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14. ABSTRACT Breast cancer metastases detection, imaging and management is limited by a key technical problem fundamental to the nature of imaging, which is that micro metastases cannot be visualized at a relevant stage, largely because most imaging is based upon structures and not molecular functions. Surgical dissection is commonly done, as a result of the fact that there is no molecular imaging tool today which can routinely sense the presence of cancer cells in lymph nodes or organs at the micro-metastases stage. The needed probe sensitivity would be in the microMolar to nanoMolar range with sub mm spatial resolution throughout the body. In this proposal, we develop a completely unique hybrid modality which will have high resolution capability through several centimeters of tissue, and all the molecular specificity of standard optical luminescence tracers, with sensitivity down to the sub microMolar level and spatial resolution below 1mm. The new approach uses high energy x-rays from a linear accelerator, LINAC, used in radiation therapy, allowing metabolic imaging and treatment in the same setting. The LINAC radiation induces Cherenkov light in the tissue, and this light can excite luminescence for molecular imaging. This study on metastatic cancer will have shown the value in a single detection paradigm of metastatic cancer in lymph nodes.					
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Addressing overlap with recently funded NIH grant R01EB024498-01 (page 14)

Specific Aims page for R01EB024498-01 (page 15)

Publications & pending publications (pages 16-69)

- 1) Dsouza A, Lin H, Gunn JR, Gladstone DJ, Jarvis LA, Pogue BW. Cherenkov-excited Multi-Fluorophore Sensing in Tissue-Simulating Phantoms and In Vivo from External Beam Radiotherapy. **Radiation Research** 2018 Feb;189(2):197-204. doi: 10.1667/RR14943.1. PMID: 29251551 (9 pgs)
- 2) S. R. Ahmed, J. M. Jia, P. Bruza, S. Vinogradov, S. Jiang, D. J. Gladstone, L. A. Jarvis, B. W. Pogue, "Radiotherapy-induced Cherenkov Luminescence Imaging in a Human Body Phantom" *Journal of Biomedical Optics* (in press, 2018) (4 pages)
- 3) J. R. Shell, E. P. LaRochelle, P. Bruza, J. R. Gunn, S. A. Vinogradov, L. A. Jarvis, D. J. Gladstone, B. W. Pogue "Comparison of Phosphorescent Agents for Noninvasive Detection Imaging of Tumor Oxygenation via Cherenkov Excited Luminescence Imaging" (submitted to **Molecular Imaging & Biology**, March 2018) (12 pages)
- 4) M. Jia, P. Bruza, D. J. Gladstone, B. W. Pogue, "Multi-point and linescan excitation from a linac for improved lateral resolution in Cherenkov-excited molecular luminescence imaging" in **submission, Optical** 2018 (7 pages)
- 5) B. W. Pogue, J. Feng, E. P. LaRochelle, P. Bruza, H. Lin, R. Zhang, J. R. Shell, H. Dehghani, S. C. Davis, S. A. Vinogradov, D. J. Gladstone, L. A. Jarvis "Maps of in vivo oxygen pressure with submillimetre resolution and nanomolar sensitivity enabled by Cherenkov-excited luminescence scanned imaging" **Nature Biomedical Engineering** (in press, for April 2018) (22 pages)

1. INTRODUCTION:

Breast cancer metastases detection, imaging and management is limited by a key technical problem fundamental to the nature of imaging, which is that micro metastases cannot be visualized at a relevant stage., largely because most imaging is based upon structures and not molecular functions. But there are no tools to effectively sense early metastases, and this lack of high spatial resolution molecular imaging is a key factor inhibiting breast metastases research and treatment. In this proposal, we develop a completely unique hybrid modality which will have high resolution capability through several centimeters of tissue, and all the molecular specificity of standard optical luminescence tracers, with sensitivity down to the sub μM level and spatial resolution below 1mm. The new approach uses high energy x-rays from a linear accelerator, LINAC, used in radiation therapy, allowing metabolic imaging and treatment in the same setting. The LINAC radiation induces Cerenkov light in the tissue, and this light can excite luminescence for molecular imaging. This Cerenkov-excited luminescence scanned imaging approach is referred to as CELSI throughout this work. We proposed to develop the two unique parts to this tool, and test it as combined with multi-probe imaging, to quantify the molecular microenvironment of cancer tumors in vivo.

The individual aims in this overall goal are (1) to optimize the setup and design of a dedicated system to sense signals. (2) To design and build a dedicated system suitable for imaging signals from in small animals on the standard radiation therapy tools. (3) To evaluate the limits on structural, metabolic and immunologic probes for molecular imaging, and (4) to complete studies on metastatic breast cancer with MeV photon imaging and treatment.

CELSI has been demonstrated at the initial in vivo feasibility stage in a lymph node imaging study and should be evaluated as a fundamentally new molecular imaging modality. We anticipate the as the ability to sense molecular concentrations at low radiation dose gets established that human use possibilities will be discovered, either as a therapy/diagnosis tool in Radiation Oncology or purely as a low dose diagnostic.

2. KEYWORDS:

Radiotherapy, Cerenkov, Cherenkov, imaging, tomography, sentinel, lymph node, metastases, detection, staging, therapy.

3. ACCOMPLISHMENTS:

- What were the major goals of the project?

Tasks	Timeline	Site 1 (PI Pogue)	Site 2 (PI Jarvis & Gladstone)
Major Task 1: Development of a high-resolution molecular imaging system	Months	Engineering	Radiation Oncology
Subtask 1: Evaluate components for optimal CELSI system - oversee system development, detector choice & evaluation - Monte Carlo modeling of detection & geometry	1-6 3-6	Bruza & Pogue	
Subtask 2: Determine the limits to detection for CELSI - Dr Pogue's lab: testing limits, SNR, CBR, for relevant targets - Dr. Gladstone's lab: evaluate the performance in linac use, testing dose requirements. - Dr. Jarvis's lab: estimate limits for lymph node detection in animals & humans	7-10 11-12		Dr Gladstone & Gollub Gunn & Jarvis
Milestones: <i>quantify possible tradeoffs on: depth, ii) radiation dose, and iii) probe concentration</i> <i>identify realistic limits for radiation dose & probe concentration for lymph node imaging</i> <i>identify realistic limits for tumor imaging with intravascular & interstitial delivery</i> <i>Publication on limits and potential for CELSI imaging (Phys. Med. Biol.)</i>			
Major Task 2: Design and build a dedicated system for CELSI imaging in small animals, to allow imaging of molecular probes in a manner which has maximal spatial, temporal and contrast resolution.		Engineering	Radiation Oncology

Subtask 1: Completion of an optimized system for delivery to Radiation Oncology	13-18	Jia & Pogue	
Subtask 2: Implementation in radiation oncology & initial animal testing.	19-21		Shell, Gladstone & Gunn
Milestones: <i>Delivery of completed system capable of 200 micron resolution & imaging 2 cm deep into tissue</i> <i>Co-author manuscript on system and construction (Optics Letters)</i>			
Major Task 3: Evaluate the quantitative limits on structural, metabolic and immunologic probes for metastatic breast cancer molecular imaging with CELSI and establishing Standard Uptake Values (SUV).		Engineering	Radiation Oncology
Subtask 1: Evaluating molecular probes for use	22-24	Pogue & Shell	Gunn, Shell & Jarvis
Subtask 2: Establish SUV value in vivo	25-28	Pogue & Shell	Gunn, Shell & Jarvis
Milestones: <i>Demonstrated limits to detection for metabolic & immunologic probes.</i> <i>Publication on CELSI & SUV of different molecular probes (Science Translational Medicine)</i>			
Major Task 4: Complete metastatic breast cancer detection & treatment with MeV photon treatment		Engineering	Radiation Oncology
Subtask 1: complete metastatic & non-metastatic imaging studies in vivo	29-34	Pogue	Gunn & Jarvis
Subtask 2: complete radiation treatment study on involved nodes	34-36		Gunn & Jarvis
Milestone #4: <i>demonstration of in vivo detection and limits to numbers of cells detectable in lymph nodes</i> <i>Publication on in vivo detection & treatment using CELSI and radiation therapy (Int. J. Rad. Oncol. Biol. Phys.).</i>			

What was accomplished under these goals?

In year 1, the Major Task 1 was largely completed, as described in the year 1 report. **What remained as the last goal of subtask 2**, to examine how this approach to imaging could be optimally deployed in animal and human lymph nodes. So, in this past year we completed a series of studies in which this goal was definitively completed for the potential for translation to human use.

In Major Task 1, Subtask 2, For human lymph node assessment, the feasibility of sensing luminescence from a tissue molecular oxygen sensor from within a human body phantom was examined using the geometry of the axillary lymph node region. Radiation sheet scanning in an epi-illumination geometry provided optimal coverage, and maximum intensity projection images provided illustration of the concept. In summary, detection of regions down to 30 mm deep was feasible with sub-millimeter spatial resolution with the total quantity of the phosphorescent sensor PtG4 near 1 nanomole. This work provided the preliminary information needed to attempt this type of imaging in vivo.

The work included a set of experiments initially focused on determining the optimum positioning of the body phantom, couch, gantry and camera for maximal sensitivity. Three orientations were investigated: i) vertical with the gantry directly above the body phantom on the couch at 180° reference angle and the camera placed directly facing the left side of the body phantom; ii) horizontal with the gantry directly facing the left side of the body phantom at 90° reference angle and a 45° front-surface mirror placed above the top surface of the body phantom, allowing horizontal mounting of the ICCD directly facing the mirror; and iii) angled with the gantry at 145° reference angle with ICCD on the same side. The angular orientation in an epi-illumination configuration was determined to be optimum and used for all subsequent experiments. In this orientation, a Cherenkov sheet was clearly visible translating through the body phantom (see Figure 2 below). The recovery of intensity was highly modulated by the depth of tissue overlying the tubes, and it was apparent that the signal was not linear with concentration because of this effect. However, with skin thickness measured by the CT scan and geometry, it is feasible to quantify these values based on depth-dependent attenuation correction.

In order to pursue a minimum radiation dose scan design, a sparse illumination was also investigated, i.e. the number of radiation sheets used was desired to be as few as possible. Here, a total of 25, 50, 100 and 250 light sheets were

used with equal spacing, i.e. with spaces 2mm, 1mm, 0.5mm, and 0.2mm, respectively. It can be seen that the image quality was substantially equivalent, even for 25 light sheets.

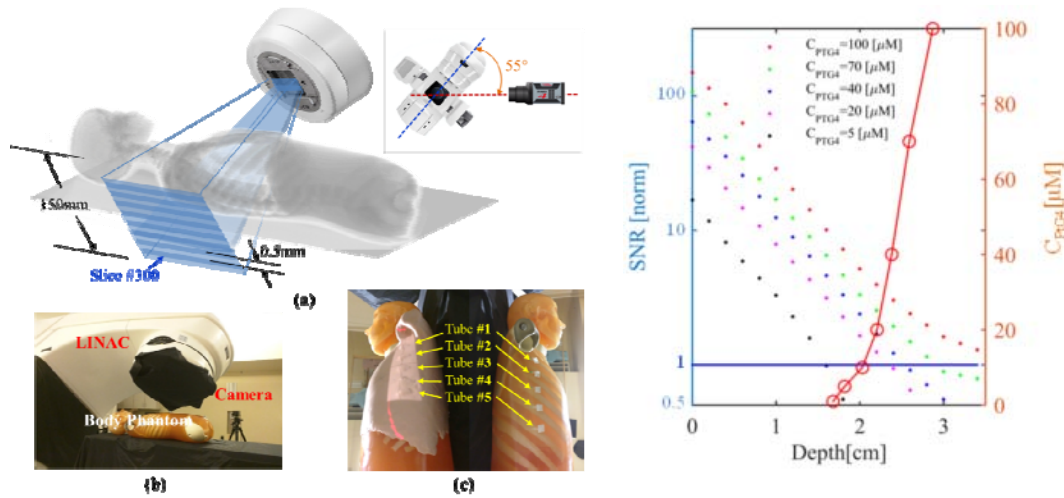


Figure 1. The measurement geometry for a body phantom is shown with (a) a schematic of the geometry, (b) a photograph of the setup, and (c) the body phantom with skin on (left) and without skin (right) as shown with five tubes fixed onto the lateral rib area. In the graph at right, a series of depth studies for CELSI recovery were done with the tubes used here, to show that imaging with sufficient Signal to noise ratio (SNR) was possible down to 2-3cm, but that the minimum detectable concentration necessarily had to increase as the depth grew (red line data & right vertical axis).

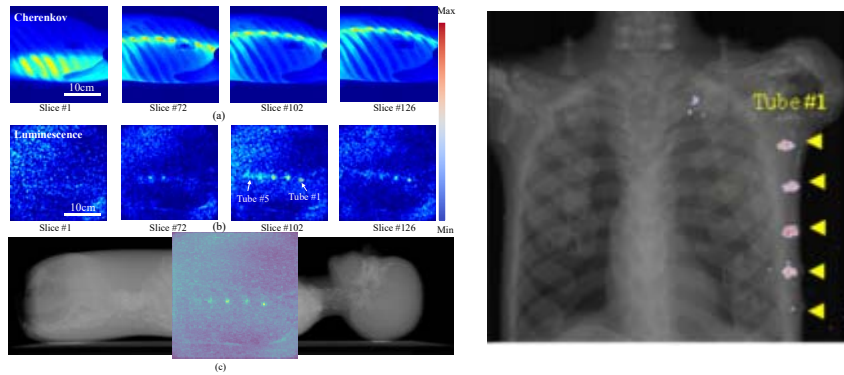


Figure 2. The imaging of the human body phantom shown in Figure 1 was completed with modeling clay skin overlying the 5 tubes of PtG4, and in the top row, 4 static time images of the linac line scan are shown at points during the vertical sweep, showing the Cherenkov emission intensity. The recovered CELSI luminescence in the middle row at each timepoint, and then in the bottom row the Maximum intensity projection is overlaid on the x-ray CT scan. The thresholded image data is shown more clearly in the image at right.

Deliverable for Major Task 1, Subtask 2: In summary, for human assessment, our conclusion is that for human imaging, we can image lymph node sized objects down to 2cm depth with as low as 10 uM concentration of PtG4 in the node, and down to 3cm depth with 100 uM concentration, and this can be done for radiation doses near 0.1 Gy, characteristic of a diagnostic x-ray CT scan.

Major Task 2, Subtask 1: Completion of an optimized system for delivery to Radiation Oncology

We have finished this goal, designing a way to optimize scan time and optimize image quality. The scan time is optimized by using multiple beams from the same linac head, by designing a multi-point or multi-line scan approach, similar to a scanning pinhole confocal microscope. The set up and sequence of studies are shown below in the figures. Briefly, the linac Multi-leaf colimator (MLC) was set to scan a spot, in phantoms and a small luminescent test object placed inside (Figure 3(a)). The scanning of the beam in smaller and smaller blocks illustrates that the Signal to Background Ratio (SBR) is better with smaller spots, because the rejection of outside light is better.

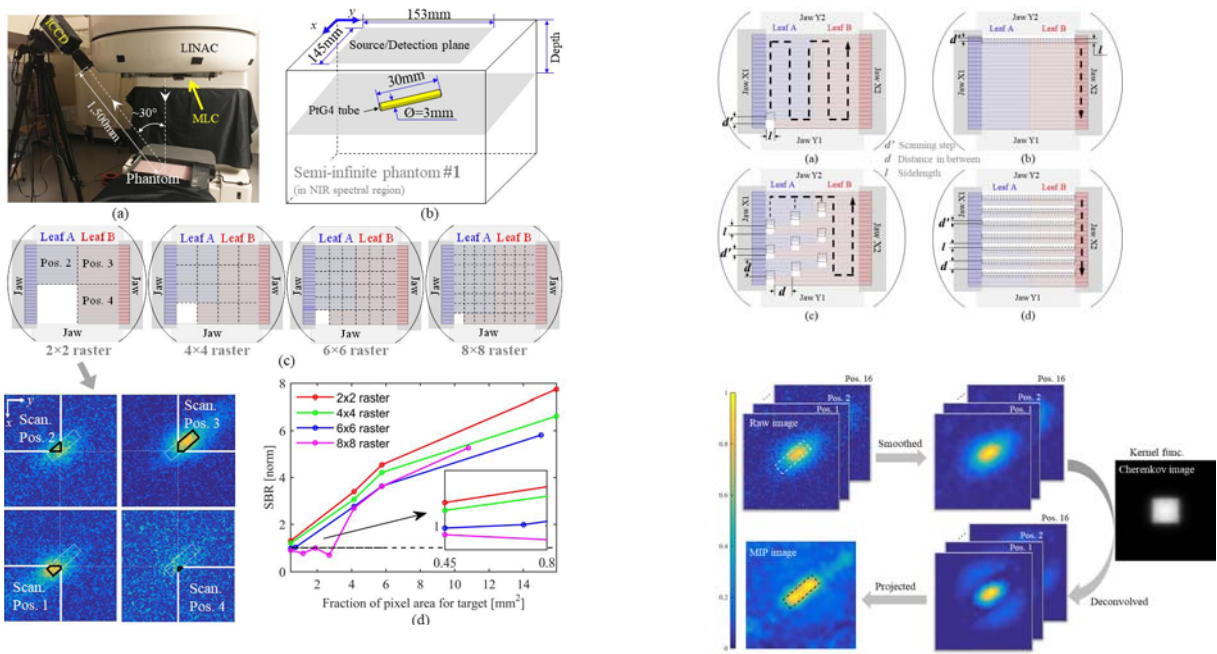


Figure 3. Experiments on the effective radiation beam size shown at left (a) the experimental setup for Cherenkov light sheet luminescence imaging; (b) the phantom geometry; (c) square-raster scanning with different pixel areas, and CELSI images from a 2x2 raster illumination, and (d) the calculated Signal to Background ratio (SBR) versus target area within an illumination square for different square size, indicating smaller is usually better. In the scanning approaches the range of scans from points or lines are illustrated at top right. Then finally in the bottom right images, the sequence of image processing to go from raw data to smoothed, to deconvolved is shown.

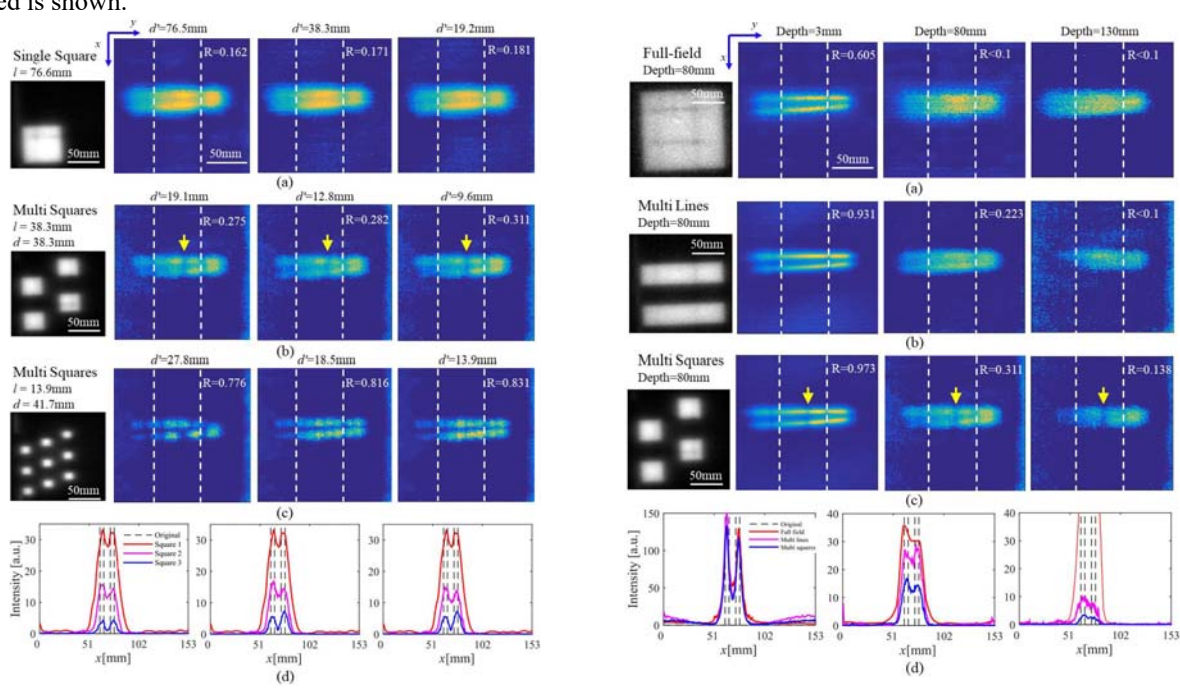


Figure 4. Cherenkov (most left) and CELSI (right color) images measured are shown as measured under the range of beam shapes shown at left. The spot scans are shown in the image at left and the comparison between a single broad beam, line scan and block scans is shown at right. The target was two lines of PtG4 embedded in tissue phantom media, which can be seen best resolved in the smaller point scan approaches. The bottom row shows the line profiles for the above scans.

Deliverable for Major Task 2, Subtask 1: We have defined our preferred approach to multipoint scanning of tissues, which optimizes for speed while providing independent sampling of the tissue at each point, and allows scanning with the same radiation dose and spatial resolution achieved as for a single point or line. This methodology is ready to be deployed in our preclinical testing.

Major Task 2, Subtask 2: Implementation in radiation oncology & initial animal testing.

A series of animal studies were carried out to verify that sufficient signal to background was obtainable for line-scan approaches. We carried out individual scans of mice injected with PtG4 and Iridium complex, to assess signal to background ratio (SBR). The optimization of the acquisition parameters were provided by a related ongoing study, and the focus here was to demonstrate sufficient SBR values to carry on with larger cohorts of mice. As shown in the Figure 5 below, both agents were imaged well (a), and the linescan approach illustrated in (b) provided SBR values near 2, for these minimal concentrations of 10 uM probe used and near 1 cGy of radiation dose. These represent true lower limits of use, and SBR will increase nearly linearly with increases of either input parameter. Finally, in a pilot study of 8 tumors, we showed that with PtG4, the tumor pO2 could be measured from the lifetime decay change (c).

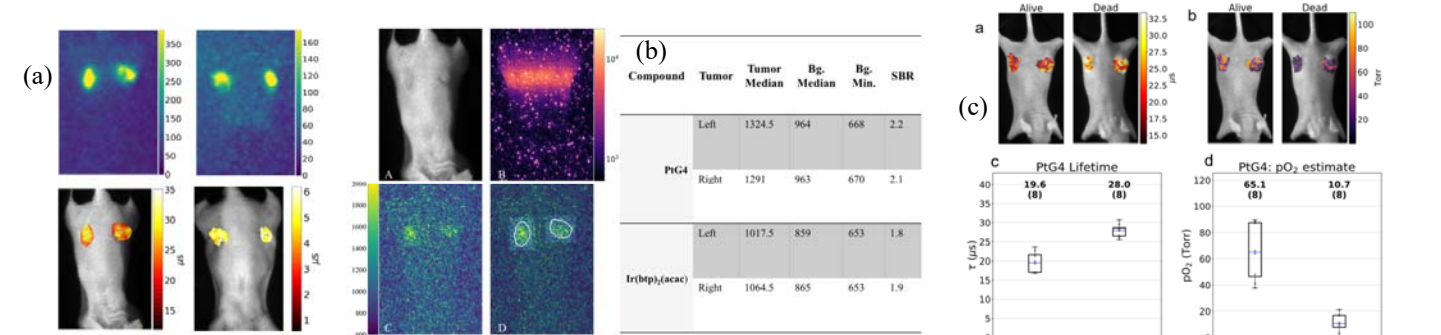


Figure 5. Images of mice in (a) show PtG4 (left) and Ir complex (right) for CELSI images (top row) and luminescence lifetimes overlaid on the white light mouse images. The linescan sequence is illustrated in (b) with lower limits on detection assessed for both PtG4 and Ir complex, with numerical data tabulated beside it to calculate the signal to background ratio (SBR). In (c) PtG4 was used with linescan sequences of CELSI to show that the luminescence lifetimes (left) with mice alive and dead show good representation of the expected values of pO₂ (65 mmHg alive and 5 mmHg dead).

Deliverable for Major Task 2, subtask 2: The scan sequences developed for phantom imaging work well in preliminary in vivo settings. Using the lower limits on PtG4 concentration (10 uM) in small volumes, and minimal radiation dose (1 cGy), Signal to Background is above 2 for PtG4. This is the lower limit, and increases in either radiation dose, PtG4 concentration will improve this. However, we also recognize that increases in depth must be accompanied by nearly linear increases in either PtG4 concentration or radiation dose in order to achieve the same SBR value.

Major Task 3, Subtask 1: Evaluating molecular probes for use: Metabolic & Immunologic

This subtask was advanced considerably this year by Dr Shell who joined the group. We completed a comparison study of all possible biocompatible **metabolic oxygen sensing luminescent agents**. We also began work on a next generation approach to imaging immune binding CELSI agents, by identifying Europium Chelate Microspheres as the ideal platform for luminescent activity and compatible with biological protein labeling. Both of these advances are described below.

Briefly, the oxygen sensing probes were chosen from a survey of the literature, and in communication with those who produce them. We identified 4 possible commercially available candidates that have claimed biological compatibility and in situ oxygen sensing with lifetimes in the range of microseconds. These were PtG4 (S. Vinogradov U Penn), Iridium complex Ir(btp)₂(acac) (Lumtec Corp), MM2 nanoparticles (Ibidi USA) and MitoID (Enzo Lifesciences). These were each compared for their efficiency i) in solution ii) in cells in vitro, and iii) in vivo (summarized in a paper in review, Shell et al, attached in appendix). In the end, only PtG4 and Ir(btp)₂(acac) were viable in vivo, which narrowed down the choice. The signal strengths were strong, and preliminary in vivo studies (Shown below) illustrated that the could both be detected with sufficient signal to background.

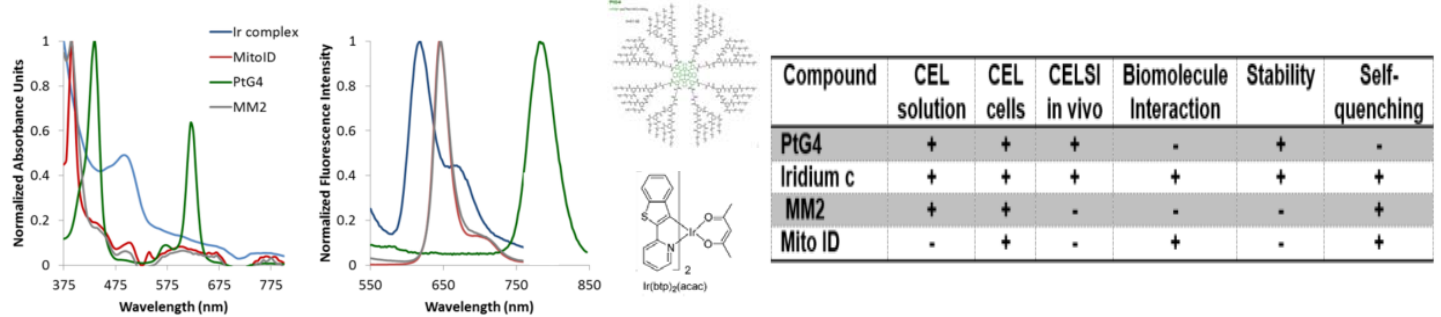


Figure 6. The 4 agents tested have their absorption and emission spectra shown (left) and the molecular structures of PtG4 and Ir(btp)₂(acac) are shown, in the middle. The summary of the testing is listed in the table at right, showing phantom, in vitro and in vivo results.

vivo results with interaction, stability and quenching effects. In the end, the in vivo results showed that only PtG4 and I(otp)2(acac) would be viable.

Additionally, we have initiated a comparison study of different ways to image **molecular tracers for immunology**. Initial studies that were done with fluorescent tracers, with fast lifetime, have not proven useful, because the temporal kinetics are too fast (1-20ns) for the comparatively slower linac pulse (3-4 us) and they could not be separated by time-gating. Spectral separation of the signals has been shown in a recent work by our group, published by Dsouza et al, (Radiation Research, 2017), but the inability to spectrally image with sufficient signal to background makes this approach unattractive. After initial screening to compare different luminescent agents, Europium chelate microspheres were found to be an optimal solution for immune sensing with CELSI, because they are highly luminescent upon UV excitation, emitting in red wavelengths, as shown in Figure 7(a). These agents were chosen for their ability to be imaged in vivo (Figure 7(d&e)), as well as be labeled with proteins such as Erbitux (Figure 7(b&c)). The signal strength has been examined and it shows the ability to image signals at the nmol concentration level, at varying sizes down to 10^{-15} moles. Given a receptor concentrations of 10 nMolar, this would indicate that we could image tumors down to $\text{vol} = 10^{-15}/10^{-8} = 10^{-7}$ L or 0.1 microliters. This is sufficient to be able to image small metastases, which is the primary goal of this work.

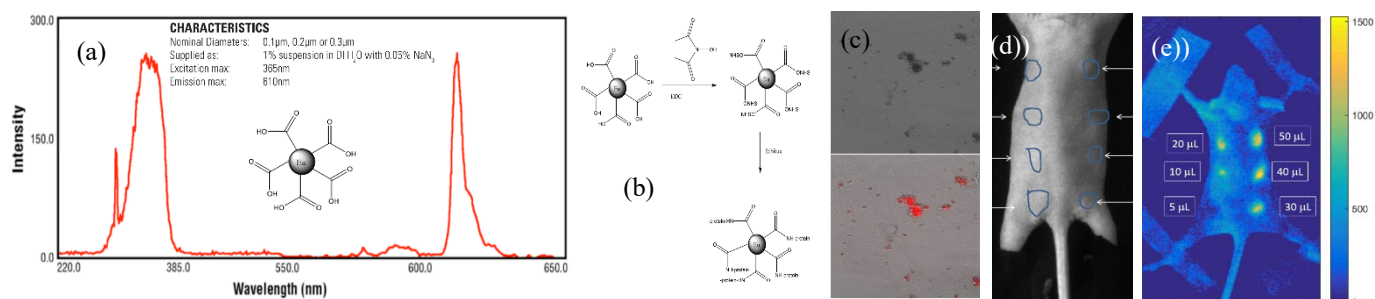


Figure 7. The Eu Chelate microsphere absorption and emission spectra are shown (a), with the chemistry produced to label them with proteins (b). After labeling with Erbitux, and purification, MCF7 cells were incubated with them to show positive uptake with EGFR expression (c). Imaging of the luminescence from CELSI excitation is shown in vivo (d) with image emission data (e). This will likely become our platform technology for immune sensing with CELSI probes in Aim 3.

Deliverables for Major Task 3, subtask 1: We have clearly identified our lead candidate molecules for Metabolic Sensing (PtG4 for pO₂) and Immunologic sensing (Eu Chelate microspheres conjugated with antibodies). These can be used in concentrations that are viable for molecular sensing in vivo (10 uM for PtG4 and nM level for Eu Chelate microspheres), and at radiation doses compatible with diagnostic imaging (near 1 cGy).

What opportunities for training and professional development has the project provided?

Mengyu Jian (post doc) obtained knowledge and mentoring about how to run the linear accelerators, how to acquire data with the cameras, and how to process the data. Jennifer Shell (Research Scientist) has been exposed to the Radiation Therapy world and trained in how to use these systems.

How were the results disseminated to communities of interest?

Our publication efforts have been quite productive this year, with 2 published, 2 near the page proof stage, and 2 more in submission/review. Dr Pogue had 4 invited conference presentations on this work at high profile academic venues, and there was at least one additional conference presentation, all listed below.

What do you plan to do during the next reporting period to accomplish the goals?

In this next year we plan to significantly advance our goals of finding the limits to lymph node size detection with different agents. We will be taking our chosen phosphors into cells and then into rodents to assess in vivo potential. We plan to test a new fast detector which has recently been introduced by Becker&Hickl which should allow superior temporal sampling and therefore improve our ability to use fluorophores with nanosecond lifetimes.

We do not plan any staffing changes in this next year, so expect to be quite productive in completing this work and publishing the results further.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project? - Nothing to report

What was the impact on other disciplines?

We demonstrated for the first time that recovery of luminescent objects could be recovered from depths near 3cm in tissue simulating media in a human body phantom. This is being published in our paper Ahmed et al, J. Biomed Optics (in press 2018), and showed that by Cherenkov excitation and data collection, can be used in a human geometry. This is a paradigm shift in how to think about molecular sensing in human tissue, and in particular if we could interrogate lymph nodes in patients with potential metastases. These proof of concept studies allow us to consider planning for human studies. The idea is quite forward looking and so while it has not penetrated any clinical practice yet, there is considerable interest in the idea, as indicated by our paper getting accepted for publication in Nature Biomedical Engineering (see appendix).

What was the impact on technology transfer? - Nothing to report to date.

What was the impact on society beyond science and technology?

The capabilities of CELSI will allow more useful detection of small objects inside tissue, such as lymph nodes involved with breast cancer. This could have wide ranging implications on how metastatic breast cancer is managed. It is hoped that this high-resolution imaging at the 0 – 3 cm depth range will provide the tool needed to interrogate lymph nodes and help surgeons make a decision about whether to resect them or not. If this works, it would reduce the number of patients with morbidity associated with lymphedema.

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change - Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them - Nothing to report.

Changes that had a significant impact on expenditures - Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents - Nothing to report

Significant changes in use or care of human subjects – Nothing to report.

Significant changes in use or care of vertebrate animals. – Nothing to report.

Significant changes in use of biohazards and/or select agents – Nothing to report.

6. **PRODUCTS:**

Journal publications published in the past year:

1. Dsouza A, Lin H, Gunn JR, Gladstone DJ, Jarvis LA, Pogue BW. Cherenkov-excited Multi-Fluorophore Sensing in Tissue-Simulating Phantoms and In Vivo from External Beam Radiotherapy. **Radiation Research** **2018 Feb**;189(2):197-204. doi: 10.1667/RR14943.1. PMID: 29251551

Journal publications in press:

2. E. P. M. LaRochelle, J. R. Shell, J. R. Gunn, S. C. Davis, B. W. Pogue "Signal intensity analysis and optimization for in vivo imaging of Cherenkov and excited luminescence" *Physics in Medicine and Biology* (**in press, 2018**)
3. S. R. Ahmed, J. M. Jia, P. Bruza, S. Vinogradov, S. Jiang, D. J. Gladstone, L. A. Jarvis, B. W. Pogue, "Radiotherapy-induced Cherenkov Luminescence Imaging in a Human Body Phantom" *Journal of Biomedical Optics* (**in press, 2018**)
4. B. W. Pogue, J. Feng, E. P. LaRochelle, P. Bruza, H. Lin, R. Zhang, J. R. Shell, H. Dehghani, S. C. Davis, S. A. Vinogradov, D. J. Gladstone, L. A. Jarvis "Maps of in vivo oxygen pressure with submillimetre resolution and nanomolar sensitivity enabled by Cherenkov-excited luminescence scanned imaging" **Nature Biomedical Engineering** (**in press, expected April 2018**) *(Note this was reported last year as a paper pending but the title has now changed, last year it was listed as: B. W. Pogue, et al "Molecular Cherenkov Excited Luminescence Scanned Imaging In Vivo: highest resolution & sensitivity for pre-clinical whole body imaging" (manuscript drafted for Nature Biomedical Engineering))*

Journal publications in submission/review:

5. J. R. Shell, E. P. LaRochelle, P. Bruza, J. R. Gunn, S. A. Vinogradov, L. A. Jarvis, D. J. Gladstone, B. W. Pogue "Comparison of Phosphorescent Agents for Noninvasive Detection Imaging of Tumor Oxygenation via Cherenkov Excited Luminescence Imaging" (**submitted to Molecular Imaging & Biology, March 2018**)
6. M. Jia, P. Bruza, D. J. Gladstone, B. W. Pogue, "Multi-point and linescan excitation from a linac for improved lateral resolution in Cherenkov-excited molecular luminescence imaging" **in submission, 2018**

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers, and presentations.

Presentations – Invited/Plenary

1. Brian W Pogue, "Optical Systems for Image Guided Therapy" Invited Talk, AAPM FOrEM on the scientific challenges of image guided cancer treatment, Arlington VA, Nov 2017.
2. Brian W Pogue, "Optical Imaging in Surgery, Radiology & Radiation Therapy" Plenary Speaker, Photonics in Imaging and Medicine Conference, Suzhou China, Sept 25 2017.
3. Brian W Pogue, "Cherenkov Excited Luminescence Scanned Imaging: The highest resolution whole body molecular imaging" Invited Talk, Advances in Optics for Biotechnology, Surgery and Medicine, Engineering Conferences International, Snowmass CO, July 2017.
4. Brian W Pogue, "Cherenkov Excited Luminescence Scanned Imaging: The highest resolution whole body molecular imaging" Invited Talk, From Light to Sound: Frontiers in Deep Tissue Imaging, Janelia Farms, Ashburn VA, June 2017

Conference papers published

- 1) J. Feng, P. Bruza, H. Dehghani, S. C. Davis, B. W. Pogue, Cherenkov-excited luminescence sheet imaging (CELSI) tomographic reconstruction Proceedings of SPIE BIOS, Volume 10049: Molecular-Guided Surgery: Molecules, Devices, and Applications III April 2017

b. **Website(s) or other Internet site(s)** - *Nothing to report*

c. **Technologies or techniques** – *Nothing to report*

d. **Inventions, patent applications, and/or licenses** - *Nothing to report*

e. **Other Products**

We have produced mostly control software and analysis tools for CELSI. These are available to any researchers interested, and are distributed via our website (www.nirfast.org).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. **What individuals have worked on the project?**

Name:	<i>Mengyu (Jeremy) Jia</i>
Project Role:	<i>Post Doctoral Researcher</i>
Researcher Identifier (e.g. ORCID ID):	<i>n/a</i>
Nearest person month worked:	<i>2.86</i>
Contribution to Project:	<i>Dr Jia worked on CELSI data collection methods and beam geometry optimization. He is the lead on data and image acquisition as well as quantitative analysis.</i>
Funding Support:	<i>This award</i>

b.

Name:	<i>Jason Gunn</i>
Project Role:	<i>Lifescience Laboratory Technician</i>
Researcher Identifier (e.g. ORCID ID):	<i>n/a</i>
Nearest person month worked:	<i>1.55</i>
Contribution to Project:	<i>Jason Gunn works on preparation of all animals, cell culture work and tissue collection and analysis.</i>
Funding Support:	<i>This award</i>

c.

Name:	<i>Jennifer Shell</i>
Project Role:	<i>Research Scientist</i>
Researcher Identifier (e.g. ORCID ID):	<i>n/a</i>
Nearest person month worked:	<i>3</i>
Contribution to Project:	<i>Dr Shell is a chemist who focuses on testing of different oxygen sensing probes, and conjugation chemistry and testing of our new immune targeting probes.</i>
Funding Support:	<i>This award</i>

d.

Name:	<i>Brian Pogue</i>
Project Role:	<i>Co-PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>n/a</i>
Nearest person month worked:	<i>2.05</i>

Contribution to Project:	<i>Dr Pogue supervises all laboratory work on data collection, experimental design and analysis of results.</i>
Funding Support:	<i>This award</i>

e.

Name:	<i>Lesley Jarvis, MD PhD</i>
Project Role:	<i>Co-PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>n/a</i>
Nearest person month worked:	<i>2</i>
Contribution to Project:	<i>Dr Jarvis supervises all biological laboratory work on tumor choices and preparation for animal work and analysis of results.</i>
Funding Support:	<i>This award</i>

f.

Name:	<i>David Gladstone</i>
Project Role:	<i>Co-Investigator</i>
Researcher Identifier (e.g. ORCID ID):	<i>n/a</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Dr Gladstone supervises all radiotherapy access and work related to beam choices, scanning, machine use and scheduling, and interpretation of results.</i>
Funding Support:	<i>This award</i>

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?-

Nothing to report

What other organizations were involved as partners? – nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from **BOTH** the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

The two reports will be uploaded with the same text, as the results are intimately related to one another, but the tasks for Jarvis and Pogue are clearly delineated in section 3 above.

9. **APPENDICES:**

- A. Statement of non-overlap with funded R01 grant.
- B. NIH R01 Specific Aims
- C. Dsouza A, Lin H, Gunn JR, Gladstone DJ, Jarvis LA, Pogue BW. Cherenkov-excited Multi-Fluorophore Sensing in Tissue-Simulating Phantoms and In Vivo from External Beam Radiotherapy. **Radiation Research** 2018 Feb;**189(2):197-204**. doi: 10.1667/RR14943.1. PMID: 29251551
- D. S. R. Ahmed, J. M. Jia, P. Bruza, S. Vinogradov, S. Jiang, D. J. Gladstone, L. A. Jarvis, B. W. Pogue, “Radiotherapy-induced Cherenkov Luminescence Imaging in a Human Body Phantom” Journal of Biomedical Optics (in press, 2018)
- E. J. R. Shell, E. P. LaRochelle, P. Bruza, J. R. Gunn, L. A. Jarvis, D. J. Gladstone, B. W. Pogue “Comparison of Phosphorescent Agents for Noninvasive Imaging of Tumor Oxygenation via Cherenkov Excited Luminescence Imaging” Molecular Imaging and Biology (in review 2018).
- F. M. Jia, P. Bruza, B. W. Pogue, “Multi-pinhole MLC scanning for Cherenkov-excited molecular luminescence achieves fast high resolution imaging up to 10mm into tissue” in submission to Optica, 2018
- G. B. W. Pogue, J. Feng, E. P. LaRochelle, P. Bruza, H. Lin, R. Zhang, J. R. Shell, H. Dehghani, S. C. Davis, S. A. Vinogradov, D. J. Gladstone, L. A. Jarvis “Maps of in vivo oxygen pressure with submillimetre resolution and nanomolar sensitivity enabled by Cherenkov-excited luminescence scanned imaging” Nature Biomedical Engineering (in press, expected April 2018)

Addressing overlap between this project and recently funded (August 1, 2017) NIH grant R01EB024498-01 (Specific Aims on next page)

In terms of the concerns related to the new NIH grant, R01EB024498-01, there is no scientific overlap. I am enclosing a copy of the specific aims and you can see that they are quite different. The work in Aims 1 and 2 are focused around development of a deep tissue imaging system for whole body imaging of rodents, rather than just lymph node imaging which is fairly superficial. The detectors needed for the deeper tissue imaging are much more sensitive than that used for the CDMRP grant proposal. Additionally, the Aim 2 of the R01 is focused on development of a stand alone system, such that it is not tethered to a clinical linac, but rather is used with a lone short pulse linac, not found in the clinic. This work is supporting Research Scientist Petr Bruza.

The R01 Funding supports the system development for the R01 grant, which has just completed 8 months of funding, as of August 1, 2017. The individuals funded to work on that R01 grant are PhD students Ramish Ashraf (who focuses on the new detection circuitry for triggering) and Ethan LaRochelle (who is focuses on general modeling of CELSI and optimization, without a focus on lymph node imaging).

This CDMRP aims are focused around system optimization and development for breast lymph node imaging, and we have completed a substantial amount of work on this. The system development for this CDMRP grant has been completed now, testing in both rodents as well as human body phantoms for lymph node detection capability, as described in our second annual report.

The CDMRP funding supports the individuals: (Jeremy) Mengyu Jia PhD (who is the lead on linac use and data collection), Jason Gunn (who is lifescience laboratory technician and focuses on preparation of animals and tissue cultures) and Research Scientist Jennifer Shell PhD (who is the lead chemist on probe identification and production). These three individuals are not supported on the R01 grant.

Specific Aims -- R01EB024498-01

There is no molecular imaging tool today which can routinely sense at the μM to nM range with sub-millimeter spatial resolution throughout the rat/mouse body, and most systems other than optical have very low animal throughput. Here we propose a fundamentally new hybrid modality with high resolution capability through several centimeters of tissue, which utilizes molecular specificity of optical tracers. The hypothesized sensitivity is level and spatial resolution near 200 microns. The rationale for this work is that all pre-clinical molecular imaging systems are limited by technical factors that reduce spatial resolution, molecular sensitivity or throughput, which are fundamental to the nature of the imaging. The new approach presented here uses thin sheets of high energy x-rays for **Cerenkov Excited Luminescence for Scanned Imaging (CELSI)**, which excite most traditional organic molecules that are used as biologically compatible molecular probes (**Fig 1**). The system detects the emission in a time-gated manner for superior SNR, and will be tested imaging the molecular microenvironment of deeply seated orthotopic cancer tumors in vivo, which cannot be easily imaged with commercially available fluorescence animal scanners (i.e. Caliper IVIS nor LI-COR Pearl). We propose a technological study of development & performance analysis in a two pronged approach. First, a basic low cost system coupled to existing LINAC sources, and secondly, we will partner with a company to create an optimal standalone system.

Hypothesis 1. A CELSI system can image through an entire rat with linear sensitivity and 200 micron spatial resolution, using molecular probes in the range of nM to μM .

Specific Aim 1. We will **optimize the hardware configuration for CELSI to sense signals for phosphorescence & fluorescence in tissue**, determining the limits to detection, using a scanned *clinical* LINAC beam, testing detector options, phantom studies, & Monte Carlo simulations. Image recover optimization will be completed with deconvolution-attenuation correction as well as NIRFAST-based diffuse tomography recovery. The system as designed will be readily implemented in any radiation oncology department with a clinical LINAC. **Deliverables:** quantify tradeoffs on i) depth, ii) radiation dose, and iii) probe concentration.

Hypothesis 2. A standalone CELSI system will provide superior performance, just as uPET, uCT, uOptical systems provide superior imaging performance relative to human-size correlate systems.

Specific Aim 2. We will **implement a commercial prototype customized system for CELSI imaging in small animals with the best features using a dedicated short pulse LINAC**, allowing superior time-gating for maximal signal to noise. The imaging with maximal spatial, temporal and contrast resolution are compared to a clinical LINAC. **Deliverables:** completed system, quantitative demo of performance specs relative to Aim 1.

Hypothesis 3. Immunologic and metabolic features of orthotopic tumors can be imaged with CELSI whereas they cannot be resolved with standard optical imaging systems.

Aim 3. We will **evaluate the quantitative limits on structural, metabolic and immunologic probes for molecular imaging with CELSI** and establish the ability to measure Standard Uptake Value (SUV) [5, 6], comparing to the best images taken with the IVIS (PerkinElmer) and Pearl (LI-COR) optical systems.

Deliverables: quantitative limits to detection for metabolic & immunologic probes, orthotopic tumors, quantitatively compared to the commercial leading optical systems.

Note, we have designed the aims so that Aims 1 & 2 are complementary and competitive, and the results of Aim 3 will finalize the performance specifications of either system. The strength is that we develop both an inexpensive and readily utilized system on a clinical LINAC (Aim 1), as well as a prototype high-performance small animal system (Aim 2). Note that CELSI has been demonstrated in vivo as well as with quantitative phantoms. We anticipate the as the ability to sense molecular concentrations at low radiation dose gets established, that human use may also be discovered, however, the invention of a high resolution, high molecular sensitivity imaging tool such as this would be extremely important in the field of clinical oncology R&D, and this is the immediate focus of this 4 year application.

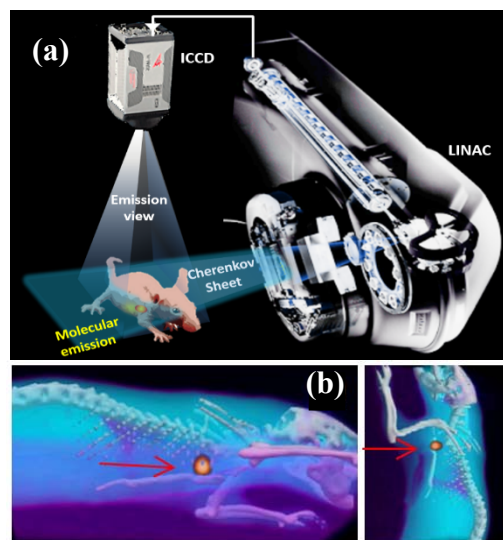


Figure 1. CELSI imaging, set up in (a), allows high resolution images (b) from a whole body rat scan, showing localization of a lymph node. The molecular sensitivity is μM -nM in nature, and spatial resolution $\sim 200 \mu\text{m}$. **This approach is analogous to light sheet microscopy, but with ability to image entire**

Cherenkov-excited Multi-Fluorophore Sensing in Tissue-Simulating Phantoms and *In Vivo* from External Beam Radiotherapy

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Cherenkov-excited Multi-Fluorophore Sensing in Tissue-Simulating Phantoms and *In Vivo* from External Beam Radiotherapy

Alisha Dsouza,^{a,1} Huiyun Lin,^{a,d,1} Jason R. Gunn,^a David J. Gladstone,^{a,b,c} Lesley A. Jarvis^{b,c} and Brian W. Pogue^{a,c,2}

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Dsouza, A., Lin, H., Gunn, J. R., Gladstone, D. J., Jarvis, L. A. and Pogue, B. W. Cherenkov-excited Multi-Fluorophore Sensing in Tissue-Simulating Phantoms and *In Vivo* from External Beam Radiotherapy. *Radiat. Res.* 189, 197–204 (2018).

In this work, Cherenkov-excited molecular sensing was used to assess the potential for simultaneous quantitative sensing of two NIR fluorophores within tissue-simulating phantoms through spectral separation of signals. Cherenkov emissions induced by external beam gamma photon radiation treatment to tissues/tissue-simulating phantoms were detectable over the 500–900-nm wavelength range. The presence of blood was demonstrated to reduce the integrated intensity of detected Cherenkov emissions by nearly 50%, predominantly at wavelengths below 620 nm. The molecular dyes, IRDye 680RD and IRDye 800CW, have excitation and emission spectra at longer wavelengths than the strongest blood absorption peaks, and also where the intensity of Cherenkov light is at its lowest, so that the emission signal relative to background signal is maximized. Tissue phantoms composed of 1% intralipid and 1% blood were used to simulate human breast tissue, and vials containing fluorophore were embedded in the media, and irradiated with gamma photons for Cherenkov excitation. We observed that fluorescence emissions excited by the Cherenkov signal produced within the phantom could be detected at 5-mm depth into the media within a 0.1–25 μM fluorophore concentration range. The detected fluorescence signals from these dyes showed linear relationships with radiation doses down to the cGy level. *In vivo* tests were successful only within the range near a μM , suggesting that these could be used for metabolic probes *in vivo* where the local concentrations are near this range. © 2018 by Radiation Research Society

INTRODUCTION

Molecular imaging *in vivo* requires both high resolution and multi-component information, ideally. Recent work in Cherenkov-excited luminescence scanned imaging (CELSI) has shown potential for sensing and imaging molecular targets with very low volumetric sensitivity in tissue. The demonstration of the concepts of CELSI has been done with *in vivo* and tissue phantom work with oxygen luminescence probes, however, developments around fluorescence reporters have previously been limited (1–3). This was extended into tissue-equivalent phantoms with two near-infrared (NIR) fluorescent reporters within the same volume. The latter issue is important, because many clinical and preclinical studies have shown the need to excite multiple fluorophores in the same tissue volume, for multi-parameter molecular imaging, either for better delineation of tumor volumes (4–7) or accurate quantification of molecular binding (8–10). In this study, the Cherenkov-excited molecular sensing was extended to test the potential for quantifying two fluorescent tracers at the same time in tissue-equivalent media. Furthermore, these signals were assessed for linearity with concentration and applied radiotherapy dose.

Cherenkov light is produced when radiation in the MeV energy range travels through tissue, as shown in Fig. 1A, giving off a continuous spectrum of light that decays roughly as the inverse-square of the wavelength (1, 11, 12) (light blue line shown in Fig. 1B). Thus, the apparent color of the Cherenkov emission is highly weighted in the ultraviolet and blue wavelengths when undistorted by the media it is in. However, in mammalian tissue, due to the high absorption of short wavelengths by blood, the signal is heavily attenuated and is largely seen as being comprised of red and NIR wavelengths. Figure 1B shows the absorption spectra of hemoglobin (weighted to 1% blood, oxygenated as HbO₂ and deoxygenated as Hb-R), as well as water and lipids, and how the Cherenkov emission spectrum would be dominant in the wavelength region of 600–900 nm, where there is a reduced absorption from either blood or water. However, at the

¹ These authors contributed equally to this work.

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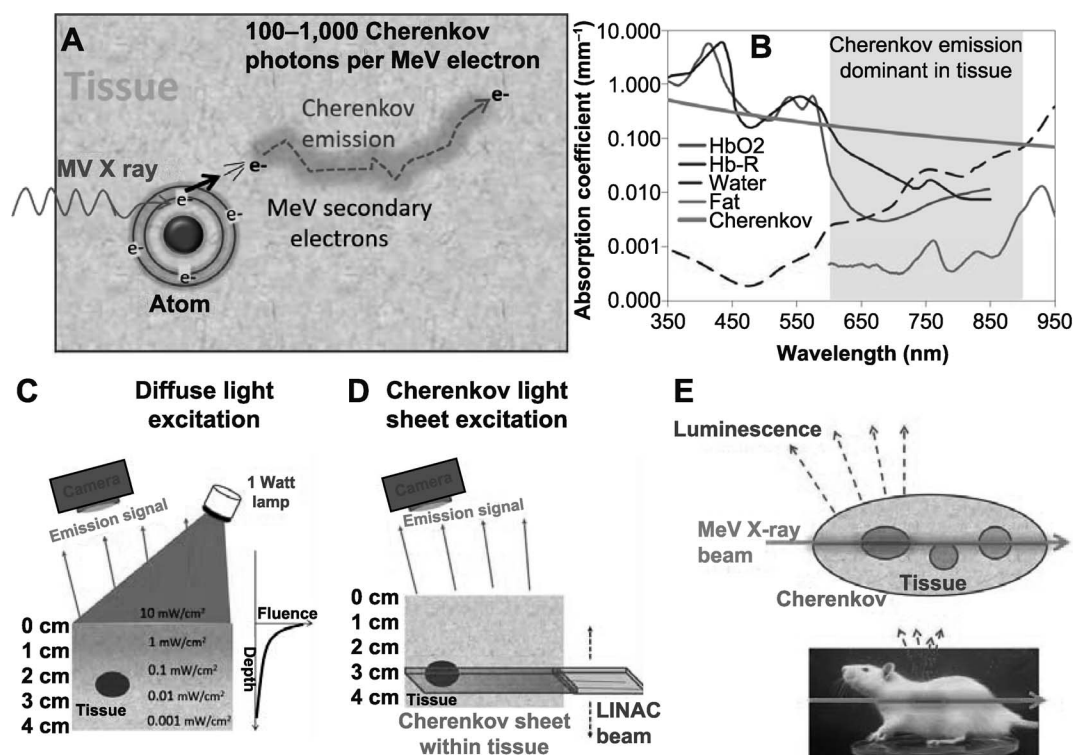


FIG. 1. The generation of Cherenkov light from secondary electrons induced by MV X-ray irradiation of tissue (panel A), with the spectra of Cherenkov light superimposed on the absorbing species present in tissue (panel B), showing the wavelength range where Cherenkov light emission dominates *in vivo*. The benefit of Cherenkov excitation by selective illumination at the point of irradiation (panel D), compared to diffuse light excitation, which decays exponentially with depth into the tissue (panel C). Finally, the entire concept of using selective illumination by MV X-ray beam to induce Cherenkov is shown (panel E), which is subsequently absorbed by local regions of fluorescence, and then the luminescence emission can be captured by a detector.

local level, the Cherenkov light can be used to excite optical absorbing species such as fluorophores, and the resulting fluorescence emissions can exit the tissue as long as the emission peak has sufficient Stokes shift to bring it out to the red-NIR diffusion transport window of tissue (2, 3, 13, 14).

The ability to spatially shape radiation beams delivered by a linear accelerator (LINAC), combined with highly sensitive optical detection instrumentation, allows for the adaptive imaging/measuring of Cherenkov-stimulated light emissions with highly resolved spatial information from the tissue (15, 16). Figure 1C and D shows a comparison of the light excitation in tissue from diffuse light versus LINAC-delivered X rays, emitting Cherenkov light selectively into the media. The diffuse light applied is attenuated exponentially with depth, while the Cherenkov light can be selectively placed anywhere by the LINAC X-ray beam. The MV X-ray radiation travels deep into tissue spinning off secondary electrons (Fig. 1A), and these secondary electrons each give off 100–1,000 optical photons by the Cherenkov effect. Taken together, the selective delivery of Cherenkov combined with localization of molecular probes, as shown in Fig. 1E, can be used to sample signals from a subsurface location, such as a lymph node for metastatic

burden estimation, or to areas for radiation treatment. The benefits of Cherenkov-excited luminescence sensing are summarized below.

1. Cherenkov light is broadband but *in vivo* 600–900-nm light has appreciable penetration.
2. This light is generated at the site of X-ray radiation dose deposition by secondary electrons, which can be throughout tissue for high-MV X-ray LINAC delivery.
3. The selective excitation can then lead to capture of the emission, with knowledge of where the signal came from within the tissue.

Thus, as has been shown in previously reported studies (1, 3), the concept of Cherenkov-excited luminescence detection can work reasonably well, depending on the probe and the tissue geometry. In the current study, spectrometer readout was used to filter the fluorescence emission from the Cherenkov light (excitation signal). This spectral decoupling of the output signals works well, as long as an estimate of contributing spectral bases is available to fit to. Here, the challenge of simultaneously detecting two molecular species from Cherenkov excitation was examined in realistic-tissue-simulating environments.

MATERIALS AND METHODS

Experimental Setup and LINAC Beam Delivery

The experimental setup was largely set by the linear accelerator (Varian Clinac 2100C; Varian Medical Systems, Palo Alto, CA), used for MeV energy delivery, in a treatment room of the Norris Cotton Cancer Center (Lebanon, NH). For all experiments, a 10×10 mm² beam was used at 6 MV photon energy. The LINAC was set up to deliver radiation in 3.25- μ s bursts at 360-Hz repetition rate, with a dose rate of 600 monitor units per minute, roughly equivalent to 600 cGy/min.

A single fiber bundle was used for light collection, and delivery to a spectrometer (Acton InSight™; Princeton Instruments, Trenton, NJ) through a 500-nm longpass filter (Thorlabs Inc., Newton, NJ). A fast-gated intensified CCD camera (ICCD, PI-MAX3; Princeton Instruments) was used at the spectrometer output for multispectral detection. The spectrometer and ICCD camera were placed just outside the radiotherapy room, so that no shielding from the radiation generated by the LINAC was necessary. The single fiber bundle (CeramOptec®, Bonn, Germany) was 15 m in length and housed nineteen 200- μ m-diameter silica fibers that conducted emitted light from the treatment region (i.e., tissue-simulating phantom surface or animal skin) to the spectrometer. The LINAC trigger SNYC output was used to trigger acquisitions by the camera at minimum trigger delay equal to the camera insertion delay of 27 ns and radiation pulse gate width of 3.25 μ s. The ICCD was cooled to a control point of -25°C and the spectrometer grating used in all experiments was 300 lines/mm, with center wavelength set to 700 nm.

Liquid Phantoms and Fluorescent Inclusions

Liquid tissue simulating “background”-simulating phantoms were prepared to contain 1% v/v intralipid concentration (Fresenius Kabi, Bad Homburg vor der Höhe) as an optical scatterer with scattering properties similar to human tissue, and 1% v/v whole porcine blood (Lampire Biological Laboratories Inc., Pipersville, PA), to match the average blood level of whole tissue (19). These were held in optically clear plastic containers approximately $60 \times 60 \times 120$ mm in size. Containers were positioned in the radiation beam, with the beam edge stopping immediately at the external boundary of the media. Immediately outside the edge of the phantom, a linear fiber array was placed in light contact with the surface, such that light emission could be directly collected from the emission, as shown in Fig. 2A and B. Typical Cherenkov-emission spectra from the two types of homogenous phantoms are shown in Fig. 2C.

Ten 2-ml centrifuge tubes filled with fluorophores, IRDye® 800CW and IRDye 680 RD (both from LI-COR® Biosciences, Lincoln, NE), at concentrations of 0.1–25 μM in 500- μl phosphate buffered saline (PBS), were used to simulate fluorescent inclusions (see Fig. 2C). Both fluorophores were present in all the tubes, and at the same concentrations, i.e., a 1:1 concentration ratio was maintained between dyes for simplicity. Typical spectra of the Cherenkov emission with the absorption and fluorescence emission spectra of the fluorophores used are shown in Fig. 3A. Each tube was then placed in the background liquid phantom, which contained PBS with 1% intralipid or PBS with 1% intralipid and 1% whole blood. Blank control tubes containing only background intralipid and blood were also prepared. The phantom setup was placed on a stirring plate, to prevent red blood cell-stacking and inhomogeneity in scatterer concentrations. The tube was positioned inside the background phantom container such that it was at a distance of 5 mm from the container wall at which the optical fiber tip was fixed. The radiation beam cross-section shape was set to 1×1 cm², and was set up to irradiate the inclusion tube from the top, with the LINAC beam isocenter set to be inside the inclusion. This provided a sufficient buildup region for the radiation beam passing through the phantom. Cherenkov emissions and fluorescence emission triggered by radiation delivery were acquired. Five frames of

continuous wavelength spectra were generated with 100–3,000 accumulations on the ICCD chip (AOC) or pulses. Since the dose rate was 10 cGy/s, and there were 360 pulses/s, this range of AOC values corresponds to roughly 2.8–83 cGy.

Spectral Fitting and Data Processing

The collected spectra were temporally median-filtered and wavelength-averaged with a moving window filter, as shown in Fig. 3B. To process the spectra for signals related to dye concentration, the acquired normalized background spectra, from Fig. 3A, were used for spectral fitting, because the signals included all distortions to the Cherenkov emission and fluorescence spectra, which could be caused by fiber and system autofluorescence and attenuation. These were thought to be minimal, but still direct measurement of these spectra on the same system was completed. Then the detected spectra were used as the input to an algorithm for spectral decoupling, based on a linear least-squares solver, fitting in the regions where each component was dominant, as shown in Fig. 3D.

In Vivo Proof-of-Concept Work: Subcutaneous Matrigel-Based Fluorescent Inclusions in Murine Model

Before the above approach could be applied and tested to quantify fluorescent-tracer ratios in animal lymph nodes, a simulated *in vivo* experiment utilizing fluorophores injected subcutaneously in a mouse was performed. In this experiment, IRDye 680RD and IRDye 800CW were mixed with clear phenol red-free and growth factor-reduced Matrigel media (Corning® Inc., Corning, NY) such that their concentration was 3 μM each. The Matrigel that was previously stored in 500- μl aliquots at -20°C was thawed in an ice tub to raise its temperature to 4°C . Care was taken during the handling to prevent overheating of the Matrigel, as this causes it to cross-link and solidify. Dyes dissolved in PBS were cooled and mixed with the Matrigel by pipetting. Insulin syringes with 27g needles were chilled on ice prior to loading. Animals were anesthetized using 1.5–2.5% isoflurane in 1.5 l/min oxygen, and placed on a heating pad. A 200- μl injection of Matrigel mixed with fluorescent dyes was administered subcutaneously such that the Matrigel solidified into a spheroid with minimal flattening. Since Matrigel is a highly viscous substance, care was given to ensure bubbles were not present in the gel.

For the spectra acquisition, the mouse was placed on a black sheet over layers of solid water phantoms (added to 1.5 cm thickness), to provide sufficient buildup region. The optical fiber position was fixed and the animal was adjusted to maintain contact between the fiber tip and the Matrigel bump (Fig. 6A). A 6-MV radiation beam was directed from below the animal such that the beam cross-section size was 1×1 cm², with the isocenter near the optical fiber tip. A background spectrum was obtained by measuring a Matrigel-free skin region away from the liver and gut, which are known sources of high autofluorescence. Spectra were acquired using the spectrometer and ICCD camera, and processed in a similar fashion as above to estimate the emission ratio of IRDye 800CW to IRDye 680RD. For the sake of simplicity in treatment, and optical fiber-based measurement, the animal was euthanized prior to any measurement by cervical dislocation. All animal work was performed under protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Dartmouth College (Hanover, NH).

RESULTS

Figure 2C shows the detected Cherenkov emissions from the background phantom with 1% intralipid and no blood and 1% intralipid along with 1% whole blood; note the change in spectral shape when blood was present. The decrease in signal at wavelengths <620 nm is apparent and

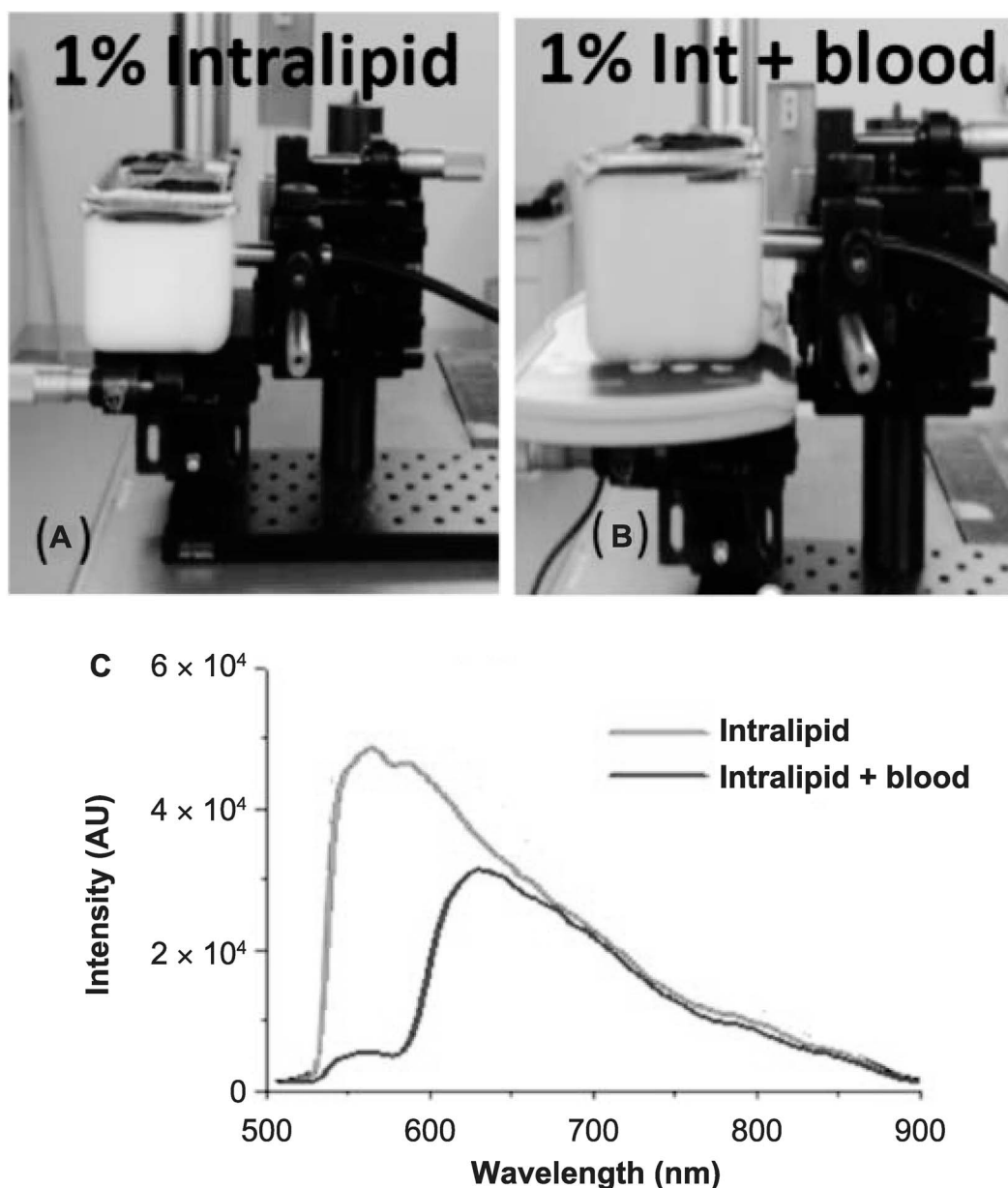


FIG. 2. The tissue phantoms used with 1% intralipid (panel A) and 1% intralipid with 1% porcine blood (panel B) are shown, set up on the radiation treatment table, with the optical fiber setup in contact with the plastic container. Representative Cherenkov spectra measured from LINAC irradiation of these phantoms is shown in panel C. The measurements were taken with a single fiber bundle coupled to the spectrometer.

matches the known attenuation of blood well. Notably, the signal loss is not strong in wavelengths above 620 nm, because the Cherenkov emission is being produced throughout the entire volume of the sample, and so the transport distance of light getting into the detection fiber was moderate. This is the rationale behind using IRDye 800CW and IRDye 680RD in this study.

Figures 4 and 5 show the processed spectra for various fluorophore concentrations (IRDye680:IRDye800 concentration was 1:1) in 1% intralipid in the background (Fig. 4) and the 1% intralipid plus 1% blood background media (Fig. 4). The IRDye 680RD measurement is on the left in both figures,

while that of IRDye 800CW is on the right in all panels in Figs. 4 and 5. The resulting integrated intensity values over the fitted fluorophore peaks for every measurement are plotted as a single point on each graph for each concentration and each radiation dose. Because of high Cherenkov background signals, there is always a lower limit on the concentration of dyes, which could be detected with any accuracy. Also, at the highest dye concentrations, there could be self-quenching of the dye, limiting the linearity of response. Concentrations above and below these extremes were eliminated from the data, to focus on reporting those data that are monotonically related to the reporter signal.

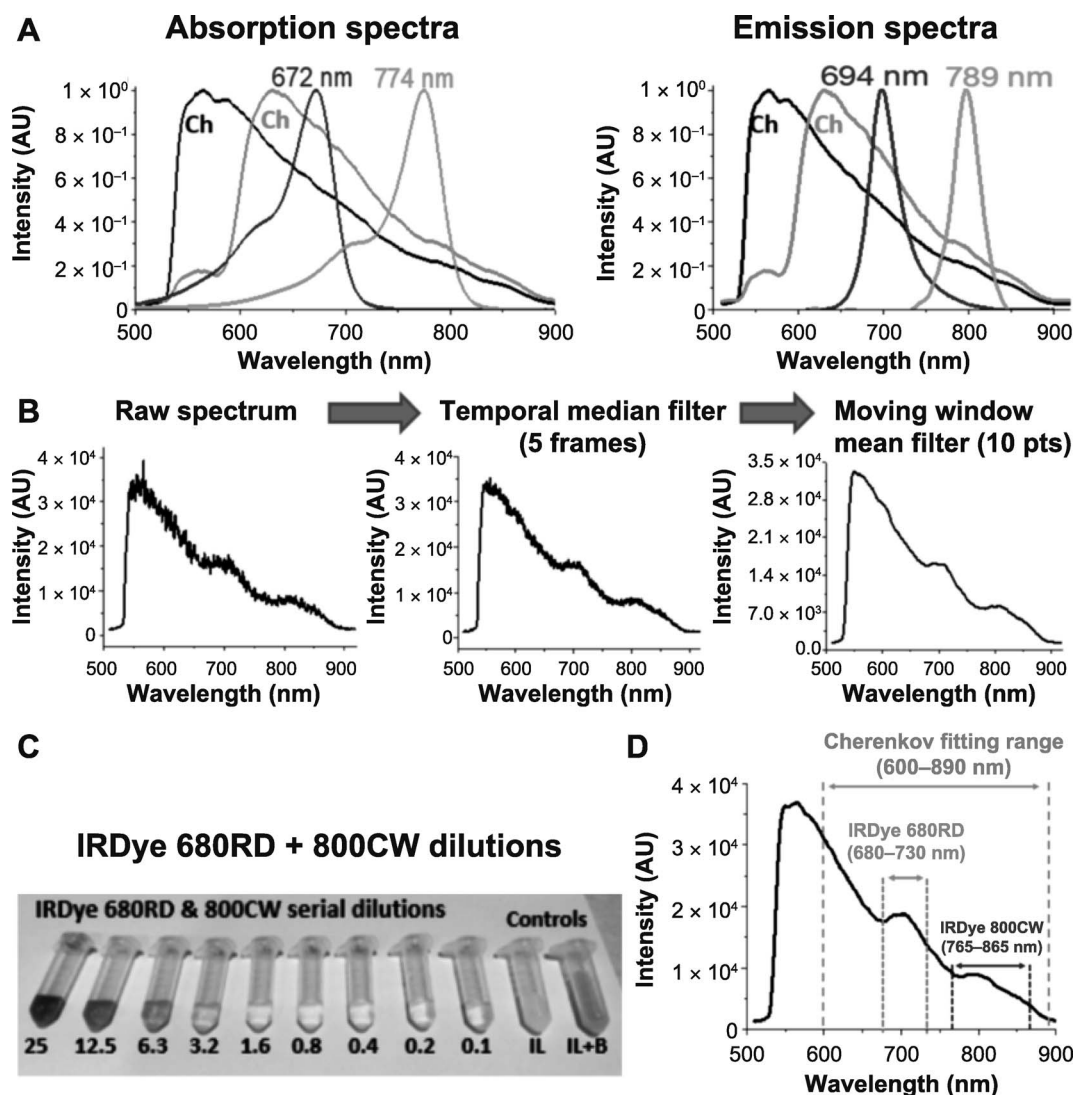


FIG. 3. Dyes in phantoms and intralipid with blood. Panel A: Absorption (left) and emission (right) spectra are shown for Cherenkov emission in intralipid (black), Cherenkov emission in intralipid and blood (red), IRDye 680RD (blue) and IRDye 800CW (pink). Panel B: Example of a raw emission spectrum from a combined phantom, with Cherenkov and the two emission peaks convolved, with temporal filtering, then moving window wavelength filtering to reduce the noise. Panel C: The tubes with serial dilutions are shown for both dyes, systematically decreased together with concentrations listed in micromolar. Panel D: Example spectrum, with the wavelength fitting regions shown, for all three components.

Figures 4A and 5A show the dependence of each fluorophore signal with respect to radiation dose used, at varied concentration of 0.1–25 μM . The signals in the blood-bearing phantoms (Fig. 5) had considerably more noise in Fig. 4, largely because the background Cherenkov of course varies with dose as well, and so the entire signal to noise of the fit degraded more significantly with less dose delivered. To better interpret the data, fits of signal versus dose were plotted in green dotted lines for these figures. In both figures, simultaneous fitting of IRDye 680RD and 800CW was possible over some range of concentrations (Fig. 4B and Fig. 5B). In comparing Fig. 4A to Fig. 5A, it is apparent that the contribution of blood merely limits the output signal by a factor of approximately 2, while the full

range of concentrations could be detected with the radiation dose used here.

Processed and fitted spectra for the murine Matrigel inclusion loaded with 3 μM IRDye 680RD and IRDye 800CW are shown in Fig. 6B. The results indicated that it was possible to resolve signals for the ratio of 800:680 down to 1, as expected.

DISCUSSION

This study focused on the particular question of utilizing fluorescent molecular probes in Cherenkov-excited molecular sensing. The results have large implications as to the utility of spectrally resolved CELSI sensing *in vivo*, and the

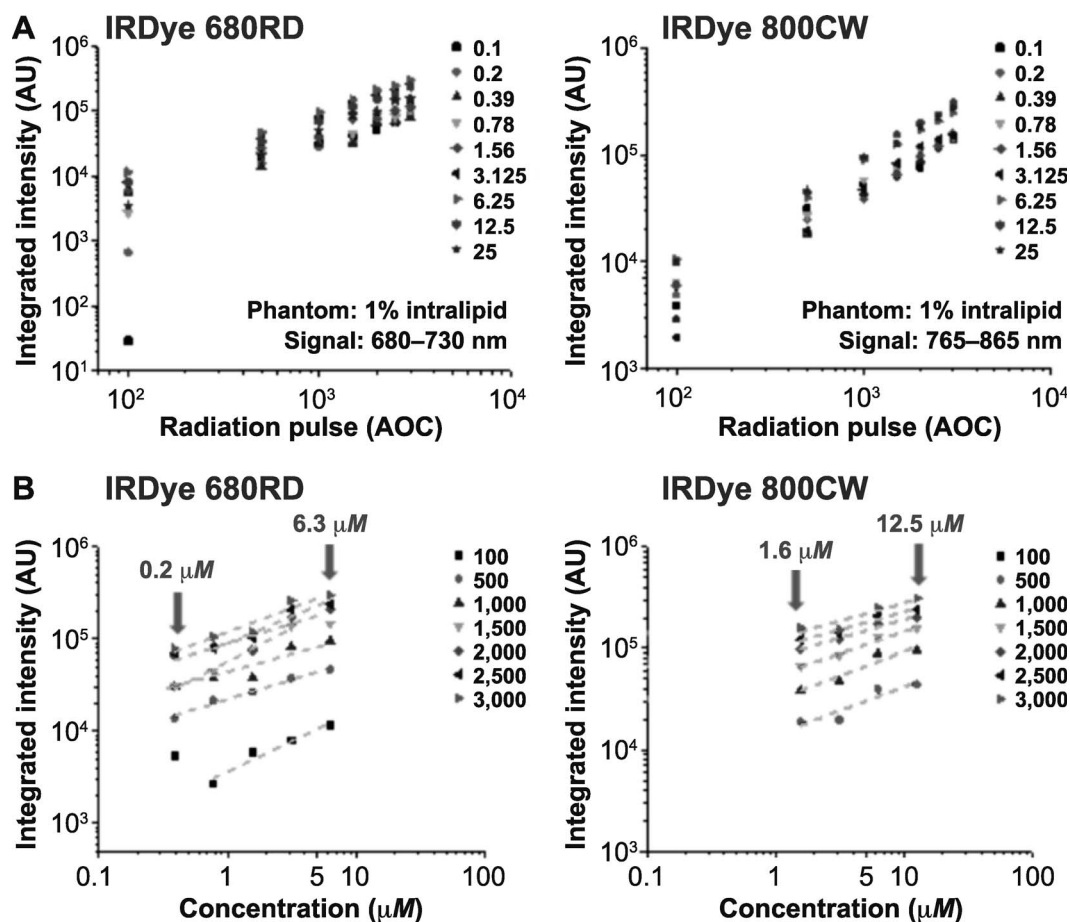


FIG. 4. The measured integrated fluorescence signal intensity from intralipid solutions are shown for IRDye 680RD (left) and IRDye 800CW (right) for varying amounts of radiation dose (panel A) at varied concentrations of 0.1–25 μM . Variation in signal with varying concentration was then examined (panel B) for varied radiation dose of 2.8–83 cGy (AOC 100–3,000). Green dashed lines represent linear fits.

uniqueness of the geometry shown in Fig. 1E, where selective areas of tissue can be probed with careful geometric and spectral choices. The concept of injecting X rays as a way to excite molecular probes *in vivo* with Cherenkov light has not been widely explored, but with the right molecular sensor, the signals can be detected from deeper than most areas that could otherwise be sampled with light. However, the range of concentration and radiation dose required for sensing will directly determine what this imaging tool can be used for, and so these two factors in particular were studied here.

Several previously reported studies have focused on phosphorescent agents, which have emission lifetimes in the tens of microseconds range, allowing for temporal gating of the signal, effectively eliminating Cherenkov completely from the signal. In such a case, luminescence can be sampled well down into the sub-nanomolar concentrations (3), because the background signal has been eliminated. In the case of time-independent spectra detection, the fluorescent signal is embedded on top of the Cherenkov background, and so the deconvolution of the two signals is dependent on the system noise and variability, and

inherently is bound to be orders of magnitude less sensitive. The data shown in Figs. 3 and 4 support this, indicating that the sensitivity range of IRDyes detected in this manner is approximately 1–10 μM in tissue-equivalent media. The upper end of the detection is likely higher than this, but just dependent on the microchemical quenching effect of the dye and the microenvironment that it is in. However, the lower end of this detection range is realistic for *in vivo* imaging, since it has more to do with the detection methodology than the tissue and dye.

Some interesting observations can be taken from the results beyond these simple limits however. For example, the effect of blood, compared to no blood, is quite subtle in the signal, e.g., the signals in Fig. 5A are not that much lower than Fig. 4A. This is perhaps obviously given by the different amounts of Cherenkov light seen in emission from the two solutions, as shown in Fig. 2C. The fact that these are not all that different is likely because the fiber collecting the light picks up through the whole volume, but the Cherenkov is launched through the whole volume as well, and because of the scattering the fiber will preferentially pick up light from near the surface. So, while the Cherenkov

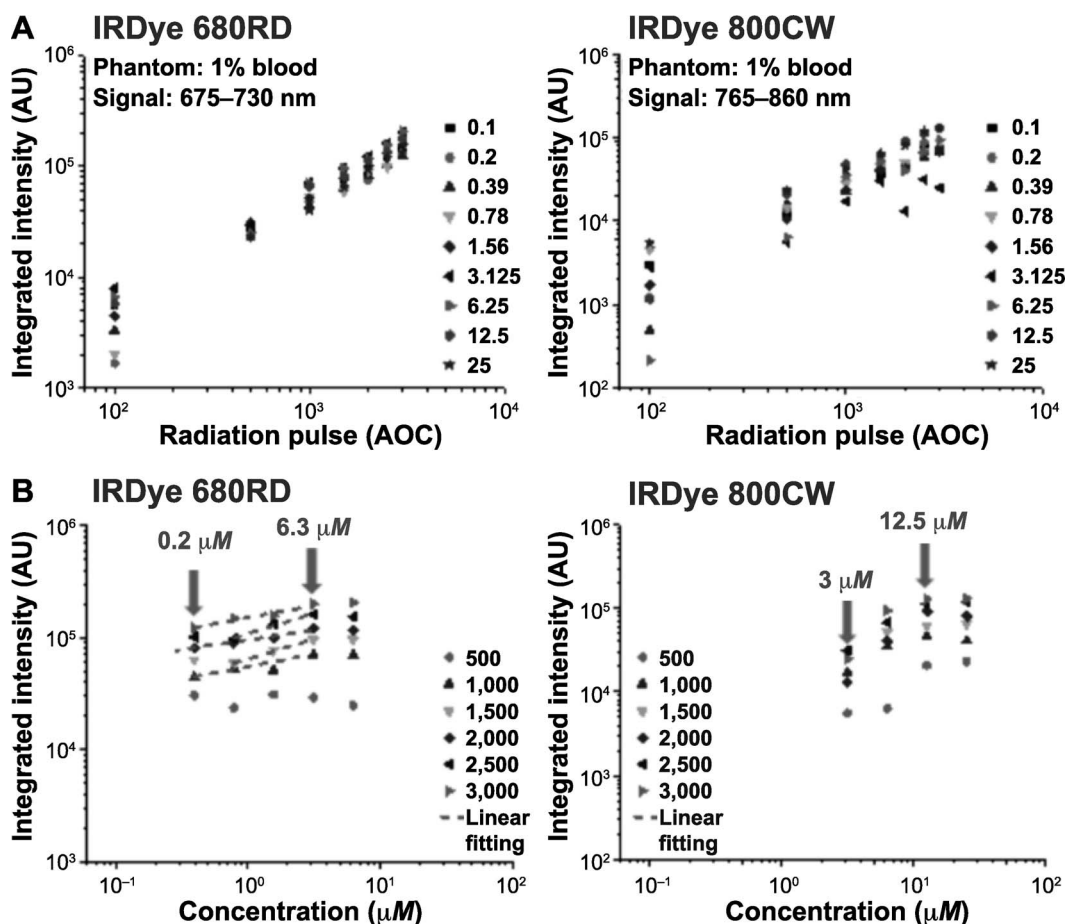


FIG. 5. The effects of integrated signal from intralipid plus blood solutions are shown for IRDye 680RD (left) and IRDye 800CW (right) for varying radiation doses (panel A) at varied concentrations of 0.1–25 μM . Variation in signal with varying concentration was then examined (panel B) for a varied radiation dose of 13.9–83 cGy (AOC 100–3,000).

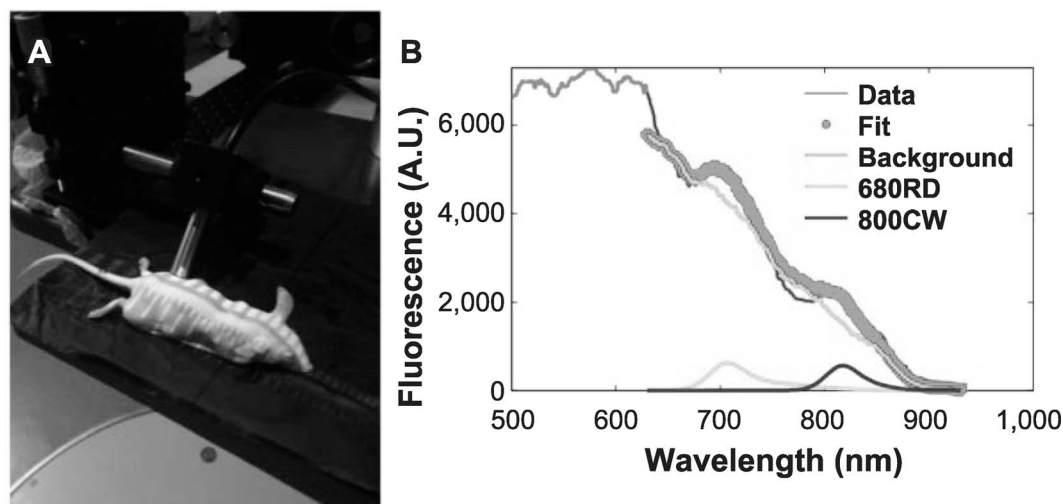


FIG. 6. Preliminary *in vivo* imaging of a nude mouse injected subcutaneously with 200 μl phenol red-free Matrigel basement membrane matrix with 3 μM IRDye 800CW and 3 μM IRDye 680RD. The mouse was positioned on the radiation treatment table and the optical fiber was placed in contact with the Matrigel injection (panel A). Panel B: Processed and fitted spectra. The measured spectrum was spectrally decoupled to estimate the contributions of each fluorophore.

light transmission through longer distances of tissue will undoubtedly be more attenuated in the NIR by blood, in the short pathlength, it does not appear to be so. Given that fluorophores are excited locally by the Cherenkov light originating right within the phantom (tissue), the distance effect of attenuation is less of an issue than just the amount of Cherenkov generated. In addition to the tissue-simulating phantom studies, the preliminary *in vivo* testing showed that a ~1:1 ratio of fluorophore concentrations was measurable when 3 μM of each dye was injected simultaneously and subcutaneously in the mouse body, suggesting that the presented approach may be promising in ratiometric fluorescent tracer quantification (8, 17).

The fact that the detectable concentration range is limited to the micromolar regime, will mean that this style of sensing will not be sufficient for immune-tagged fluorophores, where typical cell surface receptors or cell receptor concentrations are routinely in the range of 1–100 nM (18). However, most metabolic events such as enzymatic proteins, minerals and respiratory cell components are present in tissue at the micromolar range and these are primary targets to delineate cancer tumors. Thus, fluorescent sensing of these reporters should be inherently possible for CELSI sensing. Additionally, at least two fluorophores should be detectable. The two dyes used here were chosen based on the results of our previously reported study, and so were thought to be optimally separated in wavelength to allow maximal accuracy in spectral fitting (3).

CONCLUSIONS

The observations from this tissue phantom and preliminary *in vivo* study indicate that Cherenkov is attenuated by blood, but predominantly in wavelengths <620 nm, and so choosing fluorophores with emissions above these wavelengths is optimal. Demonstration of simultaneous detection of two NIR-emitting fluorophores in the Cherenkov spectrum illustrates what is achievable in future tissue studies. The concentration range feasible to detect signals is the 1–10 μM , indicating that metabolic tracer imaging with multiple reporters would be feasible with IRDyes and Cherenkov excitation. Further work to optimize the signal to background might lower this detectable range, either by better time-gating or other means, but in this current state of spectrometer-based detection, it would appear that this range is approximately the working region of use *in vivo*. The doses required are in the range of 3–80 cGy, which is at the upper end of a diagnostically useful dose.

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Radiotherapy-induced Cherenkov luminescence imaging in a human body phantom

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1 Abstract. Radiation therapy produces Cherenkov optical emission in tissue, and this light can be utilized to activate molecular probes. The feasibility of sensing luminescence from a tissue molecular oxygen sensor from within a human body phantom was examined using the geometry of the axillary lymph node region. Detection of regions down to 30-mm deep was feasible with submillimeter spatial resolution with the total quantity of the phosphorescent sensor PtG4 near 1 nanomole. Radiation sheet scanning in an epi-illumination geometry provided optimal coverage, and maximum intensity projection images provided illustration of the concept. This work provides the preliminary information needed to attempt this type of imaging *in vivo*. © 2018 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.23.XX.XXXXXX]

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1 Introduction

3 All optical imaging techniques suffer from diminished spatial resolution with increasing depth into tissue because of the severe light scattering present.¹ Yet, in recent studies it has been shown that Cherenkov-based excitation of molecular probes is a feasible way to image deep into tissues, over several centimeters, with submillimeter resolution.² One important application of this is luminescence-based sensing of oxygen over macroscopic depths into tissue. The available depth of sampling is appropriate for imaging oxygenation in lymph nodes. In this study, the feasibility of imaging oxygen in lymph node-sized objects in a human body phantom was assessed to estimate applicability of this luminescence lifetime sensing technology in human imaging.

Cherenkov radiation is produced in all tissues undergoing treatment with Megaelectron Volt photons or electrons,³ generating broadband optical radiation that can be used to excite molecular probes.^{4,5} Clinical linear accelerators (linacs) operate in pulsed mode with $\sim 4\text{-}\mu\text{s}$ bursts,^{6,7} allowing for excitation of phosphorescent probes that have 10- to 100- μs range emission decay times.⁸ Previous experiments demonstrated this luminescence imaging with Cherenkov excitation in basic tissue phantoms and in rodents,^{9,10} to image tissue oxygenation, with time-gated signals that were sensitive to the luminescence quenching by oxygen. The key advantage of the method was to image centimeters into tissue while retaining submillimeter spatial resolution. At the same time, imaging oxygen is relevant in radiotherapy since it is a radiosensitivity factor that correlates with outcome.¹¹

In this work, sheet-shaped radiation beams from a linac were used to produce Cherenkov photons, to excite luminescence of probes in a volume largely confined to the irradiation sheets. Thus, by shaping the x-ray beam into a thin sheet, images of Cherenkov-excited luminescence from a planar slice within the tissue can be observed. Analogous to light sheet microscopy, a series of luminescence images can be taken at different depths, allowing for three-dimensional (3-D) volumetric rendering. This imaging approach has been termed Cherenkov-excited luminescence scanned imaging (CELSI).^{2,10} Knowing the position of the excitation plane allows for depth-variant attenuation correction, and postprocessed 3-D images show the luminescence distribution from within the tissue.

The aim of this study was to study CELSI in a human body geometry, to demonstrate the capability of imaging local oxygenation in regions similar in size to lymph nodes, as shown in Fig. 1. Specifically, the effects of depth, concentration, and minimum radiation dose were examined here.

2 Materials and Methods

2.1 Phantoms and Geometry

Depth and concentration range evaluation experiments were carried out to assess the signal-to-noise ratio (SNR) and signal-to-background ratio for phosphorescent objects at varying depths. The phosphorescent probe used in our experiments was PtG4, which has a well-demonstrated oxygen-dependent luminescence lifetime, characterized in solution¹² as well as in our previous animal study.² Eppendorf tubes containing 0.5-mL solution of PtG4 of varying concentrations (1 to 100 μM) were positioned at different depths (0 to 3.5 cm) to assess imaging capability with concentration and depth. A semi-infinite tissue phantom, of 1% aqueous solution of intralipid and 1% bovine blood was used to mimic the optical properties of the human tissue.

A full torso PBU-50 phantom (Kyoto Kagaku America Inc., Torrance, California) was used with two sets of measurements. First, Eppendorf tubes were used with 50 μL of 25- μM solution of PtG4 (0.5 nanomoles) in phosphate buffered saline (PBS), and comparing with controls of just PBS and an empty tube. In the second set, five 0.2-mL Eppendorf tubes were used, each containing (1) 50 μL of solution of PtG4 at concentrations: 1, 5, 10, 20, and 25 μM , for total quantities of 0.05, 0.25, 0.5, 1.0, and 1.25 nanomoles, respectively. The tubes were placed in the lateral rib region of the phantom at a sufficient distance from one another to prevent spatial overlap of their signals.

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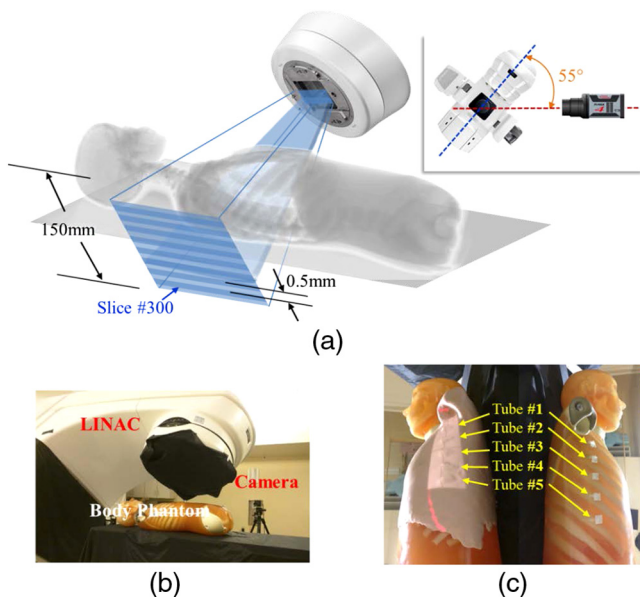


Fig. 1 Measurement geometry for the body phantom is shown with (a) a schematic of the geometry, (b) a photograph of the setup, and (c) the body phantom with skin on (left) and without skin (right) as shown with five tubes fixed onto the lateral rib area.

In both experimental sets, the phantom was imaged both with and without “skin layer” that was mimicked using tissue colored modeling clay (SuperSculpey® Original). Prior to the CELSI experiments, we validated the tissue-like optical properties of the used modeling clay by measuring its absorption and scattering coefficients.¹³ Using a custom tissue spectroscopy device, the DOSI system,¹⁴ we measured values across the spectral range of 650 to 1000 nm. The spectral range of PtG4 phosphorescence emission is peaked at 772 nm, the absorption and scattering coefficients of the clay ($\mu_a = 0.014 \text{ mm}^{-1}$, $\mu'_s = 0.55 \text{ mm}^{-1}$ match reasonable values of skin and/or adipose human tissue. A clinical x-ray CT of the body phantom, with 2.5-mm slice thickness, was obtained to facilitate postprocessing image overlay (LightSpeedCT, GE Healthcare, Chicago, Illinois).

2.2 Linear Accelerator

In each experiment, a Varian linac (2100CD, Varian Medical Systems, Palo Alto, California) was used to deliver six MV x-ray photon beams to the phantoms in the form of thin sheets. Multileaf collimators (MLC) (Millennium 120MLC, Varian Medical Systems) were used to shape and vertically translate the beam profile adaptively. Each sheet was 200-mm wide and 5-mm thick. The MLC leaves translated a total distance of 50 mm with a 0.2-mm step—this resulted in a total of 250 x-ray sheet positions along the length of the treatment area. The maximum dosage of radiation delivered by the linac was 20 MU/position, with up to 25 overlapping positions (5 mm moved in steps of 0.2 mm); this resulted in a maximum of $20 \times 25 = 500$ MU being delivered to the volume as defined by a single beam sheet—roughly equivalent to a maximum absorbed dose of 5 Gy.

2.3 Luminescence Emission Imaging

Images were captured by a gated, intensified charge-coupled device (ICCD, PI-MAX4 1024i, Princeton Instruments) with a 135-mm lens (Nikon), using the associated LightField software. An epi-illumination configuration was used, with the

linac gantry oriented at 145 deg and in the same side as the ICCD. The ICCD gate delays and widths were adjusted with 0.05 μs delay and 4 μs width for Cherenkov imaging, whereas a 4.2 μs delay and 70 μs width was used for phosphorescence imaging, and 1500 μs delay and 70 μs width used for background. Cherenkov, phosphorescence, and background images were acquired with 100 $\times$ gain on the intensifier. The image intensifier was gated by a predefined number of pulses, whereas the CCD integrated the signal prior to readout. Different values of this approach to accumulations on the chip (AoC) were used for each of the Cherenkov, phosphorescence, and background images for all body phantom CELSI experiments. Room-light images were acquired with 1 $\times$ gain and 1 AoC. An 8-MHz analog-to-digital conversion rate was used with 2×2 pixel hardware binning upon readout, resulting in 512×512 pixel images.

2.4 Image Processing

Image processing was carried out in MATLAB and Python. The protocol first involved background subtraction, followed by a depth stack (z -stack) median filter, across four frames in sequence, resulting in 250 frames. Next, each image was background corrected relative to its own residual background by subtracting the median from a background region of each image in the z -stack, followed by a 2-D (5×5) and a 3-D ($5 \times 5 \times 5$) spatial median filter. Finally, a 3-D Gaussian Filter with FWHM = 4.7 pixels was applied. Maximum-intensity projection (MIP) images were generated along the z -stack. The processed images were overlaid on the room-light image using both ImageJ and MATLAB Overlay GUI.¹⁵ Subsequently, videos were generated using ImageJ, showing the translation of the sheet beam through the region containing the simulated lymph nodes and static images of these are shown in Fig. 3.

Image display was done for 3-D views in Paraview (Kitware Inc.), using the processed phosphorescence image stack and the MIP images from the final two sets of tube experiments. Specifically, the experiments conducted with simulated skin were used for the 3-D reconstructions. The images were registered using the centroids of the Eppendorf tubes and edge of the phantom as fiducials to coalign the co-ordinate system of the CT to that of the phosphorescence images.

3 Results

In the depth and concentration ranging studies, CELSI experiments were performed for an intralipid-blood phantom embedded with PtG4-containing 0.5-mm Eppendorf tubes. The results are shown in Fig. 2, where sample luminescence and roomlight images are included in (a), and SNR is plotted in (b). The SNR has an exponential shape and the values plateau to their lowest values at deeper depths, as anticipated, because at some extreme depths the presence of the luminescent region cannot be sensed. Consequently, the SNR should be expected to asymptote to 1.0 at deep depths. The relationship between imaging depth and concentration of PtG4 for SNR = 1 is described by the circled plots in Fig. 2(b), indicating that for depths <1.7 cm there was good SNR, but below this depth, the concentration required for SNR > 1 increased dramatically. However, signal as deep at 3 cm was detectable, at concentrations just above 100 microMolar.

For the first set of geometry studies investigating phosphorescence of tubes containing PtG4 relative to control tubes containing PBS and a blank, a strong phosphorescent signal was detected. This was anticipated because the stock solution

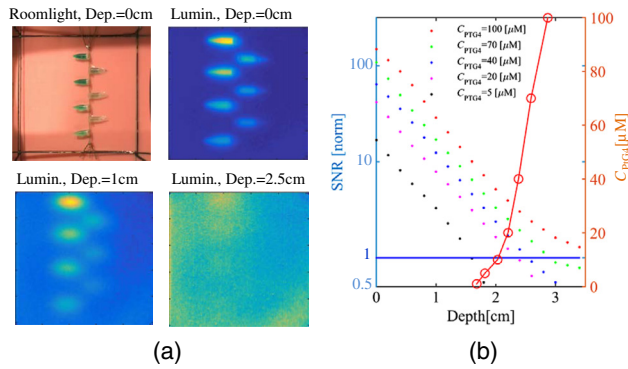


Fig. 2 Experimental results to investigate image quality versus depth for different concentrations of PtG4 (C_{PtG4}) as shown in the legend, showing (a) the room light image and luminescence images of the phantom used at three different depths in the phantom, and (b) the corresponding SNR values extracted from these images are shown as a function of depth by colored dots. In the same graph, the red line shows the C_{PtG4} at which SNR = 1 (values on left y-axis).

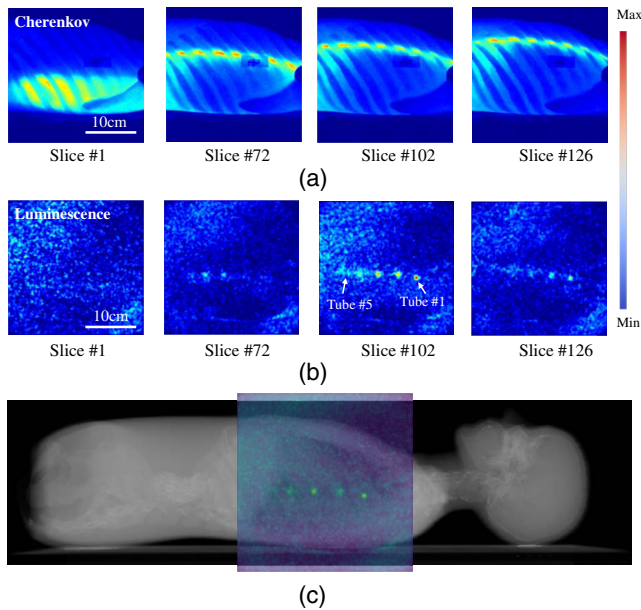


Fig. 3 Raw measurement sequences are shown for (a) Cherenkov emission and (b) luminescence photons, for the sheet at four different slice locations. In (c) a composition image is shown of the phantom CT with color overlay of the CELSI MIP image. A temporal sweep of the signals in (a) and (b) can be seen in the associated video files as the sheet moved across the phantom body.

of 25 μM was a high concentration and was placed at a depth of only 3 mm. At 144 AoC, the SNR was computed to be 59, whereas at 60 AoC the SNR was 66. Overall, the 144 and 60 AoC cases produced similar results, with very similar SNRs, for the experiments with “skin.”

For the second set of experiments investigating different concentrations of PtG4, visible phosphorescent signals were detected from the 5- to 25- μM tubes, whereas the 1- μM tube did not produce a signal with sufficient SNR to be detected. The trend in signal strength, with respect to concentrations on the MIP images was as anticipated, with greater concentrations of PtG4 yielding stronger, brighter signals, with slight variation

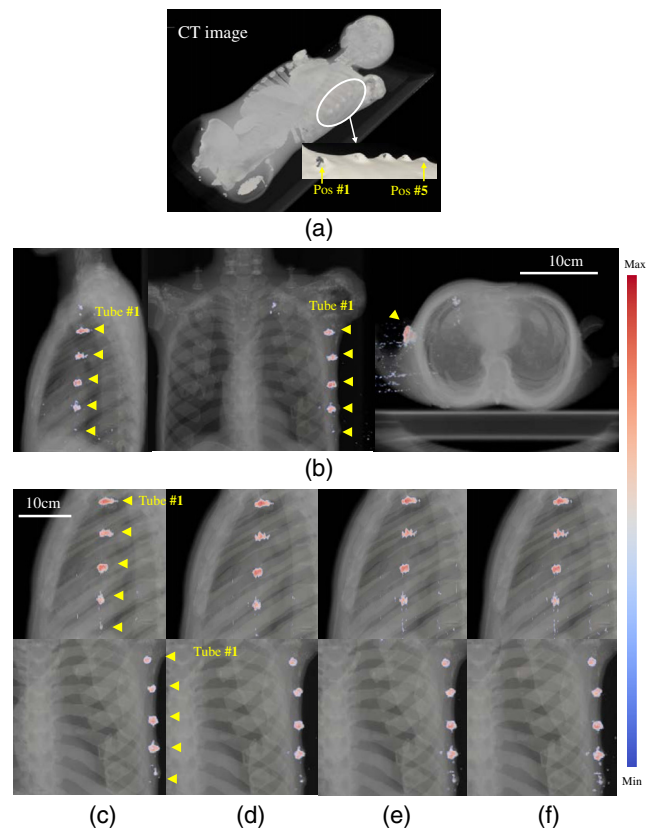


Fig. 4 3-D images of (a) CT and (b) overlaid with CELSI for three orthogonal views: coronal, sagittal, and transverse. Compositions of CT and CELSI images for varying numbers of light sheets, which correspond to less dose delivery. In these images, the number of sheets were: (c) 25, (d) 50, (e) 100, and (f) 250, respectively.

largely due to the thickness of skin overlying the tubes. Videos of the two sets of geometry studies are illustrated.

The angular sheet-beam scanning methodology that is more suitable for imaging a chest region was used, as shown in Fig. 1. Raw measurements of Cherenkov light and luminescence for four specific slice positions are shown in Figs. 3(a) and 3(b), respectively. Figure 3(c) shows a combined image of CT and luminescence maximum intensity projection (MIP). Scanned image data were reprocessed and overlaid on the 3-D volume of the CT scan, to allow visualization of the recovered positions of the tubes. The recovered images are shown in Fig. 4, with a perspective view in (a), and the three orthogonal views of the CT in (b).

To pursue a minimum radiation dose, sparse illumination was investigated, i.e., the number of light sheets was desired to be as few as possible. Here, a total of 25, 50, 100, and 250 light sheets were used with equal spacing, i.e., with spaces 2, 1, 0.5, and 0.2 mm, respectively. Figures 4(c)–4(f) show the compositions of CT and CELSI images for different numbers of light sheet. It can be seen that the image quality was reasonable, even for 25 light sheets.

4 Discussion

Initial studies of depth sensitivity have indicated that CELSI imaging would be viable for a depth of perhaps 2 to 3 cm, likely dependent upon the tissue type involved. Adipose tissues tend to have lower blood volume and consequently reduced absorption

attenuation of the light—this may allow imaging down to 3 cm. In contrast, denser tissues would have higher absorption and therefore be more limited to >2 cm.

The set of experiments conducted here were initially focused on determining the optimum positioning of the body phantom, couch, gantry, and camera for maximal sensitivity. Three orientations were investigated such as (i) vertical with the gantry directly above the body phantom on the couch at 180-deg reference angle and the camera placed directly facing the left side of the body phantom, (ii) horizontal with the gantry directly facing the left side of the body phantom at 90-deg reference angle and a 45-deg front-surface mirror placed above the top surface of the body phantom, allowing horizontal mounting of the ICCD directly facing the mirror, and (iii) angled with the gantry at 145-deg reference angle with ICCD on the same side. The angular orientation in an epi-illumination configuration was determined to be optimum and used for all subsequent experiments. In this orientation, a Cherenkov sheet was clearly visible translating through the body phantom. This orientation was also hypothesized to result in a strong phosphorescence signal from the simulated lymph nodes, and better approximated tangential breast tumor treatment protocol. The recovery of intensity was highly modulated by the depth of tissue overlying the tubes, and it was apparent that the signal was not linear with concentration because of this effect. However with skin thickness measured by the CT scan and geometry, it is feasible to quantify these values based on depth-dependent attenuation correction.¹⁰

These studies illustrate how imaging of lymph node sized objects may have potential with CELSI imaging in radiotherapy. Sensing their oxygenation is feasible, and further exploration of agents and signal analysis could lead to the next clinical trials in molecular sensing in radiotherapy.

Disclosures

The authors have no relevant financial interests in this article and no potential conflicts of interest to disclose.

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Comparison of Phosphorescent Agents for Noninvasive Imaging of Tumor Oxygenation via Cherenkov Excited Luminescence Imaging

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Short Title: Noninvasive imaging of tumor oxygenation with CELSI

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Keywords: hypoxia, optical imaging, optical probes, tumor imaging

Abstract

Purpose: Cherenkov emission generated in tissue during radiotherapy can be harnessed for tissue microenvironment imaging. Cherenkov excited luminescence scanned imaging (CELSI) provides a way to noninvasively map oxygen related signals, which is known correlate to outcomes in fractionated radiotherapy.

Procedure: In this study, four candidate phosphorescent reagents were studied for oxygen imaging, testing in a progressive series of a) in solution, b) in vitro in culture and then c) in subcutaneous tumors. In each test the signal strength and response to oxygen was assessed by decay lifetime measurement.

Results: In solution, phosphorescence intensity in response to oxygen scavenging conditions was shown for reagents MM2, PtG4, Ir(btp)₂(acac), and MitoID, with MM2 showing the most robust response, followed by PtG4, Ir(btp)₂(acac), and MitoID, respectively. However, in vitro their oxygen responses differed with Ir(btp)₂(acac) exhibiting the largest phosphorescent intensity change, followed by PtG4, MM2, and MitoID. But when used in subcutaneous tumors, it was only possible to utilize Ir(btp)₂(acac) and PtG4, with each being used at nanomole levels, to determine signal strength, lifetime, and pO₂.

Conclusions: Oxygen sensing with CELSI during radiotherapy is feasible and can estimate values from 1mm regions of tissue when used in the configuration of this study. PtG4 was the most amenable to in vivo sensing on the timescale of external beam LINAC X-rays.

Introduction

Extent of oxygenation in tumors is a known indicator of the success of radiation therapy, partly due to the oxygen enhancement ratio[1], as well as due to oxygen being a surrogate marker for other features of the tumor aggressiveness[2]. Therefore, monitoring of tumor oxygenation during fractionated radiation therapy would be advantageous to gauge the likelihood of response, or even to estimate if treatment plan alternations such as a boost to hypoxic areas might be beneficial. Despite the potential value, there has not been any clinical convergence on a method for imaging tumor oxygenation that is non-invasive, precise, and quantitative. Current methods to measure oxygen levels are either invasive and/or require complex calculations to measure tissue pO_2 [3-5]. Oxygen microelectrodes have improved the ability to measure tumor oxygen content; however, this is an invasive method that is not sensitive to small changes in oxygen levels and cannot measure oxygen in real time. Other methods used to measure tumor hypoxia such as EPR produce complex signals and require intricate processing for assessing oxygen content. In this study, the first report of oxygen-related mapping in tumors is demonstrated with Cherenkov excited luminescence scanned imaging (CELSI), which uses the inherent delivery of radiation to get maps of oxygen-dependent luminescence signal from injected chemical sensors.

Radiation treatment with MV photons or MeV electrons causes the production of Cherenkov light in tissue. This optical emission occurs when charged primary or secondary electrons pass through the dielectric medium, like tissue, at a velocity greater than the speed of light. This optical signal has been imaged to visualize surface dose in radiation therapy [6-8]. In addition, early pilot studies in tissue phantoms and individual animals have shown utilization of Cherenkov emission as the excitation source in imaging applications, including the detection of fluorophores and phosphors in conjunction with radiation therapy[9]. The potential to sense tumor oxygenation through CELSI with the phosphorescent reagent PtG4 has been shown by us, because the PtG4 phosphorescence is quenched in the presence of oxygen, reducing the observed lifetime of emission. In the presence of ambient oxygen pressure (pO_2), the PtG4 phosphorescence lifetime is 22 μs , and in low pO_2 it is 47 μs . [10, 11]

In this current study, this agent was compared to other oxygen-quenched chemical agents, and each was examined for their potential to sense tumor hypoxia by directly measuring pO_2 levels in vivo with mice bearing subcutaneous tumors[12]. In the end of the study, the pO_2 was estimated, using doses typical of fractionated radiotherapy.

Methods and Materials

Chemicals: PtG4 (platinum II G4) was provided by Sergei Vinogradov and colleagues [11]. MM2 was purchased from Luxcel Bioscience, Cork, Ireland [13], $Ir(btp)_2(acac)$ [14] was purchased from Sigma Aldrich, Saint Louis, MO and Mito-ID intracellular oxygen sensor was purchased from Enzo Life Sciences, Farmingdale, NY. MDA-MB-231 luc-D3H2LN cells were obtained from Perkin Elmer(Waltham, MA) and PANC-1 cells were obtained from ATCC(CRL-1469, Manassas, VA). DMEM, FBS, PBS, and 0.05% trypsin were purchased from Thermo Fisher Scientific (Waltham, MA). Molecular Devices Gemini XS (Sunnyvale, CA) was utilized for fluorescence plate reader measurements, Agilent Cary 50 UV-Vis (Santa Clara, CA) was utilized for absorbance spectra, Horiba Fluorolog-3(Edison, NJ) was utilized for emission spectra. Fluorescence microscopy was performed on a Zeiss LSM 800(Jena, Germany) confocal microscope.

Cell culture: PANC-1 cells and MDA-MB-231 luc-D3H2LN cells were grown in DMEM with 10% FBS in an incubator at 37°C with 5% CO_2 , and 100% humidity and divided utilizing 0.05% trypsin when desired confluency was reached.

Fluorescence Assay In Vitro: PANC-1 cells were seeded in black 96-well plates with a clear bottom at 5,000 cells/well. MM2, Ir(btp)₂(acac), and MitolD (10,20,30 µg/mL in PBS) were added to the cells and allowed to incubate for 24 h at 37°C. Media containing the reagents was removed and cells were rinsed with PBS. 10%FBS in PBS with or without glucose oxidase catalase oxygen scavenger (100 nM glucose oxidase, 1.5 µM catalase, and 56 mM glucose) was added after rinsing and the fluorescence intensity was analyzed with an excitation of 380 nm and emission of 660 nm. For limits of detection studies, 20, 2, 0.2 and 0.02 µg/mL of MM2 and Ir(btp)₂(acac) were utilized in the same fashion as described above.

Cherenkov Imaging: Cherenkov emission was induced by a linear accelerator (Varian LINAC 2100CD, Varian Medical System, Palo Alto, CA). The imaging system consisted of a time-gated intensified CCD camera (ICCD, PI-MAX4 1024i, Princeton Instruments, USA), a commercial lens (Canon EF 135mm f/2L USM). The camera was focused on a mirror that reflected the imaging field approximately one meter away. The time-gated ICCD camera was synchronized to the radiation pulses (approximately 3.25 µs duration, 360 Hz repetition rate) with the intensifier set as ×100 and turned on at a 4.26 µs or 1000 µs gate delay following each radiation pulse for phosphorescence or background measurement, and luminescence generated during 70 µs gate width (PtG4 and MM2) , or 7.77 µs (Ir(btp)₂(acac)) was integrated via this ICCD. To maximize signal and minimize background interference, the room lights were shut off throughout these studies, and all lights in the room were masked off with black cloth and black tape.

CEL studies in vitro: Concentrations of 20, 2, 0.2 and 0.02 µg/mL of MM2, Ir(btp)₂(acac), MitolD, and PtG4 were utilized 96-well plate as above. For solution studies, 300 µL of each solution with and without oxygen scavenger were utilized. For cellular studies, PANC-1 cells were seeded in black 96-well plates with a clear bottom at 5,000 cells/well. The reagents were added to the cells and allowed to incubate for 24 h at 37°C. Media containing the reagents was removed and cells were rinsed with PBS. 10% FBS in PBS was added after rinsing.

General Animal Imaging: All animal procedures were approved by the Institutional Animal Care and Use Committee, and the studies here were carried out in compliance with these approved procedures.

In vivo imaging: Briefly, 10⁵ MDA-MB-231 luc-D3H2LN cells were injected under the skin on the flank of a nude mouse. After approximately 3 weeks of growth, animals were chosen for use when their tumor was approximately 6x6x3mm³ in size. Under general anesthesia of inhaled isofluorane, 50 µL of 50 µM of each reagent was directly injected into the tumors and the animal was imaged alive and then again at 30 min after sacrifice.

Results

The set of 4 phosphorescent oxygen sensors were directly compared to determine which of these reagents would be ideal for use in CELSI in tumors. In order to be effective for CELSI, the agent needs to have a high quantum yield and a long emission lifetime due to the time gating used in the imaging experiments to separate the Cherenkov emission from the luminescence signal. These agents were chosen based upon published data and they represented the most promising mix of agents for use in vivo with longer lifetime emission from phosphorescence. The absorbance and emission spectra of PtG4 (green), MM2 (gray), Ir(btp)₂(acac) (blue), and MitolD (red) are shown in Figure 1[13, 14]. The relative brightness of the commercially available MM2, Ir(btp)₂(acac), and MitolD was assessed at increasing concentrations (10, 20, and 30 µg/mL) in PANC-1 cells in the presence and absence of ambient oxygen, through the addition of the enzymatic oxygen-scavenging system of glucose oxidase and catalase (GODCAT).

Measurements of emission were taken via fluorescence plate reader. Since the emission wavelength of PtG4 was longer than this plate reader can detect, it was not used in this preliminary study. MM2 and Ir(btp)₂(acac) exhibited the maximum signal under deoxygenated conditions at 20 µg/mL, while the MitolD intensity in the presence and absence of oxygen scavengers was lower in comparison (Figure 2a). Changes in oxygenation can be detected at concentrations as low as 0.2 µg/mL for Ir(btp)₂(acac) and 2 µg/mL for MM2, respectively (Figure 2b).

We sought to evaluate the effectiveness of these reagents to be imaged with Cherenkov excited luminescence (CEL). Solutions of PtG4, MM2, Ir(btp)₂(acac), and MitolD at decreasing concentrations (20, 2, 0.2, and 0.02 µg/mL) were placed in a 96-well plate with and without GODCAT and evaluated via epi-luminescence. The background corrected arbitrary phosphorescence intensities from this experiment at the maximum concentration of 20 µg/mL are as follows (first number for each compound represents ambient oxygen, second number represents GODCAT): MM2 (565, 1349); PtG4 (230, 1269); Ir(btp)₂(acac) (45.7, 168); MitolD (119, 150) (Figure 3a). This experiment was repeated by loading all of these reagents into PANC-1 cells with and without GODCAT and imaging via CEL. The arbitrary phosphorescence intensities from this experiment at the maximum concentration of 20 µg/mL are as follows (first number for each compound represents the signal under ambient oxygen, and the second number is the signal deoxygenated with GODCAT): MM2 (153, 376); PtG4 (181, 406); Ir(btp)₂(acac) (235, 633); MitolD (223, 288) (Figure 3b).

A murine experiment to sense tissue oxygenation was completed in mice with subcutaneous MDA-MB-231luc-D3H2LN tumors. A total of 4 tumors per compound were imaged, with local injection of 50 µL of each reagent (50 µM PtG4, 2.5 nmol, 1.75 mg per tumor and 50 µM Ir(btp)₂(acac), 2.5 nmol, 35 µg per tumor). Each mouse was imaged while alive and then repeated at 30 minutes after sacrifice. Temperature was controlled using a heating pad under each animal, throughout the study. The CELSI scan was completed vertically for these cases, with the video of the experimental data capture included in supplementary data. Images of the luminescence were captured at different delay times after the LINAC pulse. The maximum intensity projection images of CELSI from both PtG4 and Ir(btp)₂(acac) are shown in Figure 4a and b, respectively. These images were used for each mouse to create maps of emission lifetime (4d). A comparison of the intensity differences between PtG4 phosphorescence in the alive and dead mouse are shown in figure 4c. Using the Stern-Vollmer equation, we calculated the average tissue pO₂ in the tumors to be 101 mmHg (alive) and 9.6 mmHg (dead) for PtG4 and 0.98 mmHg for the tumors imaged with Ir(btp)₂(acac) (dead). The summary of total lifetimes of dead animals (4e) is shown as a box & whisker plot.

Discussion

Due to the red-shifted emission of PtG4 (772 nm) in comparison to the other reagents, it was originally hypothesized that this reagent would be the most effective for imaging in tumors, since this wavelength would achieve the most effective tissue penetration. However, we wanted to compare PtG4 with other commercially available phosphorescent oxygen sensors to identify the reagents with the best qualities for utilization in CELSI. Several factors are involved in the selection of ideal compounds. As with fluorescence imaging, the quantum yield of the compound is a significant factor which determines the efficiency of imaging. In CEL imaging time gating is used to separate Cherenkov emissions from luminescence, so luminescence lifetime is also important to consider. Medical LINACs have a 4 µs radiation pulse, and so this moderate pulse time limits the time gating to the microsecond regime, and without significant change to the LINAC or acquisition, nanosecond lifetimes would not be possible to measure. The luminescent agents which are available with microsecond lifetimes are typically phosphorescent. It has not been

feasible to detect fluorescent agents which have nanosecond lifetimes, other than through wavelength filtering and continuous wave detection[9]. The typical timing used for image acquisition that is coupled to the LINAC is depicted in Figure 5a. Experimentally the gate width is generally set between 5 - 10 times the deoxygenated lifetime (τ_0). The time between radiation pulses is one factor that will limit the maximum lifetime when choosing a phosphorescent compound. The LINAC generates radiation pulses at a repetition rate between 2.7 and 17ms (600MU/min - 100MU/min). Shorter lifetimes can be difficult to discriminate from the radiation-induced Cherenkov light. The LINAC radiation pulse does not immediately turn off, and both stray charge and Cherenkov emissions may be detected during the transition phase. Phosphorescent compounds with lifetimes in this range ($<4.5\mu\text{s}$) will be difficult to detect. A plot of the decay curves depicting lifetime measurements as a function of quantum yield for each compound used in the study is shown in Figure 5b. $\text{Ir}(\text{btp})_2(\text{acac})$ has been utilized in light emitting diode (LED) applications and has a high quantum yield of 0.33[14], which should also impart ideal imaging properties; however, its shorter fluorescence lifetime of $5.8\mu\text{s}$ [14] may limit its application in CELSI. While PtG4, MM2 and MitolD have lower quantum yields, their longer fluorescence lifetimes should be advantageous for signal detection. In addition MM2 and MitolD are nanoparticles with multiple copies of oxygen sensitive porphyrins[13], which should provide enhanced signal to noise ratio.

With optimization in mind, a comparison of commercially available oxygen sensors was completed in vitro, comparing signals in ambient oxygen to that in the presence of oxygen-scavenging GODCAT. These conditions were employed since phosphorescent oxygen sensors exhibit maximum luminescence in the absence of oxygen. PtG4 was not employed in this study since the maximum cutoff for the fluorescence emission of the plate reader is shorter than the PtG4 fluorescence emission. As expected, these reagents show a signal enhancement in the presence of GODCAT conditions, indicating a response to change in oxygenation levels. MM2 and $\text{Ir}(\text{btp})_2(\text{acac})$ gave the most robust responses in sensing oxygen via steady state fluorescence (Figure 2a). At the highest concentration utilized ($30\mu\text{g/mL}$), the signal was attenuated in comparison to the $20\mu\text{g/mL}$ samples, which could be attributed to self-quenching, although further investigation of this was not done. Due to these observations, since MM2 and $\text{Ir}(\text{btp})_2(\text{acac})$ gave the best signal, the next focus was to determine the lowest concentration of each sensor that could be utilized to detect oxygen in vitro. We found that $\text{Ir}(\text{btp})_2(\text{acac})$ had a larger dynamic range than MM2, allowing detection of changes oxygen levels in cells, with a concentration of an order of magnitude more sensitive at $0.2\mu\text{g/mL}$ of sensor, versus $2\mu\text{g/mL}$ for MM2 (Figure 2b).

We investigated the CEL both in solution and loaded into PANC-1 cells of the previously studied PtG4 as well as MM2, $\text{Ir}(\text{btp})_2(\text{acac})$, and MitolD. Both PtG4 and MM2 exhibited strong phosphorescence at $20\mu\text{g/mL}$ in solution, particularly with oxygen scavenging, when excited by Cherenkov emission, with phosphorescence intensities of 1349 and 1269, respectively, whereas $\text{Ir}(\text{btp})_2(\text{acac})$ and MitolD did not display significant phosphorescence in solution, with intensities of 250 and 150 respectively. (Figure 3a). We have previously seen excellent results with CELSI with PtG4 [7, 9, 10], and since MM2 contains multiple copies of a porphyrin grafted to a nanoparticle, enhanced signal in response to oxygen was anticipated for both of these reagents. As expected, the intracellular phosphorescence intensity was markedly decreased in comparison to the phosphorescence measured in solution. Interestingly, $\text{Ir}(\text{btp})_2(\text{acac})$ exhibits a much better phosphorescence signal in cells versus solution, giving the largest detectable signal in comparison to the other reagents (Figure 3b). This is likely due to the fact that this complex binds to albumin or another cellular protein, thereby enhancing permeability. However previous reports indicate that it is no longer sensitive to oxygen when bound to albumin in vitro, which our results and other cell studies with $\text{Ir}(\text{btp})_2(\text{acac})$ then indicate that binding to albumin is not the

mechanism that imparts enhanced signal in cells vs. solution since a response to changes in oxygenation levels was not seen [14]. The performance of MM2 was diminished intracellularly, possibly due to lower cell permeability than Ir(btp)₂(acac). In fact, Ir(btp)₂(acac) was found to penetrate cells far more rapidly than MM2 (30 min vs. 24 h). PtG4 gave a robust response to oxygenation changes in cells; however, the intensity was somewhat attenuated in comparison to Ir(btp)₂(acac). One critical advantage of PtG4 is that the dendrimer shell prevents its interaction with other biomolecules that could potentially perturb its emission lifetime and thus pO₂ estimations. This is considered to be a key factor in making this agent a linear reporter of tissue pO₂, and can be calibrated appropriately. While MitolD displays an enhanced signal in cells vs. solution, it still elicits the weakest phosphorescent response to changes in oxygen concentration. In conclusion, it seems that PtG4 and Ir(btp)₂(acac) exhibited the most robust response in cells to fluctuations in oxygen content.

PtG4 and Ir(btp)₂(acac) seemed to have promise for use in CELSI. Therefore, we investigated these reagents for sensing oxygenation levels in subcutaneous MDA-MB-231luc-D3H2LN tumors. We assessed both the phosphorescence intensity and emission lifetime in live mice as well as 30 min after sacrifice, which will allow measurements in ambient oxygen conditions and hypoxia conditions, respectively. We were able to sense both fluorescence lifetime (Figure 4) and as such calculate tissue oxygenation utilizing CELSI for both PtG4 and Ir(btp)₂(acac) at the nmol level. We found that Ir(btp)₂(acac) appeared to be more sensitive in that it required 35 µg per tumor to detect signal versus 1.75 mg per tumor for PtG4. However, we were not able to measure fluorescence lifetime or tissue pO₂ in live mice with Ir(btp)₂(acac). This is likely because the fluorescence lifetime in a fully oxygenated sample of Ir(btp)₂(acac) is shorter than we can detect since the shortest delay time feasible for imaging after the radiation pulse is 4.26µs. We also observed quenching with Ir(btp)₂(acac) at concentrations higher than 50 µM in subcutaneous tumors, as we did in the fluorescence plate reader experiments.

A summary of the results as well as a comparison of the pros and cons of the oxygen sensors we utilized for this study are illustrated in Table 1. Briefly, both PtG4 and Ir(btp)₂(acac) are stable indefinitely, while MM2 and MitolD nanoparticles have a short shelf life of only 1-2 weeks once resuspended. PtG4 has a dendrimer shell that prevents its interaction with biomolecules, allowing accurate determinations of fluorescence lifetime and pO₂ calculations. Ir(btp)₂(acac) and MitolD appear to interact with biomolecules in some way, given the difference in phosphorescent intensities discovered in solution vs. cells. Finally, we have observed that MM2, MitolD and Ir(btp)₂(acac) exhibit self-quenching at higher concentrations, where we have not seen this effect with increasing concentrations of PtG4.

Conclusion

When used in vitro at the same concentrations, the signal strengths suggest that the optimal agents would be Ir(btp)₂(acac), followed by PtG4, and then MM2 and MitolD. However, in vivo, only PtG4 and Ir(btp)₂(acac) were found to be measurable at nmol level doses in the tumors, and assessment of tissue oxygenation accomplished. On the timescale of LINAC produced x-ray imaging, PtG4 is the more ideal agent, because of the lifetime on the scale of tens of microseconds, versus Ir(btp)₂(acac) which has a lifetime of a few microseconds which hinders detection at higher tissue pO₂ values. This work represents: (1) a unique way to harness Cherenkov emission for imaging purposes in conjunction with radiotherapy, (2) a new noninvasive determination of tumor pO₂, and (3) a direct comparison of phosphorescent sensors available to probe tissue oxygenation.

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Conflict of Interest: Dr. Pogue reports grants and personal fees from DoseOptics LLC, outside the submitted work. In addition, Dr. Pogue has a patent Method And System For Using Cherenkov Radiation To Monitor Beam Profiles And Radiation Therapy pending to Trustees of Dartmouth College. Dr. Bruza reports grants from DoseOptics, LLC, outside the submitted work.

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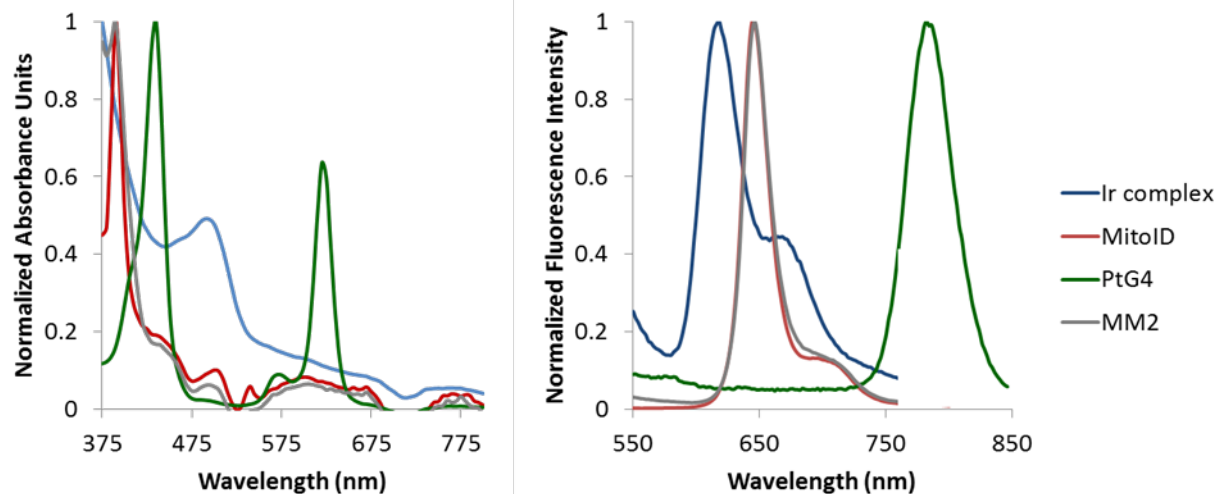
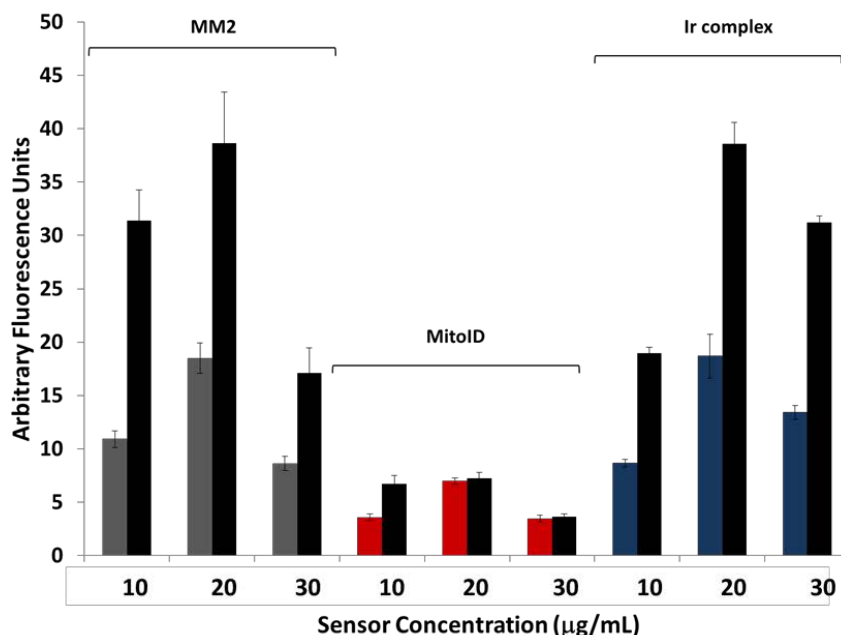


Figure 1: Absorbance and emission spectra for oxygen sensors. The absorbance and fluorescence emission spectra for Ir(btp)₂(acac) (blue), MitolD (red), PtG4 (green) and MM2 (gray).

A



B

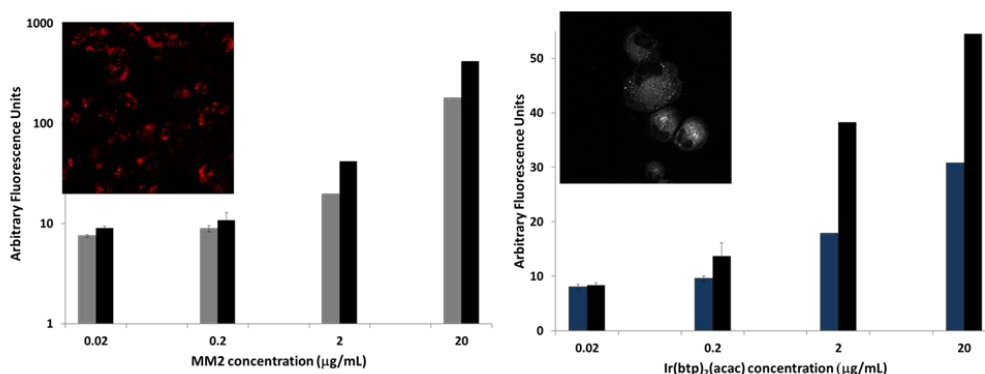


Figure 2: Comparison of commercially available oxygen sensors in PANC-1 cells. A. MM2 (gray), MitolD (red), and Ir(btp)₂(acac) (blue) were incubated with PANC-1 cells (10 µg/mL, 20 µg/mL, and 30 µg/mL) overnight. Fluorescence was measured on a fluorescence plate reader in the presence of ambient oxygen and in the presence of oxygen scavenging system of glucose oxidase and catalase (black). B. Range of detection of Ir(btp)₂(acac) and MM2. The brightest commercial oxygen sensors were explored at decreasing concentrations (20 µg/mL, 2 µg/mL, 0.2 µg/mL, and 0.02 µg/mL) to determine the lowest concentration that can be detected. Inset shows fluorescence microscopy of each sensor (20 µg/mL) loaded into PANC-1 cells. Excitation and emission were measured at 380 nm and 660 nm, respectively. The fluorescence intensity represents the average of 6 experiments +/- SEM.

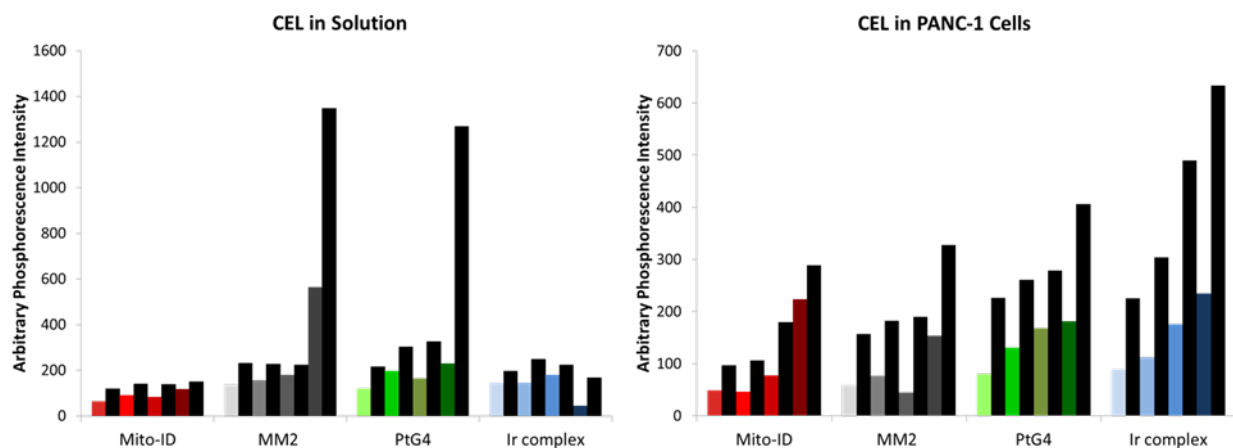


Figure 3: Comparison of Cherenkov luminescence imaging with phosphorescent oxygen sensors in solution and in PANC-1 cells. Increasing concentrations of each sensor (0.02 $\mu\text{g/mL}$, 0.2 $\mu\text{g/mL}$, 2 $\mu\text{g/mL}$, and 20 $\mu\text{g/mL}$, MitoID (red), MM2 (gray), PtG4 (green), and Ir complex (blue)) were analyzed in ambient oxygen or in low oxygen conditions with glucose oxidase and catalase (black). For investigation in PANC-1 cells, the reagents were incubated with cells overnight, rinsed with PBS, and cell culture media with or without glucose oxidase catalase was added. A 96-well plate containing solutions of oxygen sensor or PANC-1 cells loaded with oxygen sensor was exposed to 6 MeV radiation from a linear accelerator, and the phosphorescence intensity was assessed at various delay times. The plot represents the maximum intensity for each sensor.

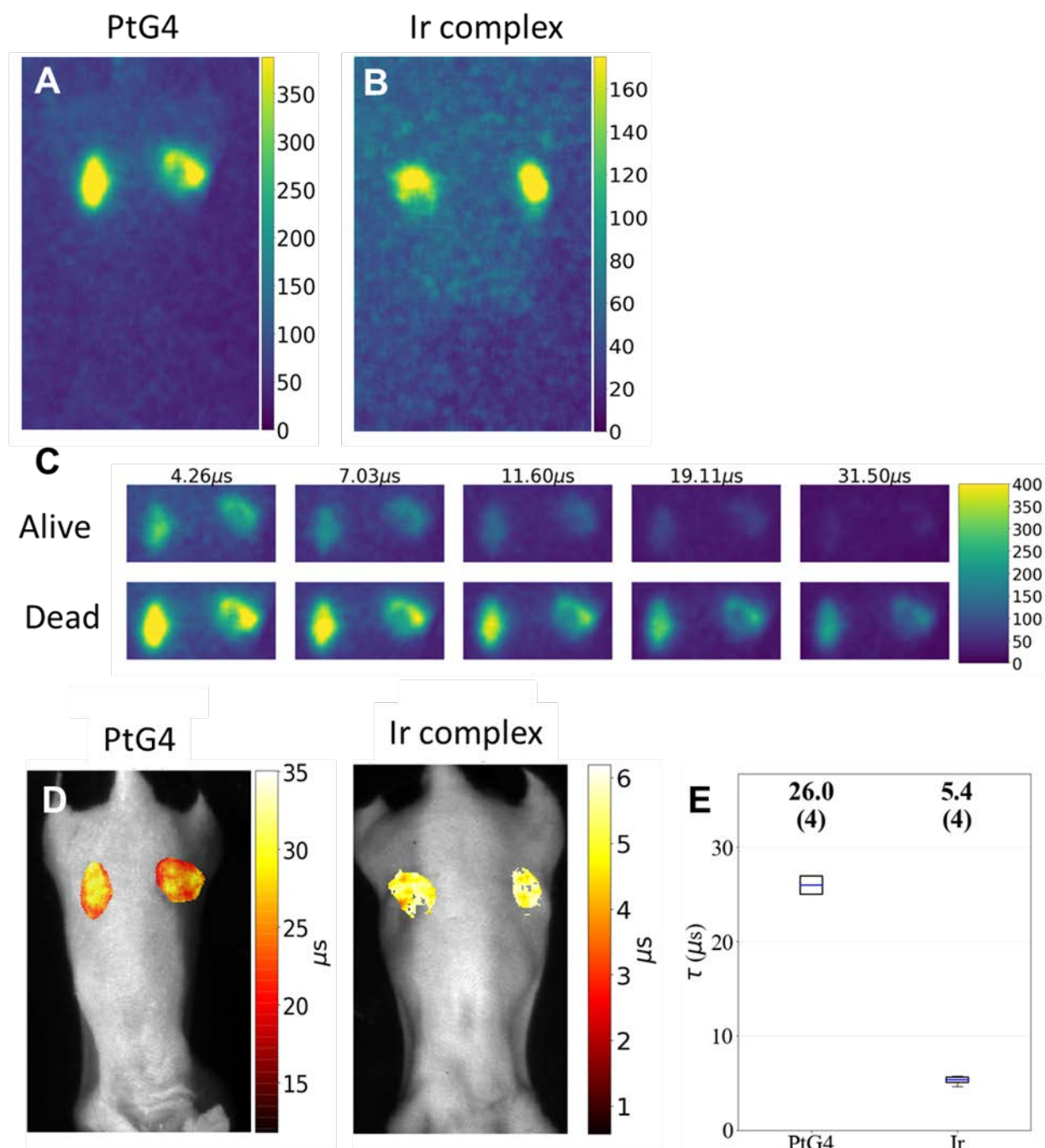


Figure 4. In vivo imaging with PtG4 and Ir(btp)₂(acac). Under anesthesia of isofluorane, 1.25 nmol of each reagent was injected into subcutaneous MDA-MB-231luc-D3H2LN tumors. The animal was imaged at various delay times to gain emission lifetime information alive and 30 min after sacrifice. A. Maximum intensity projection of PtG4 in a sacrificed animal at 4.26 μs delay time. B. maximum intensity projection of Ir(btp)₂(acac) in sacrificed animal at 4.26 μs delay time. C. comparison of PtG4 phosphorescence intensity in subcutaneous tumors alive (top, ambient oxygen conditions) and 30 min after sacrifice (bottom, low oxygen conditions). D. emission lifetime maps of sacrificed animals. E. box and whiskers plot of the emission lifetimes in sacrificed animals.

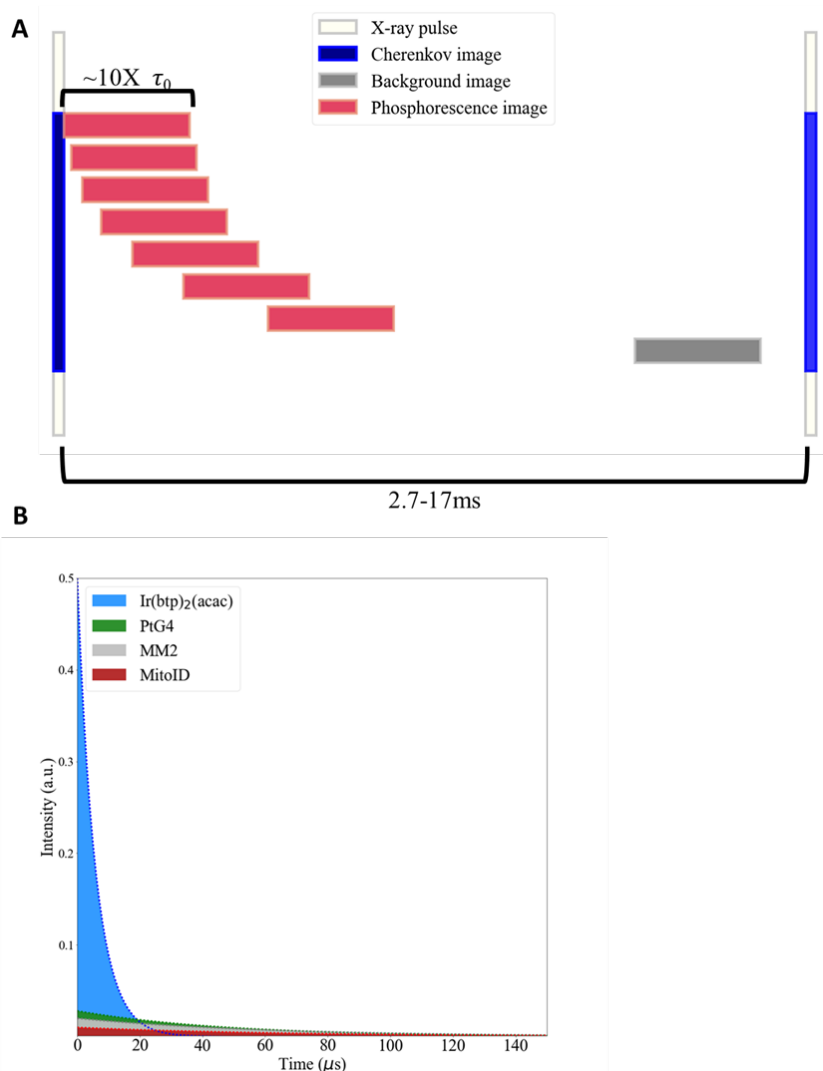


Figure 5. Considerations for CELSI. A. Cherenkov imaging timing. Experimentally the gate width is generally set between 5 - 10 times the deoxygenated lifetime (τ_0). The LINAC generates radiation pulses at a repetition rate between 2.7 and 17ms (600MU/min - 100MU/min). B. plot of fluorescence decay curves as a function of quantum yield

Compound	CEL solution	CEL cells	CELSI in vivo	Biomolecule Interaction	Stability	Self-quenching
PtG4	+	+	+	-	+	-
Ir(btp)₂(acac)	+	+	+	+	+	+
MM2	+	+	-	-	-	+
MitoID	-	+	-	+	-	+

Table 1. Side-by-side comparison of phosphorescent oxygen sensors utilized for CEL

Multi- point and line scan excitation from a linac for improved lateral resolution in Cherenkov-excited molecular luminescence imaging

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Cherenkov-excited luminescence scanned imaging (CELSI) is achieved with External Beam Radiotherapy, to map out molecular luminescence intensities or lifetimes in tissue. Just in fluorescence microscopy, the choice of excitation geometry can affect the imaging time and ultimate spatial resolution as well as contrast recovered. In this study, the use of spatially patterned illumination was systematically studied comparing point/block scans, line scans, and increasing from single beams to multiple parallel beams. The image recovery was improved by a post-processing modulation-demodulation method, which uses the ability to capture simultaneous images of the excitation Cherenkov beam shape to deconvolve the CELSI images. Experimental demonstration in the work used the multi-leaf collimator on a clinical linear accelerator (LINAC) to create the scanning patterns, and image resolution and contrast recovery was tested at different depths of tissue phantom material. As is known from microscopy, the data confirmed that the smallest illumination squares achieve optimal resolution, but at the cost of lower signal and slower imaging time. Having larger excitation blocks provides superior signal but at the cost of increased radiation dose and lower resolution. Increasing the scan beams to multiple block patterns improved the performance in terms of image fidelity, lower radiation dose and faster acquisition. The spatial resolution was mostly dependent upon pixel area with an optimized side length near 38mm and a beam pitch of $P=0.33$, and the achievable imaging depth was increased from 14mm to 18mm with sufficient resolution power for 1mm sized test objects. It is anticipated that MLC-driven CELSI can therefore allow high resolution fast imaging of molecular signals within tissue. © 2018 Optical Society of America under the terms of the [OSA Open Access Publishing Agreement](#)

OCIS codes: (170.3010) Image reconstruction techniques; (170.3880) Medical and biological imaging; (170.7440) X-ray imaging. (170.6920) Time-resolved imaging; (260.3800) Luminescence;

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1. INTRODUCTION

Radiation therapy induces Cherenkov light emission within tissue, and this signal provides potential for direct molecular sampling of the tissue microenvironment [1–4]. The approach to using Cherenkov Excited Luminescence Scanned Imaging (CELSI) was examined here, specifically varying the spatial patterns used to excite Cherenkov light from megavoltage (MV) X-rays in external beam radiation therapy (EBRT) [5, 6]. CELSI can provide three-dimensional luminescence images through layer-scanned Cherenkov light sheet, which is carried out by movements of the Multileaf Collimator (MLC) on the linear accelerator (LINAC), which is commonly used to provide conformal shaping of radiotherapy treatment beams [7]. This application has been

experimentally demonstrated in-vivo and is hopeful for clinical trial use, taking advantage of the dynamics already inherent in radiotherapy treatments [5,6]. In this study, progressive approaches to scan beam size and shape were explored to understand how incorporation with dynamic treatment plans might occur.

Since tissue partial pressure of oxygen (pO_2) is well known to affect the efficacy of radiotherapy [1,8,9], imaging this signal this has been a primary focus of previous CELSI studies. In order to image pO_2 in tissue for EBRT, megavoltage (MV) photon beams have been utilized to induce Cherenkov in thin sheets within the tissue to excite oxygen-sensitive phosphorescent probe, e.g., platinum(II)-G4 (PtG4) [10–12] via Cherenkov light emission, which efficiently absorbs blue light ($\lambda_{max}=435nm$) and emits phosphorescence in the NIR region (λ_{max}

=772nm). Epi-illumination based CELSI in tissue allows for imaging within a centimeter or more, dependent upon the fluorophore concentration and radiation dose used. The scattering-induced blurring can be compensated by deconvolution processing based on the point spread function (PSF), evaluated by radiative transport models [6, 13, 14]. The hypothesis examined in this study was that when designing this irradiation scheme, the beam shape could be chosen to optimize the spatial-resolution of recovered CELSI images. Further, it might be possible that existing clinical treatment plans allow for high resolution imaging.

Patterned illumination schemes have been investigated more recently with the emergence of compressed sensing, where computed image recovery is utilized to improve the image quality without extra radiation exposure and/or acquisition time [15]. While the ultimate extent of compressed sensing methods uses complex patterns, the methodology for this could include a simpler spatial-frequency-domain imaging (SFDI) approach or more complex techniques such as used in coded aperture imaging (CAI). Current SFDI instruments typically use projected spatially modulated light, projected onto the sample, and then knowledge of the illumination patterns are used to demodulate the images [16-18]. CAI requires a coded mask placed either, in front of the radiation beam source to shape the incident beam, and at the detector to block the transmitted beam and disambiguate scatter angles [19-21]. For both of these structured imaging modalities, the excitation beam is modulated by a projector or a coded aperture, and the measured reflectance or transmittance is demodulated in post-processing. This modulation-demodulation method (MDM) has the capacity to mathematically filter the low frequency out-of-focus scattered photons beyond the depth of field of the imaging objective. The limit of this approach with a single point which is raster scanned provides an MDM approach in which the point aperture acts as a virtual pinhole, such as in confocal microscopy. Diversification of the illumination pattern based on an MLC is clinically convenient in CELSI and could be time-saving. In particular, using coded apertures in conjunction with multiplexed illumination can reduce the acquisition time, given that projections from more than one source may be acquired in a single step [19].

Motivated by the idea of using patterned illumination schemes, the study here examined if the spatial resolution and contrast recovery of CELSI could be improved on different spatial illumination patterns and post processing with MDM. The input radiation beam was modulated and raster-scanned by programming the MLC, and the post-processing demodulation was performed by deconvolving the measured luminescence image with the kernel function of the Cherenkov image. This approach, referred to as MDM-CELSI, examined several EBRT-compatible MLC plans, and specifically how the measurement geometry affected the acquisition time, and radiation dose. Modulation of the radiation (X-ray) beam was investigated using basic illumination patterns including squares and lines, and multiple combinations of these.

2. METHODOLOGY

A. Incidence modulation

In order to utilize LINAC-based EBRT within a CELSI illumination plan, a patterned beam design must be created with consideration of the geometry and orientation the MLC, which limits what is possible. For a Varian 2100 series LINAC, since the lateral length (along the direction orthogonal to movement) of single leaf in MLC is 5mm with step sizes of 0.1mm, the smallest point excitation available realistically is on that spatial scale. The geometry of these MLCs are optimal for targeting single-connected regions or distributions along the central axis, but it can be impossible to form more complicated shapes that might be optimal, like the Hadamard matrices for example [22]. In addition, when choosing appropriate beam shapes, additional attention must be paid to the radiation dose, and beams that have multiple overlaps of the same tissue can lead to excessive dose delivery [23].

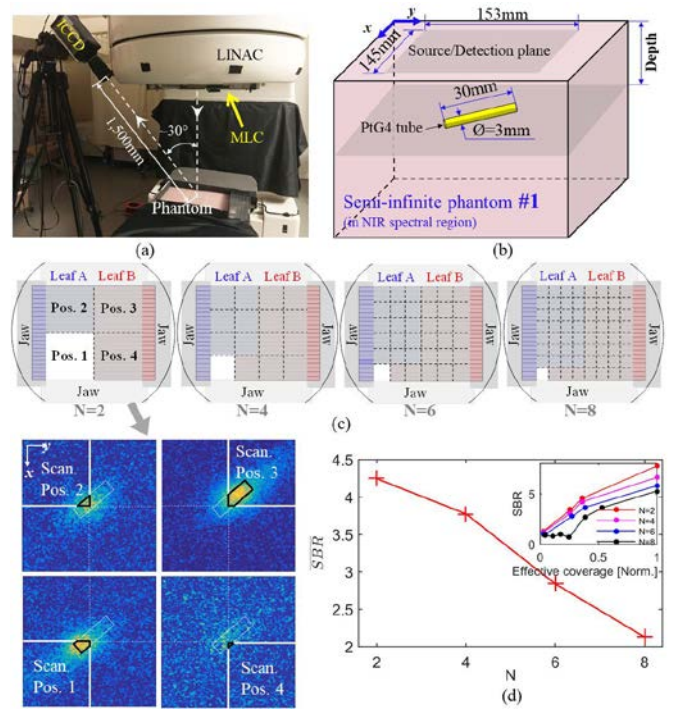


Fig. 1. The experimental setup used to investigate the effect of radiation beam size is shown in (a) with a photo of the experimental setup with LINAC, phantom and ICCD camera; (b) the internal phantom geometry; (c) the basic $N \times N$ square-raster scanning patterns tested initially with different pixel areas and the luminescence images measured for $N=2$ (below this), and (d) the calculated Signal to Background ratio (SBR) versus target area for $N=2, 4, 6$, and 8 , respectively.

The detected luminescence yield in CELSI for deeply embedded objects can be low due to the diffuse scatter of the light exiting the tissue and also due to limitations of the number of secondary electrons in the excitation volume. To achieve a reasonable SNR value, the pixelated area does need to be sufficiently large enough to generate Cherenkov light, and there is a build-up factor associated with larger beams which enhances the signal. Experimental investigations were performed by raster-scanning illuminations patterns with different sized squares, as shown in Fig. 1(c). In these experiments, the X-ray beam impinged on the tissue-equivalent phantom, embedded with a $70 \mu\text{M}$ PtG4 target (inner diameter $\phi \approx 2\text{mm}$, and length of 10mm) at a depth of $\sim 1.2\text{cm}$, which is detailed in Fig. 1(a) and (b). The source plane (*i.e.*, total region receiving radiation beam) had an area of $\sim 145 \times 153 \text{ mm}^2$, and the detection plane was tailored correspondingly. As an example, the measured luminescence images for an $N \times N$ ($N=2$) raster-scanned illumination is shown below Fig. 1(c), where the regions encircled with black lines are the pixel areas included within radiation beam, named as effective coverage. The relationship of Signal to Background ratio (SBR) with effective coverage and N is shown in Fig. 1(d), where the SBR can be found strongly dependent upon both.

2D raster-scanning illumination was next studied, where the square area could be more than 1mm^2 to afford a sufficient SNR requirement. To accelerate the acquisition process, several square-illuminations are simultaneously enabled, which allowed for reduction of total scan time. As a proof-of-concept, one type of multi-point illumination with equal spaces is shown in Fig. 2 (c), where the dashed squares represent the next scanning positions. The multi-square distribution depends on following two parameters: (1) the square side-length l , which is found by a compromise between spatial resolution and measurement SNR; and (2) the beam pitch $P = d'/l$, where d' is the step size. $P > 1.0$ denotes there is a gap in between the continuous squares and vice versa. For full-field illumination, we assume $P=1$, which indicates a contiguous scanning for adjacent positions. For multiple scanning, $d' = (Nl - d)/(N - 1)$, where d is distance in between each square, and the total scan positions should be N^2 times the number of squares in a multi-

square shape. To be comparable with the point-raster strategy, d should be large enough to avoid cross-talk effect. The minimum of d ($d_{\min}$) depends on the system SNR, which is the critical source-detector separation estimated by fluorescence Monte-Carlo simulation. For typical optical properties of normal human tissue under the wavelength $\lambda=772\text{nm}$ (PtG4 phosphorescence peak), *i.e.*, the absorption coefficient μ_a , scattering coefficient μ_s , and anisotropic factor g values common in soft tissue, at $\mu_a = 0.01\text{mm}^{-1}$, $\mu_s = 10\text{mm}^{-1}$, and $g = 0.9$, $d_{\min}$ is near 2mm.

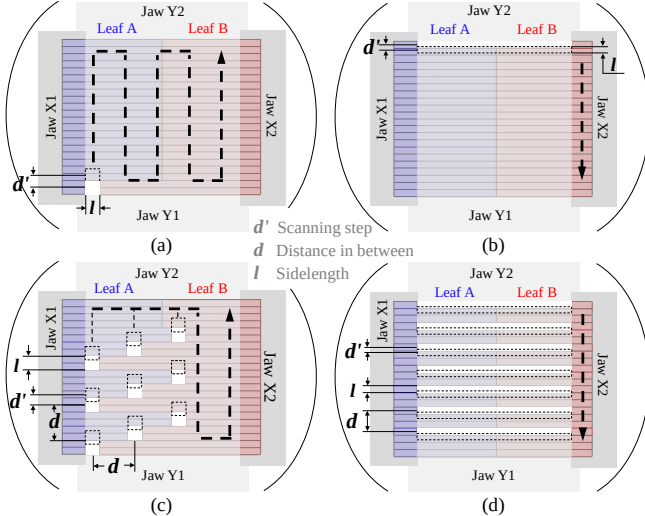


Fig. 2. Two basic illumination patterns studied are displayed from the top-view of the MLC, including: (a) a single square, raster scanned in 2 dimensions across the entire field of view, (b) a thin line, scanned in one dimension vertically down through the field, (c) a multiple-square pattern which is scanned; and (d) a multiple-line pattern scanned downwards. The blue and red rectangles represent leaves of Groups A and B, respectively, while the black dashed frames indicate movement to the next scanning positions, and the thicker dotted arrow lines indicate the total movement plan.

As an alternative way to accelerate acquisition, line-scan field illumination has been demonstrated in confocal microscopy [24], optical coherence tomography (microscopy) [25,26], and laminar optical tomography [26]. Further accelerations can be similarly reached by multiple enablement strategy, named as multi-lines illumination, as shown in Fig. 2(d). Also, the distance in between each line should be greater than d_{SNR} . Comparisons between point- and line- shaped patterns with different combinations of l and P was examined.

B. Image demodulation

The procedure of image demodulation is illustrated by the flow chart in Fig. 3. For simplicity, a 2D square-raster scan is illustrated here with 4×4 uniformly distributed scanning positions. The resulting input raw image is composed of 16 pixels corresponding to the 16 positions. The steps are: (1) the raw images were processed frame-by-frame to mainly extract the region of interest through subtracting 'background' ones, which were captured at a larger pulse delay than that of luminescence signal detection; (2) the Cherenkov image was used as the kernel function to deconvolve the corresponding luminescence images; and (3) demodulation is done by combing all the frames into a Maximum Intensity Projection (MIP) image. It is worth noting in step2 that, Luminescence and Cherenkov lights images can be successively captured by setting different delay times on the time-gated camera. In most fluorescence imaging modalities, the emission light fluence-rate has to be estimated relying on transport models, *e.g.*, diffuse approximation, radiative transport equation, and Monte-Carlo simulation. However, the exact model of emission photon migration is hard to match to high precision in practice, since optical properties are generally hard to estimate accurately, and the contribution from the fluorophore itself is generally ignored. For CELSI, the emission source is

X-ray excited Cherenkov photons, which can be captured by the same device for luminescence measurement with a different pulse delay (typically 0-3.5 μs for Cherenkov and 5-55 μs for luminescence as described below).

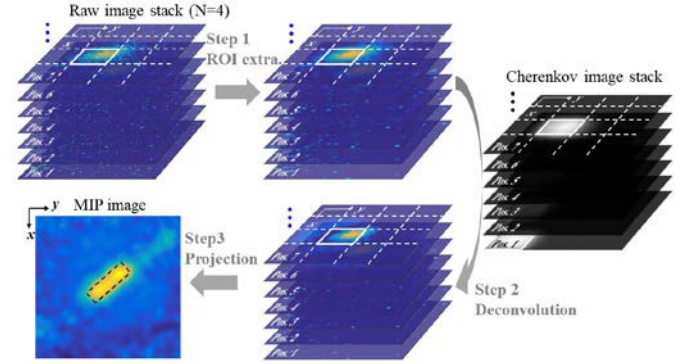


Fig. 3. Diagram of the patterned-CELSI (N=4) postprocessing sequence involving acquisition of both luminescence and Cherenkov at each of the raster scanned positions and region of interest (ROI) extraction (step 1) followed by deconvolution of the luminescence by the Cherenkov shape (step 2) and combining together for a MIP image (step 3). The dashed black square in the MIP images indicates the true position of the target.

3. RESULTS

A series of phantom studies were carried out using a tissue-equivalent phantom, which consisted of 1% Intralipid and 1% pork blood per unit volume of solids, with absorption and reduced scattering coefficients estimated to be $\mu_a=0.01\text{mm}^{-1}$ and $\mu_s'=1\text{mm}^{-1}$ in the NIR spectral region at 800nm [27,28]. The measurement geometry is shown in Fig. 1, and the phantom adopted (#2) is illustrated in Fig. 4, where two plastic capillaries (inner diameter $\phi \approx 1\text{mm}$) containing 70 μM PtG4 solution were placed with an angle of 5° in between as shown in Fig. 4. A holder, which is fixed to the wall of phantom container, was used to clamp the capillaries. Without specific notification, the depth of target is 8mm.

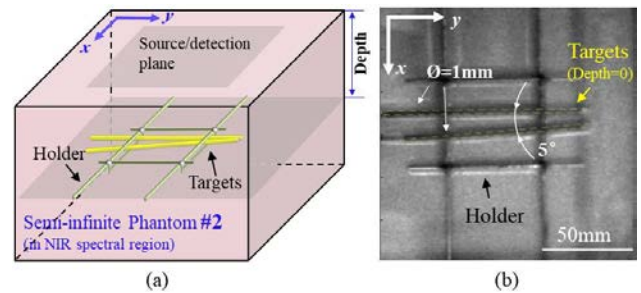


Fig. 4. Phantom experiments for image fidelity demonstration, with (a) an 3D schematic of the Intralipid-blood tissue phantom with two capillaries inside filled with PtG4 (yellow cylinder); and (b) a photography of this.

A clinical radiotherapy accelerator (Varian LINAC 2100 CD, Varian Medical System, USA) was used to generate a pulsed beam with the following parameters: photon energy of 6 MV, dose rate of 600 monitor units per minute (MU/min), at a fixed repetition rate of 360 Hz, and a $\sim 3.25\mu\text{s}$ pulse duration. The MLCs were used to shape a squared beam with sizes ranging from $14 \times 14\text{mm}^2$ to $77 \times 77\text{mm}^2$. The MLC-based raster scan was performed by delivering a custom dynamic dose plan, created in MLC code [13]. The luminescence was imaged by a gated, intensified charge-coupled device (ICCD, PI-MAX4 1024i, Princeton Instruments, USA). The camera with a FL=85mm and f/1.2 lens was mounted close to LINAC as shown in Fig. 4(a), resulting into an inclined angle of $\sim 30^\circ$ and a distance of $\sim 1,500\text{mm}$ from the sample. The luminescence signal was detected with time-gated acquisition of the ICCD (50 μs integration time and 5 μs delay after each x-ray pulse). The luminescence images were acquired with $100\times$ gain on the ICCD

intensifier, 30 pulses integrated on-chip as accumulations prior to each frame readout, and 2×2 hardware pixel binning at readout, from chip size of 1024 pixels $\times$ 1024 pixels. Cherenkov images were acquired in the same way, but with 0 pulse delay and a 3.5 μ s integration time. Measurement at an increased pulse delay of $\sim 500 \mu$ s was also acquired as 'background images' from the same FOV, which was used to subtract out stray signals in the camera and room.

A. Comparisons between line- and square- scanning pattern

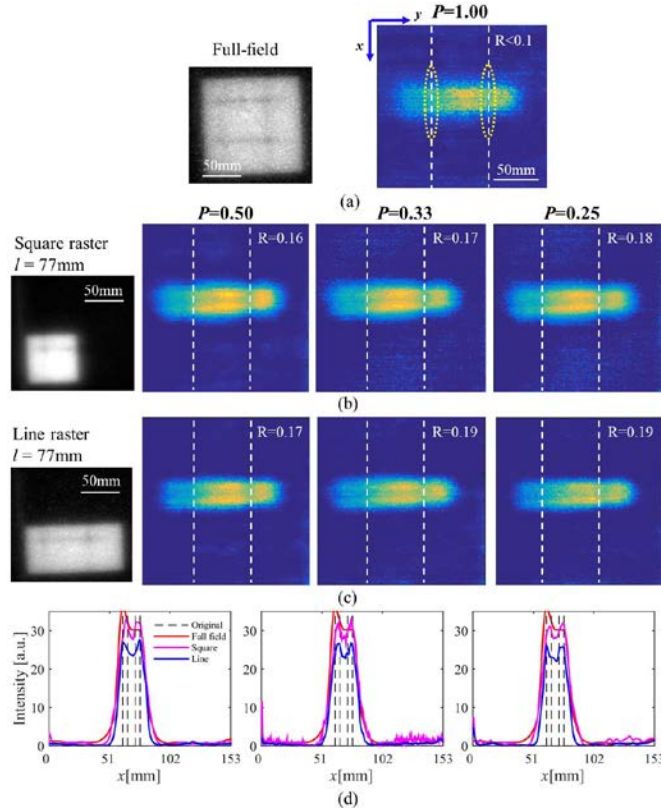


Fig. 5. Luminescence (color) and Cherenkov (greyscale left) images are shown, as measured under (a) full-field covering the entire frame, (b) a larger square 2D-raster scanned field, and (c) a thick line 1D-raster scanned. The corresponding X-profiles below (d) were averaged along the Y-axis direction for a distance in between the two white dashed lines indicated in (a)-(c). The value of R is inset in each image to qualify contrast recovery.

In the first study, line- and square- raster scanning were compared for $P=0.50, 0.33$, and 0.25 , respectively, and a fixed square size of $l=77$ mm, as shown in Fig. 5. To quantify the spatial resolution performance, a metric of success was introduced, defined as $R = (I_{\max} - I_{x=69\text{mm}}) / (I_{\max} - I_{\min})$, with $I_{\max}$ and $I_{\min}$ being the maximum and the minimum in the x-profile of the image, essentially providing a relative measure of contrast recovery. To obtain a more robust estimate of R , X-profiles for the period included by the two white dashed lines were averaged to smooth the results. With this definition, $R=1$ represents that the two targets can be completely separated, with full contrast recovery. In the characteristics of the human vision system, it is normally assumed that two targets are distinguishable for $R>0.1$ (when a 10% dip in intensity is observed between them). In addition to spatial resolution, radiation dose and acquisition time were also estimated and all are listed in Table 1. Acquisition time was calculated as: "Dose(MU)" / "Dose rate(MU/min)", which just estimated total scanning time, excluding mechanical switching issues which vary from machine to machine. We assume that Cherenkov, luminescence and background signals can be read out in one pulse cycle, which is the time period between two LINAC pulses. This can be realized with a time-resolved camera by specifying the corresponding pulse delays.

Table 1. Comparisons between line and point raster for $P=0.50, 0.33$, and 0.25 , respectively, and a fixed $l_{\text{Point}} \backslash l_{\text{Line}}=77\text{mm}$.

P	Dose (MU)			Time (s)			R		
	0.50	0.33	0.25	0.50	0.33	0.25	0.50	0.33	0.25
Full field	138			14			<0.1		
Point raster	276	552	836	124	221	345	0.16	0.17	0.18
Line raster	276	552	836	42	55	69	0.17	0.19	0.19

In general, beam projections shaped by the MLCs are clinically defined by the targeting for the tumor region. The most unresolved shape used is a squared 'full-field' shape, with its Cherenkov image shown in Fig. 5(a). It is worth noting that, the full-field region was slightly smaller than FOV to conserve the diffused edges for both Cherenkov and luminescence images, to allow for complete measurements of the kernel function by the raw Cherenkov image for the deconvolution. For the luminescence imaging, one artifact noted was that the metal-made holder could attenuate some fluorescence emission, which led to 'slightly-dimmer regions' on the PtG4 target as indicated with yellow dashed circles, but these was avoided in the analysis, and not present in the line scans analyzed, Fig. 5(d).

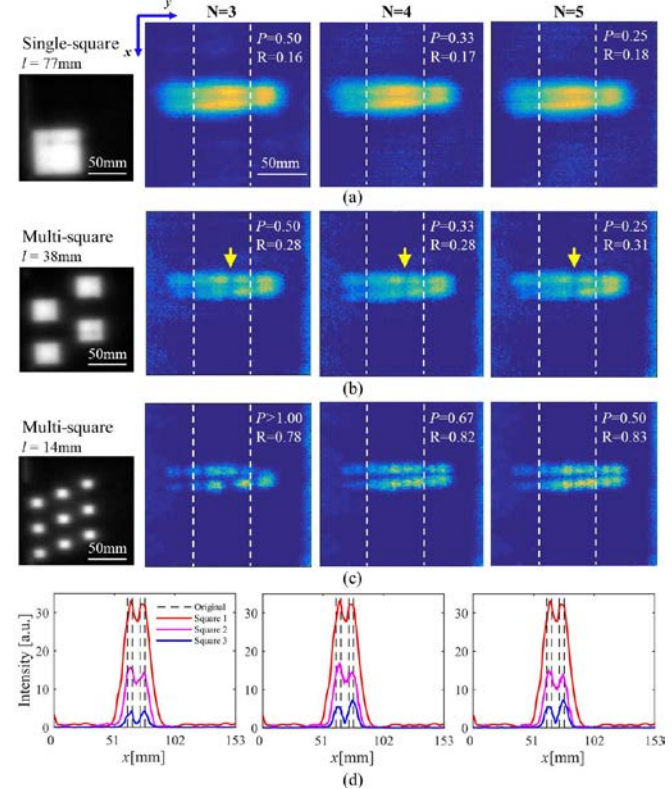


Fig. 6. Luminescence (color) and Cherenkov (greyscale left) images as measured under (a) large 77mm block beam raster scanned, (b) a series of 4 parallel 38mm square raster scanned beams, and (c) a series of 9 parallel 14mm square raster scanned beams. The corresponding X-profiles (d) were averaged along the Y-axis in between the two white dashed lines indicated in (a)-(c). The titles 'xx-by-xx' denote the number of controlling points, deciding the scanning step d' . R values recovered are overlaid on each luminescence image, showing the large improvement in R with smaller beam sizes.

Several conclusions can be drawn from Fig. 5. (1) Both square- and line- raster results show higher contrast recovery ($R>0.15$) than that of the full-field ones ($R<0.1$). Basically, the two capillary rods cannot be discriminated in the 'full-field' image. (2) Image resolution seldom varies with scanning step since no space exists in between two adjacent squares, i.e., $P<1$. However, the recovered image would degrade a lot if $P>1$, e.g., the capillary rods were found to be discontinuous in Fig. 6(c) when $P>1$. (3) In this case, the line-scan results slightly outperformed the square point-scan ones. This could be explained by the nearly

parallel direction between the capillaries placed and the illumination orientation. In other words, resolution would be reduced if the two directions were orthogonal. For example, as pointed out by the yellow arrows in Fig.6(c), some dimmed regions could be more clearly discerned in square point-scan images, which, however, are not visible in line-scan results as shown in Fig. 6(b). These regions were highly resolved in Fig. 7, and also visible in Fig. 8. An improvement to the line-scan methods would be multi-direction scanning, which could render a full-direction resolution enhancement, however, this would come at the cost of doubling the radiation dose and acquisition time.

Multi-point scanning of squares is therefore demonstrated above to be ideal compromise between optimal resolution and minimal scan time and minimal dose. Further investigations were aimed at optimization of the pattern parameters as discussed in Section 2.A, *i.e.*, l and P . Three kinds of square patterns were deliberately designed for this test, with their Cherenkov images displayed in greyscale as shown in Fig. 6, progressing from larger to smaller block sizes with separation of them sufficiently large to avoid any crosstalk. From the luminescence images in Fig. 5, R -value was not strongly dependent upon the value of d' . In comparison, the square size characterized by side-length l imposed a significant influence on spatial resolution, *e.g.*, from the images (bottom line of Fig. 5) with $R<0.2$ when $l=77\text{mm}$ to those (middle line of Fig. 5) with $R>0.77$ when $l=14\text{mm}$. However, the measurement intensity of the former cases was 5 times higher than the latter ones, which is in agreement with the conclusions of intensity versus illumination area in Section 2.1. Consequently, we applied a trade-off, where a side length of 38.3mm and $P=0.33$ were tested here.

B. Imaging for varying target depths

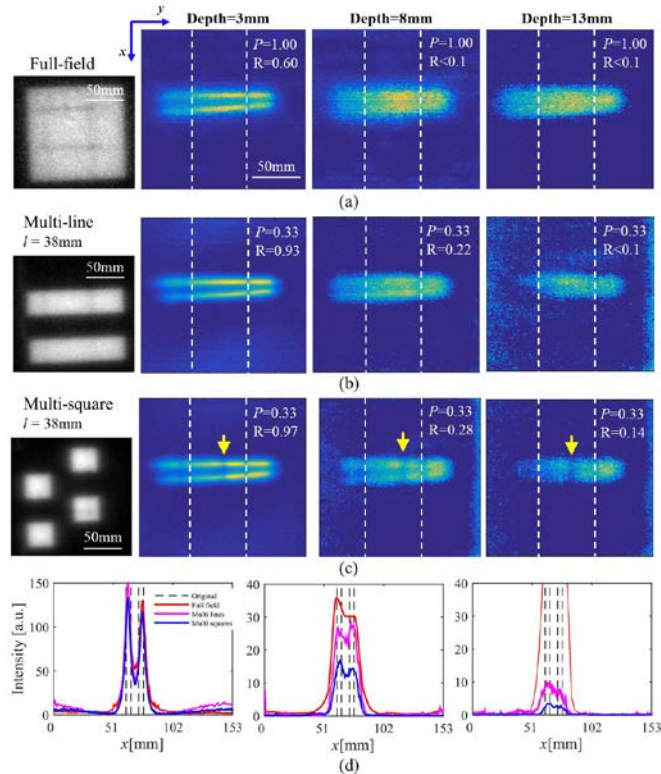


Fig. 7. Luminescence (color) and Cherenkov (greyscale left) images as measured under (a) full-field, (b) square raster scanned, and (c) line raster scanned beams, for target depths of 3, 8, and 13mm, respectively, from left to right columns. The corresponding X-profiles (e) were averaged along the X-axis in between the two white dashed lines indicated in (a)-(c). The R values of each scan are overlaid on each luminescence image, showing the large decrease with depth into the medium, but that the raster scanned square beams (c) allow sufficient R resolution down to the lowest depth of 13mm.

In this section, the effect of increasing target depth was analyzed. It can be seen from Fig. 7 that, all the methods could provide clearly discerned images for the smallest target depth. In comparison, the full-field image presents a result of lower spatial resolution as well as SNR, and an available imaging depth of less than 8mm. For the deepest target at 13mm, only the imaging result with the square beams could provide a spatial resolution of $R>0.1$. Since the deblurring strategy herein only considered Cherenkov photon scattering, the recovered images for large-depth targets embedded in a scattering medium should have diffused edges. Depth-corrections for fluorescence photon scattering have been widely developed in (fluorescence) diffuse optical tomography. Besides, depth-related image deconvolution has been previously customized for CELSI in previous work [6].

C. Modulation Transfer Function analysis

To quantify spatial frequencies that can be resolved versus depth, a phantom embedded with a single target (capillary of $\varnothing \approx 1\text{mm}$, filled with $70\ \mu\text{M}$ PtG4) was imaged to obtain the Modulation Transfer Function (MTF) from line scan data. Results as measured under both full-field and multi-square illumination are plotted in Figs. 8 (a) and (b), respectively, where the squared beam shape was scanned with an optimized parameter set at $l=38\text{mm}$ and $P=0.25$. The line spread functions (LSF) are inset correspondingly and fitted to Gaussian curves in the subplots, and demonstrate the available spatial frequencies from which information can be obtained.

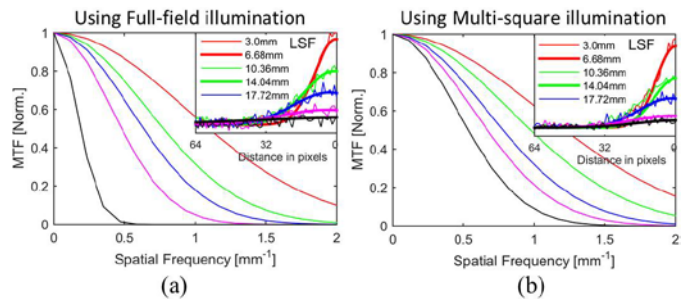


Fig. 8. Normalized Modulation Transfer Function curves estimated for (a) full-field, and (b) multi-square illuminations. The insert curves are corresponding line spread functions and their Gaussian fitting results.

Table 2. Inverse of limiting spatial frequency (10% of the MTF amplitude) defined at varying depths for full-field and multi-square illumination.

Target depth	3.0mm	6.7mm	10mm	14mm	18mm
Full field	0.51mm	0.72mm	0.88mm	1.2mm	2.9mm
Multi square	0.46mm	0.58mm	0.71mm	0.84mm	1.1mm

Spatial resolutions achievable are related to the inverse of the MTF function where there is measurable amplitude, and here it was taken as 10% of the maximum. These are defined at different target depths, as recorded in Table 2, where the effective lower limit on resolution was estimated. It can be seen from Table 2 that, the difference in spatial resolution between these two methods increases significantly from roughly 0.05mm at a target depth of 3mm to a difference of 1.8mm at 18mm. For a target depth of 14mm, both full-field illumination and the scanned multi-square method retain reasonable resolution near 1mm, but just slightly beyond this depth (18mm depth) the true capillary diameter could not be resolved with full field illumination but likely could be with the scanned multi-square approach.

D. Correction for defocused blurring

To acquire high signal intensity or measurement SNR, a large-NA lens is generally applied for fluorescence imaging, which however renders a limited depth of field (DOF). The adopted lens used in this study was FL=85mm and $f/1.2$, for instance, which provides only a DOF=9.4cm for coupling to the PI-MAX4 camera. Since defocus blur can be seen simply as the convolution between a sharp image and a blur kernel, one can solve for the sharp image again by deconvolving the blurred image with

the same point spread function (PSF) used to blur the original sharp image [6,29]. However, the PSF has to be either estimated by scaling a depth-dependent PSF, or experimentally obtained through capturing the out-of-focused target with different camera-target distance [30].

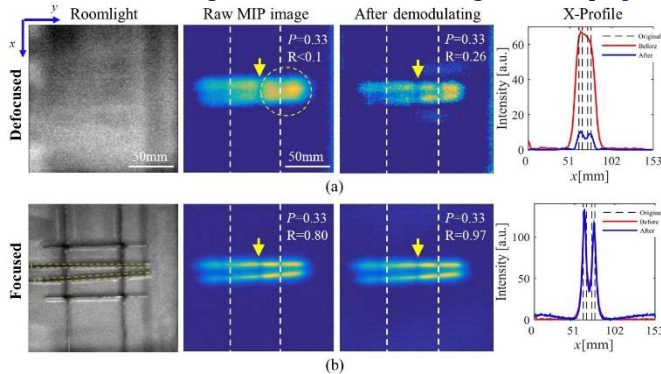


Fig. 8. (a) Focused and (b) defocused imaging results are shown. From left to right the columns are photograph, then luminescence images before and after demodulation, and the corresponding X-profiles, respectively. The X-profiles are averaged for Y-distance in between the two white dashed lines indicated.

A small target depth of 3mm was investigated to highlight the value of this deblurring by inducing a defocusing acquisition instead of photon scattering. The experimental results are shown in the upper line of Fig.8, where luminescence images before and after correction are displayed from left to right columns, respectively. As references, all-in-focus images are shown in the lower line of Fig. 8. It can be seen from the defocused image before correction, the two capillaries are hard to be separated ($R < 0.1$) and even overlapped to each other at the place indicated by the yellow dashed circle. After correction, the capillaries can be discerned with R increasing to 0.26.

4. DISCUSSION AND CONCLUSION

Based on beam scan parameters examined here, and the methodology of post processing the images, a reliable enhancement approach for CELSI imaging is proposed, with an available depth of ~ 17 mm into tissue or tissue-like medium with PtG4-dyed target. By making use of

spatial control of the LINAC beam through the programmable MLCs, a modulation of the source (*i.e.*, collimated X-ray) beam could be automatically done, as is typical for radiotherapy regimens today. Limited by the projection resolution of the MLCs, illumination patterns were investigated from basic square 2D point scans and 1D line scans. Phantom experiments showed that squared pattern outperformed linescan, achieving a similar spatial resolution while providing less radiation dose. The linescan has the potential for faster acquisition but the dose increase can be problematic. Further investigations about these pattern parameters with respect to image fidelity gave a recommended sidelength of 38mm and beam pitch of 0.33 as specified for the measurement geometry adopted in this paper. According to the MTF study, the spatial resolution at a target depth of 18mm improves from 2.90mm to 1.1mm with the optimized beam parameters. In addition to spatial-resolution enhancement, the proposed strategy was experimentally demonstrated to be capable of out-of-focus deblurring, which could be meaningful for non-contacting imaging modalities.

The ability to image pO₂ distribution during radiotherapy, as possible with molecular sensor imaged here, would provide unprecedented information about the tumor micro-environment. With the use of these and similar luminescent probes [31], the proposed approach has the potential to bring routine molecular sensing into the paradigm of radiotherapy.

For deeper application, efforts can be made towards using more optimized oxygen-sensitive probes at higher concentrations [11]. At the same time, scattering-caused blurring by luminescence photons could be compensated with the well-developed image deconvolution or reconstruction strategies for diffused optical imaging. Future work will focus on applying the proposed method to improve depth-resolved CELSI with angled incidence/detection configurations [6].

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Maps of in vivo oxygen pressure with submillimetre resolution and nanomolar sensitivity enabled by Cherenkov-excited luminescence scanned imaging

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ABSTRACT

Low signal to noise ratios and limited imaging depths restrict the ability of optical-imaging modalities to detect and accurately quantify molecular emissions from tissue. Here, by using a scanning external X-ray beam from a clinical linear accelerator to induce Cherenkov excitation of luminescence in tissue, we demonstrate in vivo mapping of the oxygenation of tumours at depths of several millimetres, with submillimetre resolution and nanomolar sensitivity. This was achieved by scanning thin sheets of the X-ray beam orthogonally to the emission-detection plane, and detecting the signal via a time-gated CCD camera synchronized to the radiation pulse. We also show with experiments using phantoms and with simulations that the performance of Cherenkov-excited luminescence scanned imaging (CELSI) is limited by beam size, scan geometry, probe concentration, radiation dose and tissue depth. CELSI might provide the highest sensitivity and resolution in the optical imaging of molecular tracers in vivo.

Introduction

Optical luminescence is the dominant preclinical imaging modality for the molecular probing of cells and tissue physiology, both in vitro and in vivo [1-3]. The sensitivity of optical detectors to small concentrations of molecular emitters and the ease of use of the detection technology make optical molecular imaging much more widely used than x-rays, ultrasound, nuclear medicine or magnetic resonance imaging (MRI). However, because of elastic scattering of light in tissue, the tissue depths accessible by optical imaging in vivo are fundamentally limited [4-6]. Although fluorescence and bioluminescence have been used extensively to elucidate intracellular signalling in vitro, their use in vivo is largely limited to bulk temporal kinetics, surface tissue imaging [7-9], or invasive/surgical measurements [7]. The inability to reconstruct the spatial origin of a detected photon generally limits non-invasive in vivo luminescent molecular imaging, where high spatial resolution has never been achieved beyond microscopy. Additionally, the decrease in signal intensity with depth into tissue is exponential, creating highly non-linear excitation during imaging, as illustrated in Figure 1(a).

Here, Cherenkov excited luminescence scanned imaging (CELSI) [10, 11] was used to sense luminescent molecular probes deep within tissue, utilizing megavolt (MV) x-ray radiation, and adopting an orthogonal excitation geometry that allows scanning the region of interest, similarly to the way fluorescent light sheet microscopy works [12], as shown in Figure 1(b). As the illuminated area is consistently at a known distance from the detector, this geometry maximises the signal to noise ratio, which tends to be the dominant factor in quantitative imaging of thick and optically turbid samples. Importantly, Cherenkov light of organic molecules is based upon the electronic energy level of the excitation beam. Thus, with high MV x-ray sheet illumination, the spatial targeting of x-rays can be optimally combined with biological organic probes [9] already widely used in animals for other imaging modalities and is translatable to humans. CELSI is intended as an optical imaging tool which can preserve the linearity of the emitted optical signal with depth, and not be overly affected by scatter dominance in tissue. Further illustration of the value of CELSI relative to other imaging modalities can be seen in the Supplementary Data, Figure S.1. The geometry used here is illustrated in Figure 1(c) and temporal and spatial parameters in Figure 1(d).

Cherenkov light is generated in tissue from the scatter-induced secondary electrons produced from x-rays, here generated by a therapeutic MV linear accelerator (LINAC), that interact with the dielectric media. The spectrum of Cherenkov light is broadband, peaks in the ultraviolet (UV), and decays in intensity (I) with a wavelength dependence of $I \sim 1/\lambda^2$, shown in Figure 1(e). Luminescence of the probe is therefore excited only within the volume that is directly in the path of the scanning radiation beam, Figure 1(b), so that all luminescent signals detected by the intensified charge coupled device (ICCD) are considered as originating exclusively from within the pathway of the linear accelerator (LINAC) beam. This geometrical arrangement adopts some of the principles of directed excitation imaging tools, such as ultrasound, where depth-dependent attenuation correction can be applied to the data because of knowledge of the excitation or emission beam depth. The limiting factor remains the intensity attenuation with distance by diffusion scatter and absorption as the emitted light passes through the tissue. The luminescent probe used here Platinum Oxyphor G4 (PtG4) is optimized for oxygen sensing, with a luminescent lifetime in the range of 22-44 microseconds, and an emission wavelength in the near-infrared [13, 14], shown in Figure 1(e) & (f).

Deep tissue Cherenkov excitation from ionizing radiation of isotopes can also excite molecular luminescence [15, 16], with the signal increasing proportionally to the emitted gamma ray energy. Similarly, radiation delivered by a medical LINAC generates Cherenkov emission within the treated tissue [17] and can be visualized for patient dosimetry studies [18], as well as excitation of molecular probes [19, 20]. This process can be utilized to excite fluorescent or phosphorescent probes in tissue to sense diagnostic molecular features, and has been demonstrated at low radiation doses [10], where high energy MV photons have the highest generation of Cherenkov, yet the lowest deposited dose level in tissue. The excitation light from Cherenkov is directed by the incident beam, and so the observation of emission need not be highly localized because the signal is backprojected along the line of the Cherenkov beam, and also enables attenuation correction to be readily applied to the signal, based upon the depth of the beam. Additionally, altering the orthogonal angle of the excitation beam to the collection plane allows some advantages in signal to background improvement, as used in thick tissue fluorescence microscopy [12, 21]. This geometry makes CELSI's approach to signal measurement much more useful for whole body imaging, as it eliminates the need to estimate the depth of the optical signal origin.

The aim of this work was to evaluate the potential for Cherenkov excited luminescence scanned imaging to directly sample partial pressure of oxygen (pO_2) in vivo for mammalian tissue, estimating the spatial resolution, depth and probe concentration sensitivity bounds, and estimate the signal to background values available for realistic tumor imaging applications.

Results

Beam Size: By measuring total luminescence signal and considering prior information about position of the scanning beam, the distribution of optical signal along the direction of scanning can be recovered. In Figure 2(a)-(c), the possible beam geometries are illustrated, where the x-ray sheet can be shaped as a broad beam covering the entire tissue volume, as is commonly used in radiotherapy, or a 2 dimensional sheet of 5mm thickness, or a pencil beam, $5 \times 5 \text{ mm}^2$ which can be raster scanned around the tissue. The benefit of a pencil beam would be the highest spatial resolution, in step sizes of 0.1mm, whereas the benefit of a broad sheet is the fast imaging acquisition. However, the choices here make a large impact on the image quality and signal strength. In Figure 2(d) a broad homogenous phantom was imaged with a large square beam $100 \times 100 \text{ mm}^2$, and then by a thin sheet beam, $5 \times 100 \text{ mm}^2$, and then a pencil beam, $5 \times 5 \text{ mm}^2$, as described in the Methods. The phantom used had an embedded luminescent square $10 \times 10 \text{ mm}^2$, at depth within it, to test for signal recovery, and the contrast to noise ratio (CNR) was plotted in the region of interest (ROI_1) relative to the background (ROI_2), in Figure 2(e), for each of the three cases at different depths of the object.

Imaging Geometry: Radiation beam energies are carefully chosen to reach specific depths in the body, and a set of beams from different gantry angles can be delivered to the patient from these planned entrance positions. In general, arbitrary angles and beam energies and dose values are chosen based upon an optimization algorithm to maximize dose to target regions and minimize dose to organs at risk of radiation damage. It is this flexibility of targeting which makes radiation-based excitation flexible for molecular imaging. The simplest angular geometries of orthogonal or lateral to the subject are shown in Figure 3(a) & (b), where the beam could be roughly orthogonal to the camera direction, for a sheet-like imaging, or aligned along it, for epi-illumination. While the range of possible angles is much more than this, these were chosen as the extremes of signal to background, as will be shown. A complete analysis of the effect of geometry upon singular value index, matrix condition number and matrix rank, for the Jacobian to be inverted, is shown in the Supplementary Information section, Figure S3, using coefficient values listed in Table S.1.

The experimental set up was photographed and is shown in Figure 3(c) and (d), and a single image of a single 1mm diameter capillary filled with $500 \mu\text{M}$ PtG4, at 5mm depth into a tissue phantom solution is shown for each geometry in Figure 3(e) and (f), respectively. The phantom was a broad flat liquid media $200 \times 200 \text{ mm}^2$, with the single luminescent capillary within it, and a scanned sheet beam was used to image with approximately 30 mGy per position of the beam. The color bar for each is the same and so the significantly higher background near 9,600 counts, leads to a contrast ratio of 1.14 and CBR value of 14% for the epi-illumination while the lower background near 2500 counts is seen for lateral illumination, leading to a contrast ratio of 3.5, and CBR of 220%. The higher background comes from the fact that the entire volume being imaged is being excited by Cherenkov, and so the signal comes from a mix of depths below the surface. Because of this observation, the lateral excitation geometry was used throughout most of the next experiments, providing a depth-selective excitation. A detailed analysis of the centroid position error and FWHM of recovered regions is shown in the Supplementary Information, in Figure S4

Dose, Concentration & Depth of Imaging: The dominant factors affecting image recovery were explored systematically by varying one parameter at a time, as shown in Figure 4. The contrast to background ratio (CBR) was used as a metric of success, assessing how this varied with radiation dose delivered, concentration of PtG4 and depth of the object into the medium. The signal to background was defined as the ratio of the target relative to off the target. This figure of merit was chosen because CELSI is a background dominated imaging geometry, as are most luminescent and fluorescent imaging modalities [22]. CELSI of PtG4 in a 1mm diameter capillary was assessed with different concentrations, depths (distance from capillary to phantom surface) and radiation doses. First using a fixed depth of 5mm, the concentrations range was varied logarithmically from $500 \mu\text{M}$ down to $0.78 \mu\text{M}$ inside a 1mm capillary, measuring CBR for a 1 second acquisition time for dose of 0.1 Gy, with the overlapping

excitation volume being $3.9 \times 10^{-6} \text{L}$, making the number of moles used vary from $2.0 \mu\text{mol}$ down to 3 nmol . The results are shown in Figure 4(a), being monotonic in shape and reducing to a CBR of 1 at about 6-8 nmol. Next the CBR was tracked for a fixed concentration of $500 \mu\text{M}$ in the 1mm capillary, for 200 nmol in the excitation volume, varying the depth down to 20mm into the phantom, also using 0.1 Gy dose, as shown in Figure 4(b). This was monotonic with depth and appears to decrease near CBR=1 well beyond the 20mm depth. Finally, the variation with radiation pulses added together was studied at a target depth of 5mm and fixed concentration of $100 \mu\text{M}$, or 2 nmol, showing monotonic dependence on dose in Figure 4(c), and decreasing to CBR of 1 at 2 radiation pulses, roughly equivalent to 1.67 mGy dose.

Scan Directions and Tomographic Reconstruction: The direction and range of scan parameters in CELSI is quite large and three particular geometries were examined as analogous to other tomographic systems, and also to compare to fluorescence tomography systems. First a study of fluorescence tomography as acquired in the epi-illumination geometry was completed, as well as transmission geometry. For this 1% noise was randomly added to the simulated forward data with either a single embedded object or the 3 objects, as shown in Figure 5. The stopping criteria for the iterative reconstruction was when either a change in projection error of less than 0.01% was reached between iterations or at the maximum iteration number of 40 was achieved. A cartesian pixel basis is 30×30 squares was used in Figure 5. These are shown in Figure 5(a) and (b) respectively with the test object shown, and reconstructed images in (d) and (e). The CELSI tomography image with lateral excitation and vertical detection is shown in the schematic (c) and reconstructed object in (f). In this case, CELSI is shown to be far superior to epi-illumination fluorescence tomography and higher contrast than transmission-based fluorescence tomography, and further numerical analysis of the Jacobian matrix improvement is shown in the Supplementary Information section, Figure S3.

Next the range of possible scans available with CELSI was explored on how it would affect image recovery. As shown in Figure 5(g)-(i), the horizontal, vertical and diagonal aspects of a scan can more fully interrogate the image space, and reconstructed images for these three respective geometries are shown in (j)-(l), with recovery from the combination of all of them shown in (m). From these simulations it is obvious that the excitation line directions distort the recovered objects in the axial direction of the source-detector lines. As with all tomography applications, the larger the range of angles sampled, the better the image recovery can become, as shown in (m), better resolving the object in all directions. In this numerical study, only 3 angles were used, because it seemed sufficient to resolve the 3 objects, yet in principle an unlimited number of angles could be used, limited only by the added dose given to the subject. So as in CT imaging, ultimately there is a dose-resolution-contrast tradeoff which dictates the imaging system performance.

Spatial Resolution: Both experiments and simulation studies were completed to assess the ultimate limits to spatial resolution with optimized conditions. The reconstruction set up and convergence criteria were the same as in the previous section, but a larger pixel basis of 100×100 pixels was used to improve quality of reconstructed images in Figure 6 (and Figure S4). Simulation studies were carried out with varying depths and using a standard resolution test, varying the distance between two small objects as illustrated in Figure 6(a). The reconstructed images are shown in with intensity in terms of the reconvened yield, as defined by the PtG4 absorption coefficient multiplied by the quantum yield of emission. The results reveal that when the depth of inclusions was smaller than 5mm, the CELSI tomography has the ability to discriminate the two inclusions with edge-to-edge distance of 0.1 mm, as plotted in Figure 6(b). When the depth of inclusions was increased to 2.5cm, the two inclusions can be also discriminated with edge-to-edge distance but nearer 0.5 mm. When the depth of inclusions was increased to 3 cm, the two inclusions can be also discriminated with edge-to-edge distance but nearer 1.8 mm. The results show that CELSI tomography can yield high spatial resolution, but that this spatial resolution does degrade with depth into the medium, as would be expected. These CELSI results can be compared to Epi-FT and Full-FT results which have significantly lower spatial resolution, ranging from a few millimeters to beyond 10mm at depth of 15 and 25 mm into the medium. Figure 6(b) shows the minimum spatial resolution as a function of depth of inclusions. Epi-FT could not reconstruct the inclusions accurately when the depth of inclusions was deeper than 15 mm, therefore, the spatial resolution was not calculated.

Experimental studies were carried out to try and match the simulations but using a simpler geometry of a single capillary rod, positioned at 5mm into the tissue simulating phantom medium. A range of capillaries with diameters varying from 1mm down to 0.1mm were used with PtG4 at $50 \mu\text{M}$ filled in them. The FWHM recovered

was extracted and the observed FWHM is plotted against the true value in (d), showing nearly perfect linearity and matching the expected value of the smallest spatial resolution tested of 100 microns.

Rodent & Phantom Imaging for Sensitivity & Resolution Testing: In order to verify that imaging in a complex tissue shape would be possible, the XFM-2 phantom was imaged with 1 mM PtG4 within 7 μ L at the tip of the cylinder, for 7 nmol total PtG4, inserted into the central part of the body. For these mouse phantom experiments, 35 radiation sheets were used, and the vertical step of each sheet was approximately 0.52 mm. The 3D forward simulation mesh contained 18496 nodes, 93818 elements and 1712 boundary nodes, and the absorption and reduced scattering coefficients for excitation and emission were both set at $\mu_a=0.007\text{ mm}^{-1}$ & $\mu_s'=1.0\text{ mm}^{-1}$. The reconstruction pixel basis was 25x25x25 voxels. The initial guess of regularization parameter was 10, and the convergence criteria to stop the iterative update was when a change in projection error was less than 0.01% between successive iterations or when the maximum number of iterations was reached at $n=10$. The remitted luminescence image from above is shown in Figure 7(a) with the Cherenkov beam in the plane of the tube, with the luminescence from the tube showing a blurry image, with full width at half maximum (FWHM) of 10.4x11.9 mm in the two orthogonal directions. This signal is super imposed on the white light image of the mouse phantom. Reconstructed images are shown in the 3 orthogonal directions superimposed on CT scan images of the phantom (in grey scale). This recovery showed good localization and recovery of the shape as might be expected, shown in Figure 7(b), with FWHM values of the reconstructed inclusion of 5.1x5.1x4.7mm in total. Note that these dimensions are less than half of the FWHM values of planar projection imaging.

To further advance and test the performance of CELSI reconstruction, an in vivo experiment with MDA-MB-231 tumors in a mouse was performed, with two tumors on the mouse hind limbs. The mouse was injected with 50 nmol of PtG4 into one tumor and 10 nmol into the other tumor, using a 50 μ M stock solution. These doses were specifically chosen to be just above, and just below the detection threshold. Given the sizes of the tumors ($9\times9\times6\text{mm}^3\sim500\text{mm}^3$), these corresponded to approximately 0.4 nM and 0.10 nM, when averaged over the tumor volume. The mouse was imaged within 30 minutes of injection. The other experimental setting was same as the phantom experiment. Figure 7(c) shows the CT scan of the animal, and (d) shows the acquired luminescent Maximum Intensity Projection image overlaid on the colorized CT scan, with this luminescence image showing very blurry localization, with FWHM of 18.9 x 14.8 mm in the two orthogonal directions. The CT images were used to generate a finite element mesh, and the optical properties of tissues in the mouse were estimated by average homogeneous values for a mouse, based upon published values [17].

For these in vivo experiments, the mouse was scanned with a sheet illumination procedure. A total of 19 irradiation sheets were used with a step between them of 0.8 mm. The forward simulation mesh contained 13185 nodes, 63852 elements and 3716 boundary nodes, and the absorption and scattering coefficients for excitation and emission were both set to $\mu_a=0.01\text{ mm}^{-1}$ and $\mu_s'=1.0\text{ mm}^{-1}$. The voxel basis for reconstruction was 25x25x25 pixels, and the initial guess of regularization parameter is 1. The convergence criteria was when the projection error changed by less than 0.01% between successive iterations or the maximum iteration number of $n=10$ was reached. Figure 7(e), (f) and (g) show the CELSI tomography reconstructed results from the 3 orthogonal views of the body interior, with reconstructed CELSI data in red. The FWHM values of the reconstructed CELSI image was $2.2\times2.2\times1.9\text{ mm}^3$. Note that these dimensions were 7-8 times smaller than with planar imaging. Thus, the tumor could be located accurately for the larger injected concentration, and the recovered size was substantially smaller than a with planar diffuse imaging, as is conventional for either epi-fluorescence or Cherenkov-excited luminescence imaging without a scanned source. For the lower concentration tumor, the recovered regions were not visible above the noise level of the reconstructed image, illustrating the detection threshold was between 0.4 and 0.1 nM total agent in the scan volume at these tumor depths. The individual mouse shown is representative of the image quality and recovery possible within this concentration range, with a rotating view of the 3D volume in the *Supplementary Information*, video 1.

Rodent Imaging for pO₂ Sensing: A final murine experiment to map out pO₂ was completed in mice with subcutaneous MDA-MB-231 tumors. A total of 4 animals and 8 tumors were imaged (2 tumors per animal), with local injection of 50uL of 25uM PtG4, a total of 1.25 nmol per tumor. Each mouse was imaged while alive and the repeated at 30 minutes after sacrifice. Temperature was controlled using a heating pad, with alive mice at 35.1 +/- 1.5°C and dead mice at 31.9 +/- 1.2°C. The CELSI scan was completed vertically for these cases, with the video 2 of the experimental data capture included in supplementary data. Images of the luminescence at different delay

times between linac pulse and emission captured are shown in Figure 8(a). These were used for each mouse to create images of lifetime (b) and with the Stern-Volmer equation, the tissue pO_2 (c). The summary of total lifetimes and pO_2 values are shown in (d) and (e), respectively, as box & whisker plots. The entire range of values of alive versus dead do not overlap, indicating a significant difference in both lifetime and pO_2 (p -value < 0.001 for each). But more importantly the range of values is $\pm 30\%$ in lifetime, and $\pm 40\%$ in pO_2 value, as would be expected in heterogeneous tumors.

Discussion

Optical imaging of tissue has the potential for such rich molecular information but image recovery has been plagued by the scattering of light in tissue, making the signal non-linear and affected by tissue shape, optical properties, layers and regions, and often unacceptably low signal to noise with any appreciable depth into the tissue. CELSI is proposed here as a way to directly use high energy radiotherapy beams to launch light directly into the tissue, and by the knowledge of where the light was placed, it can allow high resolution recovery of luminescent sources. While this approach to imaging has been introduced in just the past few years [10, 11], the factors that dictate the performance have not been exhaustively analyzed and non-linear tomography has not been applied to fully vet the imaging contrast recovery and spatial resolution. In this study, these issues have been fully examined and the capabilities of this type of imaging can now be established for pO_2 imaging of tumors. The value of a steered beam to provide localization of the excitation has inherent value to avoid organs at risk, and to multiplex the beam to multiple animals or multiple tissue volumes. The geometrical demonstrations of scanning explored in this study form the very basics of what can be done with a linac which is designed for highly conformal radiotherapy delivery.

The beam shape and angle of orientation have perhaps the largest effects upon the signal, with larger beam shapes having substantially larger signals and resulting contrast to background, as shown in Figure 3(d), presumably based upon the fact that the light scatters and builds up within the tissue. Additionally, there is an output factor for the beam which reduces with smaller beam sizes as well, and so while smaller or thinner beams provide the best spatial resolution, as shown in Figure 3, they inherently provide the lower signal intensities as well. The direction of the beam relative to the camera, has perhaps the second most important factor, as a separation of the source from the surface being imaged inherently improves the observed contrast by suppressing background signals from surface tissues, as illustrated in the images of Figure 4. For these reasons, much of the work following these two initial studies focused around using the lateral beam geometry predominantly, and with a wide sheet of radiation, to maximize build up and light irradiance in the tissue. It is this localization of the excitation beam which provides the inherent value of CELSI relative to isotope-based Cherenkov excitation studies[16, 23, 24]. In isotope-based molecular sensing, the sensitivity can be as high, or even higher, down to the sub-nanomole range, but the ability to reconstruction and localize the emission is not dictated by anything other than the detected emission. In CELSI imaging, the high precision beam knowledge, allows both deconvolution and depth-dependent attenuation correction, leaving a reconstructed signal which is highly localized in space to better than 1mm accuracy throughout the imaged volume of tissue.

Testing the key performance factors beyond these initial choices leads to a complex array of possible parameters. The radiotherapy dose, the probe concentration and the depth into the tissue each contribute to the detected signal in ways which would have a reciprocity, as illustrated in Figure 4(d). The imaging of luminescent targets is possible within the range of concentrations, doses and depths which are suitable for small animal imaging. The molecule number ranges near a μ Molar are typical for metabolites and higher concentration receptors, and the ability to resolve uptake within many millimeters of tissue is a very important application in pre-clinical imaging. Within the volume of excitation, this corresponds to sensitivity needed near μ mol to nmol levels. Radiation doses used here have been in the range of x-ray exams, so while the concept of using a linear accelerator for imaging appears dangerous from a radiation safety perspective, it is critical to appreciate that this is done with very little dose delivered. Additionally, the radiation dose is selectively deposited to only where the scanned beam passes, so radiosensitive organs or dose limiting volumes could be strategically avoided in an optimized scan.

Perhaps the key observation in this work has been establishing the feasible spatial resolution several millimeters into the animal tissue for molecular sensing. This spatial resolution is largely dictated by the precision in which the x-ray beam can be controlled, because the signal origin can be localized to where the beam excited

the luminescent agent. The imaging tests, both experimentally and computationally, support the belief that the spatial resolution is in the range of 100-300 microns for objects as deep as 5mm into the tissue. Computationally this spatial resolution appears to be resolvable down to 20mm deep, and this fundamental limit is a fascinating goal for deep tissue imaging, essentially being able to achieve near microscopic resolution deep into tissue with optical imaging. This realization is a fundamental breakthrough in molecular sensing in deep tissue.

Finally, the tests used with mouse phantoms and mouse tumors, both demonstrate the localization concepts and how the accuracy of reconstruction is superior to diffuse tomography or superficial MIP images of luminescence. This latter comparison to superficial imaging is important, because the vast majority of pre-clinical luminescent imaging is done with epi-illumination box systems where a single superficial surface image of the animal is achieved. Being able to bring optical imaging to a significantly higher whole body spatial resolution would have a profound impact for molecular imaging. The mouse phantom tested imaging in the geometry, size and average tissue optical property values of a mouse, showing excellent reconstruction of the embedded region. Then imaging in the heterogeneous tissues of a mouse is necessary since there are not realistic internal heterogeneities in the phantom. The more detailed CELSI images of tumor oxygenation as shown in Figure 9 illustrate how this methodology can be used to sense tumor pO_2 , and potentially do this with very high spatial resolution. The features mapped out in this figure have sub-millimeter lateral spatial resolution, showing the heterogeneity of the pO_2 ranging from 40-90 mmHg, as is known to be present in these tumors. The heterogeneity seen within regions and between the tumors on the left and right presumably show areas of high and low pO_2 variation. Areas that are high are presumably near major blood vessels and those with lower pO_2 near areas distal from these, as would be expected, and tumors with overall higher values are usually more well perfused than those that tend to be hypoxic. The ability to map pO_2 , pH and other features such as enzyme concentrations, protein expression, cell receptor density or metabolites would be valuable to understand responses to therapy. This methodology will be most important for higher spatial resolution molecular features, where perhaps the tumor heterogeneity is a governing factor in response.

In molecular imaging, there can be a separation between the imaging system's physical capabilities and the pharmacokinetics and localization of the biochemical targeting moiety. Yet, the value of a system is defined by the intersection of these two properties, where the physical resolution and contrast of the biochemical agent gets localized in biological features of the tissue. In the current realization of CELSI, the delivery was directly into solid tumors, avoiding the issue of plasma pharmacokinetics and tumor retention. As such, the chosen focus here has been largely technological development, combined with localized delivery of the agent to tumors. While there are clear limitations to this, the approach does still match with radiotherapy delivery as well, where the location of delivery is well planned as targeted to the treatment volume. As such, while the compound has its limitation at the site of injection, the knowledge of the oxygenation of these radiotherapy target sites is still of considerable value to radiation sensitization. Thus, we present the application of localized oxygen sensing with high resolution CELSI in this format with clear potential to be further advanced by improvements in the biotargeting of luminescent reporters. Recent studies have examined multiple agents for Cherenkov-excited sensitivity with fluorescence [14, 20, 25], and for the capability of detecting multiple emitters at the same time [26]. Yet in all of these studies, PtG4 has been shown to be the most sensitive probe in terms of lower limit on concentration. Also, with its design for oxygen sensitivity from quenching which is accessed by measurement of emission lifetime, it also provides one of the most practical tools for radiation therapy, where tissue oxygenation is known to be an important factor in therapy efficacy. However, there is also potential for other lifetime phosphor sensing agents such as pH [27, 28]

Outlook

Cherenkov Excited Luminescence Scanned Imaging (CELSI) allows pO_2 imaging at a spatial resolution possible near 0.1mm through an intact living mouse, while utilizing nanomole levels of an oxygen sensitive biological probe in several mm of tissue. Cherenkov imaging initiated by x-ray beams from a linear accelerator can allow shaped thin sheets scanning in arbitrary directions, and by time-gating of the captured luminescence this process can allow for molecular sampling of a number of agents relevant to tissue microenvironment. The depths and recovery possible are suitable for small animal imaging studies, and results from both computer simulations and experimental studies suggest that when fully automated, this type of imaging will have one of highest sensitivities and spatial resolutions for any pre-clinical whole body molecular imaging system.

Methods

LINAC & Cherenkov Imaging: Cherenkov was induced by a linear accelerator (Varian Linac 2100CD, Varian Medical System, Palo Alto, CA) based at the Norris Cotton Cancer Center, at Dartmouth-Hitchcock Medical Center. The LINAC multi-leaf collimator (MLC) provided high flexibility in shaping the output beam to make arbitrary shaped treatment fields. Leaves are 5mm wide in this LINAC and oriented in many long thin sheets that are controlled to move in and out of the output beam and can be controlled to a level of 0.1mm precision. The effect of beam shape on the signal to background recovered acquisition was studied with radiation beam sizes using a larger square beam $100 \times 100 \text{ mm}^2$, a thin sheet beam of $5 \times 100 \text{ mm}^2$, and a thin square pencil beam $5 \times 5 \text{ mm}^2$ with a vial of PtG4 of $5 \text{ }\mu\text{M}$ in the phantom. CELSI of capillary containing PtG4 $500 \text{ }\mu\text{M}$ was studied with radiation beam sizes of $40 \times 40 \text{ mm}^2$ (epi-illumination) and $5 \times 40 \text{ mm}^2$ (lateral illumination). Background was acquired with acquisition $500 \text{ }\mu\text{s}$ trigger delay. Each of these were studied for epi-illumination imaging and then the thin sheet beam was also used for lateral sheet excitation, as will be shown, for optimal contrast. To maximize signal and minimize background interference, the room lights were shut off throughout these studies, and all lights in the room were masked off with black cloth and black tape, as shown in the photographs.

Luminescent Reporter & Tissue Phantoms: NIR fluorescent phosphorescent probe PtG4 Oxyphor [27] was prepared in standard $1 \times \text{PBS}$ at $500 \text{ }\mu\text{M}$ concentration. This reporter has a strong oxygen sensitivity as described in previous work. [13, 14], as well as emission in the near infrared wavelengths, and excitation across the visible spectrum, matching Cherenkov excitation. Previous studies have examined the comparison of luminescence from phosphors and fluorophores from Cherenkov excitation, and in all studies, time-gated luminescence provides superior signal to noise, because of the superior background suppression provided by time-gating technology [25, 29, 30].

The tissue simulating phantoms were prepared using 1% Intralipid© (Fresenius Kabi, Uppsala, Sweden). A capillary with 1 mm diameter of the probe at concentrations from 0.75 to $500 \text{ }\mu\text{M}$ were placed inside the phantom at a depth which was varied between 2.5 to 20 mm from phantom surface being imaged. To study the dose efficacy for CELSI, the capillary containing the solution of the probe was irradiated with doses which varied from 360 pulses (approx. 5 Gy) down to 1 pulse (approx. 1.4 cGy) at $500 \text{ }\mu\text{M}$ concentration and 5 mm depth.

Luminescence Imaging: The imaging system consisted of a time-gated intensified CCD camera (ICCD, PI-MAX4 1024i, Princeton Instruments, USA), a commercial lens (Canon EF 135mm f/2L USM), and a tripod mounted. The camera was focused on the imaging field approximately 1 meter away. This distance was optimized through earlier studies where the signal to noise was found optimal, by moving back from the field, to avoid too much backscatter generated noise. The time-gated ICCD camera was synchronized to the radiation pulses (approximately $3.25 \text{ }\mu\text{s}$ duration, 360 Hz repetition rate) with the intensifier set as $\times 100$ and turned on at a $3.5 \text{ }\mu\text{s}$ or $500 \text{ }\mu\text{s}$ gate delay following each radiation pulse for phosphorescence or background measurement, and luminescence generated during $50 \mu\text{s}$ gate width was integrated via this ICCD. The display of images in emission were originally captured in counts per pixel, as read from the camera, and this has been calibrated to photons/ mm^2/s by calibration of the number of photons detected per count, which was previously studied and found to be 95 counts read out at the CCD per one photon incident upon the ICCD photocathode. Similarly, the camera has $13 \times 13 \text{ }\mu\text{m}^2$ pixels, and so this was converted from pixels to a mm^2 units.

Initial experiments focused on examining the signal to background issue, using a single 1mm inner diameter glass capillary tube (Hampton Research Corp, Glass number 50 capillaries, Aliso Viejo CA), filled with 500 microMolar PtG4, and imaging with the linac vs camera in two different geometries, as shown in Figure 3. In the vertical scan direction (also sometimes called epi-illumination geometry), where the linac and camera have the same perspective, higher background would be expected due to the high Cherenkov at the surface of the phantom, shown in Figure 3(c). Whereas when the linac beam was orthogonal to the camera direction, then Cherenkov is not produced at the surface, and background would expect to be lower as viewed by the camera, shown in Figure 3(d). These studies were carried out to experimentally verify this with a single capillary image.

Factors Affecting Contrast: Dose, Concentration, Depth: The signal contrast in all imaging systems is a function of key parameters and so for CELSI it was hypothesized that these key factors would be linearly related to the radiation dose used, the concentration of probe present in the tissue, and the logarithm of the depth in which the

target was located. The depth dependence on signal is likely more complex because of the light diffusion process, but might be expected to fall off over macroscopic distances with the effective attenuation coefficient of the medium, estimated by diffusion theory to be $\mu_{\text{eff}} = (3\mu_a\mu_s')^{1/2}$, where μ_a is the absorption coefficient and μ_s' is the transport scattering coefficient of the tissue. The dose delivered was varied by simple linear summation of the signal over more LINAC pulses, to effectively build up signal with delivered dose. The concentration of PtG4 was varied in solution within the embedded object, from 500 μM down to 0.78 μM using a 1mm capillary present at 5mm depth into the medium. The excitation volume of this was given by the overlap of the 5mm beam with the 1mm capillary, which was $V = \pi r^2 h = 3.9 \times 10^{-6} \text{ L}$.

The signal to background ratio was used as a metric of recovered accuracy in this study, with the simple calculation defined by the ratio of the average intensity values:

$$\text{Signal to Background} = \frac{\langle I_{\text{ROI}} \rangle}{\langle I_{\text{B}} \rangle}$$

Where I_{ROI} is the mean pixel intensity in the region of interest (ROI) and the I_{B} is the mean pixel intensity in the background, outside the ROI. In some studies, the contrast to noise ratio was used as a metric, which was defined as:

$$\text{CNR} = \frac{\langle I_{\text{ROI}} \rangle - \langle I_{\text{B}} \rangle}{\sigma_{\text{B}}}$$

Where σ_{B} was defined as the standard deviation of intensities in the background region.

Spatial Resolution Assessment: The spatial resolution of imaging was assessed with a range of glass capillary tubes of varying inner diameter, from 0.1mm up to 1.0mm (Hampton Research Corp, Glass number 50 capillaries, Aliso Viejo CA). These tubes were placed into tissue simulating phantom at 5mm depth one by one, and all were filled with 50 μM PtG4 to provide a high contrast target for resolution assessment. The sheet radiation beam was scanned laterally across the tube, to vary the signal with position, and the linescan full width at half maximum (FWHM) was recovered for each capillary experiment.

Computed Tomography Recovery: CELSI data can be scanned for different beam angles and the performance expected varies considerably based upon these choices. The range of options is so large that experimental study of these is prohibitive, and so a series of computer simulations were carried out, in particular to compare CELSI images to diffuse fluorescence tomography images where traditional light excitation is used as compared to Cherenkov excitation. Additionally, different combinations of scanning angles and different depths and sizes of objects could be simulated much more quickly than carrying out all the experiments. The computer simulations were carried out in the NIRFAST software package (available at www.nirfast.org), used to model light transport in tissue, and carry out regularized iterative reconstruction of the luminescent reporter in the medium. The recovery of the reporter was in terms of units of luminescent yield, $\eta\mu_{\text{af}}$, where η is the reporter quantum yield for phosphorescence emission and μ_{af} is the absorption coefficient of the reporter (see Supplementary Information for further explanation of the theory). This quantity is a direct report of the amount of light produced per unit volume. To assess spatial resolution of CELSI tomography, a classic resolution test was performed with two small inclusions and varying the spatial distance between them and assessing when they could not be resolved as separate objects. The edge-to-edge distance was varied from 0.1 mm to 5 mm, in the plausible range of limiting spatial resolutions given the physical constraints of the LINAC MLCs used. The yield contrast of inclusion was set as 10:1. A finer mesh with 6161 nodes and 12000 linear triangular elements was used in reconstruction. Inspired by the results of combining of scan modes, the following reconstructions were performed by summing up scan modes.

Animal Phantom Imaging: In order to assess performance in a more complex tissue geometry, a commercially available mouse phantom was used (Xfm-2, PerkinElmer Health Sciences, Waltham MA), using rod of material inserted which was filled with 1 mM PtG4, in 7 μL (7 nmol). This phantom has tissue optical properties within the NIR wavelengths 650-800nm, approximated by $\mu_a=0.003\text{-}0.008 \text{ mm}^{-1}$ and $\mu_s'=1.5\text{-}0.9 \text{ mm}^{-1}$ (manufacturer supplied data sheet). This phantom was crated for phantom studies which would allow imaging in a realistic tissue volume

and geometry that directly mimics the shape and absorption and reduced scattering coefficient values representative of murine tissue. The CELSI imaging was carried out with lateral beam excitation and vertically oriented emission capture, using a long lifetime gate on the camera, to sync out the Cherenkov light and noise, and maximize luminescence emission detection. X-ray tomography was completed on the IVIS Spectrum CT (PerkinElmer Inc, Hopkington MA) with the full field of view 120x120x30mm³, with a 150 μ m voxel scan acquired in 90 seconds.

Tumor Cell Lines: MDA-MB-231 cells were purchased directly from ATCC as a confirmed cell type and mycoplasma free, no further testing was performed, and the cells lines are not listed in the ICLAC database of cross-contaminated or misidentified cell lines. Cells were grown in culture media in an incubator at 37°C in DMEM with 10% (v/v) Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. When ready for use, cells were trypsonized, counted, spun down into a slurry, and used for inoculation into animals.

Animal Studies: All animal procedures were approved by the Dartmouth Institutional Animal Care and Use Committee, and the studies here were carried out in compliance with these approved procedures. Nude female mice were purchased at 6 weeks of age from Charles River Labs. After a week of acclimatization, animals were used for injections of 10⁵ cells under the skin on each flank of the mouse in individual 0.050 mL injections. After approximately 3 weeks of growth, when a tumor with an average of 6mm diameter tumor was observed on both flanks, then the animals were used for imaging studies. All mice were under general anesthesia of inhaled isoflurane at 1.5% in flowing air through a nose cone throughout imaging. For the tomography study, to demonstrate the detection level, a total of 0.5ml of PtG4 was directly injected into the tumors of a mouse, with one concentration of 50 nmol into one tumor and 10 nmol into the other tumor. This animal was imaged by 3D CELSI tomography at 30 minutes after injection, and then again imaged in the IVIS Spectrum CT system for x-ray tomography. The x-ray scan utilized 50 kVp standard 150 micron resolution imaging of the mouse in a 12x12x3cm³ field size. In further pO₂ imaging studies, a cohort of 4 mice were used with a tumor in each flank (2 tumors per mouse, for n=8 tumors) and used for imaging when the average dimension diameter was 6mm as measured over the two orthogonal axes.

Statistical Analysis: The differences between the alive and dead conditions, as displayed in Figure 8, with n=8 paired samples each was established by a two tailed students t-test, with alpha=0.05, and resulting p-value < 0.001. These indicate that both in lifetime as well as pO₂, there are significant differences between the values of the animals in the two conditions. However more pertinent to the value of the study is the observation of the total range of values, presented as a % of the median value. The figures 8(d) & (e) were generated using Python 3.4.3 with the library matplotlib 2.0.0. The function used to make the box plot is called boxplot available at this link: (https://matplotlib.org/api/_as_gen/matplotlib.pyplot.boxplot.html). The box extends from the lower to the upper quartile values of the data, with a line at the median. The whiskers extend from the box to show the range of the data. Flier points are those past the end of the whiskers.

Data Availability: Datasets generated and analyzed during the current study are available from the corresponding author upon email request. All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Code Availability: Image reconstruction code was custom developed for this study, and is available for download in bulk through our website www.nirfast.org, and individual MATLAB routines are available from the corresponding author.

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Figure Captions

Figure 1. Comparison of the excitation fluence loss with depth into tissue. **A**, laser/LED excitation. **B**, Cherenkov excitation generated within the tissue from a LINAC radiation beam. **C**, diagram of the experimental setup with measurement devices. **D**, a raster scan approach to image measured by the ICCD shown with gated emission detected by a spectrometer system. **E**, the absorption spectra for PtG4, and, **F**, the luminescence emission spectra for PtG4, both overlaid with the Cherenkov spectrum (blue) from single solution scans. The structure of PtG4 is illustrated in the Supplementary Information, Figure S2.

Figure 2. The configuration of the radiation beam shape and region of the tissue where Cherenkov would be generated. **a**, single broad beam throughout the 3D volume of the animal. **b**, in a 2D sheet beam, or **c**, in a 1D pencil beam. These geometric choices each affect the signal to noise possible with CELSI, and hence the sensitivity of other parameters such as minimum concentration and depth of imaging feasible and acquisition time. Images of the field light, Cherenkov emission on a phantom and the luminescence from a single square target in the phantom are shown, **d**, for the geometries in **a-c**, with duplicate images taken in separate imaging sessions showing nearly identical image quality. **e**, the contrast to noise ratio (CNR) of the target relative to the background as measured by individual scans, where each data point represents an individual acquisition. The units for the color bars on images in **d** and inset in **e** are in detected photons/cm²/s and a 1cm scale bar is in the bottom right corner of each panel.

Figure 3. The geometry of imaging camera relative to X-ray beam entrance position. **a**, a vertical (or epi-illumination) scan. **b**, a lateral sheet scan. **c** and **d**, photographs of the LINAC-camera set up for vertical and orthogonal camera and LINAC beam, similar to schematics **a** and **b**. **e** and **f**, show single frame images of PtG4 luminescence from one capillary within a broad tissue phantom for the two geometries, respectively, with color bars on the same scale for the two representing the full range available on the camera. These representative single images illustrate the high background and hence poorer contrast from the epi-illumination geometry, as seen from an individual snapshot. The signal to background values are 15% for **e** and 220% for **f** above the background level. The units for the color bars on images in **e** and **f** are in detected photons/cm²/s and 5mm scale bar is shown in the bottom right of each panel.

Figure 4. Signal to background ratio measurements from PtG4 contained within a 1mm capillary. **a**, different concentrations in the physiologically relevant range. **b**, varying depth between the capillary wall to the surface of the phantom being imaged. **c** varying radiation dose between 1 and 500 radiation pulses, with 1.67mGy/pulse. In each graph, the line scan raw data is inset. Each individual data point represents extraction of signal to background for a single scan of a test object, with varying conditions as shown in the x-axes. The red lines in each graph illustrate the linear trendline of the data on the log-log plots. **d**, the factors affecting CELSI signal strength are schematically illustrated as being reciprocal in their effect upon signal to background, illustrated by the red lines of constant value.

Figure 5. Source and detector placement effects upon reconstructed CELSI images. **a**, the Epi-fluorescence tomography (Epi-FT). **b**, the Full-FT geometry, **c**, the lateral excitation CELSI with tomographic reconstruction. The blue arrows represent the source locations, and the red arrows denote the detector locations. Reconstructed luminescence distributions corresponding to the three geometries shown in Fig 2a-c, for (d) the Epi-FT, (e) the Full-FT, and (f) the CELSI. Three angles of scanning for CELSI were examined as illustrated in (g) – (i), for horizontal, vertical and diagonal, respectively, and three test objects at different depths were used as a test field for this, with reconstructions as shown in (j) – (l). The combined set of all 3 scan geometries was used for the reconstructed image (m), showing the best preservation of the three objects space and intensities with 30 iterations in each reconstruction. These simulations were carried out once for each in individual graph shown.

Figure 6. The theoretical limits to spatial resolution in reconstructed CELSI images, shown in units of luminescent yield, $\eta_{\mu\text{at}}$, in the colorbar, for two inclusions with varied edge-to-edge distance in different depths are shown. **a**, the white scale bar in the bottom right is 30 mm. These simulations were carried out once for each location of the pair of 5mm diameter, 50 μM PtG4, inclusions. **b**, at each depth, the minimum resolvable distance was estimated for epi-illumination fluorescence tomography, diffuse fluorescence tomography, and CELSI tomography. **c**, capillaries filled with PtG4 varying from 0.1mm diameter up to 1.0 mm diameter, and were

embedded into a tissue equivalent phantom. **d**, a sheet beam scanned laterally to extract the FWHM for each sized tube. Each data point reports a single measurement, and fitting was completed with $r^2 > 0.9$ for each sample.

Figure 7. Phantom & animal tumor tomography experiments to show luminescence yield images. **a**, the luminescent yield images of a single phantom are overlaid on the top surface of the microCT image, in colorbar units of photons/cm²/s, and the reconstructed images shown with 3 orthogonal views, axial (b), sagittal (c) and transverse (d) with the colorbar reporting values for (b)-(d) in units of $\eta\mu_{af}$. The in vivo imaging study is shown from a single animal, with an x-ray CT scan and a summed intensity projection image of the luminescence overlaid on the color-coded CT scan (e) in colorbar units of photons/cm²/s. Then CELSI tomographic data is overlaid on the high resolution microCT images for axial (f), sagittal (g) and 3D perspective (g) views of the reconstruction. The location of the two MDA-MB-231 tumors grown on the hind flanks are shown by arrows, with 50 nmol PtG4 injected into the tumor with a blue arrow, and 10 nmol PtG4 was injected into the tumor with the orange arrow, and the entire body of the animal was scanned and reconstructed with CELSI. The reconstructed image shows recovery of the high concentration injection while the lower concentration injection is not recovered, to illustrate the detectable limit is in the range of tens of nanomoles. (A full rotating 3D video of this mouse is available in the Supplementary Information, Section S.7)

Figure 8. Imaging of pO₂ in subcutaneous breast adenocarcinomas. **a**, maximum intensity projection (MIP) images of CELSI are shown with increasing delays, for nude mice with MDA-MB-231 flank tumors injected with 50uL of 25uM PtG4 in each tumor, with color bar in photons/cm²/s. **b**, the pixel-wise lifetimes (τ) estimated alive, and after sacrifice as a low pO₂ control. **c**, calculated pO₂ maps via the Stern-Volmer relationship. **d**, box-plots show lifetime estimates for all tumors (n=8). **e**, the calculated pO₂ estimates for these (n=8). Measurement scans were acquired once for each animal. (A video of a single scan sequence is available in the Supplementary Information, Section S.7).

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