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Final Report: 14.3 Microbiology and Biodegradation: Deep Ultraviolet Microscopy for the Detection, Quantification, and Characterization of Microbes

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The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT

This proposal was funded for the improvement and use of a deep ultraviolet (DUV) fluorescence imaging (microscopic) system, and a coupled DUV Raman Spectrometer. This was to be used for the study of microbial cultures with several goals in mind: 1) to rapidly quantify microbes on surfaces of filters and minerals; 2) to distinguish different microbial species from one another; 3) to look for subtle changes in microbes as a function of growth conditions, and 4) to improve the fluorescence and Raman systems for higher resolution measurements. Goal 1 was accomplished with the DUV fluorescence system, and studies, and goal 2 was in the midst of its work

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Kenneth Nealson
UU	UU	UU	UU		19b. TELEPHONE NUMBER: 213-821-2271

Report Title

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ABSTRACT

This proposal was funded for the improvement and use of a deep ultraviolet (DUV) fluorescence imaging (microscopic) system, and a coupled DUV Raman Spectrometer. This was to be used for the study of microbial cultures with several goals in mind: 1) to rapidly quantify microbes on surfaces of filters and minerals; 2) to distinguish different microbial species from one another; 3) to look for subtle changes in microbes as a function of growth conditions, and 4) to improve the fluorescence and Raman systems for higher resolution measurements. Goal 1 was accomplished with the DUV fluorescence system, and studies, and goal 2 was in the midst of its work when funding was discontinued. At this point, the instrument development (improvement of resolution) was stopped at the advice of the Program Manager (Dr. Kizer), and measurements of samples with regard to goals 1 and 2 were continued until the end of the funding period.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Kenneth Nealson: Elected as Fellow of the American Geophysical Union (AGU) - 2012

Kenneth Nealson: Elected as Fellow of American Association for Advancement of Science (AAAS) - 2013

Kenneth Nealson: Noted as one of the top 1% in his field in terms of number of citations (Thomson-Reuters) -2014

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Rohit Bhartia	0.00	
FTE Equivalent:	**0.00**	
Total Number:	**1**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Annette Rowe	0.20
FTE Equivalent:	**0.20**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Kenneth Nealson	0.10	
FTE Equivalent:	**0.10**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:..... 0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Rohit Bhartia
Total Number: 1

Names of other research staff

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

Executive Summary:

Time Schedules: This report is the final report for an abbreviated full proposal that was originally scheduled for five years, but was terminated after about 2 years of support. In order to put it in perspective, a short review of the originally funded goals and time schedule are noted here, followed by what was accomplished in the 2 year period. In addition we present some supporting information that preceded the program but helps place the development in context.
In response to a request from a program manager (PM) Dr. Wallace Buchholz of the ARO, we submitted the proposal in March of 2010. The proposal was officially accepted 15 months later, in June of 2011 (given that this was a contract, USC didn't allow any money to be spent until funds arrive). Funds were received at USC 2 months later, and work was initiated in August of 2011. Fifteen months later, after three meetings with three different Program Managers, we were notified that funding was to be terminated on November of 2014, about 24 months after the funds had arrived.

Work Plan and Deliverables: The proposal as originally solicited and accepted contained two parts: I) A technology deliverable: to upgrade an existing deep ultraviolet (DUV) microscope for imaging and spectral analysis of microbes on solid surfaces; and, II) A science deliverable: to use the upgraded microscope system (and other pre-existing instruments) to analyze microbes under different growth conditions, with the goal of identifying and quantifying spectral differences that relate to the growth history of the microbes under investigation. This latter effort would be initiated while the microscope was being upgraded and continue in earnest after the upgraded instrument was tested and calibrated. In addition to the development of the microscope and the supporting instrumentation, advanced growth facilities would be used to assess the fluorescence response of cells under different growth rates, environments, and between species/genera.

To quote from the proposal's objectives section:
"... to distinguish laboratory-grown bacteria from "wild" types, and to gain information on the recent metabolic activities and histories of microbes using optical methods.", and
"... to see which kinds of growth conditions impart recognizable signals on the cells – signals that can be used to interrogate the cells for past metabolic conditions and activities."
While the instrument development portion of the work is on schedule, the status of the experimental work actually exceeds the proposed schedule and was done to accommodate the stated needs of the sponsor. The upgrades for the instrument have been completed and a newly hired postdoc from the Nealson lab, Dr. Annie Rowe, a microbiologist, has joined the team. The efforts of the group have led to initial growth rate experiments with E. coli, preliminary analysis of species/genera as a function of spectral variability, and an additional development on cell survivability during analysis resulting from telecon with sponsor. A series of experiments examining the impact of different methods of fixation on the spectral properties of the bacteria have also been initiated. It should be noted, however, the level of progress and results attainted was only possible enabled by leveraging separate funds that Drs. Ken Nealson's (USC), Rohit Bhartia (JPL) and William Hug (Photon Systems) had from internal sources and programs with related needs; distinct programs with different end-goals.

Results: Microscope assembly and testing: Over the 15-month period of this program the, the upgrades to the deep UV microscope (described in Bhartia et al., 2010) went through a design plan that optimized the balance between sensitivity, depth of focus, field of view, image quality, functionality (ease of used), and a means to enable additional functions currently incorporated in separate instruments. The development led to acquisition of parts, and development of new interfaces. The instrument is currently in the testing phase to place metrics on the performance – however it is fully functional and is being used to collect deep UV native fluorescence and visible images of microbes under a variety of experimental conditions. The details of this process required the interaction of several different key technical staff, some funded by this program, and some through other grants/contract, as described in Appendix 1. In addition to hardware, effort was placed on upgrading the image reconstruction software that combines multiple UV images.

Results: Microbiological testing: The initial testing of strains for the impact of growth conditions on spectra produced was done using Escherichia coli strain K12, one of the most well-studied organisms in captivity. We chose this organism because of the availability of mutants that could be used to test hypotheses as they arose. Initial experiments, the details of which are in the results section below lead us to the following interpretations, which will (hopefully) be tested in the future:
1. For a given strain of bacteria (in this case, E. coli K-12), there are detectable changes in spectral signatures when grown under different conditions. To discern the cause of these spectral changes will require physiological and biochemical analyses of the strains.
2. We found that the bacteria were tolerant to high UV (224 nm) flux. This is contrary to what was previously published data claiming massive bacterial killing (see data in results section). The reasons for these differences can be determined by the use of UV resistant and UV sensitive mutants in future studies.
3. We are preparing cells for studies of cell preservation, which will allow us to interact with other laboratories through fixation of cells grown under different growth conditions, fixed (killed and preserved), and then shipped to our laboratory for analysis. This work will hopefully be done in the future.

Discussion/Conclusion: This project was well on the way to meeting the stated
goals and objectives, as outlined in the original proposal. The staff was hired, and through group meetings, the coordination between the microscope assembly group, and the microbiology group was in order. Despite one tragic occurrence** that led to

the retirement of Dr. Mandy Ward, the project accomplished the initial phases of the microbiology work.

In my (KHN) opinion, it was a pity to stop the project at a point where the stated science goals were proceeding, and the information of most interest to the ARO was being gathered. In essence, while the upgrade of the microscope was accomplished, and the instrument is much improved, the science goals were only in their nascent stages. As such, it is a project that was beginning to yield exciting, very interesting and ARO-relevant results at the time it was stopped.

While the conclusions of the abruptly terminated 5yr project are minimal the 1.25 years of support continued to prove both the massive potential of deep UV native fluorescence spectroscopy for label-free microbial detection and characterization as well as a number of challenges in the technology and science. The five-year program with achievable goals was stopped after 1.25 years, and the best we can conclude is that it would have been clearly possible to achieve the goals that we set out to achieve. With the microscope in the Nealson lab (USC) , work will continue with the support of JPL and Photon Systesms at a low level (only supported by internal funding and work relevant to other funding agencies), with the hope to find another source of funding to support a highly focused effort that will continue to lead to new insights of what can be understood of microbial activity using deep UV spectroscopy.

** Dr. Mandy Ward retired early after a tragic accident in which she lost her left leg, and was unable to continue working in the lab. She made a heroic effort for a few months, but concluded it would not be possible to continue, and retired to northern California as of January 1, 2012. She played a vital role in planning early experiments, and getting lists of cultures for future analyses.

Introduction/Background:

The following report includes work that was accomplished during the course of this year, historical information about the microscope, demonstration of capability/uniqueness that was requested in conversation/meetings with the sponsor, as well as additional supporting information relevant to the task but yet to be published. One of the major accomplishments in this first year was to establish a high level plan with four distinct tasks that would lead to a better understanding of how the deep UV fluorescence microscope detect microbes, what factors influence the signal as well as a more hardware endevour of streamlined operation for ease of use and standardization of data acquisition. The four tasks have been titled as: Metabolic State and Deep UV Signatures, Strain Acquisition, Deep UV Microscope Upgrades, and Deep UV Data Analysis and Correlation to Analytical Instruments.

Origin of the project: This project began when Dr. Wallace Buchholz, a program manager (PM) at the Army Research Office (ARO) visited a poster that was presented by the Nealson laboratory (Bhartia was a graduate student in the Nealson lab at that time) at a Gordon Research Conference on Applied and Environmental Microbiology in the summer of 2008. The concept that one could image bacteria on surfaces without the addition of any vital (contaminating) stains, and without any mineral fluorescence was attractive with regard to monitoring bacteria rapidly and quantitatively, and the Nealson laboratory was asked to submit a proposal for evaluation. The proposal was submitted to Dr. Buchholz, sent out for review, and after positive reviews, a slightly revised proposal was suggested. This was done, along with a five year budget for the work that would be done jointly between the University of Southern California, the Jet Propulsion Laboratory, and Photon Systems, Inc., a small company in Los Angeles. Dr. K. H. Nealson was the PI of this grant as submitted.

Dr. Rohit Bhartia was a graduate student at USC (2007-2011), and in addition to developing the chemometric to uniquely differentiate microbes, did the development of this microscope system for his Ph.D. degree and with the help of a DURIP grant to KHN (with the support of Photon Systems and JPL),. This was the basis of the data presented at the Gordon Conference (two publications cited here, along with the thesis) and is what attracted PM Wallace Buchholz to suggest that we submit a proposal for funding. Many of the details of the building and testing of the DUV microscope are contained in the thesis of Dr. Bhartia, and in the publications cited above.

Timing: For reasons unknown to the PI, the PM of the program was changed, and somehow, though the proposal was deemed fundable and we had very good feedback, it was not funded, and we were asked to resubmit the proposal (with another revised budget one year later – it was resubmitted in March of 2010. In June of 2011 we notified that the proposal would be funded, and a “kickoff meeting” was held at USC in August of 2011 to introduce the ARO officials to our group, and our group to the ARO. Given that this was not a usual grant, but a contract, no internal funds were provided by USC, and thus, no work was allowed on the project until funds arrived, in August of 2011. During 2011 and 2012, two more meetings were held to review the project: February 7th at USC, and April 18th at Photon Systems by visiting groups from the ARO (with other visitors from various agencies), with positive feedback, and no indication of any problems provided to us. In fact, after the February meeting, a message was sent from Michelle Strand, “The others were happy with your progress. … it seems your project is fine” (5/1/12). A monthly brief progress report was sent to Dr. Strand, with the following response: “Excellent! Thanks! Keep them coming” (2/10/12).

In June (6/21/12), big changes were happening with this message from PM Strand: “Rachel has taken over this program. It appears that things are changing” -- with no hint of what that actually meant.

In July (7/12/12), we received a message from Dr. Strand “Here is the new reporting template from Rachel” -- this changed

our reporting duties from a few sentences each month to a major effort that entailed hours of work (with no one paid to do the report writing).

In September we received a telephone call from Rachel announcing that the grant was to be terminated at the end of November, and that a detailed progress report was expected in her office by the end of November.

The idea and background data:

Deep UV native fluorescence is most sensitive to aromatic compounds (Bhartia et al. 2008). For bacteria and other microbes, the primary fluorophores are the three aromatic amino acids - tryptophan (trp), tyrosine (tyr) , and phenylalanine (phe). While these amino acids are not as prevalent as alanine or glycine concentrations in a cell, these three amino acids are part of at least 15-20% of all amnio acids in vegetative bacterial cells spanning a range of genera(Lobry 1997). These three amino acids are found in all known organisms from multicellular to viral particles and in addition to structure , in some places they perform key functions in protein active sites. For example proteins for methanogenenisis , such as methylene-tetrahydromethanopterin dehydrogenase, contain phenylalanine in its active site (Ermler et al. 2002). It is not clear whether aromatic amino acids are critical or if another, non-aromatic protein, possibly less stable, can be used. However, the Shikimate pathway is the only known pathway for biological synthesis of aromatic amino acids.

It should be noted that the presence of these aromatic amino acids are, in effect, a unique biosignature - with only one exception, a very novel meteorite, abiogenic occurrence of phenylalanine has not been seen (Pizzarello and Shock 2010). Abiotic synthesis of the aromatic amino acids have only had limited corroborating evidence (Pizzarello and Holmes 2009; Sabbah et al. 2010). To understand the abiotic organic formation possibilities, meteorites provide a natural, abiotic “chemistry lab”. The diversity of organics created abiotically in meteorites consists of non-aromatic amino acids, aromatic compounds, and literally millions of other diverse organic structures. In only one meteorite sample (CR LAP02342) has there been evidence of phenylalanine and tyrosine (Pizzarello and Holmes 2009; Schmitt-Kopplin et al. 2010). While this is not evidence of extraterrestrial biology, it suggests a mechanism of abiotic aromatic amino acid synthesis. However, the low probability with which the aromatic amino acids occur outside of biological processes make for a strong biosignature that the deep UV native fluorescence analysis is ideally aligned. It is also the primary class of compounds that enable fluorescence detection of microbes.

In order to detect the aromatic amino acids found in microbial cells, the only non-invasive and non-contact methods with the necessary sensitivity are active spectroscopic methods that optically perturb the chemistry of the cells to generate a predictable spectral response. In recent years Raman scattering has become the go-to method of analysis for non-invasive chemical detection from organics in meteorite, mineralogy characterization and microbes. However, while the spectral information enabled with Raman scatter can be rich, the phenomenon is exceedingly inefficient, and with the necessary energy densities necessary to elicit a response, it does not fit the canonical definition of non-destructive analysis. The alternative methods include IR absorption methods but the sensitivities are not sufficent to observe single microbes without the use of synchroton sources or high powered lasers that also lead to thermal degradation. The “forgotten” method is native fluorescence spectroscopy. While it was first observed in 1565 (Acuña et al. 2009), it has taken a back-seat to spectroscopic methods like IR and Raman spectroscopy. In part this has been a result of a perception that all things fluoresce (minerals, organics etc), especially with excitation in the UV (254 or 365 - black-lights). To some degree this is true but only if the detection only occurs in the visible spectral region (>400 nm). However spectral analysis in the UV (260 to 400 nm), an area where specialized UV detectors are needed, there exits spectral information that provides information specific to the chemical structure of single ring amino acids and other 1 to 2 ring aromatic compounds. For these chemical species, this spectral region is not contaminated by mineral fluorescence or spectral emissions from larger organics that reside in the environment. But in order to observe these fluorescence emission bands the excitation source needs to be below the emission of the of the lowest fluorescence compound. By exciting at a wavelength <250 nm the entire fluorescence band from small aromatic compounds, including benzene and phenylalanine, are observable. Additionally, excitation <250 nm allows for use of Raman scattering for vibrational spectroscopy in a fluorescence-free region (figure 1). Since the lowest emission wavelength for fluorescence is ~270 nm (benzene), the Raman region for a 248.6 nm laser While traditional Raman that uses visible or NIR methods require high-power densities that can thermally alter samples, the deep UV enables resonance or pre-resonance effects for organics. Combining these methods has a number advantages however for the scope of this discussion, the primary one is that the native fluorescence method can help target the UV Raman methods. Since the native fluorescence is 6-orders of magnitude more efficient than even resonance Raman, it can operate at lower energies at the sample (ie larger spot sizes) and rapidly map large areas with a sensitivity of 1 cell in the beam.

Unlike bulk analysis/ingestion based methods (e.g. GC/MS, CE, HPLC, etc) the spectral patterns directly relate to the chemical composition. Additionally spectroscopic methods allow for preservation of spatial information that allow for correlation to surface morphology or chemistry. This prospects of this capability is what brought Drs. Nealson and Hug and Bhartia together. From 1997 – 2001, Prof. Nealson was in charge of the Mars Return Mission at JPL and realized the need for a means to detect microbes/life using non-contact spectroscopic methods. At the same time, Dr. Hug had established Photon Systems (10 miles from JPL) that focused developing compact deep UV lasers for Raman and fluorescence spectroscopy; both the need, the means, and realization of a solution happened to coincide. Prof Nealson and Dr. Hug, and eventually Dr. Bhartia (researcher/scientist at JPL), began a science and technology development program. The main thrust was:

1) Utilization of a deep UV sources (<250 nm) to non-invasively (label-free) detect aromatic organics – some of which are specific to microbes (tryptophan, tyrosine, and phenylalanine).
2) Minimize background fluorescence from surfaces (minerals, metals, etc) and incorporate chemometric methods to uniquely

differentiate microbes from organics.
3) Develop toward multiple spatial scales – macroscopic to microscopic in real-time. The purpose of this program was to better understand the microscopic scale analysis – a capability unique to spectroscopic methods and leads to microbial characterization in a manner currently not possible.
Since then the instrument development, spectroscopic theory/analysis, and scientific understanding have been supported by NASA, Army, DTRA, Navy, etc. and led to instruments such as the the version of the Deep UV Microscope that existed at the time this proposal. This was initiated, is a custom instrument built under a DURIP between the University of Southern California and Photon Systems Inc. (Figure 4-1). The base system is an Olympus microscope that has been modified with quartz optics and UV reflective optics, for excitation and transmission below 350 nm. It is coupled to two illumination sources, a 224.3nm HeAg, hollow-cathode laser for deep UV (DUV) epi-illumination and a white light source from Olympus attached to a secondary port on the microscope. This allows for acquisition of images illuminated by either the visible white light or deep UV laser source.

Proposed work and Results: This proposal had as its major goals the real-time quantification of microbes on solid surfaces without the use of stains, and the use of the DUV spectral system to gain insights into the past cultivation history of microbes. In order to accomplish these goals, improvements needed to be made in the microscope system that had been developed previously using funds from a DURIP grant (supplement to an AFOSR MURI proposal to KHN). Thus there were two major areas that needed to be addressed:
1. Technology – we proposed to upgrade the existing microscope
a. The upgrades to the DURIP microscope focused on optimizing the balance between sensitivity, depth of focus, field of view, image quality, functionality (ease of used), and a means to enable additional functions currently incorporated in separate instruments. These included a motorized X,Y,Z scanning stage, new objective lens, higher quality illumination path (white-light and deep UV), RGB visible camera for contextual images, and re-architecture of the laser module for future spectroscopic upgrades.
2. Microbiology
a. We proposed to use the improved microscope for rapid detection and quantification of microbes on surfaces without the need for staining
b. We proposed to test microbes for their spectral properties after growing various cultures under a variety of different conditions. When spectral changes were noted, we would then fractionate the microbial biomass to identify the molecular source responsible for the changes.
c. In response to a suggestion from the new PM (Rachel), we also initiated experiments to test the effect of DUV radiation on the survival of cells being identified using the DUV microscope.

Technology Transfer

Nothing to report

Title Page: Final Report

Deep UV Microscopy: Detection, Quantification, and Characterization of Microbes

Contract Number: W911NF110288

Principal Investigator:

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Dr. Mandy Ward**
Professor of Earth Sciences
University of Southern California
** Dr. Ward retired (6 months into the project) after a tragic accident that resulted in the loss of one leg. She lives in San Luis Obispo, CA now, and stays in contact with the project.

Foreward:

Time Schedules: This report is the final report for an abbreviated full proposal that was originally scheduled for four years of support, but was terminated after about 2 years of support. In order to put it in perspective, a short review of the originally funded goals and time schedule are noted here, followed by what was accomplished in the 2 year period. In addition we present some supporting information that preceded the program but helps place the development in context.

In response to a request from a program manager (PM) Dr. Wallace Buchholz of the ARO, we submitted the proposal in March of 2010. The proposal was officially accepted 15 months later, in June of 2011 (given that this was a contract, USC didn't allow any money to be spent until funds arrive). Funds were received at USC 2 months later, and work was initiated in August of 2011. Fifteen months later, after three meetings with three different Program Managers, we were notified that funding was to be terminated on November of 2014, about 24 months after the funds had arrived.

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Discussion/Conclusion: This project was well on the way to meeting the stated goals and objectives, as outlined in the original proposal. The staff was hired, and through group meetings, the coordination between the microscope assembly group, and the microbiology group was in order. Despite one tragic occurrence** that led to the retirement of Dr. Mandy Ward, the project accomplished the initial phases of the microbiology work.

In my (KHN) opinion, it was a pity to stop the project at a point where the stated science goals were proceeding, and the information of most interest to the ARO was being gathered. In essence, while the upgrade of the microscope was accomplished, and the instrument is much improved, the science goals were only in their nascent stages. As such, it is a project that was beginning to yield exciting, very interesting and ARO-relevant results at the time it was stopped.

While the conclusions of the abruptly terminated 5yr project are minimal the 1.25 years of support continued to prove both the massive potential of deep UV native fluorescence spectroscopy for label-free microbial detection and characterization as well as a number of challenges in the technology and science. The five-year program with achievable goals was stopped after 1.25 years, and the best we can conclude is that it would have been clearly possible to achieve the goals that we set out to achieve. With the microscope in the Nealson lab (USC) , work will continue with the support of JPL and Photon Systesms at a low level (only supported by internal funding and work relevant to other funding agencies), with the hope to find another source of funding to support a highly focused effort that will continue to lead to new insights of what can be understood of microbial activity using deep UV spectroscopy.

** Dr. Mandy Ward retired early after a tragic accident in which she lost her left leg, and was unable to continue working in the lab. She made a heroic effort for a few months, but concluded it would not be possible to continue, and retired to northern California as of January 1, 2012. She played a vital role in planning early experiments, and getting lists of cultures for future analyses.

Table of Contents:

Introduction/Background:

The following report includes work that was accomplished during the course of this year, historical information about the microscope, demonstration of capability/uniqueness that was requested in conversation/meetings with the sponsor, as well as additional supporting information relevant to the task but yet to be published. One of the major accomplishments in this first year was to establish a high level plan with four distinct tasks that would lead to a better understanding of how the deep UV fluorescence microscope detect microbes, what factors influence the signal as well as a more hardware endevour of streamlined operation for ease of use and standardization of data acquisition. The four tasks have been titled as: *Metabolic State and Deep UV Signatures, Strain Acquisition, Deep UV Microscope Upgrades, and Deep UV Data Analysis and Correlation to Analytical Instruments.*

Origin of the project: This project began when Dr. Wallace Buchholz, a program manager (PM) at the Army Research Office (ARO) visited a poster that was presented by the Nealson laboratory (Bhartia was a graduate student in the Nealson lab at that time) at a Gordon Research Conference on Applied and Environmental Microbiology in the summer of 2008. The concept that one could image bacteria on surfaces without the addition of any vital (contaminating) stains, and without any mineral fluorescence was attractive with regard to monitoring bacteria rapidly and quantitatively, and the Nealson laboratory was asked to submit a proposal for evaluation. The proposal was submitted to Dr. Buchholz, sent out for review, and after positive reviews, a slightly revised proposal was suggested. This was done, along with a five year budget for the work that would be done jointly between the University of Southern California, the Jet Propulsion Laboratory, and Photon Systems, Inc., a small company in Los Angeles. Dr. K. H. Nealson was the PI of this grant as submitted.

Dr. Rohit Bhartia was a graduate student at USC (2007-2011), and in addition to developing the chemometric to uniquely differentiate microbes, did the development of this microscope system for his Ph.D. degree and with the help of a DURIP grant to KHN (with the support of Photon Systems and JPL),. This was the basis of the data presented at the Gordon Conference (two publications cited here, along with the thesis) and is what attracted PM Wallace Buchholz to suggest that we submit a proposal for funding. Many of the details of the building and testing of the DUV microscope are contained in the thesis of Dr. Bhartia, and in the publications cited above.

Timing: For reasons unknown to the PI, the PM of the program was changed, and somehow, though the proposal was deemed fundable and we had very good feedback, it was not funded, and we were asked to resubmit the proposal (with another revised budget one year later – it was resubmitted in March of 2010. In June of 2011 we notified that the proposal would be funded, and a "kickoff meeting" was held at USC in August of 2011 to introduce the ARO officials to our group, and our group to the ARO. Given that this was not a usual grant, but a contract, no internal funds were provided by USC, and thus, no work was allowed on the project until funds arrived, in August of 2011. During 2011 and 2012, two more meetings were held to review the project: February 7th at USC, and April 18th at Photon Systems by visiting groups from the ARO

(with other visitors from various agencies), with positive feedback, and no indication of any problems provided to us. In fact, after the February meeting, a message was sent from Michelle Strand, "The others were happy with your progress. … it seems your project is fine" (5/1/12). A monthly brief progress report was sent to Dr. Strand, with the following response: "Excellent! Thanks! Keep them coming" (2/10/12).

In June (6/21/12), big changes were happening with this message from PM Strand: "Rachel has taken over this program. It appears that things are changing" -- with no hint of what that actually meant.

In July (7/12/12), we received a message from Dr. Strand "Here is the new reporting template from Rachel" -- this changed our reporting duties from a few sentences each month to a major effort that entailed hours of work (with no one paid to do the report writing).

In September we received a telephone call from Rachel announcing that the grant was to be terminated at the end of November, and that a detailed progress report was expected in her office by the end of November.

The idea and background data:

Deep UV native fluorescence is most sensitive to aromatic compounds (Bhartia et al. 2008). For bacteria and other microbes, the primary fluorophores are the three aromatic amino acids - tryptophan (trp), tyrosine (tyr) , and phenylalanine (phe). While these amino acids are not as prevalent as alanine or glycine concentrations in a cell, these three amino acids are part of at least 15-20% of all amnio acids in vegetative bacterial cells spanning a range of genera(Lobry 1997). These three amino acids are found in all known organisms from multicellular to viral particles and in addition to structure , in some places they perform key functions in protein active sites. For example proteins for methanogenenisis , such as methylene-tetrahydromethanopterin dehydrogenase, contain phenylalanine in its active site (Ermler et al. 2002). It is not clear whether aromatic amino acids are critical or if another, non-aromatic protein, possibly less stable, can be used. However, the Shikimate pathway is the only known pathway for biological synthesis of aromatic amino acids.

It should be noted that the presence of these aromatic amino acids are, in effect, a unique biosignature - with only one exception, a very novel meteorite, abiogenic occurrence of phenylalanine has not been seen (Pizzarello and Shock 2010). Abiotic synthesis of the aromatic amino acids have only had limited corroborating evidence (Pizzarello and Holmes 2009; Sabbah et al. 2010). To understand the abiotic organic formation possibilities, meteorites provide a natural, abiotic "chemistry lab". The diversity of organics created abiotically in meteorites consists of non-aromatic amino acids, aromatic compounds, and literally millions of other diverse organic structures. In only one meteorite sample (CR LAP02342) has there been evidence of phenylalanine and tyrosine (Pizzarello and Holmes 2009; Schmitt-Kopplin et al. 2010). While this is not evidence of extraterrestrial biology, it suggests a mechanism of abiotic aromatic amino acid synthesis. However, the low probability with which the aromatic amino acids occur outside of biological processes make for a strong biosignature that the deep UV native fluorescence analysis is ideally aligned. It is also the primary class of compounds that enable fluorescence detection of microbes.

In order to detect the aromatic amino acids found in microbial cells, the only non-invasive and non-contact methods with the necessary sensitivity are active spectroscopic methods that optically perturb the chemistry of the cells to generate a predictable spectral response. In recent years Raman scattering has become the go-to method of analysis for non-invasive chemical detection from organics in meteorite, mineralogy characterization and microbes. However, while the spectral information enabled with Raman scatter can be rich, the phenomenon is exceedingly inefficient, and with the necessary energy densities necessary to elicit a response, it does not fit the canonical definition of non-destructive analysis. The alternative methods include IR absorption methods but the sensitivities are not sufficent to observe single microbes without the use of synchroton sources or high powered lasers that also lead to thermal degradation. The "forgotten" method is native fluorescence spectroscopy. While it was first observed in 1565 (Acuña et al. 2009), it has taken a back-seat to spectroscopic methods like IR and Raman spectroscopy. In part this has been a result of a perception that all things fluoresce (minerals, organics etc), especially with excitation in the UV (254 or 365 - black-lights). To some degree this is true but only if the detection only occurs in the visible spectral region (>400 nm). However spectral analysis in the UV (260 to 400 nm), an area where specialized UV detectors are needed, there exits spectral information that provides information specific to the chemical structure of single ring amino acids and other 1 to 2 ring aromatic compounds. For these chemical species, this spectral region is not contaminated by mineral fluorescence or spectral emissions from larger organics that reside in the environment. But in order to observe these fluorescence emission bands the excitation source needs to be below the emission of the of the lowest fluorescence compound. By exciting at a wavelength <250 nm the entire fluorescence band from small aromatic compounds, including benzene and phenylalanine, are observable. Additionally, excitation <250 nm allows for use of Raman scattering for vibrational spectroscopy in a fluorescence-free region (figure 1). Since the lowest emission wavelength for fluorescence is ~270 nm (benzene), the Raman region for a 248.6 nm laser While traditional Raman that uses visible or NIR methods require high-power densities that can thermally alter samples, the deep UV enables resonance or pre-resonance effects for organics. Combining these methods has a number advantages however for the scope of this discussion, the primary one is that the native fluorescence method can help target the UV Raman methods. Since the native fluorescence is 6-orders of magnitude more efficient than even resonance Raman, it can operate at lower energies at the sample (ie larger spot sizes) and rapidly map large areas with a sensitivity of 1 cell in the beam.

Unlike bulk analysis/ingestion based methods (e.g. GC/MS, CE, HPLC, etc) the spectral patterns directly relate to the chemical composition. Additionally spectroscopic methods allow for preservation of spatial information that allow for correlation to surface morphology or chemistry. This prospects of this capability is what brought Drs. Nealson and Hug and Bhartia together. From 1997 – 2001, Prof. Nealson was in charge of the Mars Return Mission at JPL and realized the need for a means to detect microbes/life using non-contact spectroscopic methods. At the same time, Dr. Hug had established Photon Systems (10 miles from JPL) that focused developing compact deep UV lasers for Raman and fluorescence spectroscopy; both the need, the means, and realization of a solution happened to coincide. Prof Nealson and Dr. Hug, and eventually Dr. Bhartia

(researcher/scientist at JPL), began a science and technology development program. The main thrust was:

1) Utilization of a deep UV sources (<250 nm) to non-invasively (label-free) detect aromatic organics – some of which are specific to microbes (tryptophan, tyrosine, and phenylalanine).
2) Minimize background fluorescence from surfaces (minerals, metals, etc) and incorporate chemometric methods to uniquely differentiate microbes from organics.
3) Develop toward multiple spatial scales – macroscopic to microscopic in real-time. The purpose of this program was to better understand the microscopic scale analysis – a capability unique to spectroscopic methods and leads to microbial characterization in a manner currently not possible.

Since then the instrument development, spectroscopic theory/analysis, and scientific understanding have been supported by NASA, Army, DTRA, Navy, etc. and led to instruments such as the the version of the Deep UV Microscope that existed at the time this proposal. This was initiated, is a custom instrument built under a DURIP between the University of Southern California and Photon Systems Inc. (Figure 4-1). The base system is an Olympus microscope that has been modified with quartz optics and UV reflective optics, for excitation and transmission below 350 nm. It is coupled to two illumination sources, a 224.3nm HeAg, hollow-cathode laser for deep UV (DUV) epi-illumination and a white light source from Olympus attached to a secondary port on the microscope. This allows for acquisition of images illuminated by either the visible white light or deep UV laser source.

Proposed work and Results: This proposal had as its major goals the real-time quantification of microbes on solid surfaces without the use of stains, and the use of the DUV spectral system to gain insights into the past cultivation history of microbes. In order to accomplish these goals, improvements needed to be made in the microscope system that had been developed previously using funds from a DURIP grant (supplement to an AFOSR MURI proposal to KHN). Thus there were two major areas that needed to be addressed:

1. Technology – we proposed to upgrade the existing microscope
    a. The upgrades to the DURIP microscope focused on optimizing the balance between sensitivity, depth of focus, field of view, image quality, functionality (ease of used), and a means to enable additional functions currently incorporated in separate instruments. These included a motorized X,Y,Z scanning stage, new objective lens, higher quality illumination path (white-light and deep UV), RGB visible camera for contextual images, and re-architecture of the laser module for future spectroscopic upgrades.
2. Microbiology
    a. We proposed to use the improved microscope for rapid detection and quantification of microbes on surfaces without the need for staining

b. We proposed to test microbes for their spectral properties after growing various cultures under a variety of different conditions. When spectral changes were noted, we would then fractionate the microbial biomass to identify the molecular source responsible for the changes.
c. In response to a suggestion from the new PM (Rachel), we also initiated experiments to test the effect of DUV radiation on the survival of cells being identified using the DUV microscope.

Technology Development

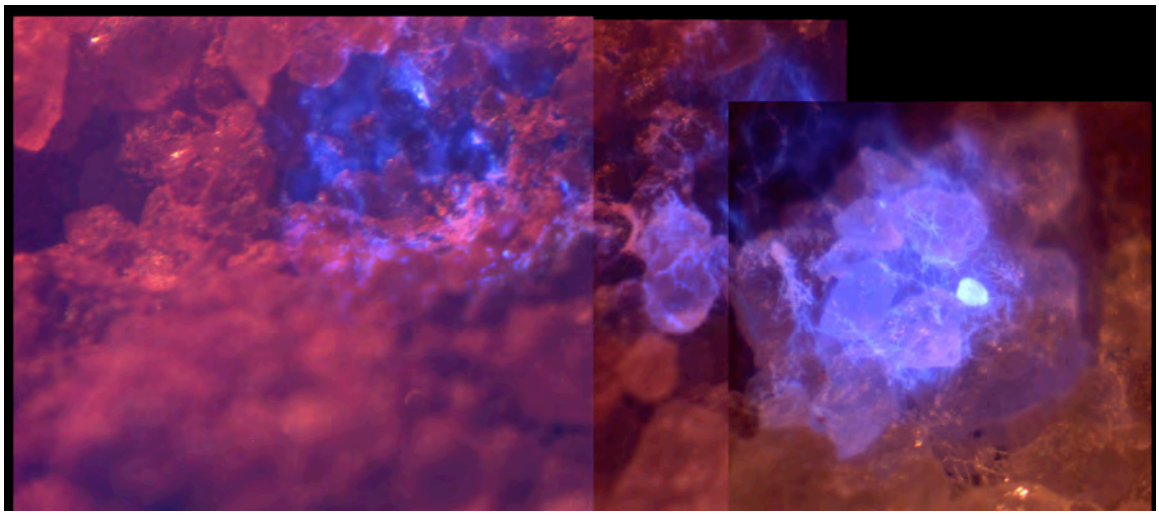

Figure 1. *First deep UV image of microbes on silicates from that Antarctic Dry Valleys. While the first "deep UV fluorescence microscope", it only observed in the near UV (350nm) – to visible spectral region.*

Over the last 12 years, the deep UV microscope has gone through 3 previous iterations of development before arriving at the current generation. The development began as a fortunate side-effect of deep UV resonance Raman spectroscopy. It began as a means of targeting where Raman spectra should be taken since the materials that were fluorescent would likely contain aromatic ring structures. Based on the broadband fluorescence features observed when targeting the early Raman spectrometer (figure 1), in 2002 JPL/PSI began to look into native fluorescence imaging in the wavelength bands where many of the microbial features and corresponding aromatic amino acids and proteins fluoresce (<400 nm). However at the time, the only available detector solution for these wavelengths were low efficiency (QE:0.15) ICCDs using Gen II Multichannel Plate (MCP)/phosphor screens. Much like a PMT (Photomultiplier Tube), photons that illuminate the detector are converted to photoelectrons and amplified by ~100-500x and bombards a phosphor that upconverts the energy to visible photon detectable by a CCD. Due to coupling losses between the components, the resulting image is not of the highest quality. However, with this instrumentation, we demonstrated the first deep UV native

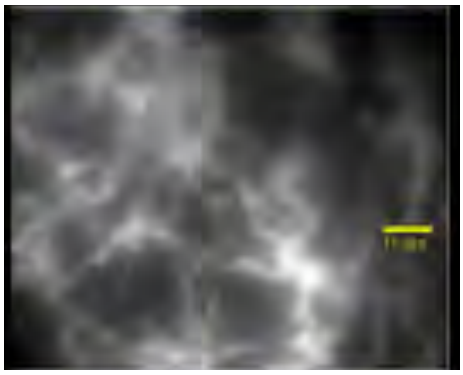

Figure 2. *Deep UV image of microbes on silicates from that Antarctic Dry Valleys using Gen 2 of deep UV microscopes. This observed the deep UV fluorescence emissions but was broadband rather than discrete banded emissions.*

fluorescence images of bacterial cells. Unfortunately, in addition to the detector, the optics available were not ideal. Since the optics used fused silica lenses, the change in index of refraction from the visible light used to focus the instrument in the visible was not the focus for the deep UV laser/UV images. In addition, since the images were collected over 300 - 380 nm, chromatic aberration effected the image quality. However the development led to an understanding of how improve the design and also demonstrated the proof of concept. Furthermore, it was the first incarnation of a deep UV multiband (hyperspectral) native fluorescence image (figure 2).

The 3rd generation of the microscope (figure 3) was the first time that a deep UV native fluorescence spectroscopy and imaging has advanced to providing a highly efficient means of obtaining spatial distribution of chemical composition without the need to physically or chemically interact with the sample (ie label free).

Figure 3. 3rd Gen Deep UV Native fluorescence Microscope.
The microscope is a custom design that enables native fluorescence imaging with epi-illumination using a 224.3nm laser (Photon Systems, Inc.). To reduce chromatic aberrations, an all reflective optical system was used. The detector is a photon counting UV sensitive EMCCD

To demonstrate the ability to image microbial cells on opaque surfaces, only one

spectral band centered at ~ 320 nm was needed (Bhartia et al. 2010). However, in order to uniquely distinguish cells versus organics and even differentiate cells based on aromatic amino acid content, multiple spectral bands are required; combining chemistry to morphology. In many ways, combining spectral bands to spatial information is similar to the early digital cameras that combined three visible colors, red, green, and blue. However while filter selections for digital cameras were driven by color reproduction to match what the reflectance information the eye observes, the deep UV hyperspectral mapping/imaging methods are more complicated, requiring more than three bands and false color representation. More importantly, the Rayleigh scatter that is being observed by a camera is more efficient than native fluorescence. Active spectroscopic methods, such as native fluorescence, provide their own illumination source and require some level of power/unit area to detect the spectroscopic response. For the deep UV native fluorescence instruments the illuminated diameter can range from 20 μm to as large as 7 mm; which diameter beam depends on a variety of factors including desired sensitivity, standoff distance, imaging versus mapping, feature size and scan area.

Filter Position	Filter and specifications
1	Open
2	C : 320 nm; 20 nm FWHM –Chroma Technology
3	C: 340 nm; 20 nm FWHM –Semrock
4	C: 360 nm; 40 nm FWHM –Chroma Technology
5	C: 387 nm; 11 nm FWHM – Semrock
6	C: 438 nm; 20 nm FWHM - Semrock

Table 1. Bandpass filters in the DUV Fluorescence Microscope.
C: Center Wavelength; FWHM: Full Width Half Maximum.

To illuminate the sample with either the deep UV laser or the white light source, a custom, 270 nm long-pass dichroic filter at 45 degrees injects the light on to the sample through a 52x reflective objective. The white light source is able to use this filter using the 2-4% broad band reflection inherent to the filter. A 52x reflective-objective lens is used to illuminate and collect the fluorescence without chromatic aberrations that would occur with a quartz compound lens. The objective lens is a Schwarzchild lens that observes a 150 x 150 μm area with white light illumination. However, with the DUV laser, a 30 μm diameter spot is illuminated.

White light reflectance and native fluorescence images are taken using a motorized 6 position filter wheel preceded by a 248nm long – pass filter to remove any DUV Rayleigh scatter from the sample. The filter wheel includes the filters described in Table 1. A DUV sensitive EMCCD (Electron multiplied Charge Coupled Device) was used for detection at all wavelengths stated in Table 1. The EM portion of this CCD allows the detector to amplify the signal prior to the A/D register. The result of this pre-digitization amplification is avoidance of read-noise that typically occurs with high frame-rate cameras. The native fluorescence images presented, use a constant electron multiplied

(EM) gain of 100x. Each image triggered by the DUV laser was gated to the pulse duration of the DUV laser with exposure time of 100 μs such that dark noise was reduced.

The upgrades to the DURIP microscope (figure 3) focused on optimizing the balance between sensitivity, depth of focus, field of view, image quality, functionality (ease of used), and a means to enable additional functions currently incorporated in separate instruments. These translate to 5 phases shown in Table 2. The Kohler illumination and visible camera path required for both higher contrast imaging and fewer visible artifacts to enable future integration of autofocusing capabilities, requires integration of custom interface plates. Additionally, the Olympus COTS components assume the use of an ocular module between the visible CCD and the deep UV imager. The inclusion of this extra module causes a mismatch in the back focal length of objective lens to the visible camera and the deep UV CCD focal planes resulting in an image z-plane shift between the detectors. However, given that the Z-axis is motorized, a software solution was to be developed to accommodate the image plane shift.

Table 2. *Deep UV Microscope Hardware Development Phases:*

Phase	Description	Expected Completion Date
1	Mechanical Integration and testing of XYZ stage	Feb 2012 (completed)
2	Mechanical Integration of white light Kohler illumination + Visible color camera, new reflective objective (36x) and necessary mechanical modifications for integration of new reflective objective.	Sept 2012
3	Initial testing of microbial spectral features (microbes grown by USC and Photon Systems) to refine number of bandpass filters required to enhance spectral separation.	Oct 2012
4	Integration and testing of next generation high speed filter wheel for rapid multi band image acquisition. Also includes optical modifications to avoid pixel shift.	Late Oct 2012
5	Integration of laser beam shaping optics to optimize illumination area – trade space between power densities necessary to detect versus cell death results. At this phase, the mechanical hardware portion is complete and the effort will be to integrate automation to enable a simplified image collection process.	Nov 2012

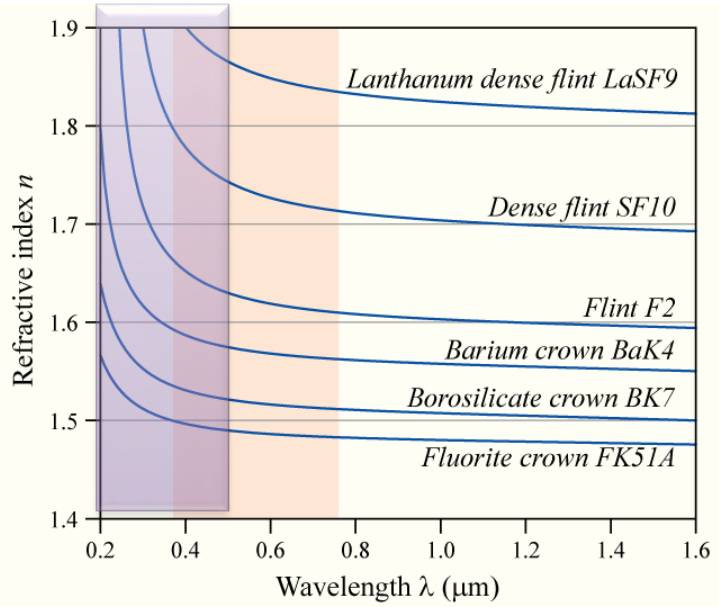

Figure 4. *Refractive index of some materials. The spectral region that the deep UV instruments operate over is highlighted in purple and corresponds to a region where the refractive index changes rapidly.*

A major change to the microscope was the use of a 36x reflective objective lens as opposed to the 52x objective lens. During the design phase, there was discussion of whether a non-reflective lens could be used. The limiting factor is the issue of chromatic aberrations stemming from index of refraction of traditional and non-tradition material options (figure 4). However the effective cost are complexities in alignment, limitation of magnifications, and limited field of views. There are optical lenses that attempt to correct for the rapidly changing refractive index, however, the focus between wavelengths of interest is ~5% of the focal length. Recent developments that Dr Bharitia has been leading on a NASA program has shown some new optical designs that may enable the use of lenses with wide field of views and enables a variety of magnification, however it is currently in there development phase – an initial version will be available in mid-2013 and while not optimized for the deep UV microscope, can be tested for imaging purposes.

Figure 5. *Completed Deep UV microscope*

While there are limitations, the 36x objective lens advanced the previous 52x lens in both image performance and ease of use. With the new lens, the calculated μm/pixels with the

estimated back focal length was ~170nm/pixel. Using a micrometer, the actual pixel resolution was ~185nm/pixel. The difference is likely in the estimation of the back focal length from 250 mm to 240 mm. The transitioning to this lens increased the depth of focus from <1μm to 1.2-1.5μm and to increase the working distance from 1.9mm to 8.6mm. The initial results also suggest that the 36x objective lens has a higher contrast ratio than the original 52x objective lens. This is likely associated to the unavoidable over-magnification occurring with the 52x objective lens.

As stated above, the microscope has been completed on schedule (figure 5).

Microbiological Experiments:

Current microbiological experiments have focused on the model organism *E. coli* K12. Cultures of these organisms have been routinely grown under controlled/constant environmental conditions in a chemostat (uniform growth rate) or in batch culture where substrate concentrations (and growth rates) are in flux throughout the growth cycle. Experiments were performed in M9 minimal media[1] with glucose as the limiting growth factor. Minimal media was utilized to mitigate the effects of background fluorescence that may occur in complex or protein rich media. A variety of growth conditions were testing including variation temperature for batch and chemostat cultures, and changes in hydraulic residence time (HRT) in chemostat cultures which functionally result in changes in growth rate. The major goal of this work was to compare the effect of growth conditions on the spectral responses and variability of spectral responses. Earlier work with batch grown *E. coli* cells suggested that there is a significant amount of cell-to-cell variability in spectral responses. We hypothesized that these are largely a result of variable growth conditions and rates in the batch system and that growth in chemostats could mitigate some of this variability. Additionally, by controlling growth rates in a chemostat, we hypothesized that we could begin to quantify the spectral that arise as a result of growth rate.

Effect of growth conditions on spectral response

As previously observed, batch grown *E. coli* samples exhibited significant variability in spectral features across cells imaged (Figure 16). In general, cell size and fluorescence intensity tended to vary to a greater degree in batch vs chemostat samples (personal observation, A. Rowe, example illustrated in Figure 6 A&B, D&E) which could be explained by the variability in growth conditions in batch compared to chemostat cultures. Quantifying the mean pixel intensity for a random selection of data points within each cell per image demonstrates that the variation observed (and/or standard deviation from the mean value) is up to six-fold larger in batch compared to chemostat samples (Figure 6 C&F): standard deviation range from 600-1400 and 100-450 for batch and chemostat cultures respectively.

[1] Sambrook, J., et al. "Molecular Cloning." 2nd Ed., Vol. 3. 1989

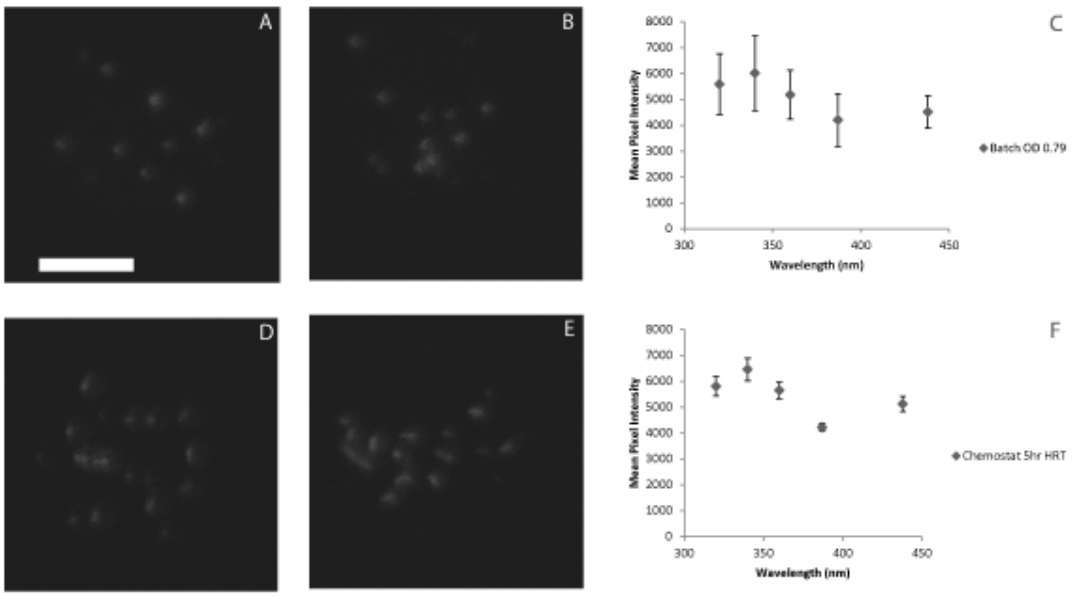

Figure 6. *DUV images for spectral emissions at 340 nm for batch (OD 0.79, A&B) and chemostat (5hr HRT 37^{o}C, D&E) grown E. coli cells. Emissions at 340 nm exhibited the greatest variability across samples. The mean pixel intensity for all cells observed for corresponding growth conditions (Batch 37^{o}C OD 0.79 [n=45] and 5hr HRT 37^{o}C [n=55]) in for emission spectra at 320, 340, 360, 387, and 438 are shown. Error bars depict standard deviations from the mean. Scale bar = 10µm.*

The variability in mean pixel intensities of emission spectra limits the ability to resolve significant differences in emission spectra with varying optical density (OD) (Figure 7). This is likely due to the variability of total spectral features at any given time within batch samples. Compared directly with data gleaned from chemostat cultures under similar growth conditions (i.e., 37^{o}C, M9 media 0.04% glucose), we observe not only the difference in standard deviations associated with each sample type, but we note that significant differences can be resolved between chemostat cultures grown at different rates for emission wavelengths of 320, 340 and 360 nm (Figure 7).

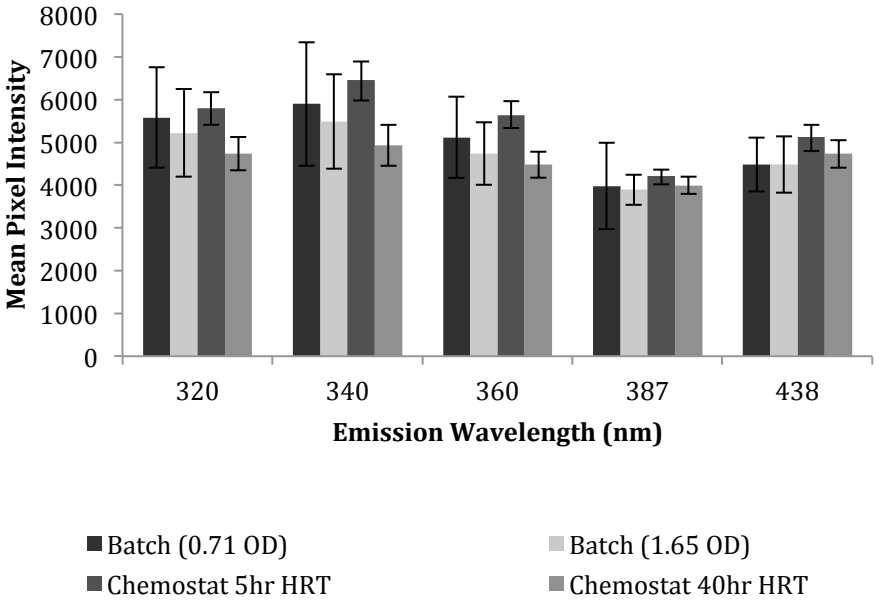

Figure 7. *Mean pixel intensity for sample batch and chemostat grown cells. Mean pixel intensity based on three random points per cell for n=45-60 cells. Error bars represent one standard deviation from the mean.*

These quantitative observations match the qualitative assessment performed early on comparing chemostat cultures grown at 30°C for 5, 10 and 20 hour residence times. Focusing on the 320, 340 and 360 nm wavelength emissions, we observed a distinct relative enrichment in 340 nm emission spectra (observed as enrichment in the red channel, Figure 8). We expect that this enrichment is likely due to an enrichment in tyrosine residues. However, direct determination of amino acid content is difficult to perform on bulk sample. Bulk quantification of tryptophan samples using the Acid-Ninhydrin method[2] did not resolve difference in tryptophan content for *E. coli* cells grown under various conditions that were not a result of differences in optical density (data not shown). Other methods, such as fast protein liquid chromatography, will be

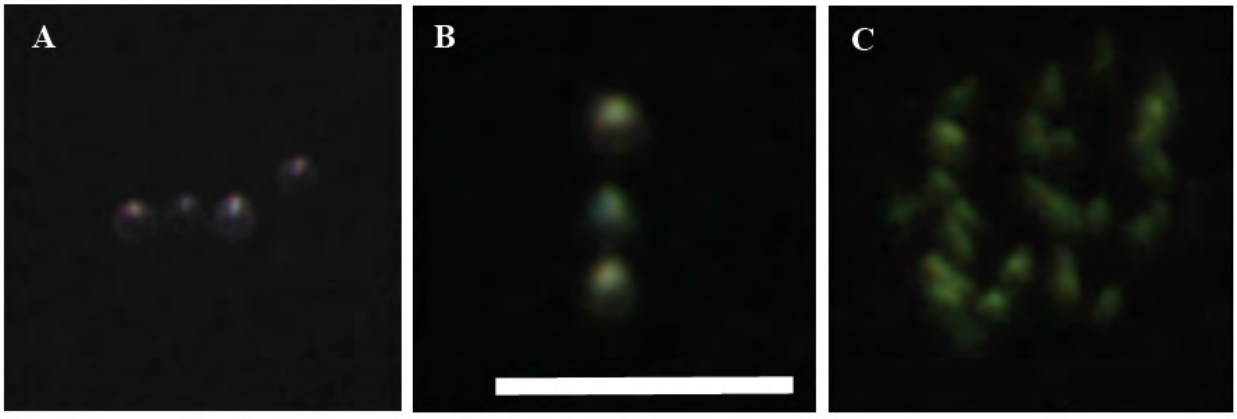

Figure 8. *Growth rate appears to affect the spectral characteristics of chemostat-grown cells. E. coli images taken with the deep UV microscope under various growth rates/conditions: chemostat culture grown at 30°C, and a 5-hour HRT (A), chemostat culture grown at 30°C, and a 10-hour HRT (B), and chemostat culture grown at 37°C, and a 20-hour HRT. Scale bar = 10μm.*

[2] Pinter-Szakacs, M, and I. Molnar-Perl, J. Agric.. Food Chem.1990, 38, 720-726

attempted in the future to begin to determine what factors are driving spectral shifts.

Future work along these lines will focus on comparing other bacterial species, grown under identical growth conditions to those utilized for *E. coli* growth in order to compare variation in spectral features across species. Though changes in the spectral features of *E. coli* have been observed, it remains to be seen if these trends are: 1) present in other bacterial species, and 2) affect the ability to distinguish bacterial species based on spectral features.

Spectral Features of Bacterial Cells (spores and species variability)

As stated earlier, the published data for deep UV images have used only one fluorescence emission band at 320 nm and for the most part only showed 0.4% of the total information. The latter aspect appears astounding, however it highlights a challenge of the image reconstruction process. For each band, the data acquired are 16-bit data (0-65535). However, for any given color ramp (black to gray or black to color of choice), the human eye can only perceive 256 shades. This compounded by the fact that there are six detection bands, each with 16-bits of data. Fortunately, for most samples the dynamic range of the signal does not span the entire 16-bits and therefore a smaller "window" can be mapped over 8-bits - thus losing less information.

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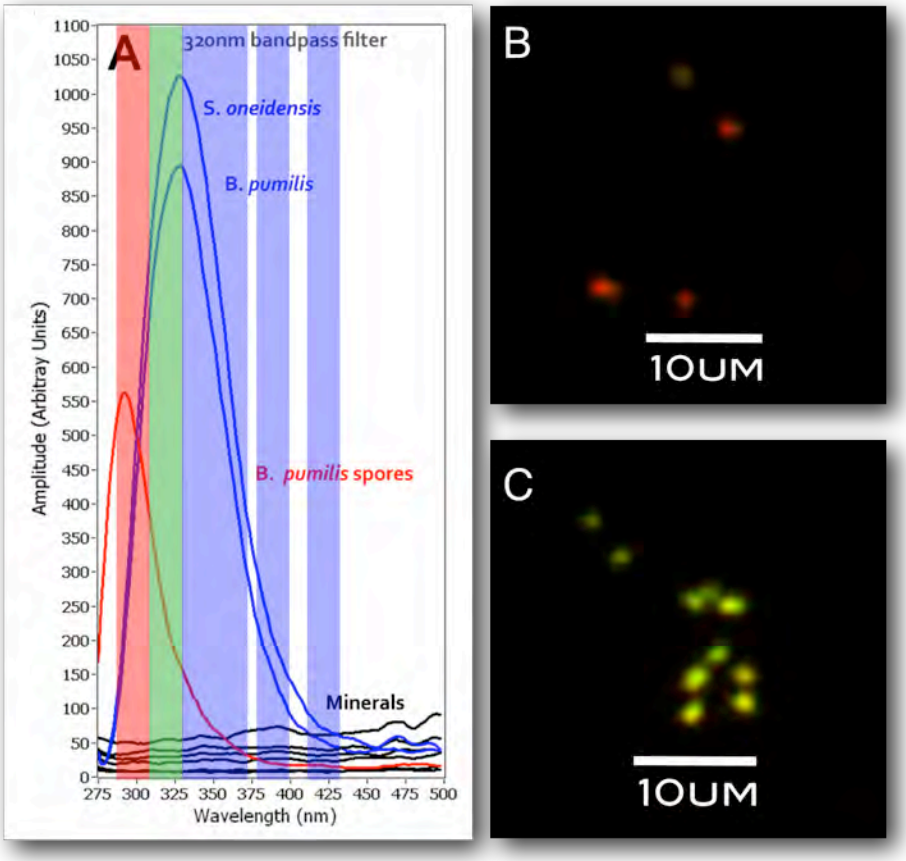


Figure 9. Color conversion for Deep UV Hyperspectral Images and Sample Images of Bacterial Cells and Spores
(A) 2D emission spectra of a variety of minerals and rocks. Red, Green and Blue bars show the spectral bands that were combined to color the fluorescence images. B) fluorescence image of bacterial spore; B. pumilis: C) fluorescence image of E. coli.

For the 224nm excited images, the RGB (red, green, and blue are the combination of 5 fluorescence bands: 320nm - red; 340nm: green; sum of 360 - 430 nm: blue). Spectral differences are indicated by color and show clear differentiability between cells and spores.

To form hyperspectral images, the band are colorized as show in figure 9A. Using this method, single ring organics, such as tyrosine and phenyalanine, appear red, tryptophan appears green and NADH and other flavins appear blue. Since the deep UV microscope images are colorized differently than the raster scanning maps, color comparisons are not possible.

Figure 9B is a deep UV native fluorescence image of *E. coli.* The green/yellow color suggests that the major emission is at 340 nm and associated to be dominated by tryptophan. However, there is a red component that is shifting the color to a light yellow, suggesting that tyrosine and phenylalanine contribute to the signature but to a lessser degree than tryptophan (trp). In comparison, figure 9C the bacterial spores fluorescence emission is shifted, a region coded red. The spectral shift results from a significant concentration of tyrosine and dityrosine and a lack of significant concentrations of tryptophan.

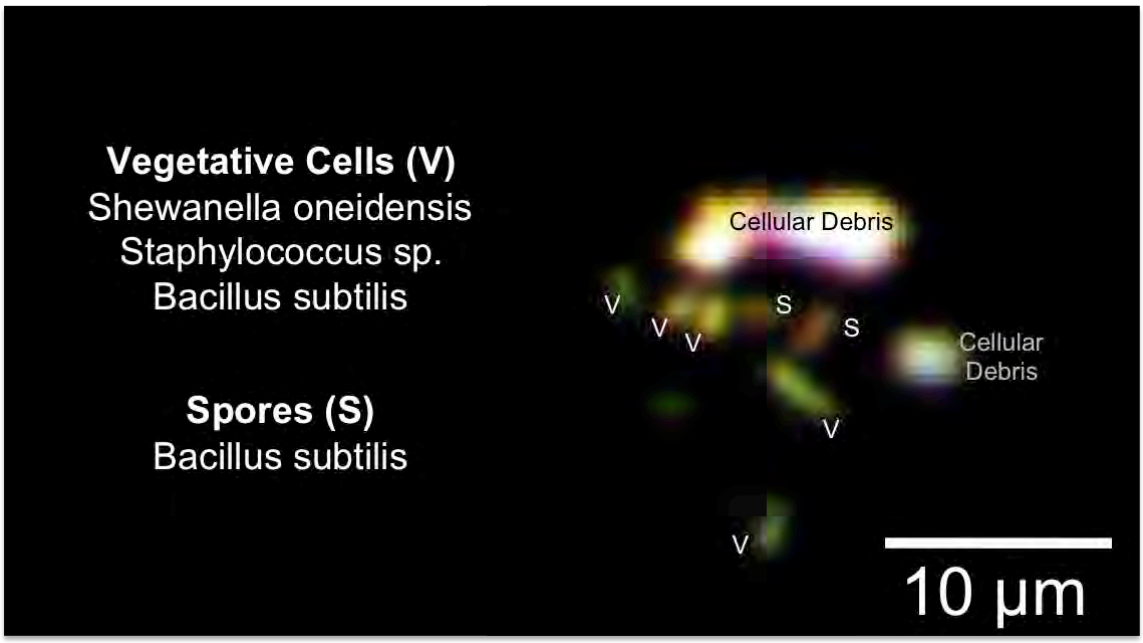


Figure 10. Deep UV Native Fluorescence Hyperspectral Image of Mixed Bacterial Cells
The bacterial cells shown are identified as spore or vegetative cell. Size/morphology would suggest that the Bacillus is the elongated green cell, Shewanella is the small green cell and Staphylococcus are the small yellow cells. The white regions are cell debris.

Figure 10 is a group of cells placed on an inert substrate and shows the variability of fluorescence emission for different cells of different genera. The current data analysis suggests that the variability is associated to aromatic amino acid variability that may correlate to the G+C% content of the cell. Given that there can be variations in these

amino acid concentrations as function of growth medium and rate, we have yet to determine the extent of classification with G+C% is possible.

Microbial Detection on Surfaces

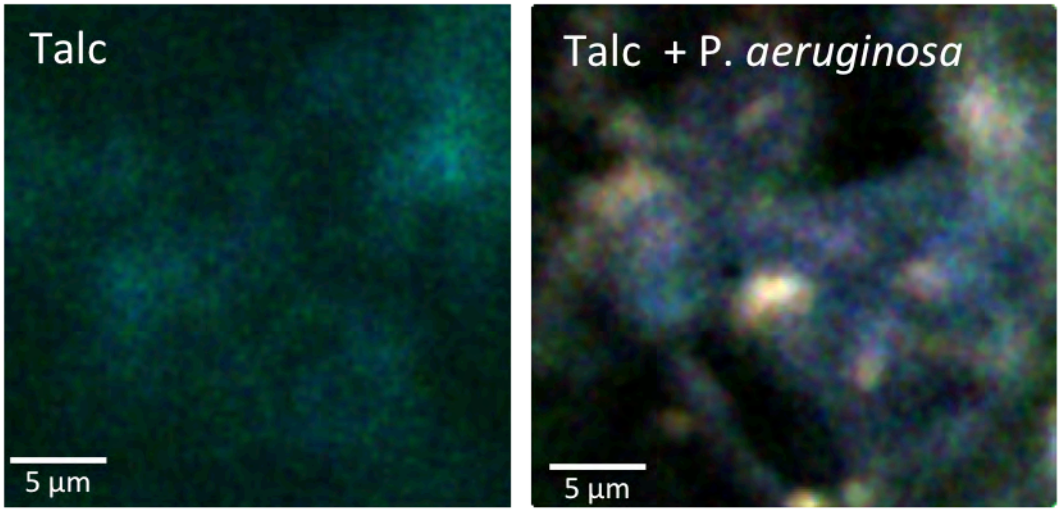


Figure 11. *Deep UV native fluorescence images of Talc (left) and Talc + Pseudomonas aeruginosa (right). The cells are distinct from the background fluorescence of the talc. The cells are 1-3 µm long bright features. The talc fluorescence is likely naturally occurring trace organics embedded in the talc (magnesium silicate) however these do not interfere with the fluorescence emission from the microbes. 150 nm spatial resolution.*

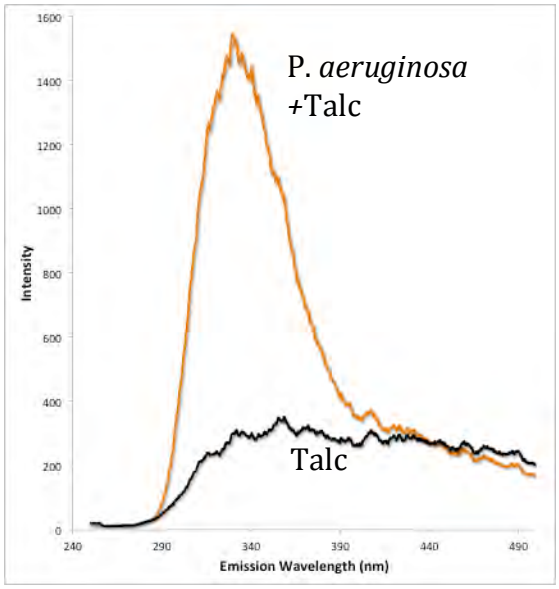


Figure 12. High resolution deep UV spectrum of Talc alone and Talc + P. *aeruginosa*. The number of cells in the field of view is <10 cells.

Detection of biological materials on surfaces requires a highly sensitivity methodology since the number of cells being observed can be much less than the total number of cells present in the sample. Additionally, the matrix/substrate surrounding the biological material can obscure the signal of the threat. Finally, the variations in sample topography of powders can be requires a focus tolerant optical design which prohibits the use of high numerical aperture optics traditionally used by Raman or fluorescence methods for biological detection. *Unlike NIR or visible Raman methodologies that have been previously suggested, the deep UV the native fluorescence and Raman spectral efficiencies are enhanced for proteins/toxins and microbes and allow for instrument*

designs that couple high sensitivity detection, without interference from the background, using focus tolerant optics.

Examples demonstrating the ability to detect biological materials on inorganic and organic backgrounds are shown in Figures 11-13. Figure 11 is a deep UV excited native fluorescence hyperspectral image that pictorially demonstrates the ability to detect microbes on an inorganic surface using the organics native to the bacterial cells[3]. Figure 12 is a high resolution native fluorescence spectrum from the image data in figure 11 and demonstrates the a 5:1 signal to *background* with <10 cells in the field of view. As expected, the distribution of microbes on the talc is extremely heterogeneous (observed by microscopic imaging) and in many talc grains, no cells are present. For perspective, given an average talc grain size of 10-20 μm, perfect distribution of microbes with a 1000:1 inorganic/biological ratio would lead to 1-2 cell/grain. With an interrogated volume of 1e-4 mm3 (~50 x 50 x 50 μm), there would be an average of 8 cells in the beam.

Figure 13 demonstrates the ability to detect a few number of cells (<10) on an organic background (growth media) using both native fluorescence and deep UV Raman spectroscopy. This is an important capability, as the media used by terroristic groups to grow the cells will not necessarily be removed and cells buried in this organic rich background need to be detectable. Additionally, the ability to detect a few cells in this organic rich media will mitigate false alarms from hoaxes that capitalize on easily available media but no biological component.

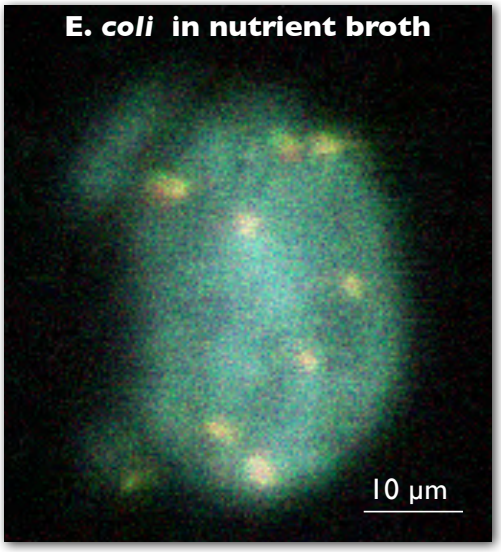


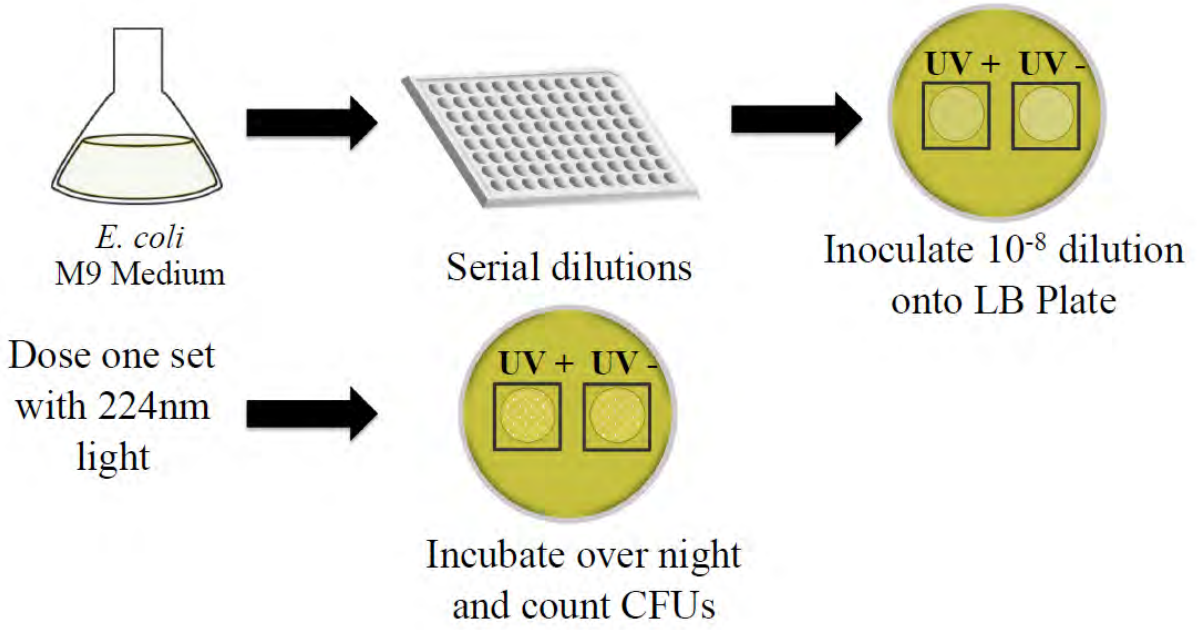
Figure 13. *A) Detection E. coli bacterial cells in media using Deep UV native fluorescence imaging showing that the defining spectral features associated to the microbes enable detection and differentiation of bacterial cells*

[3] DUV native fluorescence images in figures 2 and 4A were created using three spectral bands centered at 320, 340, and 360 nm colored red, green, and blue respectively.

Bacterial Survivability with DUV spectral analysis

In order to assess the effects of DUV exposure to cells, *E. coli* cells grown in M9 media were serially diluted and plated in triplicate (per dilution) on Luria Broth (LB) Agar plates (as illustrated in Schematic 1) . The effects of DUV light on colony forming units (CFUs) was quantified.

Schematic 1. *Protocol for conducting E. coli viability experiments.Cells were grown overnight in M9 medium. The overnight culture was serially diluted and two separate aliquots of the 10^8 dilution (of CFU per mL) were inoculated onto LB plates. One spot was dosed with 224nm light. The plates were incubated overnight, and CFUs were counted the following day.*



Half of the dilutions were exposed to a 224 nm laser either 30 or 200 µm in diameter and allowed to grow over night. Surprisingly, exposure to DUV light did not statistically effect the growth of CFUs (Table 3). The average cell numbers observed in the control samples (including the removal of an outlying data point*), fall within the range of cells observed in DUV exposed samples though a marginal (20%) decrease was observed. However, performing a Kruskal-Wallis ANOVA test demonstrated the no statistically significant difference was observed between experimental and control samples, even when including outlying data point in analysis (p values of 0.2 and 0.3 omitting and including outlier respectively).

Table 3. *CFU counts for culturability of DUV exposed samples. Raw data and averages presented. *indicates data point omitted as an outlier two standard deviations above the mean (14.7 ± 4).*

	Unscanned	**200 µm**	**30 µm**
Power Exposure (µJ/900µm2)	**0**	**0.01**	**0.35**
CFUs	13	12	10
	11	11	10
	15	12	14
	12		
	14		
	23*		
Average	13	11.7	11.3
Standard Deviation	1.6	0.58	2.3
Percent reduction in CFUs	0	20	23
Percent Uncertainty	12	3.5	12

Planned experiments for cell preservation and spectral analyses

Given that we have been able to observe spectral difference in cells grown under different growth conditions one of the natural next questions is how long until the environmental conditions that prevail during sampling begin to result in shifts in spectral features and more importantly are there methods we can use to preserve the spectral features of cells we want to quantify. This is especially important for the application of DUV microsopy to environemental samples, where sampling itself can alter the state or cause death or damage to cell by exposing them to abnormal or toxic environmental conditions (e.g., oxygen to an anaerobe). In order to assess the effects of different preservation and fixation methods we will be taking our well-characterized *E. coli* chemostat cultures and comparing the spectral features observed between living (unpreserved) samples and a variety of different methods for preservation including: flash freezing, freeze drying (lyophilization), and chemical fixation using compounds such as formalin, paraformaldehyde and ethanol. For each fixation technique, time coursed will be performed to observe the maintenance of spectral features over time.

Discussion:

The discussion of this proposal is primarily about what could have been. It is clear that the microscope that was assembled is a major improvement over the one that was used for the thesis work of Dr. Bhartia. It is easier to use, higher sensitivity (better SNR), increased depth of focus, wider field of view, better image quality (both UV and Visible), and has a means to enable additional functions currently incorporated in separate instruments. The original microscope, described in the articles cited above (Bhartia papers & Thesis) contain the details of the microscope that we began with, and the changes made are outlined in the attached monthly reports, as noted above.

One advantage we have with our collaboration is that between the three sites (USC, JPL, and Photon Systems), we have a wide array of equipment and facilities for microbiology, optical research, and assembly and testing. Using this combination of skills and talent on one hand, and excellent facilities on the other, we were able to rapidly push the technology forward. The system has attracted great interest from the scientific community around the world.

The things that now need to be done are in the category of "obvious" – especially as they were already objectives and goals of the five year proposal that was submitted:

1. Establish a data base of different microbes under different growth conditions
2. Analyze these data to see if a "history of cultivation" can be gleaned from such spectral data
3. When changes are seen, fractionate the cells to identify those components responsible for the spectral change(s) ***
4. Understand the effect of DUV radiation, and investigate the mechanism of killing.
5. Develop a system for cell fixation that leads to no spectral changes (or predictable changes) so that interactions with other labs can take place.

One of the tasks that requires additional attention is the means by which we identify the chemical/biochemical causes of the spectral variability. The analyses traditionally used are insensitive to the level of changes being observed at the single cell level; in bulk the effects are at the 2% levels. The supporting instrumentation at USC, JPL, Photon Systems, and other collaborating institutions in the LA area, have need means to perform the necessary analysis with the level of precision required.

In addition the last issue is an important one, as many labs are growing microbes under controlled conditions, and this is a time-consuming project in which extensive meta-data, if collected can lead to a systems approach to organism identification. We hope to eventually couple our efforts with those of many microbial laboratories, adding another method to detect and characterize microbes on surfaces.

While it was not a part of this proposed work, we are also developing a series of Raman systems for coupling to the microscope(s), so that more than just spectral data can be obtained, and this is an obvious, and important, step to move from visual imaging to chemical imaging.

Conclusions:

While the conclusions of the abruptly terminated 5yr project are minimal the 1.25 years of support continued to prove both the massive potential of deep UV native fluorescence spectroscopy for label-free microbial detection and characterization as well as a number of challenges in the technology and science. The five-year program with achievable goals was stopped after 1.25 years, and the best we can conclude is that it would have been clearly possible to achieve the goals that we set out to achieve. With the microscope in the Nealson lab (USC) , work will continue with the support of JPL and Photon Systesms at a low level (only supported by internal funding and work relevant to other funding agencies), with the hope to find another source of funding to support a highly focused effort that will continue to lead to new insights of what can be understood of microbial activity using deep UV spectroscopy.

Other Comments:

Personal comments: In nearly 40 years of research, I have never had an experience like this one – with funding not arriving for over a year after it was approved, with continuous changing of program officers, and an almost total lack of advice concerning reporting requirements: until we were advised that we need extensive reports written on a monthly basis. I am not against such reports in principle, but it should be made clear from the start so it can be budgeted. There was no one on our team with time to put together such reports without compromising the science or instrument development. In addition, from a biological perspective, this is invention of work – it often takes a month to just get a microbial culture system growing properly, so that one can believe the data that are obtained. Then to have it abruptly stopped with really no explanation was disheartening to say the least. I believe that this was a project with great potential applications in many areas of interest to the ARO, and I hope that someday, someone will continue it.

Other devices: As noted above, we have the advantage in this collaboration of having access to a wide variety of other spectral and optical equipment. It was asked in the final report to have details concerning these other devices so they could be assembled or purchased. Each of these was either purchased directly from a vendor (fluorimeter and Raman microscope), or developed at Photon Systems/JPL as part of other funded projects. Thus, we have NOT added details of the assembly of each instrument, as such information is the property of other funding agencies, and was not part of this proposal.

Related Presentations

- **KHN** presented DUV seminar at the Marine Biology Lab Microbial Diversity Course, summer, 2011.
- **KHN** presented DUV seminar at the USC Geobiology Course, summer 2011.
- **ES** presented lab demonstration of DUV system at USC Geobiology course, Summer 2011
- **KHN** presented DUV seminar at the Max Planck Inst. For Marine Microbiology in May of 2012
- **RB** finished his Ph.D. thesis work and passed his Ph.D. defense in November of 2011
- **WH** presentation on Deep UV instrumentation for Chem/Bio/Explosive detection at PITTCON March 2012
- **RB/WH** presentation on Deep UV detection of Chem/Bio/Explosive detection at Department of State March 2012
- **RB** invited paper/publication on Deep UV detection of organics for planetary science SPIE Security and Defense April 2012
- **WH** paper/publication on fusion of Deep UV native fluorescence and resonance Raman data for improved detection of Chem/Bio/Explosives at SPIE Security and Defense April 2012
- **RB/WH** FACSS/ScIX conference Oct 2012, invited presentation on fusion of deep UV native fluorescence and Raman spectroscopy for microbial detection and differentiation.

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Appendix 1: Interim/Monthly Reports

Monthly Technical and Funding Status Report – July 6 to Aug 3 2012

Executive Summary

During the month of July a contract signed between USC and Photon Systems on July 13 2012 for 95K with a period of performance (POP) from July to November 2012. This supports PSI efforts on spectral characterization of microbes and image acquisition with the microscope. Additionally it supports PSI's efforts to conduct microbial growth experiments with USC. This month highlights include partial completion of the next phase in the microscope hardware development, initial spectral and image comparisons of chemostat and batch cultures on both Deep UV microscope and a fluorescence spectrofluorimeter.

Task Activities

Metabolic State and Deep UV Signatures

Prior to this month, the chemostat and batch cultures of *E.coli* have not been growing well in the defined medium (M9). This defined media is preferred over nutrient rich versions to minimize effects on the intrinsic microbial spectral signatures. The efforts at USC and PSI during the month of July have resulted in bacterial growth. However, the cell densities are low (<<1e6 cells/ml) are are not amenable to microscopic examination. The effort during this coming month will focus on changing the carbon source concentration to alleviate this issue.

As stated in last telecon, we were attempting growth studied at USC and then transporting samples to PSI. However given the possibility of sample alteration during transport, USC has now moved a chemostat to Photon Systems and an initial

batch of E.coli are growing. Photon Systems has also initiated batch cultures in the same M9 medium for comparison. Data from both chemostat and batch cultures were recently acquired by PSI on the spectrofluorimeter and the microscope with results that are still being analyzed. However initial findings show a major spectral intensity difference between the two growth methods, where the batch cultures appear to have a higher quantum yield (fluorescence cross-section). With the microscopic banded images, there are apparent spectral differences between the two growth conditions where the batch cultures appear to have a more intense emission at 360 nm and 438 nm relative to the chemostat cultures. Additionally, the spectrofluorimeter data of batch samples that were started days apart started, there appears to be a red-shifting near UV emission that correlates to older cultures. These data may be showing the effect of cell age where batch cultures are older cells in stationary phase and the chemostat has continuously dividing (young) cells. However, at the moment this is speculative and further tests and more spectral and image data are required before conclusions can be made.

Strain Acquisition
Nothing new to report this month. However the stock supply of microbes at Photon Systems are show in Table 1.

Table 1.

Kingdom	Class	Genus	Species	Strain
Bacteria	γ-proteobacteria	*Shewanella*	*putrefaciens*	CN32
				W3-18-1
			oneidensis	MR-1
		Marinobacter	*aqueaolei*	VT8
		Escherichia	*coli*	K12
				-
		Pseudomonas	*putida*	F1
	Actinobateria	*Mycobacterium*	*vanballenii*	Pyr-1
	Bacilli	*Bacillus*	*pumilis*	-
			atropheaus	-
			subtilis	-
	Clostridia	*Clostridium*	*sporogenes*	-
Fungi	Agaricomycetes	*Phanerochaete*	*chyrsosporium*	-

Given the unique growth requirements for each of these microbes, current deep UV image and spectral analysis of all genera is not possible. For the initial studies the efforts are placed on the *E.coli* for ease of testing. We expect that transition to testing Bacillus species will occur in the next three months.

However, aside from the growth experiments, we will be using a subset of cells to understand viability after deep UV illumination. For these experiments, samples will

be illuminated by the 224.3 nm deep UV laser and viability will be assessed by both culturing and ATPase luminometry. Initial results should appear in Mid October.

Upgrades to the Deep UV Microscope

In response to discussions over last months telecon and previous discussions, we have broken down the deep UV microscope hardware development into a series of discrete phases that extend to November 2012 (Table 2). The development has progressed to an initial portion of phase 2. However integration of the Kohler illumination and visible camera path required for both higher contrast imaging and fewer visible artifacts to enable future integration of autofocusing capabilities, requires integration of custom interface plates. Additionally, the Olympus COTS components assume the use of an ocular module between the visible CCD and the deep UV imager. The inclusion of this extra module causes a mismatch in the back focal length of objective lens to the visible camera and the deep UV CCD focal planes resulting in an image z-plane shift between the detectors. However, given that the Z-axis is motorized, a software solution may be able to accommodate the image plane shift but a small design analysis in the next month will determine the complexity of the a mechanical solution and be used to assess the most cost/time effective path.

In addition to Kohler illumination, the new 36x reflective objective lens that was described at the February site visit was installed and tested. The calculated μm/pixels with the estimated back focal length was ~170nm/pixel. Using a micrometer, the actual pixel resolution was ~185nm/pixel. The difference is likely in the estimation of the back focal length from 250 mm to 240 mm. The reason for transitioning to this lens was to increase the depth of focus from <1μm to 1.2-1.5μm and to increase the working distance from 1.9mm to 8.6mm. The initial results also suggest that the 36x objective lens has a higher contrast ratio than the original 52x objective lens. This is likely associated to the unavoidable over-magnification occurring with the 52x objective lens.

Table 2. Deep UV Microscope Hardware Development Phases (Photon Systems):

Phase	Description	Expected Completion Date
1	Mechanical Integration and testing of XYZ stage	Feb 2012 (completed)
2	Mechanical Integration of white light Kohler illumination + Visible color camera, new reflective objective (36x) and necessary mechanical modifications for integration of new reflective objective.	Sept 2012 (partially complete)
3	Initial testing of microbial spectral features (microbes grown by USC and Photon Systems) to refine number of bandpass filters required to enhance spectral separation.	Oct 2012
4	Integration and testing of next generation	Late Oct 2012

	high speed filter wheel for rapid multi band image acquisition. Also includes optical modifications to avoid pixel shift.	
5	Integration of laser beam shaping optics to optimize illumination area – trade space between power densities necessary to detect versus cell death results. At this phase, the mechanical hardware portion is complete and the effort will be to integrate automation to enable a simplified image collection process.	Nov 2012 (End-date of current PSI contracts from USC)

The microscope software control development schedule will be presented in an extended Gantt chart in the annual report.

Deep UV Data Analysis and Correlation to Analytical Instruments
As stated in the *Metabolic State and Deep UV Signatures* task, the *E.coli* samples from multiple batch and the chemostat cultures were scanned on a commercial spectrofluorimeter that resides at PSI. This instrument provides high resolution (2nm spectral resolution) excitation/fluorescence emission maps and 2D fluorescence spectra (0.2 nm spectral resolution) at selectable excitation wavelengths that can be used to determine the optimum excitation and emission bands that can be used differentiate the samples of interest.

The current purpose of the high-resolution spectral data is two-fold. In the near-term, it will be used to determine whether additional emission band are necessary to capitalize off of spectral information in more narrow bands or emission bands that extend to 600 nm. Additionally the data provides a means to correlate spectral trends observed in the microscope; example of which is the spectral anomaly's that appear and shift with older batch cultures.

There is also a long-term goal to develop a deep UV native fluorescence database for every microbe and for every growth condition we run. This is not a high priority task but it is important to realize that data collection uses standard protocols that were formulated in 2008 and therefore relatable to all previous datasets. We plan on incorporating these types of protocols for the deep UV microscope to support future development of spectral and morphological analysis of microbes.

Schedule

Key Events in July and for August to October 2012

Contract from USC to PSI for 95K; POP July – Nov 2012	7/13/2012
Chemostat operational at PSI	~7/25/2012

Phase 2 (partial) Microscope operational	7/31/2012
Monthly Report	8/6/2012
Monthly Telecon (Status Review Meeting)	8/10/2012
Annual Report	8/17/2012

Deliverables and Milestones Status for Aug to Oct 2012 Reporting Periods

Deliverable/Milestones	Expected Date	Responsible Institution(s)
Annual Report	Aug 2012	PSI/USC
Increase cell density for experiments by changing the carbon source concentrations	Aug 2012	PSI/USC
Implement Solution for Image plane shift between UV and visible imaging detectors	Aug 2012	PSI
Collect spectral/image data on exponential phase batch cultures and chemostat cultures to better understand observed spectral shifts	Aug 2012	PSI
Integration of the Kohler illumination and visible camera path	Sept 2012	PSI
Begin batch culture work on Bacillus species	Sept 2012	PSI
Refinement of number/position of bandpass filters on deep UV Microscope	Oct 2012	PSI
Cell viability assay post deep UV illumination	Oct 2012	PSI
Integration and testing of next generation high speed filter	Oct 2012	PSI

Cost Information

Cost Status

(all values are stated in total burdened costs)

At Photon Systems there have been two separate contracts from USC in the amounts of 150K (contract start date Oct 2011) and 95K (contract start date July 2012). For the first 150K contract, 83% of the funds have been expended and the remaining funds are $26,993. There were no charges in the month of July. From the new 95K contract, 20% ($18,669) was expended in July with $76,331 remaining to be expended by November 2012.

The breakdowns of cost by task are shown below and for the moment, include only the Photon Systems expenditures.

Metabolic State and Deep UV Signatures
The Photon Systems portion of this task is being supported by the new $95K contract from USC. The efforts placed on this task in July totaled ~$5400.

Upgrades to the Deep UV Microscope
This task is supported by two separate contracts from USC to Photon System one was an initial $150K for the design, acquisition of the hardware, and included development of the program plan. The 95K contract in July from USC to Photon Systems also partially supports this development task in terms of integration, optical/mechanical enhancements, and software control development. For the efforts in July, Photon Systems expended ~$11800 from $95K contract.

Strain Acquisition
No Effort was placed on this task other than the time to inventory the Photon Systems Stocks supply.

Deep UV Data Analysis and Correlation to Analytical Instruments
For July efforts data collected on the spectrofluorimeter and spectral analysis efforts on microbial batch and chemostat samples, Photon Systems expended ~$1000 from the $95K contract.

Monthly Technical and Funding Status Report – August 4 to September 7 2012

Executive Summary

During the month of August, progress was made in both hardware development and cell viability experiments. Improvements in hardware modifications focused on the design of the adapter plate to integrate the new dedicated white light Kohler illumination module with the current deep UV illumination path.

During the last teleconference there was an expressed desire to acquire microbial viability as a function of the deep UV laser. While there was an experimental design using ATPase was discussed over the telecon, there was a necessary change to provide some preliminary results while the details of the ATPase assay were better understood. Thus the results presented are more appropriately referred to as "culturability curves" for *E. coli* as a function of laser power on the target. Initial results indicate a modest effect on cell culturability.

In addition, new deep UV microscope images with higher cell densities in batch cultures were obtained via modifications in medium composition. Increased cell densities with the chemostat growths were also attempted with mixed success.

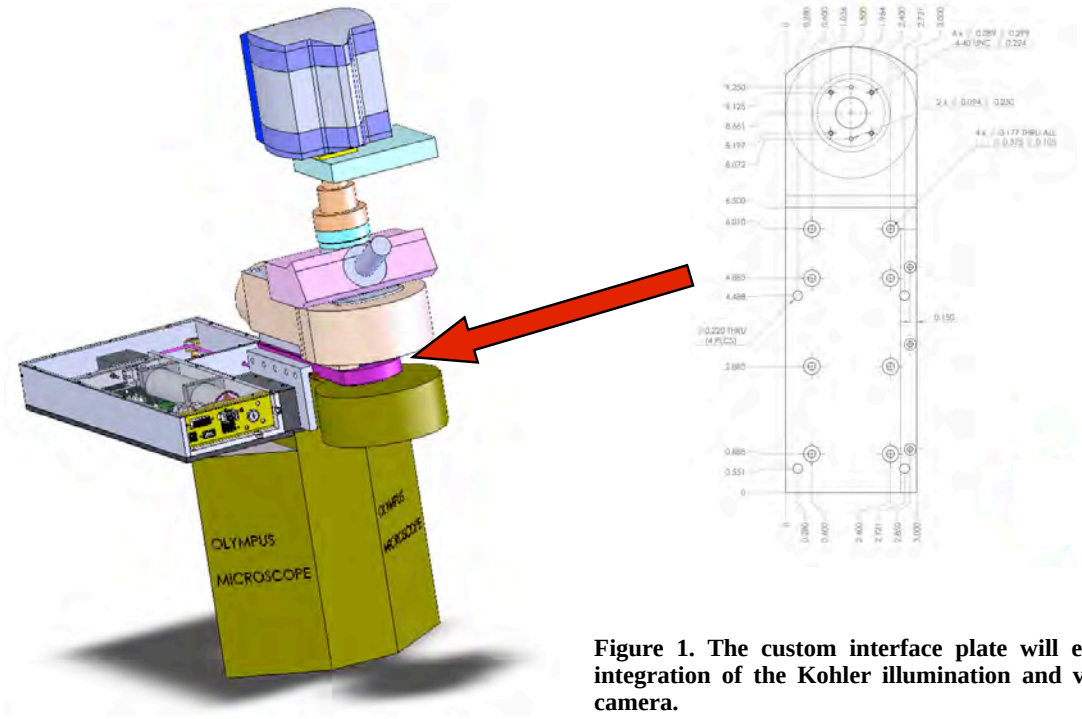


Figure 1. The custom interface plate will enable integration of the Kohler illumination and visible camera.

Task Activities

Upgrades to the Deep UV Microscope

As discussed in last month's report, custom interface plates are required for integration of the Kohler illumination and visible camera, as well as enabling future integration of autofocusing capabilities. Design of these custom interface plates has been completed

(Fig. 1). Fabrication and installation should be completed by mid-September 2012.

Design analysis concerning the mismatch in the back focal length of the objective lens to the visible camera and the deep UV focal planes has been placed on hold until the interface plate has been incorporated.

This month's work effort also included training A. Rowe (Postdoctoral Scholar, USC) on the current microscope. While the procedure for using the microscope is still manual and intensive, training A. Rowe is providing user feedback that will enable further improvement in human factors and control software ergonomics.

Metabolic State and Deep UV Signatures

As stated in the previous report, chemostat and batch cultures of *E. coli* were not achieving high population densities using a published recipe for M9 medium. Culturing efforts at USC and PSI during this month have focused on amending the medium composition to increase cell densities. Using an M9 recipe from Sambrook (Table 1), cell densities > $1x10^8$ cells/mL were achieved in chemostat cultures, and densities ca.$1x10^9$ cells/mL were achieved in batch cultures. These densities are more amenable to examination with the deep UV microscope, and initial imaging work has been carried out with this standard medium composition. The work effort for this coming month will be to assess the spectral uniformity of cells in chemostat cultures using the standard M9 medium.

Table 1. Composition of M9 medium

Component	in 1L of ddH_2O, pH = 7.0
Na2HPO4	12.8 g
KH2PO4	3.0 g
NaCl	0.5 g
NH4Cl	1.0 g
1M MgSO4	2.0 mL
0.1M CaCl2	1.0 mL
0.1M FeSO4	1.0 mL
20% Glucose	10.0 mL
LPT solution*	5.0 mL

*Per 1L: 20g L-leucince, 20g L-proline, and 0.05g Thiamine
Recipe from: Sambrook, J., et al. "Molecular Cloning." 2nd Ed., Vol. 3. 1989

We will also begin work comparing cells from chemostats to cells in batch culture. This work will aim to determine if there is a growth stage in batch cultured-cells that is spectrally similar to chemostat cultures. As reported previously, data collected from chemostat and batch cultures suggest differences in the quantum yield (fluorescence cross-section) between the two systems. However, this data was collected when the batch cultures were at high density. At this point, the cells in a batch culture would be in stationary phase and anaerobic. It is possible that there may be similarities in quantum yield and spectral structure at earlier growth stages.

During this month's telecon, the issue of cell damage as a function of deep UV exposure was discussed. There was a a discussion on the use of ATPase assays to determine whether the deep UV led to cell death or effects in culturability as a response to a stressed induced state. However, the experimental protocol that was discussed had cells be placed into a 96 well plate and irradiated with a dose equivalent to the deep UV microscope methods of cell imaging. Since the power density for the deep UV microscope is ~ 200pJ/cm2 (or using the the illuminated diameter: 191 nJ/900 μm^2), illuminating a single well of the 96 well plate would require 60,000 pulses. Given the pulse rate, this would take a little over 3 hours to reach the microscope equivalent dose; a timeframe in which

the cells can repair themselves and have many doublings.

As a result of these calculations and additional concerns of the the precision of the ATPase assay, we focus on an experiment that provided some preliminary analysis on the effect of the deep UV on culturability. This was accomplished by dosing *E. coli* cells with varying pulses from a 224nm laser. *E. coli* cells were inoculated into M9 medium and grown overnight in batch culture; cell counts indicated that the batch culture densities were approximately $1x10^{9}$ cells/mL. Triplicate samples from the overnight cultures were then serially diluted in a 96 well plate using M9 medium with no carbon source (Fig. 2). The 10^{-8} dilutions were used to inoculate LB plates so as to have a countable number of colony forming units (CFUs). Two aliquots were spotted onto each plate: one to be UV exposed, and a non-exposed control. Because the aliquots applied to the LB plates dried in approximately 8mm diameter spots, the area over which the cells dried was rastered with a laser to ensure that every cell received an equivalent UV dose. A Microbial and Organic Surface Analyzer/Image Reconstructor (MOSAIC, Photon Systems, Inc.) was used to raster scan the surface.

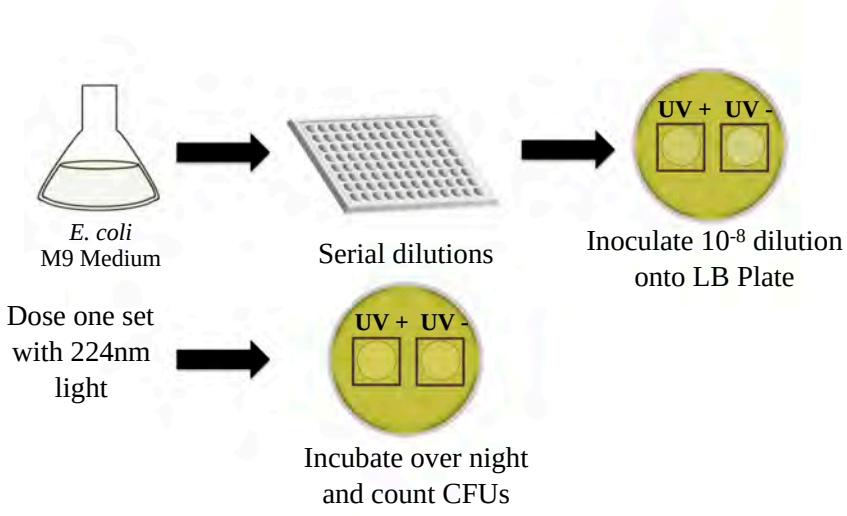


Figure 2. Protocol for conducting *E. coli* viability experiments. Briefly, cells were grown overnight in M9 medium. The overnight culture was serially diluted and two separate aliquots of the 10^{-8} dilution were inoculated onto LB plates. One spot was dosed with 224nm light. The plates were incubated overnight, and CFUs (Colony Forming Units) were counted the following day.

MOSAIC is a scanning system that incorporates a Targeted Ultraviolet Chemical Sensor (TUCS, Photon Systems, Inc.) to interrogate surface samples. The TUCS uses the same 224nm laser found in the deep UV microscope, however, because of the optical design, the energy from a TUCS is applied over a much larger area (~200 μm). As such, targets on the microscope stage receive approximately 40 times more energy than targets excited with a TUCS. To approximate the dosage received from the microscope using MOSAIC, each area was scanned with an 85% percent overlap, and the laser pulsing at 5 Hz. The overlap, coupled to the instrument scan speed and the laser pulse rate increased the does in each 200 μm spot by 49x. While this experimental design did not deliver the dosage all at once, it was reasoned that the frequency of the laser and the scan time did not allow

sufficient time for cellular repair mechanisms to respond to incurred cell damage. A second set of scans with no overlap were also run to provide a baseline for how a single pulse from the laser would affect colony growth.

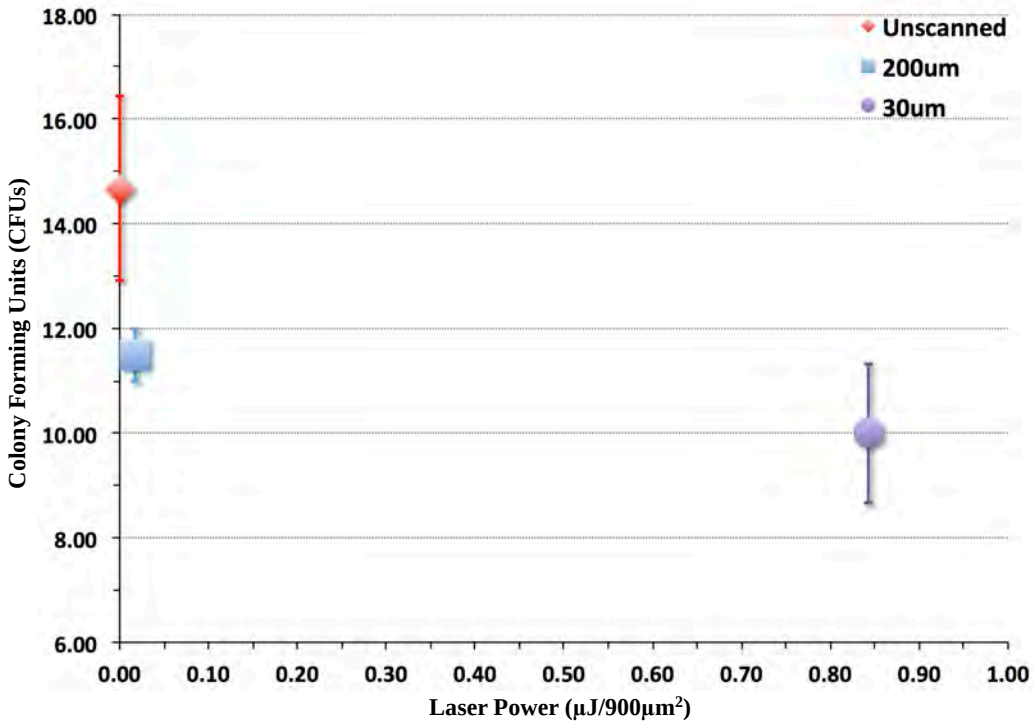


Figure 3. *E. coli* displays a modest reduction in colony formation as a function of laser power. Colony formation decreases asymptotically as a function of laser power on the surface. Colony forming units were counted from the 10^{-8} serial dilutions. Red diamond = undosed colonies; blu square = colonies dosed with 0.02 µJ/900µm^2; purple circles = colonies dosed with 0.84 µJ/900µm^2. Error bars indicate one standard deviation from the mean.

Table 2. Percent reduction in CFUs as a function of laser power

Power on Sample (µJ/900µm^2)	0	0.02	0.84
Percent reduction in CFUs	0 +/- 12.03	21.59 +/- 4.35	31.82 +/- 13.33

Cultures exposed to UV excitation show an asymptotic decrease in the number of CFUs (Fig. 3, Table 2). There is a 20% drop in the number of colonies formed in the areas exposed to a 200µm laser spot, which is equivalent to 0.02 µJ/900µm^2 of power on the sample. An increase in the laser power to 0.84 µJ/900µm^2 results in another 10% decrease in colony formation. It is not clear why the decrease in CFUs between 0.02 µJ/900µm^2 and 0.84 µJ/900µm^2 is not as marked as the decrease between the undosed and dosed populations, nor is the biochemical cause for the decreased colony formation currently understood. Although UV exposure may have killed the cells, it is also possible that UV exposure has induced a physiologic state that is akin to what has been described as viable but not culturable (VBNC). Cells in a VBNC state have typically been exposed to some external stressor resulting in cells that retain some metabolic capability, but no longer divide.

Future work in this area will involve conducting ATPase assays and live-dead cell staining on both UV-exposed and non-exposed cells to determine if the decrease in CFUs

for the UV-exposed populations is due to destruction of cells or induction of an altered physiologic state due to UV exposure. We will also alter the experimental design so that the samples are placed on a quartz slide prior to exposure. While this may increase the overall cell loss due to desiccation, it will more closely approximate the conditions currently used for collecting samples with the deep UV microscope.

chedule

As stated in the previous report, a Gantt chart for the remainder of PSIs contract has been constructed, showing the microbe control and development schedule (Fig. 4).

Key events and milestones for August through November 2012

Event/Milestone	Expected Completion Date	Responsible Institution
Annual Report	Sept 2012 (Completed)	PSI/USC
Monthly Report	Sept 2012 (Completed)	PSI/USC
Growth Medium Optimization	Aug/Sept 2012 (Completed)	PSI/USC
Mechanical integration of white light Kohler illumination	Sept 2012	PSI
Assessment of variations in spectral signature as a function of nutrient composition	Oct/Nov 2012	PSI/USC
Begin batch culture work on Bacillus species	Oct 2012	PSI
Effect of Deep UV exposure on cells	Oct 2012	PSI
Installation of visible color camera	Oct 2012	PSI
Monthly Report	Oct 2012	PSI
Integration and testing of of next generation filter wheel	Nov 2012	PSI
Testing of microbial spectral features	Nov 2012	PSI
Characterization of Bacillus species with Hitachi F4500 Spectrofluorimeter	Nov 2012	PSI
Monthly Report	Nov 2012	PSI/USC

Cost Information

Cost status (all values are stated in total burdened costs)

The cost status information discussed below includes only expenditures by Photon Systems, Inc. (PSI).

At PSI there are two separate contracts from USC in the amounts of $150K (contract start date Oct 2011) and $95K (contract start date July 2012). For the first $150K contract, 90% of the funds have been expended and the remaining funds are $14,707. From the second $95K contract, 29% ($27,369) was expended in August with $48,962 remaining to be expended by November 2012.

Upgrades to the deep UV microscope

This task is supported by two separate contracts from USC to PSI. The first contract was

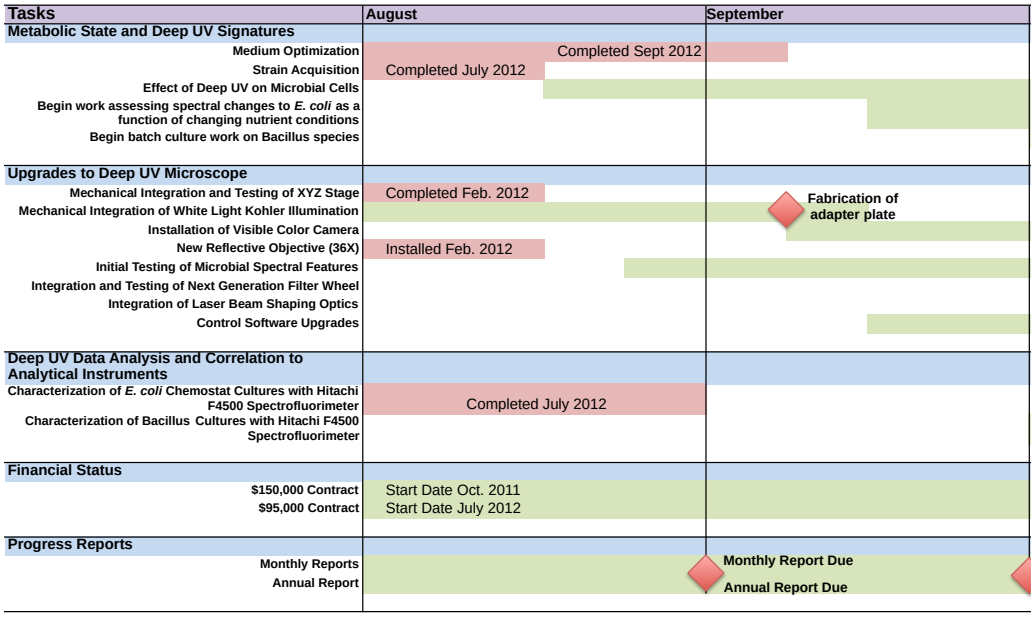
an initial $150K for design work and hardware acquisition. This contract also included development of the program plan. For efforts in August, PSI expended approximately $10,000 from the $150K contract.

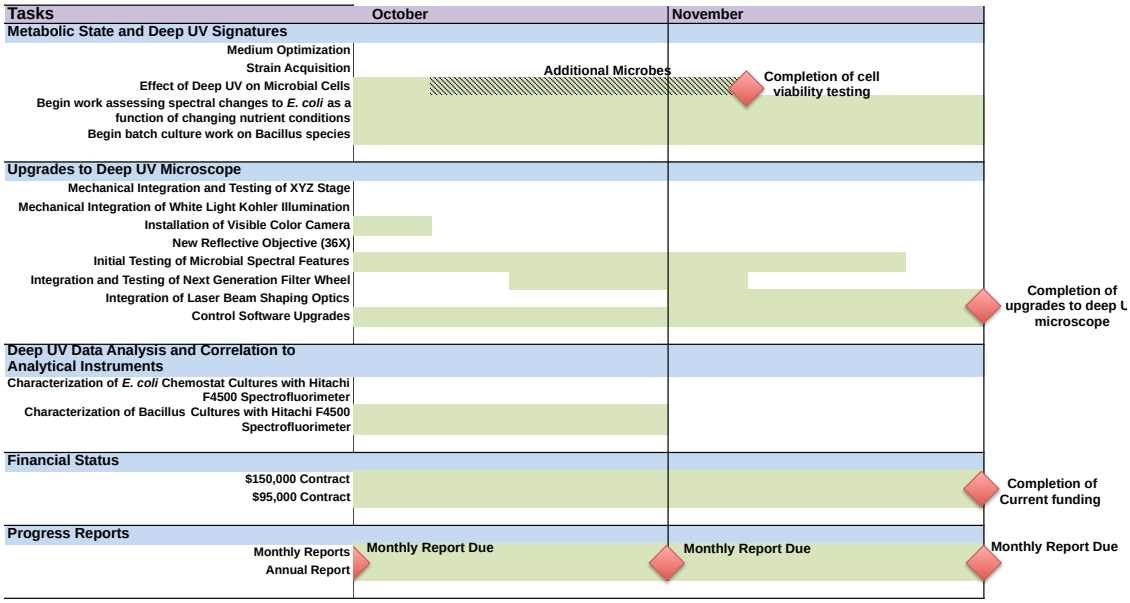
The $95K contract in July from USC to Photon Systems also partially supports this development task in terms of integration, optical/mechanical enhancements, and software control development. For the efforts in August, Photon Systems expended approximately $9,700 from the $95K contract.

These costs include work done reconditioning the 224nm laser source, design and fabrication of the Kohler illumination adapter plate and design analysis of the mismatch between the visible camera and deep UV CCD focal planes.

Chemostat work, viability experiments and training

For August, approximately $17,669 was expended at PSI from the $95K contract for conducting experimental work related to chemostat cultures, conducing cell viability experiments, and training for USC personnel on the deep UV microscope.


Tasks
August
September
Metabolic State and Deep UV Signatures
Medium Optimization
Completed Sept 2012
Strain Acquisition
Completed July 2012
Effect of Deep UV on Microbial Cells
Begin work assessing spectral changes to E. coli as a function of changing nutrient conditions
Begin batch culture work on Bacillus species
Upgrades to Deep UV Microscope
Mechanical Integration and Testing of XYZ Stage
Completed Feb. 2012
Mechanical Integration of White Light Kohler Illumination
Fabrication of adapter plate
Installation of Visible Color Camera
New Reflective Objective (36X)
Installed Feb. 2012
Initial Testing of Microbial Spectral Features
Integration and Testing of Next Generation Filter Wheel
Integration of Laser Beam Shaping Optics
Control Software Upgrades
Deep UV Data Analysis and Correlation to Analytical Instruments
Characterization of E. coli Chemostat Cultures with Hitachi F4500 Spectrofluorimeter
Completed July 2012
Characterization of Bacillus Cultures with Hitachi F4500 Spectrofluorimeter
Financial Status
$150,000 Contract
Start Date Oct. 2011
$95,000 Contract
Start Date July 2012
Progress Reports
Monthly Reports
Monthly Report Due
Annual Report
Annual Report Due


Tasks
October
November
Metabolic State and Deep UV Signatures
Medium Optimization
Strain Acquisition
Additional Microbes
Effect of Deep UV on Microbial Cells
Completion of cell viability testing
Begin work assessing spectral changes to E. coli as a function of changing nutrient conditions
Begin batch culture work on Bacillus species
Upgrades to Deep UV Microscope
Mechanical Integration and Testing of XYZ Stage
Mechanical Integration of White Light Kohler Illumination
Installation of Visible Color Camera
New Reflective Objective (36X)
Initial Testing of Microbial Spectral Features
Integration and Testing of Next Generation Filter Wheel
Integration of Laser Beam Shaping Optics
Control Software Upgrades
Completion of upgrades to deep U microscope
Deep UV Data Analysis and Correlation to Analytical Instruments
Characterization of E. coli Chemostat Cultures with Hitachi F4500 Spectrofluorimeter
Characterization of Bacillus Cultures with Hitachi F4500 Spectrofluorimeter
Financial Status
$150,000 Contract
$95,000 Contract
Completion of Current funding
Progress Reports
Monthly Reports
Monthly Report Due
Monthly Report Due
Monthly Report Due
Annual Report

Monthly Technical and Funding Status Report – September 07 – October 07, 2012

Executive Summary

During the month of September, progress was made in both hardware development and cell imaging work. Hardware modifications include installation of the adapter plate that enables mechanical integration of white light Kohler illumination with the current deep UV Kohler illumination. Imaging work progressed on two fronts: 1) as part of our effort to image different bacterial strains on different substrates, four different strains and a spore were imaged in talc mixtures on steel substrates; 2) *E. coli* grown in chemostats under different residence times and temperature regimes were collected and imaged. Results of bacterial fluorescence on talc mixtures will be compared to fluorescence from pure samples to determine the influence of talc on microbial spectral signature. Results from the chemostat work will be will be analyzed in the coming month to assess any spectral variation as a function of growth conditions. Finally, the viability experiments carried out in August were given a more thorough statistical treatment. This work suggests that there is so statistically significant difference between control and dosed populations.

Task Activities

Hardware modifications

As discussed in the previous two monthly reports, custom interface plates are required for integration of the Kohler illumination and visible camera, as well as enabling future integration of autofocusing capabilities. Fabrication was completed in late September, and installation was completed at the end of September (Fig. 1).

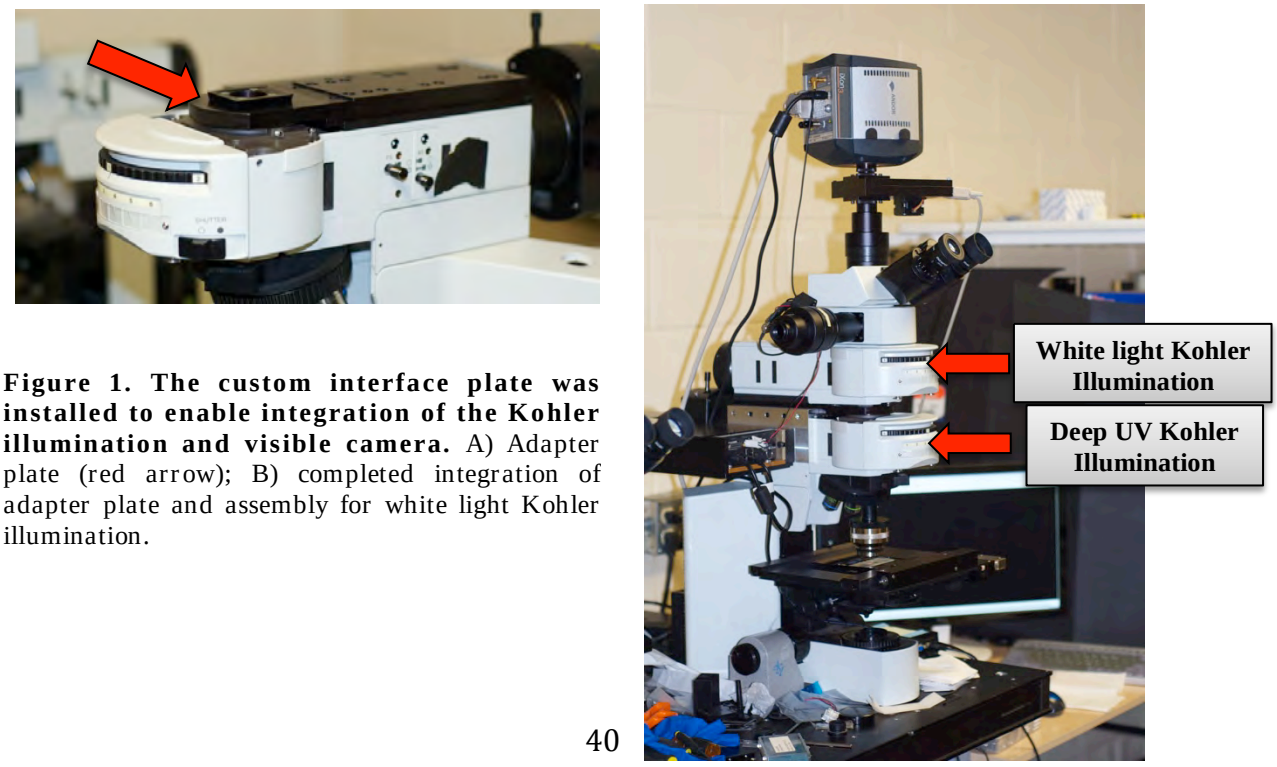


Figure 1. The custom interface plate was installed to enable integration of the Kohler illumination and visible camera. A) Adapter plate (red arrow); B) completed integration of adapter plate and assembly for white light Kohler illumination.

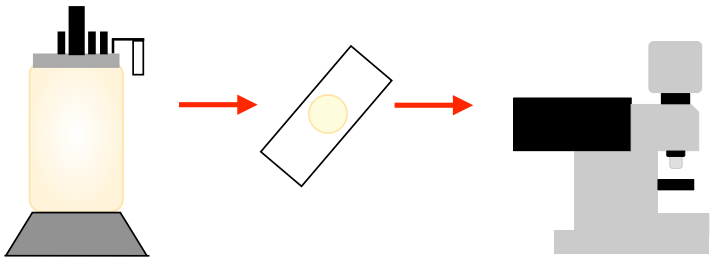


Figure 2. Chemostat samples grown under different growth conditions were imaged with the deep UV microscope. Aliquots of media were taken directly from the chemostat and imaged to collect fluorescence spectral information of *E. coli* in different growth rates.

Chemostat work

In the previous report, we indicated that PSI/USC had established a chemostat protocol for obtaining densities of *E. coli* that were usable for the current microscope imaging studies. To reiterate, although PSI's deep UV instrumentation has single-cell detection capabilities, imaging with the microscope is limited to diameters of approximately 40µm. At that spatial resolution, localizing a cell from an aliquot with a density of less than 10^6 cells/mL, spread out onto a slide, would be extremely challenging.

During the last month, we began work imaging *E. coli* under different residence times and temperatures in order to influence the growth rate of cells in the chemostats. The rationale was that a number of tyrosine containing proteins, such as chaperonins, ribosomes and synthase enzymes might undergo upregulation as a function of growth rate[4,5,6]. Because deep UV fluorescence of microbes is based on excitation of aromatic amino acids, a change in the concentration of these molecules should lead to changes in fluorescence intensity and variations in spectral patterns. Briefly, imaging was carried out on samples collected from the chemostat, using aseptic techniques. A subsample of approximately 10µL was then applied to a fused silica microscope slide, and imaged with the deep UV microscope (Fig. 2). Data has been collected for a variety of residence times

Table 1. Matrix of experimental variables for imaging *E. coli* chemostat cultures

	Residence Time (hours)		
Temperature (deg. C)	20	10	5
25			
37			

[4] Price, NC. et al., The aromatic amino acid content of the bacterial chaperone protein groEL (cpn60). FEBS. 292(1,2): 9-12. 1991.
[5] Tribe, DE., et al. Constitutive and repressible enzymes of the common pathway of aromatic biosynthesis in *Escherichia coli* K-12: regulation of enzyme synthesis at different growth rates. J Bacteriology. 127(3): 1085-1097. 1976.
[6] http://www.uniprot.org/uniprot/P0A7X3

and temperatures (Table 1). The residence time of the chemostat is a measure of how often the solution in the reactor vessel turns over. At shorter residence times, the bacteria divide more quickly to maintain a steady population, effectively increasing their growth rate.

A qualitative assessment of the collected images (Fig. 3) suggests that there is an effect on the spectral characteristics of the *E. coli* cultures as a function of growth rate. Cells maintained in chemostats with longer residence times have a "greener" hue compared to cells incubated at longer residence times. With microscope images, color is assigned to a pixel based on a three-dimensional coordinate derived from the spectral pattern at that point. In the case of microbes, the majority of fluorescence information comes from the combination of tryptophan, tyrosine and phenylalanine. The color theory used to assign a color to a given coordinate results in tyrosine-rich samples having a red color, while samples with higher relative concentrations of tryptophan tend to have greener hues. Qualitatively, it appears that cells in an accelerated growth-state have higher amounts of tyrosine relative to tryptophan and phenylalanine compared with slower growing cells, based on the assigned colors (Fig. 3). This could be due to either an up-regulation of tyrosine relative to tryptophan and phenylalanine, or do a down-regulation of tryptophan and phenylalanine with respect to tyrosine. During the coming month, we will analyze the spectra from the collected images and couple this with assays to assess the correlation between any spectroscopic differences and tyrosine, tryptophan and phenylalanine concentration.

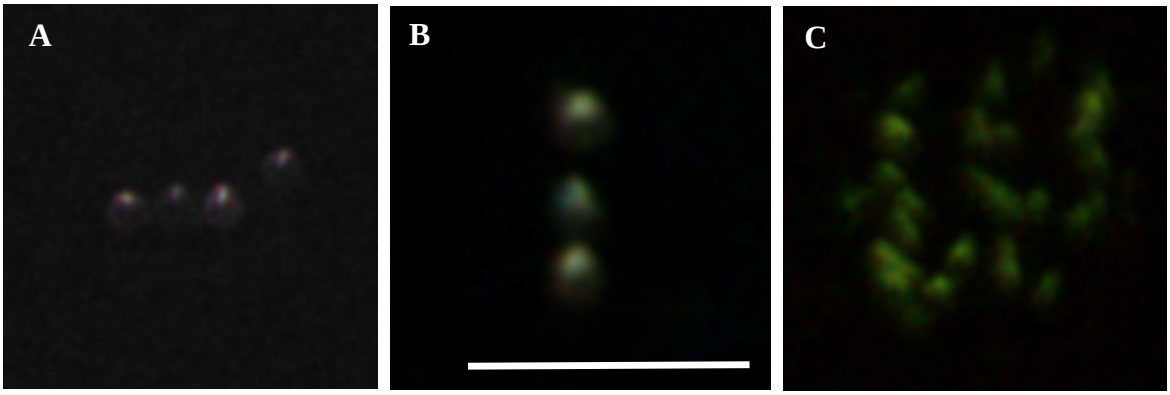


Figure 3. Growth rate appears to affect the spectral characteristics of chemostat-grown cells. *E. coli* images taken with the deep UV microscope under various growth conditions. Data will be analyzed to assess any variation as a function of growth rate. A) *E. coli* chemostat culture grown at 25 ^{0}C, and a 5-hour residence time; B) *E. coli* chemostat culture grown at 25 ^{0}C, and a 10-hour residence time; C) *E. coli* chemostat culture grown at 37 ^{0}C, and a 20-hour residence time. Scale bar = 10μm.

Imaging of bacteria and Talc

Based on discussions during last month's telecom, we have begun work imaging several different bacterial strains on a variety of surfaces (Table 2). This will create a database of known organisms and allow us to understand the effect that substrates have on microbial fluorescence signatures, as well as helping us understand the limits, if any, on our ability to detect microbes on various surfaces.

Work was done imaging mixtures of bacteria and talc on stainless steel surfaces. The four different strains of bacteria listed in Table 2 were grown in TSB. Talc solutions

were prepared by placing 100mg of talc into 1mL of 1% saline solution. Two different

Table 2. Bacterial strains and substrates to be used for the remainder of this program

	Substrates					
Bacterial strains	Talc	Stainless Steel	Copper	Teflon	Clothing/Fiber	Organic-rich medium
E. coli	X					
B. atrophaeus	X					
B. subtilis	X					
P. *aeruginosa*	X					

sample sets were used (Fig. 5). One set was mixed directly into talc, while a second set of microbes were washed to remove the TSB prior to mixing with talc. 10uL aliquots of each sample were applied to a stainless steel coupon and allowed to dry. The dried samples were then imaged with the deep UV microscope.

Interrogation using deep UV fluorescence indicated that pure talc produced very low intensity, spectrally distinct, fluorescence signatures compared to the microbe-talc mixtures (Fig. 4). Additionally, microbe-talc-TSB mixtures were spectrally different from the washed (non-TSB) samples. This data suggests that deep UV fluorescence can distinguish between abiotic powders, mixtures containing only organics, and mixtures containing organics and microbes. Further work will involve obtaining spectral data for the variants in Table 2. In addition, we are currently working on refining a spore induction and purification technique. Once that is accomplished, spores will be imaged on various substrates as well.

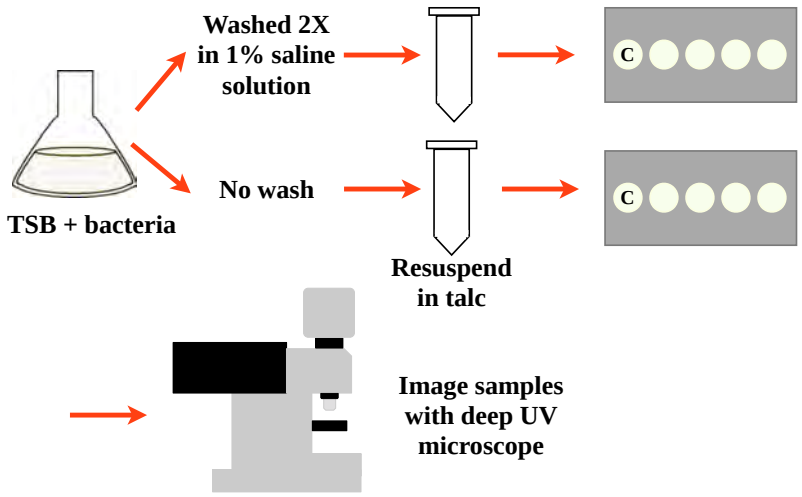


Figure 4. Protocol for imaging bacteria-talc mixtures. Bacteria were grown in TSB, then split into 2 sets. One set was washed in saline solution to remove any TSB, while the other set was not. Both were then mixed with a talc solution and then applied to a stainless steel coupon. Each group had a negative control (C) for reference.

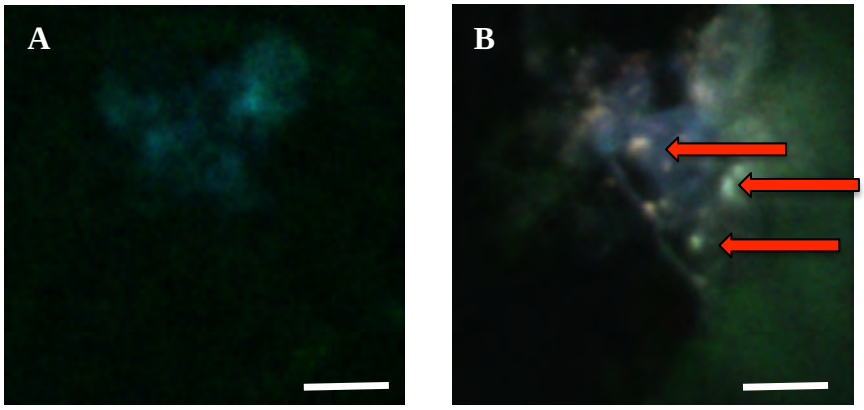


Figure 5. Pure talc has a different spectral signature compared to talc mixed with bacteria. A) Talc in saline solution on a stainless steel substrate; B) talc mixed with *P. aeruginosa* (red arrows) washed in saline solution. Arrows in B point to microbes. Scale bar = 10 µm.

Viability Experiments

Last month's report included discussion of results from experiments designed to assess cell culturability as a function of UV dose. Initial analysis indicated that there was an approximately 30% decrease in culturable cells when they were dosed with the equivalent of a 30um spot. A more thorough analysis of the data, however, indicated that there was an inadvertent omission of a data point in the 30µm spot-equivalent data (Table 3). Inclusion of this data point resulted in a reduction in culturable cells was no different than when the cells were dosed with a 200 µm spot (Table 3).

We also revisited the control dataset, which had one data point that was much larger than the rest (Table 3). A Dixon's Q-test indicated that this value could be considered an outlier with 95% confidence. If the point is treated like an outlier and removed, the mean CFUs for all three groups is almost identical (Table 4), indicating that there is no effect on cell culturability under the tested conditions. However, because our overall dataset is not that large, it may be premature to remove this point at the present time.

Table 3. Data for culturability study conducted by PSI/USC

	Unscanned	200µm	30µm
Power on Sample (µJ/900µm^2)	0	0.01	0.35
CFUs	13	12	10
	11	11	10
	15	12	14**
	12		
	14		
	23*		
Average	14.67	11.67	11.33
Standard Deviation	4.32	0.71	2.31
Percent Reduction in CFUs	0	20.45	22.73
Percent Uncertainty	12.03	3.5	11.76

*data point may be considered an outlier; **data point omitted from the previous month's report

To get a sense of how different the datasets were, a Kruskal-Wallis Analysis of Variance was run on the complete dataset. This test is a good way of determining how unique multiple, independent sets of data are. Based on the analysis, these data sets do not appear to be statistically different, based on a chi-squared threshold of 90% (Table 5). This was the case whether or not we used the putative outlier in the control dataset. Thus, contrary to what we initially reported, at this time there is no indication that we are causing measurable damage to the cells. These experiments will be rerun, however, the experimental set up will be altered to more closely approximate the method used when we take imaging data.

Data collected by PSI/USC was also compared to published data on UV-mediated inactivation. Cells dosed by deep UV under the PSI/USC study received approximately 38mJ/cm^2/laser pulse. In comparison, work done by Kumamoto et al[7]. described spectral alterations that were achieved only upon reaching doses of 400J/cm^2. Additionally, a study carried out by Vermeulen et al[8] described a 6 -log kill at 250nm excitation with 1.5mJ/cm^2. While the power used in our work was lower than that described in Kumamoto, it is higher than that described Vermeulen.

Table 4. Dixon's Q-test for data point 23

	Unscannec	200µm	30µm
Dixon's Q-test fo	0.667		
	0.740		
	0.625		
	0.560		
Average CFUs withou	13	11.67	11.33
Standard Deviation withou	1.58	0.71	2.31
Percent Reduction in CFU	0	10.26	12.82
Percent U	5.44	3.5	11.76

Table 5. Kruskal-Wallis ANOVA

	With point 23	Without point 23
Chi-squared	3.30	2.34
Degrees of freedom (d.f.)	2	2
p-value	0.19	0.31
Chi-squared, 95%, 2 d.f.	5.99	5.99
Chi-squared, 90%, 2 d.f.	4.61	4.61

The data reported last month appears to contradict Vermeulen et al., and may be a result of the dosing process. In PSI/USC study, the cells were pulsed by a laser firing at 5Hz, with a 100µs pulse and 250ms "gaps" between pulses. Vermeulen et al. dosed the cells with 0.2mW/cm^2 *continuously* for up to 30s (i.e., total dose = 1.5mJ/cm^2). It is

[7] Kumamoto, Y., et al. Deep UV resonant Raman spectroscopy for photodamage characterization in cells. Biomedical optics express, 2(4), 927–936. 2011.

[8] Vermeulen, N., et al. The bactericidal effect of ultraviolet and visible light on Escherichia coli. Biotechnology and Bioengineering, 99(3), 550–556. 2008.

possible that these 250ms gaps provide enough time for chemical repair to occur, such that, while a bond may be broken upon dosage with deep UV, the molecules remain in close enough proximity that the bonds are able to reform. Additionally, because the laser is pulsed, there is a probability that the same molecule does not receive more than one dose, as each subsequent pulse of photons may not transect through the same space. These concepts will be explored in more detail over the next two months.
It is important to reiterate that the work carried out by PSI/USC only described effects on cultivability
, not cell death. There is a good likelihood that some of the exposed cells were in a viable but not culturable (VBNC) state. However, understanding whether cells are viable after microscopic interrogation is currently not possible and thus we are using a larger spot diameter instrument. This system, in its current design only allows for a dose of 38mJ/ cm^2. While this is significantly less than the microscope, it is 30x more than what has been stated to cause 6 -log of kill[5]. Our results are drastically different and demonstrate statistically insignificant changes in cell viability. While this does not directly state the effect of the microscope, it suggests that pulsed illumination may not be as damaging as continuous illumination.

Cost Information

Cost status (all values are stated in total burdened costs) The cost status information discussed below includes only expenditures by Photon Systems, Inc.

(PSI).

At PSI there are two separate contracts from USC in the amounts of $150K (contract start date Oct 2011) and $95K (contract start date July 2012). For the first $150K contract, 100% of the funds have been expended. From the second $95K contract, 74% ($70,000) was expended through September 2012, with $25,000 remaining to be expended by November 2012.

Upgrades to the deep UV microscope

This task is supported by two separate contracts from USC to PSI. The first contract was an initial $150K for design work and hardware acquisition. This contract also included development of the program plan. For efforts in September, PSI expended the remaining $14,707 from the $150K contract.

The $95K contract in July from USC to Photon Systems also partially supports this development task in terms of integration, optical/mechanical enhancements, and software control development. For the efforts in September, Photon Systems expended approximately $24,000 from the $95K contract.

These costs include fabrication and installation of the Kohler illumination adapter plate and subsequent realignment the visible camera and deep UV CCD focal planes.

Chemostat work, talc work and viability experiments

For September, approximately $24,000 was expended at PSI from the $95K contract for conducting experimental work related to chemostat cultures, experimental work related to collection of fluorescence data of microbes on talc and data analysis of the cell viability experiments.