AWARD NUMBER: W81XWH-15-1-0367

TITLE: Understanding Microbial Sensing in Inflammatory Bowel Disease Using Click Chemistry

PRINCIPAL INVESTIGATOR: Dennis L. Kasper, Ulrich von Andrian

CONTRACTING ORGANIZATION: Harvard College, President and Fellows of

Boston, MA 02115

REPORT DATE: October 2016

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	30 Sept 2015 – 29 Sept 2016

4. TITLE AND SUBTITLE

Understanding Mircrobial Sensing in Inflammatory Bowel Disease Using Click Chemistry

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0367

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S) Dennis L. Kasper, Ulrich von Andrian

uva@hms.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
President & Fellows of Harvard College
AND ADDRESS(ES)
25 Shuttuck Street
Boston, MA 02115-6027

8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Materiel 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 surface molecules of bacteria contribute significantly to the host's response to both pathogens and commensals. However, the technology available to track these molecules in host cells and tissues remains primitive. To address this limitation, we have developed an expanded metabolic labeling approach that chemically tags lipopolysaccharide, capsular polysaccharide, and peptidoglycan simultaneously in live anaerobic commensal bacteria. This technology enabled us to track the entry of differentially labeled surface molecules from live, luminal bacteria into specific host intestinal immune cells and their subsequent degradation in host phagocytes. Notably, this approach also enabled live imaging of the tagged bacterial surface molecules from endogenous commensals in the intestine of the living murine host. These tools will make it possible for investigators to decipher the role of specific bacterial surface molecules in host response.

15. SUBJECT TERMS

Bioorthogonal click-chemistry, bacterial cell wall, bacterial outer membrane, peptidoglycan, lipopolysaccharide, endotoxin, capsular polysaccharide, inflammatory bowel disease,microbiome

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. **INTRODUCTION:**. In this proposal, we will apply and expand our bioorthogonal click-chemistry strategy to simultaneously label an unprecedented three components of the bacterial cell wall: PSA, lipopolysaccharide (LPS), and peptidoglycan (PGN). With this approach, we will be able to track a TLR2 ligand (PSA), a TLR4 ligand (LPS), and a ligand for NLR signaling (PGN) in living bacteria and their host and to follow these molecules into specific host immune cells in order to define the pathways by which protective or inflammatory responses develop. This methodology will also allow us to visualize the spatial and temporal changes in bacterial product acquisition within diseased tissue. Beyond adding to the basic knowledge of host-commensal communication, an understanding of microbial sensing in the gut will allow us to design new treatments for IBD patients. Our long-term objective is to use fluorescently labeled commensal bacteria as a less invasive tool for the study of IBD in patients. This approach leverages the Kasper lab's expertise in commensal microbiology and carbohydrate chemistry with the von Andrian lab's skills in fluorescence microscopy and immunology to develop a new interdisciplinary platform for imaging and tracing bacterial products in the gut.

1. **KEYWORDS:** Bioorthogonal click-chemistry, bacterial cell wall, bacterial outer membrane, peptidoglycan, lipopolysaccharide, endotoxin, capsular polysaccharide, inflammatory bowel disease, microbiome, microbiota, carbohydrate chemistry, fluorescent microscopy, 2-photon microscopy, confocal microscopy

2. **ACCOMPLISHMENTS:**

There were three specific aims in our proposal.

Aim 1. Incorporate non-natural sugars and amino acids into lipopolysaccharide, capsular polysaccharide, and peptidoglycan for live bacterial-cell labeling.

Aim 2. Use click labeling to elucidate the acquisition and distribution of commensal material *in vitro* and in host intestinal tissue.

Aim 3. Use live intravital microscopy to monitor changes in bacterial-component acquisition during chemical and pathogen-induced colitis.

We have made significant progress on all three specific aims as summarized below. The Kasper lab has done the chemistry, microbiology and worked with the von Andrian lab on the immunology and microscopy. All work was done between the two labs at Harvard Medical School

For our first target (Specific Aim 1), we focused on a macromolecule that is present in nearly all bacteria, including commensals, and is known to contribute to host intestinal health and disease progression. The PGN component of bacteria seemed an obvious choice as it is a highly conserved

structural feature of most bacterial phyla. PGN is sensed by the innate NOD-like receptors in mammalian cells, and the strong link of mutations in these receptors to inflammatory bowel disease (IBD) highlights their importance in maintaining healthy commensal–host interactions. The promiscuity of PGN biosynthesis was recently exploited to install non-natural fluorescent D-amino acids into the PGNs of many aerobic bacteria (Fig 1a). Since cells use L-amino acids for protein synthesis, this method makes the specific labeling of PGN possible. We wanted to determine whether this approach could be used to label and track anaerobic commensal bacteria.

Incubation of anaerobic cultures with the fluorescent D-amino acid hydroxyl coumarin carbonyl-amino-D-alanine (HADA) resulted in the successful labeling of a wide range of anaerobic and facultative commensal bacteria, including *Bacteroides fragilis*, *Bacteroides vulgatus*, *Parabacteroides merdae*, *Clostridium clostridioforme*, *Clostridium ramosum*, *Enteroccocus faecalis*, *Escherichia coli*, and *Bifidobacterium adolescentis* (Figs. 1a and 1b). In fact, we have yet to find an anaerobic or facultative bacterium that cannot be labeled by growth with HADA. As a control, we incubated the bacteria with the L-enantiomer of the fluorescent amino acid referred to as HALA; the minimal background we observed supported the specific labeling of the PGN layer by D-amino acid incorporation. This labeling is both time and concentration dependent, as previously reported, although overnight incubation with HADA was optimal, a finding similar to our previously reported metabolic labeling conditions with GalNAz.

Given the success of *in vitro* PGN labeling, we examined whether the labeled bacteria could be imaged and traced within the natural niche of the host intestine. *B. adolescentis* and *E. faecalis* were labeled as described and administered to mice via oral gavage and direct intestinal injection, respectively. As shown in Fig. 1c and 2a, the bacteria retained the PGN label and were successfully imaged in tissue sections of the host small intestine and colon. *E. faecalis* in particular was found close to the tissue in the proximal colon and in the vicinity of $CD11c^+$ antigen-presenting cells (APCs, Fig. 1c). We also synthesized the fluorescein-based D-amino acid fluorescein-D-lysine (FDL) and labeled *C. clostridioforme* in culture, whose imaging in the murine colon (Figs. 2b and 2c) demonstrated the capacity for multicolor labeling.

Since exogenous D-amino acids are not used by mammalian cells, we reasoned that feeding conventionally raised mice the fluorescent D-amino acid might allow selective labeling of endogenous bacteria (Specific Aim 2). We administered HADA by oral gavage to specific pathogen–free (SPF) mice and analyzed the intestinal lumen by flow cytometry and microscopy. Confocal imaging of tissue histology slices from HADA-gavaged mice showed robust labeling of the commensal bacteria already present in the lumen of the small intestine and the colon 2 h and 4 h, respectively, after gavage (Fig. 1d). By contrast, the HALA-gavaged controls showed little background.

Since our approach labels living bacteria, we also employed multiphoton intravital microscopy (Specific Aim 3) to view the dynamics of host–microbiota interaction in real time. We have extensive experience in the use of this

technique for the investigation of lymphocyte trafficking in living animals; to our knowledge, however, it has not been used to follow bacterium-derived components in the host. Mice were given HADA by oral gavage; after 16 h, the animals were anesthetized and intestinal loops were surgically extracted and mounted for imaging. Our results showed robust labeling of the commensal microbes in the small and large intestines.

There have been two postdoctoral fellows working on this project and the work has afforded significant training in solving the chemical, immunologic, and microbiologic aims of this grant. One fellow from the Kasper lab has done the chemistry, microbiology and worked with the second fellow from von Andrian lab on the immunology and microscopy. Teaching has come from several sources including advise from PI's and senior lab members, conferences, and regular lab meetings.

We have nothing to report yet on dissemination of knowledge to the community but intend to have a manuscript by the next reporting period.

We will continue to advance all three specific aims. We will follow fluorescent labels into specific immune cells and pay special attention to macrophages and dendritic cell uptake. We will also begin to study uptake in the setting of inflammation. Finally, we will develop the labeling techniques further for lipopolysaccharides of gram-negative bacteria.

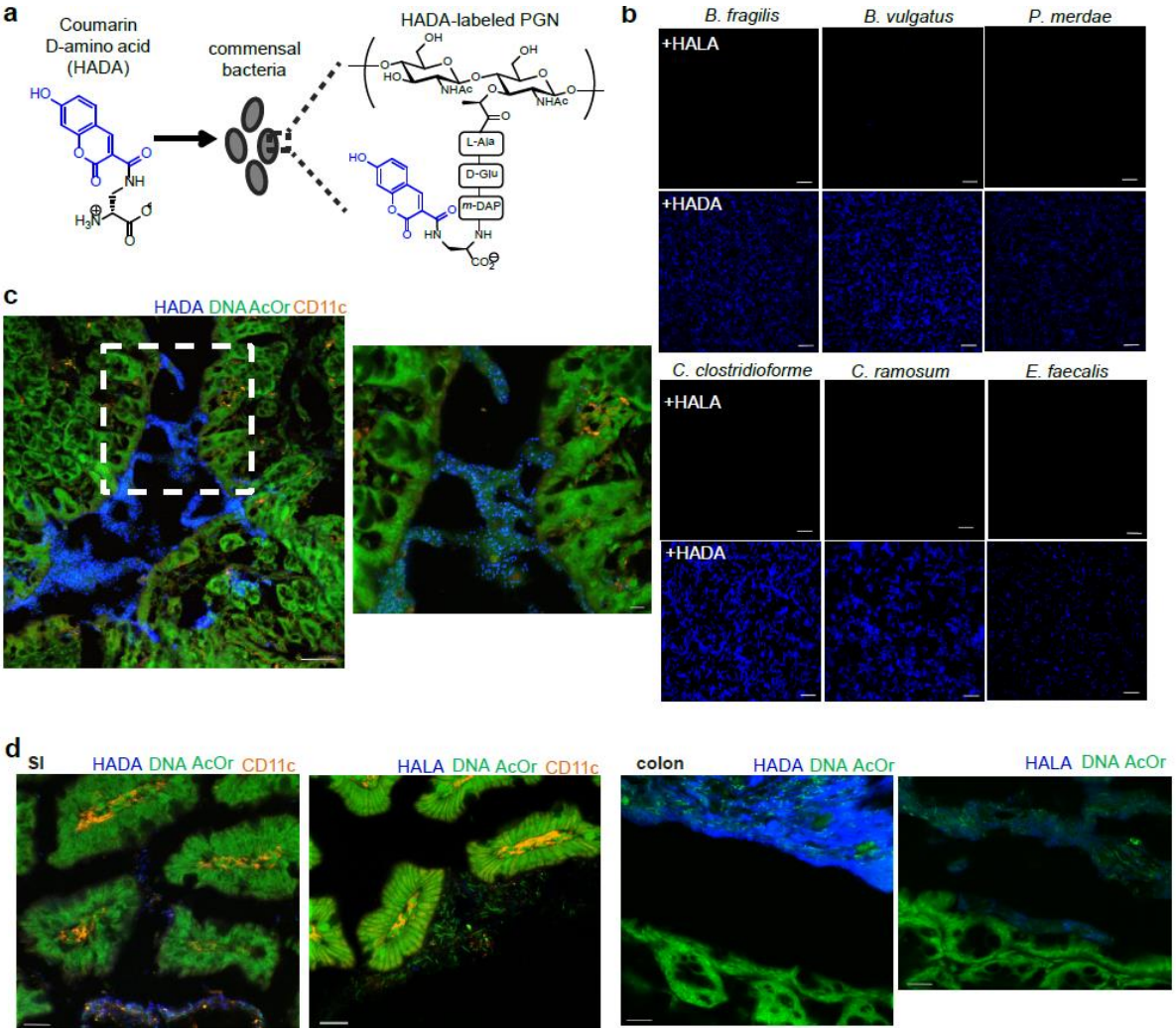
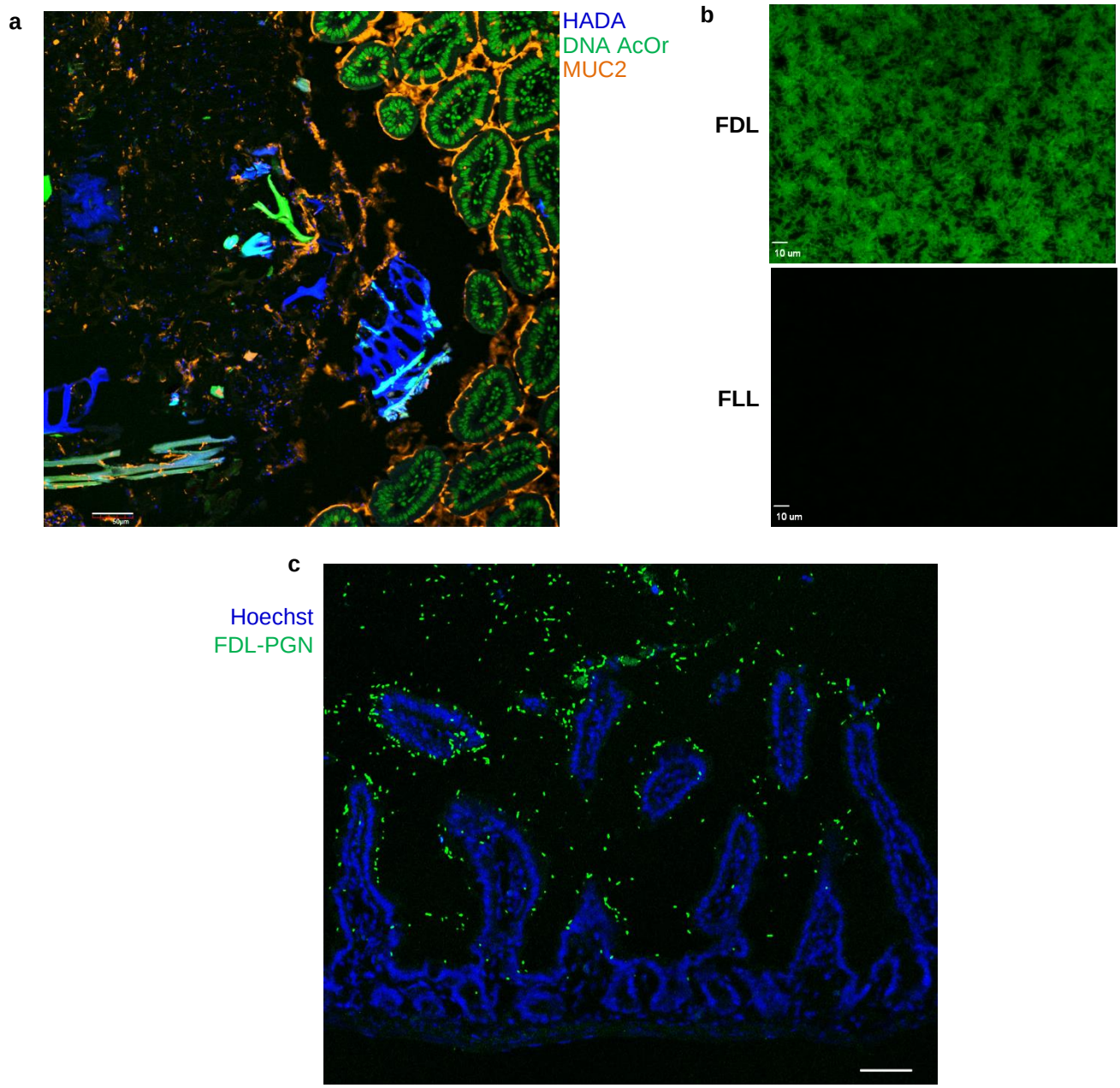


Figure 1. Fluorescent D-amino acid labels PGN in commensal bacteria.

(**a**) Schematic of metabolic labeling of the peptidoglycan of commensal bacteria with fluorescent D-amino acid derivatives. The addition of the fluorescent coumarin moiety is highlighted in blue. (**b**) Images of various commensal bacteria grown overnight in medium with a fluorescent amino acid: HADA or (as a negative control) HALA. Scale bar, 10 μm. (**c**) Confocal image and expanded region (right) of frozen, fixed tissue slice of mouse colon 1 h after injection of HADA-labeled *E. faecalis*. AcOr, acridine orange. Scale bar: left, 50 μm; right, 10 μm. (**d**) Confocal images of Carnoy's-fixed, paraffin-embedded tissue slices from mice 2 h (small intestine [SI], left) or 4 h (colon, right) after oral gavage with HADA or (as a negative control) HALA. Scale bar, 20 μm.

Figure 2. Fluorescent D-amino acid labeling of gram-positive bacteria



(**a**) Confocal image of HADA-labeled *B. adolescentis* in Carnoy's-fixed murine small intestine 1 h after direct luminal injection. Scale bar, 50 µm. (**b**) Images of *C. clostridioforme* grown overnight in 1 mM fluorescein D-lysine (FDL) or fluorescein L-lysine (FLL). Scale bar, 10 µm. (**c**) Confocal image of FDL-labeled *C. clostridioforme* in formalin-fixed and cryosectioned murine small intestine 1 h after direct luminal injection. Scale bar, 50 µm.

4. **IMPACT:**

The development of the HADA labeling is an important discovery that will enable research considerably be being able to follow essentially any bacteria using fluorescent techniques into the host or other environments.

This chemistry is also a potential method for delivery of antibiotics directly to the target in the bacterial cell. We are exploring the possibility of commercial impact of this approach.

Nothing to report on impact beyond science and technology

5. **CHANGES/PROBLEMS**

Nothing to report

6. **PRODUCTS**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATION

Name:	*Dennis Kasper*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.8*
Contribution to Project:	*Dr. Kasper supervised this project, organized and planned the labeling technology, microbiology and worked with Dr. von Andrian on planning immunology*
Funding Support:	*-*

Name:	*Ulrich von Andrian*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*.6*
Contribution to Project:	*Dr. von Andrian worked with Dr. Kasper and monitored scientific progress. His general role included directing, supervising and coordinating the research efforts in his lab*
Funding Support:	*-*

Name:	*Francesca Gazzaniga*
Project Role:	*Research Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Gazzaniga was a postdoctoral fellow working on this project and worked on cellular uptake of labeled microbes*

Funding Support:	-

Name:	*Lesley Pasman*
Project Role:	*Research Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Pasman is a postdoctoral fellow working on this project and worked on labeling microbes in vivo*
Funding Support:	-

Name:	*Shakir Edwards*
Project Role:	*Animal Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Mr. Edwards is a technician who maintained the gnotobiotic mouse facility which was a major source of mice for these experiments*
Funding Support:	-

Name:	*Kailyn Stefan*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Ms. Stefan is a graduate student and was responsible for the metabolic labels and the click chem reagents as well as*

	labeling the microbes in vitro
Funding Support:	-

Name:	*Bella Printseva*
Project Role:	*Research Administrator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2.4*
Contribution to Project:	*Dr. Printseva is the laboratory manager and is responsible for ordering supplies, maintaining COMS and IACUC approvals and for maintaining FACS for these studies*
Funding Support:	-

Name:	*Munir Mosaheb*
Project Role:	*Research Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Mosaheb is a postdoctoral fellow working on this project. He has been investigating the role of specifically labeled (metabolically/click/chemistry labeling) bacterial component in the gut during the progression of colitis and to elucidate the acquisition of these components by immune cells in the intestine*
Funding Support:	-

Name:	*Pavel Hanc*
Project Role:	*Research Fellow*
Researcher Identifier	

(e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Hanc is a postdoctoral fellow working on this project. Using bacteria labeled by click chemistry in the gut, he has been looking at the role of B cells in gut homeostasis and its role in establishing colitis*
Funding Support:	*-*

Name:	*Shaida Omid*
Project Role:	*Lab Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Ms. Omid coordinated the housing, breeding and procurement of all mouse strains used for this project in the von Andrian lab*
Funding Support:	*-*

Name:	*Lauren Jones*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*.8*
Contribution to Project:	*Ms. Jones was the laboratory technician and assisted the postdocs with their projects and Ms. Omid with the care and maintenance of the mouse strains. She also was in charge of ordering supplies*
Funding Support:	*-*

7. **continued**

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**

9. **APPENDICES**

Nothing to report