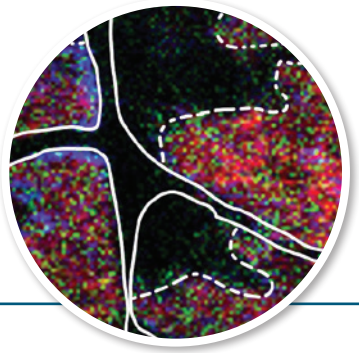

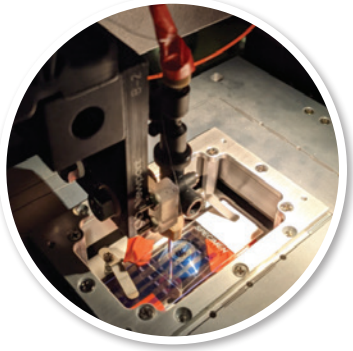

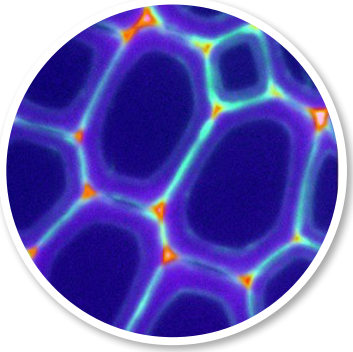

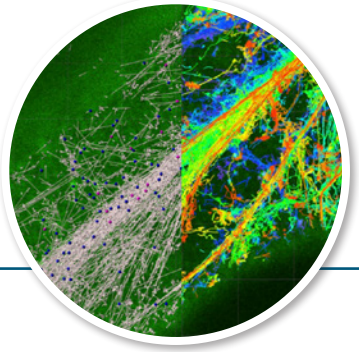

Biomolecular Characterization and Imaging Science

Bioimaging Science Program 2021 Principal Investigator Meeting Proceedings

U.S. DEPARTMENT OF ENERGY | Office of Science

Office of Biological and Environmental Research

June 2021

Biomolecular Characterization and Imaging Science

Bioimaging Science Program
2021 Principal Investigator Meeting

February 22–23, 2021

Program Manager

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Office of Science
U.S. Department of Energy

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About BER

The Biological and Environmental Research (BER) program advances fundamental research and scientific user facilities to support Department of Energy missions in scientific discovery and innovation, energy security, and environmental responsibility. BER seeks to understand 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.

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Preface

As part of the 2021 Biological Systems Science Division (BSSD) Principal Investigator (PI) Meeting, the Bioimaging Science program (BSP), within BSSD's Biomolecular Characterization and Imaging Science portfolio, held its annual PI meeting virtually February 22–23.

BSP's mission 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.

BSP convenes annual PI meetings to bring together its contributing investigators to review progress and current state-of-the-art bioimaging research. Holding the BSP meeting as part of the broader BSSD PI meeting allowed researchers to interact with the extended Genomic Science program community. This convergence provided a platform for networking and exchange of ideas, helping to forge new multidisciplinary collaborations among investigators from the two sister programs.

An important highlight of the BSP meeting was the keynote presentation "Enhancing Fluorescence Microscopy with Computation" by Dr. Hari Shroff of the NIH National Institute of Biomedical Imaging and Bioengineering. All the BSP PIs made presentations describing their research focus and progress, and these were followed by round-table discussions of each project. The meeting's proceedings provide an outline of the program's current state and potential future directions and opportunities.

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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

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 Hancock, The Pennsylvania State University

Time-Resolved 3D Multi-Resolution Microscopy for Real-Time Cellulase Actions *In Situ*
Haw Yang, Princeton University

***In Planta* Multimodal Single-Molecule Imaging to Study Real-Time Turnover Dynamics of Polysaccharides and Associated Carbohydrate Metabolites**
Sang-Hyuk Lee, Rutgers University

Development of Broadband Infrared Nanospectroscopy of Biological Materials in Fluid
Tina Jeoh, University of California–Davis

Inorganic Voltage Nanosensors as Tools for Bioelectricity Studies in DOE-Relevant Bacteria and Their Communities
Shimon Weiss, University of California–Los Angeles

Tracking Lignocellulosic Breakdown by Anaerobic Fungi and Fungal Cellulosomes
Michelle O'Malley, University of California–Santa Barbara

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

Spatiotemporal Dynamics of Photosynthetic Metabolism in Single Cells at Subcellular Resolution
Jeffrey Cameron, University of Colorado–Boulder

Quantum Dot Toolkit for Multimodal Hyperspectral Bioimaging
Prashant Nagpal, University of Colorado–Boulder

Live-Cell, Quantum Dot–Based Tracking of Plant and Microbial Extracellular Vesicles
Jeffrey L. Caplan, University of Delaware

Correlative Imaging of Enzyme and Metabolome Dynamics for Yield and Titer Co-Optimization in Biofuel-Producing Microorganisms
Andreas E. Vasdekis, University of Idaho

Development and Implementation of an *In Situ* High-Resolution Isotopic Microscope for Measuring Metabolic Interactions in Soil Mesocosms
Elizabeth A. Shank, University of Massachusetts Medical School

Expanding the Utility and Range of Quantum and Polymer Dots for Multiplexed Super-Resolution Fluorescence Imaging in Plants
Gary Stacey, University of Missouri–Columbia

Hyperspectral Light-Sheet Raman Imaging of Leaf Metabolism
Keith Lidke, David Hanson, Jerilyn Ann Timlin, and Jamey Young University of New Mexico

Metaoptics-Enabled Multifunctional Imaging
Paul Bohn, Anthony Hoffman, and Joshua Shrout University of Notre Dame

Multiparametric Optical Label-Free Imaging to Analyze Plant Cell Wall Assembly and Metabolism
Marisa S. Otegui and Kevin W. Eliceiri, University of Wisconsin–Madison

National Laboratories

Detecting Chemical Signals in the Soil with 4DMAPS, an Integrated Aptasensor Assembly
Marit Nilsen-Hamilton, Ames Laboratory

A Quantum-Enhanced X-ray Microscope
Sean McSweeney, Brookhaven National Laboratory

Development of a Full-Field X-ray Fluorescence Imaging System for Near Real-Time Trace Element Microanalysis of Complex Biological Systems
Ryan Tappero, Brookhaven National Laboratory

3DQ Microscope: A Novel System Using Entangled Photons to Generate Volumetric Fluorescence and Scattering Images for Bioenergy Applications
Ted A. Laurence, Lawrence Livermore National Laboratory

Illuminating the Rhizosphere: Developing an Adaptive Optics, Multiphoton Microscope for 3D Label-Free Live Imaging of Microbes and Organic Matter in Soil and Roots
Peter K. Weber, Lawrence Livermore National Laboratory

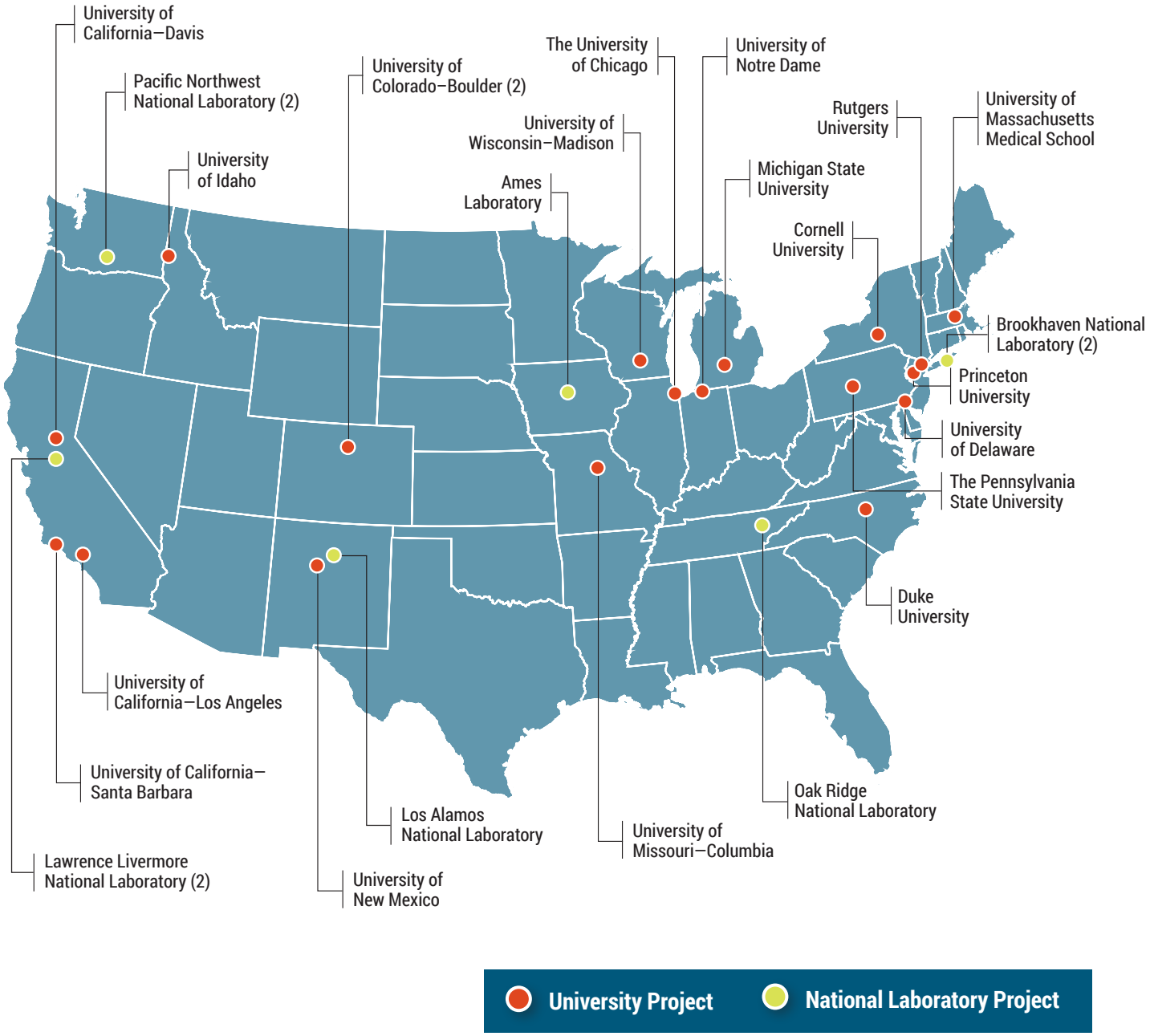
Quantum Ghost Imaging of Water Content and Plant Health with Entangled Photo Pairs
James Werner, Los Alamos National Laboratory

Intrinsically Coregistered Chemical Imaging of Living Plant and Microbial Systems via 3D Nonlinear Optical Mapping and *In Situ*–Liquid Extraction–Mass Spectrometry
John F. Cahill, Oak Ridge National Laboratory

Probing Photoreception with New Quantum-Enabled Imaging
James E. Evans, Pacific Northwest National Laboratory

Multimodal Chemical Imaging Across Scales to Visualize Metabolic Pathways in Live Plants and Microbial Systems
Scott Lea, Pacific Northwest National Laboratory

Project Map


University of California—Davis
Pacific Northwest National Laboratory (2)
University of Idaho
University of Colorado—Boulder (2)
University of Wisconsin—Madison
Ames Laboratory
The University of Chicago
University of Notre Dame
Michigan State University
Rutgers University
University of Massachusetts Medical School
Cornell University
Brookhaven National Laboratory (2)
Princeton University
University of Delaware
The Pennsylvania State University
Duke University
University of California—Los Angeles
University of California—Santa Barbara
Lawrence Livermore National Laboratory (2)
Los Alamos National Laboratory
University of New Mexico
University of Missouri—Columbia
Oak Ridge National Laboratory
University Project
National Laboratory Project

Executive Summary

The U.S. Department of Energy's 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 portfolio of DOE's Office of Biological and Environmental Research (BER)—currently sponsors multidisciplinary research at 9 national laboratories and 19 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, and whole organisms. BSP researchers are developing instruments and imaging systems from the ground up and are enhancing existing capabilities with new or transformational improvements. These novel capabilities, design-based technologies, and improved or innovative uses of established methodologies will enable new fundamental discoveries and provide solutions to challenges in plant and microbial systems biology. These challenges cross a range of scales—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 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.

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 and 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. These techniques include surface-enhanced Raman scattering (SERS), stimulated Raman scattering (SRS), hyperspectral stimulated SRS (hsSRS), and tip-enhanced Raman scattering (TERS), as well as 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 non-invasive 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 national laboratories. These projects encompass state-of-the-art quantum-based techniques such as quantum-enhanced X-ray microscopy, quantum ghost imaging, three-dimensional (3D) quantum microscopy, and quantum-enabled imaging using entangled photons. Individual research programs focused on multidisciplinary projects are complemented by research and development at DOE-sponsored user facilities, which are building and applying various technologies, such as ion microscopy and full-field X-ray fluorescence imaging.

BSP researchers are further enhancing co-application of mass spectrometry and spectrochemical imaging capabilities to yield highly selective, sensitive, and quantitative chemical maps that identify 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.

Researchers also 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,

as well as 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 particular 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 growing microbial and plant systems. Also anticipated are new insights into the fundamental biology of many macro events, such as nutrient utilization and community and ecosystem interactions that include soil water retention caused by the presence or absence of particular organisms or biomass. This comprehensive 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, BSP researchers are increasing imaging throughput rates and resolution of single cells through quantitative phase imaging (QPI) combined with light-sheet fluorescence microscopy-based optical quasi-lattice technology. Dark-field fluorescence-based hyperspectral imaging is enabling the collection of high signal-to-noise images and will allow multiplex collections of multi-fluorophore 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 sub-diffraction, TERS, coherent anti-Stokes (CARS), SRS, SERS, spatially offset Raman spectroscopy (SORS), shifted-excitation Raman difference spectroscopy (SERDS), and cavity-dumped SERS. BSP researchers also have developed a multimodal microscope integrating CARS, SRS, and two-photon excitation systems and 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. 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. Also developed was a wide-field CARS microscope for rapid and simultaneous acquisition of CARS images across an entire field of view.

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 that travel through 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, as well as the detection of riboswitches that act as metabolite reporters using quantitative phase imaging that leverages a light-sheet fluorescence technique 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 resources. The subject organisms include plants, bacteria, fungi, and their combinations. Teams are developing sophisticated instruments to image metabolism in a single organism, gene expression, and regulatory molecules (e.g., microRNAs, quorum-sensing molecules, and protein kinases) that operate in intact 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 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 these specialized agents. BSP-supported researchers are developing instruments that will address the critical need of measuring these features directly, enabling a future in which molecular signatures can be tracked in real time and over 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.

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

The functional dimensional scales in biological systems are vast, spanning molecules to multiorganismal 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 3D tracking with high-speed AFM and optical tweezers to control molecules or microbes, enable force measurements, or 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.

Quantum-Enabled Techniques

BSP has recently added state-of-the-art quantum-enabled bioimaging projects at national laboratories. Quantum-enhanced X-ray microscopy uses entangled X-rays beams. With the ghost imaging technique, samples are illuminated using less-intense beams with energy more suitable for maintaining biological integrity. Furthermore, the quantum nature of the imaging process enables visualization of details impossible to detect with classical methods. BSP researchers also are developing a new microscope using entangled photon

pairs to visualize water, lignocellulose, and lipids in plants. To probe samples, this system uses a wavelength that can be 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.

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.

Moving Toward More Complex Systems

Although many of the initial samples BSP researchers use to test new instruments and methods may be from canonical model systems, the program should continue to evaluate and adapt to real-world biosystems as well. For example, label-free identification of microbes obtained from the environment remains a grand challenge in biology, so extending BSP-developed label-free approaches to such microbes in the long term is a next frontier.

In addition, 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 feedstock yields, enhancing lignocellulosic deconstruction, or boosting feedstock sustainability and plant drought tolerance. Organisms under study include:

- Living plants — *Arabidopsis thaliana*, *Medicago truncatula*, *Brachypodium distachyon*, *Populus* sp., *Pinus taeda*, and *Zea mays*.
- Microbial chemotrophs — *Bacillus subtilis*, *Yarrowia lipolytica,* and *Pantoea* sp.
- Microbial phototrophs — *Cyanothece* sp., *Rhodopseudomonas palustris, Ostreococcus tauri, Chlamydomonas reinhardtii*, 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

Multidisciplinary Research Teams

Biological imaging is inherently transdisciplinary, and successful teams need to continue to reflect this approach to advance BSP programmatic goals. Multidisciplinary 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, nanoscience, computer science, structural biology, biochemistry, plant physiology, microbiology, genomic science, ecology, soil science, and biogeochemistry. This cross-disciplinary approach will be a critical step toward connecting phenotypes with genotypes and translating laboratory-developed technologies into the natural environment.

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 as well as 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.

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 as well as 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) 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.

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 the simultaneous marking, spatially resolved tracking, and sensing of multiple players (e.g., elements, isotopes, enzymes, metabolites, and other molecular biomarkers) in a given 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 in 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 are typically 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 cryo-electron microscopy or nano-secondary 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:

- Integrating bioimaging techniques with advanced probes and delivery mechanisms that expand the monitoring capability for important biotargets ranging from key metabolites to molecular and genomic biomarkers.
- Developing new or improved conventional or quantum-enabled imaging technologies capable of monitoring biological systems in their natural states or as they respond to environmental perturbations and stressors.
- Developing new or improved biosensing and bioimaging approaches that enable real-time data collection across the full range of relevant spatial scales in the laboratory and under field conditions.
- Correlating multimodal dynamic and static "snapshot" imaging methods, both destructive and nondestructive, to provide a holistic understanding of chemical-structural-functional linkages.
- Establishing cross-platform protocols for sample preparation, calibration, indexing and spatial registration, data verification, and correlation to increase the suite of complementary analyses that can be conducted on a given sample or suite of samples.
- Developing methods to increase throughput for more mature imaging technologies that can be used for new applications.

Expanding BSP's Impact and Interactions

Community Access to BSP-Developed Technologies Through User Facilities

User accessibility to new BSP technologies and approaches will be a key factor for the program's success and longevity. Deploying some BSP imaging capabilities 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 injects 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

A new mechanism that allows supplemental funding could foster even more direct cross-fertilization and interaction between the GSP and BSP research communities. The envisioned new class of funding could supplement the 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 funding 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 labora-

tory 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.

Abstracts

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

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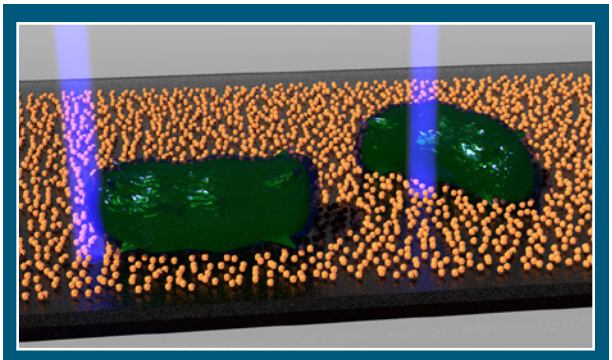
Research Plans and Progress: This project's research aims to combine quantum materials synthesis, bacterial synthetic biology, and multimodal single-entity imaging to quantitatively study how hybrid quantum dot (QD)–bacteria systems convert light to value chemicals at the single- to subcell level, with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

On quantum materials, the project focused on developing semiconductor cadmium sulfide (CdS) thin films on indium tin oxide (ITO) as photosensitizers; for that, CdS's energy gap and redox potential can be tuned by its size and surface chemistry. The project examined partial surface oxidation and ligand-exchange processes and characterized them using spectroscopy and photoelectrochemical measurements. The project also studied using PEDOT:PSS between the ITO electrode and the QD thin film to ensure that photoexcited electrons flow toward the microbe, thus focusing on photoreduction (rather than photooxidation) processes in the QD/microbe hybrids.

On bacterial biology, the team has completed a systematic survey of thermodynamic constraints on electromicrobial conversion of CO_2 and electricity to bioproducts, encompassing microbes that uptake electricity by H_2-oxidation (*Ralstonia eutropha*) and by extracellular electron uptake (*Shewanella oneidensis*). Team members demonstrated that both methods of electron uptake have comparable high maximum conversion efficiencies. This theoretical analysis allowed for building a 10-point roadmap for the development of electromicrobial production technology. The project has also identified genes encoding an electron uptake pathway in *S. oneidensis*. Using a high-throughput screening, researchers discovered 150 genes that affect electron uptake; four of them are indispensable. This set of genes provides a portable electron uptake module, transferrable to highly engineerable microbes to enable electron uptake and power CO_2 fixation.

On multimodal single-entity imaging in the *R. eutropha* chromosome, researchers have tagged the membrane-bound hydrogenase (MBH), the soluble hydrogenase (SH) and/or PhaP1 that decorates the surface of biomass PHB granules, with a (photoactivatable) fluorescent protein. Under H_2/CO_2/air lithoautotrophic growth, researchers determined: (1) the intracellular concentrations of MBH and SH; (2) MBH and SH concentrations both have strong positive correlations with PHB accumulation, with SH having slightly stronger correlation; and (3) biomass accumulation remains unchanged upon deleting MBH but decreases by ~95% upon deleting SH, suggesting SH's role in supplying reducing equivalents toward biomass synthesis.



In this schematic of bacterial cells sitting on a layer of quantum dots (QDs) on top of a transparent electrode, focused laser beams excite local regions on the QD layer. The excited electrons can be donated to the bacteria for subsequent reduction of CO_2 to biomass, in which a photoelectrochemical current is generated. The QDs and the bacterial and photoelectrochemical processes can all be imaged. Courtesy Tobias Hanrath, Cornell University.

Team members further examined the photoelectrochemical current across single semiconductor-cell interfaces for individual *R. eutropha* cells in contact with a semiconductor film. With CdS (n-type), researchers measured single-interface photoelectrochemical currents at anodic conditions to quantify the cells' ability to accept photogenerated holes (i.e., donate electrons). Many cells show enhanced or suppressed photocurrent relative to CdS films alone, suggesting pronounced cell-to-cell heterogeneities and highlighting the need of single-entity experiments. Researchers examined one-on-one correlations between cell-induced photocurrent changes and the characteristics of the associated single cells (e.g.,

cell size/shape, the amounts of hydrogenase/PHB). Team members observed a clear correlation between cell-induced photocurrent changes and cell size. The team also employed Cu_2WS_4 (p-type) and measured single-interface photocurrents at cathodic conditions. Many cells are associated with cathodic photocurrent enhancement, indicating that under this condition most cells exhibit strong electron-accepting capabilities.

Current and/or Anticipated Accomplishments and Deliverables:

1. Image analysis software to find electron uptake genes in *S. oneidensis*: github.com/barstowlab/macroscope-imageanalyzer.
2. Code for calculating electromicrobial production efficiency: github.com/barstowlab/rewiredcarbon.
3. Paper: Salimijazi, F., et al. 2020. "Constraints on the Efficiency of Engineered Electromicrobial Production," *Joule* **4**(10), 2101–30. DOI: 10.1016/j.joule.2020.08.010.
4. Preprint: Rowe, A. R., et al. 2021. "Identification of a Pathway for Electron Uptake in *Shewanella oneidensis*," *bioRxiv*. DOI: 10.1101/2021.01.12.426419.

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 research will transform the study of hybrid inorganic-bacterial systems for energy and chemical conversions. The proposed experiments should break new scientific grounds and open unforeseen opportunities.

Plasmonics-Enhanced Optical Imaging Systems for Bioenergy Research

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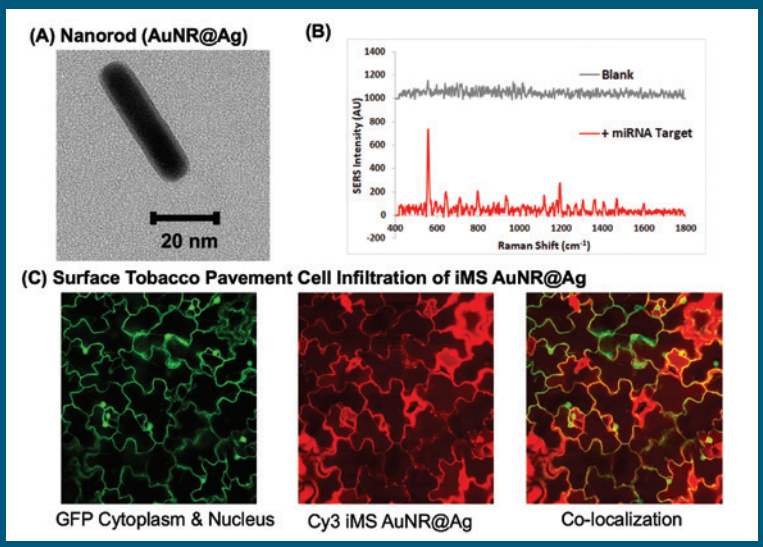
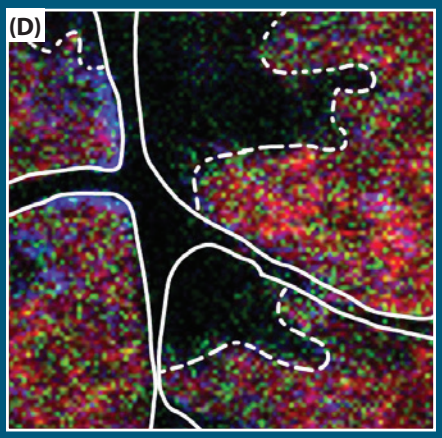
The goal of this project is aimed at addressing the DOE Funding Opportunity Announcement need to develop innovative and improved imaging instrumentation that can enable visualization and quantitative characterization of biomarkers and their dynamic role in cellular functions in living plants relevant to DOE bioenergy programs.

Research Plans and Progress, Including Objectives and Goals for the Project Period: 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 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 inverse molecular sentinels (iMSs) that can be monitored using surface-enhanced Raman scattering (SERS). The team is currently developing innovative imaging technologies for visualization and quantitative characterization of biomarkers related to molecular processes and cellular function within living plants, namely *Multimodal Optical Sensing and Imaging Combinatory (MOSAIC) System*. The advanced MOSAIC system will provide much-needed biofuel research tools such as elucidating the regulation of the pathway to synthesize photosynthetic terpenes more efficiently for biofuel production and tracking pathways of carbon fixation in plants.

Current and/or Anticipated Accomplishments/ Deliverables for the Project Period: The project has developed a strategy for efficient delivery of iMS nanoprobes into plant cells using silver-coated gold nanostars (AuNR@Ag) for SERS sensing. Figure panels A and B show the transmission electron microscopy (TEM) image of AuNR@Ag (A) and the SERS detection of microRNAs (miRNAs) (B) using AuNR@Ag-iMS nanoprobes. Also shown is the confocal imaging coregistration of iMS nanoprobes inside tobacco cells (C).





(A) TEM image of silver-coated gold nanostars (AuNR@Ag). **(B)** SERS spectra of AuNR@Ag-iMS in the presence (bottom spectrum) or absence (top spectrum) of target miRNAs. **(C)** Representative confocal microscopy images of Cy3-labeled AuNR@Ag-iMS infiltrated into tobacco plants expressing GFP fluorophore in the cytoplasm. **(D)** Overlay of gold (Au; green), zinc (Zn; blue), and manganese (Mn; red) distributions in XRF confocal image of 200 μ × 200 μ area of leaf treated with AuNR@Ag. White lines drawn as an aid to identify four leaf cells within the field of view. Dashed white lines drawn to delineate where the confocal plane of the image transitions from inside to outside of the cell. Courtesy Tuan Vo Dinh, Duke University; and Ken Kemner, Argonne National Laboratory.

Researchers demonstrated that AuNR@Ag iMS have a high degree of cellular penetration in the leaf, as they were effectively colocalized with the intracellular green fluorescent protein (GFP) in the pavement cell cytoplasm. The X-ray fluorescence (XRF) confocal image (see figure, panel D) also indicates that AuNR@Ag are located predominantly inside leaf cells. These results confirm that the AuNR@Ag iMS accumulate in the intracellular spaces of cells due to one of their dimensions being smaller than the plant cell wall exclusion limit. The team is currently working on imaging iMS in plant cells using hyperspectral surface-enhanced Raman imaging (HSERI) and shifted-excitation Raman difference spectroscopy (SERDS) that allow sensitive detection of miRNA and mRNA targets in complex background conditions while retaining high spatial, spectral, and temporal resolution within the plants.

Potential Benefits/Applications of DOE-Funded Research for DOE and Dissemination and Deployment of Bioimaging Technology to Public and Private Sector for Generic Biological Imaging Use by the Broader Scientific Community: This project will be applied to research on next-gene ration 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 efficiency and reduce costs. The project's novel tools will greatly facilitate studies on the regulatory mechanism for photosynthetic terpene production in plants.

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

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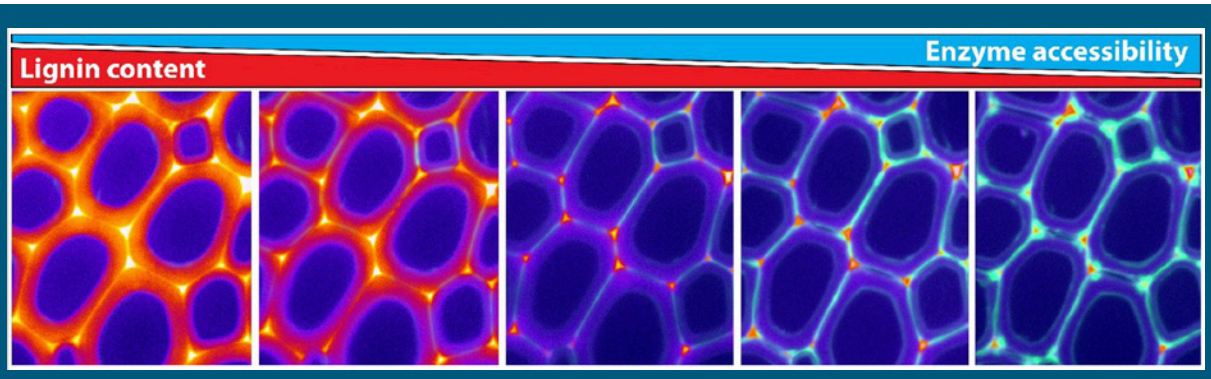
Research Plans and Progress: This project aims to develop a new generation of stimulated Raman scattering (SRS) imaging tools to quantitatively analyze plant cell wall constitutes with high sensitivity. The project is particularly interested in developing a correlative imaging method in addition to the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

Current and/or 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.

Quantify biomass susceptibility to pretreatment chemistry. The project's recent studies (Zhang et al. 2021; Zhang, Ding et al. unpublished data) have demonstrated that the three-dimensional (3D) structure of plant cell walls exhibits different susceptibility to different pretreatment chemistry and temperature. Project researchers found in acid-catalyzed γ-Valerolactone (GVL) pretreatments relatively high lignin removal occurred in the compound middle lamellae (CML) area of the cell walls with HCl-GVL, allowing increased cellulose accessibility and high enzyme digestibility; whereas, H_2SO_4-GVL removed a small amount of lignin in all cell wall layers at 100˚C, resulting in low enzyme digestibility (see figure). These results suggest that fine tuning of the chemistry and severity may further improve the pretreatment efficiency in which advanced imaging techniques could further provide new insights into mechanistic understanding of biomass deconstruction.

Identify modified lignin structure in mutant plants. Lignin is a major impediment in the deconstruction and fractionation of plant biomass to its soluble monomeric constituents for producing biofuels and biomaterials. Modification of the lignin biosynthetic pathway has proven to be an effective means of reducing biomass recalcitrance but can often result in impaired growth. Project researchers used hyperspectral SRS to map the Raman signal—specifically representing engineered lignin and the distribution pattern in cell wall layers. The study suggests that both physical and chemical modification of the cell wall could contribute to the observed improvements in sugar yields. (Shen et al. 2019)

Characterize the fundamental structure of cellulose in native state and cell wall structure in mutant plants with cellulose synthase mutations. Cellulose microfibrils, which form the mechanical framework of the plant cell wall, are synthesized by the cellulose synthase complex in the plasma membrane. A correlative imaging method has been used to characterize native cellulose microfibril



This image shows *in situ* imaging of lignin removal and enzyme accessibility. The same area of poplar cell walls pretreated by HCl-GVL for 5, 15, 30, 45, and 60 minutes (left to right) showing reducing lignin signal (red) and increasing accessibility of cellulose (cyan). Reprinted with permission from Elsevier from Zhang, J., et al. 2021. "Visualizing Plant Cell Wall Changes Proves the Superiority of Hydrochloric Acid Over Sulfuric Acid Catalyzed γ-Valerolactone Pretreatment," *Chemical Engineering Journal* **412**(15), 128660. DOI: 10.1016/j.cej.2021.128660.

structure (Song et al. 2020), and cellulose synthase mutants *in vivo.* (Park et al. 2019; Park and Ding 2020)

Potential Benefits and Applications: It has long been known that multiple polysaccharidases and accessary enzymes are required to effectively deconstruct pre-treated lignocellulosic biomass. However, many studies on enzyme hydrolysis have been conducted based on individual enzymes or specific enzyme mixture with specific pretreatment methods. The challenge remains to build a predictable model of enzyme kinetics that can explain the dynamics of the complex cell wall structure and the synergistic reactions of multiple enzymes. The project's development in advanced imaging techniques has enabled 3D in-depth mapping and quantification of the physicochemical properties of cell wall constituents in their native state and during enzymatic hydrolysis, a process which will be correlated with enzymatic activities by tracking cellulases on biomass *in situ*. The team has tackled the early stage of this research and will continue in a collaborative effort to understand the molecular mechanism of synergistic reactions among cellulases, hemicellulases, and accessary enzymes (Ding and Bayer 2020).

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Single-Molecule Imaging of Lignocellulose Deconstruction by SCATTIRSTORM Microscopy

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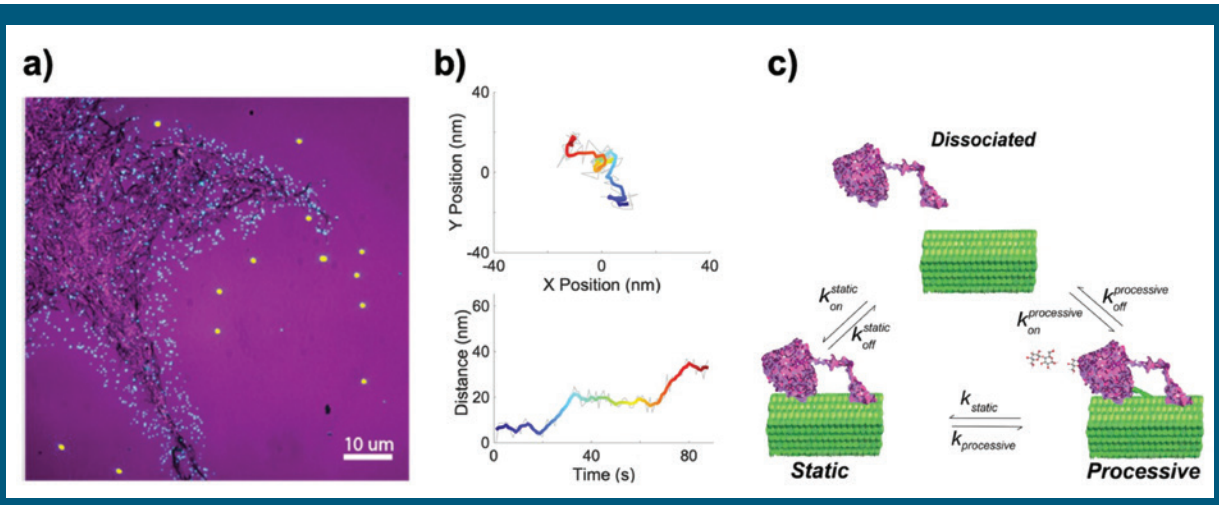
Research Plans and Progress: The goal of this project is to build a multimodal optical microscope to measure the binding, processive degradation, and pausing behaviors of cellulases as they interact with and degrade both synthetic and naturally occurring lignocellulosic walls. To achieve this, the project is using high spatiotemporal single-molecule imaging to track cellulases while visualizing specific molecular components of cellulose, lignin, and hemicellulose, all of which make up their lignocellulose substrate. The initially proposed microscope combines interferometric scattering (iSCAT), which provides unprecedented spatiotemporal resolution; total internal reflection fluorescence (TIRF), which provides single-molecule resolution of multiple fluorophore-labeled molecules; and stochastic optical reconstruction microscopy (STORM), which allows for three-dimensional super-resolution imaging of intact plant cell walls during degradation. The team has constructed the microscope and demonstrated high stability and spatiotemporal resolution for TIRF. The team has implemented interference reflection microscopy (IRM), which is an alternate strategy from iSCAT that allows for a larger field of view. Researchers have discovered that cellulose can be visualized label free with high spatiotemporal resolution using IRM, and that IRM and TIRF can be used together to simultaneously image cellulose immobilized on a glass coverslip and quantum dot (QD)–labeled Cel7a enzymes binding and moving along this cellulose substrate (see figure, panel a).

Current/Anticipated Accomplishments and Deliverables for Project Period: The microscope has been built and calibrated, and it is being used for single-molecule experiments. The team has incorporated both z-focus drift correction by active feedback, and x-y drift correction by using Tetraspeck fiduciary markers stuck to the glass coverslip. By stepping the piezo stage in known increments and using point-spread-function fitting, the team has established that the standard deviation of measured position is 1.5 nm. The project has collected 1,000-second movies at 1 frame/s of 11,117 QD-labeled Cel7a molecules binding and moving along bacterial cellulose. Many static binding events (<10 nm displacement) were observed, with exponentially distributed durations indicative of a first-order off-rate; the mean duration of these static events was 98.9 seconds. Processive movement was observed with a mean run length of 39 nm



(a) Superimposed image of cellulose imaged by interference reflection microscopy and quantum dot-labeled Cel7a imaged by total internal reflection microscopy. Yellow spots are TetraSpeck fiduciary markers. (**b)** Single-molecule tracking with nanometer-scale resolution showing x-y position (top) with color-coded time and distance versus time (bottom). (**c)** Three-state model of Cel7a binding and moving along cellulose showing rate constants that can be measured by single-molecule microscopy. Reprinted under a Creative Commons license (CC BY 4.0) from Haviland, Z. K., et al. 2021. "Nanoscale Dynamics of Cellulase TrCel7A Digesting Cellulose," *bioRxiv*. (Preprint) DOI: 10.1101/2021.02.18.431891.

at a velocity of 3.2 +/- 2.7 nm/s (mean +/- SD, N = 1,112 segments) (see figure, panel b). These processive runs were interspersed by static segments of similar duration as the wholly static binding events. Enzyme molecules landed into either a processive or a static state, and processive runs were terminated by either dissociation or entering a static state. Although transient jumps were observed (>10 nm displacement within two frames), two-dimensional diffusion of enzymes on the surface of the cellulose was not observed, even for binding events of hundreds of seconds. From these measurements, the team constructed a three-state model (see figure, panel c) in which Cel7a can interact with cellulose either in a static state or processive state, and the molecule switches reversibly between these two states and can bind and unbind from either state. In this formulation, the rate-limiting step that limits turnover by the enzyme is transition out of the static state, either by dissociating from the cellulose or by entering a processive state. These experiments are ongoing and are expanding to different cellulose substrates and different cellulases.

Potential Benefits/Applications for DOE and Other Research: There is scant single-molecule data of cellulases degrading cellulose substrates and increasing the temporal and spatial resolution of cellulase dynamics will greatly enhance understanding of the basic mechanism of enzymatic cellulose degradation. The project's microscope will apply cutting-edge microscopy tools developed in the motor protein and related fields to the plant biology and bioenergy communities.

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

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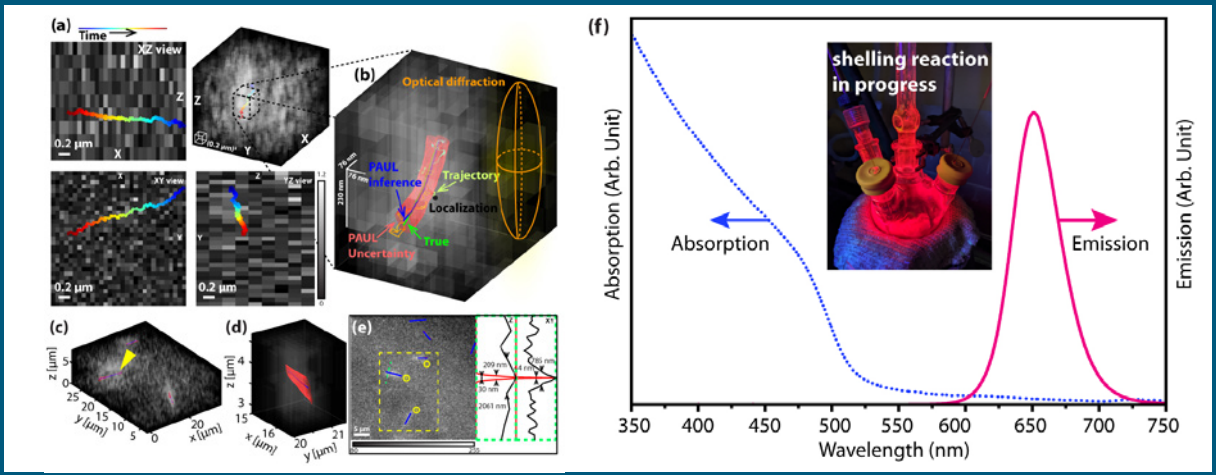
Research Plans and Progress, including Objectives and Goals for the Project Period: This project aims to build time-resolved 3D multi-resolution microscopy (TR-3DMRM) to observe real-time actions of cellulase to gain better insights into their processivity during catalytic activities. The plan involves two major components. One is to build the two-submodule microscope, 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). The other is to develop suitable protocols and assays for the application of 3DMRM on a cellulose-cellulase system. This involves cellulase and substrate development characterization, single-molecule assay for TR-3DMRM and 3DMRM optimization and assay. Progress has been made on both the instrumentation and the assay development components. The Snee Group has developed new, faster syntheses for a spectrum of nonblinking bright quantum dots (QDs) for this purpose and is currently finalizing characterization for publication. On the instrument side,



This image shows 3D super line-localization by prior-apprised unsupervised learning (3D-PAUL) and new customizable quantum dot (QD) synthesis. **(a)** Simulated noisy microscope image of a curvilinear structure overlaid with a high-localization-precision trajectory of a moving particle (color coded in time). The three 2D images are maximum intensity projections (MIPs) along each direction, where pixel values are normalized to [0, 1] and displayed in [0, 1.2] for better visualization. A 3D view is shown with each voxel represented as a translucent cuboid. **(b)** Zoom-in of the 3D image, showing the end result of PAUL inference (blue) and uncertainty (pink), together with the true curve used in the simulation (green). Also shown for scale comparison are the size of the optical diffraction (yellow, plotted as full width at half maximum), 3DSPT localization (black), and the size of a voxel (76 × 76 × 230 nm, white). Experimental demonstration (c–d). **(c)** 3D view of a region of interest obtained by multifocal microscopy where each voxel is rendered as a translucent cuboid in real dimensions. **(d)** Zoom-in of the region indicated by arrow in (c), showing the PAUL-inferred centerline and the bounding curves. **(e)** MIP of the raw image, with PAUL results overlaid (blue: PAUL-inferred central position; red: PAUL uncertainty). A subimage size of 32 × 32 × 9 within a total 16 division schemes is used in PAUL analysis. Yellow box is a region of interest (ROI) that contains three visible features labeled as A, B, and C. Along the annotated dashed green line and vertically at the crossing between the green line and the detected curve, image profile cross sections are plotted (black), together with the PAUL-inference uncertainty (red) and the true object dimension (purple dot). **(f)** A set of representative absorption and emission spectra of wavelength and luminescence lifetime tunable non-blinking QDs. Panels a–e: Adapted from Yin, S., et al. 2020. "Three-Dimensional Super Line-Localization in Low Signal-to-Noise Microscope Images via Prior-Apprised Unsupervised Learning (PAUL)," *Proceedings SPIE, Applications of Digital Image Processing XLIII* 11510, 115101L. Panel f: Courtesy Preston Snee.

Yang Lab has completed the TG-RT3DSPT instrumentation and is in the process of finishing up data acquisition for proof-of-principle datasets for publication. As soon as the manuscript is submitted, construction of 2PLSM-FLIM (Year-2/Q1) will be immediately carried out and integrated with TGRT3DSPT (Year-2/Q2). On the assay side, Tien Lab has generated dye-labeled cellulases from commercial sources as well as high-quality cellulose fibers. Yang Lab has also completed development of a novel super line-localization method based on machine statistical learning for both 2D and 3D line structures (two papers published, see figure, left panel). This capability is critical for resolution matching between two 3DMRM modules (e.g., placing the cellulose line images on the same resolution footing as the 10-nm tracked cellulases).

Current and/or Anticipated Accomplishments/Deliverables for the Project Period: Currently, the Snee Group has developed a new, faster synthesis of "giant" CdSe/CdS as well as type-2 CdZnSe/CdS quantum dots and is currently examining similar chemistry for other systems. Importantly, type-2 gQDs will enable optimal TG-RT3DSPT performance due to the nanomaterial's record-breaking 100s ns to ms excited state lifetimes (see figure, right panel). Tien Lab has generated dye-labeled cellulases that will be used by Snee Lab to construct cellulase-gQD 1-1 conjugates. Tien and Snee Labs will then work together to develop assays using such 1-1 conjugates. Construction of the TG-RT3DSPT is complete, and a manuscript is being prepared. Tien Lab has focused most recent efforts on substrate development and characterization. In addition to isolation of cellulose from *Cladophora*, bacterial cellulose has been isolated from *Gluconoacetobacter hansanii*. Comparison of this preparation with *Cladophora* and Avicell shows that cellulase activity, as measured by cellobiose production is much higher. The team has also characterized the cellulose in regard to concentration of reducing enzymes and degree of polymerization.

Potential Benefits/Applications of DOE-Funded Research for DOE and Dissemination and Deployment of Bioimaging Technology to Public and Private Sector for Generic Biological Imaging Use by the Broader Scientific Community: 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 multimodel 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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In Planta Multimodal Single-Molecule Imaging to Study Real-Time Turnover Dynamics of Polysaccharides and Associated Carbohydrate Metabolites

Principal Investigators: Sang-Hyuk Lee[1] (PI), Shishir Chundawat,[1] Eric Lam,[1] and Matthew Lang[2]

Collaborators: Wellington Muchero,[3] Sai Venkatesh Pingali,[3] and Laura Fabris[1]

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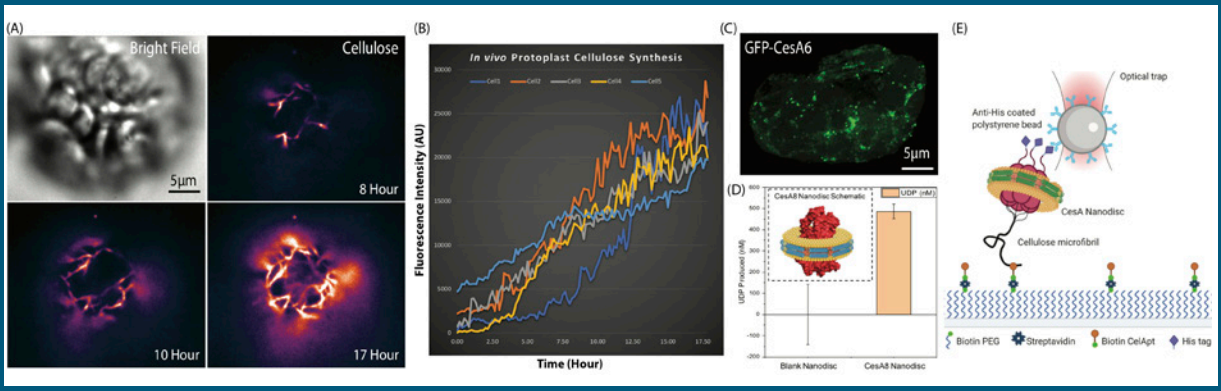
Research Plans and Progress: This project aims to study plant cell wall polysaccharide synthesis by applying multimodal single-molecule manipulation/imaging techniques to protoplast cells. The team plans to conduct: (1) optical tweezers-based force spectroscopy of cellulose synthesis by cellulose synthase complex (CSC) both *in vitro* and *in vivo*; (2) real-time single-particle tracking of CSC; and (3) SERS sensing of sugar metabolites using plasmonic gold nanostars (GNS) attached to CSC.

Current and/or Anticipated Accomplishments/Deliverables: First, the project has succeeded in real-time visualization of *in vivo* cellulose fiber network growth/development on a live protoplast's membrane surface at high resolution continuously for a full day using total internal reflection fluorescence (TIRF) microscopy (see figure, panels A–B). This was made possible by establishing a workflow with *Arabidopsis*/poplar protoplasts isolation, cell wall regeneration with minimal manipulation steps, *in situ* cellulose labeling with CBM-Alexa dyes, and integration of environmental condition (lighting and temperature) control into the microscopy platform. Second, the project has established methods to fluorescently label CSC proteins inside protoplasts via transient gene expression techniques. The team has successfully labeled CSC enzymes with green fluorescent protein (GFP) and imaged them inside plant protoplasts (see figure, panel C). The team is currently optimizing the condition that is compatible with both cell wall regeneration and CSC-GFP expression in order to image real-time dynamics of CSC and cellulose on protoplast membrane. Third, the project has succeeded in purifying single CSC enzymes as nanodiscs for *in vitro* characterization of single cellulose synthase enzyme complexes (see figure, panel D). Single-molecule optical tweezers assay with CSC nanodisc is currently under development (see figure, panel E). Fourth, a new LabView software has been developed for control of multimodal microscopy instruments. The modular and field-programmable gate array (FPGA)-based architecture of this software will enable ultrafast and robust integration of multimodal instrument controls and data acquisition schemes while allowing easy adaptation to future changes or upgrades of the instrument. Fifth, the team has conducted extensive and systematic numerical study of GNS that elucidates the complex relationship between GNS geometry and SERS performance. The



(A, B) Live *Arabidopsis thaliana* protoplast cell wall cellulose fibers synthesis acquired using Total Internal Reflection Fluorescence (TIRF) microscopy. **(C)** Confocal microscopy z-stack image shows GFP-CesA6 fluorescent puncta in protoplast cells. **(D)** *In vitro* cellulose synthesis activity (i.e., UDP formed from UDP-glucose) by reconstituted CesA8 protein nanodiscs. **(E)** *In vitro* optical tweezers assay schematic under development to test CesA8 complex. Courtesy Sang-Hyuk Lee, Shishir Chundawat, and Matthew Lang.

project will ultimately combine all these accomplishments to perform multimodal hybrid imaging assays that will reveal *in vivo* CSC dynamics, cellulose synthesis, and cell wall development on protoplast membrane.

Potential Benefits/Applications: Project research will reveal *in vivo* plant cell wall polysaccharide synthesis processes with unprecedented molecular-level detail through the concurrent characterization of dynamics and function of a single enzyme complex as well as intracellular metabolite flux. The results from this project will greatly advance the mechanistic and holistic understanding of *in vivo* cell wall synthesis, which will accelerate the development of better transgenic crops for bioenergy-related applications.

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

Principal Investigators: Tina Jeoh[1] (PI) and Hoi-Ying Holman[2]

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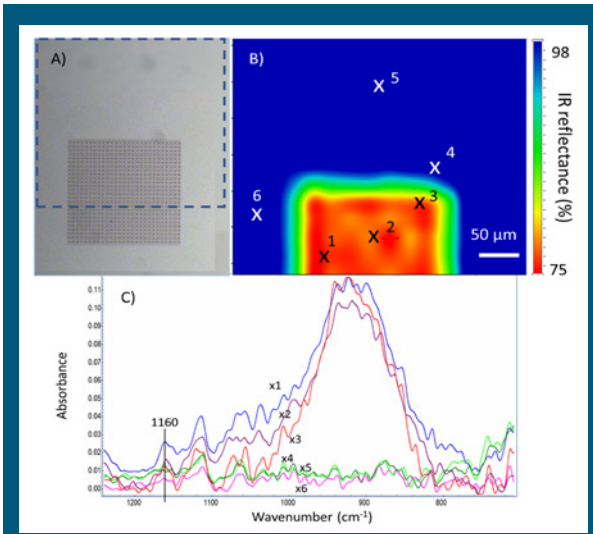
Research Plans and Progress: This research aims to develop a label-free, nanometer-scale and time-resolved imaging technique to study surface reactions in aqueous biological reactions. State-of-the-art nanoscale imaging (i.e. nano-Fourier transform infrared; nano-FTIR) can map topography and chemical composition at nanometer spatial resolution, but it is limited to dry samples. This project is overcoming limitations of near-field energy delivery and extensive background scattering in aqueous samples by integrating plasmonic infrared nanofocusing gratings with microfluidics. This technique is being applied to nanoscale mapping of cellulose surface fibrils undergoing cellulase hydrolysis toward solving the mechanisms of cellulose hydrolysis.

Despite weathering considerable challenges this year due to the ongoing pandemic, the team has made exciting progress in achieving mid-infrared (IR) signal enhancement of sparsely deposited cellulose samples on newly designed and prototyped light-trapping nanophotonic gratings (see figure). In the previous year, the project demonstrated far-field IR imaging of wet cellulose in a novel humidity-controlled microfluidics device (MFD). Ongoing work integrates the plasmonic surfaces with the MFD to achieve the goal of enabling IR nanospectroscopy of wet biological samples.

Accomplishments/Deliverables: A prototype of the mid-IR plasmonic gratings with a highly dilute sample of cellulose nanofibrils deposited on the surface is shown in figure panel A. The cellulose nanofibrils, with dimensions of ~5 nm (thickness) and 10 µm (length) is optically transparent and not visible in figure panel A. In regions outside the plasmonic structures, the presence of cellulose is undetectable from the FTIR spectra (see figure, panel C, x4, x5, and x6). In regions within the plasmonic gratings (x1, x2, and x3), however, considerable improvement in the signal-to-noise ratio (SNR) enhances characteristic peaks of the cellulose backbone (e.g. 1,160 cm^{-1}, 1,060 cm^{-1}, and 1,035 cm^{-1}). Near-field imaging of this sample in the nano-IR system is ongoing to validate the presence of cellulose nanofibrils and to test the near-field SNR enhancement.



Signal enhancement of cellulose fibrils in the 900 to 1,200 cm^{-1} wavenumber region deposited on light-trapping nanophotonics plasmonic gratings structured onto the imaging surface. **(A)** optical image of the sample surface showing regions with and without plasmonic structures; **(B)** total infrared reflectance signal in the region corresponding to the dashed box in (A); **(C)** FTIR spectra obtained from the corresponding locations in (B). The peak at 1,160 cm^{-1} corresponds to absorption by the glycosidic bonds of cellulose. Courtesy Hoi-Ying Holman and Xiangchao (Jude) Zhu.

Potential Benefits/Applications: Successful integration of the plasmonic surfaces with microfluidics will enable both far- and near-field IR imaging of wet biological samples.

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

Principal Investigator: Shimon Weiss[1]

Collaborators: Robert P. Gunsalus,[1] Robert Clubb,[1] and Evan Miller[2]

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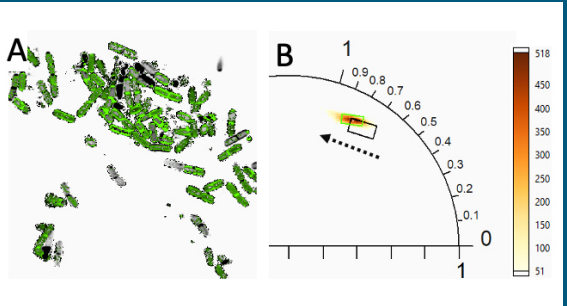
Research Plan: To make headway in quantitatively understanding microbial metabolism and how cells communicate within communities, there is a need for improved tools that are able to map their bioelectrical circuits. Existing tools for reading resting membrane potential (Δψ) in bacterial cells and bacterial communities are limited. Engineered quantum dots (QDs) and nanorods (NRs) display a large quantum-confined Stark effect (QCSE) at room temperature that is observable on the single-particle level. Such nanoparticles could be used as efficient nanoscale membrane potential nanosensors (MPNs) by monitoring changes in quantum yield, lifetime, and/or emission spectrum in response to Δψ changes.

Progress: (1) The team is continuing work on MPN synthesis and functionalization methods for delivery and targeting to bacterial membranes. (2) In parallel, the team has scaled up the production of BeRST, a silicon-rhodamine voltage-sensitive fluorophore, and explored synthetic routes for modified carbo-rhodamines, which show promising monoexponential decays in fluorescence lifetime measurements. (3) The project initiated a limited pilot study to isolate and culture several previously undescribed microbial strains of environmentally relevant origin for future tests with fluorescent dye delivery and monitoring to mimic *in situ* environmental sites. Several cell types were able to utilize cellulose derived from freshwater and marine plant types that can thrive under conditions of lower temperature and osmolarity—properties not typical of currently targeted model organisms including *Escherichia coli*, *Bacillus subtilis,* and the common cellulose-degrading microbe *Clostridium thermocellum*. (4) In collaboration with École Polytechnique Fédérale de Lausanne (EPFL), the team continues to develop detectors and methodology for widefield imaging of Δψ in bacterial communities.

Current and/or Anticipated Accomplishments and Deliverables: Preliminary studies of *B. subtilis* and *E. coli* staining with BeRST and other non-Nernstian dyes have shown that gram-positive bacteria are the easier ones to use for starting work. Perturbation of the membrane potential of *B. subtilis* by carbonyl cyanide m-chlorophenylhydrazone (CCCP), an ionophore that is readily detected by fluorescence lifetime analysis using the phasor approach, (as illustrated in the figure) while intensity measurements remain difficult to interpret. This result motivates the project's pursuit of more efficient approaches to fluorescence lifetime imaging microscopy (FLIM) data acquisition and analysis. On the first front, the team will perform preliminary FLIM measurements with a microlens-equipped single-photon avalanche diode camera (SwissSPAD2) and a moderate-power pulsed laser. The next step will be to extend these measurements to the more sensitive SwissSPAD3 detector and, hopefully, a more powerful laser. To speed acquisition, as few as possible time-gated images are needed, and thus part of the team's effort has been invested in developing the theory for phasor analysis of sparse data. The development of these assets will allow researchers to confidently tackle the study of biofilms in combination with temperature-controlled microfluidics for better control of cellular environment and long-term monitoring.

Potential Benefits and Applications: The tools developed during this project could be used to learn how bacteria interact with one another within DOE-relevant communities such as rhizospheres, soil, syntrophic assemblages, microbial fuels, and consortia that dismantle complex polysaccharides, among others.



CCCP effect on *B. subtilis* membrane potential: **(A)** Cells labeled with BeRST exposed to a puff of CCCP show no noticeable membrane staining change but a marked fluorescence lifetime change as shown in the phasor plot in B. **(B)** The green ROI in the phasor plot corresponds to the green-colored pixel in A, (i.e., bacterial cell membrane). The black rectangle represents the region of the plot where the cells' phasors were located prior to addition of CCCP. The shift from the black rectangle to the green rectangle represents voltage-dependent change in BeRST lifetime. Courtesy Yung Kuo, Xavier Michalet, Robert P. Gunsalus, Robert Clubb, Evan Miller, and Shimon Weiss.

Tracking Lignocellulosic Breakdown by Anaerobic Fungi and Fungal Cellulosomes

Principal Investigators: Michelle O'Malley[1] (PI) and James Evans[2] (Co-PI)
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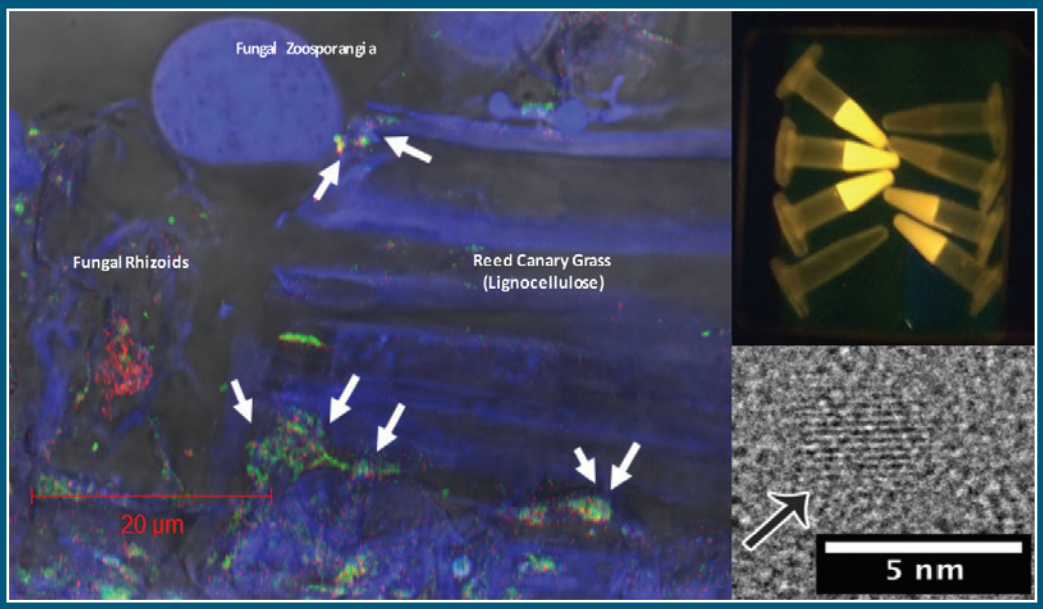
Research Plans, Progress, and Objectives: Anaerobic fungi degrade plant biomass through invasive, filamentous growth and the secretion of multiprotein biomass-degrading complexes called fungal cellulosomes. Despite their potential for lignocellulosic bioprocessing, no nondestructive, real-time imaging tools exist to probe anaerobic fungi or the action of their cellulosomes across spatial and temporal scales. The project will develop 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. This project's approach benefits from a suite of new genomic, transcriptomic, and proteomic data obtained for multiple strains of anaerobic fungi, which 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 cryo-electron microscopy (cryo-EM). As a complementary approach, team members will advance genetic tools for the anaerobic fungi to conjugate QD probes onto cellulosome components *in vivo*, further enabling hypothesis testing of protein function in genetically recalcitrant anaerobic systems.

Current and/or Anticipated Accomplishments/Deliverables for the Project Period: Despite COVID-related research shutdowns, the team was able to successfully generate four nanobodies raised against isolated



(Left) Immunofluorescence of the anaerobic fungus *Piromyces finnis* colonizing reed canary grass, where blue indicates DNA staining, red indicates staining of an anti-ScaA (scaffoldin) antibody, and green indicates staining of a dockerin-containing anti-GH48 antibody. Colocalization of the scaffoldin and dockerin (arrows) is seen near the grass, indicating fungal cellulosome degradation. **(Top right)** High-resolution transmission electron microscopy (HRTEM) image of a functionalized gold nmQD grown in the Evans Lab showing an overall core diameter of around 3 nm. **(Bottom right)** Example series showing the effect of various functionalizations on overall nmQD emission intensity following exposure to 405-nm light. Courtesy James Evans, Will Chrisler, and Stephen Lillington.

cellulosomes. The team now has those nanobodies expressed from both cell-based and cell-free sources and has purified both fluorescent and nonfluorescent nanobodies. The team is performing biochemical and structural characterization of these nanobodies to confirm the epitope recognition and specificity while also working toward cell-free expression of individual components of the larger fungal cellulosome complex and structural work on those components and the natively isolated intact cellulosome complex. The team plans to leverage these developments along with the new ability to transform fungal zoospores and begin imaging the localization of native cellulosome *in situ* using multimodal optical approaches.

Potential Benefits/Applications of DOE-Funded Research: 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.

Understanding Plant Signaling via Innovations in Probe Delivery and Imaging

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Research Plans and Progress, Including Objectives and Goals for the Project Period: The team is (1) optimizing nanofiber arrays to deliver signaling probes/biomolecules to plant cells and (2) building a robotic fiber optic microscope and image analysis platform that enables iterative, nondestructive measurements to be made and compared. These tools are being developed together with imaging experiments aimed at understanding receptor-mediated peptide trafficking and responses relevant to plant cell growth and longevity. The goals for the project period are (a) further optimize the microscope; (b) improve and test different nanofiber array designs for delivering probes to plants; (c) use fluorescent peptide probes based on the growth-regulating peptide phytosulfokine (PSK) in uptake and mobility tests; (d) construct transgenic plants to facilitate study of PSK trafficking and response mechanisms; and (e) determine and validate the transcriptional changes due to PSK-induced signaling related to growth versus longevity. The pandemic caused a delay in optimizing the microscope that will be addressed in the next project period.

Current and/or Anticipated Accomplishments/Deliverables for the Project Period:

Microscope. The project has imaged a cell-impermeable fluorescent dye introduced to a *Populus deltoides* leaf using the nanofiber arrays, as well as a green fluorescent protein (GFP)-tagged reporter in a transgenic *Arabidopsis thaliana* plant, at subcellular resolution (<5 µm). Ongoing efforts in image processing should improve the quality of microscope images. These initial experiments demonstrate the ability to introduce probes into a live plant and image the signal nondestructively with a microscope. They set the stage for imaging biologically relevant biomolecules. The team designed and is constructing an optical mount that will allow (1) secure but repositionable attachment of the imaging fiber; (2) precise and repeatable X-Y positioning of the sample probe; and (3) precise Z translation to achieve ~1 µm positional control and will maintain stable focus on the specimen. This stage will subsequently be equipped with a leaf clip and is a prelude to robotic control.

Nanofibers. The team successfully used vertically aligned carbon nanofiber arrays to deliver and get expression of DNA constructs with reporter fusions in various plant tissues. Team members are working on a device to semiautomate nanofiber use in plant tissue. Results on the versatility of the nanofiber arrays for DNA and probe delivery to different plant tissues are being prepared for publication.

Biological Materials/Deliverables. The project's designed active and inactive fluorescent versions of PSK work as expected. Project research has shown that PSK is internalized into cells. The team has constructed the needed crosses of receptor-GFP plants into different backgrounds (mutants lacking receptors or the ability to produce active PSK) for trafficking studies. The team performed a time series transcriptomic analysis of root and shoot responses to PSK and identified the tissue-specific pathways impacted by this hormone. Researchers will build new reporters, based on findings, to use with the microscope. The project performed a detailed developmental analysis of plants that cannot make or respond to PSK and quantified developmental and physiological phenotypes.

Potential Benefits/Applications of DOE-Funded Research for DOE and Dissemination and Deployment of Bioimaging Technology to Public and Private Sector for Generic Biological Imaging use by the Broader Scientific Community:

1. A major advance will be 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 probes within plants, and realizing the ability to track their movement nondestructively.
2. 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.

Spatiotemporal Dynamics of Photosynthetic Metabolism in Single Cells at Subcellular Resolution

Principal Investigators: Jeffrey Cameron (PI) and Ivan Smalyukh (Co-PI)
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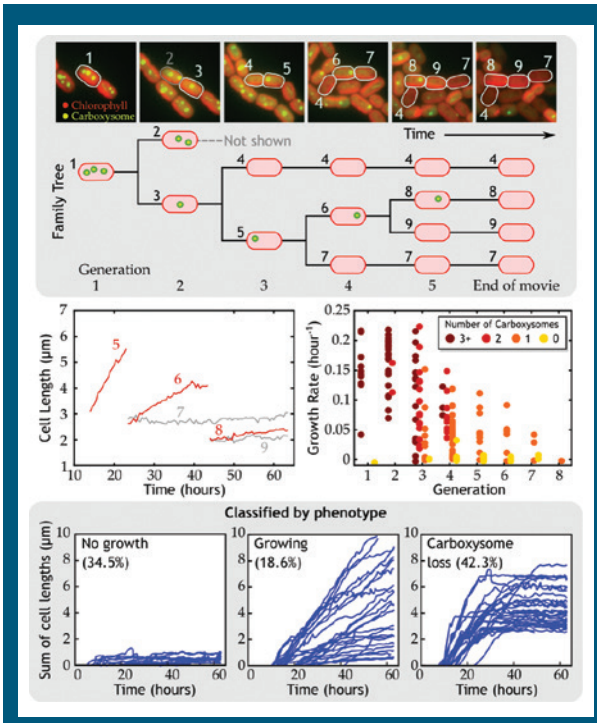
Research Plans and Progress: The project's objective is to design and build a multimodal nanoscopy system to generate adaptive 3D images with high-resolution, real-time, dynamic, label-free chemical imaging of metabolic processes in photosynthetic organisms. Various strains of cyanobacteria will be utilized for system benchmarking and calibrating of resolution and sensitivity. The project will then apply the system to generate a dynamic spatio-temporal map of photosynthetic metabolism with a focus on tracking CO_2 fixation and conversion into biomass. The project is currently on track to complete each its milestones.

Current and Anticipated Accomplishments and Deliverables: The team has completed the initial design/build phase of the project and is now integrating custom imaging modalities into a single multimodal imaging platform. The team has developed a robust sample preparation pipeline that enables the reproducible growth of single-cell derived microcolonies and tracking of individual carboxysomes, protein organelles essential for carbon-fixation in cyanobacteria (see figure). This enables multigenerational tracking of the position and activity of single carboxysomes and has revealed a subpopulation of ultraproductive carboxysomes and that inactive carboxysomes are degraded (Hill et al. 2020).

Potential Benefits/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 and optical nanomanipulation while studying actively growing DOE-relevant biological systems. This study will provide mechanistic insights on the subcellular location and regulation of photosynthetic pathways and identify potential opportunities to engineer and improve these pathways for the production of food, fuel, and other high-value chemicals that will benefit society and the environment. 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, the team has provided the foundation and molecular understandings for engineering more active and stable photosynthetic systems (Hill et al. 2020; Dahlgren et al. 2021; and Hurley et al. 2021).



The carbon-fixing activity of individual carboxysomes can be measured in single-cell lineages of actively growing cyanobacteria using time-lapse microscopy. Individual carboxysomes can be tracked over multiple generations and correlated with single-cell growth kinetics over time to generate population-level data at single-cell and single-organelle resolution. Courtesy Jian W. Tay, Nicholas C. Hill, and Jeffrey C. Cameron.

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Quantum Dot Toolkit for Multimodal Hyperspectral Bioimaging

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A long-standing goal toward advanced understanding of plant and microbial systems for bioenergy applications involves characterization and quantification of multiple complex biological processes *in vivo*. Such quantification includes determining the specific copy number and function of enzymes, tracking metabolic pathways, monitoring the associated transport of materials (metabolites) within cells or across cellular membranes, and specifically activating the selected pathways with desired flux. Using the proposed quantum dot (QD) toolkit in uncovering molecular biology of interest using *in vivo* high-throughput, nondestructive, real-time tracking of subcellular components in living cells requires multiple simultaneous modes (multimodal) of imaging. In the last progress update, the project had shown: (1) QD toolkit with more than 100 spectrally distinct, chemically functionalized, biocompatible QDs ranging from a 400–750 nm wavelength using mono, binary, ternary, and quaternary QDs; (2) a range of spectrally tunable filters, easily tunable and programmed, generated hundreds of spectrally distinct filters in a narrow spectral range *reproducibly* for high-throughput, real-time, hyperspectral imaging; (3) progress in conducting several single-cell imaging and quantification of subcellular processes; and (4) first demonstration of "nanobugs" for light-mediated metabolic processes in different DOE-relevant bacteria using size-dependent redox chemistry and metabolic manipulation with different-sized gold nanoclusters.

In this period, the project will present new results showing: (1) expansion of the optical window of bioimaging to near-infrared (800–1,500 nm) to exploit windows of optical transparency I and II using different-sized gold nanoclusters to enable deep imaging modalities for thick plants and rhizosphere imaging; (2) formation of FASTmers, or coupled peptide nucleic acid (PNA)-QD biohybrids, which enable specificity of attachment to targeted macromolecules and metabolites (sequence-specific attachment with minimal off-targeting); (3) imaging and regulation of transcriptional inhibition and activation using high-throughput FAST platform for bioinformatic design of specific sequences, rapid synthesis of designed PNAs and peptides, and attachment to desired QDs (using modularity and integration of both components); (4) testing of these FASTmers (biohybrid PNA-QD molecules) in PCC 7002 and other genetically intractable DOE-relevant microbes (aerobic and anaerobic) to show desired gene expression modulation and tracking; and (5) first demonstration of light-directed modulation of bacterial metabolism using QDs to show desired light-modulated removal of metabolic bottlenecks (light control of metabolic cycles in nonphotosynthetic bacteria).

These demonstrations can pave the way for expanding these studies to other microbial and plant systems for bioenergy production using the proposed QD toolkit and multimodal imaging developed here.

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

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

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

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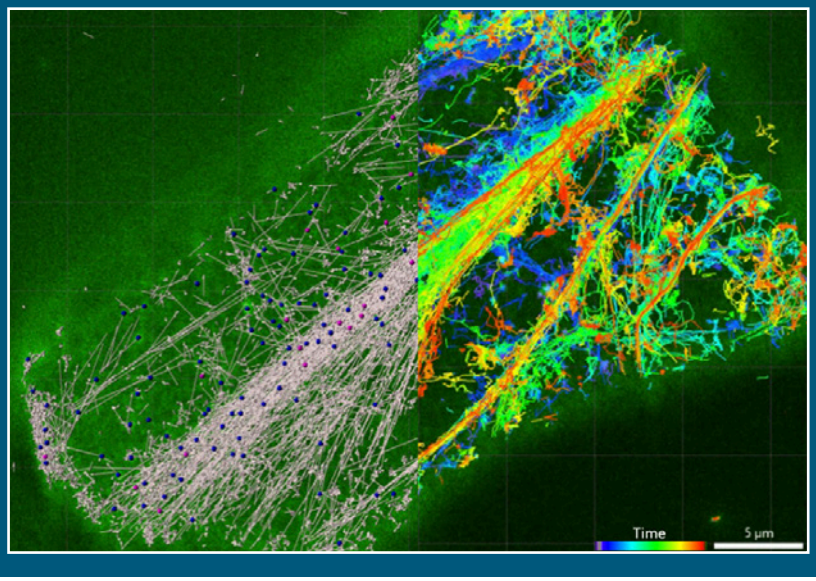
Research Plans and Progress: The project aims to develop new quantum dot (QD)-enabled methods for studying the role of extracellular vesicles (EVs) in the plant immune system. The methods development part of the project is focused on creating QD-based molecular beacons for the detection of small RNAs (sRNAs). The project's goal is to improve upon basic molecular beacon design by improving sensitivity, stability, and accuracy for *in vivo* detection of sRNAs. These will be used to study the role of EVs in the interspecies communication between the host plant, *Sorghum bicolor,* and its fungal pathogen *Colletotrichum sublineola.* The QD molecular beacon sensors will be loaded into EVs by electroporation or membrane-penetrating peptides. The project will take advantage of the superior multiplexing properties of QDs to make QD molecular beacon sensors for different types of sRNAs found in EVs from both sorghum and *C. sublineola.* A major goal is to determine if there are different subclasses of EVs carrying specific sRNAs. Specific QD molecular beacons will be complemented by other fluorescence labeling of all EVs, using direct conjugation of dyes and transgenic sorghum expressing fluorescent protein EV markers. Combined with the ability to detect QDs by light microscopy, electron microscopy, and magnetic resonance imaging, the project will conduct a multiscale examination of EV uptake, localization, and long-distance movement in sorghum and *C. sublineola.*

Current and/or Anticipated Accomplishments and Deliverables: The project has created a protocol for the isolation of EVs from sorghum. Isolated sorghum EVs have been sent for mass spectrometry (MS) analysis to detect potential protein cargos. Five EV cargo candidates have been chosen based on two MS experiments, and fluorescent protein fusion constructs will be generated and transformed into sorghum in the current project period. MS experiments were prioritized in the prior project period to identify marker line candidates. In this project period, the sorghum EV isolation will be repeated and sent for sRNA-seq analysis to characterize their small RNA content. This work is essential to generate the candidates for the QD molecular beacons.

Further characterization of QDs suggests that those of CdSe/ZnS are best suited for the study. They are excitable with a lower toxicity laser line (488 nm), while all other QDs tested require near-ultraviolet light excitation. Therefore, the team expects that a CdSe/ZnS molecular beacon will be a deliverable in this project period, and the QD molecular beacon will be made ratiometric using Quasar 670 dye conjugation for quantitative measurements of the sRNA, Mir396A. Other sensors will be made based



Plants expressing Pen3:GFP label intracellular and extracellular vesicles. Their movement was captured with variable angle epi-fluorescence, and images show preliminary analysis in Imaris software. The left side of the image shows the spots identified and the tracks of their movement at a single time point. The right side shows those to capture GFP+ objects over the full time-lapsed dataset. Courtesy Tim Chaya.

on sRNA-seq analysis that will also be delivered in this project period.

A second EV isolation protocol was developed for arugula to provide a consistent supply of plant EVs. Arugula can be locally purchased, effectively eliminating the time and cost of growing numerous plants. Arugula EVs will be used to optimize the uptake of QDs into EVs via electroporation. The team has developed a method using BHQ-10 to quench any QDs not inside EVs. That quenching method will be used to quantify and improve the efficiency of electroporation.

Lastly, the project is characterizing *C. sublineola* infection into the susceptible sorghum line, BTx623, to determine the spore concentration and the timing of fungal infection. These experiments are being conducted to prepare for live-cell tracking of EVs. Tracking the uptake of sorghum EVs loaded with QDs into *C. sublineola* will be conducted in this project period.

Potential Benefits and Applications: Plant EVs may play a critical role in pathogen defense and cell wall biogenesis. They have been well characterized in *Arabidopsis*. This project will examine sorghum EVs with the potential for improving this important biofuel crop. In the process, the project will create new QD-enabled technologies and methods that can be used to study EVs from any organism.

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] and Scott E. Baker[2]

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

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

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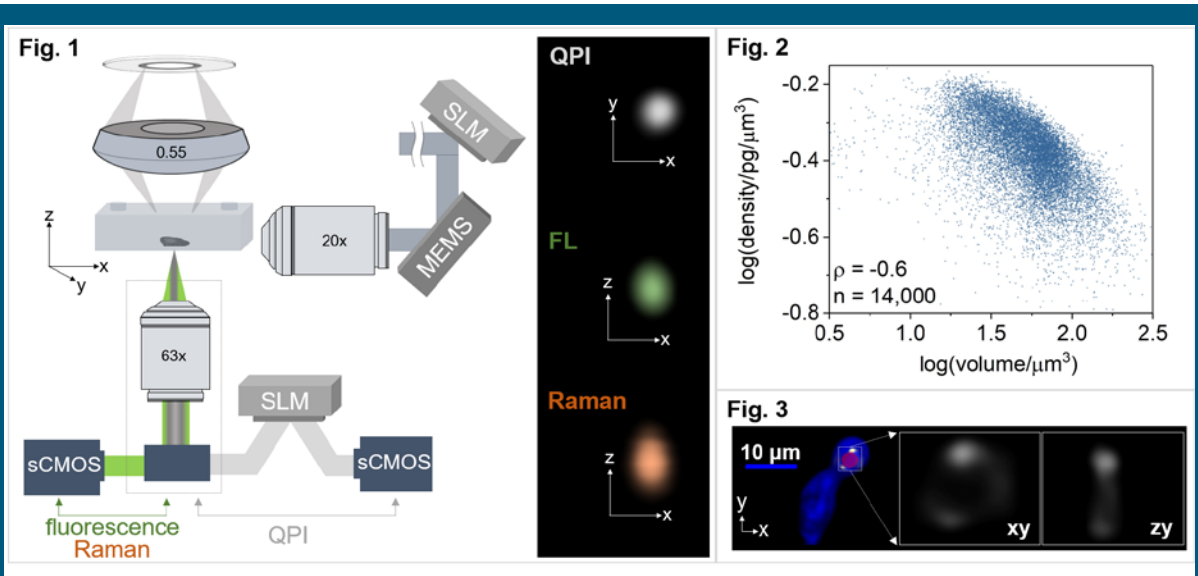
Research Plans: To accelerate the efforts of strain optimization in biofuel production, the team is constructing an imager that quantifies yields, titers, and the cellular compartmentalization of metabolic pathways in single living cells. This approach relies on the integration of quantitative-phase imaging (QPI) with light-sheet fluorescence and Raman imaging for quantifying enzyme, metabolite, and nutrient uptake dynamics with the aid of machine learning and dedicated Raman and fluorescent biomarkers.

Current and Anticipated Accomplishments: In the context of hardware, the project has expanded QPI's utility in imaging the metabolism of a single cell in microfluidics by integrating it with light-sheet fluorescence microscopy (see figure, panel 1). Here, the implementation of an accelerating Airy beam as the illuminating light-sheet yielded more than fivefold greater imaging throughput rates than diffraction-limited systems. The project has now expanded this integrative platform in two ways. First, the team has engineered a detection scheme that is congruent with the Poisson nature of sparse photons, thus enabling the decrease of irradiance requirements by one order of magnitude than modern light-sheet systems. Second, the team has integrated a Raman imaging scheme that decreases the required excitation densities by replacing traditional spectrometers with light-sheet excitation from a tunable laser. In the context of machine learning, organelles in QPI images can now be recognized without staining at >90% accuracy using desktop machines. In the context of biomarker



(Fig. 1) Integrative fluorescence-Raman Airy light-sheet with quantitative-phase imaging and representative images of a 1 μm polystyrene bead. **(Fig. 2)** Non-TAG dry-density of *Yarrowia lipolytica* as a function of cell volume. **(Fig. 3)** 3D integrative imaging of *Y. lipolytica* (blue: optical phase; red: lipid droplet (LD); white: GFP-erg6) denoting the LD's non-uniform coating by erg6. Courtesy Regents of the University of Idaho. Fig. 1 and Fig. 3: Reprinted under a Creative Commons Attribution 4.0 International License (CC BY 4.0) from Subedi, N. R., et. al. 2020. "Integrative Quantitative-Phase and Airy Light-Sheet Imaging," *Scientific Reports* **10**, 20150. DOI: 10.1038/s41598-020-76730-x. Fig. 2: Reprinted under a CC BY 4.0 license from Vasdekis, A. E., et al. 2019. "Eliciting the Impacts of Cellular Noise on Metabolic Trade-Offs by Quantitative Mass Imaging," *Nature Communications* **10**, 848. DOI: 10.1038/s41467-019-08717-w.

development, the project has augmented its palette of strains with GFP-tagged enzymes partaking in the tricarboxylic acid cycle (TCA) cycle and lipid biogenesis pathways by over threefold, including strains with two enzyme reporters in one cell. Similarly, the team has investigated deuterated glucose as a Raman tag, finding sufficient incorporation into key Raman bands. Importantly, these imaging platforms have been applied to unmask key information about cellular metabolism, namely: (a) cell density is not necessarily constant with size as previously postulated (see figure, panel 2), and (b) cellular noise impacts the localization of metabolic reactions within a cell (see figure, panel 3).

Primary next steps in the project are to: (1) complete the development of a metabolite biosensor using riboswitch-controlled reporters, (2) further increase the accuracy of organelle detection by machine learning, and (3) validate the imager by investigating how cellular noise impacts metabolism.

Benefits and Applications: By minimizing photobleaching and phototoxicity, this project will unmask key multivariate molecular information pertaining to the metabolism of single cells. Further, the imager is developed on standard inverted microscopes and utilizes open-access software, thereby making the project's technologies more accessible to the broader scientific community.

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

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

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Research Plans and Progress: The planet's ecosystems rely on the activities of soil microbes, yet the ability to observe their metabolic activities within soil is limited by the tools available to study their molecular interactions *in situ*. This project aims to create an instrument that integrates fluorescence microscopy, Raman spectroscopy, and nanospray desorption electrospray ionization mass spectrometry imaging (nanoDESI-MSI) to investigate the microbial activities and molecular transformations occurring in soil and model soil environments. **Aim 1:** Develop a test system for validation. **Aim 2:** Construct a high-resolution isotopic microscope that integrates Raman, nano-desorption electrospray ionization mass spectrometry, and fluorescence imaging for *in situ* measurements of carbon transformation. **Aim 3:** Interrogate polysaccharide decomposition in model and native soils.

Current and Anticipated Accomplishments: Substantial progress has been made regarding instrument assembly and optimization. Software has been completely rewritten and tested to address major bugs with the nanoDESI program and landing of the shear force probe (essential for collecting data from irregular surfaces). Maintaining a constant signal and liquid bridge throughout line scans across samples proved to be a major challenge, which the team addressed by changing solvent flow rate and line-scan speed. The project has successfully landed the shear force probe on a dried-down *Bacillus subtilis* colony and observed distinct molecular signatures from individual spot readings. Inconsistency issues with soft samples (agar) are still being resolved, but manually obtaining spot readings is possible. The team has optimized preparation conditions for both laser desorption ionization (LDI) and matrix-assisted laser desorption ionization (MALDI) samples [by varying gold thickness (LDI) and spraying parameters and number of dihydroxybenzoic acid (DHB) matrix passes (MALDI) to provide the highest number of annotation matches to existing metabolite databases. Conducting matched LDI and MALDI imaging on a single colony, the team found substantial (~67%) overlap between the datasets; LDI identified more small molecules and neutral lipids, while MALDI better identified surfactin species. Molecular annotations were confirmed using liquid extraction surface analysis (LESA) with tandem mass spectrometry. In parallel, the team has been optimizing the Raman experiments (using different growth media, conditions, and timepoints) to visualize microbial metabolic activity [via heavy water (D_2O) incorporation] in *B. subtilis* and *B. cereus*. Identifying appropriate calibration of the Raman lasers for these experiments will allow cross-institutional validation of this multimodal instrument. The team has also made progress in identifying soil bacteria capable of selfish carbohydrate uptake, characterization of which is planned using the instrument. The project is currently optimizing bacterial isolation protocols from soil, including using Nycodenz gradients, sonication, and fluorescence-activated cell sorting. The team is refining carbohydrate incubation protocols based on previous studies to establish appropriate concentrations and time of incubation. Related work includes developing additional fluorescently labeled polysaccharide probes to those already in hand, including chitosan, a polysaccharide present in some fungal biomass that is degraded by *B. subtilis*; the team is confirming stability of this reagent. Finally, the project is generating novel fluorescent reporter strains for extracellularly exported polysaccharide processing enzymes to visualize enzyme production along with carbohydrate uptake in future experiments using the project's instrument.

Potential Benefits/Applications: MSI, Raman, and fluorescent capacities provided by this instrument (housed at EMSL 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 by the team will not only allow validation of this multimodal imaging platform but are also applicable to a broad range of scientific questions about the key roles that microbes play in global carbon cycling.

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

Principal Investigators: Gary Stacey[1] (PI), Zeev Rosenzweig,[2] Marcin Ptaszek,[2] Galya Orr,[3] Christopher Anderton,[3] Mowei Zhou,[3] and Dehong Hu[3]

Institutions: [1]University of Missouri–Columbia, [2]University of Maryland–Baltimore County, and [3]Environmental Molecular Sciences Laboratory at Pacific Northwest National Laboratory

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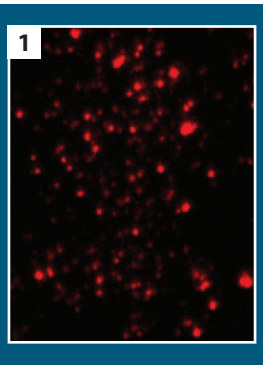
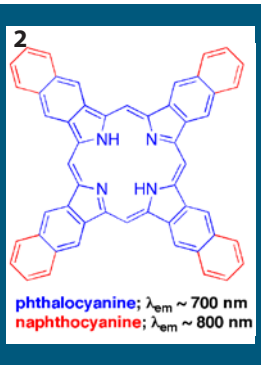
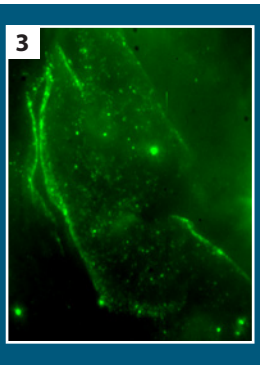
The emission spectra of plant pigments currently limit the number of fluorescent colors that can be imaged simultaneously and, therefore, the number of cellular components that can be imaged within a single experiment. Hence, developing fluorescent tags and a microscope system that can image beyond the visible range into the near-infrared (NIR) would greatly enhance plant imaging capabilities. Extending imaging into the NIR would support the interrogation of multiple proteins and molecules simultaneously, but it would inevitably decrease spatial resolution due to the increase of the diffraction limit of light. To address this need, the project developed a fluorescence microscope that seamlessly integrates imaging in the visible and NIR regions and applied it to screen a series of polymer dots (Pdots) while optimizing imaging parameters (see figure, panel 1). The project focused over the past year on the synthesis, spectroscopic characterization, and application of hydroporphyrin-containing Pdots in confocal and super-resolution imaging studies. The Pdots imaging studies revealed that dye photostability is not sufficient for successful fluorescence imaging studies that utilize high-flux laser sources for excitation. Hence, the team changed focus to phtalocynanine and naphthocyanine NIR dyes (see figure, panel 2), which exhibit higher photostability and offer opportunities for structural modification to enable emission wavelength tenability between 700 and 875 nm. With these phtalocyanine-containing Pdots, the team recently demonstrated the imaging of single Pdots (see figure, panel 1) and characterized their photo-blinking and suitability for single particle-based super-resolution fluorescence microscopy. These Pdots are now being used to tag antibodies against membrane receptors, such as FLS-2 (plant receptor for bacterial flagellin), for studying receptor dynamics in wildtype and protein acyltransferase (PAT)-deletion transgenic plants (see figure, panel 3). Data suggest that the acylation state of a number of plant receptor proteins determines, in part, their localization within plasma membrane nanodomains. Future experiments are focusing on perfecting quantum dots (QDs) and Pdots that fluoresce in the NIR. Biological experiments using these imaging tools will focus on examining the dynamic nature of receptor movement and degradation at a single-molecule level as a result of environmental perturbations.

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. Hence, they 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, including 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.







Panel 1: An image of single phthalocyanine Pdots, where each pixel is 160 × 160 nm. Excitation = 440 nm; emission= 690–730.
Panel 2: Structures of phthalocyanine and naphthocyanine dyes.
Panel 3: Localization of FLS-2 in an *Arabidopsis* leaf cell, lacking PAT5 and PAT9 function.

Hyperspectral Light-Sheet Raman Imaging of Leaf Metabolism

Principal Investigators: Keith Lidke (contact), David Hanson, Jerilyn Ann Timlin, and Jamey Young
Institution: University of New Mexico
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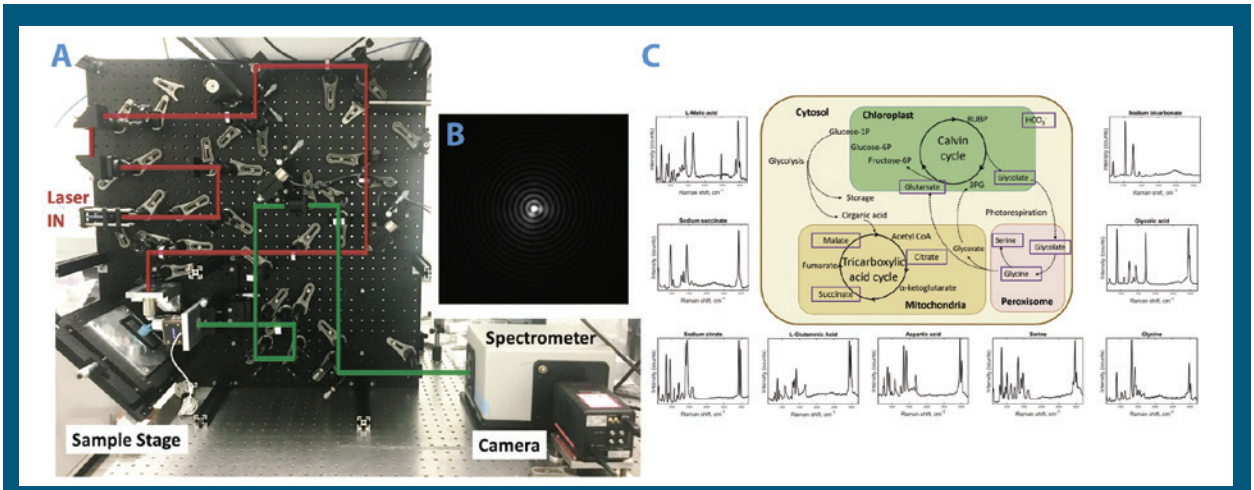
Research Plans and Progress: The project's hyperspectral light-sheet Raman microscope has been assembled (see figure panels A, B) and a custom-designed, volume-phase holographic grating for the spectrometer has been commissioned. The project is designing ^{13}C and ^{18}O labeling experiments and plans for sample collection and analysis to be conducted in spring 2021. Characterization of leaf structure and physiology included: (1) development of a Raman library of key cellular substrates and metabolites (see figure, panel C); (2) measurement of gas exchange for selected C3, C2, and C4 plants; (3) analysis of chloroplast position in the bundle sheath cells of C2 species in response to oxygen concentrations; (4) redesign of a Licor 6800 chamber for labeling and freeze-quenching in high and low oxygen conditions; (5) quantification of the expression level of the glycine decarboxylase subunit P (GLDP) by quantitative reverse transcription PCR (RT-qPCR) using the cell enrichment method to identify cell types after freeze-quenching isotope-labeled leaf materials; (6) quantification of chloroplasts and mitochondria investment in mesophyll and bundle sheath cells of the C3 and C2 species; and (7) collection of hyperspectral data on chloroplast and pigment composition of mesophyll and bundle sheath cells across the abaxio-adaxial and proximodistal axis of primordia, developing, and mature leaves of sorghum and rice.

Current and/or Anticipated Accomplishments/Deliverables for the Project Period: The project anticipates full functionality and characterization of the microscope and will proceed with imaging leaf and algae samples. The project will analyze isotope labeling trajectories of leaf metabolites involved in Calvin cycle, glycolysis, tricarboxylic acid cycle (TCA) cycle, and amino acid metabolism. These data will be used to assess photorespiration and photosynthetic fluxes in C2/C3/C4 plants and provide validation data for Raman imaging. The project will perform $^{18}O_2$ labeling of the leaves with different photosynthetic types and using cell enrichment methods to identify cell-specific distribution and concentration of the key photorespiratory metabolites.

Potential Benefits/Applications: The project will develop a better fundamental understanding of leaf metabolism and validate novel tools to measure *in planta* metabolic fluxes. Energy and food crop productivity can be improved through engineering photosynthesis such as replicating C4 and related pathways in crops and other ways to reduce photorespiration. The project's imaging system and the group of species to be examined will demonstrate the power of our technology for assessing and understanding the effectiveness of these attempts to re-engineer photosynthesis.



(A) Hyperspectral Light-Sheet Raman Microscope showing excitation path (red) and Raman detection path (green). **(B)** Cross section of Bessel beam at a conjugate image plane. **(C)** A cartoon summarizing biochemical reactions in a photosynthetic cell and the corresponding Raman spectra of some metabolites. Courtesy Sandeep Pallikkuth and Roxana Khoshravesh.

Metaoptics-Enabled Multifunctional Imaging

Principal Investigators: Paul Bohn, Anthony Hoffman, and Joshua Shrout
Institution: University of Notre Dame
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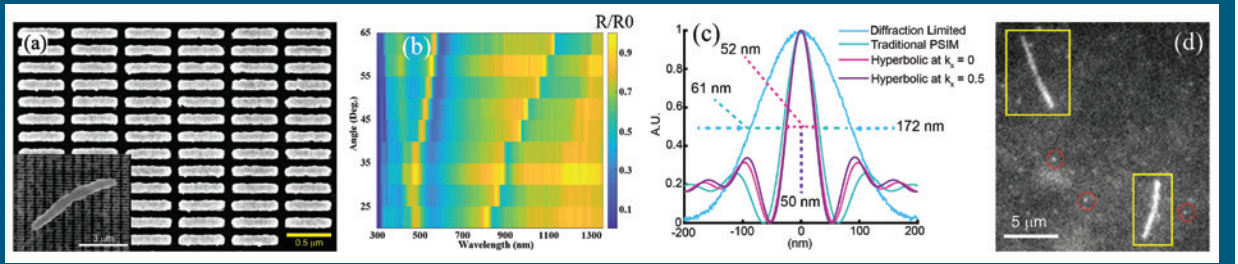
Research Objectives: The project is developing enhanced imaging tools by pursuing two overarching technical goals: (1) the development of new metaoptics-enabled approaches to imaging and spectroscopic characterization, and (2) the development of tools to control the chemical environment of a microbial sample with nanometer-scale precision.

Accomplishments/Deliverables for the Project Period:

Metaoptics. To confine hyperbolic modes spatially, the team fabricated finite-length nanoridge arrays with subwavelength spacing in both axes, (see figure, panel a). The large array efficiently couples free-space light to multiplasmon modes of the nanoridge. These multiplasmon modes are engineered to exhibit a large effective modal index at 458 nm. Angle- and polarization-dependent spectroscopic ellipsometry was employed to characterize the optical metasurfaces. Figure panel b shows the measured transverse magnetic reflectance with the incident wavevector along the long axis of the ridge. For an incident angle around 30°, two higher-order modes hybridize, giving rise to a coupled mode, which exhibits hyperbolic dispersion. The confined nature of the hyperbolic mode enables super-resolution imaging, as the team has shown numerically for a random distribution of quantum dots (d = 9 nm) with deep subwavelength spacing. Structured illumination microscopy (SIM) reconstruction using these metasurfaces produces images with 3.44x resolution enhancement compared to the diffraction limit, (see figure, panel c), giving a resolution of 52 nm at λ = 458 nm; a result superior to other state-of-the-art approaches in SIM.

Spectroelectrochemistry of Myxococcus xanthus. Potential-dependent dynamics of the bacterium *M. xanthus* was studied using combined surface-enhanced Raman microspectroscopy and electrochemistry. While potential did not affect the formation of dormant fruiting body spores, the Raman bands from 1,500 to 1,600 cm^{-1} showed clear potential dependence, reflecting redox activity flavoproteins in the outer membrane of the cell. Because the observed Raman band modulation was convoluted with silver oxide behavior from the silver nanoparticle used for surface enhancement, the team devised a thin-layer electrochemical cell with 10 µL volume using ITO and screen-printed electrodes to trap the bacterial cells without silver (Ag) nanoparticles. These experiments paved the way for studying the behavior of *M. xanthus* drop-cast on an Ag-based metasurface (see figure, panel a). The epi-illumination fluorescence image (458 nm excitation; see figure, panel d) illustrates the ability to successfully image green fluorescent protein (GFP)-tagged *M. xanthus* without lysing. The underlying Ag structures produce native photoluminescence, (see red circles in figure, panel d), which impacts the ability to exploit hyperbolic dispersion for super-resolution fluorescence. The team is currently working to develop computational filtering schemes to separate the two sources of fluorescence.

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



(a) SEM image of 500 nm nanoridge array metasurface. (Inset) Single *Myxococcus xanthus* cell on metasurface. **(b)** Wavelength-, angle-, and polarization-dependent reflection for metasurface in (a). **(c)** Comparison of the spatial resolution of quantum dots using different super-resolution techniques. **(d)** Fluorescence image of GFP-tagged *M. xanthus* drop-cast on silver metasurface. Courtesy J. Haug, M. Palei, H. Do, V. Sundaresan, C. Madukoma, A. J. Hoffman, J. D. Shrout, and P. W. Bohn.

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

Principal Investigators: Marisa S. Otegui and Kevin W. Eliceiri

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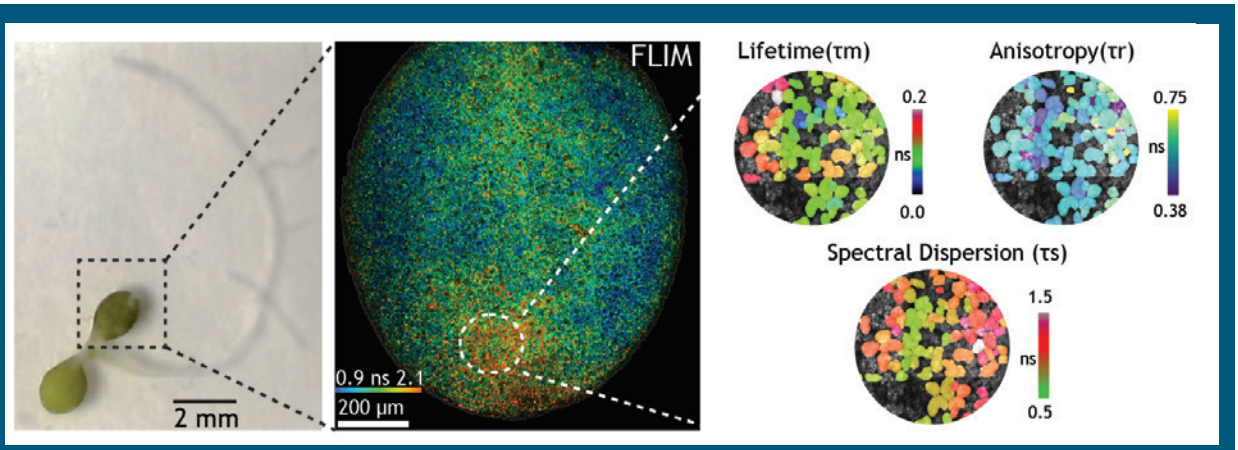
Research Plans and Progress: Plant tissues are often considered not ideal for fluorescence imaging because of the pervasive intrinsic fluorescence of many plant metabolites and the intricate interactions with light of the many semicrystalline polymers at the cell wall. The project aims to take advantage 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. The team is working to combine these imaging capabilities with computational tools that enable correlated registration, integration, and analysis. This fully integrated, multiparametric optical system when complete would be the first of its kind. The team is working 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) develop an accessible imaging platform and associated open-source software able to extract and integrate fingerprints from fluorescence-associated (multispectral emission, lifetime, and polarization), wide-field polarimetry, second harmonic generation (SHG), and stimulated Raman scattering signals (SRS); (2) determine unique combination of fingerprints for various cell wall components and selected metabolites; and (3) 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.

Current Accomplishments/Deliverables:

Progress to date includes:

1. Development of a fully functional open-source multiphoton scanning system
    a. Added open-source fluorescence lifetime imaging microscopy (FLIM) acquisition – OpenScan (manuscript in preparation)
    b. Added open-source FLIM Analysis package – FLIMJ (Gao et al. 2020)



The image shows hyperdimensional microscopy imaging of *Arabidopsis thaliana*. The Brightfield image shows one cotyledon, the zoomed image shows the FLIM image of the bottom of one leaf, and the single-cell zoom of three principal modalities of hyperdimensional imaging including fluorescence lifetime, fluorescence anisotropy, and fluorescence spectrum—measured as temporal dispersion. The images do not correspond to the exact location but are shown as an abstract graphical view of the imaging modalities and the imaging size. The scale bar and look-up tables are provided in the image. Courtesy Jenu Chacko, Marisa Otegui, and Kevin Eliceiri, University of Wisconsin–Madison.

c. Added full automation and real-time image analysis using Jupyter notebooks

2. Implementation of a fast two-channel time domain-based FLIM system with polarization control
   a. Published fast FLIM optimization based on non-parametric Bayesian model (Wang et al. 2019)
   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 (Sagar et al. 2019)
   b. Ongoing work to utilize fiber dispersion for fast hyperdimensional contrast imaging (*see item 4.*)
4. Hyperdimensional imaging microscopy (HDIM) 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 (Chacko et al. 2021)
5. Development of a novel scheme to detect forward and backward SHG.
   a. Implemented and validated for cellulose and starch (manuscript in preparation)
6. Development of CRISPR/CAS9-edited lines expressing mutated silicon transporters (in progress)

Potential Benefits/Applications: Understanding the assembly and deconstruction of cell walls in grasses is very important for bioenergy-related purposes. 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, the team 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.

Papers Published in Last Funding Year

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Gao, D., et al. 2020. "FLIMJ: An Open-Source ImageJ Toolkit for Fluorescence Lifetime Image Data Analysis," *PLoS ONE* **15**(12), e0238327. DOI: 10.1371/journal.pone.0238327.

Sagar, M. A. K., et al. 2019. "Optical Fiber-Based Dispersion for Spectral Discrimination in Fluorescence Lifetime Imaging Systems," *Journal of Biomedical Optics* **25**(1), 1–17. DOI: 10.1117/1.JBO.25.1.014506.

Wang S., et al. 2019. "Nonparametric Empirical Bayesian FrameWork for Fluorescence-Lifetime Imaging Microscopy," *Biomedical Optics Express* **10**(11), 5497–5517. DOI: 10.1364/BOE.10.005497.

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

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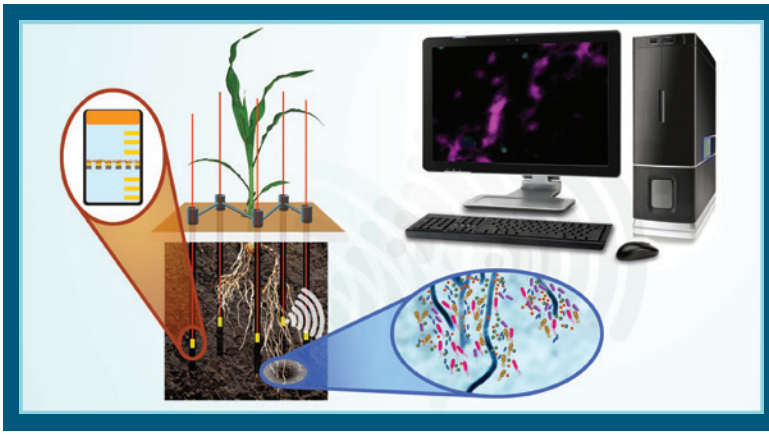
Interactions between plants and microbes define the growth and health of plants. Most of these interactions involve plant-derived and microbially derived chemical and biochemical signals that travel through the rhizosphere. These chemical communications shape the metabolic activities of the rhizosphere microbial community. In turn, they can influence how the microbial community and plants interact. Despite the importance of these communications to agriculture, the environment, and development of clean energy sources, little is known about these activities in the rhizosphere.

Research Plans: The 4D analysis of molecules by aptamers in soil (4DMAPs) project will provide a means of probing the distributions and time-dependent changes of these chemical communicators and their changes in response to perturbations of the system, such as those that occur with variations in nutrients, water, temperature, and pathogen presence. Molecular sensors located in and close to the rhizosphere will detect specific communicating molecules and relay that information to the 4DMAPS instrument, which will integrate input from numerous electrochemical sensors distributed throughout the soil and be vertically mobile to produce real-time images of the locations and concentrations of signaling molecules. The molecular specificities of the sensors will be provided by aptamers, which will be selected to recognize specific molecules known to be secreted by plants or microbes in the rhizosphere.

Progress: The overall objective this past year has been to expand the sensing capabilities of the 4DMAPS aptasensor and develop the capability of obtaining an image of released communicators. To that end, the project has achieved the following: (1) an aptasensor capable of simultaneous imaging of multiple molecules; (2) an aptasensor that detects the quorum-sensing signal molecule C4-homoserine lactone in soil conditions of salt and pH; (3) sequences (cDNAs) of two endoglycosidases that are secreted from plant roots cloned with one protein expressed and purified for use as an aptamer target; (4) aptamers that recognize the bacterially produced iron-chelating pyoverdine; (5) a novel chemical synthesis approach to synthesize the universal quorum-sensing signal furanosyl borate diester (autoinducer 2), which is now a target for aptamer selection; and (6) a genome-enabled microbiome that produces targeted microbial chemical signals for use in a synthetic hydrogel-based soil mimic for growing plants.

Potential Benefits/Applications: The goal of this project is to develop an instrument that can conduct real-time imaging of chemicals in the rhizosphere that govern the plant's nutrition and health and the metabolic activities and interspecies communication potential of microbes colonizing roots. Although this early period of development will create an instrument in a laboratory setting, the long-term goal is to create an instrument with the flexibility of being fitted with a variety of sensors, which can be used in the field in experimental and agricultural applications to monitor nutrient transformations and microbial metabolic activities in the rhizosphere that contribute to the growth of healthy, productive plants.



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.

A Quantum-Enhanced X-ray Microscope

Principal Investigators: Sean McSweeney[1] (PI), F. Alexander,[1] L. Berman,[1] A. Fluerasu,[1] A. Nomerotski,[1] T. Paape,[1] and C. Da Via[2]

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

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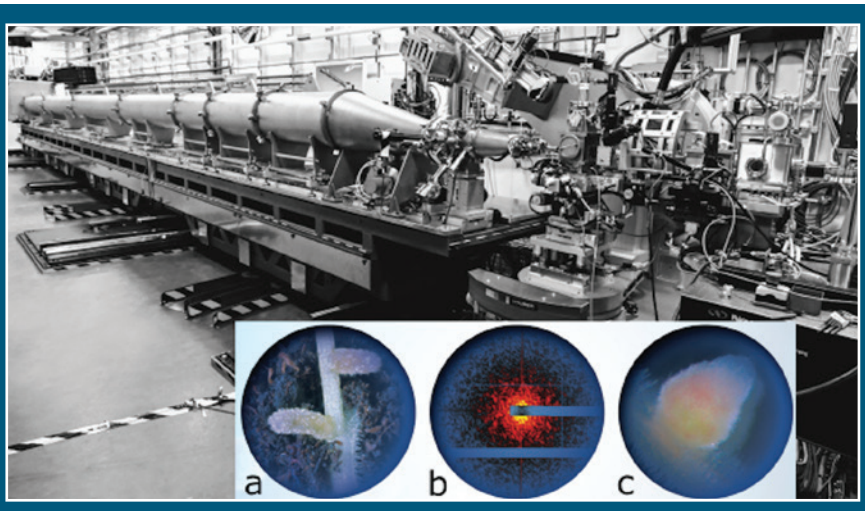
Imaging of systems has an extraordinary ability to convince and inform. However, high-accuracy measurements require the production of images with a high signal-to-noise ratio. Typically, this is achieved using high input flux, but for living cells a high incident dose complicates the image by inducing radiation damage leading to unwanted artifacts. This issue represents the essential compromise made in designing a biological imaging experiment: the experimenter must choose between precision of the image and damage to the sample.

The use of quantum properties of light, in this case X-rays, offers a new opportunity for imaging in the use of quantum correlations of the (two-photon) system to retrieve the image. This approach has powerful implications in applications where the sample would normally require high-intensity beams to be imaged but would be destroyed by them. With "ghost imaging," a sample could be illuminated by less intense beams with a more suitable energy without being modified during the experiment. Further, the quantum nature of the imaging process would allow the visualization of details impossible to detect with classical methods. This project's objective is to develop a mechanism to perform ghost imaging of biological samples using X-rays. The goal is to build upon research in the optical regime by creating an X-ray equivalent microscope using entangled X-ray beams produced at the BNL National Synchrotron Light Source II (NSLS-II). Key to the experiment are the use of coherent X-rays from the NSLS-II, the recent successful research in nonlinear media for entangled X-ray generation, and the use of ultrafast pixelated detectors with excellent detection efficiency.

The delivery of an X-ray quantum microscope is built upon four pillars: experimental methods, nonlinear media, biological systems, and data analysis. During this initial reporting period, efforts have been focused on establishing the team and baseline set of measurements, specifically:

Experimental Methods: The team has created an optical system for benchtop experiments to act as a guide for X-ray work. In both cases, efforts are to establish ghost imaging from photon correlations generated from chaotic sources (type-2 ghost imaging). This conceptually less sophisticated experiment will allow for progress while more complex needs are met.

Nonlinear Media for Parametric Down-Conversion: In the X-ray region, available media have very low cross sections for the conversion. A key element of the research program is to investigate new systems capable of higher efficiency generation of entangled X-rays. Research



The Quantum Enhanced Microscope project at Brookhaven National Laboratory makes use of the quantum nature of X-ray beams in various forms. The NSLS-II Coherent Hard X-ray Scattering (CHX) beamline (shown in background) is the source. The research team will study *Medicago truncatula* and its symbiotic interactions with *Sinorhizobium medicae* and *S. meliloti* **(panels a and c)** as models systems for studying the genetics of nodulation and nitrogen fixation in bacteria. First experiments have been performed using the coherent speckle patterns **(b)** developed at CHX. Courtesy Sean McSweeney, BNL.

is underway, and novel media will be tested as they become available.

Biological Systems: There is growing interest in the role microbes play in increasing host-plant resilience to stressful or toxic conditions. *Medicago truncatula* is the model legume species that will be studied in its symbiotic interactions with *Sinorhizobium medicae* and *S. meliloti*. Together, the *Medicago-Sinorhizobium* system provides a powerful experimental biological system to study molecular- and biochemical-level processes using genomics, ionomics, and imaging approaches. Staff have been recruited, and sample preparation is underway.

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 low count rates, types of noise, and experimental realities. The project has established a multidisciplinary analysis team to support this area.

The ultimate goal of this project is to achieve the potential of ghost imaging for X-ray image reconstruction of biological samples. Further advantages of this approach are apparent: (1) Due to the time correlation used, dynamical snapshots of the samples will be possible. (2) By choosing the correct X-ray wavelength for illumination, X-ray fluorescence will be stimulated in metals of relevance to biological function, and so recorded. (3) Due to the penetrating power of X-rays, thick samples or otherwise optically opaque samples can be imaged *in vivo*. The team thinks of this approach as multicolor, temporal X-ray microscopy.

Development of a Full-Field X-ray Fluorescence Imaging System for Near Real-Time Trace Element Microanalysis of Complex Biological Systems

Principal Investigators: Ryan Tappero[1] (PI), David (Peter) Siddons,[1] Mourad Idir,[1] Sunny Liao,[2] Jenny Bhatnagar,[3] and Rytas Vilgalys[4]

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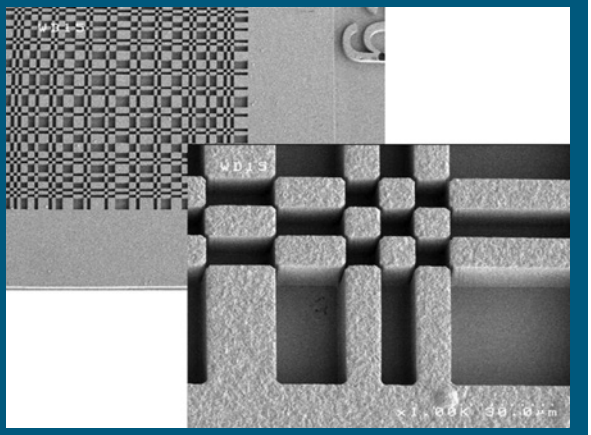
Research Plans and Progress: To develop a full-field X-ray fluorescence imaging system (FFFI) for studying spatial and temporal dynamics of trace elements in complex biological systems. Conventional X-ray fluorescence (XRF) imaging is performed in a scanning-probe modality. A limitation of scanning XRF is the time required to record images one pixel at a time. This project aims to produce a full-field scheme for XRF imaging enabled by development of a new imaging detector capable of recording both a photon "hit" and its energy (i.e., spectrometer). Such a detector system will transform XRF imaging by enabling studies of dynamics and transport processes and studies requiring high throughput for adequate statistics or replication (e.g., mutant screening to link genotype and phenotype). Understanding detailed interactions among synergistically functioning organisms, particularly fungi and roots, provides a scientific driver for this bioimaging technology development.

Current and/or Anticipated Accomplishments/Deliverables: (1) Benchmark measurements of *Pinus-Sullius* model system (compatible/incompatible pairs); (2) simulations of different detector magnification solutions (Wolter, coded aperture, polycapillary); (3) pink beam imaging tests with polycapillaries and MURA (coded aperture) using TimePix3 sensor; (4) FFFI detector ASIC design (130 nm process) and redesign (65 nm process); (5) FFFI detector readout system design and circuit for handling of charge-shared events; and (6) preparations for scanning the MURA to beat the 10 µm resolution specification.



The goal of the project is to develop a full-field X-ray fluorescence imaging (FFFI) system for studying spatial and temporal dynamics of trace elements in complex biological systems at the micrometer scale. A key aspect of FFFI is to configure an optical system that provides achromatic image magnification. To this end, the team has designed and fabricated a novel coded aperture (MURA-73) with high efficiency (50% transmission). Reprinted under a Creative Commons Attribution License (CC BY) from Siddons, D. P., et al. 2020. "A Coded Aperture Microscope for X-ray Fluorescence Full-field Imaging," *Journal of Synchrotron Radiation* **27**(6), 1703–706. DOI: 10.1107/S1600577520012308.

Potential Benefit/Applications: Project seeks to deliver a first-generation, working prototype of a FFFI detector system for *in situ* and near real-time monitoring of nutrients and trace elements in complex, heterogeneous materials such as soil and sediments. Subminute temporal resolution is expected for detection of first-row transition elements (e.g., Fe, Cu, and Zn). Dynamics and transport processes on this timescale can be studied. Another expected outcome is a design blueprint for future development of a second-generation, sub-10-micron resolution model of the FFFI detector system that could be deployed on benchtop X-ray sources.

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

Principal Investigators: Ted A. 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] and Xiyu Yi[1]

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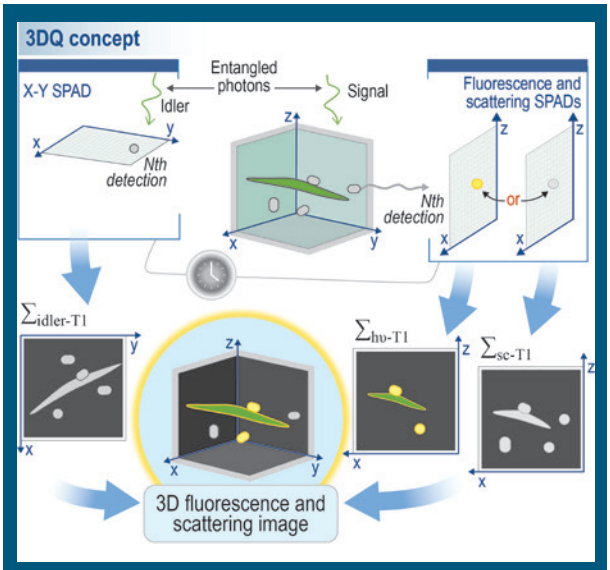
Goals and Objectives: The research goal is to develop a 3D imaging modality that uses quantum-entangled photon pairs to obtain more information about fluorescence and scattering events than is available in standard fluorescence or scattering measurements. The entangled photons will enable a microscope 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. Based on this, the team envisions a new, 3D quantum (3DQ) microscope that uses quantum-entangled light to provide 3D optical imaging at high frame rates. The project will apply this microscope to dynamic host-bacterial interactions in bioenergy algal pond and plant systems.

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) arrays; and (4) adapt existing biosensors and bioenergy systems to apply and benchmark the 3DQ capability against light-sheet microscopy and spinning-disk confocal microscopes. During this project period, the team will implement a Phase 1 system using a lower-resolution microscope, an initial visible quantum-entangled light source based on an individual beam path with standard nonlinear crystals, first-generation synchronized 2D SPAD arrays with 1,024 total pixels, and benchmark studies of host-bacterial interactions using existing light-sheet and confocal systems.

Accomplishments: In the first four months of the project, the team acquired laser sources, crystals, and optics necessary to generate quantum-entangled photons at visible wavelengths. The team established an additional optics laboratory for this project with LLNL support. A new postdoc with expertise in spontaneous parametric down-conversion excitation of two-photon fluorescence was hired. An initial 2D SPAD array is under development by EPFL for immediate testing and integration into LLNL optical microscopy setups. The team built microcosms and initiated pilot experiments for the algal- and plant-bacterial systems. CSM provided LLNL with nano oxygen sensors for the initial experiments with scanning confocal and lattice light-sheet microscopes and initiated nanosensor design for the 3DQ.



Quantum-Enabled Techniques. BSSD researchers are developing quantum imaging modalities, such as the three-dimensional quantum microscope illustrated here, which will use entangled photon pairs to obtain information beyond standard fluorescence or scattering measurements. Courtesy Lawrence Livermore National Laboratory.

Potential Benefits and Applications to DOE Research: This project will support DOE research on bioenergy systems, both expanding the application of live imaging and developing new tools for high-speed, 3D imaging. Live imaging will provide new data on microbial interactions. New nanosensors will provide *in situ* geochemical data. High-speed, low-power imaging will enable low-distortion, extended live imaging. In addition, rapid 3D optical imaging of fluorescence and scattering processes is a very general need that has applications to biological, biomedical, and materials systems.

Illuminating the Rhizosphere: Developing an Adaptive Optics, Multiphoton Microscope for 3D Label-Free Live Imaging of Microbes and Organic Matter in Soil and Roots

Principal Investigators: Peter Weber (PI), Janghyuk Lee, Liliana Dongping Wang, Diana Chen, Sonny Ly, Ted Laurence, Lisa Poyneer, Mark Ammons, Keith Morrison, Rachel Hestrin, Erin Nuccio, and Jennifer Pett-Ridge
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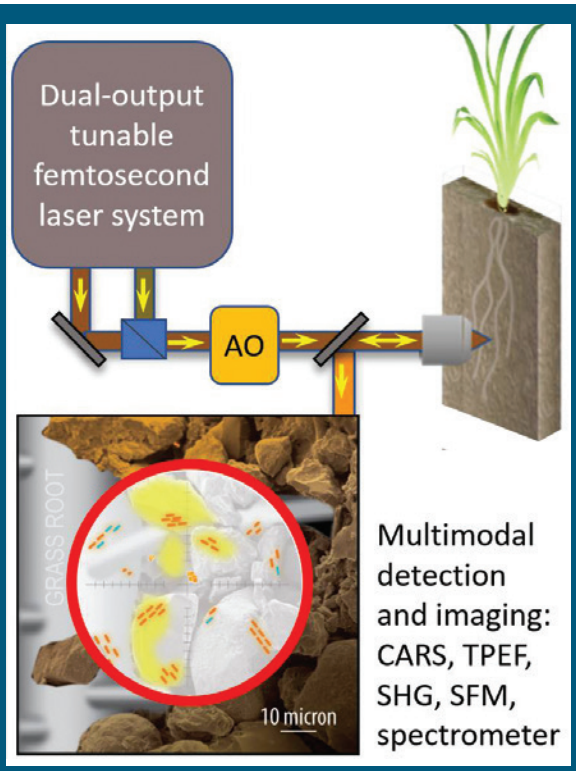
Goals and Objectives: The purpose of this research is to advance the ability to visualize plant-microbe-mineral interactions in the rhizosphere to study carbon cycling, sustainable food and fuel production, and environmental processes, including contaminant transport. This project is using adaptive optics (AO) and label-free multiphoton microscopy and spectroscopy to develop a new microscope that overcomes the challenges of optical imaging in mineral and soil matrices. Project objectives are: (1) develop a label-free method of visualizing microbial cells and organic matter in mineral and soil matrices; (2) develop adaptive optics for optical imaging in mineral and soil matrices; (3) design and build an integrated AO-multiphoton microscope capable of working with plant-scale rhizospheres; and (4) apply the AO-multiphoton microscope to rhizosphere and soil pilot studies. The goals of this performance period were to initiate studies of rhizosphere microorganisms and to apply AO modeling and algorithms to soil-surrogate samples.



The Lawrence Livermore National Laboratory (LLNL) rhizosphere microscope is designed to image microorganisms and organic matter in roots and soil. Courtesy LLNL.

Accomplishments: This year, the project reached two major milestones. One milestone was that the microscope team made the multimodal multiphoton microscope fully operational, allowing the project to initiate studies to characterize the optimal modes for imaging rhizosphere fungi and bacteria within mineral and root matrices. The team used the microscope's coherent anti-Stokes Raman scattering (CARS), two-photon excitation fluorescence (TPEF), second-harmonic generation (SHG), sum-frequency mixing (SFM), fluorescence lifetime imaging (FLIM), and spectroscopy capabilities to image a range of simplified samples including symbiotic fungi and bacteria in minerals and roots.

The second milestone was that the AO team implemented a testbed to develop and optimize the sensorless AO correction algorithm to control the high-resolution spatial light modulator (SLM). The project has developed a GUI platform, where team members can control the hardware (SLM, camera, and photodetector), implement the AO correction algorithm, conduct imaging and AO correction experiment, and save experimental data for postprocessing. Experiment-wise, the team has optimized the AO testbed optical system and carried out low-order AO corrections on synthetic soil samples, with the Strehl ratio tripled. Next steps are to perform high-order AO corrections and then integrate the AO system with the multimodal, multiphoton microscope.

Benefits and Applications: This research enables direct imaging of rhizosphere and soil processes with an emphasis on plant-microbe-mineral interactions. The multimodal, multiphoton microscope can image live symbiotic fungi and bacteria within roots and minerals. TPEF gives broad imaging contrast. CARS provides chemical images of organic and inorganic compounds. Some minerals produce SHG and SFM photons. FLIM can deconvolve TPEF from other signals. Spectroscopy characterizes the detected signal in detail. Adaptive optics will increase sensitivity and depth of imaging. These capabilities will enable advanced live, label-free imaging in the rhizosphere and soils.

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

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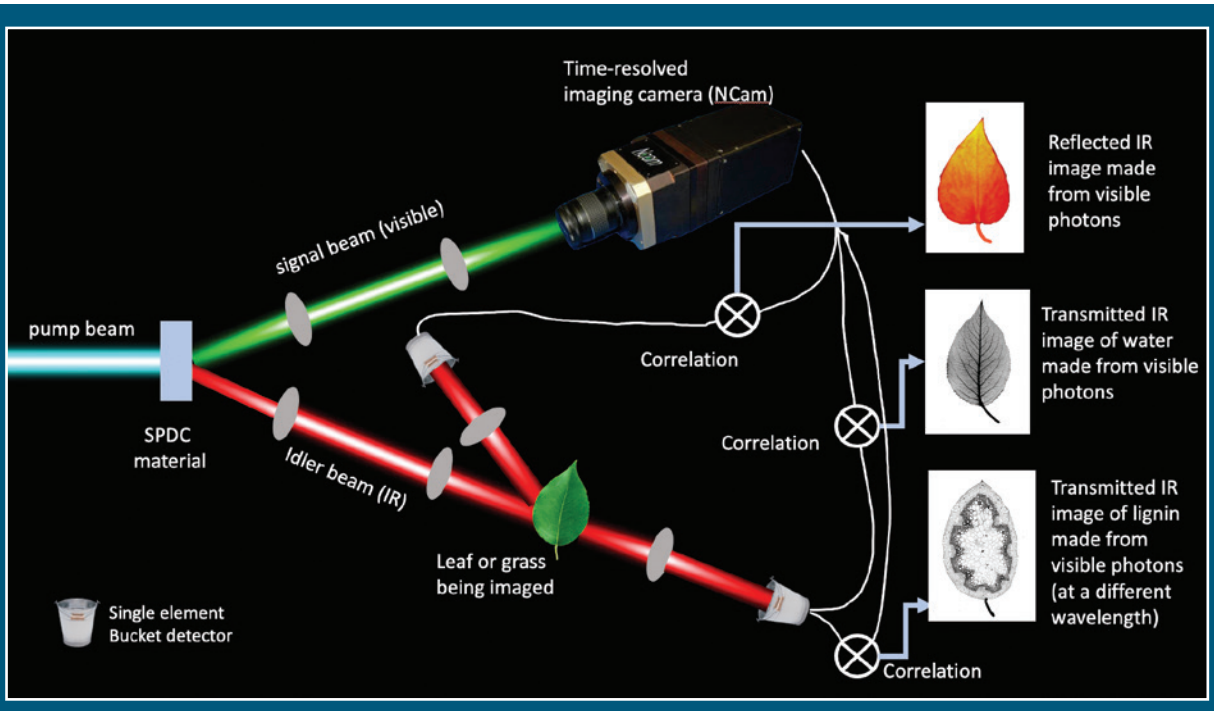
This program is developing a new microscope that uses entangled photon pairs to visualize water, lignocellulose, and lipid content in plants. A primary power of this quantum ghost imaging approach is that the wavelength used for probing the sample can be in the near- or mid-infrared (where vibrational fingerprinting to identify key molecular species is possible) and the detection/imaging is done with visible light, for which high-efficiency and low-noise imaging detectors are available (see figure). Using two detectors and correlating photon arrival times can greatly reduce noise in image formation. As such, images can be formed under extremely low light conditions—approximately an order of magnitude less light intensity than starlight. The project will advance this promising technique to its full potential by using a unique LANL-developed detector technology. In particular, the team will exploit a unique time-resolved, single-photon counting imaging detector that will enable measuring coincidence photon events with an order of magnitude better timing resolution (~100 ps) over the current state of the art (several nanoseconds). These new imaging approaches will be tested in transmission and reflection geometries on two plant species that demonstrate different mechanisms and pathways for carbon storage: a grass (sorghum) and a dicot (*Camelina*). The initial focus is on measuring an important and largely abundant plant constituent with large near-infrared (IR) and mid-IR absorption features—water. However, the plan is to mature this technology toward simultaneous measures of plant lignin/cellulose, lipid, and protein content over the course of the proposed research, ultimately leading to more informative measurements of plant environmental responses.

The initial phase of this project is primarily devoted to microscope development at a single wavelength (1,450 nm) overlapping a water absorption band (Milestones 1.1 and 1.2). The team is acquiring and assembling several key components: a pump laser, a spontaneous parametric down-conversion (SPDC) crystal, and a single element bucket detector. The team also has been considering trade-offs between system magnification



Schematic of a quantum ghost imaging microscope. Images of water content, lignocellulose, or lipid content will be formed from infrared (IR) absorption measurements using entangled photon pairs. This technique allows images to be formed at an extremely low, nonperturbative light dose. Courtesy James Werner, Los Alamos National Laboratory.

and desired spatial resolution and have decided to focus initial instrument developments at imaging a 1-cm by 1-cm area of a plant or grass leaf at a nominal 100-micron resolution. The project expects to demonstrate imaging water content at this single frequency by the end of the fiscal year. Additionally, the team has begun IR absorption measurements of plant specimens using a commercial Fourier Transform IR (FTIR) microscope. This preliminary work with commercial instrumentation is helping to establish image analysis and sample preparation pipelines needed for future work. In terms of staffing, the project has identified and is in the process of hiring a full-time postdoc with a strong background in entangled photon source development. The project has also successfully converted a postdoc, Duncan Ryan, to a staff scientist working full-time on developing and building this new microscope capability.

This work has substantial benefit to the broader scientific and biomedical community. In particular, there are drawbacks to fluorescence microscopy, which is a powerful and widely used method to explore biomolecular processes. For fluorescence imaging, the introduction of probes to label targets of interest (while enhancing signal) can perturb the underlying biomolecular processes one is trying to observe. Furthermore, the use of high-intensity laser excitation can also cause photodamage to or perturbation of the sample under investigation. The label-free, low-light imaging methods developed here can readily and easily be applied to a wide range of biological systems of biomedical or scientific importance that are currently being studied by conventional fluorescence microscopy methods.

Intrinsically Coregistered Chemical Imaging of Living Plant and Microbial Systems via 3D Nonlinear Optical Mapping and *In Situ*-Liquid Extraction-Mass Spectrometry

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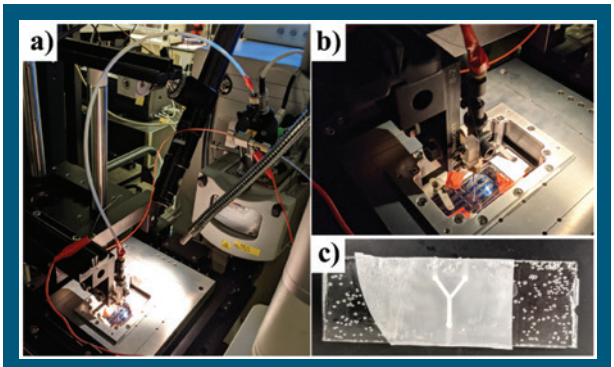
Research Plans: This project aims to develop a multimodal imaging platform integrating novel and existing analytical capabilities whose synergy yields 3D spatiotemporal chemical information in the bulk and at the interface in biological systems *in situ*. Project objectives are to (1) develop new bioimaging modalities, vibrational sum-frequency generation (vSFG) microscopy and *in situ*-liquid extraction mass spectrometry (*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/Deliverables: The project's new technology, *in situ*-LE-MS, continues to be developed for enabling chemical imaging of living biosystems by MS. The technique was the recipient of an R&D 100 award, which recognizes emerging technologies of note in that year. The *in situ*-LE-MS technique has been further validated by establishing sensitivity limits while sampling from water and different media compositions. The system has been applied for chemical imaging of *Pantoea* YR343 biofilms grown inside microfluidic devices. Methods have been developed to reduce the negative impacts of high salt concentrations present in cell media while also maintaining improved sensitivity of the technique. To facilitate coupling of the *in situ*-LE-MS and nonlinear optical imaging modalities, a novel tethered LE-MS sampling design was developed that enables long liquid transfer distances without sacrificing the performance metrics of the *in situ*-LE-MS system. The tethered LE-MS approach allowed for an increase in probe distance from the mass spectrometer by >3 times, facilitating incorporation of this device with nonlinear optical imaging modalities viable.

Simultaneously, the team's novel wide-field CARS microscopy system has been advanced further by leveraging a total internal reflection (TIR) excitation scheme to enable rapid, wide-field imaging with enhanced surface sensitivity. The platform now combines CARS, two-photon fluorescence, second harmonic generation, and SFG modalities on the same platform. The multimodal imaging system was validated using model systems and has been successfully applied for live cell imaging of *Hydrangea quercifolia* pollen and *Pantoea* YR343 for the first time.



Photographs of **(a)** the combined nonlinear wide-field and *in situ*-LE-ME imaging system, **(b)** the sampling area, and **(c)** the multimodal-enabled flow cell. Courtesy John F. Cahill, Oak Ridge National Laboratory.

The team recently constru cted a modified *in situ*-LE-MS system to combine with the wide-field multimodal microscope. A novel multimodal imaging-enabled flow cell has been designed and fabricated. This flow cell enables simultaneous acquisition by both nonlinear optical and MS imaging modalities. The combined imaging system is currently being validated against model systems.

Potential Benefits/Applications: The *in situ*-LE-MS imaging modality allows broad chemical analysis of the liquid flow inside a microfluidic device without affecting its operation. Recent developments improving the sensitivity and flexibility of the approach enable coupling with various imaging modalities. The developments in TIR-enabled wide-field multimodal technology enable users to access complementary chemical and structural information for various species near interfaces. This approach to TIR-enabled wide-field imaging is expected to provide insight into fragile bacterial films and their interactions with other species in the rhizosphere in a time-resolved and chemically selective manner.

Probing Photoreception with New Quantum-Enabled Imaging

Principal Investigators: James E. Evans[1] (PI), Patrick El-Khoury,[1] and Robert Boyd[2]
Institutions: [1]Pacific Northwest National Laboratory (PNNL) and [2]University of Rochester
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Research Plans and Progress: The project was funded less than six months ago and is therefore still in the process of ramping up and hiring. The three-year program will develop a new hybrid quantum-enabled imaging platform that combines advances in adaptive optics, quantum entanglement, coincidence detection, ghost imaging, quantum phase microscopy, and multidimensional nonlinear coherent spectromicroscopy to characterize photoreception in phytotropin and phytochrome proteins. The project's approach has three main aims, which are intended to be developed 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.

Current and/or Anticipated Accomplishments/Deliverables for the Project Period: During the current project period, the team anticipates installing a Leica Dmi8 optical microscope with fluorescence, coherent anti-Stokes Raman scattering (CARS), stimulated Raman scattering (SRS), and multiparametric (MP) imaging modes. This will be the base microscope for Aims 1 and 2 and possibly Aim 3 (still under evaluation). The team will also install a PicoEmerald optical parametric oscillator and two Coherent lasers to be used in Aims 1–3. The team will initiate work on both the ghost imaging and the quantum phase contrast imaging with milestones of developing the theory and numerical simulation for these aims as well as developing the control software for the necessary spatial light modulator. Finally, the team will perform cell-free expression of PhyB and PhyC phytotropin proteins and begin their structural and spectroscopic characterization to advance Aim 3.

Potential Benefits/Applications of DOE-Funded Research: This work 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 offices of DOE. All results are expected to be published in peer-reviewed journals. Because the principal investigator is a staff member of the Environmental Molecular Sciences Laboratory (EMSL), a DOE user facility located at PNNL, the new capabilities will also be made accessible to other researchers through the EMSL user program as the technologies described in this proposal mature. This tactic will facilitate widespread dissemination to the global scientific community and enhance overall impact.

Multimodal Chemical Imaging Across Scales to Visualize Metabolic Pathways in Live Plants and Microbial Systems

Principal Investigators: Scott Lea[1] (PI), Patrick El-Khoury,[1] Christer Jansson,[1] Victoria Orphan,[2] and Sam Hazen[3]

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Research Plans and Progress: This project is developing a novel next-generation spectral imaging platform to image and identify biomolecules involved in both microbial and plant metabolic processes through different chemical contrasts and across multiple length and time scales. The spectroscopic methods include a combination of Raman and fluorescence scattering, as well as reflected/transmitted light extinction spectroscopy in a single optical setup. This technology will be tested on two model systems of relevance to BER's bioenergy and environmental microbiology research thrusts. Using the first system, the team will explore the role of microbial communities in controlling carbon cycling. Through the second system, the team will strive to advance the existing knowledge of plant metabolic processes influencing cell wall composition, synthesis, function, and deconstruction for dedicated bioenergy biomass crop development. In both cases, the team anticipates that the proposed first-of-its-kind BioImager will significantly advance the existing fundamental understanding of basic metabolic processes in live microbial and plant systems.

Current and Anticipated Accomplishments: To date, the team has constructed the multimodal spectral imaging platform and has dedicated personnel to further develop the system and run measurements on a day-to-day basis. The setup is equipped with three optical axes (top, bottom, and oblique) that support optical absorption/dark field, fluorescence, and Raman imaging using various light sources including (in)coherent white light and several narrow-band lasers. With this setup, the team has been able to detect absorbance signals less than 10 mOD at low incident powers (few microwatts) using integration times on the order of 5 milliseconds per pixel in a point scanning hyperspectral imaging scheme. While one can get full point spectra every 5 milliseconds, delayed stage-detector communication currently limits the collection of spatiospectral images to about one minute per full frame (50 x 50 pixels). Therefore, the team incorporated a line-scanning detector into the setup, whereby hyperspectral images are recorded line by line. This approach affords recording full hyperspectral image cubes in a matter of seconds, particularly for hyperspectral optical absorption. Furthermore, the hyperspectral optical imager is now also equipped with an atomic force microscope (AFM) to allow correlated, colocalized multimodal hyperspectral imaging and topographic atomic force microscopy measurements. The team is currently implementing software changes that will allow for simultaneously recorded spectral images and topography (via AFM).

In addition to instrument development, the team is benchmarking its system on single-cell systems and plant cell wall composition. The team is growing *Brachypodium* mutants from seeds in hand and will be chemical imaging and analyzing these samples over the next few months. Team members are also generating sample sets for the anaerobic methane-oxidating archaea and sulfate-reducing bacteria microbial community symbionts.

Potential Benefits: The project anticipates the platform will result in unprecedented molecular-level insight into metabolic pathways in microbial communities and plants. This imaging technology will be able to nondestructively image biomolecules in their native environments, from the macroscale in plants down to nanoscale in microbes. Achieving this goal will provide technology critical to the understanding of the intra- and intercellular metabolic controls in plants and microbes relevant to carbon cycling, bioenergy production, and biogeochemical transformations and will allow researchers to accelerate engineering of plants and microbial communities for improved performance. By leveraging the capabilities of the BioImager, the project would be able to build a knowledgebase positioned to deliver information for modeling biomass accumulation in plants under future climate scenarios and for genome-engineering approaches aimed at improving the yield and/or saccharification potential of lignocellulosic biomass.

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