Biomolecular Characterization and Imaging Science

Bioimaging Science Program 2022 Principal Investigator Meeting Proceedings

U.S. DEPARTMENT OF **ENERGY** | Office of Science

Biological and Environmental Research Program

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Bioimaging Science Program
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Program Manager

Paul Sammak
Biological and Environmental Research Program
Office of Science
U.S. Department of Energy

Meeting Co-Chairs

James Evans
Pacific Northwest National Laboratory

Theodore Goodson
University of Michigan

Andreas Vasdekis
University of Idaho

Shiva Abbaszadeh
University of California at Santa Cruz

About BER

The Biological and Environmental Research (BER) program advances fundamental research and scientific user facilities to support U.S. Department of Energy missions in scientific discovery and innovation, energy security, and environmental responsibility. BER seeks to understand U.S. biological, biogeochemical, and physical principles needed to predict a continuum of processes occurring across scales, from molecular and genomics-controlled mechanisms to environmental and Earth system change. BER advances understanding of how Earth's dynamic, physical, and biogeochemical systems (atmosphere, land, oceans, sea ice, and subsurface) interact and affect future Earth system and environmental change. This research improves Earth system model predictions and provides valuable information for energy and resource planning.

Cover Images

Image 1: Photobleached cell, see p. 12; **Image 2:** Root hair cell, see p. 21; **Image 3:** X-ray beamline configuration for production of entangled X-ray photons, see p. 25; **Image 4:** Light-sheet propagation upon scattering, see p. 15; **Image 5:** White light reflected image of an aspen leaf, see p. 28.

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Contents

Preface

The mission of the U.S. Department of Energy's (DOE) Biological and Environmental Research (BER) program's Bioimaging Science Program (BSP) is to understand the translation of genomic information into the mechanisms that power living cells, communities of cells, and whole organisms. The goal of BSP is to develop new imaging and measurement technologies to visualize the spatial and temporal relationships of key metabolic processes governing phenotypic expression in plants and microbes. The extended goal of dynamic imaging is to functionally connect cellular components and interdependent organisms. Information about the time and place of chemical reactions *in situ* can identify causal relationships between biological activators and downstream effectors.

Technologies are inspired by their eventual application to biological hypothesis-driven research. BSP supports fundamental imaging research for proof-of-concept studies of novel untested methods and devices and for working prototypes and biological validation studies relevant to BER bioenergy and environmental research. The role of bioimaging in the larger portfolio includes visualizing spatiotemporal expression patterns of natural or engineered pathways, tracking specific metabolic pathways between intact living cells, and illuminating causal interrelationships among plant tissue layers or microbial colonies in the rhizosphere.

BER continues its support of use-inspired optical and electron methods with funding announcements for academic, commercial, and DOE national laboratories. A recent addition extends BER support to quantum sensing and imaging approaches for circumventing fundamental limitations of classical optical techniques. Limitations include statistical photon noise, light scattering in thick samples, photo-damage of cellular chemistry, and reporters that perturb molecular function. Bioimaging enhanced with quantum phenomena will benefit from improvements in detector sensitivity, image resolution, and noise reduction and from repetitive imaging of fragile samples without damage. Methods that use light beyond the visible range might be necessary to see new features of biological shape and movement.

To determine the potential of quantum science for bioenergy and basic biology research of the BSP, the BER program sponsored a workshop at the National Academies of Sciences, Engineering, and Medicine and published the proceedings, "Quantum Science Concepts in Enhancing Sensing and Imaging Technologies applications for Biology" to identify both promising quantum detection methods and quantum-based biological processes. DOE supports five large-scale quantum information science (QIS) centers across the Office of Science to develop materials, computation, and communication network capabilities. BER participates by providing partial support for one of these centers to develop high-performance instruments and quantum sensors.

BSP held its annual PI meeting virtually February 28–March 1. Contributing investigators are convened to review progress and current state-of-the-art bioimaging research. Holding the 2022 BSP meeting as part of the broader Genomic Science Program (GSP) PI meeting allowed researchers to interact with the extended GSP community. This convergence provided a platform for networking and exchange of ideas with experts in other technologies and in target BSP application areas, helping to forge new multidisciplinary collaborations among investigators from the sister programmatic areas within BER's Biological Systems Science Division.

An important highlight of the BSP meeting was the keynote presentation by Nobel Laureate Dr. Joachim Frank on *Time-Resolved Macromolecular Imaging using Cryo-EM*. He discussed microfluidic mixing and fast freezing to capture nonequilibrium intermediate states during molecular binding and conformational changes. The action of molecular machines can be captured at nanometer resolution and millisecond discrimination.

BSP PIs made presentations describing their research focus and progress in plenary sessions on bioimaging science and on quantum-enabled bioimaging science research projects. BSP research at universities and DOE laboratories is presented in this report. A final discussion of the BSP was organized by meeting plenary session chairs, who prepared the following Executive Summary of current BSP research, research challenges, future opportunities, and potential ideas for expanding the BSP's impact and interactions.

Paul Sammak, Ph.D.
Program Manager, Biophysicist
Biological and Environmental Research
Office of Science, U.S. Department of Energy
Paul.Sammak@science.doe.gov
301.903.4071

Executive Summary

Prepared by the Plenary Session Chairs: James Evans (Pacific Northwest National Laboratory), Theodore Goodson (University of Michigan), Andreas Vasdekis (University of Idaho), Shiva Abbaszadeh (University of California at Santa Cruz), and Paul Sammak (U.S. Department of Energy)

Overview of Current BSP Research

- Expansion of New and Existing Technologies
- Multimodal Microscopy Techniques
- Raman and Mass Spectrometry–Based Approaches
- Imaging Using Nucleic Acids
- Tracking Molecules *in Situ* and in Real Time
- Characterizing Diverse Molecules Across Scales
- Quantum-Enabled Techniques
- Imaging Complex and Deep Tissue Systems
- Organisms Under Study

Research Challenges and Future Opportunities

- Dark-Matter Imaging
- CRISPR-Cas Genome Editing and Multiscale Optogenetics

- Multidisciplinary Research Teams
- Further Integration of Technologies into Multimodal Hybrid Instruments
- Cross-Platform Data Fusion and Integration
- Advances in Data Management and Analytics
- AI-Guided Experiments and Image Acquisition
- New Probes and Quantum-Enabled Techniques to Expand Investigations
- Field-Deployable Capabilities for Whole Organisms and Complex Communities
- Correlative Frozen or Fixed-Sample
- Imaging Summary of Opportunities and Needed Developments

Expanding BSP's Impact and Interactions

- Summary of Opportunities and Needed Development
- Community Access to BSP-Developed Technologies Through User Facilities
- Bioimaging Science Program Annual Meeting
- Additional Cross-Program Interactions and Community Engagement

The U.S. Department of Energy's (DOE) Bioimaging Science program (BSP) supports fundamental research to develop and apply new and enhanced bioimaging and measurement capabilities that enable scientists to study the biological functions of plant and microbial systems relevant to bioenergy research. The program—within the Biomolecular Characterization and Imaging Science (BCIS) portfolio of DOE's Biological and Environmental Research (BER) program—currently sponsors multidisciplinary research at nine national laboratories and 25 universities (see List of Funded Projects and map, pp. vi–vii) with the goal of understanding the mechanisms that power living cells, communities of cells, whole organisms, and ecosystem processes. BSP researchers are developing instruments and imaging systems and are enhancing existing capabilities with new or transformational improvements. These novel capabilities

and innovative uses of established methodologies will enable new fundamental discoveries and provide solutions to challenges in plant and microbial systems biology—from single molecules to small unicellular organisms to complex microbial and fungal community interactions with plants. Together, BSP-supported researchers are creating an extensive and versatile chemical imaging toolbox enabling real-time dynamic imaging of metabolic pathways, material transport within and between cellular organelles, plant-root and organism interactions, enzyme functions, and cellular structures. The combination of novel quantum dot technology coupled with new approaches to quantum light technology may provide large enhancements in the detection limits of biological systems important to BER. A major emphasis has been the use of a multipurpose toolbox of complementary technologies to enable very low light–sensitive excitation for

reduced photobleaching of biological material. Some of the major challenges remaining for bioimaging are deep imaging; ultralow light level detection of dark matter—namely metabolites, proteins, and organelles—and dedicated databases for retrieving information per measured signature.

Overview of Current BSP Research

Expansion of New and Existing Technologies

BSP has significantly expanded since its inception in 2015. The program recently added an extensive range of novel bioimaging technologies and cutting-edge sensing approaches including super-resolution microscopy, hyperspectral light-sheet imaging, adaptive optics, code-aperture methods, quantum entanglement, dynamic positron emission tomography imaging, quantitative phase imaging, correlative imaging, and holographic force spectroscopy. These new technologies are complementary to and synergistic with ongoing developments in instrumentation involving molecular, optical, fluorescence, Raman, and nonlinear optical techniques. The ongoing techniques include surface-enhanced Raman scattering (SERS), stimulated Raman scattering (SRS), coherent anti-Stokes Raman scattering (CARS), hyperspectral stimulated SRS (hsSRS), and tip-enhanced Raman scattering (TERS), nano-Fourier transform infrared (FTIR), and X-ray microscopies.

BSP researchers are developing spectroscopic techniques to image dynamic events and molecular processes *in situ*, enhancing various combinations of nondestructive and destructive approaches to image laboratory-prepared or fixed samples, and creating inorganic voltage nanosensors to study bacterial communities. Optical modalities are noninvasive and include infrared/ultraviolet absorption and adaptive optics multiphoton microscopy, fluorescence, and Raman techniques (e.g., conventional, nonlinear, and plasmonics-enhanced). Recently, BSP added quantum-enabled bioimaging science research projects at both national laboratories and universities. The national laboratory projects encompass state-of-the-art, quantum-based techniques utilizing quantum entanglement and coincidence measurements to image microbes, fungi, and plant biosystems. While three of the four national laboratory projects focus on quantum-entangled photons for light microscopy applications, one project is developing a quantum-

enhanced X-ray microscope at the NSLS-II synchrotron. All four projects are employing ghost imaging methods, however unique design features distinguish the various projects and will create new capabilities for three-dimensional quantum microscopy, super-resolution ghost imaging, and quantum phase contrast imaging.

The BSP program has also supported research led by universities regarding the use of quantum-enabled techniques. Quantum entanglement has been shown to imply correlations that are much stronger than those allowed by classical models. Among the many applications of nonclassical states of light, nonlinear microscopy has the potential to make an impact in broad areas of science from physics to biology. Here, the microscopic image created by the fluorescence selectively excited by the process of the entangled two-photon absorption is reported. In combination with novel spectroscopic capabilities provided by non-classical light excitation, this is of critical importance for sensing and plant biological applications because reduced light levels can be directed to the sample, reducing photodamage, scattering, and improving detection. The program has seen the development of an entangled two-photon microscope, which can image organic and biological materials with extremely small numbers of input photons. The program has also witnessed the development of ghost imaging approaches, which may provide detailed information regarding biological systems of interest to BER. The advantages of the ghost imaging are the lower input flux, remote or discrete detection, and high resolution (lower signal to noise). These techniques involving nonclassical light excitation and detection schemes are still being developed. In addition to the work with entangled two photon excitation microscopy and ghost imaging, there are analogous phenomena using X-rays that have the potential for deeper penetration without photodamage, which has limited the use of X-rays for high-resolution biological imaging. Moving forward, the combination of these approaches with novel creation of quantum dots with specific interactions with plant and microbial biological systems would be a great benefit to the program.

Outside the quantum realm, BSP researchers are further enhancing co-application of mass spectrometry and spectrochemical imaging capabilities to yield highly selective, sensitive, and quantitative chemical maps of intra- and extracellular molecular gradients

and the distributions, abundances, and fates of stable isotopes, natural elements, and metabolites. Using conventional microscopies for correlated structural and chemical imaging, this work supports simultaneous observation and interpretation of the biological function of living plant and microbial systems, with the overall goal of eliciting how microscale interactions within and between cells can lead to large-scale ecosystem phenomena, such as carbon cycling.

Some BSP researchers are significantly expanding the performance and impact of label-based and label-free sensing and imaging technologies by developing unique probes, such as quantum and polymer dots, and plasmonic nanoprobes equipped with various bioreceptors (e.g., antibodies, aptamers, and gene probes). These probes can specifically detect important biomarkers—including metabolites, proteins, and genomic markers—related to specific processes and metabolic pathways in microbial and plant systems relevant to bioenergy research. Development of these unique probes and sensors is expanding the applicability of the new instrumentation by enabling researchers to dynamically track targeted cells, organelles, enzymes, biomarkers, and small molecules and to test and validate cellular processes and genome-based models of cellular metabolism.

With the new instrumentation and optical probes developed under BSP sponsorship, these investigations are expected to result in a better understanding of the spatial and temporal distributions of metabolites associated with living microbial and plant systems. Also anticipated are new insights into the fundamental biology of many events occurring at single-cell and macro levels, such as nutrient utilization and community and ecosystem interactions, (e.g., soil water retention caused by the presence or absence of particular organisms or biomass). This comprehensive BSP portfolio will improve understanding of the molecular underpinnings of a diverse array of biological and environmental processes.

Multimodal Microscopy Techniques

New BSP instruments span a wide range of modalities. Microscopy approaches include optical methods such as luminescence, confocal, adaptive optics multiphoton, fluorescence scattering, reflected/transmitted light extinction spectroscopy, entangled photon, and total internal reflection fluorescence

(TIRF). Also included are full-field X-ray fluorescence, imaging, polarimetry, entangled X-ray imaging, and novel single-molecule sensing methods, such as stochastic optical reconstruction microscopy (STORM) and photo-activated localization microscopy (PALM). In addition, by combining quantitative phase imaging (QPI) with light-sheet fluorescence and Raman microscopy when using optical quasi-lattice technologies, BSP researchers are increasing single-cell imaging throughput rates and resolution. Dark-field and fluorescence-based hyperspectral imaging is enabling the collection of high signal-to-noise images and will allow multiplex collections of multifluorophore images. While these imaging approaches focus on events inside cells, an alternative imaging approach uses aptamers as sensors to image specific molecular species present around cells. These imaging modalities will be complemented by advanced technologies such as high-speed atomic force microscopy (AFM), interferometric scattering microscopy, infrared, and vibrational sum frequency generation. Researchers also are applying plasmonic infrared nanofocusing gratings combined with microfluidics to map cellulose surface fibrils with cellulose at the nanoscale.

Raman and Mass Spectrometry–Based Approaches

Other important portfolio components are various Raman spectroscopy–based approaches, including spontaneous, far-field subdiffraction, TERS, coherent anti-Stokes Raman (CARS, including broadband CARS), SRS, SERS, spatially offset Raman spectroscopy (SORS), shifted-excitation Raman difference spectroscopy (SERDS), time-resolved SRS and CARS, and cavity-dumped SERS. BSP researchers also have developed a multimodal microscope integrating CARS, SRS, and two-photon excitation systems with adaptive optics. The combination of SERDS with hyperspectral Raman imaging (HSRI) demonstrated the possibility of directly imaging microRNA biotargets in intact living plants under ambient light conditions.

Added to these imaging modalities will be a capability that enables researchers to capture samples for profiling metabolites using several forms of mass spectrometry, including laser ablation electrospray ionization mass spectrometry (LAESI-MS) and LAESI Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS) using a 21 Tesla magnet. Raman-activated cell sorting (RACS) for isolating single cells that are active in polysaccharide degradation

followed by 16S rRNA sequencing for species identification is also being developed. Further, to provide 3D spatiotemporal chemical information in bulk and at the interfaces of biological systems, BSP researchers developed a nonlinear optical mapping and *in situ* liquid extraction–mass spectrometry (LE-MS) capability utilizing a porous membrane microfluidic surface in combination with a continuous LE sampling probe. A wide-field CARS microscope for rapid and simultaneous acquisition of CARS images across an entire field of view was also developed.

Imaging Using Nucleic Acids

In a different approach, BSP researchers are developing electrochemical impedance spectroscopy with nucleic acid aptamer sensors. This technology will enable scientists to monitor nutrient transformations and microbial metabolic activities in the rhizosphere that contribute to plant growth and health and to investigate plant-microbe interactions that involve chemical communications in the rhizosphere. Other applications of nucleic acids to image plant and microbial activities include the detection of microRNAs using silver-coated gold nanorods and SERS sensing, and the detection of riboswitches that act as metabolite reporters using quantitative phase imaging that leverages a light-sheet fluorescence te chnique and Raman imaging.

Tracking Molecules in Situ and in Real Time

BSP researchers are focusing on understanding a variety of biological systems for better controlling plant health and growth to improve bioenergy sources. Subject organisms include plants, bacteria, fungi, and their combinations. Teams are developing sophisticated instruments for single-organism imaging of metabolism, gene expression, and regulatory molecules (e.g., microRNAs, quorum-sensing molecules, and protein kinases) as they operate in intact living organisms or are involved in communication between organisms. Several approaches are underway to capitalize on the advantageous spectroscopic properties offered by semiconductors, polymers, and quantum dots. Nuclear-based imaging technologies such as positron emission tomography (PET) enable scientists to visualize and quantify the movement of radiolabeled nutrients, plant hormones, and other signal molecules within intact live plants. However, the widespread use of these technologies has been hampered by access to the limited number of facilities that have the unique

capabilities to produce the radiolabeled versions of these agents. BSP-supported researchers are developing instruments that will address the critical need of measuring those molecular-level activities directly, enabling a future in which molecular signatures can be tracked in real time and across time periods consistent with the biological processes under study. These developments will include the capability of visualizing biosystems as they respond to external stressors and perturbations such as nutrient starvation and chemical exchanges. BSP researchers also are using synthetic rhizosphere microhabitats, transparent soil microcosms, and versatile nanofluidic and microfluidic imaging and sampling devices simultaneously to cultivate and analyze biosystems from single cells to complex communities.

BSP researchers are also developing low-cost, modular PET detector modules that can be scaled up easily to form different geometries, which could grow PET imaging capabilities and increase sensitivity for dynamic imaging of isotopes with the short half-life required for capturing plant and soil systems.

Characterizing Diverse Molecules Across Scales

BSP research teams are developing capabilities to study molecular signatures and processes that are highly diverse and cover a broad range of length scales. The functional dimensional scales in biological systems are vast, spanning molecules to multi-organismal systems. Because these systems are hierarchical in nature, activities on longer length and time scales are built on activities and structures on shorter length and time scales. Therefore, processes must be fully explained at the molecular level to be fully understood at the organismal or multiorganismal level. Recognizing this need, BSP supports some innovative cross-scale imaging approaches that include plasmonic nanoprobes to track single molecules. Also supported is (1) 3D tracking with high-speed AFM and (2) optical tweezers to control molecules or microbes, enable force measurements, and track molecules such as cellulose synthase as it moves along the membrane or cellulase as it moves along cell walls. These studies will answer important questions regarding the mechanisms of cellulose synthesis and degradation. Understanding such mechanisms will, in turn, enable the development of biomass feedstocks that more readily can be converted to biofuels and bioproducts.

In addition to cellulose and lignin synthesis and degradation, other processes and targets have been actively investigated. These include atomic isotopes, metabolites, plant hormones, quorum-sensing molecules, silica, trace elements, redox metabolism, microbial electron transfer, membrane potential, intercellular trafficking, microRNAs that regulate lignification, and enzymes and other proteins secreted by plants.

Quantum-Enabled Techniques

As mentioned above, the BSP has recently added state-of-the-art, quantum-enabled bioimaging projects at both national laboratories and universities. The possibility of performing imaging and sensing tasks that are nearly impossible using classical methods has made quantum entanglement a powerful resource for the development of novel methods and applications in materials and biology. The need for the development of next-generation technologies utilizing entangled quantum light has progressed in the last five years.

With ghost imaging, samples are illuminated using less-intense beams with energy more suitable for maintaining biological integrity. The quantum nature of the imaging process enables visualization of details impossible to detect with classical methods. BSP researchers also are developing a quantum-enhanced X-ray microscope, which uses entangled X-rays beams, and a new optical microscope that uses entangled photon pairs to visualize water, lignocellulose, and lipids in plants. To probe samples, the latter system applies a wavelength in the near- or mid-infrared range where vibrational fingerprinting to identify key molecular species is possible. Detection and imaging are then performed with visible light using high-efficiency and low-noise imaging detectors. An entangled two-photon microscope will provide greater context in which many of the possible enhancement schemes can be employed. In the already realized entangled fluorescence scanning microscope, fluorescence is selectively excited by the entangled two-photon absorption in organic solid-state films. Microscopic images of numerous organic aggregates in drop-cast films under entangled two-photon excitation have been obtained. The excitation flux used to create entangled photon-excited images was found to be six orders of magnitude lower than that necessary for classical light two-photon excitation. A nonmonotonic dependence of the image on the delay between the components of the entangled photon pair has

been demonstrated. The specific delay dependence associated with the inherent quantum interference effects specific to the entangled photon absorption process in the material demonstrates a nonclassical light origin of the image. The teams working in this direction and collaborating with scientists making novel quantum dot and organic chromophore assemblies may couple these two directions for optimal performance.

Research teams are developing a high-quality 3D imaging modality that uses quantum-entangled photon pairs to obtain more information on fluorescence and scattering events than is available with standard fluorescence or scattering measurements. The system uses two separate 2D detectors to obtain three- and four-dimensional information about the same photon, providing 3D optical imaging at high frame rates to monitor dynamic host-bacterial interactions in bioenergy algal pond and plant systems.

Also under development is a hybrid quantum-enabled imaging platform that combines advances in adaptive optics, quantum entanglement, coincidence detection, ghost imaging, quantum phase-contrast microscopy, and multidimensional nonlinear coherent (nonentangled) photons and four-wave mixing. This system will enable researchers to visualize photoreception in phytotropin and phytochrome proteins and other quantum coherent processes that occur naturally within biosystems, improving the ability to track ultrafast protein dynamics and the flow of metabolites between biological compartments in real time.

While each of these new instruments are developing unique quantum-enabled imaging regimes, there remains an opportunity to further develop quantum-enhanced, super-resolution microscopy for synthetic biology applications coupled to appropriate and repeatable single photon sources that accelerate image acquisition.

Imaging Complex and Deep Tissue Systems

Although many of the samples BSP researchers use to test new instruments and methods may be from canonical model systems, the program continues to evaluate and adapt to real-world biosystems, including signature detection in natural ecosystems. For example, label-free identification of microbes and small molecules, such as metabolites, directly in soils or deep into the plant tissue—where multiple

scattering renders them invisible by conventional technologies—remains a grand challenge in biology. As such, extending BSP-developed technologies towards natural environments in the long term represents the next frontier.

In this context, while focusing on high-resolution imaging, some BSP-supported projects are applicable to more complex biological systems and challenges relevant to bioenergy and the environment, such as understanding quorum sensing, improving lipid feed-stock yields, enhancing lignocellulosic deconstruction, or boosting feedstock sustainability and tolerance to environmental stress, (e.g., water and nutrient limitation). One such effort develops broadband CARS imaging of root architectures, nodule development, and various carbon sources. Here, coherent rejection of out-of-focus signals will enable deep penetration directly in soils. A similar effort sets out to adapt multimodal light-sheet microscopy to imaging the metabolic interactions between plants and arbuscular mycorrhizal fungi that occur deep inside the root cortex. Here, optical lattices and quasi-lattices with tailored self-healing properties are coupled to photon-sparse imaging strategies to enable image reconstruction with high spatial frequency components.

Organisms Under Study

- Living plants
  - *Arabidopsis thaliana, Medicago truncatula, Brachypodium distachyon, Populus* sp., *Pinus taeda*, and *Zea mays*
- Microbial chemotrophs
  - *Bacillus subtilis, Yarrowia lipolytica, Azotobacter*, and *Pantoea* sp.
- Microbial phototrophs
  - *Cyanothece* sp., *Rhodopseudomonas palustris, Ostreococcus tauri, Chlamydomonas reinhardtii, Anabaena* sp. and *Synechococcus* sp.
- Systems for studying plant-microbe interactions
  - Arbuscular mycorrhizal symbioses, *Glycine max* with *Bradyrhizobium japonicum*, and *Suillus brevipes* with *P. taeda*

Research Challenges and Future Opportunities

Dark-Matter Imaging

A plethora of small molecules, such as metabolites and transcripts, but also organelles and genomic dynamics,

remain invisible in most biological systems. This is either because their optical signatures are unknown or because their signal levels are buried below noise and resolution limits. As such, there is a need for developing characterization and imaging methods that can quantify such entities by location and concentration, either directly in their natural environment (e.g., soils), or within single living cells. This calls for the development of new methods that can identify fine chemical and physical structures *in vivo*—such as transient spectroscopy, ultrasensitive detection, and robust super-resolution microscopy (e.g., anti-bunching)—and open-access libraries for depositing specific chemical or scattering signatures. The latter requires the development of databases of (1) expected spectral signals for various chemical signatures, and (2) synthetic imaging systems, (e.g., soils, leaves, and roots) for charting the underlying scattering properties. Importantly, the properties of such dark, biological matter require multiscale integration, technologies capable of high-throughput and analyses of enhanced statistical significance, and sorting capabilities.

CRISPR-Cas Genome Editing and Multiscale Optogenetics

The ability to modify microbes *in situ* within their native environments would be revolutionary to modulate microbial community activities. The CRISPR interface could be used to knock down pathways, manipulate biochemical pathways, and validate label-free chemical imaging. This is an alternative to making gene deletions when metabolites may be essential. Since deletions can affect the system in ways that may cause the resulting data to not be informative, the ability to alter activity on the short term could provide important insights. An iteration of this could be to try ectopic activation or to add a switch that can be used to turn a particular pathway on or off. Additionally, introduction of optogenetic tools to activate or perturb one cell within a community would be powerful. It is also suited for making functional mosaic plants to probe signaling interactions between one organ and other parts of the plant or how groups of cells communicate.

Multidisciplinary Research Teams

Biological imaging is inherently transdisciplinary, and successful teams need to continue to reflect this approach to advance BSP programmatic goals. Multi-disciplinary teams are needed to integrate imaging

results with the corresponding genomic, proteomic, lipidomic, and metabolomic changes within cells to further understand biological complexity and heterogeneity. Achieving this understanding requires combining the expertise from researchers in conventional and quantum-enabled imaging technology. Projects that combine a novel component into conventional approaches are most impactful for moving the field forward. Combinations of established disciplines including nanoscience, computer science, structural biology, biochemistry, plant physiology, microbiology, genomic science, ecology, soil science, and biogeochemistry will provide clear purpose and use-inspired tool development. This cross-disciplinary approach will be a critical step toward connecting phenotypes with genotypes and translating laboratory-developed technologies into the natural environment. The possibility to link the resources at EMSL with synchrotron and neutron facilities is important.

Further Integration of Technologies into Multimodal Hybrid Instruments

BSP-supported development of individual imaging techniques is making significant strides. These technologies range from complementary targeted and untargeted methods to destructive and nondestructive imaging modalities (e.g., optical, scanning probe, mass spectrometric, X-ray, and ion-based approaches) that cover a wide range of spatial and temporal scales. The recent addition of cutting-edge, sophisticated, and laboratory-based imaging methods (e.g., quantum entanglement and super-resolution techniques such as STORM and PALM) strongly complement the sensing and imaging approaches more suitable for general laboratory and field use.

In addition to pursuing advances within each of these techniques, a major programmatic focus moving forward should be on making the developments robust, easy to use, and accessible to the BER research community. One approach toward meeting this goal could be to further integrate these different and complementary approaches into hybrid all-in-one instruments. Multimodal spectral imaging in a single and user-friendly setup across nano-, micro-, meso-, and macroscopic spatial domains will be a useful and versatile tool for future users. There is also a need to develop highly specialized, sophisticated instrumentation for fundamental research in the laboratory and portable and easy-to-use instrumentation for large-

scale monitoring applications in the field. Previously unachievable studies of microbes, plants, and other species in their environments will be possible due to the new capabilities provided by these instruments. Results of these studies are expected to reveal new insights on how to optimize development of sustainable bioenergy resources.

There is a need for high-throughput-screening imaging applications that enable us to target areas of interest with multimodal methods. This could potentially help address statistically significant challenges observed in imaging methods. The variability and variance in a biological system can be quite high. As a result, increasing throughput might be needed to obtain statistically significant numbers.

There is an opportunity to integrate multimodal imaging and spatially resolved omics data to understand plant cellular processes. Separately, there is also an opportunity for multimodal imaging using scanning probes to enhance image acquisition and correlate with additional modalities, like electrochemistry. Developing microscopy systems that enable "upright" sample imaging will allow gravity to act and other gradients to form in a more native way. In addition, development of biosensors will allow signals to be spatially tracked without perturbing the system.

Cross-Platform Data Fusion and Integration

With BSP's expansion and the rapid increase in monitoring modalities, data integration across multiple technologies and approaches remains a high priority. Data fusion (i.e., linking complementary data from different techniques) will produce a more holistic picture and better understanding of the biological systems being imaged. Facilitating cross-platform bioimaging systems will require indexing and registering images (e.g., multifunctional tracers, probes, and sensors to serve as cross-platform fiducial markers) and meaningfully co-referencing and co-registering disparate datasets for the same sample but of different formats, magnifications, or resolutions. Also needed are models capable of integrating multimodal data spanning a wide range of spatial and temporal scales to effectively extract causality from observations and understand complex biological phenomena. Other important advances are integrated data processing algorithms, visualizations, and modeling, which are key components for properly interpreting the

diverse sets of imaging data, omics-based organismal models, and other information emanating from BER genomics research.

Advances in Data Management and Analytics

To enable effective extraction of critical biological and environmental information from experimental data, major advances are needed in data storage, processing, and visualization. BSP's long-term goal is to develop enabling capabilities that can generate spatially and time-resolved snapshots of relevant cellular metabolism, including both primary and secondary metabolites, genomic biomarkers, and internal and secreted compounds. Achieving real-time data collection and interpretation of these integrated data will lead to major advances in bioimaging technology that will improve monitoring and phenotyping of plant and microbial systems and expand the understanding of molecular and genomic pathways in both the laboratory and in complex natural environments. These advancements will require new methods and algorithms to handle increasingly challenging volumes of data, along with automated and machine learning approaches to rapidly analyze this data and identify biologically and environmentally meaningful signals.

Of interest is a central clearinghouse for archiving experimental and simulation data that incorporates a standardized output and imaging framework for different and potentially widely adoptable analytical modalities. Such a data repository could be independent or integrated with the DOE Systems Biology Knowledgebase (KBase; Kbase.us) and take advantage of advances in artificial intelligence to extract patterns from raw data for improved organization, interpretation, and representation.

Another opportunity for improving data interpretability is to leverage computer science (CS) graduate programs to help accelerate image processing or data analysis pipelines for the large datasets collected within BSP. Many CS programs require students to gain access to and experience with real-world data by building new software or other algorithms for more effective analytics. Using the plethora of BSP data, principal investigators (PIs) could sponsor CS graduate students to develop the next frontier of bioimaging analytics tools.

There is a lack of sufficient microbial and plant metabolite databases. Available commercial libraries are primarily focused on human and mouse signals. The Global Natural Products Social Molecular Networking (GNPS) knowledgebase for mass spectrometry data (gnps.ucsd.edu) is one exception for bacterial metabolites, but it is very erratic in terms of what is included. In addition, some of the plant databases are not open to researchers. Making them more widely available would be helpful.

The question of achieving statistically significant numbers of images for understanding relevance of the observation on the phenotype remains an issue for many techniques. This is particularly true for synchrotron-based imaging techniques.

AI-Guided Experiments and Image Acquisition

There is an opportunity for imaging strategies using AI to decrease negative impact on biological systems and increase image acquisition speeds. AI-guided experiments and image acquisition could be used to encompass probe/stimulus delivery, observation, area, and frequency. This would include (but not be limited to) artificial intelligence (AI), machine learning (ML), and compressed-sensing strategies. The BSP program should continue to push on deep-imaging and large-scale statistics through high-throughput data collection and statistical modeling to understand fundamental heterogeneity in response (cell-to-cell, molecule-to-molecule). There is opportunity for image reconstruction schemes, including AI, to leverage established spectral and spatial databases for enabling imaging capabilities that can be utilized by the broader community.

New Probes and Quantum-Enabled Techniques to Expand Investigations

In parallel with BSP's instrumentation development efforts, there is also a critical need for probe development that enables identification, sensing, and functional imaging of various targets within complex biological systems, ranging from key metabolites to molecular and genomic biotargets (e.g., mRNA, microRNA, proteins, and regulatory small molecules). Relevant key advances would include simultaneous marking, spatially resolved tracking, and sensing of multiple players (e.g., elements, isotopes, enzymes, metabolites, and other molecular biomarkers) in a specific biological system. BSP's wide range of biosensing and imaging capabilities are expected to provide the essential flexibility to broaden the scope

of investigations, opening new possibilities to discover yet-unknown key biomarkers or intermediates.

Probing a sample inherently perturbs it, yet methods based on selective probe-induced perturbations of key biotargets or metabolic pathways of specific organisms could provide opportunities to investigate and understand biological processes that otherwise would be difficult to unveil. BSP researchers are also pursuing an approach to minimize perturbation: the incorporation of quantum-enabled science and technologies. The potential of using ghost imaging for bioimaging applications is intriguing because this approach can image a sample by detecting a photon that never interacted with the sample. Furthermore, the ability of quantum-entangled, two-photon imaging to provide higher detection efficiency and decrease the total photon flux needed to observe a high-contrast image, and thereby permit very low-dose imaging that could minimize photodamage effects, would facilitate longer-term, time-resolved imaging of biosystems. Deeper penetration by X-rays combined with X-ray–entangled imaging will enable imaging in thicker biological samples. The development and integration of these and other quantum-enabled imaging technologies or sensors into the BSP portfolio could significantly expand the range of scientific questions the program addresses.

Field-Deployable Capabilities for Whole Organisms and Complex Communities

Another important challenge for the near term is the extension of laboratory-based approaches into applications for whole organisms and plants in their natural environments and under field settings. This expansion will require incorporating the dynamics of microbially driven biogeochemistry (e.g., within the rhizosphere, biofilms, and other key biological interfaces) into the imaging process. Although there has been progress in imaging genomic biotargets in living plants, advances are needed for imaging complex native microbial communities to decipher their organization and the multiple metabolic processes occurring simultaneously in space and time. Concerted efforts will also be needed to develop the ability to probe inherent signals within nontractable microbes in the environment and to create pathways that enable *in situ* microbial synthesis of probes for assaying function and activity. Furthermore, in addition to sophisticated lab-based analytical methods, portable instrumentation and

practical techniques will allow the detection of weak optical signals from whole-organismal data containing strongly interfering background signals such as fluorescence, ambient light, vibrations, and fixed-pattern noise encountered under field conditions.

Correlative Frozen or Fixed-Sample Imaging

Finally, it is important to realize the benefits of combining additional approaches that may be destructive or applicable only to frozen or fixed samples, which have historically been outside the scope of the BSP portfolio. Many current BSP capabilities are based on optical approaches that empower real-time or *in situ* observations of living systems, but they do not provide a complete picture of the sample or a whole-cell context. Some science questions require more holistic imaging and analysis to decipher complex associations within or between living cells. Combining current BSP approaches with sequential downstream frozen or fixed-sample correlative imaging (such as cryoelectron microscopy or nanosecondary ion mass spectrometry) can provide additional spatial, ultrastructural, or chemical context needed for critical scientific breakthroughs related to cellular sensing and metabolite response, flow, and fate. Such multimodal and correlative imaging approaches should be encouraged within BSP to accelerate the understanding of biosystem complexity and organization and their impact on dynamics.

Summary of Opportunities and Needed Developments

In summary, several advances are needed in key areas:

- Fine/sensitive detection of metabolites and dark matter.
  - New technologies (e.g., chemical imaging or transient absorption spectroscopy).
  - Dedicated databases for retrieving information per measured signature.
- Improving throughput rates versus resolution trade-offs: Imaging from single molecules to cells, organisms, and ecosystem processes.
- Extremely low–light levels on the sample.
  - Need for classical, quantum, and computational (AI-guided experiments, compressive sensing) approaches (Henderson limit in cryo-TEM).
- Probes: New redox-active, FISM–smFISH for (gram positive) microbes, optogenetics, genome/evolution imaging, CRISPR-interference (indirect), protection

against probe perturbation, DNA barcoding in imaging, post-mortem for following cells in a population.

- Expanding imaging dimensionality: special, temporal, modalities trade-offs.
- Automation: from bench (bioreactor, soils) to the imaging system and vice versa (*in situ*, live-cell sorting through dedicated microfluidics or optofluidics).
- Integration with existing DOE facilities (e.g., X-ray, cryo-EM, mass spectrometry).
- Quantum super-resolution imaging: coincidence-throughput-dimensionality trade-offs (e.g., SOFI, antibunching).
- Systematic approaches to ensure cross-validation, reproducibility, and better understanding of light/electron/ion effects on molecules.
- Synthetic imaging systems, [e.g., synthetic soils, leaves, and roots, anaerobic microenvironments, (micro)gravity control, EcoFAB-like environments].
- Systems of interest: plants, synthetic biology chassis, mixed cultures with species tracking, aerobic/anaerobic, motile microorganisms, algae, fungi, bacteria.
- Correlating multimodal dynamic and static snapshot imaging for holistic understanding of chemical-structural-functional linkages: combining both destructive and nondestructive methods, live-cell dynamics followed by correlative EM, MS, ion imaging or analysis, and visual proteomics.

Potential Ideas Discussed for Expanding BSP's Impact and Interactions

Community Access to BSP-Developed Technologies Through User Facilities

User accessibility to new BSP technologies and approaches is a key factor for the program's success and longevity. Deploying some BSP imaging capabilities analogous to DOE scientific user facilities would expand the research community's access to these technologies, thereby increasing their impact. Such an approach would also facilitate continued technological developments through the important user-developer feedback loop and the synergistic interactions between imaging scientists and facilities. These interactions would expand the scope of research being conducted using BSP-developed capabilities.

Bioimaging Science Program Annual Meeting

BSP's annual PI meeting provides an important avenue for the program to increase the cross-platform, cross-disciplinary, and multiscale synergies needed to achieve its goals. Scheduling this meeting proximal to the DOE Genomic Science program (GSP) annual PI meeting creates invaluable opportunities for synergistic interactions with that community. Furthermore, inviting imaging experts external to BSP as keynote speakers interjects novel perspectives and approaches into discussions during the program's annual meeting. Additional interactions across BSP's research teams (e.g., through teleconferencing or web conferencing) could help maintain this interactive momentum and catalyze new directions of investigation.

Additional Cross-Program Interactions and Community Engagement

Additional comments from meeting attendees envision opportunities to foster even more direct cross-fertilization and interaction between the GSP and BSP research communities. The envisioned opportunity could provide travel and supply costs of embedding a graduate student or postdoctoral researcher from a GSP-funded research group into a BSP-funded research group for 1 to 6 months. This arrangement would stimulate more direct collaboration and cross-talk between the two programs, yielding benefits for both. For GSP researchers, this collaboration would give them access to cutting-edge technology that otherwise may have been beyond reach, leading to new scientific discoveries. For BSP researchers, it would provide access to new science and samples they could use for adapting, benchmarking, and evaluating the performance of their newly developed instrumentation and methods. This mechanism would be very similar to DOE's Office of Science Graduate Student Research opportunity. However, instead of enabling researchers to pursue part of their graduate thesis research at a DOE national laboratory or user facility, it would support GSP researchers who want to visit and use the new technologies developed by BSP-funded groups at universities and national laboratories.

Finally, the creation of a bioimaging capability portal could enhance BSP's impact on a wider community of scientists who could use the program's bioimaging approaches. As part of outreach to BER researchers, the portal would detail BSP's diverse technological approaches, highlight the applications for which they are best suited, and provide a forum for information dissemination, tutorials, and training opportunities.

Bioimaging Science Program Projects

Universities

Multimodal Single-Cell/Particle Imaging and Engineering for Energy Conversion in Bacteria

Peng Chen, Cornell University

Plasmonics-Enhanced Optical Imaging Systems for Bioenergy Research*

Tuan Vo-Dinh, Duke University

Deep Chemical Imaging of the Rhizosphere

Marcus Cicerone, Georgia Institute of Technology

Real-Time Imaging and Quantification of Plant Cell Wall Constituents Using Cavity-Dumped Stimulated Raman Scattering (cdSRS) Microscopy*

Shi-You Ding, Michigan State University

Single-Molecule Imaging of Lignocellulose Deconstruction by SCATTIRSTORM Microscopy*†

William O. Hancock, Pennsylvania State University

Time-Resolved 3D Multiresolution Microscopy for Real-Time Cellulase Actions *In Situ**

Haw Yang, Princeton University

***In Planta* Single-Molecule Imaging and Holographic Force Spectroscopy to Study Real-Time, Multimodal Turnover Dynamics of Polysaccharides and Associated Carbohydrate Metabolites*†**

Sang-Hyuk Lee, Rutgers University

Development of High-Throughput Light-Sheet Fluorescence Lifetime Microscopy For 3D Functional Imaging of Metabolic Pathways in Plants and Microorganisms

Mark Kasevich, Stanford University

Ultrasensitive High-Resolution Label-Free Nonlinear Optical Microscopy for Imaging Plant-Microbe Interactions *In Vivo*

Na Ji, University of California–Berkeley

Development of Broadband Infrared Nano-Spectroscopy of Biological Materials in Fluid*†

Tina Jeoh, University of California–Davis

Tracking Lignocellulosic Breakdown by Anaerobic Fungi and Fungal Cellulosomes

Michelle O'Malley, University of California–Santa Barbara

Inorganic Voltage Nanosensors as Tools for Bioelectricity Studies in DOE-Relevant Bacteria and Their Communities

Shimon Weiss, University of California–Los Angeles

Nondestructive, Three-Dimensional Imaging of Processes in the Rhizosphere Utilizing High-Energy Photons

Shiva Abbaszadeh, University of California–Santa Cruz

Understanding Plant Signaling via Innovations in Probe Delivery and Imaging*

Jean T. Greenberg, University of Chicago

Quantum Dot Toolkit for Multimodal Hyperspectral Bioimaging

Jeffrey Cameron, University of Colorado–Boulder

Spatiotemporal Dynamics of Photosynthetic Metabolism in Single Cells at Subcellular Resolution*

Jeffrey Cameron, University of Colorado–Boulder

Live-Cell, Quantum Dot–Based Tracking of Plant and Microbial Extracellular Vesicles

Jeffrey Caplan, University of Delaware

Correlative Imaging of Enzyme and Metabolome Dynamics for Yield and Titer Co-Optimization in Biofuel Producing Microorganisms*

Andreas Vasdekis, University of Idaho

Integrative Imaging of Plant Roots During Symbiosis with Mycorrhizal Fungi

Andreas Vasdekis, University of Idaho

Development and Implementation of an *In Situ* High-Resolution Isotopic Microscope for Measuring Metabolic Interactions in Soil Mesocosms

Elizabeth Shank, University of Massachusetts Chan Medical School

Biological Imaging Using Entangled Photons

Theodore Goodson, University of Michigan

Expanding the Utility and Range of Quantum and Polymer Dots for Multiplexed Super-Resolution Fluorescence Imaging in Plants

Gary Stacey, University of Missouri

Hyperspectral Light Sheet Raman Imaging of Leaf Metabolism*†

Keith Lidke, University of New Mexico

Metaoptics Enabled Multifunctional Imaging*

Paul W. Bohn, University of Notre Dame

Novel *In Vivo* Visualization of Bioenergy Metabolic and Cellular Phenotypes in Living Woody Tissues

Leslie Sieburth, University of Utah

Multiparametric Optical Label-Free Imaging to Analyze Plant Cell Wall Assembly and Metabolism*

Marisa S. Otegui, University of Wisconsin-Madison

* No-cost extension projects
† Abstract not submitted

* No-cost extension projects

† Abstract not submitted

Project Map: Current Projects and No-Cost Extensions

University of California–
Berkeley

University of California–
Davis

Lawrence Livermore
National Laboratory

Pacific Northwest
National Laboratory

University
of Idaho

University
of Utah

University of
Colorado–Boulder

Michigan State
University

University of
Notre Dame

University
of Chicago

University of Wisconsin–
Madison

Rutgers
University

University
of Massachusetts
Chan Medical School

University
of Michigan

Cornell
University

Brookhaven
National Laboratory

Princeton
University

University
of Delaware

Duke
University

Pennsylvania State
University

University of California–
Los Angeles

University of California–
Santa Barbara

University of California–
Santa Cruz

Stanford
University

Los Alamos
National Laboratory

University of
New Mexico

University
of Missouri

Ames
Laboratory

Georgia Institute
of Technology

Oak Ridge
National Laboratory

University Project   **National Laboratory Project**

Current Projects

Multimodal Single-Cell/Particle Imaging and Engineering for Energy Conversion in Bacteria

Principal Investigator: Peng Chen

Institution: Cornell University

Email: pc252@cornell.edu

Research Plans and Progress: This project aims to combine quantum materials synthesis, bacterial synthetic biology, and multimodal single–entity imaging to quantitatively study how hybrid quantum dot (QD)-bacteria systems convert light to value chemicals at the single-cell to subcellular level, with the overall goal of gaining insights to guide the engineering of QDs and bacterial genetics for more efficient bioenergy conversion (see figure, next page).

One effort reduced the complexity of QD-microbe interactions to focus on the specific charge transport phenomena at the interface of a photoexcited QD and microbial cell membrane. The team developed a bioelectronic platform that couples microbial outer membranes with colloidal QDs in a standard three-electrode electrochemical device. The working electrode consisted of indium tin oxide (ITO) glass, conductive polymer, and microbial outer membrane–supported lipid bilayers (SLBs). The electrolyte contained controlled concentrations of QDs and native biological redox mediators in an aqueous buffer, and platinum (Pt) was used as a counter electrode. The model bioelectronic platform used *Pseudomonas aeruginosa* outer membranes, CdSe QDs, and pyocyanin redox mediators. The team reported a comprehensive electrochemical analysis of the thermodynamics and kinetics at the interface of QDs and microbial outer membranes, providing critical new insight into the pathways for charge transport from photoexcited QDs through redox mediators and the outer membrane of the SLB (submitted to *ACS Applied Material Interfaces*).

In another, the project used multimodal, single-molecule, super-resolution fluorescence imaging to study *Ralstonia eutropha* cells in fixing CO_2 into bioplastic polyhydroxybutyrate (PHB), using either H_2 or photoexcited electrons from semiconductor particles as the energy source. Results showed that under H_2+CO_2 lithoautotrophic growth, soluble hydrogenase is essential for PHB production. In contrast, membrane-bound hydrogenase is nonessential for PHB production but plays a facilitating role in forming more PHB when the cellular level of soluble hydrogenase is higher. The project's use of multimodal single–entity imaging further implemented single-cell photoelectrochemical current mapping and showed that single *R. eutropha* cells can sustain nanoampere-level photocathodic current on thin-film photoelectrode, in which both membrane-bound and soluble hydrogenases play key roles. This level of current far exceeds the capability of H_2-mediated electron transport, and the team found it occurring via non-H_2-mediated pathways, which opens the possibility of using *R. eutropha* in integrated hybrid systems for bioenergy conversion. The team also showed that a single cell's capability of uptaking electrons directly from a semiconductor is linearly correlated with cellular levels of membrane-bound and soluble hydrogenases (manuscript in preparation).

Potential Benefits and Applications: This research will provide quantitative knowledge to understand the basic materials and biological factors as well as guiding principles to engineer and improve such systems. If successful, this project's research will transform the study of hybrid inorganic-bacteria systems for energy and chemical conversions. The project's experiments should break new scientific grounds and open unforeseen opportunities.

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Multimodal Single-cell/particle Imaging and Engineering for Energy Conversion in Bacteria
Peng Chen (PI), Tobias Hanrath, and Buz Barstow (Cornell University)

A Bioelectronic platforms to study charge transfer at photoexcited QDs/microbial membrane interfaces (Hanrath group)

B System-wide genetics to uncover genes for electron transport (Barstow group)

C Multi-modal single-entity imaging to elucidate energy conversion pathways in microbial-inorganic hybrids (Chen group)
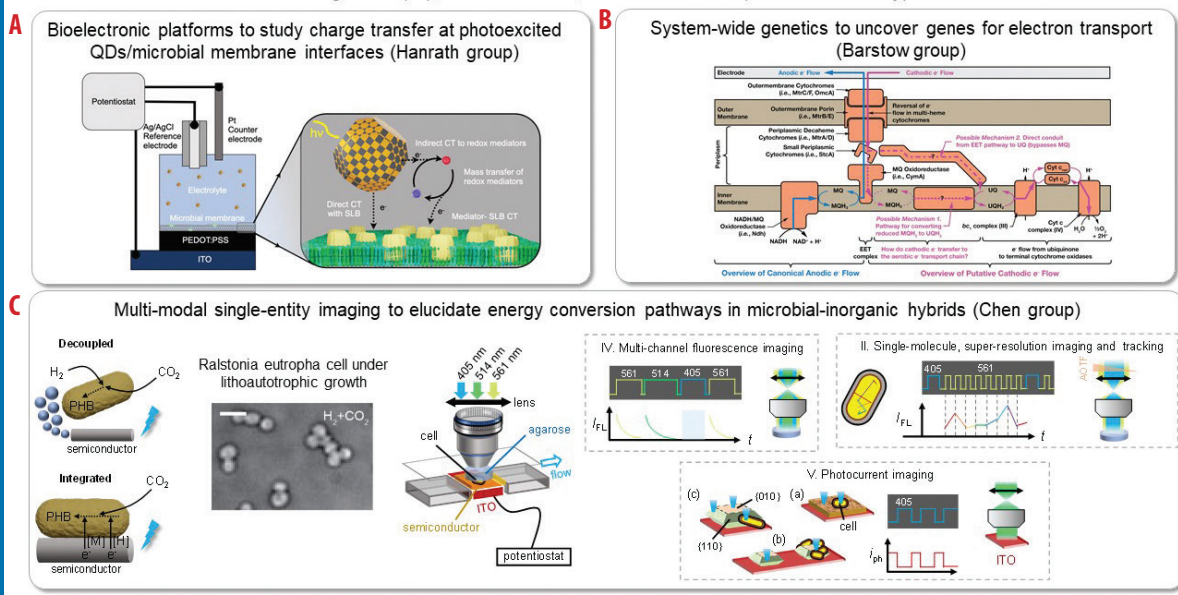
(A) Design of bioelectronic platform to investigate charge transfer at the quantum dot (QD) microbial-supported lipid bilayer interface. The inset illustrates specific interactions between QDs, redox mediators, and the microbial-supported lipid bilayer. **(B)** Electron uptake in the model electroactive microbe *Shewanella oneidensis* MR-1 cannot be fully explained by reversal of its extracellular electron-transfer pathway. The canonical anodic extracellular electron-transport (EET) pathway for electron deposition is shown in light blue and the putative cathodic extracellular electron-uptake (EEU) pathway is shown in pink. Known electron-transfer pathways are denoted with solid lines, while speculated transfer pathways are shown as dashed lines. Two possible mechanisms for transfer of cathodic electrons from the Mtr EET complex to the ubiquinone pool and onto terminal cytochrome oxidases are highlighted. The project speculates that two of the proteins identified in this work (SO_0400 and SO_3662) could form part of possible mechanism 2. **(C)** Schematics of the project's multimodal single-entity imaging approach to interrogate the hybrid QD-bacteria system for energy conversion, including multichannel fluorescence microscopy at the single-cell/single-molecule level and single-cell photoelectrochemical microscopy. [Courtesy (A)Tobias Hanrath and Mokshin Suri, Cornell University, (B) Reprinted under a Creative Commons Attribution 4.0 International License (CC BY 4.0) from Rowe, A. R., et al. 2021. "Identification of a pathway for electron uptake in *Shewanella oneidensis*," *Communications Biology* **4**, 957. DOI:10.1038/s42003-021-02454 and Barstow group, Cornell University, and (C) Peng Chen group, Cornell University.]

Deep Chemical Imaging of the Rhizosphere

Principal Investigator: Marcus Cicerone

Institution: Georgia Institute of Technology

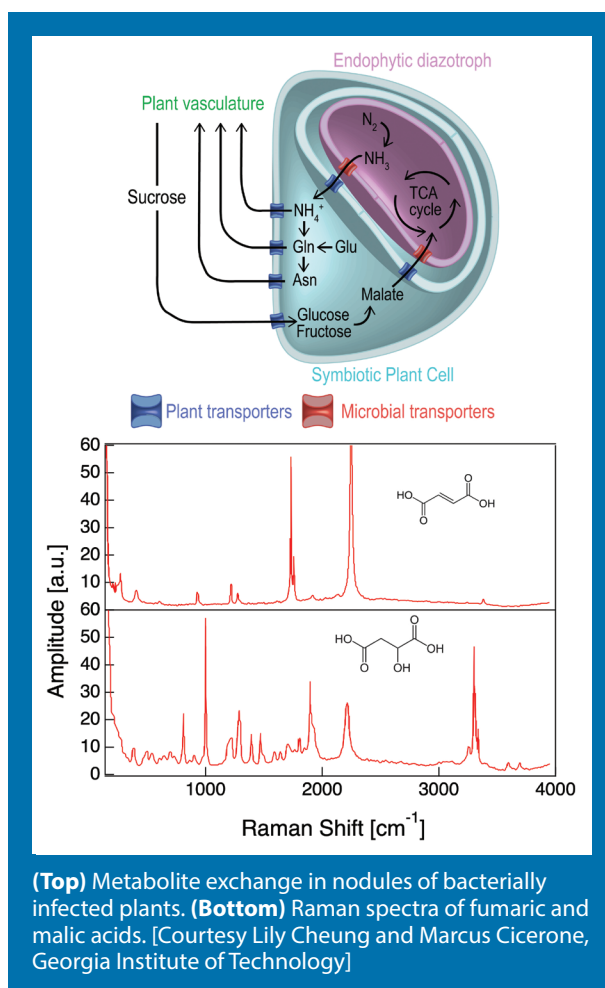**Email:** cicerone@gatech.edu

Research Plans and Progress: (1) Instrument Development. A major goal of this project is to develop an instrument based on coherent anti-Stokes Raman scattering (CARS) and time-gated dynamic scattering (TGDS). Instrument development requires generating spectrally broad coherent light and designing signal generation and collection approaches. The team has made the following progress:

- Purchased the instrument laser.

- Reconfigured 1,600 ft² of laser laboratory space shared between Cicerone and Robles Labs to accommodate instrument development and imaging under this project.

- Recruited two graduate students and one postdoc to develop instrumentation and signal analysis protocols.

- Begun to acquire library Raman spectra for metabolites the team will track.

The postdoctoral researcher has created a software framework for acquiring and displaying the CARS signal. One of the graduate students is developing a theoretical framework to use for optimizing coherent, broad-spectrum light generation. Both students are becoming familiar with existing custom-built instrumentation in the project's laboratories that will inform the development of the new instrument.

(2) Plant Models. Additionally, the team has successfully recruited and trained a graduate student on sterile plant and bacteria culture. They have secured all plant and bacteria samples necessary for experiments in Years 1 and 2: A wild-type *Medicago truncatula* (sequenced ecotype R108) plants and four *Tnt1* retrotransposon insertion mutant lines with a knockout of either *MtSWEET11* or *MtSWEET1* genes. These sugar transporters have been implicated in the interaction of *Medicago* with nodule-forming and free-living nitrogen-fixing bacteria, respectively. Nitrogen-fixing bacteria samples include the nodule-forming *Sinorhizobium meliloti* and the free-living *Azotobacter*. The team also constructed autoclavable and humidity-controlled plant growth chambers for the sterile germination and handling of seeds. This will allow recording of root architecture and nodule development before transferring to soil or soil mimetic systems and imaging.

Potential Benefits and Applications: This project is building and demonstrating a label-free microscope to image

(Top) Metabolite exchange in nodules of bacterially infected plants. **(Bottom)** Raman spectra of fumaric and malic acids. [Courtesy Lily Cheung and Marcus Cicerone, Georgia Institute of Technology]

metabolic activity and chemical exchange between plants and bacteria, deep within thick living plant roots and their associated rhizosphere microbial communities. The instrument developed will be reasonably reproduced to enable other DOE BER–supported researchers to visualize the transport of materials during plant-microbe and microbe-microbe interactions and track metabolic pathways. It will allow team members to improve their understanding of the allocation of carbon compounds from photosynthesis, the mechanisms controlling nutrient fluxes in the rhizosphere, and new microbial metabolic pathways in the production of biofuels and chemicals that could reasonably be duplicated by other plant scientists and used in DOE-funded research. In the first four months of funding, the project has focused on developing instrumentation, model plants, and model soils.

Development of High-Throughput Light-Sheet Fluorescence Lifetime Microscopy for 3D Functional Imaging of Metabolic Pathways in Plants and Microorganisms

Principal Investigator: Mark Kasevich

Institution: Stanford University

Email: kasevich@stanford.edu

Research Plans and Progress: This project is developing techniques for functional 3D fluorescence lifetime microscopy (FLIM) using its recently demonstrated electro-optic (EO)-FLIM method. EO-FLIM employs electro-optic modulators to combine high throughput and high sensitivity for lifetime imaging in wide field, overcoming the throughput bottleneck of traditional FLIM methods and improving acquisition speeds by up to five orders of magnitude. The project's goal is to demonstrate a practical high-speed FLIM platform for light-sheet microscopy of metabolic pathways and plant-microbiome interactions. The aim is to enable new biochemical contrast mechanisms for fluorescence imaging by exploiting the nanosecond time domain—using both fluorescent labels and endogenous autofluorescence.

Accomplishments and Deliverables: The EO-FLIM method has been developed starting from proof-of-concept demonstrations (Bowman et al. 2019). Subsequent improvements enabled widefield optical gating at 40 MHz using a resonant drive technique. This allowed widefield FLIM of single molecules with high sensitivity in applications such as super-resolution microscopy and FRET (Bowman and Kasevich, 2021). The program has focused on continuing development of the EO-FLIM technique by building two microscopy platforms: a light-sheet microscope and widefield single-molecule microscope. These systems will incorporate multispectral units to allow simultaneous capture of lifetime, spectral, and polarization information in widefield images. Progress in the current performance period includes:

1. Completed component sourcing and began integration of light-sheet FLIM (see Fig. a, next page), widefield FLIM (see Fig. b), and excitation laser systems.

2. Completed phase-stable resonant Pockels cell design at 40 MHz with active cooling (see Figs. b and c).

3. Expressed and purified library of fluorescent proteins for use in multiplexed FLIM imaging (see Fig. d). Lifetimes are in good agreement with database values.

4. In progress: Instrument control and analysis pipeline.

5. In progress: Preparation of biological samples for light-sheet and widefield imaging.

Potential Benefits and Applications: Fast widefield FLIM will enable exciting opportunities for biophysical measurements and label-free autofluorescence imaging. Due to the all-optical nature of the EO-FLIM imaging path, multiple imaging dimensions can be measured for each acquired photon without trade-offs. Spatial coordinates, wavelength, polarization, time-resolved anisotropy, and nanosecond lifetime can all be measured at the frame rate of an sCMOS camera (>100 frames/sec on bright samples). This provides a wealth of information to enhance optical measurements such as FRET and single-particle tracking. In samples such as plant tissues, multidimensional detection provides a valuable means to image multiple biological molecules spatiotemporally by separating the signals from fluorescence-labeled and endogenous autofluorescent molecules using fluorescence lifetime signatures. One target application is the real-time imaging and characterization of extracellular vesicles. Combining widefield imaging with spectroscopic capabilities of EO-FLIM may allow not only tracking of single vesicles, but also obtaining of an optical signature of their chemical contents.

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EO-FLIM System Integration.
(a) Lightsheet FLIM system under construction using a modified commercial base.
(b) Widefield EO-FLIM optics path comprising a custom resonant Pockels cell (top left), image splitting optics (bottom left), and a low-noise sCMOS camera (bottom right).
(c) Resonantly driven 40 MHz Pockels cell with thermal stabilization and motorized tuning.
(d) Phase traces with best fit lifetimes indicated are plotted for solutions of fluorescent proteins. These traces are all taken within a single spectral channel, demonstrating potential for lifetime-based multiplexing. [Courtesy Adam Bowman, Stanford University]

Ultrasensitive High-Resolution Label-Free Nonlinear Optical Microscopy for Imaging Plant-Microbe Interactions *In Vivo*

Principal Investigators: [1]Na Ji (PI), Trent R. Northen,[2] and John P. Vogel[2]

Institutions: [1]University of California–Berkeley and [2]Lawrence Berkeley National Laboratory

Email: jina@berkeley.edu, trnorthen@lbl.gov, and jpvogel@lbl.gov

Research Plans and Progress: Visualizing root morphology at cellular and subcellular resolution is critical for understanding plant growth and the accompanying structural changes, as well as their interactions with microbes in the rhizosphere. Optical microscopy based on second harmonic generation (SHG) and third harmonic generation (THG) provides high-resolution images of plant-microbe interaction without extrinsic labeling but causes photodamage with limited imaging resolution at depth. This project aims to combine several advanced optical techniques with SHG and THG microscopy to reduce photodamage and improve imaging resolution and depth for live plant roots and microbes grown in microfabricated ecosystems (EcoFABs).

The team has manufactured EcoFABs for imaging live *Brachypodium distachyon* roots and microbes and successfully acquired SHG and THG images at subcellular resolution. The team is designing homodyne mixing modules for SHG and THG microscopes. Once assembled and incorporated into the microscopes, the modules will generate a reference signal, which will interfere with the weaker sample signal to increase detection sensitivity. This should allow a substantial reduction in excitation power and minimize photodamage for chronic *in vivo* imaging of roots. Researchers have tested the application of adaptive optics to SHG and THG microscopy and have improved signal strength and image resolution from tissue samples. They will apply adaptive optics to SHG and THG imaging of root samples and expect to observe improvements in image quality there as well. To test whether longer wavelength excitation will reduce photodamage and increase imaging depth, the project has acquired the optics needed for the detection of SHG and THG signals of longer wavelengths. The team has used project microscopes to image root samples with fungal colonies and confirmed that label-free imaging of fungi using THG is feasible. In addition, researchers have imaged root samples mixed with bacteria and found that they can measure the distribution of bacteria surrounding roots based on the intrinsic THG signal from roots and bacteria alone.

Potential Benefits and Applications: The project's combined efforts of microscopists and plant biologists will provide the first insights into the mechanisms, localization, and sequence of colonization of live plant roots by growth-promoting fungi and bacteria and their effect on root cellular and subcellular morphology and exudates. Given the widespread use of SHG and THG microscopy for label-free imaging, the project's methodologies will be of interest to the broader scientific community and can be applied to studying other biological samples.
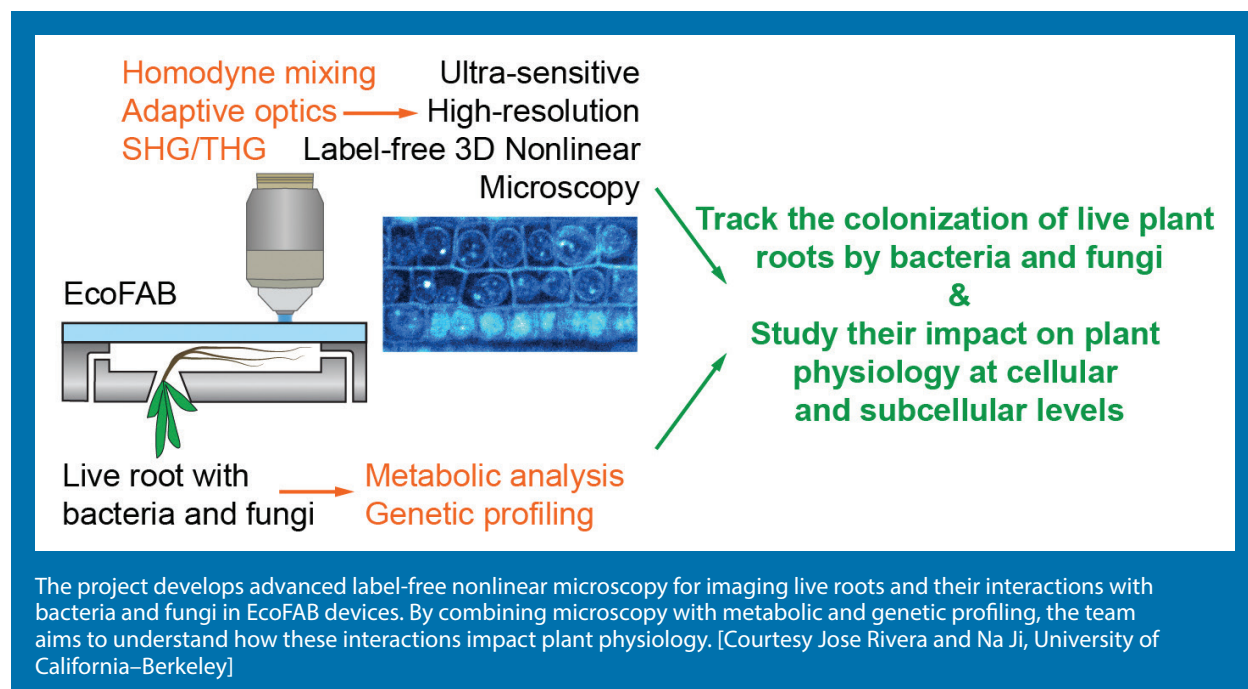
The project develops advanced label-free nonlinear microscopy for imaging live roots and their interactions with bacteria and fungi in EcoFAB devices. By combining microscopy with metabolic and genetic profiling, the team aims to understand how these interactions impact plant physiology. [Courtesy Jose Rivera and Na Ji, University of California–Berkeley]

Tracking Lignocellulosic Breakdown by Anaerobic Fungi and Fungal Cellulosomes

Principal Investigators: Michelle O'Malley[1] (PI) and James Evans[2]

Institutions: [1]University of California–Santa Barbara and [2]Pacific Northwest National Laboratory

Email: momalley@ucsb.edu and james.evans@pnnl.gov

Research Plans, Progress, and Objectives: Anaerobic fungi degrade plant biomass through invasive, filamentous growth and the secretion of multiprotein biomass-degrading complexes called fungal cellulosomes. Despite their potential for lignocellulosic bioprocessing, no nondestructive, real-time imaging tools exist to probe anaerobic fungi or the action of their cellulosomes across spatial and temporal scales. This project is developing new bioimaging approaches to learn how anaerobic fungi orchestrate biomass degradation through their unique multiprotein cellulosomes—and how these fungi are able to access carbohydrate biopolymers encased in lignin. Multiplexed imaging tools based on the synthesis of novel quantum dot (QD) nanobody fusions targeted at different components of fungal cellulosomes will reveal where cellulosome components are localized within the fungal ultrastructure and at the interface with lignocellulose. The project's approach benefits from a suite of new genomic, transcriptomic, and proteomic data obtained for multiple strains of anaerobic fungi. This data enables the synthesis of custom QD and nanobody probes to localize cellulosomes and track their dynamics without suffering photobleaching effects. The team will also leverage a cell-free production pipeline to reconstitute QD-tagged fungal cellulosomes in vitro and characterize enzyme rearrangement, kinetics, substrate breakdown, and high-resolution structure via cryoelectron microscopy (cryo-EM). As a complementary approach, the project is advancing genetic tools for the anaerobic fungi to conjugate QD probes onto cellulosome components *in vivo*—a process which further enables hypothesis testing of protein function in genetically recalcitrant anaerobic systems.

Anticipated Accomplishments and Deliverables: In the current project period, novel nanobody tools were synthesized and deployed to image native fungal cellulosomes. Antibodies raised against key fungal cellulosome components were also used to define the localization patterns of cellulosomes in mature fungal mats versus fungal zoospores, revealing direct connections between cellular life stage progression and the regulation of cellulosome production. A preliminary cryoEM structure of a native fungal cellulosome was achieved, as well as a proof-of-concept for transformation of fungi with flavin-based anaerobic reporter proteins for in vivo labeling of cellulosome components.

Potential Benefits and Applications: Overall, this project will establish new state-of-the-art bioimaging capabilities to observe cellulosome dynamics and localization *in situ* with label-based and label-free approaches and will reveal critical attributes of fungal cellulosomes that can be engineered and exploited for biobased fuel and chemical production. Ultimately, the bioimaging capabilities will fold into the Environmental Molecular Sciences Laboratory (EMSL) user program and be accessible to the general research community with applications well beyond fungal cellulosome dynamics.

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(A) Schematic of the affinity chromatography setup used to find the nanobody binding partners and representative Western blot data showing the double dockerin-GH48 protein in the bound eluent. (B) ELISA binding curves of three double dockerin variants. K_D's: WT −2.6 ± 1.2 nM; W28F − 5.2 ± 2.4 nM; W35F − 0.4 ± 0.5 nM. (C) Preliminary, low-resolution cryo-EM structure of a native cellulosome complex from the anaerobic fungus *Neocallimastix californiae* grown on switchgrass. [Courtesy Stephen Lillington, James Evans, and Michelle O'Malley]

Inorganic Voltage Nanosensors as Tools for Bioelectricity Studies in DOE-Relevant Bacteria and Their Communities

Principal Investigators: Shimon Weiss[1] (PI), Robert P. Gunsalus,[1] Robert Clubb,[1] and Evan Miller[2]

Institutions: [1]University of California–Los Angeles and [2]University of California–Berkeley
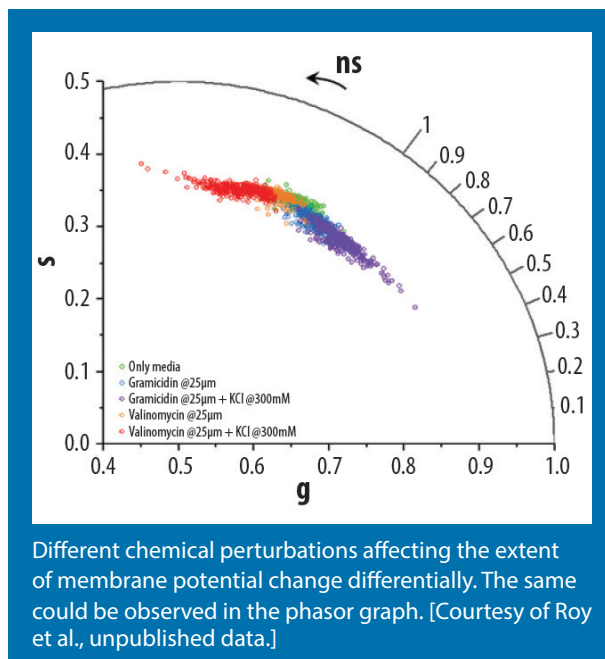
Email: sweiss@chem.ucla.edu

Research Plans and Progress: (1) With iterative testing and optimization of synthetic protocols, substantial single-particle voltage sensitivity (~2% spectral Stark shift, up to ~30% ΔF/F per 160 mV) could be achieved at room temperature for engineered nanoparticles. (2) Biomaterial-derived surface ligands that satisfy anisotropic facet-selective coating have been developed. The hybrid bionanomaterial results in effective compartmentalization beyond nonspecific staining. membrane potential (MP) changes in self spiking–and patched-HEK293 cells—patched cortical neurons can be clearly visualized with these hybrid materials. (3) Organic nanoparticles, which utilize fluorescence resonance energy transfer (FRET) for sensing local electric field changes, have been developed in parallel. With nanodisc-based organic nanoparticles, MP recordings from individual targeted sites (synapses and spines) have been realized. (4) A library of photoinduced electron transfer (PeT)-based VoltageFluors (VF) dyes (e.g., VF2.1Cl and BeRST) has been screened for bacterial membrane staining and Δψ sensitivity. (5) Synthesis of BeRST derivative (3-sulfono replaced by 3-carboxy) has been explored and optimized. This will allow improved localization and orientation of dyes in bacterial membranes, while retaining the high-voltage sensitivity of the parent compound. (6) With gram-positive model system *Bacillus subtilis*, utilizing VF2.1Cl, single-cell level end point Δψ measurements as a function of chemical perturbations have been done. A library of chemicals (at varying concentrations) has been used: (a) CCCP (a protonophore), (b) Valinomycin (a K+-specific pore-forming ionophore), (c) Gramicidin (a K+-specific channel-forming ionophore), and (d) KCl. These proof-of-concept experiments demonstrate that the lifetime measurements of VF dyes provide an efficient way to measure ΔΨ changes optically and quantitatively in bacterial cells. A phasor-based analysis of the FLIM data has been performed. (7) Exploration work towards engineering surface pili to bind MPNs / VF dyes for increased efficiency of membrane targeting is in progress. (8) Several new microbial strains (closely related to *Clostridium thermocellum*), with thinner cell envelope and more susceptible to manipulation and insertion of lipophilic probes, have recently been isolated and cultured. The new strains are currently being screened for substrate use and stocked for preservation. (9) In collaboration with École Polytechnique Fédérale de Lausanne (EPFL), the project is

Different chemical perturbations affecting the extent of membrane potential change differentially. The same could be observed in the phasor graph. [Courtesy of Roy et al., unpublished data.]

continuing its effort towards the development of detectors (microlens-equipped, time-gated SPAD camera (SwissSPAD3) and methodology (phasor-based real-time data processing) for wide-field imaging of Δψ in bacterial communities.

Anticipated Accomplishments and Deliverables: The proof-of-concept experiments based on VF dyes and chemical perturbation of Δψ will be extended from confocal FLIM measurements to wide-field FLIM measurements. The effect of cellulose degradation and glucose metabolism on Δψ changes will be probed. Wide-field FLIM setup will be combined with temperature-controlled microfluidics for better control of cellular environment and long-term monitoring. With this new capability, Δψ changes in bacterial biofilm will be imaged and recorded under steady-state conditions and dynamically under nonequilibrium and spatial gradient conditions of nutrient limitation, substrate consumption, and environmental stressors.

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Nondestructive, Three-Dimensional Imaging of Processes in the Rhizosphere Utilizing High-Energy Photons

Principal Investigators: Shiva Abbaszadeh[1] (PI), Weixin Cheng,[1] Craig S. Levin,[2] and Adam S. Wang[2]

Institutions: [1]University of California–Santa Cruz and [2]Stanford University

Email: sabbasza@ucsc.edu

Research Plans and Progress: Soil structure, described as either the status of aggregation or distribution and networks of pore spaces, controls Earth system functions. Due to the inadequacy of soil structure data from analysis of intact soils, soil structure has not been successfully incorporated into current Earth system models. This inadequacy includes three key aspects: (1) a major challenge in scale integration from the micron scale to the ecosystem scale; (2) difficulties in functional connectivity between soil structural properties and functional processes (e.g., water, carbon, and nitrogen cycles), and (3) lack of dynamic observations and measurements of soil structural changes in response to disturbances at various temporal scales. To address these challenges, this project aims to develop a system combining positron emission tomography (PET) and computed tomography (CT) to make quantitative, *in situ*, 3D images of dynamic rhizosphere phenomena.

The goals of the first year of the project are to develop a section of the PET scanner, create a radiotracer infrastructure for ^{11}C radioisotope labeling and imaging at Stanford University, and downselect complementary metal-oxide-semiconductor (CMOS) readout electronics for the amorphous selenium (a-Se) detector for X-ray imaging. Within the first 4 months of the project's inception on August 15, 2021, the team added two graduate students and a postdoc. PET system development has been initiated in collaboration with Stanford University. Potential CMOS candidates have been identified, and a collaboration with Lawrence Berkeley National Laboratory has been established to test a-Se on the CMOS readout.

Anticipated Accomplishments and Deliverables: The modular PET system design was finalized to simplify construction, disassembly, troubleshooting, repair, and maintenance. The system footprint was optimized for plant imaging with ^{11}C-labeled reagents to account for space constraints inside the fume hood (130x60x80 cm^3) where experiments would be conducted. A box-shaped scanner geometry (see Fig. 1a) was selected among various configurations due to proven high sensitivity and the flexibility to vary the field of view (FOV) if required. The team redesigned the "intermediate" circuit boards that serve as a connective bridge between the cadmium zinc telluride (CZT) detectors, high voltage distribution board, and RENA3 readout ASICs using rigid flex printed circuit board (PCB) design (see Fig. A, next page).

The design of 11C labeling and tracing chamber (see Fig. B) was finalized. This design includes a top labeling chamber (20 cm tall, 10 cm diameter acrylic) and a bottom soil column (10 cm tall, 10 cm diameter acrylic). The top chamber is made to fit on the top edge of the bottom one and sealed/fixed by using a rubber sleeve seal. The team included a 3-way solenoid valve fitted to the air circulating system of the top chamber to introduce the [11]CO$_2$.

For CT, three different CMOS chips (Topmetal II, RD53B, 1k $\times$ 1k ROIC by KA Imaging) were identified. The team verified the fabrication process and developed two prototype detectors utilizing the CMOS chips by directly depositing multilayer a-Se detector on active area of the CMOS chips (Fig. C). The test of the prototype detector will be accomplished by August 14, 2022, and the team will complete a portion of the PET system.

Potential Benefits and Applications: With low-cost a-Se–based technology for micro-CT and with the continuous reduction of CZT cost reaching scintillators, a PET/CT system based on CZT and a-Se will be the appropriate choice for imaging the rhizosphere in terms of cost, performance, and complexity. With an accessible PET/CT system for rhizosphere imaging, researchers from the community will be able to probe the processes and interactions within the rhizosphere to answer key questions, such as how underground fungal networks are connected and use the same water source and nutrients.

(A) Positron emission tomography scanner. **(B)** Design of a ^{11}C labeling and tracing chamber. **(C)** Development of high voltage a-Se detector on top of RD53B (left) and Topmetal II (right) CMOS ROIC. [Courtesy Shiva Abbaszadeh]
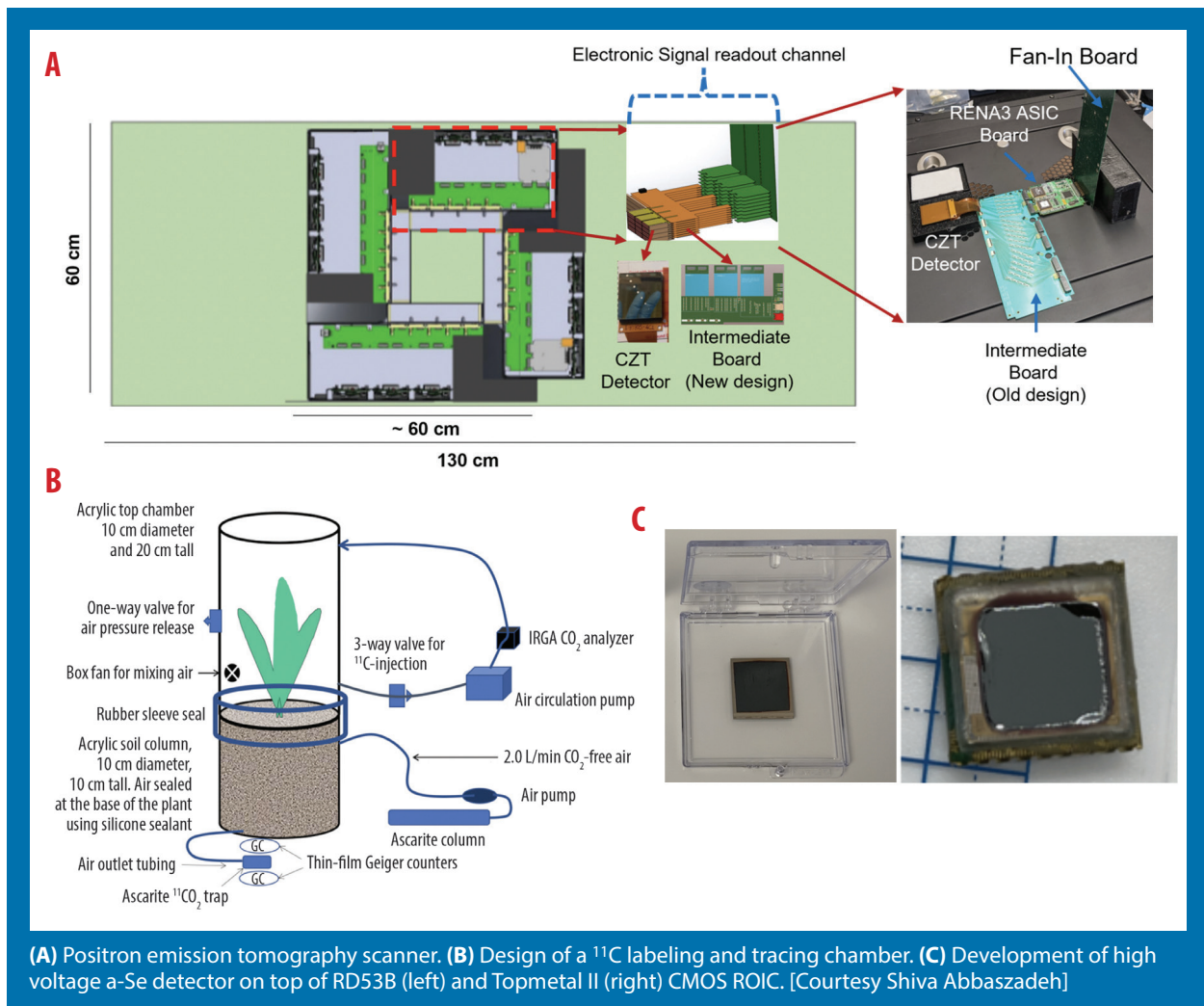
Quantum Dot Toolkit for Multimodal Hyperspectral Bioimaging

**Principal Investigators:** Jeffrey Cameron and Anushree Chatterjee

Institution: University of Colorado–Boulder

Email: jeffrey.c.cameron@colorado.edu and chatterjee@colorado.edu

Research Plans and Progress: The objectives of this project are to develop a multifunctional quantum dot (QD) toolkit for imaging and manipulating microbial metabolism. Spectral tuning and surface modification of quantum dots enable specific targeting and hyperspectral imaging of labeled enzymes. Multicolor QDs can be multiplexed and employed to localize multiple proteins in live cells and can be used as fiducial markers in correlative light-electron microscopy. These tools are being applied to the study of carbon assimilation in cyanobacteria (see figure) and biomass degradation in thermophilic anaerobes. The project is on track to complete each of the project milestones.

Anticipated Accomplishments and Deliverables: The project has completed the initial design and testing of multiple QDs and generated and characterized compatible cyanobacterial strains for targeted live-cell imaging. The team has improved its imaging pipeline to enable multidimensional imaging of cells and QDs using multiphoton excitation with a tunable laser. By imaging and manipulating metabolism in single microbial cells, researchers will be able to generate a novel map of metabolic pathways at the subcellular level. The project is currently focused on multiplexed labeling for high-resolution imaging of metabolic processes using light and correlated cryoelectron microscopy and tomography that will provide a new view of cellular structure and function.

Potential Benefits and Applications: QDs are versatile tools for biological imaging and manipulation of biological processes for the benefit of society and the environment. Because the spectral properties, size, composition, and surface functionalization can be independently controlled, QDs can be tuned for specific applications. Because cyanobacteria contain many native pigment-protein complexes, imaging with traditional chemical or genetically encoded fluorophores can be challenging due to spectral overlap or bleaching. QDs are highly stable and can be tuned to sit within the spectral window of the target organism (see figure). In addition, photoelectrons derived from specific
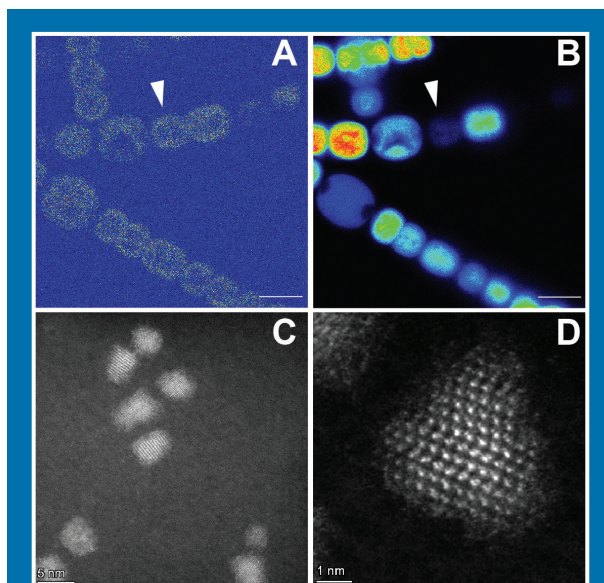
Stable interactions between CdSe/ZnS quantum dots (QDs) coated with polyacrylic acid/MPEG (MW 750) and photosynthetic cyanobacterial cells. Stable emission of QD (550 nm) **(A)** is retained after specific photobleaching of chlorophyll fluorescence in a single cell **(B)**. Arrow indicates single cell that was photobleached with the laser scanning unit. Note that QD signal remains stable after photobleaching and marks the position of the cell. Scale bars are 5 μm. **(C and D)** Quantitative analysis of QD size and composition using Scanning Transmission Electron Microscopy (Titan Themis) showing CdSe core structure. [Courtesy Anton Avramov, Christian Brininger, Sadegh Yazdi, Preston Snee, and Jeffrey Cameron]

QDs could be used to inject electrons into specific sites of electron transport chains *in vivo*, providing an opportunity to accelerate or short-circuit metabolic pathways including photosynthesis. Furthermore, using multicolor and multi-element QDs, it is possible to perform correlated light-cryoelectron microscopy (cryo-EM) studies for ultra-high-resolution spatial maps of cellular metabolism. These studies will provide new insight into critical biochemical pathways important for energy metabolism and new tools and methods that will enhance biological imaging in complex organisms relevant for bioenergy.

Live-Cell, Quantum Dot–Based Tracking of Plant and Microbial Extracellular Vesicles

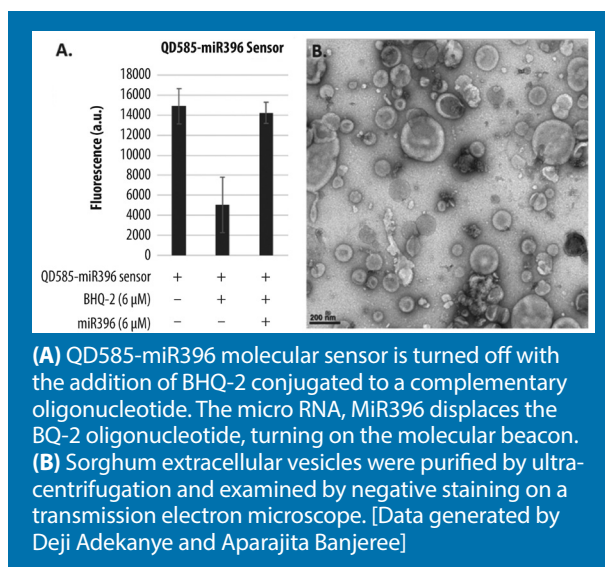**Principal Investigators:** [1]Jeffrey L. Caplan (PI), Roger W. Innes,[2] and B.C. Meyers[3]

Institutions: [1]University of Delaware, [2]Indiana University, and [3]Donald Danforth Plant Science Center

Email: jcaplan@udel.edu

Research Plans and Progress: This project aims to characterize extracellular vesicles (EV) released from *Sorghum biocolor*. Plant EVs help transport both small RNA (sRNA) and proteins to the extracellular spaces. Similarly, fungal pathogens also release EVs, and EVs may be involved in interspecies communication during plant-fungal interactions. To study the function of EVs, this project has developed improved methods for the isolation of EVs from both sorghum and its fungal pathogen *Colletotrichum sublineola*. With this part of the project complete, the experimental focus is on tracking the transfer of EVs between sorghum and *C. sublineola*. For these studies, quantum dot (QD)-enabled approaches for tracking EVs and their specific sRNA cargo are being developed. A QD-based molecular beacon for the detection of specific sRNA was created using click chemistry. The goal is to take advantage of the superior photophysical properties of QD molecular beacons for tracking and sensing sRNA. EVs isolated from sorghum and *C. sublineola* will be loaded with QDs using cell-penetrating peptides (CPP) and fluorescent dyes. These will be used to examine the process of EV uptake and localization.

Anticipated Accomplishments and Deliverables: This project has successfully made a QD-based molecular beacon for sRNA detection using a highly efficient, copper-free click chemistry approach. CdSe/ZnS QDs modified with dibenzo-cyclooctyne (DIBO) were used to conjugate azide-modified oligonucleotides that were complementary to the microRNA, miR396. These QD molecular beacons were purified by HPLC and examined on a spectrofluorimeter to demonstrate that they can detect miR396 *in vitro* (see figure, panel 1A). A QD electrophoretic mobility assay (QEMSA) is being used to purify a more homogenous population of QD molecular beacons, and these will be used for single-molecule and *in vivo* studies.

This project has developed an improved protocol for the isolation and characterization of EVs from sorghum (see figure, panel 1B). The project's new protocol has resulted in yields that are sufficient for cryoelectron tomography (cryo-ET)

(A) QD585-miR396 molecular sensor is turned off with the addition of BHQ-2 conjugated to a complementary oligonucleotide. The micro RNA, MiR396 displaces the BQ-2 oligonucleotide, turning on the molecular beacon. **(B)** Sorghum extracellular vesicles were purified by ultracentrifugation and examined by negative staining on a transmission electron microscope. [Data generated by Deji Adekanye and Aparajita Banjeree]

and have been submitted to the Laboratory for BioMolecular Structure (LBMS) at Brookhaven National Laboratory. A major aim of this project is to conduct live-cell tracking of EVs using QDs and complementary fluorescent labeling approaches. An azide-modified CPP is being synthesized to implement the same copper-free click chemistry approach used to generate the project's QD molecular beacon. As a counterstain to QDs, a robust pair of fluorescent dyes for the lumen and membrane of EVs have been identified. Sorghum lines with fluorescent protein EV markers will be generated. Five protein candidates and EV markers from other plant systems were chosen to label using the fluorescent tagging of full-length proteins (FTFLP) method for internal labeling with fluorescent proteins.

Potential Benefits and Applications: Extracellular release of vesicles is an important component for cell wall biogenesis and defense against plant pathogens. This project has developed a method to extract EVs from sorghum, which can be adapted to study EVs from other important monocot biofuel crops.

Furthermore, the project's QD-based molecular beacons and related tools to detect EVs can be used to study the function of EVs in other biological processes important for biomass production.

Integrative Imaging of Plant Roots During Symbiosis with Mycorrhizal Fungi

Principal Investigators: Andreas Vasdekis[1] (PI), Scott E. Baker,[2] Demetrios Christodoulidis,[3] and Maria Harrison[4]

Participants: Luke Sheneman[1] (senior personnel), Haokun Luo[3] (graduate student), Lochlann Dunn[1] (research associate)

Institutions: [1]University of Idaho, [2]Pacific Northwest National Laboratory, [3]University of Central Florida, and [4]Boyce Thompson Institute

Email: andreasv@uidaho.edu

Research Plans and Progress: To accelerate understanding of the symbiosis between crops and mycorrhizal fungi, this project is constructing an optical system that images the development of each symbiont, localizes key metabolic pathways, and quantifies nutrient exchange rates. To accomplish this, the team is integrating quantitative-phase with light-sheet Raman and fluorescence imaging. These three modalities will be specifically constructed to overcome the innate spatiotemporal broadening that the illumination undergoes deep inside the root, as well as form subcellular-resolution images from ultralow signals. Hardware development will be supported by light-sheet computational analyses, deep learning for image reconstruction, and the development of tailored gene-encoded biomarkers.

Anticipated Accomplishments: Since its beginning in September 2021, this project has focused on three aspects. First, computationally assessing how different light sheets propagate in simulated scattering environments. Team members observed that light sheets relying on Gaussian beams fail to propagate upon scattering; however, the accelerating Airy beam can self-heal and reform upon scattering (see Fig. 1, next page), thus solidifying its value in deep-tissue imaging. Second, leveraging previous work with near-infrared (NIR) Airy beams (Subedi et al. 2021) and enhancing the diffraction-free path of the Airy beam. This effort yielded image sizes that are greater than 400 µm in the illumination direction and, as such, 20-fold higher imaging throughput rates than Gaussian-based light sheets. Third, determining that photon-sparse light-sheet imaging can detect chemical bonds in homogeneous and inhomogeneous samples with less than one photon per pixel. This finding evidences the utility of ultralow-light imaging, expanding the team's previous work in fluorescence to chemical microscopy (Sanchez et al. 2021). The immediate next steps in this project are to (1) experimentally assess and improve the self-healing properties of NIR Airy beams in various scattering media; (2) design and construct a light-sheet microscope compatible with plant root imaging; and (3) expand quantitative-mass imaging investigations of plant roots, as shown in the example in Fig. 2, next page.

Potential Benefits and Applications: Most bioenergy crops form mutualistic associations with mycorrhizal fungi, thus bestowing crops with access to nutrients that are limited in most arable lands, but also sequester atmospheric CO_2. The optical system is expected to provide new insight into the interactions between plants and mycorrhizal fungi and to contribute to a long-standing DOE goal in energy prosperity, namely: to achieve a predictive understanding of biosystems through transformative instrumentation solutions. Further, the optical system will be realized in standard inverted microscopes and harness open-access software, thus making it more accessible to the broader scientific community.

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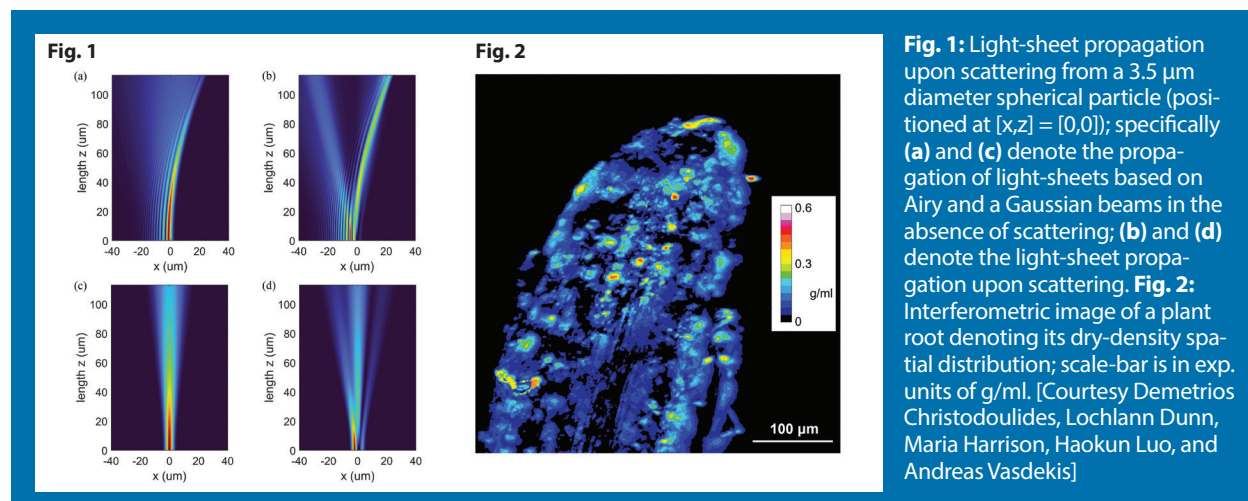
Fig. 1: Light-sheet propagation upon scattering from a 3.5 µm diameter spherical particle (positioned at [x,z] = [0,0]); specifically **(a)** and **(c)** denote the propagation of light-sheets based on Airy and a Gaussian beams in the absence of scattering; **(b)** and **(d)** denote the light-sheet propagation upon scattering. **Fig. 2:** Interferometric image of a plant root denoting its dry-density spatial distribution; scale-bar is in exp. units of g/ml. [Courtesy Demetrios Christodoulides, Lochlann Dunn, Maria Harrison, Haokun Luo, and Andreas Vasdekis]

Development and Implementation of an *In Situ* High-Resolution Isotopic Microscope for Measuring Metabolic Interactions in Soil Mesocosms

Principal Investigators: Elizabeth Shank[1] (PI), Christopher R. Anderton,[2] Venkateshkumar Prabhakaran,[3] David Berry,[4] and Carol Arnosti[5]

Institutions: [1]University of Massachusetts Chan Medical School, [2]Environmental Molecular Sciences Laboratory, [3]Pacific Northwest National Laboratory, [4]University of Vienna, [5]University of North Carolina

Email: elizabeth.shank@umasmed.edu, christopher.anderton@pnnl.gov, venky@pnnl.gov; david.berry@univie.ac.at, and arnosti@email.unc.edu

Research Plans and Progress: The nanospray desorption electrospray ionization (nanoDESI) sampling interface was successfully implemented on a vibrationally isolated cart and interfaced with a 21T-FTICR-MS (see figure, next page). Isotopic fine-structure data for naturally occurring isotopes were obtained from direct spot analysis of *Bacillus subtilis* colonies, allowing for high confidence in molecular annotations with resolution >830 k (at m/z 825). Absorption mode data, a nontrivial solution to the raw output of FTICR-MS, was implemented for improved mass resolution and sensitivity relative to conventional (i.e., magnitude mode) data processing. This work is currently under review for publication. In parallel, researchers have been creating bacterial cell standards of stable isotope–labeled (^{13}C, ^{2}H) *B. subtilis* cells for interlaboratory comparability of Raman microspectroscopy. They are collecting spectra employing the bacterial cell standards using both 533 and 632 nm Raman lasers to ensure the reproducibility of the data regardless of which laser wavelength is used. The team has identified a wide range of lab strains capable of growing on the soil polysaccharide, pullulan, as a sole carbon source that can be used for further analysis on the multimodal instrument. In addition, researchers have devised a soil-processing protocol (including blending, low-speed centrifugation, and a Nycodenz gradient) to isolate wild microbes from soil that are putatively involved in "selfish" utilization of pullulan. They are in the process of using 2H$_2$O-activity labeling coupled with Raman-activated cell sorting (RACS) to identify those soil bacteria actively involved in degrading pullulan (based on their 2H$_2$O incorporation). Preliminary experiments have indicated the feasibility of this approach and are allowing the project to build a targeted collection of bacteria with which researchers can generate bacterial communities and study metabolic interactions in soil mesocosms using the project's multimodal instrument. In conjunction, the team has developed a protocol to successfully label chitosan, a sparingly soluble polysaccharide that is highly relevant in soil systems. Initial degradation experiments are underway, and access to this chitosan will broaden the scope of examinations of carbon degradation processes in soil.

Anticipated Accomplishments: The implementation of nanoDESI with 21T-FTICR-MS for detection of signals from *B. subtilis* colonies demonstrated the necessary mass-resolving power and sensitivity for the desired application of resolving ^{2}H- and ^{13}C-enriched species. Furthermore, the use of a vibrationally isolated cart will allow for the parallel integration of Raman spectroscopy and fluorescence imaging. Raman integration with the cart is ongoing; the bacterial standards assembled will provide the necessary reagents to ensure interlaboratory comparability of the Raman microspectroscopy results. Assembling a collection of known pullulan-degrading bacteria as well as newly isolating pullulan-degrading soil bacteria by RACS will be important to development of representative soil mesocosms for interrogation with nanoDESI and multimodal imaging techniques. The team anticipates complementing these results with microscopy and flow cytometry to confirm selfish pullulan uptake. Upon completion of the instrument, the project anticipates exploiting its capabilities through the generation of polydimethylsiloxane microcosms containing purified carbohydrate sources inoculated with known bacteria to begin to dissect carbohydrate degradation, uptake, and competition dynamics in microbial communities.

Potential Benefits and Applications: The nanoDESI mass spectrometry imaging, Raman, and fluorescent capacities provided by this instrument (housed at the Environmental Molecular Sciences Laboratory to facilitate broad community usage) will enhance understanding of the microbial and metabolic interactions occurring in soil communities that are relevant to carbon degradation. The protocols and experimental reagents (bacterial and labeled carbohydrates) being developed will not only allow validation of this imaging platform across multiple imaging modalities, but also will be applicable to a broad range of scientific questions about the key roles that microbes play in global carbon cycling.

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Goal of the Project
Develop and implement an *in situ* high-resolution multimodal microscope that integrates fluorescence microscopy, Raman microspectroscopy, and nanoDESI mass spectrometry for measuring metabolic interactions in soil mesocosms
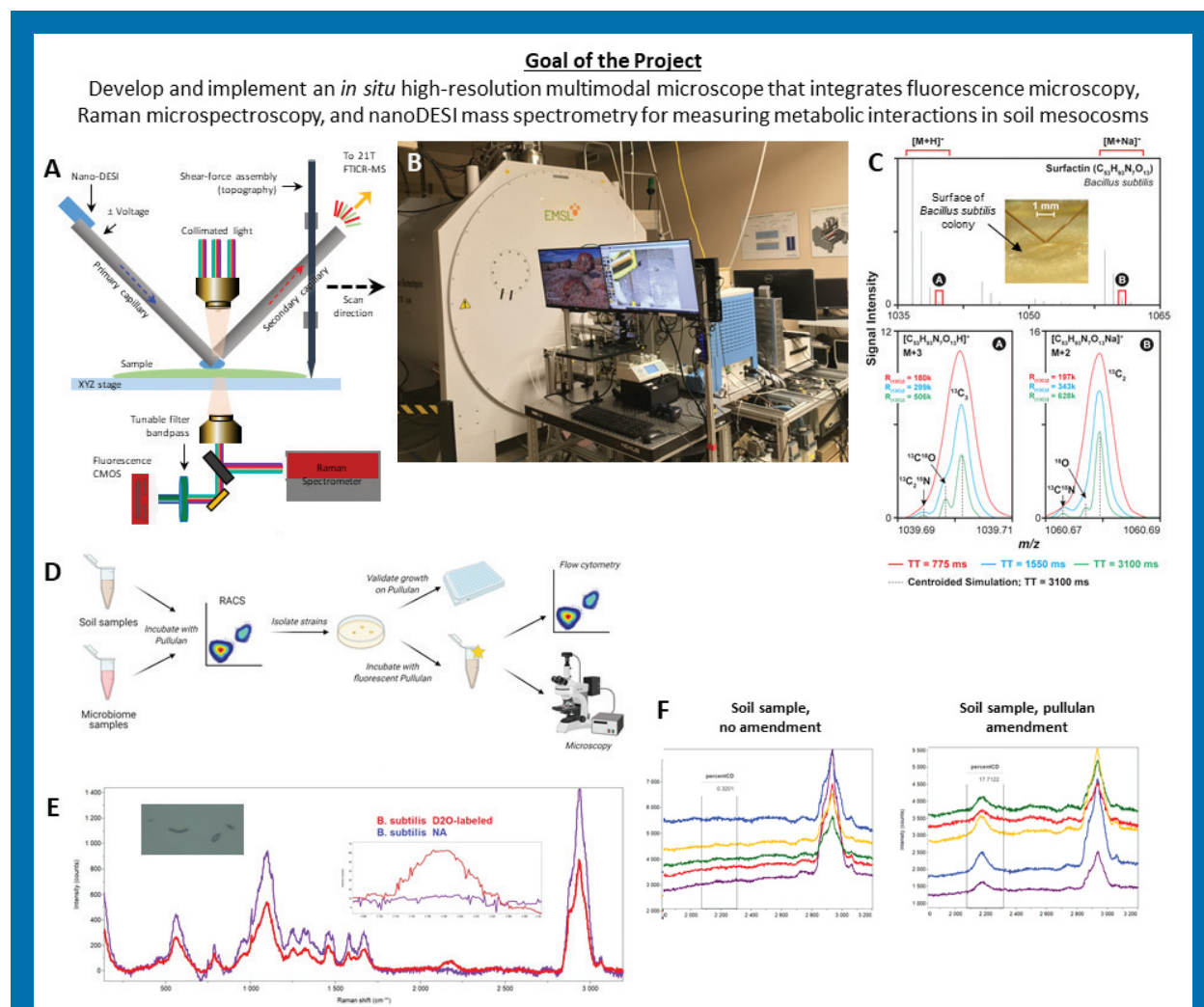
(A) Schematic of the planned configuration of the multimodal microscope. **(B)** Vibration-isolated nanoDESI platform, which consists of a 24x36-inch damped optical breadboard positioned in front of the 21T-FTICR-MS in EMSL at Pacific Northwest National Laboratory. **(C)** Measurement of isotopic fine structure (IFS) of surfactin from *Bacillus subtilis* using nanoDESI-21T-FTICR-MS. (TT: transient time; M: monoisotopic mass; R: resolution) **(D)** Experimental workflow for Raman-activated cell sorting (RACS) of bacterial samples. **(E)** Raman spectra collected from *B. subtilis* cells grown for three hours at 37° C in MSN with 50% ^{2}H$_2$O, before washing and spotting onto aluminum slide. **(F)** Raman spectra from single bacterial cells isolated from soil through RACS experiment. Those cells incubated with pullulan (right) show a Raman peak at ~2,200 cm^{-1} indicative of incorporation of ^{2}H$_2$O due to their metabolic activity. [(A) Courtesy Venkateshkumar Prabhakaran and Christopher Anderton. (B) Courtesy Gregory Vandergrift. (C) Reprinted with permission from American Chemical Society from Vandergrift, G. W. et al. 2022. "Imaging and Direct Sampling Capabilities of Nanospray Desorption Electrospray Ionization with Absorption-Mode 21 Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometry," *Analytical Chemistry*. Article ASAP. DOI: 10.1021/acs.analchem.1c05216. © 2022, American Chemical Society. (D) Courtesy Katarzyna Dubiel. (E and F): Courtesy Georgi Nikolov and Hamid Rasoulimehrabani]

Biological Imaging Using Entangled Photons

Principal Investigators: Theodore Goodson III[1] (PI), George C. Schatz,[2] Jennifer Morrell-Falvey,[3] and Yingzhong Ma[3]

Institutions: [1]University of Michigan, [2]Northwestern University, and [3]Oak Ridge National Laboratory
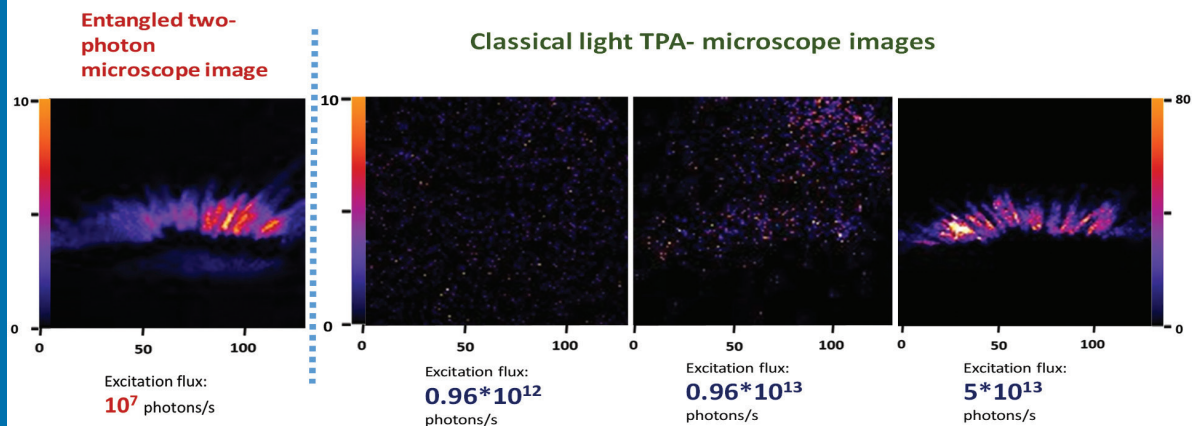
Email: tgoodson@umich.edu

Research Plans and Progress: The objective of this project is to improve the capabilities for direct, *in vivo* visualization of biological processes taking place over various time and length scales while reducing detrimental issues that cause damage to the biological system. Phototoxicity is particularly problematic for gaining a comprehensive understanding of biological function, which often necessitates prolonged imaging measurements. The overarching goal of this project is to seek the full theoretical potential of entangled two-photon fluorescence microscopy, such that not only are its capabilities comparable to or surpassing those achievable with the corresponding classical modalities, but they are also attained with extremely low excitation intensity.

Studies underway provide a unique combination of novel technical innovations between the University of Michigan and Oak Ridge National Laboratory (ORNL), fundamental understanding and theoretical predictions between the university participants, as well as validation of the novel imaging capabilities through probing complex biosystems in the rhizosphere primarily carried out at ORNL. The team will focus on advancing entangled two-photon fluorescence microscopy for ultralow-light bioimaging to protect against photobleaching in biological systems (see figure, next page). Detailed technical work is underway to upgrade the entangled photon source as well as aspects of the entangled two-photon microscope at the university. A similar microscope construction with advances in dispersion compensation and adaptive pulse shaping is underway at ORNL. The effort is motivated by the fact that entangled photon absorption is extremely sensitive to the excitation characteristics of the entangled photon pairs such as entanglement time and area, and optimization of these parameters thus offers an effective means to maximize the entangled two-photon response for any given molecule. The project's initial development focused on adaptive pulse shaping for the pump laser for entangled photon generation, as ideal spectral and temporal characteristics of such a pump laser for entangled two-photon spectroscopic and microscopic remained unexplored. An understanding gained through this effort will enable further extension of such a pulse-shaping approach to optimal control of entangled photon pairs.

Potential Benefits and Applications: An objective at the early stage of this project is to provide understanding of the structure-function relationships for the entangled two-photon absorption properties of complex biomolecules. Team members have identified a remarkable enhancement of entangled two-photon absorption through controlling the bandwidth of the entangled photon pairs. This is underway at the university.

Entangled Two Photon vs Classical Two Photon Microscope Excitation

Entangled two-photon microscope image

Classical light TPA- microscope images

Excitation flux:
10^7 photons/s

Excitation flux:
$0.96*10^{12}$ photons/s

Excitation flux:
$0.96*10^{13}$ photons/s

Excitation flux:
$5*10^{13}$ photons/s

Entangled two-photon microscopy offers nonlinear imaging capabilities with excitation intensity **six orders of magnitude (10^6) lower** than that necessary for **classical light.**

Scanning microscope image obtained with entangled photon excitation (left) in comparison with classical light two-photon excitation at different excitation fluxes. Excitation flux (average) is shown under respective microscope images. Sample: drop-cast film of bis(styryl)benzene derivative (Bu_2/OMe) (see Supporting Information). Color bar intensity scales are given in numbers of fluorescence photons per pixel. Spatial scale is indicated in pixels at the bottom of each image. The resolution per pixel is 6.14 µm. Chemical structure of bis(styryl)benzene derivative (Bu_2/ OMe) is shown below the images. [Reprinted with permission from Varnavski, O., et al. 2020. "Two-Photon Fluorescence Microscopy at Extremely Low Excitation Intensity: The Power of Quantum Correlations," *Journal of the American Chemical Society* **142**(30), 12966–75. DOI: 10.1021/jacs.0c01153. © 2020 American Chemical Society.

Expanding the Utility and Range of Quantum and Polymer Dots for Multiplexed Super-Resolution Fluorescence Imaging in Plants

Principal Investigators: Gary Stacey(PI) and Zeev Rosenzweig

Institution: University of Missouri

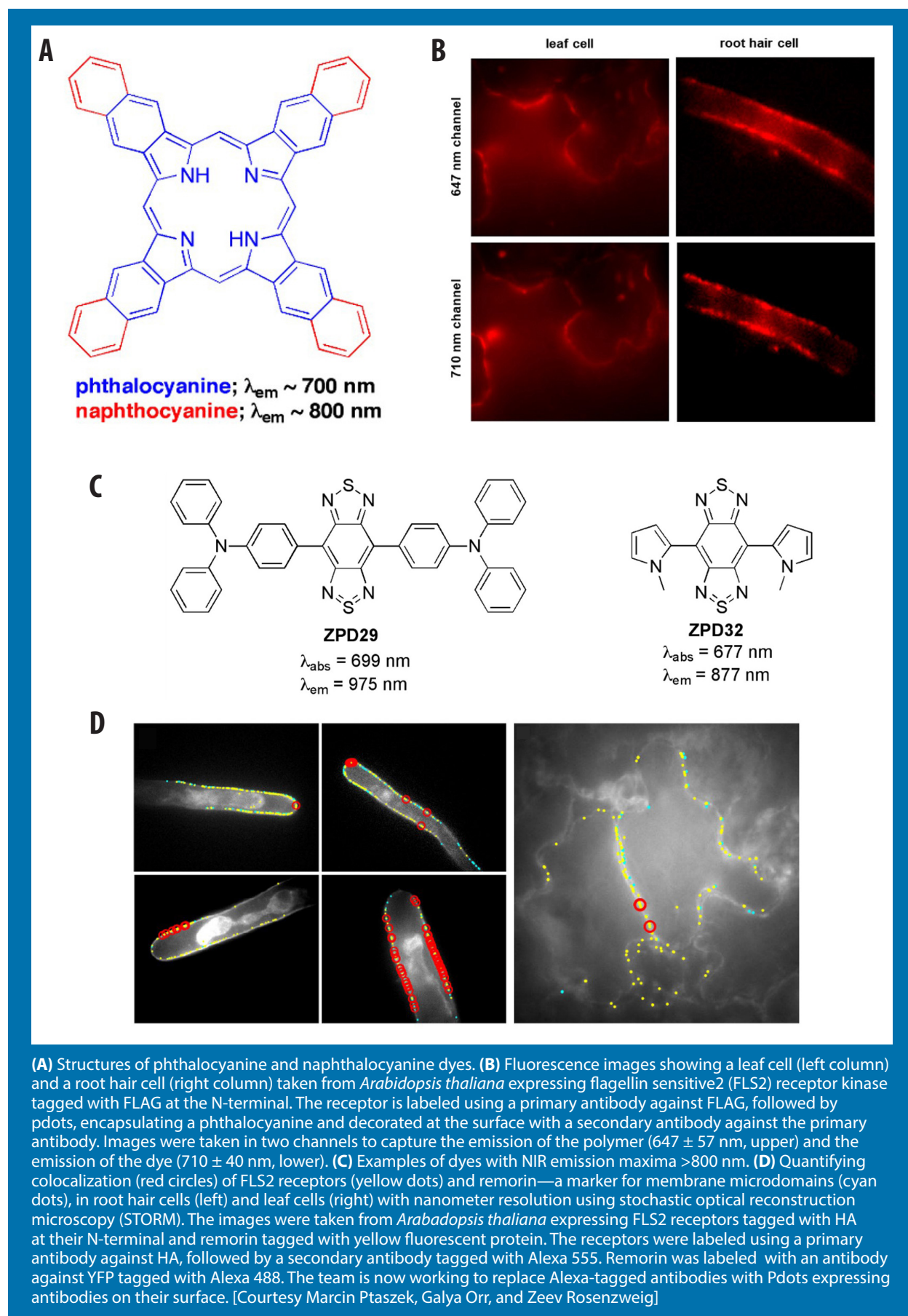**Email:** staceyg@missouri.edu and zrosenzweig@umbc.edu

Research Plans and Progress: Objective 1: Proteomic analysis of plant receptors. The team has succeeded in labeling membrane receptors with fluorescent polymer dots (Pdots). To realize receptor labeling, researchers genetically engineered root and leaf plant cells with the capability to express FLAG-tagged flagellin (FLS2) membrane receptors (see figure, next page). This year, the team is producing genetically engineered plants in which two or more proteins are labeled (e.g., FLAG, HA). This will enable the use of near-infrared (NIR) emitting Pdots in multiplex cellular imaging.

Objective 2: Assemble a super-resolution fluorescence microscopy system for integrated multiplexed imaging in the visible and NIR regions. During the last year, the team completed the construction of a NIR fluorescence imaging system. The microscopy system was constructed at Pacific Northwest National Laboratory (PNNL) and is available for the community via the PNNL Environmental Molecular Sciences Laboratory (EMSL) user program. This year it will be used for multiplexed super-resolution imaging of membrane receptors using NIR-emitting Pdots.

Objective 3: Synthesis of QDs/Pdots with visible and NIR emission for multiplexed imaging. Last year, the team synthesized hydrophorphrin-doped Pdots and tuned their emission peaks between 640 and 820 nm. Researchers also synthesized phthalocyanine-doped Pdots with emission peaks ranging between 700 and 875 nm. This year, the team will test new NIR-emitting dyes and prepare Pdots with longer emission wavelengths ranging from 850 to 1100 nm. Researchers will also prepare Pdots with dye molecules with two emission peaks and with two NIR dyes to further expand multiplex imaging capabilities.

Potential Benefits and Applications: Practical use of bioenergy crops will require plants with high yield and tolerance to a variety of biotic and abiotic stresses. Plants are rooted in place and, hence, have evolved mechanisms to recognize environmental threats and respond. Environmental sensing is largely localized to the plasma membrane, where a plethora of receptors and associated proteins form complex and dynamic interactions in response to specific environmental stimuli. Paraphrasing the funding opportunity announcement, the innovative approaches the team is using include QD-based imaging approaches and complementary optical imaging instrumentation for observation and characterization of multiple complex biological processes. These include development of probes functionalized with specific, active molecules to bind with specific cellular targets that will enable dynamic localization and imaging to validate hypotheses related to cellular signaling while dramatically enhancing the ability to measure processes in and among living cells.

(A) Structures of phthalocyanine and naphthalocyanine dyes. (B) Fluorescence images showing a leaf cell (left column) and a root hair cell (right column) taken from *Arabidopsis thaliana* expressing flagellin sensitive2 (FLS2) receptor kinase tagged with FLAG at the N-terminal. The receptor is labeled using a primary antibody against FLAG, followed by pdots, encapsulating a phthalocyanine and decorated at the surface with a secondary antibody against the primary antibody. Images were taken in two channels to capture the emission of the polymer (647 ± 57 nm, upper) and the emission of the dye (710 ± 40 nm, lower). (C) Examples of dyes with NIR emission maxima >800 nm. (D) Quantifying colocalization (red circles) of FLS2 receptors (yellow dots) and remorin—a marker for membrane microdomains (cyan dots), in root hair cells (left) and leaf cells (right) with nanometer resolution using stochastic optical reconstruction microscopy (STORM). The images were taken from *Arabadopsis thaliana* expressing FLS2 receptors tagged with HA at their N-terminal and remorin tagged with yellow fluorescent protein. The receptors were labeled using a primary antibody against HA, followed by a secondary antibody tagged with Alexa 555. Remorin was labeled with an antibody against YFP tagged with Alexa 488. The team is now working to replace Alexa-tagged antibodies with Pdots expressing antibodies on their surface. [Courtesy Marcin Ptaszek, Galya Orr, and Zeev Rosenzweig]

Novel *In Vivo* Visualization of Bioenergy Metabolic and Cellular Phenotypes in Living Woody Tissues

Principal Investigators: Leslie Sieburth[1] (PI), Rajesh Menon,[1] Chang-Jun Liu,[2] and Andrew Groover[3]

Institutions: [1]University of Utah; [2]Brookhaven Science Associates, LLC; [3]U.S. Forest Service Pacific Southwest Research Station

Email: sieburth@biology.utah.edu

Research Plans and Progress: The ability to directly visualize structures and metabolic processes in living cells has been central to major advances in understanding complex biological processes in agriculture and medicine. Wood formation is the biological process that produces biomass, and features of the resulting wood cells influence both growth rates, drought resistance, and conversion properties for bioenergy production. Tissues and cell types associated with wood formation lie so deep inside the trunk that they cannot be directly visualized using traditional microscopy methods. As a result, fundamental questions related to differentiation of wood-forming cells, cell-wall biosynthesis, metabolic processes, signaling, and transport of materials within and among living cells remain unaddressed. This project is developing methods for live-cell imaging of the vascular cambium and its cellular derivatives with the long-term goal of visualizing cells and metabolic processes associated with lignification, vessel element differentiation, and development of tension wood.

The project is developing two strategies for tissue visualization. The first uses a surgical cannula (diameter = 0.22 mm) to access and image the tissues of interest, and it has been successful for live-cell imaging of mouse brain tissues (Kim et al. 2014; Kim et al. 2015; Kim et al. 2017). The cannula pipes excitation light into the sample and then collects the emitted fluorescence and redirects it back to a camera (see figure, panel a, next page). The camera's collected image is scrambled, necessitating an additional computational postprocessing step that recovers the image's spatial details. The team applied this cannula method to BCECF AM—stained stem sections and successfully reconstructed images using two methods. A linear-algebraic approach (matrix inversion via Tikhonov regularization) produced the images shown in figure panel b, while a deep neural network trained using reference images captured from the same stained sections and then applied to reconstruct images from the cannula's data, are shown in figure panel c.

Team members are also developing a method to directly visualize the cells of interest, called the Utah Miniscope (Aharoni et al. 2018; Campos, Walker, and Mollard, 2020). The miniscope sidesteps computational image reconstruction, as the lens is embedded in the tissue where the camera can capture images directly. The project's initial Miniscope uses a 1 mm-thick gradient-index (GRIN) lens (see figure, panel
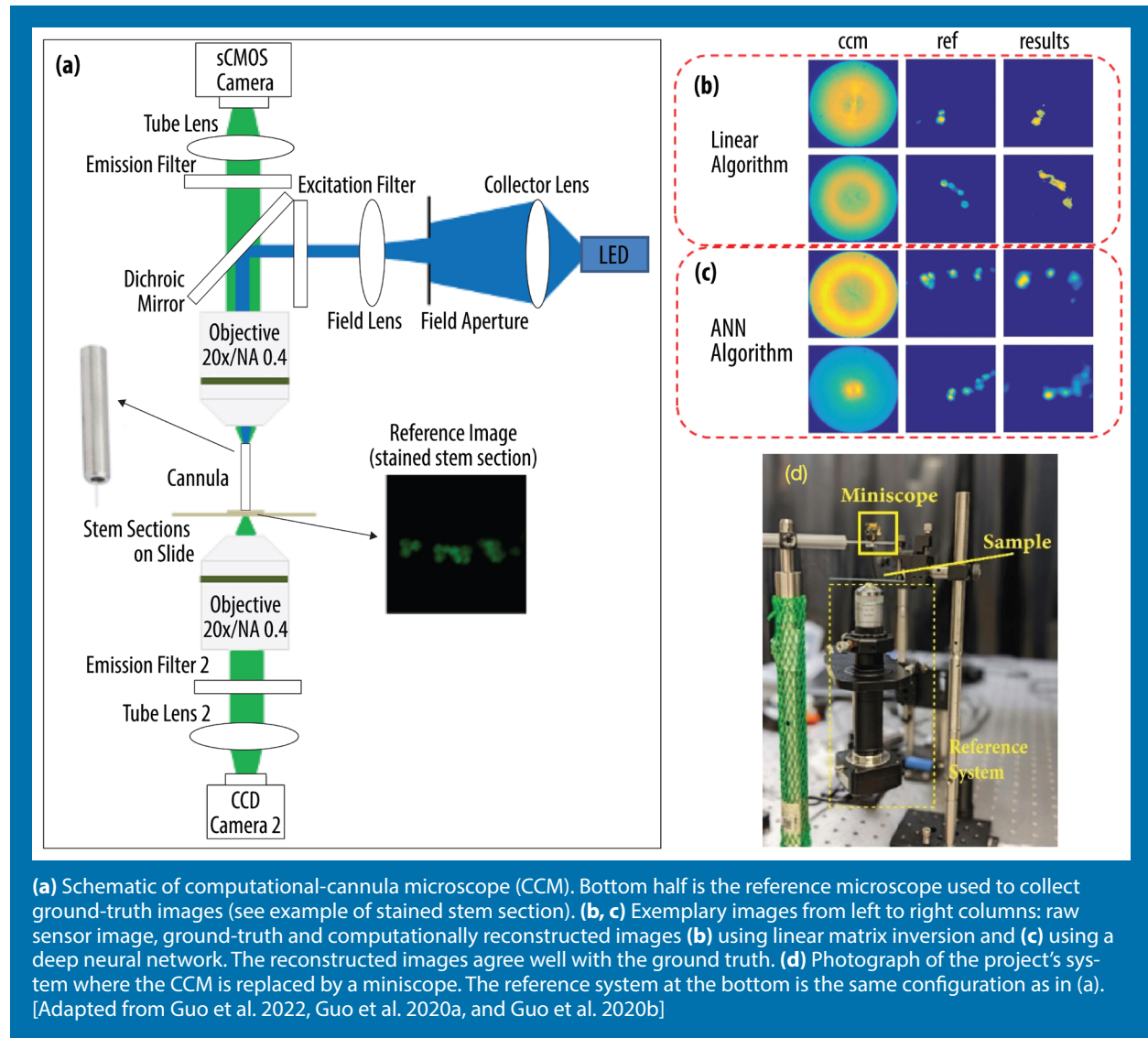
d). Thus, it sacrifices tissue trauma for minimal computation, which might be advantageous when the signal-to-noise ratio is small.

The next steps are optimizing these methods, applying them to transpiration stream-applied stains, and exploring use of autofluorescence combined with computational hyperspectral imaging. Project outputs will include these new approaches for live-cell imaging in plants; new insights into cell differentiation within woody stems; and impact on growth rates, drought-stress response, and conversion properties.

Potential Benefits and Applications: Visualizing structures and metabolic processes in the living cells that produce biomass, specifically wood, has not been possible because of the inaccessibility of these tissues. However, characteristics of wood influence many processes, including the ability to convert biomass to energy, plant growth, and resilience in the face of drought and climate change. This project is developing novel methods for carrying out these visualization methods with the aim of revealing fundamental aspects of wood formation and its associated metabolic processes. The project's goals include special characterization of the lignification process, vessel element differentiation, and the developmental processes that lead to production of tension wood.

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(a) Schematic of computational-cannula microscope (CCM). Bottom half is the reference microscope used to collect ground-truth images (see example of stained stem section). **(b, c)** Exemplary images from left to right columns: raw sensor image, ground-truth and computationally reconstructed images **(b)** using linear matrix inversion and **(c)** using a deep neural network. The reconstructed images agree well with the ground truth. **(d)** Photograph of the project's system where the CCM is replaced by a miniscope. The reference system at the bottom is the same configuration as in (a). [Adapted from Guo et al. 2022, Guo et al. 2020a, and Guo et al. 2020b]

A Quantum-Enhanced X-Ray Microscope

Principal Investigators: Sean McSweeney[1] (PI), Lonny Berman[1], Cinzia Da Via[2], Andrei Flurasu[1], Andrei Nomerotski[1], Timothy Paape[1], and Elisha Siddiqui[1]

Institutions: [1]Brookhaven National Laboratory and [2]Stony Brook University

Email: smcsweeney@bnl.gov

Research Plans and Progress: In the reporting period, this project has begun the practical implementation of a platform to perform ghost imaging of biological samples using X-rays. The team has established the administrative details to make regular access to the National Synchrotron Light Source II (NSLS-II) beamline CHX facile. Researchers have engaged with leading experts—pioneers in the field—to allow for discrimination between the various X-ray optical schemes available. The delivery of an X-ray quantum microscope is built upon four pillars: experimental methods, nonlinear media for generation of entangled photons, biological systems, and data analysis. During this initial reporting period, the project has focused on establishing the team, protocols, and a baseline set of measurements, specifically:

Experimental methods. The team created an X-ray optical system for first experiments (see figure, next page). Following advice, they established ghost imaging from photon correlations generated from chaotic sources. These measurements have allowed refinement of beamline configuration, design of measurement protocols, and establishment of feedback loops with the data analysis tasks outlined below. Further enhancements of the X-ray optics are to be implemented.

Nonlinear media for generation of entangled photons. In the X-ray region, available media have very low cross sections for the conversion. The team has procured and tested high-quality diamond single crystals to produce entangled X-rays. The first measurements have been made, and data analysis is underway. In parallel, re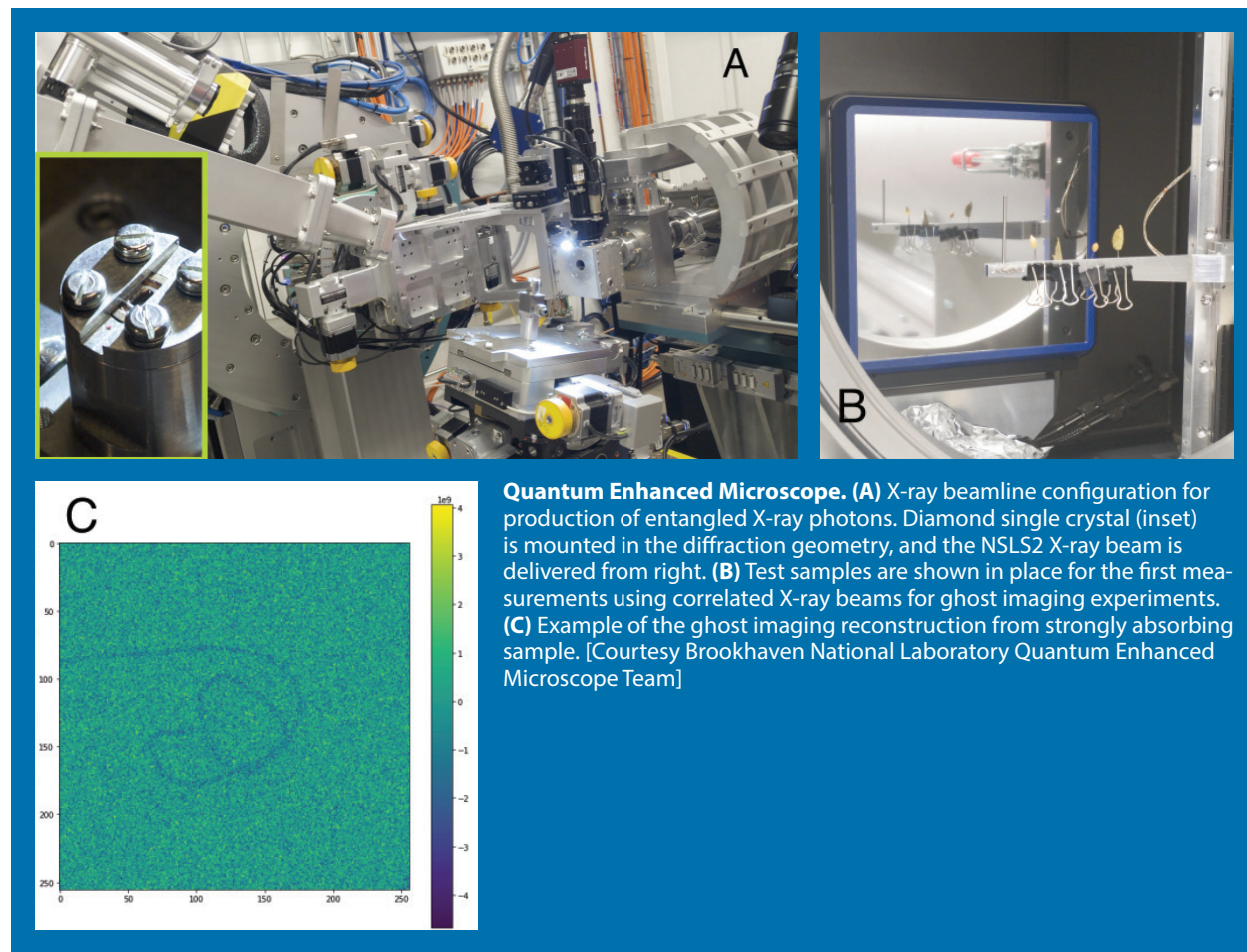searchers will investigate new systems capable of higher-efficiency generation of entangled X-rays; these novel media will be tested as they become available.

Biological systems. The model system to verify imaging of *Medicago truncatula* is the model legume species that the team will study in its symbiotic interactions with *Sinorhizobium medicae* and *S. meliloti*. Experiments have started to prioritize investigation once the prototype system is validated; adaptation of the growth chambers to the beamline will be needed, and prototypes will be tested during the next period.

Data analysis. While the mathematics of image formation from both type-1 and type-2 are well documented, the applicability to X-ray imaging will bring surprises due to the low count rates and the types of noise, both experimental realities. The project has established a test suite implementing the most usual ghost imaging reconstruction algorithms, which are validated against simulated and experimentally derived data. Outcomes of these analyses were fed back into X-ray measurements.

Potential Benefits and Applications: The project's use of the quantum properties of light, X-rays, offers a new opportunity for imaging, in that the use of quantum correlations of the (two-photon) system all retrieve the image with minimal dose requirements—this approach has powerful implications in biological and environmental science applications, where the sample would normally be damaged by the X-rays during imaging. In exploiting the correlation of photons inherent to ghost imaging, a sample can be illuminated by less intense beams and thus unmodified during the experiment. Further, the quantum nature of the imaging process will allow higher signal-to-noise and better spatial resolution of thick samples.

This project's ultimate goal is to achieve the potential of ghost imaging for X-ray image reconstruction of *in vivo* thick samples or otherwise optically opaque biological samples. With baseline measurements made, the team aims to expand the approach to enable dynamic snapshots of the samples and by stimulation of X-ray fluorescence for additional information. Results will be published in peer-reviewed journals.

Quantum Enhanced Microscope. (A) X-ray beamline configuration for production of entangled X-ray photons. Diamond single crystal (inset) is mounted in the diffraction geometry, and the NSLS2 X-ray beam is delivered from right. **(B)** Test samples are shown in place for the first measurements using correlated X-ray beams for ghost imaging experiments. **(C)** Example of the ghost imaging reconstruction from strongly absorbing sample. [Courtesy Brookhaven National Laboratory Quantum Enhanced Microscope Team]

The 3DQ Microscope: A Novel System Using Entangled Photons to Generate Volumetric Fluorescence and Scattering Images for Bioenergy Applications

Principal Investigators: Ted Laurence[1] (PI), Tiziana Bond,[1] Claudio Bruschini,[2] Kevin Cash,[3] Edoardo Charbon,[2] Matthew A. Horsley,[1] Shervin Kiannejad,[1] Paul Mos,[2] Erin Nuccio,[1] Ty Samo,[1] Michael Wayne,[2] Peter K. Weber,[1] Steven Yang,[1] Xiyu Yi,[1] and Jake Yoo[1]

Institutions: [1]Lawrence Livermore National Laboratory (LLNL), [2]École polytechnique fédérale de Lausanne (EPFL), [3]Colorado School of Mines

Email: laurence2@llnl.gov

Research Plans and Progress: The goal of this project is to develop a 3D imaging modality that uses quantum-entangled photon pairs to obtain more information about fluorescence and scattering events than is available in standard fluorescence or scattering measurements. The entangled photons will enable the project's microscope to use two separate 2D detectors to obtain 3D and 4D information about the same photon absorption/fluorescence emission event or scattering event in the sample, providing 3D imaging without scanning.
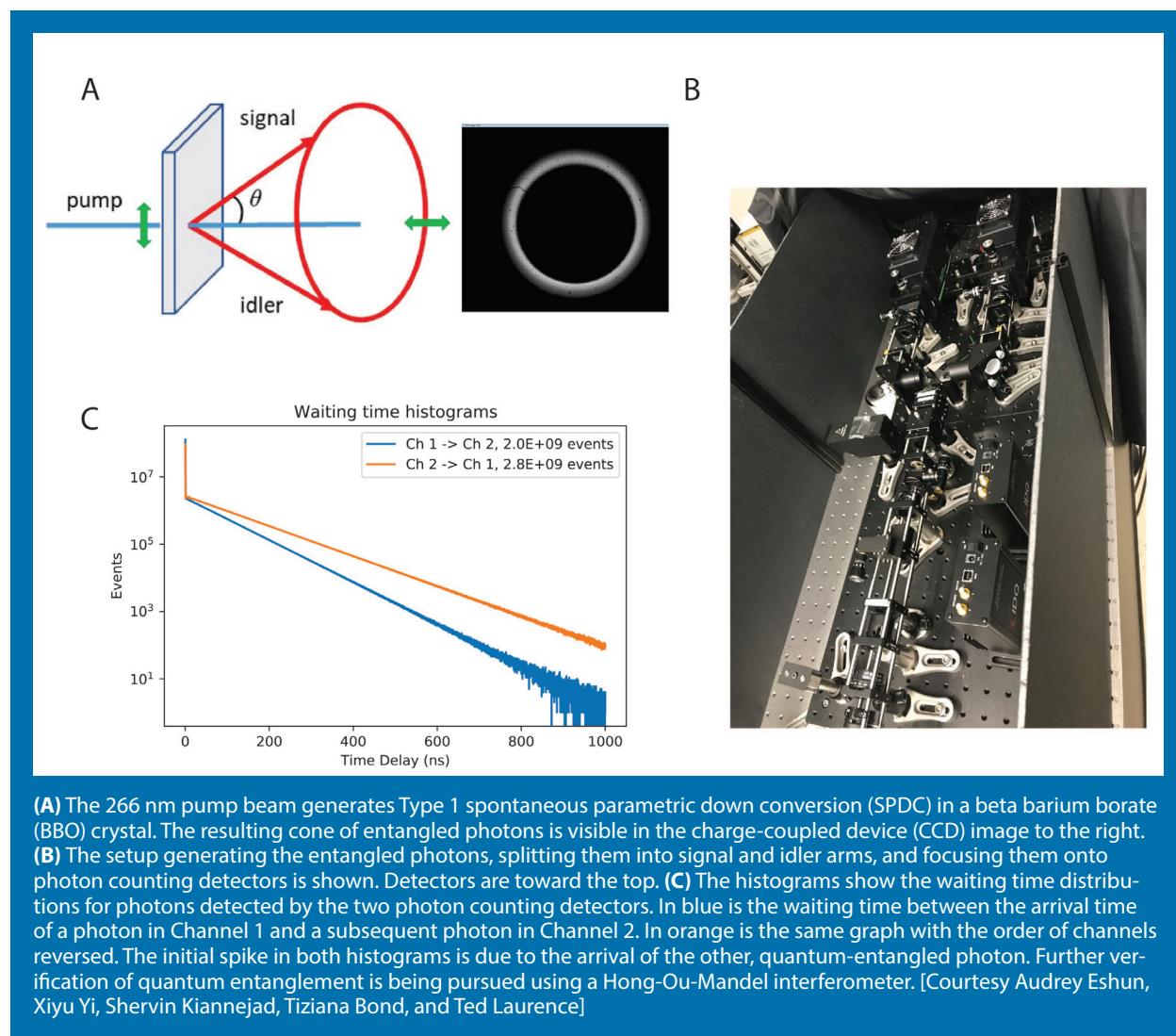
This project includes four parallel technical objectives that will culminate in benchmarking against state-of-the art microscopes: (1) implement a novel optical system to capture simultaneous imaging data across x-, y-, and z-axes using quantum-entangled light; (2) produce and test high-power, quantum-entangled light sources at visible wavelengths; (3) detect coincidence with two high-speed 2D photon-timing single-photon avalanche diode (SPAD) detector arrays; and (4) adapt existing biosensors and bioenergy systems to apply and benchmark the 3D quantum (3DQ) capability against light-sheet microscopy and spinning-disk confocal microscopes.

During this period the project is implementing the Phase 1 system using a lower-resolution microscope and the first-generation synchronized 2D SPAD arrays with 1,024 total pixels. The team will finish initial measurements of fluorescence excited by entangled photons that will inform the final design of the Phase 1 3DQ microscope. Researchers will then assemble and test the 3DQ microscope and characterize the operation of the microscope. They will model the imaging capabilities using the quantum-entangled Fourier optics formalism. The team will begin design efforts for the higher-resolution, larger field-of-view Phase 2 3DQ microscope.

Anticipated Accomplishments: In the past year, the project reached a major milestone in technical objective 2: The team developed a source of visible quantum-entangled photons based on Type-I SPDC and a 266 nm laser as shown in the figure (next page). Researchers were able to obtain ~108 entangled photon pairs per second with moderate laser powers while limited by saturation of current detectors. They detected the time correlation associated with the entangled photons. They have finished designing and are assembling the microscope setup for technical objective 1. For technical objective 3, the team tested an initial 2D SPAD array under development by EPFL, and EPFL is now integrating two correlated detectors for delivery. Initial oxygen sensors were developed and published for technical objective 4, and initial baseline fluorescence images were obtained for the algal-bacterial and plant systems under study.

Potential Benefits and Applications: This project envisions a new 3DQ microscope that uses quantum-entangled light to provide 3D optical imaging at high frame rates. The team will apply this microscope to dynamic host-bacteria interactions in bioenergy algal pond and plant systems. The 3D imaging capability developed in this project will have applications well beyond bioenergy research. Rapid 3D optical imaging of fluorescence and scattering processes is a very general need that has applications to biological, biomedical, and materials systems.

(A) The 266 nm pump beam generates Type 1 spontaneous parametric down conversion (SPDC) in a beta barium borate (BBO) crystal. The resulting cone of entangled photons is visible in the charge-coupled device (CCD) image to the right. **(B)** The setup generating the entangled photons, splitting them into signal and idler arms, and focusing them onto photon counting detectors is shown. Detectors are toward the top. **(C)** The histograms show the waiting time distributions for photons detected by the two photon counting detectors. In blue is the waiting time between the arrival time of a photon in Channel 1 and a subsequent photon in Channel 2. In orange is the same graph with the order of channels reversed. The initial spike in both histograms is due to the arrival of the other, quantum-entangled photon. Further verification of quantum entanglement is being pursued using a Hong-Ou-Mandel interferometer. [Courtesy Audrey Eshun, Xiyu Yi, Shervin Kiannejad, Tiziana Bond, and Ted Laurence]

Quantum Ghost Imaging with Entangled Photon Pairs of Water Content and Plant Health

Principal Investigator: James Werner

Institution: Los Alamos National Laboratory

Email: jwerner@lanl.gov

Research Plans and Progress: During the past year, this project has made substantial progress. In terms of staffing, the project hired (1) a postdoctoral research associate with expertise in entanglement source characterization, (2) a full-time postdoctoral research associate with expertise in plant biology and microscopy, and (3) an early career staff scientist with expertise in studying drought tolerance in plants.

Experimentally, the project has made significant headway on quantum ghost imaging (QGI) microscope development. The team began by fully characterizing the spectral properties and power stability of a 405 nm pump laser acquired specifically for this project. Measuring the central wavelength stability and bandwidth of this laser was necessary to specify a custom periodically poled nonlinear optical crystal for entangled photon generation at the project's first selected wavelengths (1,450 nm idler photon, 558 nm signal photon). The custom crystal has been received and installed in an oven for temperature tuning of its index of refraction. The project expects to soon have spectral and temporal characterization of the entangled photon pairs using an InGaAs single-photon avalanche photodiode (SPAD) to detect idler photons and a silicon SPAD for detection of the visible photons. Temporal correlation of the photon pairs will be used to optimize the number of entangled photon pairs generated per second. The team has also explored the potential to perform hyperspectral ghost imaging. This involves spectrally resolving the energy of the photon reaching the bucket detector. To this end, the project has ordered a compact, low-loss Fourier transform infrared (FTIR) spectrometer unit (a NIREOS GEMINI interferometer) to be integrated into the QGI system. Primary milestones for this year included completely staffing the project, which was accomplished, and performing QGI of water absorption in leaves at 1,450 nm, which is expected to be demonstrated soon.

While QGI microscope development continues, researchers have begun to explore what type of information IR absorption microscopy can yield about plant health and vitality. In particular, they have started to perform conventional FTIR microscopy on plant samples in a reflection geometry on a Bruker Hyperion FTIR microscope. The mid-FTIR spectrum is being analyzed by principal component analysis to better assess and classify plants that are healthy and well-hydrated from those grown under more demanding and environmentally stressful conditions. The project's FTIR leaf images (see figure) and available databases of hyperspectral images also are being used to test which type of image processing (spatial or spectral) is useful for the much lower photon number images that will be obtained by QGI. To aid the FTIR microscopy work, researchers have begun to grow sorghum and *Camelina* in EcoFab devices (Jabusch et al. 2021).

Research Plans and Progress: This project has begun to disseminate its work to the broader scientific community to enable better use of its progress by the public and private sectors. This includes invited virtual oral presentations to Brookhaven National Laboratory (June 2021) and to a Defense Threat Reduction Agency Quantum Biology Workshop (December 2021). Publications (see References) include methods to calibrate cameras for quantitative microscopy. The project is hopeful the camera-calibration methods will be of use to DOE and other research scientists interested in performing quantitative fluorescence microscopy.

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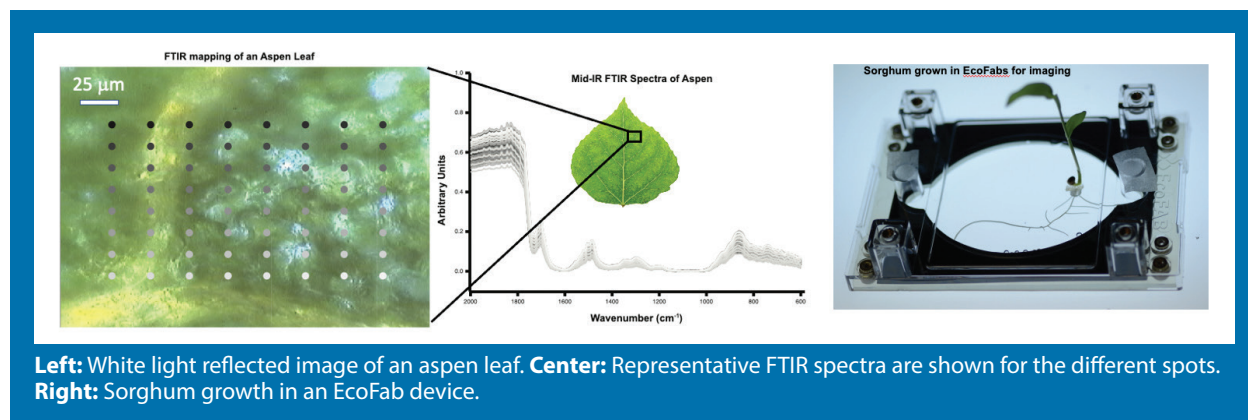
Left: White light reflected image of an aspen leaf. **Center:** Representative FTIR spectra are shown for the different spots. **Right:** Sorghum growth in an EcoFab device.

Probing Photoreception with New Quantum-Enabled Imaging

Principal Investigators: James Evans[1] (PI), Patrick El-Khoury,[1] and Robert Boyd[2]

Institutions: [1]Pacific Northwest National Laboratory (PNNL) and [2]University of Rochester

Email: james.evans@pnnl.gov

Research Plans and Progress: This three-year project will develop a new hybrid quantum-enabled imaging platform that combines advances in adaptive optics, quantum entanglement, coincidence detection, ghost imaging, quantum phase-contrast microscopy, and multidimensional nonlinear coherent spectromicroscopy to characterize photoreception both at the protein and whole-cell levels. The project's approach is to develop three aims in parallel. The first two aims focus on developing new quantum imaging approaches in which entangled photons will be employed to investigate biological samples with increased spatial resolution (Aim 1) and detection sensitivity (Aim 2) while permitting lower flux or sample interrogation with lower-energy photons. Aim 3 focuses on using coherent (nonentangled) photons and four-wave mixing to visualize photoreception and other quantum coherent processes occurring naturally within biosystems to better track ultrafast protein dynamics and the flow of metabolites between compartments in real time.

During the past year, the team has made critical progress on all three research aims, including acquiring the front-end laser that will be the foundation for Aims 1 and 2. The biggest development of the past year was in Aim 3, where team members completed the design, construction, and qualification of a femtosecond laser system for time-resolved four-wave mixing to probe bioquantum coherent states. The present application of this system involves tunable two-color, three-pulse, time-resolved coherent anti-Stokes Raman scattering (tr-CARS) in the range from 700 to 3,100 cm^{-1}.
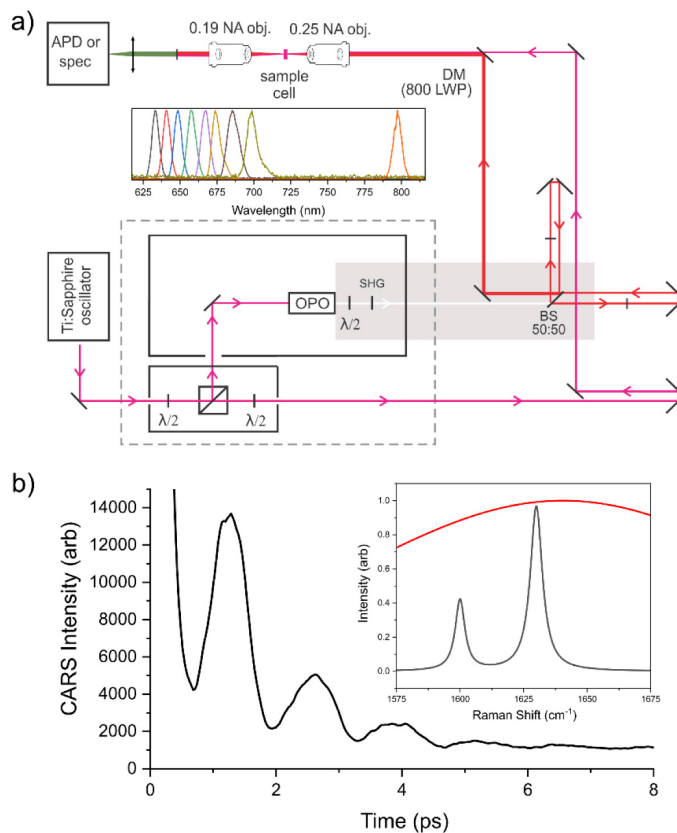
The figure (next page) shows an acquired tr-CARS signal where the time profile exhibits quantum beats that are separated by ~1 ps. A Fourier transform of the time profile recovers the vibrational modes involved in the prepared quantum coherence (see figure, panel b inset, black curve). As shown, this analysis recovers the familiar symmetric and antisymmetric 1,600 and 1,630 cm^{-1} C=C stretching modes of styrene. Tuning the two-pulse convolution window will allow different vibrations to be individually targeted. Note, when all the pulses coincide, the peak intensity of the first vibrational recursion is two orders of magnitude smaller than the tr-CARS signal at time zero and would normally be undetectable. This highlights a primary advantage of executing coherent Raman in the time domain, where the nonresonant contribution may be filtered out. The next stage of Aim 3 will involve demonstrating tr-CARS for imaging photoreception in biological samples at the ensemble level. In parallel, the apparatus will be paired with a high-speed scanning optical microscope to bridge the dimensional gap between ensembles and individual cells.

Anticipated Accomplishments and Deliverables: During the current project period, the team installed a confocal microscope with fluorescence, CARS, stimulated Raman scattering (SRS), and multiphoton (MP) imaging modes. The project acquired the front-end laser, which is the foundation of ghost imaging and quantum-phase contrast imaging development (Aims 1 and 2). In Aim 3, researchers developed femtosecond tr-CARS that can detect quantum beats separated by only 1ps (as highlighted above). Researchers also performed cell-free expression and purification of phytotropin proteins and are in the process of their structural and spectroscopic characterization.

Potential Benefits and Applications: This project will have immediate and broad applications to biosystems of interest to the DOE BER research program. Namely, the team will visualize photoreception in microbial systems with high spatial resolution using minimally invasive and ultralow-power approaches, all while implementing novel imaging approaches derived from novel quantum properties of light and biomolecules. The advancements made in quantum imaging should also be broadly impactful to emerging quantum information science thrusts within other DOE offices. All results are expected to be published in peer-reviewed journals. Furthermore, because the principal investigator is a staff member of the Environmental Molecular Sciences Laboratory (EMSL, a DOE user facility at PNNL), the new capabilities will also be made accessible to other researchers through the EMSL user program as the technologies described in this project mature. This will facilitate widespread dissemination to the global scientific community and enhance overall impact.

Development of tr-CARS for probing quantum biology states at PNNL

a)

b)

(a) Schematic of the two-color, three-pulse, time-resolved coherent anti-Stokes Raman scattering (tr-CARS) apparatus and the frequency tuning range of the system (inset). **(b)** Acquired tr-CARS signal exhibiting quantum vibrational coherent recursions corresponding to the 1600 and 1630 cm^{-1} C=C stretching modes (inset). [Courtesy Kevin Crampton and James Evans]

No-Cost Extension Projects

Plasmonics-Enhanced Optical Imaging Systems for Bioenergy Research

Principal Investigators: Tuan Vo-Dinh[1] (PI), Tai-Ping Sun[1], and Kenneth M. Kemner[2]

Institutions: [1]Duke University, [2]Argonne National Laboratory

Email: tuan.vodinh@duke.edu

Research Plans and Progress: Monitoring gene expression in whole plants is a key requirement in many important fields, ranging from fundamental plant biology to biofuel development. However, current methods to monitor gene expression in plants require sample extraction and cannot be performed directly *in vivo*. To overcome these limitations, the project has developed *in vivo* imaging and biosensing of nucleic acid biotargets using plasmonic nanoprobes referred to as inversed molecular sentinels (iMS) that can be monitored using surface-enhanced Raman scattering (SERS). The team is developing innovative imaging technologies, referred to as Multimodal Optical Sensing and Imaging Combinatory (MOSAIC) system for visualization and quantitative characterization of biomarkers (microRNA, mRNA) related to molecular processes and cellular function within living plants. The advanced MOSAIC system will provide much-needed tools for biofuel research.

Anticipated Accomplishments and Deliverables: Tobacco plants were infiltrated with nanoprobes based on gold nanorods coated with silver (AuNR@Ag). Figure panel A (see next page) shows the transmission electron microscopy (TEM) images demonstrating intracellular accumulation of nanorods capable of penetrating the plant cell wall. The red box shows the region of interest, and the red arrows indicate the nanorods accumulating inside a cell identified by the presence of the cell wall and organelles. As a co-registration technique, X-ray fluorescence (XRF) imaging was also applied to the TEM sample (see figure, panel B). Potassium signals from the cellular structures and osmium signals from the staining permit accurate identification of the cell wall.

The XRF technique allows for the acquisition of the gold signals from the TEM sample to localize the nanorods. When performed in tandem, these techniques provide absolute evidence of intracellular accumulation of nanorods (AuNR@Ag) in plant cells. The team functionalized the nanorod with iMS probes to serve as a biosensor for miR397b, a microRNA that was shown to reduce lignin polymer formation in *Arabidopsis* cell wall. Figure panel C shows successful *in vivo* detection of miR397b in tobacco leaves after intracellular infiltrating of nanorod-iMS probes and injection of miR397b targets. The team used shifted-excitation Raman spectroscopy (SERDS) to extract intrinsically weak Raman signals under ambient light and complex background conditions, which are often encountered in field applications. The results demonstrate the possibility of *in vivo* and intracellular detection of microRNAs injected in plant leaves under ambient light conditions without the need of sample extraction and processing.

Potential Benefits and Applications: This project will be applied to research on next-generation biofuels, which aim to use nonfood biomass, such as lignocellulose in plant wastes or hydrocarbon produced by photosynthesis in plants and certain microbes. Current production of cellulosic and hydrocarbon biofuels is far from optimal and requires further research to improve the efficiency and reduce costs. The project's novel tools will greatly facilitate studies on the regulatory mechanism for photosynthetic terpene production in plants. This is the first demonstration of SERDS/SERS signal detection inside of tobacco pavement cells using plasmonic nanosensors *in vivo*. The newly developed biosensing and imaging techniques will allow the team to monitor the expression pattern of microRNA targets such as miR397b in the vasculature of developing plants without the need for sample extraction. The stand-off SERDS technique will also allow monitoring of miR397b expression remotely in whole plants in their natural state.

(A) Transmission electron microscopy images of AuNR@Ag inside a tobacco cell. Red arrow indicates presence of the AuNR@Ag. **(B)** X-ray fluorescence images of the AuNR@Ag inside the region of interest shown in the red square of (A). **(C)** Tobacco leaves treated with AUNR@Ag iMS and the resulting SERDS signal from the nanosensor turning ON, demonstrating *in vivo* detection of miR397b microRNA target in whole plant leaves. [Courtesy the Vo-Dinh Laboratory at Duke University and the Fitzpatrick Institute of Photonics in collaboration with the Sun Lab (Duke University) and Kenneth M. Kemner (Argonne National Laboratory)]

Time-Resolved 3D Multiresolution Microscopy for Real-Time Cellulase Actions *In Situ*

Principal Investigator: Haw Yang

Institution: Princeton University

Email: hawyang@princeton.edu

Research Plans and Progress: The goal of this project is to build the time-resolved 3D multiresolution microscopy (TR-3DMRM) to elucidate the kinetic and chemical factors affecting cellulase action on lignocellulose *in vitro* and, in the future, *in situ*. This goal is fulfilled through two aims: (A) Develop the new TR-3DMRM, a combination of a time-gated real-time 3D single-particle tracking (TG-RT3DSPT) and a two-photon laser-scanning microscope with the capability of a fluorescence-lifetime imaging microscope (2PLSM-FLIM); and (B) Develop suitable protocols and assays for the application of 3DMRM on cellulose-cellulase system. This involves giant quantum dots (gQDs) synthesis, cellulase and substrate development characterization, and single-molecule assay for TR-3DMRM and 3DMRM optimization and assay.

Progress has been made on both the instrumentation and the assay development components. On the instrumentation side, the Yang Lab has developed the TG-RT3DSPT (Zhao et al. 2021; see figure). The proof-of-principle experiment showed that a maximum ~100-fold improvement in signal-to-background ratio was achieved by tracking the single gQDs in solutions containing dye-stained cellulose. The translational diffusion coefficient of the fastest tracked particle was ≈3.3 µm^2/s, more than sufficient to resolve the events in the cellulose-cellulase system. Compared with previous works, the newly developed system shows a 33-fold improvement in terms of the mobility of the particles that can be tracked. The construction of 2PLSM-FLIM is ongoing. After the critical evaluation, 2PLSM-FLIM will be integrated with TG-RT3DSPT to finish up Aim A.

On the assay side, because the original co-principal investigator (Ming Tien) retired, much of the assay development was shifted to the Snee Lab, including generating the 1-1 gQD-enzyme conjugates. The Snee Lab has generated CdZnSe/CdS type II gQDs with long photoluminescent lifetime (>100 ns) and high two-photo cross section, which is favored by the experiments carried out using TR-3DMRM. They have developed the capability to water-solubilize gQDs with silica (called CdZnSe/CdS@SiO$_2$) with near unit efficiency and maximize biological compatibility by surface functionalization. The Yang Lab has also made progress on 3DMRM optimization and assay, in that a method has been developed to extract subpixel information for low signal-to-noise ratios that enables video-rate super-line localization and resolution matching between two 3DMRM modules. This method is currently being upgraded for compatibilities on 3D image stack with nonisotropic point-spread function and voxel shape, an anticipated situation in the coming experiments.

Potential Benefits and Applications: This project will reveal *in situ* single-cellulase actions in real time and thus facilitate the basic scientific understanding of cellulose degradation. Such understanding will lead to a greater impact for biofuel and energy-related applications. In addition, the application scope of the new tool built for this study—the next-generation 3DMRM platform—can be extended to more complicated environments beyond the cellulose-cellulase system. While the original 3D multiresolution microscope (Yang Lab, Professor Kevin Welsher at Duke University, published work), a technique made possible through prior DOE funding, already enables more direct scientific discoveries by its powerful multimodal approach and high spatial and temporal resolutions, the current generation is also applicable to scenarios with high background, which is not uncommon for systems requiring dye-labeling of the substrate or the environment.

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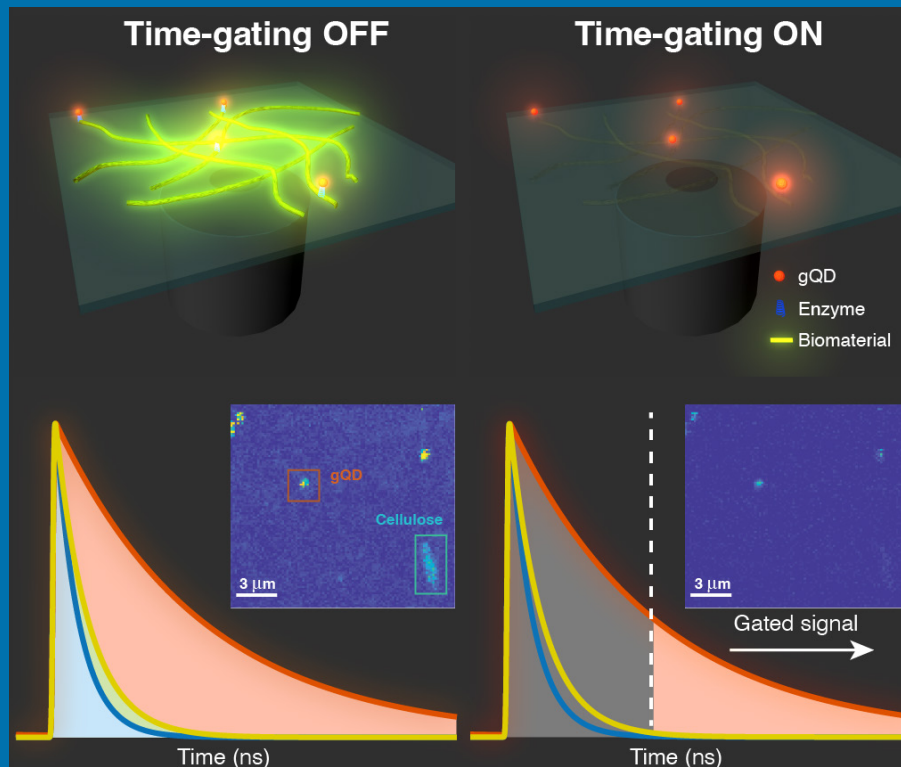
Conceptual drawing for the time-gated, real-time 3D single-particle tracking (TG-RT3DSPT) technology. Cartoon on the top panel shows an example of a TG-RT3DSPT experiment on an enzyme-biomaterial system, where enzymes are labeled with non-blinking quantum dots (gQD) with long photoluminescence lifetime, whereas the biomaterials are labeled with organic dyes with short fluorescence lifetime. When lifetime gating is on, the short-lifetime fluorescence background signals from the dye-stained biomaterial are suppressed, which significantly improves the signal-to-background ratio (top left: time-gating off; top right: time-gating on). Bottom panel shows the mechanism for the time-gating on the time domain. Without time-gating (bottom left), the experimental raster scan image (insert) records the optical response from both gQDs and dye-stained cellulose. By setting proper time-gating (bottom right, with the vertical dotted line indicating the gate position), the short-lifetime signal is largely suppressed, and only the long-lifetime signal from gQD is observed (insert). [Reprinted under a Creative Commons License (CC BY 4.0) from Zhao, T., et al. 2021. "Leveraging Lifetime Information to Perform Real-Time 3D Single-Particle Tracking in Noisy Environments," *The Journal of Chemical Physics* **155**(16), 164201. DOI:10.1063/5.0063634]

Understanding Plant Signaling via Innovations in Probe Delivery and Imaging

Principal Investigator: Jean Greenberg

Institution: The University of Chicago

Email: greenbe@uchicago.edu

Research Plans and Progress: This project is (1) optimizing nanofibers to deliver DNA expression constructs to plant cells and (2) developing and using a custom-built fiber optic microscope and image analysis platform that enables iterative, nondestructive measurements to be made and compared. These tools are being developed together with research aimed at understanding receptor-mediated trafficking of the growth-promoting PSK peptide and responses. The goals are (a) to use the microscope to image the trafficking of fluorescent bioactive peptides under different conditions; (b) to improve and test different nanofiber designs for delivering probes to plants, including the delivery of DNA constructs to curved tissue; and (c) to further discover and validate the transcriptional changes due to PSK-induced signaling.

Anticipated Accomplishments and Deliverables:

- **Microscope.** The team upgraded the fiberoptic microscope to optimize plant stabilization and imaging. The microscope includes two LED light sources plus a new white LED for brightfield imaging and interchangeable fiberoptic lenses with different magnifications. They created a mount to allow upright imaging, fabricated a 3D-printed leaf clip, and mounted the fiber on an extensible arm with a five-axis (X Y Z plus pitch and yaw) for precise sample manipulation and fine focus to obtain high-resolution, iterative micrographs using live plants. With these refinements, the team has observed trafficking of PSK-TAMRA from *Arabidopsis* roots to leaves, with clear accumulation of the probe at cell periphery.

- **Nanofibers.** The project successfully used vertically aligned carbon nanofiber arrays (VANCFs) to deliver and get expression of DNA constructs with reporter fusions in various plant tissues. The team has quantified DNA delivery via VANCFs in transformed plants, using confocal microscopy and image analysis tools (Fiji/ImageJ). From this analysis, researchers saw a significantly higher integrated density of green fluorescent protein (GFP) in plants transformed with fibers in comparison to the controls (+Fibers, -DNA; -DNA, -Fibers; and -Fibers, + DNA). They have used both wet and semi-dried DNA on the fibers for successful delivery in plants, further showing the versatility of the method. A manuscript describing this work is currently in preparation for publication. Additionally, the project had a new user proposal accepted at the Center for Nanophase Material Sciences (CNMS) for 2022–2023. The team is now actively working with CNMS to create longer fibers and transfer these fibers to a flexible PDMS substrate to expand the utility of the fibers.

- **Biological Materials and Deliverables.** The project has shown that PSK is internalized into cells and traffics from roots to shoots. The team has constructed the needed crosses of receptor-green fluorescent protein (GFP) plants into different backgrounds (mutants lacking receptors or the ability to produce active PSK) for trafficking studies. They performed a time series transcriptomic analysis of root and shoot responses to PSK and identified the tissue-specific pathways impacted by this hormone. The team is building new reporters, based on its findings, to use with the microscope. Researchers performed a detailed developmental analysis of plants that cannot make or respond to PSK and quantified developmental and physiological phenotypes.

Potential Benefits and Applications:

- A major advance is the iterative, nondestructive imaging of peptide signaling responses in plants that are highly relevant to improving traits for energy applications. This includes documenting changes in growth parameters and cell longevity and the accompanying signaling events, imaging of probes within plants, and the ability to track their movement nondestructively.

- Nanofibers for introducing nonpermeable probes and biomolecules into plant cells will permit researchers to accelerate the discovery of plant signaling response components in many plant species in response to many stimuli/environmental conditions. These fibers serve the dual goal of providing fiducial markers for the iterative imaging developed.

Understanding Plant Signaling via Innovations in Probe Delivery and Imaging
### Jean T. Greenberg et al. The University of Chicago

A. Fiber-optic microscope. A1: Set-up for upright microscopy, with microscope body (left), optical module (center) and PC (right). A2: Close-up of objective lens with surrounding "leaf compressor" to flatten the specimen gently. A3: Remote set-up with fiber optic mounted on an extensible arm with leaf/tissue clip and mini-stages for X-Y-Z and pitch-yaw control of fiber position.
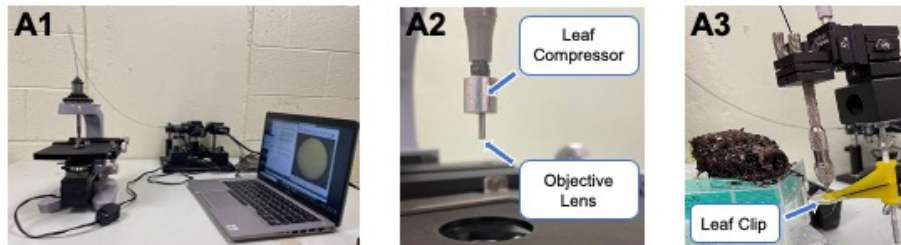
B. Imaging peptide trafficking in intact Arabidopsis plants.
Example: Tracking a mobile bioactive peptide (PSK) non-destructively: Arabidopsis leaves 90–150 min after root treatment with 10µM PSK-TAMRA. PSK moves from the roots and accumulates at cell periphery, probably at the plasma membrane (B1/B2) and within vascular cells (B3/B4).

TAMRA fluorescence   Brightfield   TAMRA fluorescence   Brightfield
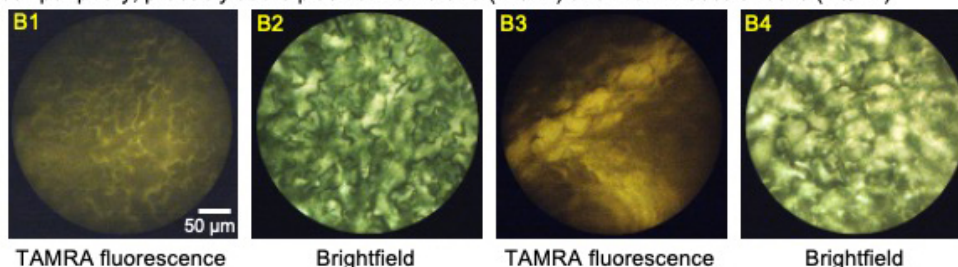
C. Imaging roots (C1) and a PSK receptor fusion to GFP in a leaf (C2).

D. Identifying a PSK response marker. Plants lacking the ability to make PSK due to a *tpst* mutation were treated with 10 nM PSK and profiled by RNA-seq.

E. Wet and semi-dry methods for delivering DNA constructs to plant cells using carbon nanofibers.
Fluorescence images of leaves transiently transformed with UBQ:YFP 48 h after delivery using 200 ng of DNA dried onto fibers for 15 minutes (semi-dry method) or 1 µL (200 ng) of DNA placed on leaf surface prior to impalement. Images after impalement with fibers. Scale bars = 20 µm * background stomata signals. Arrows point to nanofibers.

[Courtesy Jessica Morgan, Dian Lu, Bernard Abakah, Christopher Erb, Edward Offei, Thomas Ntim, Robert F. Standaert, Jennifer L. Morrell-Falvey, Joanna Jelenska, and Jean T. Greenberg]

Metaoptics-Enabled Multifunctional Imaging

Principal Investigators: Paul Bohn (PI), Anthony Hoffman, and Joshua Shrout

Institution: University of Notre Dame

Email: pbohn@nd.edu

Research Plans and Progress: The project is developing enhanced imaging tools by pursuing two overarching technical goals: (1) Development of new metaoptics-enabled approaches to imaging and spectroscopic characterization; and (2) Development of tools to control the chemical environment of a microbial sample with nanometer-scale precision.

Accomplishments:

- **Metaoptics.** Plasmonic hyperbolic metasurfaces have emerged as an effective platform for manipulating the propagation of light. The team has developed confined modes on arrays of silver nanoridges that exhibit hyperbolic dispersion and used these to demonstrate and model a super-resolution imaging technique based on structured illumination microscopy (SIM). A spatial resolution of ~75 nm at 458 nm was obtained, which is 3.1 times better than an equivalent diffraction limited image. This work emphasizes the ability to engineer the properties of confined optical modes and to leverage those characteristics for applications in imaging. The team expects this work to lead to improved approaches for super-resolution imaging using designed sub-wavelength structures that are applicable to many problems of interest to DOE.

- **Spectroelectrochemistry of *Myxococcus xanthus*.** *M. xanthus*, a common soil bacterium with a complex life cycle, is known for production of secondary metabolites. However, little is known about the effects of nutrient availability on *M. xanthus* metabolite production. In the past year, the team utilized confocal Raman microscopy (CRM) to examine the spatiotemporal distribution of chemical

signatures secreted by *M. xanthus* and their response to nutrient availability (i.e., metabolic state). Ten distinct spectral features were observed by CRM from *M. xanthus* grown on nutrient-rich medium. However, when *M. xanthus* was constrained to grow under nutrient-limited conditions, it developed fruiting bodies, and the accompanying Raman microspectra were dramatically altered. Reduced metabolic states were accompanied by reduced, or completely eliminated, characteristic Raman features at 1140 cm^{-1}, 1560 cm^{-1}, and 1648 cm^{-1}. In their place, a feature at 1537 cm^{-1} was observed, this being tentatively assigned to a transitional phase important for cellular adaptation. In addition, principal component analysis heat maps were used to illustrate how fruiting bodies in the center coexist with motile cells at the colony edge.

Potential Benefits and Applications: The experimental results together with numerical demonstration of super-resolution provide a pathway for super-resolution imaging of *M. xanthus* using simplified nanoridge arrays. The nanoridge geometry can be controlled to implement various super-resolution imaging paradigms.

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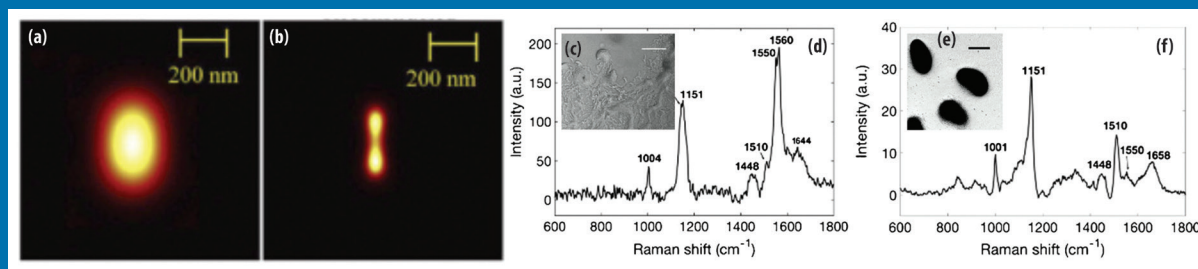
(a, b) Demonstrates the ability to distinguish two quantum dots placed 170 nm apart center-to-center, with the intensity line profile taken vertically through the dot locations provided on the right that shows two clear peaks. **(c)** Confocal laser scanning microscopy (CLSM) image of the advancing colony edge, and **(d)** corresponding average CRM spectrum for *M. xanthus* after 5 days on nutrient-rich 1% casitone. **(e)** CLSM image of the fruiting bodies, and **(f)** corresponding average CRM spectrum for *M. xanthus* after 5 days on TPM starvation agar. Scale bars represent 100 μm. [(a, b) adapted from Haug, J., et al. (c,d) adapted from Do, H., et al. 2022]

Detecting Chemical Signals in the Soil with 4DMAPS, an Integrated Aptasensor Assembly

Principal Investigators: Marit Nilsen-Hamilton (PI), Larry Halverson, George Kraus, Pranav Shrotriya, and Olga Zabotina

Institution: Ames Laboratory

Email: marit@iastate, larryh@iastate, gakraus@iastate, shrotriy@iastate, zabotina@iastate

Research Plans and Progress: The 4DMAPS project is building the capability of monitoring communication molecules in the rhizosphere in real time and *in situ*. The concept is to use aptamer-enabled electrochemical sensors to detect local concentrations of communicating molecules in the rhizosphere and to use this capability to understand how the communications between the plant and its microbiome help to maintain the health of the community. The project's goals are to monitor molecules that support the microbial community for quorum sensing and iron acquisition, with autoinducers 1 and 2 representing the former and pyoverdines representing the latter.

Anticipated Accomplishments and Deliverables: Autoinducer 1 molecules are homoserine lactones, of which C4HSL is representative. An aptamer-recognizing C4HSL was incorporated into a sensor and C4HSL diffusion was monitored through a gel mimic of the soil environment. Numerical models to predict C4HSL diffusion matched well with the experimental data and the combination of numerical and experimental data provided us with estimate of the gel diffusion properties, which were used to create a 4D map of C4HSL diffusion in the soil mimic gel. To provide an economical means of gathering the data, a microprocessor-based sensor array reader was developed and used to monitor the C4HSL diffusion at increased temporal frequency.

Whereas AI-1 is a gram negative–bacterial signaling molecule, the boron-furan-derived "universal" autoinducer-2 (AI-2) is used for quorum sensing by both gram-negative and gram-positive bacteria. This molecule was synthesized by a novel direct synthetic method and is now being used to isolate aptamers for integration into a sensor. Several microbial community members were found to produce AI-2.

In parallel with building sensing capability, researchers optimized the synthetic soil system to minimize the maize "rapid growth phenotype" that results in stunted growth. This required finding the right genotype and seed germination conditions. The project has also identified a microbial community of 31 members to create the plant microbiome with *Zea mays*, which will be used for testing the 4DMAPS sensors *in situ*. Whole genome sequencing with short and long reads is moving forward with an ensemble strategy to assemble all the microbial genomes of 31 species. In this collection, researchers have established bacteria cross-species interaction networks to explore the possible role of diffusible molecules that potentially shape the structure and function of the microbial community in the synthetic soil-microbiome-plant experimental system. For example, microbes were identified that enhanced growth of the poor rhizosphere colonist *Exiguiobacterium* species, which produces copious amounts of the "universal" AI-2 autoinducer.

Pyoverdines are produced by *Pseudomonads* for sequestering iron and are important for maintaining species balance in the microbial community. The team has synthesized the pyoverdine chromophore and developed procedures for fractionating and characterizing siderophores from bacterial pools and for controlling pyoverdine production by the microbial community. Researchers have also identified positive and negative interactions between members of the community that are influenced by nutrient level and iron bioavailability. To measure pyoverdine in the rhizosphere, researchers have isolated aptamers that recognize the chromophore and others that recognize other parts of the pyoverdine, pf5. Sensor construction with these aptamers to measure pyoverdine is underway.

Potential Benefits and Applications: The team's studies are directed to obtaining a predictive, systems-level understanding of plant-microbial interactions. Researchers anticipate that their studies using *in situ* and real-time monitoring by aptasensors will result in direct insights into roles of exploitation versus contest competition and shared public goods (metabolites secreted into the environment) in modulating cooperative or competitive behaviors that influence the assembly, maintenance, or function of root microbiomes. Measuring the levels of public goods (from plants and microbes) *in situ* over time and space provides a unique means of evaluating the microbial community, its metabolic activities, and the plant's responses, which cannot be easily identified with static imaging approaches. This real-time *in situ* approach to studying molecular interactions in the rhizosphere is poised to provide many opportunities for solving critical challenges in energy security and environmental stewardship by real-time analysis of organismal interactions within plant-microbe biosystems.

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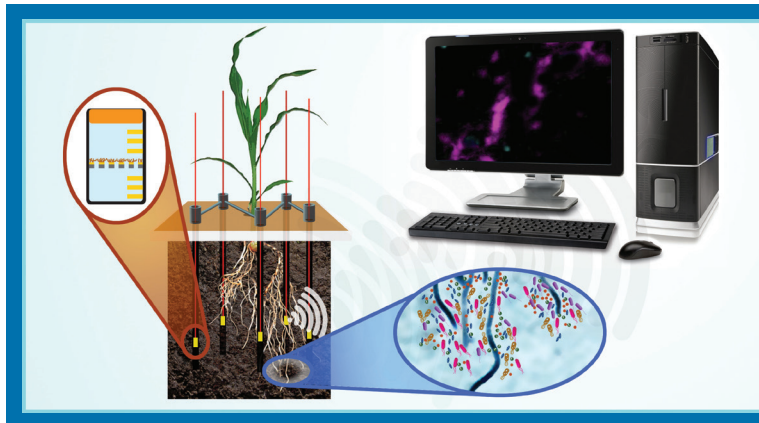
4DMAPS aptasensors are attached to moveable rods in shafts around a plant root. The sensors report wirelessly on the concentration of a specific molecular component to a receiver. The collected data are integrated to show where around the plant root a particular chemical compound is located and how this changes with time. [Courtesy Ames Laboratory]

Real-Time Imaging and Quantification of Plant Cell Wall Constituents Using Cavity-Dumped Stimulated Raman Scattering (cdSRS) Microscopy

Principal Investigator: Shi-You Ding

Institution: Michigan State University

Email: sding@msu.edu

Research Plans and Progress: This project aims to develop a new generation of stimulated Raman scattering (SRS) imaging tools to quantitatively analyze plant cell-wall constitutes with high sensitivity. The team is particularly interested in developing a correlative imaging method in addition to the multimodality of current microscopic platform to enable in-depth characterization of dynamic changes in cell wall chemistry and nanoscale structure.

Anticipated Accomplishments and Deliverables: The developed imaging platform has been applied to study different biological systems, such as lignocellulosic biomass processed by thermochemical and enzymatic approaches, and cell wall biosynthesis in plants.

- **Development of multimodal platform for imaging living plants.** The team has focused on developing new methods suitable for characterizing plant cell-wall biosynthesis in living plants. Researchers used a correlative imaging approach (see figure, next page) that combines state-of-the-art hyperspectral SRS techniques, fluorescence-based single molecule spectroscopy, and atomic force microscopy (AFM). The result overlays chemical information onto plant tissue structures, reveals nanoscale morphological features in the cell walls, and provides insights into their dynamic changes in nanoseconds. These nondestructive bioimaging approaches allow researchers to visualize plants at multiple (1) spatial resolutions, from single molecules (cellulolytic enzymes, cellulose synthases, and metabolites) to inter- and intracellular interactions; (2) chemical resolutions, from

bonding confirmations (linkages of polysaccharides and lignins) to crystalline/noncrystalline structures (cellulose microfibrils); and (3) temporal resolutions, from days (plant growth and enzymatic deconstruction of biomass) to nanoseconds (catalytic activities of cell wall synthase and cellulolytic enzymes).

- **Imaging cell wall structure in modified glucuronoxylan (GX) plants.** Using image analysis, the team shows that microfibril width is increased by as much as three times in the severe GX mutants compared to the wild type, and the degree of directional dispersion of the fibrils is approximately doubled in all three mutants. Findings show that these changes correlate with both altered nanomechanical properties of the secondary cell walls (SCW), as observed by AFM, and with increases in enzymatic hydrolysis. Results from this study indicate the critical role that normal GX composition has on cellulose bundle formation and cellulose organization as a whole within the SCWs (Crowe et al. 2021).

Potential Benefits and Applications: The multimodal imaging systems and nondestructive and correlative imaging approaches developed in this project provide new insights into biosystems design of energy crops. The ability to identify the chemical specialty of metabolites *in vivo*, overlay fine chemical features onto the ultrastructure of the plant cell walls, and track molecules in living plants allows researchers to measure and evaluate bioengineered plants at multiple length scales.

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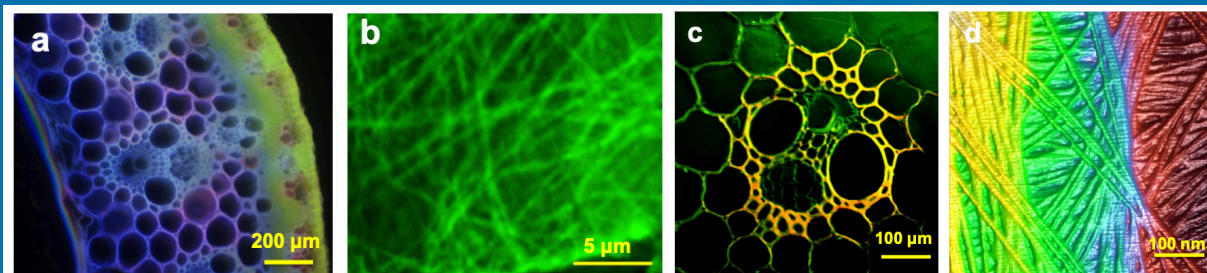
Correlative imaging of switchgrass (*Panicum virgatum L.*) cell walls using nondestructive approaches. Cross-sections of fresh switchgrass stems were manually cut and imaged using fluorescence microscopy **(a)** to show the autofluorescence at the tissue scale in different cells and cell walls, which may attribute to lignin, polyphenols, chloroplasts, and cuticles. **(b)** Super-resolution fluorescence microscopy to show the cellulose microfibrils network on the cell-wall surface labeled by CtCBM-GFP (a family 3 cellulose binding module tagged with green fluorescence protein, which specifically recognizes crystalline cellulose). **(c)** Stimulated Raman scattering microscopy showing the distribution of cellulose (green) and lignin (red) in different types of cell wall in the vascular bundle area. Note the different lignin contents in the different types of cells, in the same cell type but located in different sides of the vascular bundle, in the same cell but different sidewalls, and in the same wall but different layers. **(d)** Atomic force microscopy showing cellulose microfibrils and bundles at the sub-nanometer scale. [(a) Courtesy of S.-Y. Ding and DOE BioEnergy Science Center. (b) Reprinted under a Creative Commons License (CC-BY-NC-ND 4.0) from Ding, S.-Y., et al. 2018. "Versatile Derivatives of Carbohydrate-Binding Modules for Imaging of Complex Carbohydrates Approaching the Molecular Level of Resolution," *BioTechniques* **41**(4), 435–43. DOI:10.2144/000112244. (c, d) Courtesy Ding et al., unpublished data.

Spatiotemporal Dynamics of Photosynthetic Metabolism in Single Cells at Subcellular Resolution

Principal Investigators: Jeffrey Cameron (PI) and Ivan Smalyukh

Institution: University of Colorado–Boulder

**Email:** jeffrey.c.cameron@colorado.edu; ivan.smalyukh@colorado.edu

Research Plans and Progress: The research objective is to design and build a multimodal nanoscopy system to generate adaptive 3D images with high-resolution, real-time, dynamic label-free chemical imaging of metabolic processes in photosynthetic organisms. Various strains of cyanobacteria will be utilized for system benchmarking and calibrating of resolution and sensitivity. Researchers will then apply the system to generate a dynamic spatiotemporal map of photosynthetic metabolism, with a focus on tracking CO_2 fixation and conversion into biomass. Each of the proposed objectives has been completed.

Current and Anticipated Accomplishments and Deliverables: Researchers have completed the design/build phase of the project and integration of the custom imaging modalities into a single multimodal imaging platform. This enables multigenerational tracking the position and activity of single carboxysomes and revealed a subpopulation of ultraproductive carboxysomes and that inactive carboxysomes are degraded (1). Label-free chemical imaging is enabled with the newly integrated CARS modality (Figure 1, next page).

Potential Benefits and Applications: The multifunctional nanoscope developed in this proposal will be the first integration of these capabilities in a single setup and will enable an entirely new class of experiments that take advantage of high-resolution resolution and optical nanomanipulation while studying actively growing DOE-relevant biological systems. Cyanobacterial carbon fixation is highly efficient due to the biophysical CO_2-concentrating mechanism. By uncovering the life cycle of the cyanobacterial carboxysome and developing a new system to measure the spatial and temporal organization of metabolism, researchers have provided the foundation and molecular understandings for engineering more active and stable biochemical systems (1, 2, 3). Label-free chemical imaging with CARS offers a new way to screen high-throughput libraries and investigate metabolic pathways *in vivo*.

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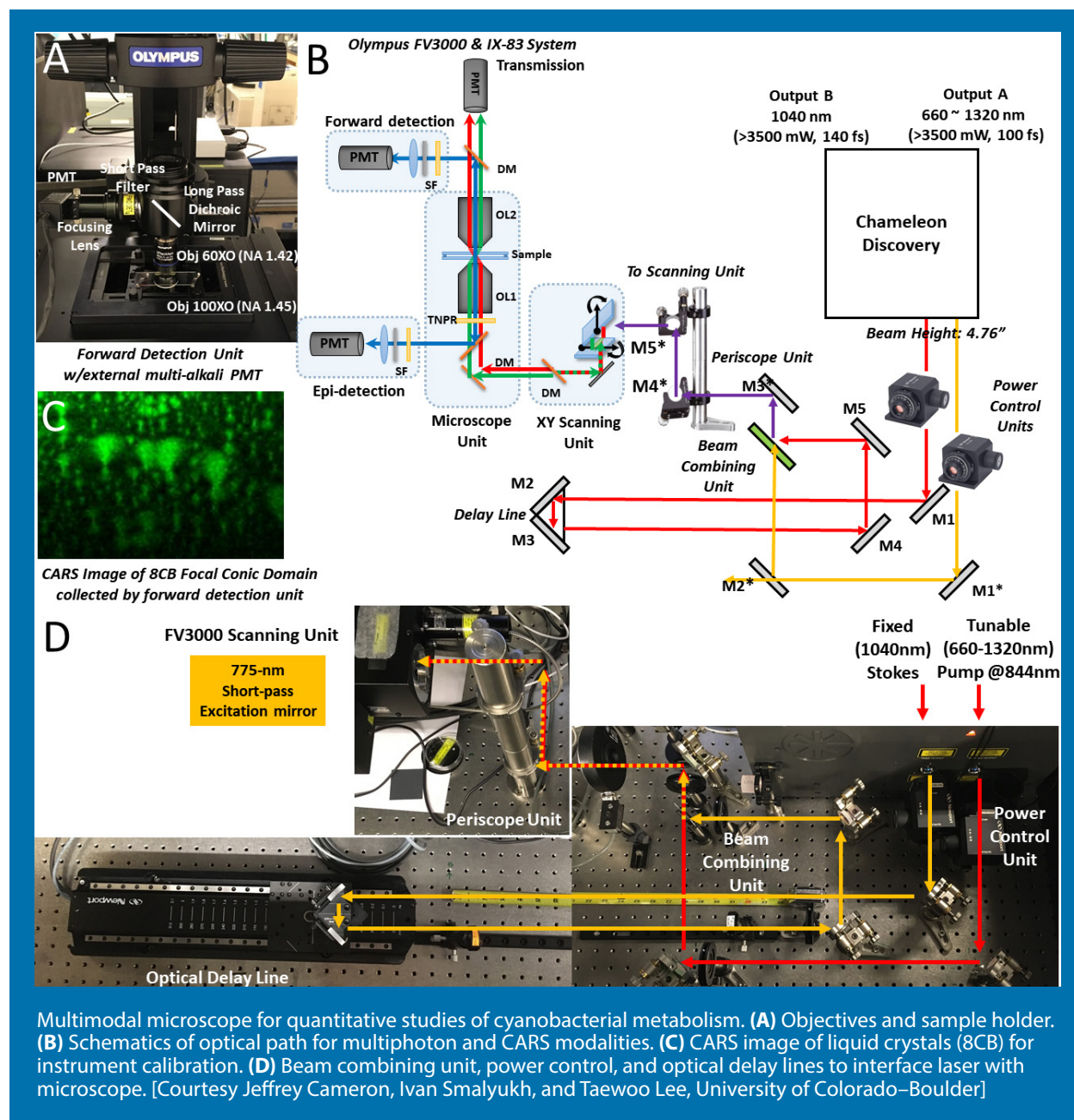
Multimodal microscope for quantitative studies of cyanobacterial metabolism. **(A)** Objectives and sample holder. **(B)** Schematics of optical path for multiphoton and CARS modalities. **(C)** CARS image of liquid crystals (8CB) for instrument calibration. **(D)** Beam combining unit, power control, and optical delay lines to interface laser with microscope. [Courtesy Jeffrey Cameron, Ivan Smalyukh, and Taewoo Lee, University of Colorado–Boulder]

Multiparametric Optical Label-Free Imaging to Analyze Plant Cell Wall Assembly and Metabolism

Principal Investigators: Marisa S. Otegui (PI) and Kevin W. Eliceiri

Institution: University of Wisconsin Madison

Email: otegui@wisc.edu and eliceiri@wisc.edu

Research Plans and Progress: Plant tissues are often considered not ideal for fluorescence imaging because of the pervasive intrinsic fluorescence of many plant metabolites and the intricate interactions with light of the many semi-crystalline polymers at the cell wall. This project is taking advantage of this observed shortcoming by developing a label-free, optical microscopy platform for characterizing multiple fingerprints of important cell wall components and stress-related conditions, at subcellular scale resolution. The new imaging system can collect fingerprints from both emitted and scattered light that can inform on the chemical nature, subcellular distribution, anisotropy, and molecular environment of multiple cell-wall components in intact plant tissues. Researchers are combining these imaging capabilities with computational tools that enable correlated registration, integration, and analysis. This fully integrated, multiparametric optical system will be used to address biological problems connected to cell-wall assembly in grasses. This includes a focus on developmental and environmental variation of cell-wall impregnation with silica, lignin, suberin, and cutin in different tissues and cell types. The research plan comprises three main goals: (1) To develop an accessible imaging platform and associated open-source software able to extract and integrate fingerprints from fluorescence-associated (multispectral emission, lifetime, and polarization), widefield polarimetry, second harmonic generation (SHG), and stimulated Raman scattering signals (SRS); (2) To determine unique combination of fingerprints for various cell-wall components and selected metabolites; (3) To analyze the process of cell-wall silicification in grasses and determine how silicification affects cell-wall properties and lignin, cutin, and suberin deposition in other cell types under differ stress conditions.
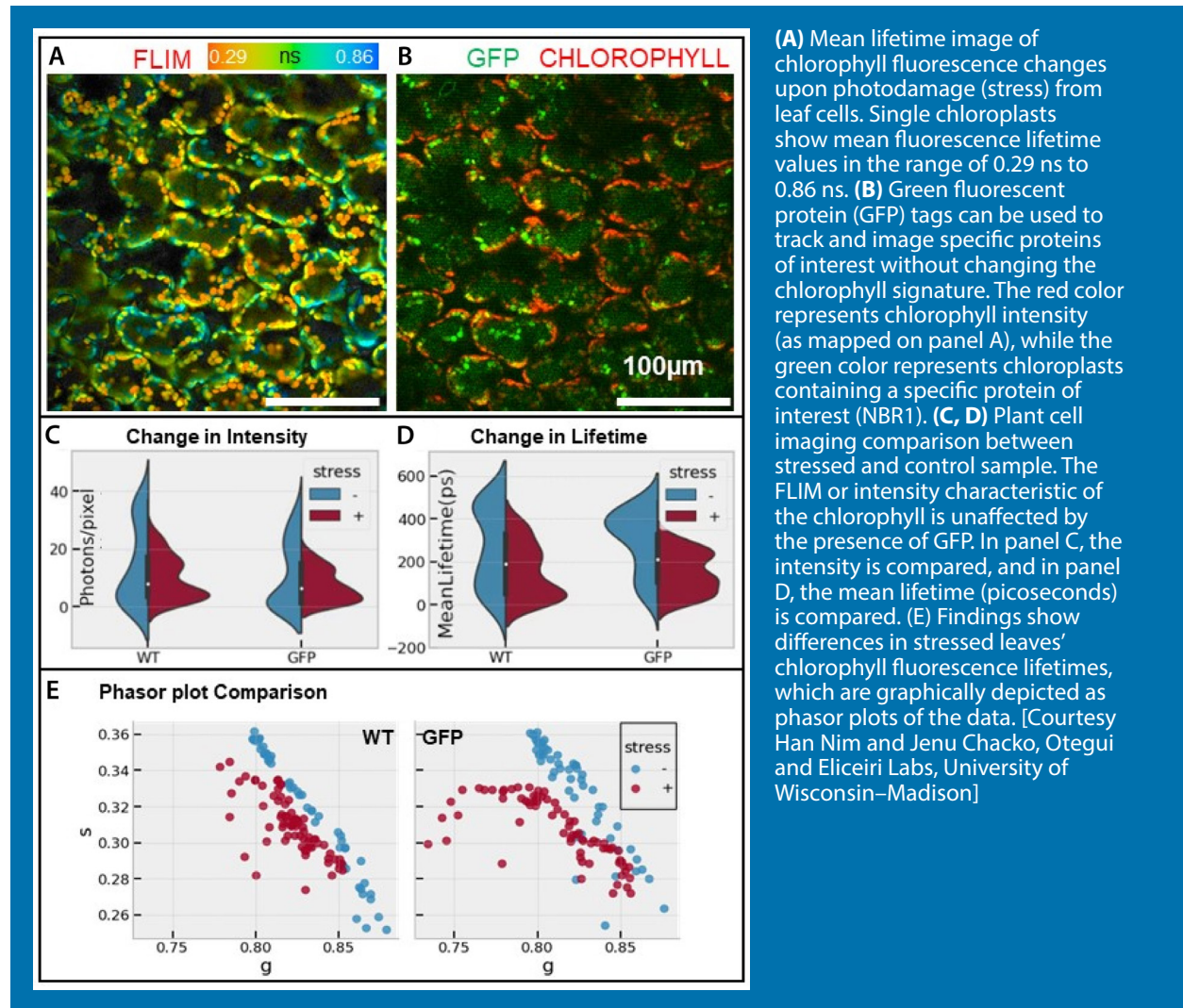
Accomplishments and Deliverables:

(1) Development of a fully functional open-source multi-photon scanning system.
   a. Added open-source fluorescence lifetime imaging (FLIM) acquisition - OpenScan (manuscript in preparation)
   b. Added open-source FLIM analysis package – (FLIMJ)
   c. Added Python support to FLIM analysis through PyImageJ-FLIMJ (Gahm, et al. 2021)
   d. Developed open-source FLIM denoising algorithm for faster acquisition (manuscript in preparation)
   e. Developed slow scanning modes and low-resolution modes for stage and galvo scanning for hyperspectral Raman imaging and fast registration with fluorescence.
   f. Developed open-source python based FLIM collection and analysis package based on FLIMLib

(2) Implementation of a fast two-channel, time-domain-based FLIM system with polarization control.
   a. Published fast FLIM optimization based on nonparametric Bayesian model
   b. Polarization-based imaging of autofluorescence (manuscript in preparation)

(3) Development of a novel fiber-based spectral detector.
   a. Published spectral detection proof of principle
   b. Published fast, hyperdimensional, contrast imaging using fiber-based FLIM (Chacko, et al., 2021).

(4) Hyperdimensional Imaging (HDIM) microscope for multiparametric signature registration was built to record emission spectrum, fluorescence lifetime, and rotational anisotropy.
   a. Fast biochemical separation of fluorescent species was achieved in MP imaging
   b. Tested correlated, Raman-fluorescence imaging on pollen grain samples. (manuscript in preparation)

(5) Development of a novel scheme to detect forward and backwards second harmonic generation.
   a. Implemented and validated for cellulose and starch (manuscript in preparation)
   b. Implemented wide area detector for deep tissue imaging (manuscript in preparation)

(6) Development of CRISPR/CAS9-edited lines expressing mutated silicon transporters (in progress.)

(7) Tested chloroplast metabolic imaging of *Arabidopsis* leaves (in progress).

Potential Benefits and Applications: Understanding the assembly and deconstruction of cell walls in grasses is very important for bioenergy. Grass cell walls have many chemical singularities, including high content of silica. The extent of cell-wall silicification is inversely correlated to lignin accumulation. However, how the two processes are coordinated is not known. As an example of what the new device will be able to accomplish, researchers will analyze patterns of cell wall silicification in maize and sorghum and determine how silicification affects cell wall properties and lignin and suberin deposition in other cell types under differ stress conditions.

(A) Mean lifetime image of chlorophyll fluorescence changes upon photodamage (stress) from leaf cells. Single chloroplasts show mean fluorescence lifetime values in the range of 0.29 ns to 0.86 ns. (B) Green fluorescent protein (GFP) tags can be used to track and image specific proteins of interest without changing the chlorophyll signature. The red color represents chlorophyll intensity (as mapped on panel A), while the green color represents chloroplasts containing a specific protein of interest (NBR1). (C, D) Plant cell imaging comparison between stressed and control sample. The FLIM or intensity characteristic of the chlorophyll is unaffected by the presence of GFP. In panel C, the intensity is compared, and in panel D, the mean lifetime (picoseconds) is compared. (E) Findings show differences in stressed leaves' chlorophyll fluorescence lifetimes, which are graphically depicted as phasor plots of the data. [Courtesy Han Nim and Jenu Chacko, Otegui and Eliceiri Labs, University of Wisconsin–Madison]

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Intrinsically Co-Registered Chemical Imaging of Living Plant and Microbial Systems via 3D Nonlinear Optical Mapping and *In Situ* Liquid Extraction-Mass Spectrometry (LE-MS)
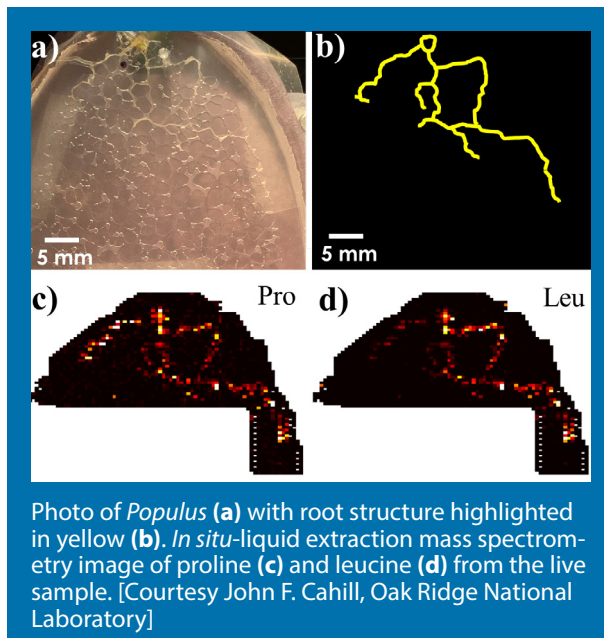
Principal Investigator: John F. Cahill

Institution: Oak Ridge National Laboratory

Email: cahilljf@ornl.gov

Research Plans: This project aims to develop a multimodal imaging platform integrating novel and existing analytical capabilities whose synergy yields 3D spatiotemporal chemical information in the bulk and at the interface in biological systems *in situ*. Research objectives are to (1) develop new bioimaging modalities, vibrational sum-frequency generation (vSFG) microscopy and *in situ*–LE-MS, (2) couple vSFG, coherent anti-Stokes Raman spectroscopy (CARS), and *in situ*–LE-MS imaging modalities into a singular, co-registered, multimodal imaging system, and (3) measure the dynamic chemical environment of a living biofilm and through imaging of stress-induced rhizosphere dynamics occurring among plant roots, microbial colonies, and soil.

Accomplishments and Deliverables: The team has successfully acquired *in situ*–LE-MS imaging data from a living plant root for the first time, demonstrating proof of concept. *Populus trichocarpa* cuttings were grown for several weeks in a soil-mimic, rhizosphere-on-a-chip device. The devices utilize an optically clear porous membrane surface that enables spatially resolved chemical characterization anywhere along the root structure using *in situ*–LE-MS. More than 12 amino acids were measured simultaneously in the root rhizosphere. Moreover, the technique was validated to be nondestructive to the plant, which continued to grow after several chemical images were acquired. The high chemical specificity of mass spectrometry enables each amino acid to be confidently identified despite being in a mix of molecules supplied by the media. The chemical images clearly show spatial alignment with the plant root for most amino acids. However, several amino acids exhibit different spatial distributions around the plant root than other amino acids. This suggests the spatial and chemical distribution of amino acids in the rhizosphere is heterogenous and is specific to each individual amino acid. Additionally, differences in amino acid composition across the root imply heterogeneous release of amino acids based on their identity, whether by direct exudation or other processes.

The team has also demonstrated a multimodal microscope platform capable of measuring second harmonic generation,

Photo of *Populus* **(a)** with root structure highlighted in yellow **(b)**. *In situ*-liquid extraction mass spectrometry image of proline **(c)** and leucine **(d)** from the live sample. [Courtesy John F. Cahill, Oak Ridge National Laboratory]

two-photon fluorescence, sum-frequency generation, and CARS images. Researchers have been able to image the alive/dead status of individual bacteria while acquiring complementary information on molecular vibrations. This provides additional contrast to image data. These advances were recently published and resulted in a patent application. The team's expertise in this field was recognized by an invitation to write a perspective on nonlinear imaging.

Potential Benefits and Applications: The *in situ*–LE-MS imaging modality enables, for the first time, detailed measure of the spatial distribution of dozens of exudated metabolites simultaneously without modification or destruction of the plant. Similarly, the microscope platform provides complementary chemical and spatial information from live samples. These capabilities enable resolution of the spatial, temporal, and chemical questions inherent to plant-microbe interactions in the rhizosphere. Technological developments have resulted in patent applications and awards. Together, these imaging technologies enable currently unobtainable insights into the chemical dynamics and localization in living systems such as plant-microbe dynamics occurring in the rhizosphere.

Correlative Imaging of Enzyme and Metabolome Dynamics for Yield and Titer Co-Optimization in Biofuel Producing Microorganisms

Principal Investigators: Andreas E. Vasdekis[1] (PI), Armando G. McDonald[1], Luke Sheneman[1], Scott E. Baker[2]

Participants: Nava R. Subedi[1] (postdoc), Gurkeerat Kukal[1] (student); Erin Bredewig[2] (staff)

Institutions: [1]University of Idaho, [2]Pacific Northwest National Laboratory

Email: andreasv@uidaho.edu

Research Plans: To accelerate biofuel production and related synthetic biology efforts, the project is developing an optical imaging platform that quantifies yields, titers, and compartmentalization of metabolic pathways in single, living cells. To this end, researchers have integrated quantitative-phase imaging (QPI) with both fluorescence and Raman light-sheet microscopy, thus, independently quantifying enzyme, metabolite, and nutrient fate dynamics, with the aid of deep learning and dedicated biomarkers.

Accomplishments and Deliverables: The team has developed a hardware platform that integrates QPI with both light-sheet fluorescence and Raman microscopy (see figure 1, next page). Here, the illumination sheet relies on an Airy beam quasi-lattice, enabling both minimal phototoxicity and 5-fold higher throughput rates than competing methods. Researchers have further extended this platform by integrating a detection architecture that is congruent with imaging at the Poisson limit and using the particle nature of light. This integration enabled the reduction of sample irradiance by 100x compared to state-of-the-art light-sheet microscopy, thus, eradicating photobleaching (see figure 2, next page) and unmasked dim fluorescent objects, typically buried below detection limits (see figure 3, next page). Moreover, the team developed software that segments cytosolic organelles in label-free QPI images using deep learning, as well as reconstructs Poisson images. Similarly, researchers have augmented the library of strains expressing GFP-tagged biomarkers by 3-fold, including strains that co-express >1 reporters. The team has also validated the use of deuterated Raman tags in quantifying the fate of nutrients in biofuel-producing microorganisms. Finally, researchers have applied these strategies to unmask how cellular noise impacts metabolism, specifically: [a] the spatiotemporal fluctuations of cytosolic crowding levels, previously thought to be constant; and [b] the localization of metabolic reactions. Remaining steps are to further validate bioimaging strategies by quantifying the impacts of cellular noise on metabolic trade-offs and rates.

Benefits and Applications: This project develops an integrative imaging platform that performs multivariate, molecular-level imaging of single living cells with minimal photobleaching and phototoxicity. Further, the developed hardware is compatible with standard inverted microscopes, while all related software tools are open access, thus, broadening accessibility within the bioimaging scientific community.

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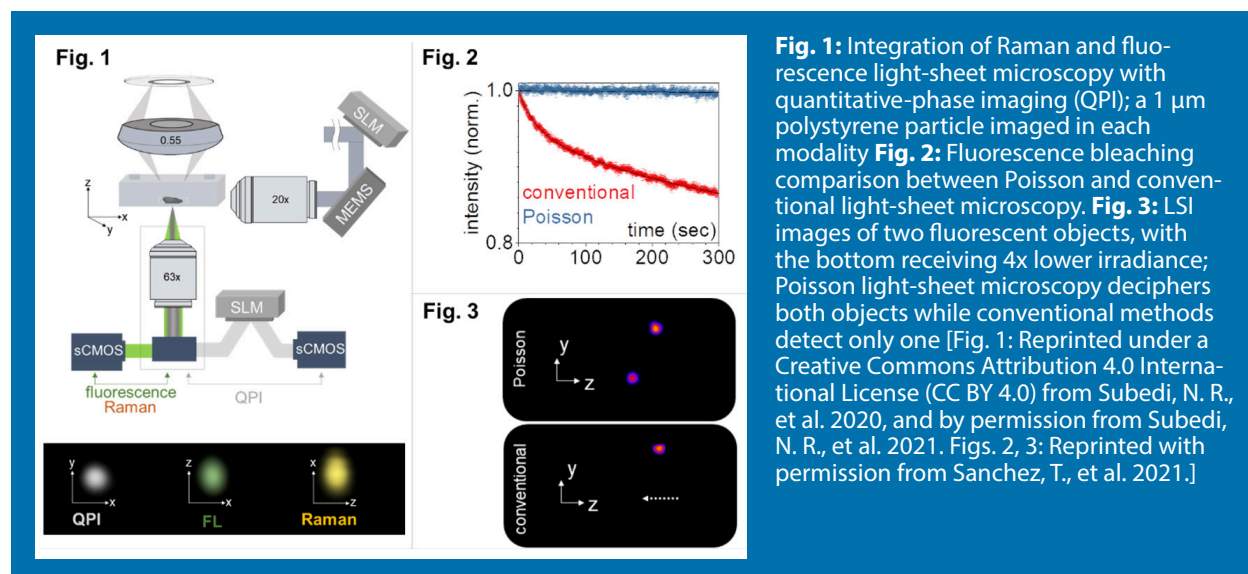

Fig. 1: Integration of Raman and fluorescence light-sheet microscopy with quantitative-phase imaging (QPI); a 1 μm polystyrene particle imaged in each modality **Fig. 2:** Fluorescence bleaching comparison between Poisson and conventional light-sheet microscopy. **Fig. 3:** LSI images of two fluorescent objects, with the bottom receiving 4x lower irradiance; Poisson light-sheet microscopy deciphers both objects while conventional methods detect only one [Fig. 1: Reprinted under a Creative Commons Attribution 4.0 International License (CC BY 4.0) from Subedi, N. R., et al. 2020, and by permission from Subedi, N. R., et al. 2021. Figs. 2, 3: Reprinted with permission from Sanchez, T., et al. 2021.]

2022 Bioimaging Program Principal Investigators

Current Projects — University

Shiva Abbaszadeh
University of California–Santa Cruz
sabbasza@ucsc.edu
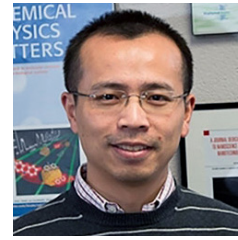
Jeffrey Cameron
University of Colorado–Boulder
jeffrey.c.cameron@colorado.edu

Jeffrey L. Caplan
University of Delaware
jcaplan@udel.edu

Peng Chen
Cornell University
pc252@cornell.edu

Marcus Cicerone
Georgia Institute of Technology
cicerone@gatech.edu

Theodore Goodson III
University of Michigan
tgoodson@umich.edu

Na Ji
University of California–Berkeley
jina@berkeley.edu

Mark Kasevich
Stanford University
kasevich@stanford.edu

Michelle O'Malley
University of California–Santa Barbara
momalley@ucsb.edu

Elizabeth Shank
University of Massachusetts Chan Medical School
elizabeth.shank@umassmed.edu

Leslie Sieburth
University of Utah
sieburth@biology.utah.edu

Gary Stacey
University of Missouri
staceyg@missouri.edu

Andreas Vasdekis
University of Idaho
andreasv@uidaho.edu

Shimon Weiss
University of California–Los Angeles
sweiss@chem.ucla.edu

Current Projects — National Laboratory

James Evans
Pacific Northwest National Laboratory
james.evans@pnnl.gov

Ted Laurence
Lawrence Livermore National Laboratory
laurence2@llnl.gov

Sean McSweeney
Brookhaven National Laboratory
smcsweeney@bnl.gov

James Werner
Los Alamos National Laboratory
jwerner@lanl.gov

No Cost Extensions — University

Paul W. Bohn
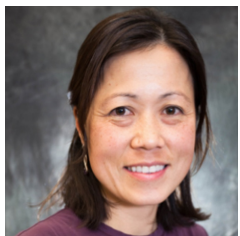*University of Notre Dame*
pbohn@nd.edu

Shi-You Ding
Michigan State University
sding@msu.edu

Jean T. Greenberg
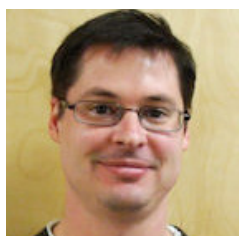*University of Chicago*
jgreenbe@uchicago.edu

William O. Hancock
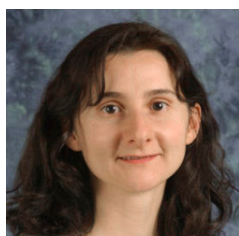*Pennsylvania State University*
wohbio@engr.psu.edu

Tina Jeoh
University of California–Davis
tjeoh@ucdavis.edu

Sang-Hyuk Lee
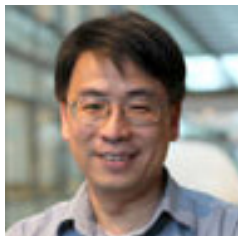*Rutgers University*
shlee@physics.rutgers.edu

Keith Lidke
University of New Mexico
klidke@unm.edu

Marisa S. Otegui
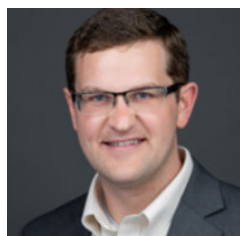*University of Wisconsin-Madison*
otegui@wisc.edu

Tuan Vo-Dinh
Duke University
tuan.vodinh@duke.edu

Haw Yang
Princeton University
hawyang@princeton.edu

No Cost Extensions — National Laboratory

John Cahill
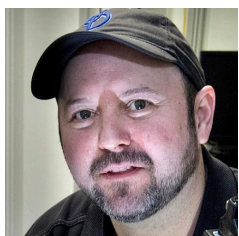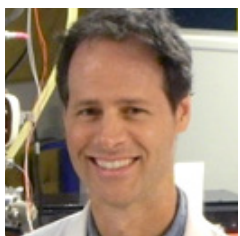*Oak Ridge National Laboratory*
cahilljf@ornl.gov

Scott Lea
Pacific Northwest National Laboratory
scott.lea@pnnl.gov

Marit Nilsen-Hamilton
Ames Laboratory
marit@iastate.edu

Ryan Tappero
Brookhaven National Laboratory
rtappero@bnl.gov

Peter Weber
Lawrence Livermore National Laboratory
weber21@llnl.gov