AWARD NUMBER: W81XWH-20-1-0101

TITLE: Can Altered Bioenergetics Drive Antibiotic Persistence in Low-Oxygen Cond

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CONTRACTING ORGANIZATION: University of California, Santa Barbara

REPORT DATE: MARCH 2022

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
MARCH 2022	ANNUAL	03/01/2021 - 02/28/2022

4. TITLE AND SUBTITLE

Can Altered Bioenergetics Drive Antibiotic Persistence in Low-Oxygen Conditions?

5a. CONTRACT NUMBER
W81XWH-20-1-0101

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this project is to engineer genetically encoded fluorescent biosensors, which can be imaged in low-oxygen (or fully anaerobic) conditions; and apply these sensors to examine how ATP levels differ in aerobic vs. low-oxygen conditions for cells treated with antibiotics. The current toolbox of genetically encoded sensors requires oxygen to produce fluorescence. To address this issue, we will develop our sensors based on flavin-binding fluorescent proteins, which do not require oxygen to produce light. Our work will therefore establish a new sensing technique for basic research in anaerobic living systems and potentially yield new insights on how ATP correlates with antibiotic action in model bacteria.

15. SUBJECT TERMS

Fluorescent reporters, genetically encoded ATP sensors, flavin-binding fluorescent proteins, anaerobic imaging, anaerobic microorganisms, antibiotic response, persister

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	11	**19b. TELEPHONE NUMBER** *(include area code)*

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1. INTRODUCTION

The goal of this project is to engineer genetically encoded fluorescent biosensors, which can be imaged in low-oxygen (or fully anaerobic) conditions; and apply these sensors to examine how ATP levels differ in aerobic *vs*. low-oxygen conditions for cells treated with antibiotics. The current toolbox of genetically encoded sensors requires oxygen to produce fluorescence. To address this issue, we will develop our sensors based on flavin-binding fluorescent proteins, which do not require oxygen to produce light. Our work will therefore establish a new sensing technique for basic research in anaerobic living systems and potentially yield new insights on how ATP correlates with antibiotic action in model bacteria.

2. KEYWORDS

Reporter genes, genetically encoded fluorescent sensors, flavin-binding fluorescent proteins, anaerobic living systems, antibiotic drugs, proteases, persister cells

3. ACCOMPLISHMENTS

What were the major goals of the project?

Aim 1: Engineer an oxygen-independent, genetically encoded ATP sensor
- Major Task 1: Engineer a 4-HT-inducible allosteric fluorescent sensor based on AFP
- Major Task 2: Engineer an ATP-inducible fluorescent reporter based on the AFP- ERLBD fusion
- Major Task 3: Use AnViAS to detect ATP in bacteria treated with various chemical and environmental modulators of metabolism

Aim 2: Image/quantify ATP distribution in antibiotic-treated bacteria.
- Major Task 4: Optimize time lapse fluorescence imaging for single cell studies
- Major Task 5: Time lapse fluorescence imaging of bacterial bioenergetics and persistence in different oxygen levels

What was accomplished under these goals?

In this project period, we made several significant advances towards completing Major Tasks 1-3 in Aim 1 and established methods to begin work on Aim 2. Building on the engineering groundwork developed in the last period, our primary goal here was to establish a broadly applicable approach for constructing fluorescent sensors based on iLOV, a moderately bright oxygen-independent fluorescent reporter. These sensors will be initially used to image cellular energetics but will be expandable to other targets. Unlike creating sensors from the green fluorescent protein (GFP), there is limited knowledge on the engineerability of iLOV – for example if iLOV can accommodate insertions; and whether iLOV may be split, circularly permuted or shortened by truncations. *Addressing this technological gap would pave the way for all future sensor engineering efforts based on iLOV*. Accordingly, in this project period, we made key advances towards this goal by systematically testing various engineering strategies keeping in mind the end-goal being of developing sensors with an optimal combination of both large analyte-driven fluorescence change and peak fluorescence. The key outcomes are briefly summarized below and detailed in later sections.

(1) Our initial search for allosteric hotspots in the iLOV backbone identified the region between residues Gly95 and Glu96 as the most viable point for engineering sensors by insertion of analyte binding-domains. This finding was further supported by analysis of the iLOV crystal

structure. Insertion of an ATP-binding domain at this location allowed us to construct a preliminary ATP sensor with ~ 15% ATP-induced change fluorescence.

(2) We engineered a circularly permuted variant of iLOV by connecting the original N and C terminals; and introducing new ends at the Glu96 and Gly95 positions identified above. This variant will serve as a new template for constructing additional iLOV-based ATP sensors by directly inserting the permuted reporter within the backbone of the ATP-binding domain.

(3) We developed a truncated version of iLOV by systematically identifying and deleting non-essential amino acids. This modified reporter has a smaller footprint compared to full-length iLOV and provides a new avenue for simplifying our sensor engineering efforts.

(4) In parallel with the above efforts, we are exploring a new approach for imaging cellular energetics on the basis of detection of protease activity. Given that many proteases depend on ATP for their activity, iLOV-based protease sensors could provide an alternative avenue for monitoring ATP fluctuations with high sensitivity.

In the following sections, we provide a detailed description of major research activities, key scientific findings, and significant results pertaining to this project period.

Towards engineering anaerobically-visible biosensors based on iLOV

Towards sensor engineering by domain insertion. Previously, we engineered 108 constructs consisting of the estrogen receptor ligand binding domain (in short, ER) inserted at every position in the iLOV backbone. The ER domain was chosen to explore the effect of insertions within iLOV on the basis of this domain's ability to convert from an open to a closed state upon binding a cell-permeable chemical, 4-hydroxytamoxifen (4-HT). We cloned all 108 constructs in *E. coli* and measured fluorescence in the presence and absence of 4-HT. The majority of insertions (89/108) lead to a sharp decrease in fluorescence relative to native iLOV, making these sites inviable for sensor construction. While a handful of inserts (5/108) exhibited moderate fluorescence in the range of 4-7 % of native iLOV emission, the high variability in fluorescence (coefficient of variation > 10%) precluded their use for further engineering. Only 2 constructs, involving ER insertions between Lys94 & Gly95 and Gly96 & Glu96, showed a desirable combination of fluorescence (~5% of native iLOV) and low variability. These residues are located in the Asp-Gln-Lys-Gly-Asp loop, which also shows high crystallographic B-values and low conservation score relative to other flavin-binding fluorescent proteins. Taken together, the Gly95 position was deemed to be the most attractive site in iLOV for sensor engineering. **This represents a major finding, reminiscent of the discovery (largely by serendipity) of the N144-Y145 region in GFP, which served as a "hot-spot" for creating a vast repertoire of GFP-based sensors by circular permutation and split reporter technology**.

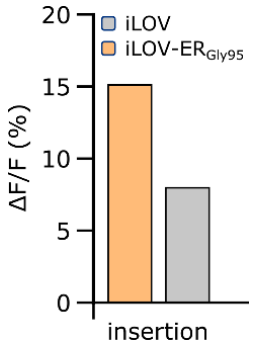

Figure 1. Initial ATP sensor formed by inserting an ATP binding subunit between Gly85 & Glu96 in iLOV

We next explored the possibility of creating an ATP sensor by inserting the ATP-ε subunit from *B. subtilis* immediately after residue Gly95 in full-length iLOV. The initial construct was able to detect cyanide-induced ATP depletion with a ~15.1 % change in fluorescence (-ΔF/F), which exceeds the signal change obtained with wild type iLOV (-ΔF/F ~ 8%) by approximately 2-fold (**Fig. 1**). However, the peak fluorescence was only 2.77 % that of wild type iLOV, which we propose to optimize by linker mutagenesis.

Towards sensor engineering by circular permutation. Based on the above findings, we reasoned that Gly95 could serve as a viable site for circularly permutating iLOV by joining the original termini and creating new N′ and C′ ends at Glu96 and Gly95 respectively. We engineered 3 circularly permuted iLOV constructs by varying the length of the linker joining the original termini (**Fig. 2A**). We also formed 3 additional constructs in the same fashion with miniSOG, a moderately brighter variant of iLOV (**Fig. 2B**). We cloned these reporter candidates in *E. coli* and measured whole cell fluorescence. The brightest variant (henceforth referred to as cpiLOV) showed a 42.3 ± 0.6 % (*N = 3, p = 0.005, 2-sided t-test*) increase in fluorescence relative to untransformed *E. coli* cells and ~10-fold increase in transiently transfected model cell lines relative to untransfected cells (**Fig. 2C**). **The discovery of cpiLOV is a significant development as it provides us with a new template for sensor engineering, akin to how the development of cpGFP enabled the creation of a breadth of molecular sensors (including ATP indicators) by leveraging protein allostery**.

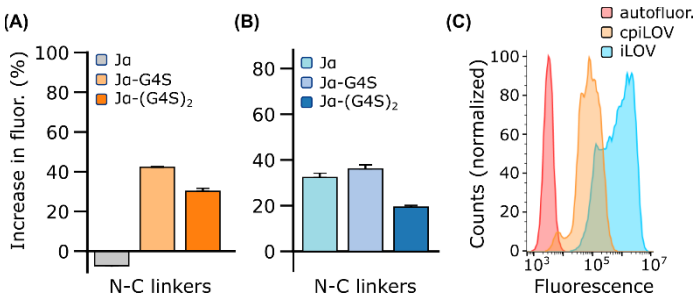

Figure 2. Development of circularly permuted anaerobic reporters by protein engineering. Circular permutation and testing of (A) iLOV and (B) a brighter variant called miniSOG. The best-performing variant connects the original N and C ends with a Jα-(G4S)2 linker and produces and 42% fluorescence increase in *E. coli* and (C) ~ 10-fold increase in model cell lines.

Towards sensor optimization by protein truncation. Reporter proteins with a small size are desirable because of their reduced footprint, which simplifies sensor engineering and grafting of ligand-binding domains. To this end, we explored the possibility of engineering a shorter iLOV variant (henceforth, nanoLOV) by systematically removing amino acids from the N and C ends (**Fig. 3A-B**), editing internal loops (**Fig. 3C**), a helix with high crystallographic B-values (**Fig. 3E**), and deleting glycine and proline residues (**Fig. 3D**). After screening 23 constructs, we identified 3 avenues to reduce the size of iLOV – deletion of Gly95 and removal of 5 amino acids each at each end. Taken together, the resulting mutations will make nanoLOV the only fluorescent reporter (to our knowledge) under 100 amino acids, thereby providing a more malleable template for sensor engineering.

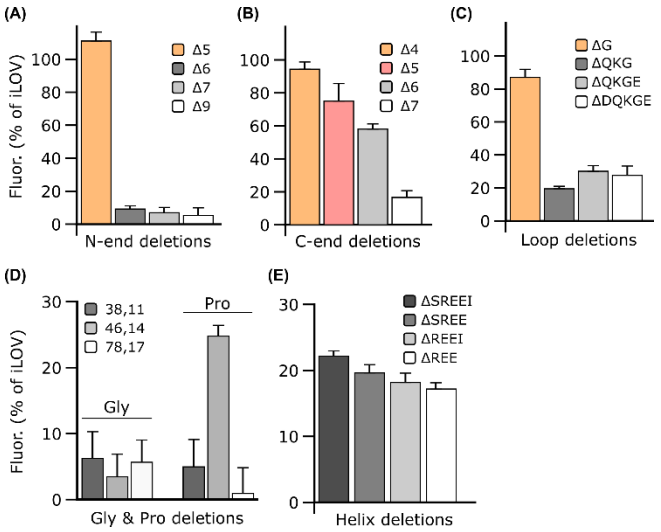

Figure 3. Development of shorter iLOV reporters to enable sensor engineering. To reduce the size of iLOV, we explore (A) N-terminal truncations (B) C-terminal truncations (C) deletions in the DQKGE loop region (D) single deletions of several Gly and Pro residues and (E) deletion of regions in a high B-value helical region, SREEI.

Towards sensor engineering based on protease activity. As an alternative approach for engineering ATP sensors, we are developing methods to connect iLOV fluorescence with

protease activity. Many intracellular proteases are dependent on ATP for their activity, for example, ClpXP, ClpAP, and Lon protease, thereby allowing protease sensing to serve as a proxy for energetic status of a cell. We recently found that attaching the heavy chain of the eukaryotic iron storage protein, ferritin (FtH), at the N terminus of iLOV leads to > 50 % drop in fluorescence, likely due to interference with protein folding (**Fig. 4**). Based on this, we reasoned that removal of ferritin by protease cleavage should allow for improved iLOV expression and fluorescence. Our first approach will therefore focus on fusing FtH to iLOV, separated by a protease cleavage site. As a second approach, we will leverage the circular permutation mechanism, covalently joining the original N and C ends of iLOV with rigid linker and by bringing the modified N′ (Glu96) and C′ (Gly95) ends in proximity by fusing dimerizing peptides to their termini (**Table 1**). We have found that one such construct leads to a near-complete quenching of iLOV fluorescence. To increase fluorescence, we will introduce protease cleavage sequences either in the N-C linker region or between the junctions of the dimerizing domains and the modified N′ and C′ ends. In this way, we propose to build four permuted constructs (**Table 1**), test them for fluorescence turn-on in the presence of a model protease (known as TEVp), and eventually move to an ATP-dependent protease like Lon. To this end, we have already formed 2/4 of the above constructs as well as the ferritin-based (**Fig. 4**) construct by Gibson assembly. We incorporated these constructs in constitutive expression vectors and engineered cell lines expressing TEVp from an inducible promoter.

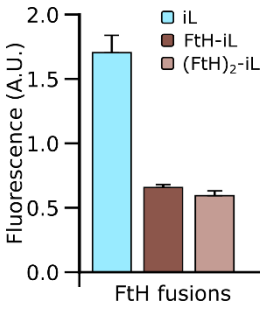

Figure 4. iLOV fusions to FtH.

Table 1. iLOV constructs for protease-based sensing of energetics

N–C linker	N′ (E96) fusion	C′ (G95) fusion
ENLYFQ|S-Jα	E3: $(EIAALEK)_3$	K3: $(KIAALKE)_3$
ENLYFQ|S-Jα	Kzip:ALKKELQANKKELAQLKW ELQALKKELAQ	Ezip:EQLEKKALEKKLAQL WKNQALEKLAQ
cpiLOV: Jα-(G_4S)	E3-ENLYFQ|S	K3-ENLYFQ|S
cpiLOV: Jα-(G_4S)	Kzip-ENLYFQ|S	Ezip-ENLYFQ|S

What opportunities for training and professional development has the project provided?
Nothing to report

How were the results disseminated to communities of interest?
The project has so far resulted in one publication and one conference presentation, which are described below (Section 6).

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

(1) Randomized linker mutagenesis was successful in producing libraries with moderate rises in peak fluorescence. Therefore, we will continue to optimize the current ATP-sensing construct (**Fig. 1**) with linker mutagenesis and FACS-based screening for chemically-induced changes

in cellular fluorescence. The screening workflow will comprise 2 rounds of positive sorting (*i.e.*, high-ATP state), followed by a negative sort (*i.e.*, low ATP state), and finally, an additional round of positive sorting to identify clones that recover fluorescence after ATP is restored.

(2) In parallel, we will explore a second approach for engineering ATP sensors by directly inserting engineered cpiLOV between amino acids 107 and 110 in the *B. subtilis* ATP-ε subunit, effectively sandwiching cpiLOV between the 2 α-helices of the ATP-ε domain. The premise for this approach is based on the successful construction of fluorescent ATP sensors (oxygen-dependent) by insertion of cpGFP in ATP-ε, and further reinforced by our discovery of a viable cpiLOV construct (**Fig. 2**). In the absence of ATP, the two helices in ATP-ε are more than 10 nm apart; but move closer to ~ 2.5 nm upon ATP binding. This large ATP-induced change in conformation could potentially allow ATP-binding induced fluorescence changes in cpiLOV. Constructs will be optimized and screened by linker mutagenesis as described above.

(3) Given that the engineering space of the iLOV reporter is largely unexplored, innovative techniques are required to identify best-performing sensor constructs (especially to increase sensitivity). To this end, we are exploring the possibility of imaging protease activity as a proxy for cellular bioenergetics. We have already cloned several constructs (**Table 1, Fig. 4**), which we will first test with a model protease (TEVp) before moving to other proteases that are more directly connected to the cellular energetic status.

(4) Successful sensor constructs identified by any (or all) of the above steps will be transferred to nanoLOV (**Fig. 3**) with the goal of reducing the total size of the sensor and consequently its footprint as well as metabolic burden due to over-expression in cells.

(5) As improved sensors become available from the above workflow, these will be expressed in cells and tested for ATP response (in some cases, protease response) both by FACS and fluorescence imaging. Best-performing constructs will be further tested in cells under various levels of environmental oxygen and in the presence *vs*. absence of antibiotics, and finally under combinations of various oxygen levels and antibiotic treatment conditions. These experiments will be designed to confirm (or possibly, refute) proposed correlations between oxygen status, ATP levels, and the effect of drug molecules.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

The activities accomplished during this project period established the core methodologies for developing fluorescent biosensors to probe cellular bioenergetics (as well as other analyte targets) independent of oxygen availability. To our knowledge, this work represents the first systematic exploration of protein engineering methods towards constructing genetic sensors based on flavin-binding fluorescent proteins. Two immediate advances will ensue if our efforts in the remainder of this project are successful. First, this research will establish a one-of-its-kind methodology for monitoring bioenergetics with implications ranging from disease management to anaerobic bioproduction. Second, the project will introduce a sensor-engineering paradigm that can be adapted to design sensors for any analyte much in the same way as current GFP-based tools. However, because these reporters will not depend on oxygen to produce light, they may be used to study a much broader range of biological problems than currently possible with GFP.

What was the impact on other disciplines?

The project is likely to have a major (and admittedly, initially unexpected) impact on two other biotechnology disciplines: sensor engineering for low-oxygen systems and design of chemically-inducible optogenetic tools. (1) This project has led us to explore and identify several avenues for constructing biosensors based on iLOV. Given the acute paucity of sensors that operate in low oxygen conditions, the work performed here is anticipated to have a major impact on the ability to perform molecular imaging in living systems when oxygen levels run low (*i.e.*, hypoxia). (2) Furthermore, the smaller size of the nanoLOV construct could also provide a critical practical utility for applications hindered by the size of GFP – for example, packaging of viral gene delivery vectors and protein-trafficking studies. (3) The final anticipated impact is based on the ability of certain iLOV variants to serve as optogenetic tools where the goal is to control biological processes using light. In the course of this work, we realized that the methodology developed above could be extended to invent a new class of optogenetic LOV constructs that are responsive to a cell's internal state (*e.g.*, bioenergetic status). This would then establish an entirely new paradigm in synthetic biology where light could be used to manipulate biological functions on the basis of the internal biochemical state of a cell. For instance, ATP-responsive optogenetic LOV proteins could be used to selectively activate kill switches in exhausted cells experiencing low levels of energy. This general principle can have wide-ranging impact in disciplines ranging from bioproduction to cell-based biomedicines. We are keen to build on the conceptual and methodological advances ensuing from this project to pursue both the ideas delineated here (pending successful securing of research funds).

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

We have not implemented any major changes to the workflow as originally proposed except for a few strategic modifications as noted below:

- In addition to exploring insertion of the ATP-binding domain in iLOV, we are also exploring the possibility of constructing sensors by inserting cpiLOV in the ATP-binding domain. These efforts, while successful for engineering ATP-sensing GFPs, have been previously hindered due to the lack of circularly permuted iLOV variants. Our successful discovery of cpiLOV (**Fig .2**) now lets us explore this powerful avenue for sensor engineering.
- For similar reasons as above, we are also exploring the potential of protease-based sensors as an alternative approach to probe cellular energetics. The goal here is to design constructs that give maximum fluorescence changes, while also emitting light significantly above baseline levels, thereby permitting detection with high sensitivity. *The underlying principle of sensor optimization remains unchanged from our original goals*.
- One key challenge we face relates to the low brightness of iLOV and even weaker emission of engineered variants. As a result, screening of sensor candidates in *E. coli*

cells has been difficult because the fluorescence levels are often close to the noise floor. This limitation can easily overwhelm efforts to identify viable ATP-responsive mutants. To remedy this problem, we are exploring the use of model mammalian cell lines that could allow for the production of orders of magnitude more reporter protein, thereby allowing even small signal changes to be easily intercepted. *The basic sensor principles and operating mechanisms, however remain unchanged and successful constructs will be tested in E. coli cells as originally proposed*.

Actual or anticipated problems or delays and actions or plans to resolve them

On account of COVID-19 and the omicron surge, we have experienced delays due to intermittent suspension of research activity, loss of personnel, and delivery interruptions. Accordingly, we have requested and were graciously granted a 1-year No Cost Extension (NCE).

6. PRODUCTS

Publications, conference papers, and presentations

Journal publications.

Anderson, N.T., Weyant, K.B., Mukherjee, A. Characterization of flavin binding in oxygen-independent fluorescent reporters. *AIChE J*. 66(12). (2020) doi: 10.1002/aic.17083 (acknowledgement of federal support: yes)

The above work characterizes basic characteristics of fluorescence response in iLOV and determines how this response may be modulated based on flavin-binding. This concept is related to the mechanistic principles underlying the development of allosteric ATP biosensors and is important for the characterization of sensor constructs as proposed in the SOW (Major Task 2).

Conference presentations.

Anderson, N.T. (speaker), Mukherjee A. Engineering genetically-encodable oxygen-independent fluorescent reporters based on LOV proteins. American Chemical Society (ACS) Annual Meeting, San Diego, CA, 2022

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on this project?

Name	Nolan Anderson
Project Role	Graduate Student
Nearest person month worked	12
Contribution to project	Engineering allosteric iLOV-based ATP sensors, engineering cpiLOV, iLOV truncation, engineering protease-sensors as a proxy for bioenergetics monitoring, cellular expression and testing, *in vitro* protein characterization
Funding Support	UCSB Chancellor's fellowship, NIH

Name	Harun Ozbakir
Project Role	Postdoctoral Scholar
Nearest person month worked	7
Contribution to project	Establishing and optimizing general procedures for reporter protein expression and purification (*e.g.*, ultrasonication,

	FPLC techniques), design and planning of insertion library screening, establishing general protein engineering and library protocols (*e.g.*, linker mutagenesis, high efficiency electroporation), preliminary FACS optimization, provision of training to Graduate Student (above) in these procedures, engineering alternative ATP sensors based on ATP-dependent riboswitches (as proposed in potential pitfalls & alternative planning).
Funding Support	

Name	Arnab Mukherjee
Project Role	PI
Nearest person month worked	1.25
Contribution to project	Overall supervision, data analysis & reporting
Funding Support	NIH, UCSB startup funds

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

In this period, the PI received two seed fundings on unrelated projects

- $16,139 from the UCSB Academic Senate Faculty Research Grant
- $150,000 from the National Institutes of Health

What other organizations were involved as partners?
Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS
Nothing to report

9. APPENDICES
Nothing to report