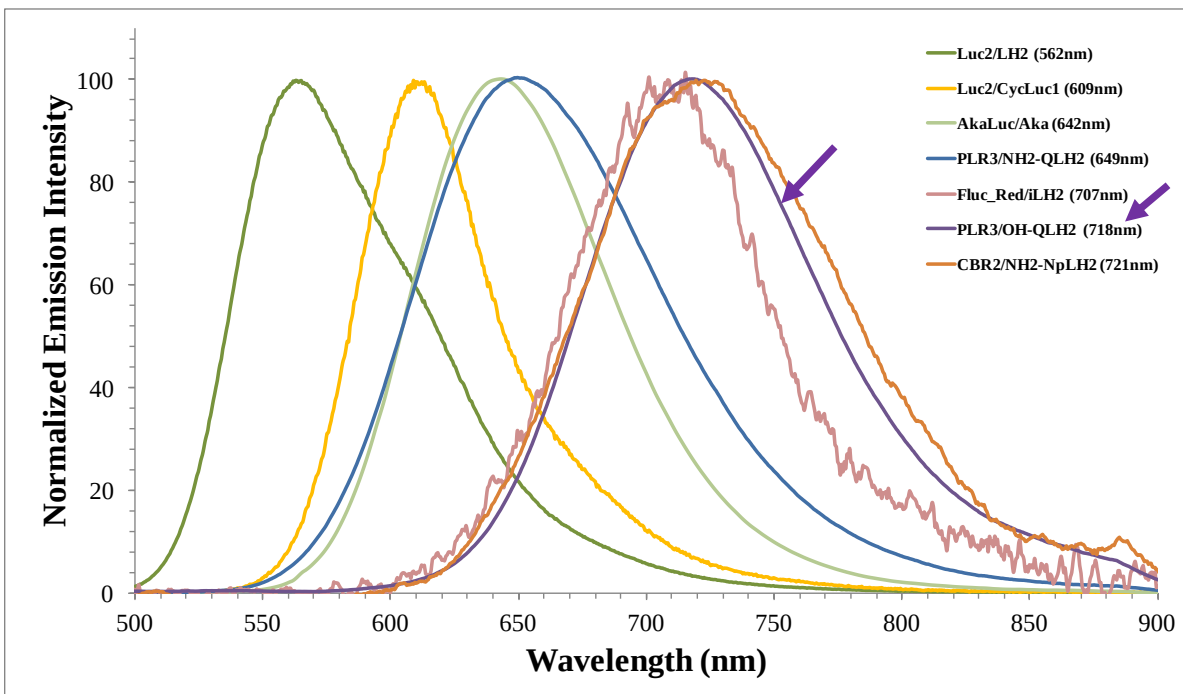


Figure 3. Normalized BL emission spectra of purified luciferase/substrate pairs at 37 °C.

approximately 625 nm maximum bioluminescence that can be achieved with the natural substrate LH₂ (*Table 1*), no matter which enzyme is used to produce light emission. In contrast, there were 8 examples of BL maxima in the range 642 nm to 750 nm produced by 6 of the substrates: Aka, iLH₂, NH₂-NPLH₂, OH-NPLH₂, NH₂-QLH₂, and OH-QLH₂. Our OH-QLH₂ analog paired with the highly engineered Luc variant PLR3 and the commercially developed CBR2 and NH₂-NpLH₂ combination produced broad BL emission spectra with maxima of ~720 nm. All of these substrates contain extended conjugation compared to LH₂ and presumably the respective oxyluciferins, the actual sources of BL.

Focusing on our goal to make highly efficient biological sources of nIR light with λ_{max} values > 690 nm and with minimal emission in the visible for use as biomarkers, we measured the % BL emission > 690 nm of the luciferase/substrate combinations and adjusted the data for their relative *in vitro* specific activities. The top three enzyme/substrate pairs (*Figure 4c*) had BL λ_{max} , % visible, and % > 690 nm emission values of: PLR3/OH-QLH₂ (718 nm, 2%, 76%); CBR2/NH₂-NpLH₂ (721 nm, 3%, 73%); and Red_Fluc/NH₂-NpLH₂ (694 nm, 8%, 62%). PLR3/OH-QLH₂ and CBR2/NH₂-NpLH₂ are excellent sources of nIR and their greater BL maxima are clearly the major contributing factor.

While the intensities of these sources are low compared to the Luc2-LH₂ pair, for *in vitro* applications it should be possible to significantly improve the sensitivity mainly by simply increasing the concentrations of the enzymes and of substrates.

Table 1. BL emission and relative activities of Luc-substrate pairs.										
LUC/SUBSTRATE	LH₂	NH₂-LH₂	Cycluc1	Aka	iLH₂	4'-BrLuc	NH₂-NpLH₂	OH-NpLH₂	NH₂-QLH₂	OH-QLH₂
Luc2										
Specific Activity ^a	100 ± 4	9.1 ± 0.48	8.3 ± 0.19	1.9 ± 0.01	0.07 ± 0.001	8.3 ± 0.78	0.18 ± 0.003	b.d.	0.08 ± 0.001	0.03 ± 0.003
Biolum λ _{max} (nm) ^b , 23	562 (84)	603 (85)	609 (61)	677 (90)	709 (96)	617 (70)	708 (135)	b.d.	678 (139)	720 (154)
Biolum λ _{max} (nm), 37 °C	600,574 (105)	602 (80)	606 (64)	674 (88)	708 (99)	618 (70)	706 (126)	b.d.	678 (130)	719 (133)
RedFluc										
Specific Activity ^a	50.7 ± 1.5	27.40 ± 0.63	14.25 ± 3.3	2.6 ± 0.12	0.17 ± 0.01	37 ± 0.48	0.91 ± 0.01	b.d.	1.10 ± 0.01	1.93 ± 0.11
Biolum λ _{max} (nm) ^b , 23	612 (60)	595 (73)	605 (68)	671 (77)	718 (95)	616(66)	694 (112)	b.d.	660 (117)	715 (107)
Biolum λ _{max} (nm), 37 °C	613 (63)	594 (76)	604 (69)	670 (83)	720 (85)	616 (75)	694 (116)	b.d.	659 (122)	715 (107)
Akaluc										
Specific Activity ^a	0.10 ± 0.01	0.84 ± 0.02	4.17 ± 0.21	1.62 ± 0.03	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	598 (73)	588 (75)	595 (70)	642 (93)	682 (95)	613 (81)	682 (116)	b.d.	b.d.	b.d.
Biolum λ _{max} (nm), 37 °C	597 (73)	587 (78)	593 (70)	643 (89)	681 (104)	614 (82)	685 (131)	b.d.	b.d.	b.d.
Mut51										
Specific Activity ^a	1.87 ± 0.02	1.77 ± 0.07	4.85 ± 0.22	0.61 ± 0.05	0.03 ± 0.001	1.9 ± 0.08	0.06 ± 0.004	b.d.	b.d.	b.d.
Biolum λ _{max} (nm) ^b , 23	568 (101)	598 (78)	608 (69)	672 (84)	708 (109)	617 (77)	701 (118)	b.d.	665 (122)	b.d.
Biolum λ _{max} (nm), 37 °C	612 (86)	594 (79)	608 (69)	670 (86)	714 (102)	618 (76)	700 (124)	b.d.	668 (115)	b.d.
Fluc_Red										
Specific Activity ^a	47.11 ± 2.6	11.52 ± 0.32	4.13 ± 0.18	1.04 ± 0.02	0.14 ± 0.02	9.5 ± 0.2	0.14 ± 0.001	b.d.	0.04 ± 0.003	0.17 ± 0.005
Biolum λ _{max} (nm) ^b , 23	602 (76)	595 (80)	603 (60)	662 (85)	707 (93)	616 (73)	690 (125)	727 (122)	660 (141)	701 (121)
Biolum λ _{max} (nm), 37 °C	604 (68)	593 (76)	603 (65)	661 (88)	707 (86)	616 (74)	693 (125)	733 (136)	665 (117)	703 (123)
CBR2										
Specific Activity ^a										
Biolum λ _{max} (nm) ^b , 23	52.1 ± 0.26	10.1 ± 0.24	2.69 ± 0.26	0.62 ± 0.03	0.14 ± 0.002	5.4 ± 0.37	0.42 ± 0.01	0.48 ± 0.01	0.25 ± 0.001	0.89 ± 0.01
Biolum λ _{max} (nm), 37 °C	619 (64)	600 (72)	622 (66)	575,684	727 (87)	619 (73)	718 (117)	749 (116)	688 (123)	721 (118)
PLR3										
Specific Activity ^a	74.3 ± 8	13.7 ± 0.95	13.2 ± 0.24	0.82 ± 0.08	0.21 ± 0.01	44 ± 2	0.27 ± 0.001	0.004 ± 0.0001	1.23 ± 0.003	2.82 ± 0.06
Biolum λ _{max} (nm) ^b , 23	613 (61)	593 (72)	606 (62)	667 (88)	723 (109)	615 (63)	693 (110)	755 (104)	649 (111)	718 (107)
Biolum λ _{max} (nm), 37 °C	611 (62)	593 (76)	602 (65)	665 (91)	718 (108)	615 (69)	691 (103)	751 (107)	649 (113)	716 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified Luc2 (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Data are expressed as the mean ± standard deviation and are reported relative to the Luc2/LH ₂ value and were corrected for the spectral response of the CCD detector. ^b BL emission spectra measured in <i>in vitro</i> assays were obtained as described in Materials and Methods of main text. Bandwidths at full width at half-maximum values are shown in parentheses. b.d.; below detection having a relative specific activity of < 0.03.										

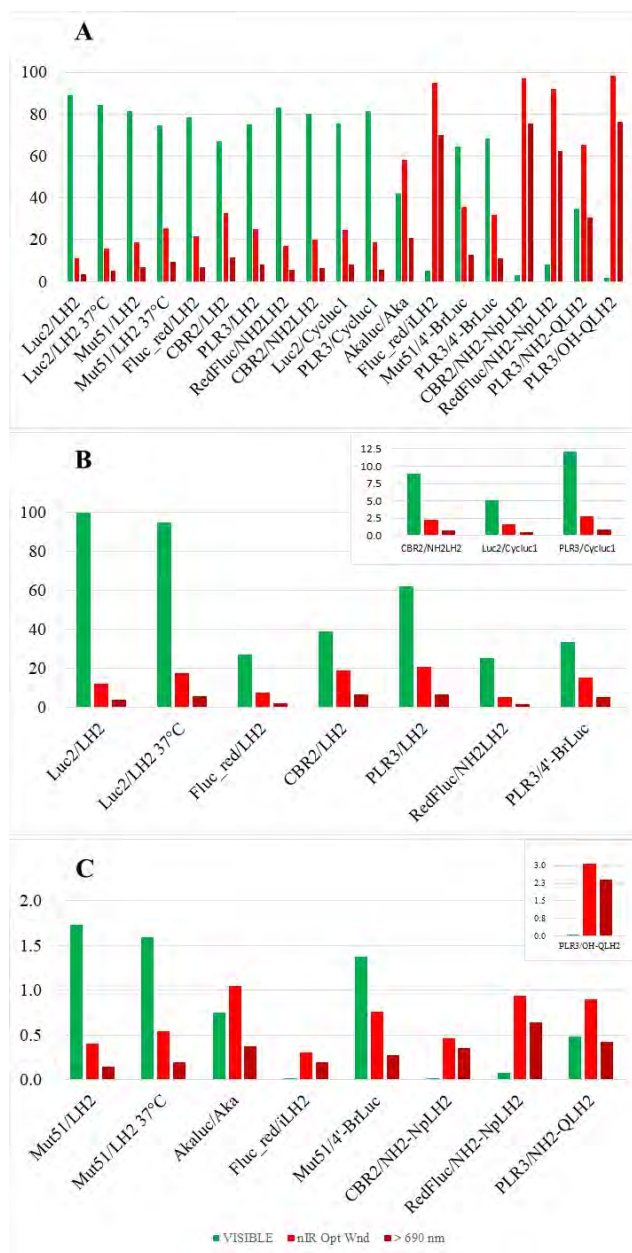


Figure 4. Relative BL emission wavelength distribution of purified Luc protein - substrate pairs. BL reactions were monitored under glow (slow decay) conditions with an IVIS Spectrum III instrument. For each Luc - substrate combination (A) its relative percent visible (500 nm – 640 nm, green), nIR window (650 nm – 810 nm, red), and nIR only (690 nm – 810 nm, maroon) emission recorded through filters is plotted. In (B) and (C) the data in panel A are multiplied by the relative specific activity data (Table 1) in which the Luc - LH₂ pair at 23 °C is defined as 100.

1C. Directed evolution of PLR3-QLH₂ pair. With the most promising pair, our PLR3/OH-QLH₂, in hand the remaining major challenge was to increase the sensitivity, that is the signal strength, of this amazing biological source of nIR light. To address this deficiency, ongoing efforts to produce better substrate analogs and to improve on the PLR3 enzyme's ability to produce bright BL using a Directed Evolution approach that specifically targeted the substrate

binding region of PLR3. The intent was to improve sensitivity with minimal loss of long wavelength maximum.

Directed Evolution (DE) is a method of protein engineering that mimics the process of natural selection. The idea is that in a controlled, laboratory environment DE can be used to develop proteins towards a specified goal. DE can be used to increase a protein's thermostability, specific activity, affinity, overall stability, solubility, etc. The process includes subjecting a gene to multiple rounds of random mutagenesis creating a library of variants, selecting those variants with the desired function, and amplification by generating a template for the next round.

Table 2: BL emission maxima and specific activities of Luc variants (mutation combinations) from the PLR3 N2-region GeneMorph® Library with OH-QLH ₂			
Luciferase	Mutations	Specific Activity ^a	BL, 23 °C ($\lambda_{\text{max}} \pm 2 \text{ nm}$) ^b
PLR3	T214A/A215L/I232A/G246A/F250H/Y255F/S284T/F295L/E354K/I457V/A482G/H489K/A503N/K543Q	3.00E+06 (100)	718 (107)
PLR3/ C47T	PLR3/C258S/M265T/V366M	9.30E+06 (310)	710 (107)
^a Specific activities were obtained from assays at pH 7.4 and 23 °C by monitoring and integrating signal intensity for 2 min using purified proteins (2.5 µg), 50 µM substrate, 1 mM ATP, and 3 mM MgSO ₄ . Flash height (FH) specific activities were calculated using the max peak height obtained. Data were corrected for the spectral response of the CCD detector. Activities relative to PLR3 are shown in parentheses. ^b Data were obtained as described in Materials and Methods. Bandwidths at full width at half-maximum values are shown in parentheses.			

The general DE methodology we used involved: (1) improving the PLR3 template by random mutagenesis (error prone PCR) of the substrate binding/active site region; (2) rational site-directed mutagenesis and site-saturation mutagenesis; (3) insertion of PCR product into bacterial hosts and growing colonies; (4) screening for BL red-shift and/or increase in BL intensity upon addition of OH-QLH₂; (5) picking positive (bright) colonies; (6) growing colonies in liquid cultures for a second round of screening to eliminate false positives; (7) DNA sequencing to identify positive mutations; (8) repeating the process for several rounds adding multi-site combination mutagenesis, site saturation mutagenesis, and additional rational design to fine tune the results. The related experimental details can be found below in the “Technical Updates” section.

The results of the time-intensive DE results were encouraging. The best PLR3 variant that was developed called PLR3/C47T (*Table 2*) was a hybrid of the 2 best double mutants that

we discovered. Site directed mutagenesis results of the 4 amino acid changes allowed us to delete one change that was slightly deleterious. Unfortunately, site-saturation mutagenesis of the 3 identified residues C258S, M265T, and V366M did not improve on the properties of PLR3/C47T. With the new variant, a significant 3-fold improvement in brightness was obtained at the expense of a modest 7 nm blue shift in BL maximum emission. As shown below (Figure 5) increasing the concentration of PLR3/C47T 10-fold produced a signal as bright as the Luc2/LH₂ pair, one of the brightest known BL systems, and with extraordinary high nIR content as photographed with a nIR camera. However, additional work will be required to decrease the emission decay of the OH-QLH₂ reaction with PLR3/C47T.

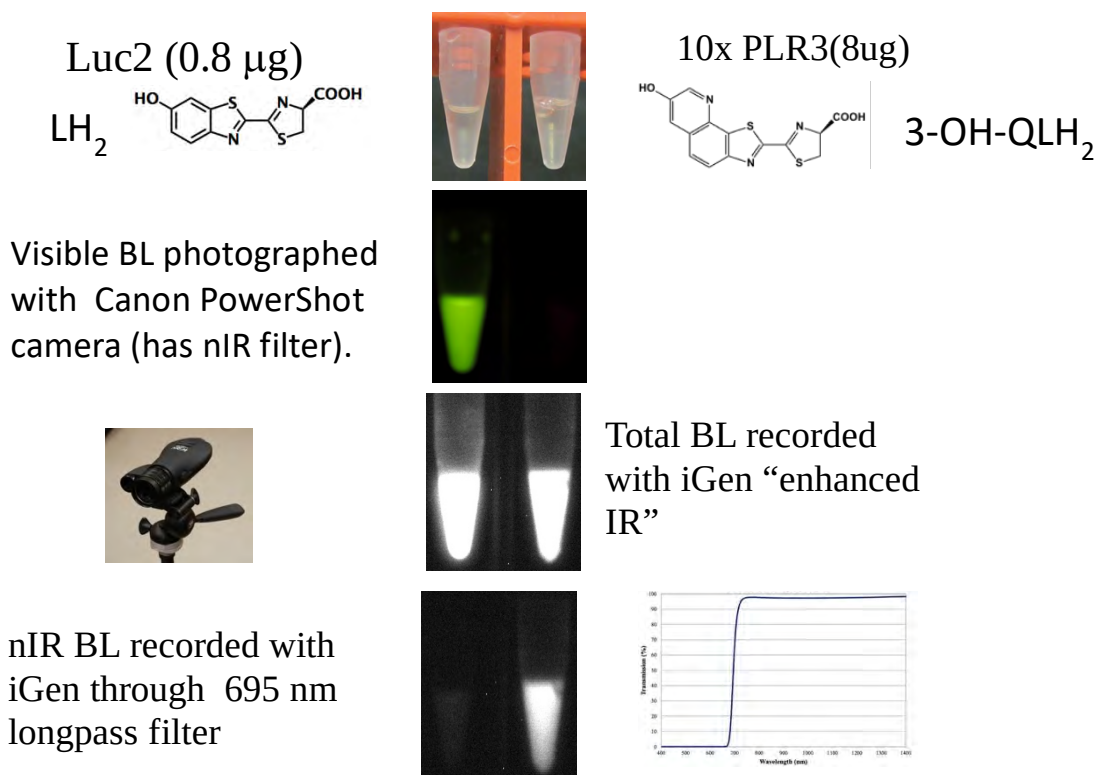
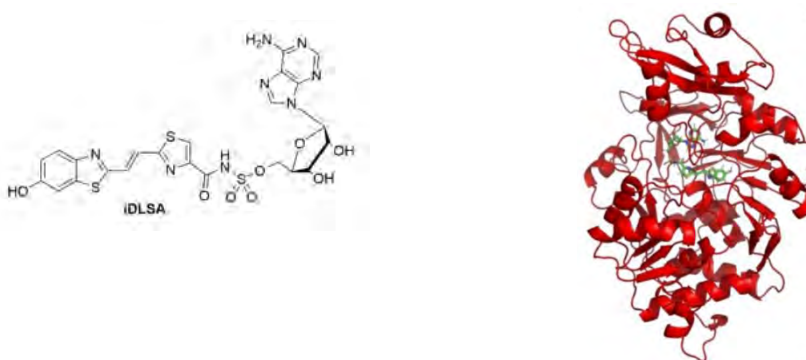


Figure 5. Relative brightness and nIR content of Luc2/LH₂ and PLR3/C47T - OH-QLH₂. The iGen is an inexpensive nIR camera.

2A. Collaborative Studies on infraluciferin (iLH₂). We undertook a collaborative crystallographic study with the Anderson lab (University College London) with a nIR emitting luciferin (iLH₂)-based inhibitor of *P. pyralis* luciferase called 5'-O-[N-(dehydroinfraluciferyl) sulfamoyl] adenosine (iDLSA), based on the structure of iLH₂ (Figure 2). The study was aimed at improving the ability to predict efficient new infra-LH₂ analogs (Publication 6). Analysis of the 3D structure of Luc2 (wild-type) luciferase complexed with iDLSA (PDB: 6HPS) identified amino acid residues R337, S314, S347, and I434 as targets for mutagenesis to enable larger substrate to bind productively to the active site.



In fact, one of these residues was mutated (R337S) and was essential to the ability of the improved beetle luciferase CBR2's ability to bind NH₂-NpLH₂. Unfortunately, mutations at position 337 did not improve PLR3 or variants based on it. The other target residue could potentially lead to better versions of PLR3/C47T.

2B. Collaborative Studies demonstrating the wide BL spectral range of infraluciferin (iLH₂).

In this project conducted in conjunction with the Jathoul, Anderson and Murray labs (*Publication 10*), a study of the basic BL properties of beetle luciferases with iLH₂ was conducted. This study expanded on our investigation of 7 luciferases with iLH₂ to include additional thermostable *P. pyralis* based enzymes as well as with luciferases from Elaterids *Pyrearinus termitiluminans* (Eluc) and *Pyrophorus plagiophthalmus*. The iLH₂ was demonstrated to have an incredibly wide BL (~185 nm) emission range from a λ_{max} of 536 nm in the green with Eluc to a λ_{max} of 721 nm in the nIR with click beetle enzyme CBR. This range with a single substrate offers great promise for future bioengineering of improved systems for bioluminescence based imaging of multiple analytes.

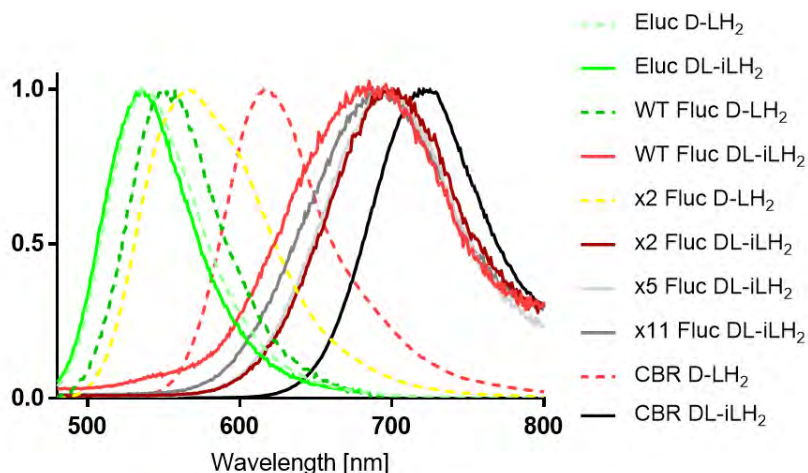


Figure 6. Expanded BL spectral range with iLH₂ and for comparison LH₂. Normalized BL emission spectra were measured from reactions of substrates with 0.2 mM luciferins, 5 μ M enzymes, and 2 mM ATP.

II. To develop and characterize new bioluminescence systems.

1. Preliminary characterization of the bioluminescence system of *Orfelia fultoni*. Bioluminescence in the New Zealand glowworm caves is a very uncommon example of light

emission from Diptera (flies). In the US, the only presently known example is that of *Orfelia fultoni* fly larvae found in the southern Appalachian region. We collected 300 specimens in May 2019 in Highlands, NC and preliminarily characterized putative luciferase-luciferin chromatographic fractions from size exclusion chromatography (Figure 7). We shared our results with collaborator Vadim Viviani who continued the luciferase characterization (Publication 7). We then isolated the fluorescent compound whose spectral properties are consistent with it being the actual light emitter, the Orfelia oxyluciferin (Figure 7). We initiated high resolution mass

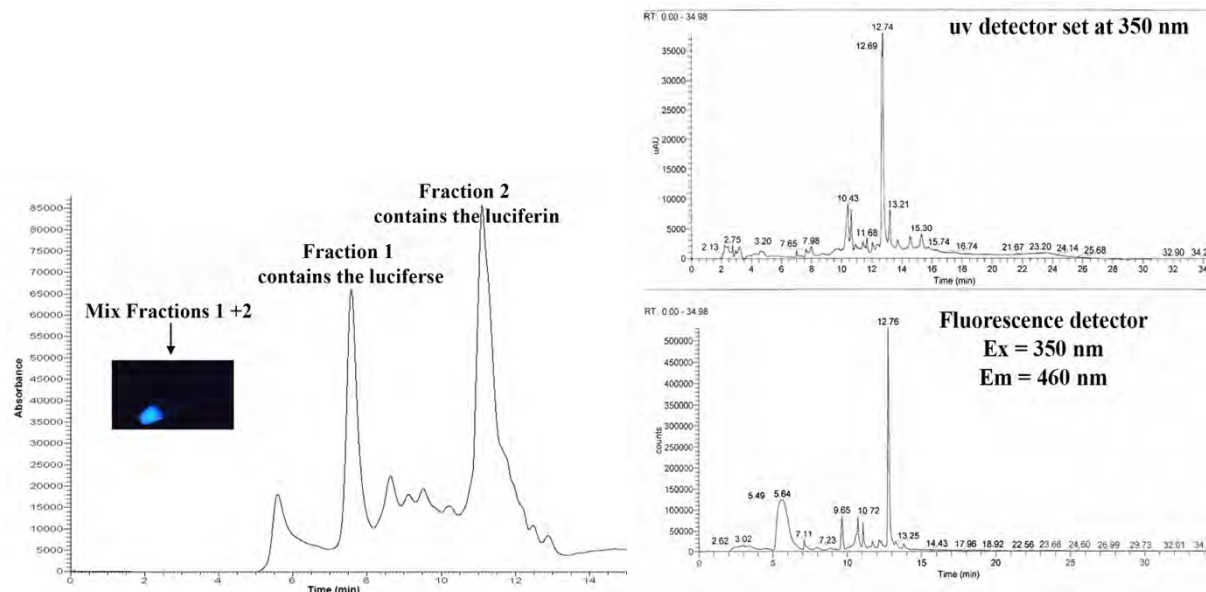
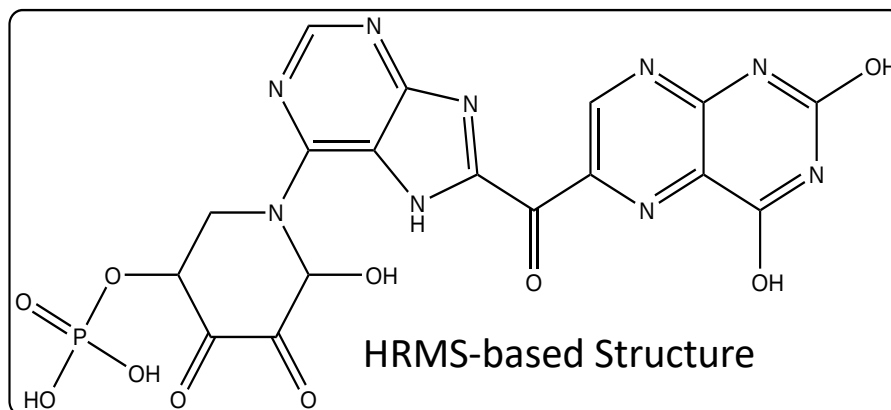


Figure 7. (left) BL of mixture of putative luciferase and luciferin fractions separated by SEC chromatography. (right) UV and Fluorescence properties of putative oxyluciferin isolated by HILIC chromatography match those of *Orfelia* bioluminescence.

spectrometry (hrms) studies on the putative BL emitter. The positive ion MS results are shown in Figure 8 and the proposed structure is presented below.



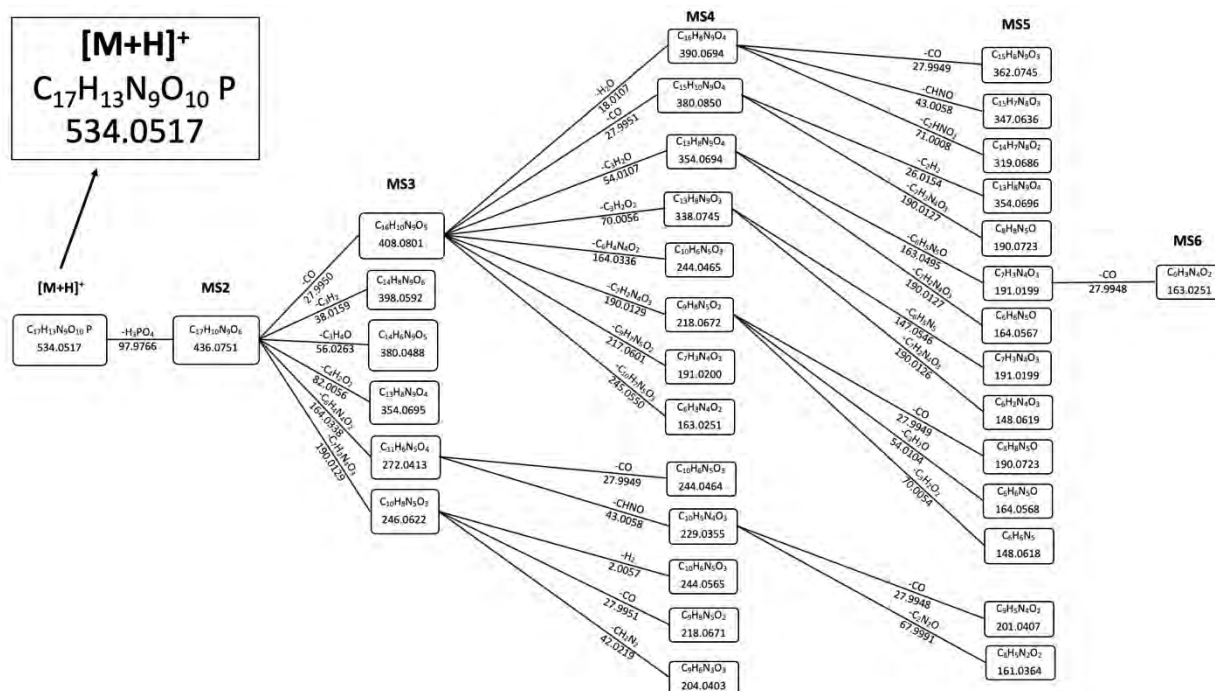


Figure 8. HRMS analysis of putative Orfelia oxyluciferin.

We next established a collaboration with a group of scientists, led by Yampolsky & Tsarkova (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences in Moscow), to conduct NMR experiments (800 MHz with cryoprobe) to further the Orfelia oxyluciferin structure determination process. This required developing a 2 step chromatography protocol to make a highly purified sample starting with extracts of the organism. While some additional progress was made, including confirmation of the lumazine ring and the proposal of an alternative cyclic carbohydrate fragment, our collaborators encountered serious sample precipitation problems probably associated with sample degradation during the long NMR experiments. This required us to prepare additional sample, however, we were unable to do so because College restrictions on travel due to Covid policy prevented us from collecting additional specimens.

2. Structural basis for the bioluminescence properties of *Photinus scintillans* luciferase. We had previously cloned an orange-light emitting luciferase from *Photinus scintillans* (PsnWT) and determined that only 6 of the 56 amino acid differences compared to the common *pyralis* species (PpyWT aka Luc2) account for the 50% higher specific activity, greater stability to heat, aggregation and acidic pH, and the +14 nm shifted light emission. To better understand the structural basis for the altered properties, we determined the crystal structure of a complex of Pps6, the variant containing the 6 mutations, and bound inhibitor 5'-O-[(N-dehydroluciferyl)-

sulfamoyl]-adenosine (DLSA), a luciferyl-adenylate analog (*Figure 9B*) with Andrew Gulick's lab, SUNY Buffalo (*Publication 3*).

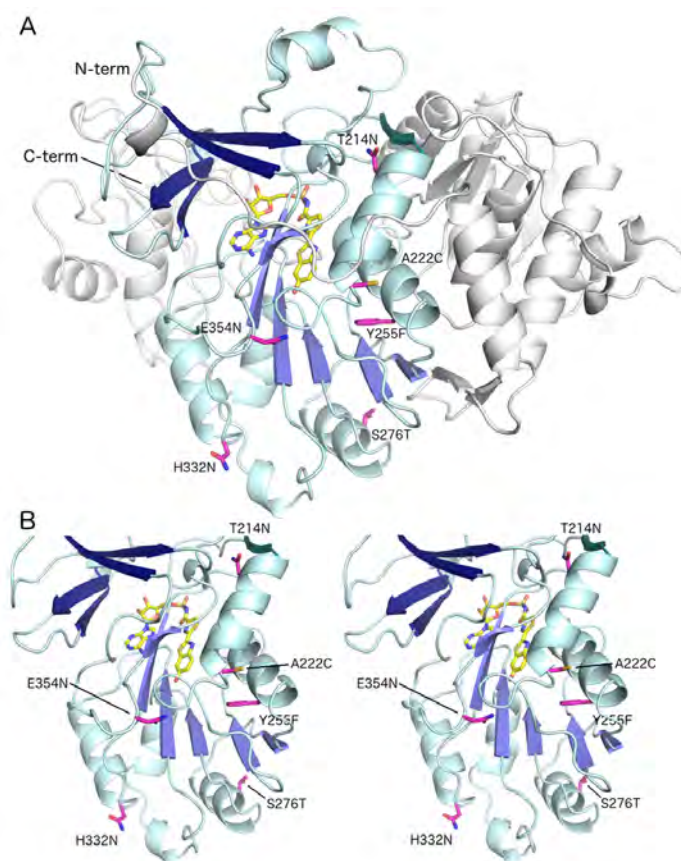


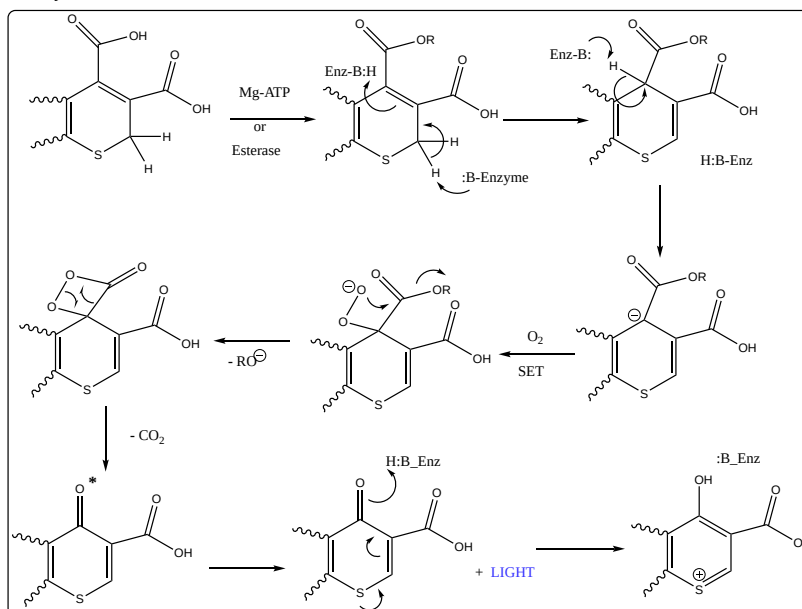
Figure 9. Structure of Pps6-DLSA complex. A. Ribbon representation of the Pps6 protein (top). The N2 core (residues 192-440) of the protein containing the substitutions between PpyWT and PsnWT is colored blue, while the N1- and C-terminal regions are grey. The molecule of DLSA is bound in the core of the protein. B. Stereorepresentation of the location of the six residues (magenta) in Pps6 that are mutated to the residue present in PsnWT. The protein is rotated slightly compared to the view of panel A.

The major finding of the +14 nm BL emission shift attributed to the single change Y255F. The proposed basis of the BL emission shift associated with the Y255F change is a disruption of the key H-bonding network resulting from the absence of the phenolic side chain group. In the *L. cruciata* Luc structure (PDB Code: 2D1S), Y255 makes a H-bond to a H₂O, which is also bonded to N229 and S284 (PpyWT numbering). This network of hydrogen bonds further connects to E311 and ultimately to the phenolate group of the luciferin analog. In the Pps6 protein, there are only two, poorly ordered water molecules in this cavity. The highly ordered water network of the *L. cruciata* protein necessary for 560 nm BL is incompatible with the Y255F mutation. We also note that the position of F255 in the Pps6 structure is essentially the same as the aromatic side chain of the homologous Y257 in the *L. cruciata* structure suggesting that the mutation had any other effect than to disrupt the H-bond network.

3. Insights into the bioluminescence process of *Odontosyllis undecimdongata*, a marine polychaetae that emits blue-green light. I was part of a group of scientists led by Yampolsky & Tsarkova (Russian Academy of Sciences), to determine the structure of the luciferin from the bioluminescent marine fireworm *Odontosyllis*, only the 10th known luciferin, and to propose a pathway for its biosynthesis and putative mechanisms of light emission (*Publication 4*). Marine polychaete *Odontosyllis undecimdongata*, commonly known as fireworms, possess bright blue-green bioluminescence. Luciferin-luciferase reaction of these polychaetes was first demonstrated in the middle of the 20th century, but the key components of *Odontosyllis* bioluminescent system have never been identified. The structure elucidation of the novel luciferin and oxyluciferin emitter was accomplished by 1D and 2D NMR spectroscopy, mass-spectrometry and X-Ray diffraction studies. were performed on the isolated substances and revealed luciferin to be an unusual tricyclic sulfur-containing heterocycle. The *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of *Odontosyllis* bioluminescent system components allow us to propose a chemical transformations pathway during enzymatic and non-specific oxidation of luciferin (structures shown below).



I proposed the mechanism below to account for the detailed transformation leading to light emission by *Odontosyllis undecimdonata*.



Before participating with the project team, we had undertaken studies to identify the substrate luciferin and oxyluciferin emitter of *O. phosphorea* specimens provided by Dimitri Deheyn (Scripps Oceanographic Institute). While we did identify a fluorescent compound and determined a partial structure, the material we investigated was not a bioluminescence metabolite. The problem with our investigation was that we were severely material limited. In fact, knowing the structures of the luciferin and oxyluciferin, and using fresh samples of *O. phosphorea* provided by Dimitri, we could only find traces of the oxyluciferin and we were unable to definitively identify the luciferin using our most sensitive by LC/MS analysis.

4. Bioluminescence biosensor for endocrine-disrupting chemicals. Our collaborators in the Roda lab (University of Bologna, Italy) developed a two yeast system bioluminescence-based biosensor for endocrine-disrupting compounds in a cell phone based device (*Publication 5*). The methodology required two highly sensitive (bright) BL enzymes using two luciferases that reacted with separate substrates that could not cross react. One of the luciferase-luciferin pairs was the commercially available NanoLuc-furimazine. Our role in the project was to develop the second enzyme-substrate pair for their application. We accomplished this by screening and testing luciferase variants with firefly luciferin. The optimal luciferase variant was PLR2 an enzyme that had been constructed as a chimeric luciferase from the N-domain of *P. pyralis* luciferase and the C-domain of *L. italica*. Additional mutagenesis produced PLR2 a thermostable luciferase with BL maximum emission at 560 nm that when paired with firefly luciferin produced bright BL signals. PLR2 is resistant to pH shifting and thermostable, important properties for cell-based assays.

5. Development of an ultrasensitive bioluminescence-based method for the important metabolic target monoacylglycerol lipase. In a collaboration with the Ciuffreda lab (Università degli Studi di Milano, Italy) that involved graduate student Matteo Miceli working in our lab for several months, a novel bioluminescence-based assay was developed based on our PLR2 luciferase (*Publication 8*). The Monoacylglycerol lipase (MAGL) substrate 6-*O*-arachidonoylluciferin, a D-luciferin derivative, was synthesized, chemically characterized, and used as highly sensitive substrate for MAGL in this assay. This new method is based on the enzymatic cleavage of arachidonic acid with luciferin release using human Monoacylglycerol lipase (*hMAGL*) followed by its reaction with a chimeric luciferase, PLG2, to produce bioluminescence (*Figure 10*). Enzymatic cleavage of the new substrate by MAGL was demonstrated and kinetic constants, K_m and V_{max} have been determined. 6-*O*-arachidonoylluciferin has proved to be a highly sensitive substrate for MAGL. The bioluminescence assay (LOD 90 pM, LOQ 300 pM) is much more sensitive and should suffer fewer biological interferences in cells lysate applications than typical fluorometric methods. The assay was validated for the identification and characterization of MAGL modulators using the well-known MAGL inhibitor JZL184. The use of PLG2 displaying

distinct bioluminescence color and kinetics, should offer a highly desirable opportunity to extend the range of applications to cell-based assays.

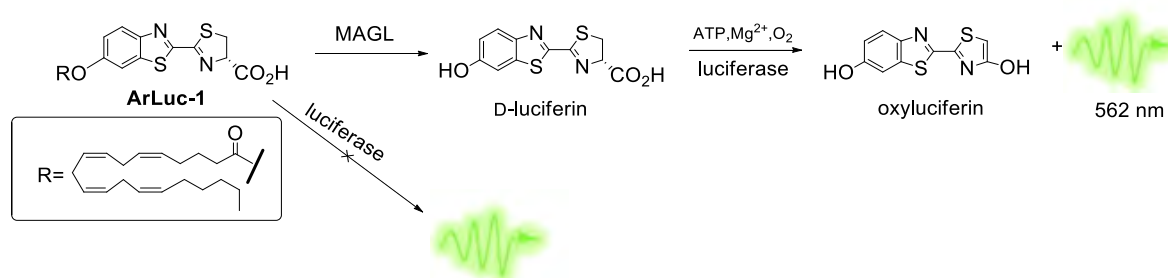


Figure 10. Bioluminescence reactions of compound ArLuc-1. The substrate chemical structure and biochemical reactions leading to 562 nm peak light emission (λ max) are shown.

6. A method for imaging bioluminescent CD8 T cells in an anti-tumor immunity study. We participated in a study lead by Yina Huang (Dartmouth University School of Medicine) by enabling methodology for the bioluminescence-based imaging of CD8 T cells expressing the Pmel T receptor and our PpyRE9 luciferase reporter gene. (*Publication 9*). The PpyRE9 luciferase is a *P. pyralis* variant that contains 9 mutations that red-shift bioluminescence, confer excellent thermostability at 37 °C, and produces light with maximum emission at 617 nm. The red light emission provides better transmission of BL through cells. A major result of this study was the identification of a novel role for dendritic cells and that the CD8 T cells remain in close proximity to dendritic cells in the skin.

Impacts

Development of the principal discipline(s) of the project. We have developed new enzymes (luciferases) that emit light (bioluminescence) in the presence of a natural substrate (firefly or beetle luciferin) or synthetic substrate analogs and ATP. While we made great progress in our main goal of producing an efficient system that serves as a biological source of near Infrared light, other enzymes made in our project have been developed by collaborators to create biosensors that detect important chemicals that are related to disease processes. Additionally, our work has contributed to the basic understanding of how light is produced by living organisms.

Development of other disciplines. Our systematic comparative study of beetle luciferase-substrate pairs that produce near Infrared light will positively impact the large community of scientists, including pharmaceutical scientists engaged in drug development, involved in *in vitro* bioluminescence imaging studies to monitor disease associated processes including cancer.

Development of human resources. In funded and non-funded roles, five research technicians (4 women and one man), five undergraduates (4 women, one of color, and one man), and a male international graduate student participated in this project. Three of the technicians moved on to careers at Pfizer Central Research, Groton, CT. The other two currently have research positions in my lab. The international student completed his PhD in Italy and the undergraduates have all graduated. Two are in PhD programs including the woman of color, one is in dental school, one is working in a pediatric clinic and will likely go on to medical school, and one is in a genetic counseling graduate program.

Physical, institutional, and information resources that form infrastructure. With equipment funding for this project, we obtained an IVIS Lumina III Imaging System. This instrument has been made available to the chemistry and biology departments at Connecticut College. The equipment was essential to the progress we made in developing our own luciferase variant-substrate analog system that is an excellent source of near Infrared light that produces little visible light. Additional published results (*Publication 11*) will benefit investigators working in the field of *in vivo* bioluminescence imaging.

Impact on society beyond science and technology. The future impacts of this project will be accomplished by the continued work of the 11 participants who were trained to think critically.

Changes

Delays

We experienced delays over an approximately 2 year period due to Covid restrictions at Connecticut College. I estimate that our research productivity was decreased ~40%, but we did receive a one year no cost extension. However, we were unable to continue the *Orfelia* bioluminescence system identification project because we missed 3 years of collection trips due to Covid-related travel restrictions.

Technical Updates

With one exception, all of the technical details not reported above are available in the four Annual Reports submitted previously and/or in the 11 listed publications. Therefore, the materials and methods related to the directed evolution studies are included here.

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) and ATP (disodium salt hydrate) from Sigma-Aldrich (St. Louis, MO, USA); Glutathione Sepharose 4B media and pGEX-6P-2 vector from GE Healthcare (Piscataway, NJ, USA); Ni-NTA

Agarose resin (Valencia, CA, USA); and XL10-Gold Ultracompetent Cells from Agilent Technologies. Plasmids containing *luc* genes for the following were obtained as generous gifts: CBR2opt (CBR2) (ATG-1929)⁵ in the pF4Ag vector from Keith Wood (Addgene plasmid # 108712; <http://n2t.net/addgene:108712>; RRID: Addgene_108712). Akaluc in the pCDH-EF1-MCS-T2A-copGFP vector was obtained from Gene Dynamics, LLC (Portland, OR, USA).

The PLR3 Luc protein was expressed in *Escherichia coli* strain BL21(DE3) pLysS from the pGex-6P-2 vector. The PLR3 gene was constructed by inserting the G246A and F250H mutations into the PLR1 gene.

The substrates NH₂-NpLH₂ and OH-QLH₂ were synthesized by Brian Huta and Ben Fort in the Branchini laboratory (Connecticut College, New London, CT, USA). TokeOni, also known as AkaLumine-HCl (Aka), was purchased from Sigma Aldrich (St. Louis, MO, USA) as ((*S*)-2-((1*E*,3*E*)-4-(4-(dimethylamino) phenyl) buta-1,3-dien-1-yl)-4,5-dihydrothiazole-4-carboxylic acid) The following substrate was a generous gift: potassium salts of Beetle LH₂ ((*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid) from Promega (Madison, WI, USA).

General Methods. All Luc gene sequences were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT, USA). The luciferase genes were expressed in the pGEX-6P-2 expression vector and the purified enzymes contain the N-terminal peptide extension GlyProLeuGlySer-. Concentrations of purified proteins were determined using a NanoDropTM Lite Spectrophotometer. Typical protein yields were 7-10 mg per 0.25 L culture.

Mutagenesis. Site-directed and site-saturation mutagenesis were performed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Initially, the PLR3 gene was the template for mutagenesis.

Screening and picking. Screening of the GeneMorph® Libraries were carried out using an IVIS Spectrum III (Perkin Elmer) with an 8 second exposure, FOV B, and OPEN filter settings. The DNA was transformed into XL10-Gold Ultracompetent Cells and plated onto Luria–Bertani (LB) media/Agar plates containing 0.1 mg/mL ampicillin and nitrocellulose, placed on top of the agar. The plates were incubated at 37°C to for ~24 h. The nitrocellulose sheet was then placed in 600 µL of 0.07 mg/mL of OH-QLH₂ in 100mM sodium citrate buffer (pH 5.50). After a ~30 s incubation, BL was measured at 22°C. Signals were monitored for 8 min with a measurement at 1-minute intervals. Data were analyzed with the Living Image 4.7 Software (Perkin Elmer) after selecting the appropriate region of interest (ROI) Luc–substrate activity was recorded in radiance (p/s/cm²/sr). The colonies with brightness at least 2 - 4-fold greater than the control were picked and grown overnight in LB media with 0.1 mg/mL ampicillin.

Protein Expression and Purification. Glutathione S-transferase (GST) fusion constructs of all enzymes in the pGEX-6P-2 bacterial expression vector were expressed in *Escherichia coli* strain BL21(DE3) pLysS as previously described. Cultures (0.25 L in Luria–Bertani media with 0.1 mg/mL ampicillin) were grown in 1 L flasks at 37°C to mid-log phase ($A_{600} = 0.5-0.7$) and then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and incubated at 22°C for 18-20 h. Cells were harvested by centrifugation at 4°C and then frozen at –80°C for 15 min. Cell

pellets were resuspended in 25 mL of phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (DTT). After the addition of lysozyme (2.5 mL of 10 mg/mL solution in PBS), the cells were lysed by sonication (three 10 s bursts at setting 3 of a Virsonic model 475 instrument) and treated with DNase (5 μ g/mL) and RNase (10 μ g/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20,000 $\times$ g for 45 min. Proteins were further purified using Glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT (CB; cleavage buffer) for 18–20 h at 4°C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N₂ for long-term storage at –80°C or stored at 4°C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Bioluminescence Emission Spectra. BL was initiated by mixing equal volumes (0.25 mL) of a solution of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄ with a solution of 50 mM Tricine pH 7.4 containing 12.5 μ g of enzyme and 0.1 mM of the indicated analog in a quartz cuvette. All solutions were pre-warmed to 23°C. The final concentrations of the mixture (0.5 mL) in 50 mM Tricine pH 7.4 were 0.4 μ M enzyme, 50 μ M of the indicated analog, 1 mM ATP, and 3 mM MgSO₄. Emission spectra were acquired at 23°C after a 1 min delay with a Horiba Jobin-Yvon iHR 320 imaging spectrometer equipped with a liquid N₂ cooled CCD detector. Data were collected over the wavelength range 450–925 nm, with the excitation source turned off and the emission slit width set to 10 nm and were corrected for the spectral response of the detector using a correction curve provided by the manufacturer.

In Vitro Specific Activities. All assays were performed in triplicate in white 96-well microtiter plates containing 2.5 μ g of purified enzyme and 50 μ L of 0.1 mM analog in 50 mM Tricine pH 7.4. BL was initiated by the automated injection of the assay mix containing 50 μ L of 50 mM Tricine pH 7.4 containing 2 mM ATP and 6 mM MgSO₄. Signals were monitored over 2 min using a Synergy 2 microplate luminometer (BioTek, Winooski, VT, USA). Data were integrated and corrected for the spectral response of the Hamamatsu R928 PMT detector.

K_m Measurements. Values of K_m for the substrates/analogues were determined from BL activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. The equipment used to make these measurements has been previously described. Data were collected in 0.525 mL reactions in CBA, pH 7.4. Reactions were initiated by injection of 0.12 mL of a solution of Mg-ATP (9 mM). K_m values were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola.

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