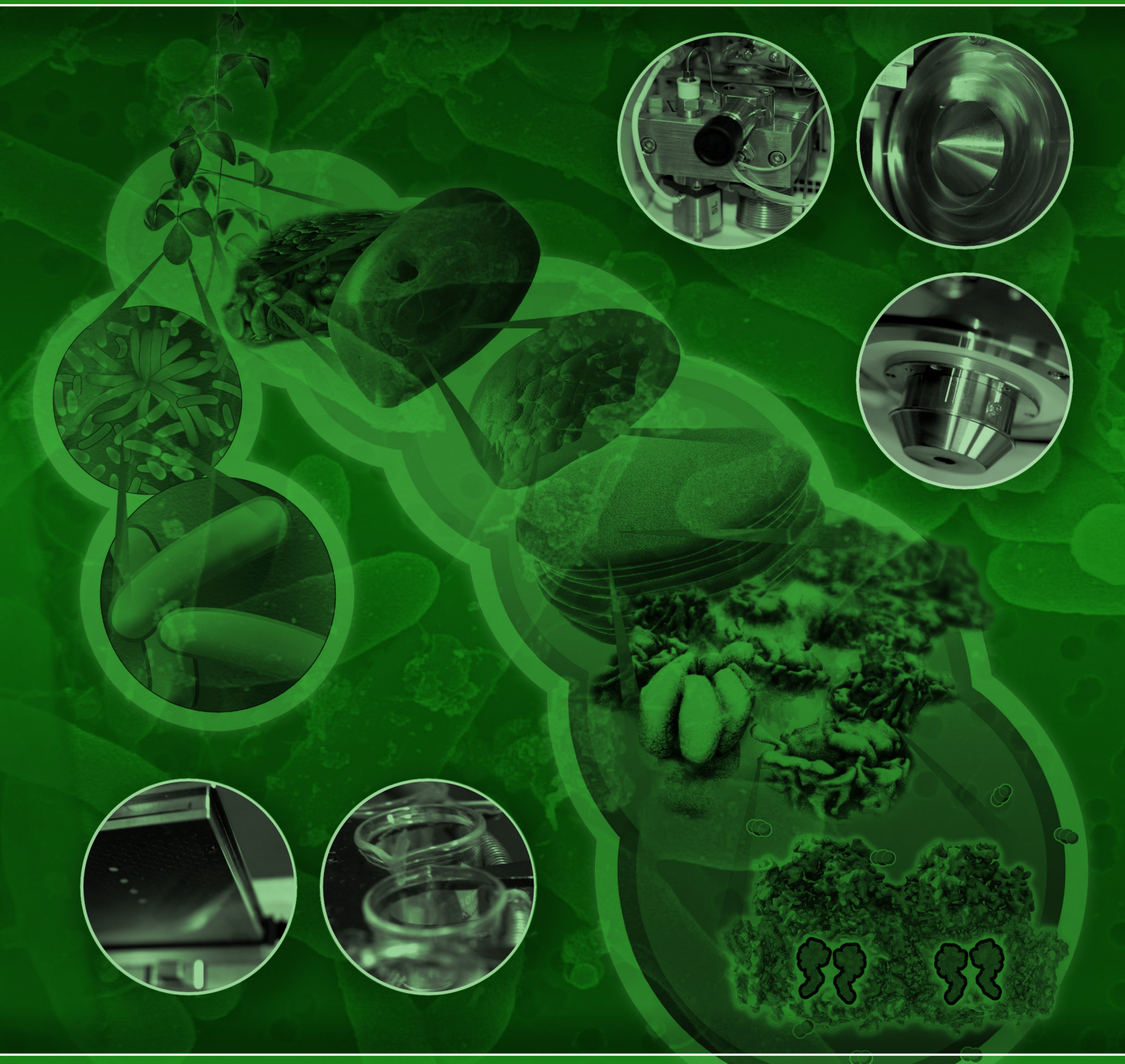
Mesoscale to Molecules:
Bioimaging Science Program
2018 Principal Investigator Meeting Proceedings



U.S. DEPARTMENT OF ENERGY | Office of Science

Office of Biological and Environmental Research

Mesoscale to Molecules: Bioimaging Science Program

Principal Investigator (PI) Meeting

February 28–March 1, 2018

Gaithersburg, Maryland

Program Manager

Prem C. Srivastava
Office of Biological and Environmental Research
Office of Science
U.S. Department of Energy

Meeting Co-Chairs

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Cover illustration: Metabolic processes integrate genetically programmed molecules into structures that span different physical scales (left: microbe and microbial community, right: plant). Imaging and measurement technologies that can resolve multiple key metabolic processes over time within and among cells will enable the linking of molecular-scale information to whole-cell, systems-level understanding. [Plant component image adapted from Iowa State University figure, p. 2, of BER. 2015. *New Bioimaging Approaches for Plant and Microbial Systems*. Microbe and microbial community images adapted from Fig. 2.1 of BERAC. 2013. *BER Virtual Laboratory: Innovative Framework for Biological and Environmental Grand Challenges; A Report from the Biological and Environmental Research Advisory Committee*, DOE/SC-0156. Instrument photos courtesy Basil Nikolau, Iowa State University.]

These proceedings are available at science.energy.gov/ber/community-resources/2018_Bioimaging_Program_PI_Meeting.pdf.

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Preface

The 2018 annual Mesoscale to Molecules: Bioimaging Science Program Principal Investigator (BSP-PI) Meeting was held February 28–March 1, 2018, at the Sheraton Tysons Hotel, Tysons Corner, Virginia. The program's mission is to understand translation of genomic information into the mechanisms that power living cells, communities of cells, and whole organisms. The goal of BSP is to develop new imaging and measurement technologies to visualize the spatial and temporal relationships of key metabolic processes governing phenotypic expression in plants and microbes.

The program convenes annual PI meetings to bring together its contributing investigators to review progress and current state-of-the-art bioimaging research. This year's BSP-PI meeting was held consecutively at the same venue with the Genomic Science Program PI meeting. Holding the two PI meetings conjointly, provided a platform for networking, stimulating discussions, and exchange of ideas, helping to forge new collaborative alliances among investigators from the two sister programs. Important highlights of the meeting were two keynote presentations: "Advanced Fluorescence Microscopy for Opaque Samples," by Dr. Na Ji, University of California, Berkeley, and "The Promise and Challenges of 3D Super-Resolution Optical Microscopy and Single-Molecule Tracking in Cells as Probes of Structure and Dynamics," by Dr. William E. Moerner, Stanford University. The meeting's proceedings provide an outline of the program's current state and potential future directions and opportunities.

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List of Funded Projects

Universities

Multifunctional Plasmonics Nanoprobes for Cellular Sensing and Imaging

Tuan Vo-Dinh, Duke University

Integrated and Dynamic Multispectroscopic *In Situ* Imaging of Plant Metabolism at the Level of Subcellular Components

Basil Nikolau, Iowa State University

Development of Biosensors to Measure the Spatial and Temporal Concentration Profiles of Inorganic Phosphate in Plants During Arbuscular Mycorrhizal Symbiosis

Wayne K. Versaw, Texas A&M University

Multiscale Dynamics of Water Regulation by Bacteria in Synthetic Soil Microsystems

Leslie M. Shor, University of Connecticut

Development and Refinement of an *In Situ* "Molecular Microscope" Utilizing Ultrahigh-Resolution Mass Spectrometry

Gary Stacey, University of Missouri, Columbia

The Transparent Soil Microcosm: A Window into the Spatial Distribution and Dynamics of Carbon Utilization and Microbial Interspecies Interactions

Elizabeth Shank, University of North Carolina at Chapel Hill

Development of a Novel High-Precision SIMS Platform for Elemental and Isotopic Characterization of Microbial Cells at a Systems Level

David Fike, Washington University in St. Louis

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Emily Smith, Ames Laboratory

Small Worlds

Ken Kemner, Argonne National Laboratory

Systems Biology Through an Integrated Multimodal Imaging and Analysis Framework

James Evans, Pacific Northwest National Laboratory

Multimodal Nanoscale Chemical Imaging of Intra- and Inter-Cellular Biomolecules Involved in Microbial Interactions

Scott Lea, Pacific Northwest National Laboratory

Executive Summary

The Mesoscale to Molecules Bioimaging Science Program (Bioimaging Program), within the Department of Energy's (DOE) Office of Biological and Environmental Research (BER), sponsors research at national laboratories and universities that addresses grand challenges for biology. These projects are centered on developing a better understanding of the spatial and temporal distributions of key metabolites and biomarkers associated with microbial and plant systems related to bioenergy and environmental systems. The Bioimaging Program is developing new and improved instruments, which are being designed and constructed from the ground up, adapted from other scientific disciplines, or upgraded with new capabilities to create a comprehensive and versatile toolbox for imaging biological dynamics and chemistry. These approaches involve (1) hypothesis-based approaches focused on solving particular problems for plant biology, biofuels, and biogeochemistry and (2) broad-based systems and design-based technologies that can lead to new discoveries. Scientists developing both approaches keep in mind that versatility, transferability, and portability will help foster widespread adoption by the larger science community for future field and laboratory studies.

Modalities spanning electron, ion, optical, Raman, and X-ray microscopy and spectroscopy are being used with dynamic, *in situ*, cryogenic, or fixed samples. Analysis includes both nondestructive and destructive approaches for holistic bioimaging. A major focus for the Bioimaging Program comprises new optical developments advancing multimodal optical and fluorescence microscopy and Raman microspectroscopy. These activities are complemented by innovative applications of electron and ion microscopy as well as X-ray absorption spectroscopy and microscopy and femtosecond crystallography at DOE-sponsored user facilities. Further enhancements of imaging mass spectrometry capabilities are yielding highly specific, sensitive, and quantitative chemical maps that identify intra- and extracellular gradients. These maps also determine the distribution; abundance; and fate of stable isotopes, natural elements, and metabolites. This work supports simultaneous observations using conventional microscopies for correlated structural and chemical imaging and the interpretation of biological function.

Complementary novel fluidic devices are creating controlled environments to visualize how biosystems sense and respond to environmental changes. New tracers, probes, and sensors are further expanding the impact of the new instrumentation and fluidic devices by allowing dynamic tracking of targeted cells, organelles, enzymes, biomarkers, and small molecules. To capture different spatial resolutions afforded by the various techniques, researchers are pursuing label-based and label-free imaging approaches to visualize complex pathway dynamics occurring in living biosystems while also detecting chemical signatures at different scales. These new technologies will help track cellular responses to environmental perturbations, spatially localize complex mechanistic pathways, and visualize structural dynamics of macromolecules.

In addition to pursuing advances within each of these techniques (e.g., optical, electron, X-ray, and ion-based approaches), the program should continue to focus on integrating these different approaches either in hybrid all-in-one instrumentation or by linking the resulting complementary data from multiple instruments. Such cross-platform imaging requires the incorporation of methods that permit indexing and registration of images (e.g., multifunctional tracers, probes, and sensors to act as cross-platform fiducial markers). This cross-platform approach will enable disparate datasets of different formats, magnifications, or resolutions for the same sample to be overlaid and co-referenced in a meaningful fashion. Successful realization of all the technologies described above will produce a diverse set of bioimaging data. Researchers recognize that integrated data processing algorithms, visualization, and modeling are key components for properly interpreting and connecting these imaging data to omics-based models of the organisms or the pore-scale lattice Boltzmann models and root-scale water flux models being developed to describe these biological systems.

Finally, biological imaging is inherently transdisciplinary; thus, successful teams should continue to reflect this approach by integrating research communities such as imaging, technology development, nanoscience, and computation with those of structural biology, genomic science, ecology, plant science, and biogeochemistry. Transdisciplinary research will enable scientists to take critical steps toward translating laboratory-developed technologies into applications for the natural environment. This process will address the need to dynamically image complex, and often unknown, native microbial populations, allowing scientists to investigate community organization, interorganismal relationships, and co-occurrences of multiple metabolisms and processes in space and time.

Program Summary

Since its inception, BER's Mesoscale to Molecules Bioimaging Science Program has sponsored research at 7 national laboratories and 16 universities across the United States. All the projects are working toward addressing a grand challenge for biology—to enable *in situ* and dynamic bioimaging across a range of spatial and temporal scales. Because no single instrument spans the complete spatiotemporal landscape for the bioimaging needs of bioenergy and the environment, the program is developing and employing multiple new instruments, methods, and techniques to bridge current gaps.

At the heart of any bioimaging approach platform is core instrumentation. New instruments are being developed, and existing instruments are being applied (or adapted

from other scientific disciplines) and upgraded to complement other modalities. With this core, the program strives to create a versatile and powerful toolbox for imaging biological dynamics and chemistry occurring across scales ranging from small unicellular organisms to complex microbial and fungal community interactions with plants. Due to their noninvasiveness and ability to record dynamic events, in many cases nondestructively, optical methods have dominated past use of *in situ* bioimaging methods. Consequently, new developments will advance confocal, super-resolution and subdiffraction, interferometric, multifocal, hyperspectral, and infrared scanning near-field optical microscopies, as well as Raman microspectroscopy (e.g., spontaneous, far-field subdiffraction, surface-enhanced scattering, tip-enhanced, and stimulated or coherent anti-Stokes scattering). All are a major focus for the Bioimaging Program. Both label-based and label-free imaging approaches are being commissioned to visualize complex pathway dynamics occurring in living biosystems while also detecting chemical signatures.

Other approaches in which scientists have achieved important progress include X-ray absorption spectroscopy, nanotomography and macromolecular femtosecond crystallography involving the DOE-sponsored synchrotron and X-ray free electron laser (XFEL) facilities. Under development are correlative X-ray fluorescence and X-ray transmission microtomography for imaging bacterial distributions within opaque soil aggregates. Enabling insights into metabolite identities, spatial distributions, and rates of metabolic activity with single-cell spatial resolution are *in situ* elemental, isotopic, and molecular imaging [e.g., atmospheric pressure laser ablation electrospray ionization (LAESI) and matrix-assisted laser desorption ionization (MALDI), 21 Tesla Fourier transform ion cyclotron resonance, and secondary ion mass spectrometry (SIMS)].

Along with new instrumentation that provides the framework for capturing dynamic movies or time-lapsed images, new fluidic devices are enabling higher-throughput and reproducible *in situ* microscopy studies designed to visualize how biosystems sense and respond to induced perturbations such as nutrient starvation and chemical exchanges. Also being employed are synthetic rhizosphere microhabitats, transparent soil microcosms, and versatile nanofluidic and microfluidic imaging and sampling devices that permit simultaneous cultivation and analysis of biosystems, from single cells to complex communities. In many of the instruments described above, these fluidic devices are empowering *in situ* or correlative observations simply by creating controllable chambers in which biosystems can thrive while the chambers protect the sample from inherent instrument constraints. For example, the need for many electron, ion, and X-ray microscopes to maintain high-vacuum conditions that optimize imaging beam stability and resolution typically prevents *in situ* observations. However, the incorporation of fluidic devices into these imaging modalities is heralding new avenues of research. Simultaneously, quantitative tracking and comparisons being performed on multiple strains in equivalent environments during controlled chemical exchanges or depletions are providing insight into specific protein expression levels as a function of nutrient availability.

Finally, new tracers, probes, and sensors are expanding the impact of the new instrumentation and fluidic devices described above by allowing dynamic tracking of targeted organelles, enzymes, and small molecules. Now being designed, engineered, and implemented are inverse molecular sentinels, caged Raman tags, fluorescent probes, functionalized quantum dots, engineered fluorescent proteins, optogenetic regulators, and Förster resonance energy transfer (FRET) inorganic phosphate and root exudate biosensors. The ultimate goal is to sense and track lipids, metabolites, enzyme activity, mRNA, and microRNA in living biosystems, in correlation with controlled changes to the local chemical or physical environments or using light-based control of expression. A common challenge in tracking the fate and distribution of metabolites in biological systems is clearly the small size and varied nature of biomolecules. In many cases their small size prevents physical labeling with extrinsic tags, while their relatively rapid transit and broad concentration range complicate analytical measurements. Thus, considerable efforts are ongoing to minimize or avoid any alteration of normal cellular function or mechanisms of small-molecule uptake, ultracellular localization, metabolism, or fate when exposed to novel tracers, probes, and sensors, as well as to develop untargeted and unlabeled approaches.

While any one of the platforms described above can generate highly informative data of biosystem dynamics, those recognized as significantly more powerful are the multimodal, correlative, and integrative approaches. New hybrid instruments in development allow simultaneous acquisition of dynamics, chemistry, topology, and ultrastructure within the same instrument, and most of these projects involve multimodal or correlative imaging using a suite of separate instruments. Modalities spanning electron, ion, optical, Raman, and X-ray microscopy and spectroscopy are being used with dynamic, *in situ*, cryogenic, or fixed samples and analyzed with both nondestructive and destructive approaches for holistic bioimaging. A requirement for such cross-platform imaging is the incorporation of methods that permit indexing and registration of images to enable meaningful superimposition and co-registration of disparate datasets of different formats, magnifications, chemical specificities, or resolutions for the same sample. Multifunctional tracers, probes, and sensors are being actively exploited as cross-platform fiducial markers that provide both direct indexing and registration. For example, glycine-conjugated quantum dots are helping to identify a rhizospheric microbial community structure and environmental ligands because they are detectable by fluorescent, electron, and X-ray imaging modalities.

Realizing all the approaches described above will produce a diverse set of bioimaging data. Integrated visualization and mod-

eling are recognized as key components for properly interpreting and connecting these imaging data to omics-based models of the organisms or pore-scale lattice Boltzmann models and root-scale water flux models. Such models are expected to improve the mechanistic understanding of how biological systems not only are impacted by their environment, but also directly change and modify the larger ecosystems of which they are a part.

Combined in a comprehensive portfolio, the resulting capabilities will enable the imaging of key metabolites and molecular biomarkers (e.g., proteins, mRNA, and miRNA) across the hierarchies and dimensions of biological systems to provide understanding of a diverse array of biological and environmental processes. Although the primary focus of the Bioimaging Program is to expand bioimaging frontiers, the program also plans to validate new technologies through real-world biological applications relevant to bioenergy and the environment. Examples are understanding and controlling quorum sensing, improving lipid feedstock yields, enhancing lignocellulosic deconstruction, or boosting feedstock sustainability and plant drought tolerance. Many organisms are being investigated to understand nutrient utilization and community and ecosystem interactions such as soil water retention due to the presence or absence of particular organisms or biomass. Among these organisms are (1) living plants (e.g., *Medicago truncatula* and *Brachypodium distachyon)* and (2) microbes [e.g., chemotrophs (*Bacillus subtilis* and *Yarrowia lipolytica*), phototrophs (*Cyanothece*, *Rhodopseudomonas palustris*, *Ostreococcus tauri*, and *Chlamydomonas reinhardtii*), and methylotrophs (*Methylobacterium*)], as well as plant-microbe interactions (e.g., arbuscular mycorrhizal symbioses and *Glycine max* with *Bradyrhizobium japonicum*). These studies will lead to a better understanding of spatial and temporal metabolite distributions associated with growing microbial and plant systems.

Recommendations and Potential Future Directions

Biological imaging is inherently transdisciplinary, and the research and development process aimed at advancing DOE programmatic goals for the Mesoscale to Molecules Bioimaging Science Program needs to continue to reflect this approach. Advances in biological imaging require integrating the expertise of the imaging community with that in the technology development, nanoscience, and computational communities. Moreover, reaching across biological disciplines is essential to incorporate structural biologists and genomic scientists; connect phenotyping and genotyping; and integrate imaging results with the corresponding genomic, proteomic, lipidomic, and metabolic changes within cells. Similarly, coordination with ecologists and biogeochemists is needed to better mimic the intrinsic multiphase complexity of soils in laboratory experiments as scientists move toward deploying imaging approaches in the natural environment.

The Bioimaging Program spans a broad spectrum of approaches, including complementary targeted and untargeted, destructive and nondestructive imaging modalities (e.g., optical, electron, scanning probe, X-ray, and ion-based approaches) that cover various spatial and temporal scales. In addition to pursuing advances within each of these respective techniques, a major focus of the program moving forward should center on the integration of these different approaches. These linkages of the resulting complementary data will create a more holistic picture of the biological systems being imaged. There also is a critical need to develop selective probes that allow identification, sensing, and functional imaging of various targets ranging from key metabolites to molecular and genomic biotargets (e.g., mRNA and miRNA) in complex biological systems. Relevant key advances include the simultaneous marking, tracking, and sensing of multiple players (e.g., elements, isotopes, metabolites, and other molecular biomarkers) in a given biological system. Compiling these capabilities will also provide the essential flexibility to broaden the scope of investigations, opening new possibilities to discover still 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 particular organisms could provide unique means to investigate and understand complex native communities.

A major near-term challenge is translation of laboratory-developed approaches into applications for the natural environment. This process will involve the need to incorporate the dynamics of microbially driven biogeochemistry (e.g., within the rhizosphere, biofilms, and other key biological interfaces). The ability to image complex native microbial communities will enable deciphering of their organization and multiple metabolic processes co-occurring in space and time. Essential components include the development of approaches to harness inherent signals within nontractable microbes in the environment and the creation of pathways for microbial synthesis of probes for assaying function and activity.

The ultimate goal of such an approach is to generate spatially resolved snapshots of relevant cellular metabolism, including both primary and secondary metabolites, as well as internal and secreted compounds. The real-time collection and interpretation of these integrated data will constitute a major advance in bioimaging technology—one that will provide new understanding for monitoring and understanding phenotyping in the laboratory and in complex natural environments. This advancement will require new approaches and algorithms to handle increasingly challenging volumes of data and automated means to sift through the data to identify biologically and environmentally meaningful signals. Also needed are new models capable of incorporating multimodal data from across a range of spatial and temporal scales to most effectively extract causality from observations and understand the emergence of complex phenomena.

To reach these goals, advances in several areas are needed, including these key developments:

1. Advanced probes that expand the monitoring capability for important biotargets ranging from key metabolites to molecular and genomic biomarkers.

2. Associated delivery mechanisms (e.g., micro- and nanofluidics) to more comprehensively sample key compounds and biomolecules.

3. New or improved imaging technologies capable of watching systems grow and evolve in their natural state, while acquiring real-time data across the full spectrum of relevant spatial scales.

4. Cross-platform protocols for sample preparation; indexing and spatial registration; and cross-platform calibration, data verification, and correlation to increase the suite of complementary analyses that can be conducted on a given sample or suite of samples.

Along with the aforementioned developments, improvements are needed in data storage, processing, and visualization to enable effective extraction of critical biological and environmental information from the experimental data. Of special 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 Knowledgebase (KBase) system and take advantage of advances in artificial intelligence designed to extract patterns from raw data for improved organization, interpretation, and representation.

The annual BER Bioimaging Program's principal investigator (PI) meeting provides a useful platform for the program to increase horizontal (cross-platform) and vertical (cross-scale) synergies needed to achieve its goals. Scheduling this meeting proximal to the DOE Genomic Science Program annual principal investigator meeting creates invaluable synergies, and the inclusion of outside expert speakers ensures the injection of novel perspectives and approaches into discussions. Additional cross-team interactions (e.g., through teleconferencing or web conferencing) will help maintain this interactive momentum and catalyze new directions of investigation. Moreover, the creation of a Bioimaging Capability portal, detailing the diverse technological approaches and highlighting the applications for which they are best suited, would enable the program to impact a wider community of scientists who could make use of new bioimaging approaches for their research. In particular, the creation of a "boot camp" (e.g., appended to relevant annual conferences) that targets young PIs, postdoctoral scholars, and graduate students from outside the current pool of DOE-affiliated researchers could dramatically expand the reach of the Bioimaging Program and have a transformational impact on the training of the next generation of scientists.

Abstracts

Small Worlds

Principal Investigators: Ken Kemner and Mark Hereld
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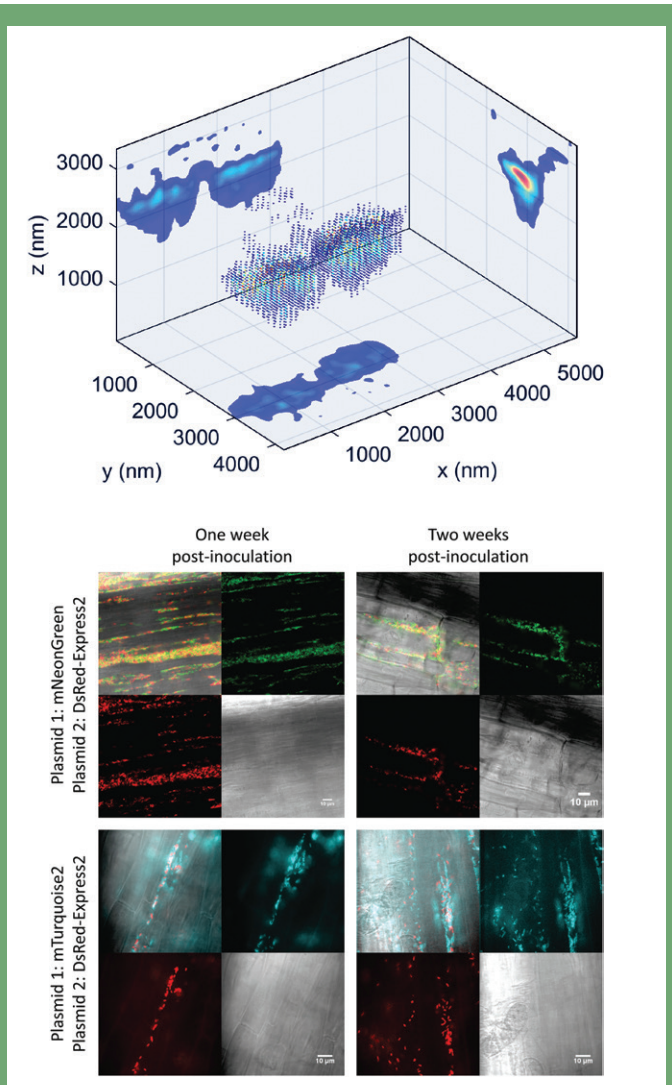
Research Plans and Progress: To develop a new multimodal imaging capability for studying complex multiagent processes in cells and systems of cells across physical and temporal scales. A scientific driver of understanding detailed interactions among synergistically functioning organisms, particularly bacteria and roots, provides a focus for imaging technology development that will enable the development of models that make it possible to enhance the growth and health of a wide range of plants and to image microbial structure and function within soils. To create this new experimental capability, the project is developing *three-dimensional* (3D), *multimodal imaging systems* capable of targeting several elements of a process at once. The combination of these imaging systems with supporting (1) *multiagent molecular sensors* and (2) *software* for image reconstruction, volumetric data fusion, and quantitative analysis will enable scientists to target complex processes in a wide range of biological systems.

Current and/or Anticipated Accomplishments/Deliverables: Progress to date includes: (1) introduction of multiple self-labeling fluorescent proteins within multiple rhizobacteria species and cloning of metabolite sensors for root exudates and reactive oxygen species sensing, (2) initiation of the development of a 3D X-ray fluorescence tomography microscope for imaging quantum dot–labeled bacteria within opaque soil aggregates, (3) development of root-microbe microfluidic interaction chamber integrated into optical microscopes, (4) development of microscope for video rate capture of 3D inter- and intracellular dynamics from snapshot frames, (5) development of computational algorithms for reconstructing 3D volumes from snapshots, (6) demonstration of 2 nm precision 3D particle tracking using interferometric microscopy, and (7) method for preparation and processing of robustly labeled root-microbe samples for correlative fluorescence and tomographic electron microscopy.

Potential Benefits/Applications: A platform for studying a range of complex dynamic processes in cellular and intercellular systems in 3D in transparent and opaque media. This platform will systematize creation of sensor systems capable of simultaneously tracking, sensing, and controlling several aspects of a complex process in a single experiment. It also will enable correlation of image volumes by providing nanoscale markers (quantum dots) that function across modalities.



(Top) Results from a reconstruction of 3D snapshot multifocus microscopy (MFM) image of a *P. fluorescens* SBW25 bacterium chemically fixed during late stages of cell division. Courtesy Xiang Huang, Argonne National Laboratory (ANL). (Bottom) Confocal and brightfield images of 1 week– and 2 week–old tomato roots colonized with *P. fluorescens* SBW25 double labeled with mini-Tn7 transposon and plasmid. The fluorescent protein expressed by each genetic component is indicated on the left side. In each montage, the lower right square is the brightfield image, the lower left is the DsRed-Express2 image, the upper right is the mNeonGreen image, and the upper left is the overlay of all three channels. Courtesy Rose Wilton, ANL.

Multifunctional Plasmonics Nanoprobes for Cellular Sensing and Imaging

Principal Investigators: Tuan Vo-Dinh[1] (PI), Zhen-Ming Pei,[1] Tai-Ping Sun,[1] and Kenneth Kemner[2]
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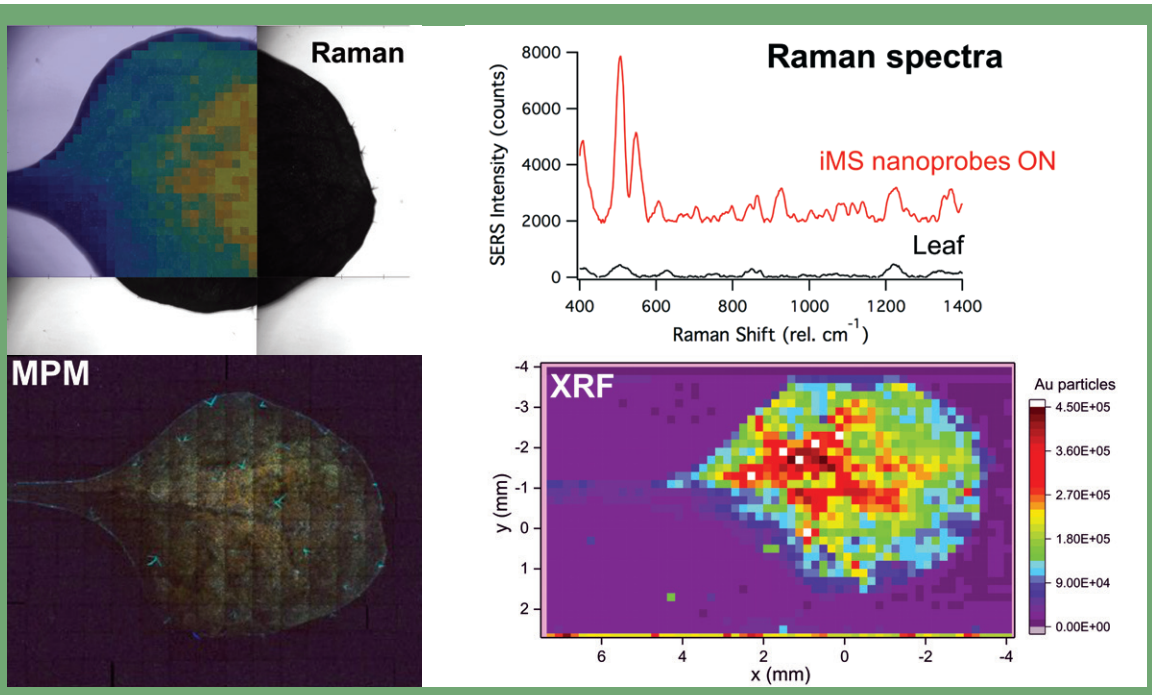
Project Summary: The goal of this project is aimed at addressing the DOE FOA need to "develop and apply selective, nonperturbative probes to measure the spatial and temporal concentration profiles of nutrients, metabolites, signaling molecules, extracellular matrices, and other biomolecules," particularly as they are related to studies of plant and microbial biosystems relevant to DOE bioenergy programs.

1. Research plans and progress, including objectives and goals for the project period. Our team has developed multifunctional plasmonics-active nanoprobes, called inverse molecular sentinel (iMS), for the detection of important plant biomarkers such as miRNAs (e.g., miR156, which regulates plant flowering time) relevant for biofuel research. We have successfully performed *in vivo* imaging of iMS nanoprobes in whole plant leaves. Multimodal imaging of iMS nanoprobes was obtained by combining X-ray fluorescence microscopy (XRF) and multiphoton microscopy (MPM) with Raman spectroscopy. XRF and MPM were used for the localization of the nanoprobes in whole leaves, while surface-enhanced Raman scattering (SERS) imaging was used for the detection of miRNAs through the iMS nanoprobes. The functional imaging capabilities of the nanoprobes were demonstrated by recording SERS maps and localization of the iMS nanoprobes infiltrated in leaves with and without target miRNAs.

The figure below shows the data obtained from a leaf infiltrated with iMS nanoprobes and miRNAs. In this figure, the MPM and XRF images provide the physical locations of the iMS nanoprobes and the Raman functional map exhibits the SERS signal from the iMS turned on by the presence of miR156. The results demonstrate the possibility of SERS monitoring and imaging the presence of miRNA targets in whole plant leaves.

2. Current and/or anticipated accomplishments/deliverables for the project period. We have successfully demonstrated the possibility of using the SERS sensing and imaging technology for sensing the inverse molecular sentinels in detecting specific nucleic acid biotargets in whole plant leaves. Currently, we are applying the iMS nanoprobe technology for direct detection of miRNAs in transgenic plants *in vivo*. This knowledge will provide significant insights into novel ways to manipulate plant growth to increase biomass for renewable energy sources.

3. Potential benefits/applications of DOE-funded research for DOE and dissemination and deployment of bioimaging technology to public and private sectors for generic biological imaging use by the broader scientific community. This study will contribute significantly to effective strategies in order to better control flowering time and increase biomass accumulation for biofuels and crops. MiRNAs play important roles in regulating diverse developmental processes in plants. Recent studies showed that alteration of specific miRNA expression could result in increased biomass and/or lignin content of the plants. It is important to monitor and understand the regulation of these miRNAs during plant development in order to optimize alteration of their expression to increase lignocellulosic biofuels.



(Top left) SERS mapping of iMS nanoprobes in plant leaf. (Top right) SERS signal of iMS nanoprobes turned on by miRNA156. (Bottom Left) Multiphoton microscopy (MPM) luminescence imaging showing the iMS gold nanostars in plant leaf. (Bottom right) XRF image localizing the iMS nanoprobes in plant leaf.

Integrated and Dynamic Multispectroscopic *In Situ* Imaging of Plant Metabolism at the Level of Subcellular Components

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This project is applying integrated molecular imaging technologies to monitor membrane lipid remodeling in plant cells, which affect photosynthetic energy capture and hence is a major determinant of plant biomass productivity. The molecular imaging technologies are being applied to specifically image the remodeling of membrane lipid topologies, which occur during the process of autophagy. During this process, the spatial distribution of subcellular regions within plant cells is reprogrammed to accommodate the assembly of a new organelle, the autophagosome, during an environmentally induced stress. The imaging technologies combine fluorescence, Raman, and mass spectroscopy to image the cellular and subcellular level distribution of specific target lipid molecules. And these imaging technologies are being developed in the context of a computational platform that integrates multispectral imaging with genome-scale models. An integrated strategy to address the goals of the project are addressing project-defined tasks as indicated below.

Task 1: Genetic and biochemical analysis of defined autophagy and lipid metabolism genes. We have focused on identifying specific lipid molecules that are affected by the autophagy-induced dynamics of cellular membranes. These specific lipid molecules are the targets for imaging via the technologies developed in Tasks 2–4. To date we have completed the lipidomics analysis of a large collection of mutants that express changes in the autophagy pathway and have thereby identified specific target lipids for molecular imaging. In parallel, RNA-seq analysis of these mutants is in process to define the genes and pathways that are responsible for the autophagy-induced lipid changes.

Task 2: Develop and apply *in situ* optical imaging platforms. We have completed a study that used multimodal Raman and mass spectrometric imaging methods to evaluate the cellular-level biochemical changes that occur from silencing the phytoene desaturase (*pds*) gene. The multimodal imaging method localized carotenoid distribution and revealed spatial differences that are normally lost in the spatial averaging that occurs when carotenoids are analyzing via established extraction-based methods. Because Raman and mass spectrometric signals are complementary, we gained access to high–spatial resolution data via the Raman signal and high-resolution chemical data via the mass spectrometric signal.

Task 3: Chemical synthesis and tuning of self-destructing fluorophores for the *in situ* visualization of dynamic events. A new class of fluorescent chemical imaging probes capable of *in situ* imaging have been synthesized. In particular, we have designed and synthesized a new class of photocages derived from BODIPY dyes capable of dynamic fluorescence imaging using visible light. These probes release compounds with visible light irradiation with wavelengths spanning the visible and entering the near-IR. The team has demonstrated a versatile photocage that can be easily linked to nucleophile-containing molecules to generate a probe that targets specific cellular organelles. Furthermore, novel photoactivatable BODIPY compounds have been developed that allow super-resolution imaging (i.e., stochastic optical reconstruction microscopy) of cellular structures. These probes can be activated and bleached with a single low-power visible laser, making them ideal for *in situ* imaging of living cells.

Task 4: Spatial mapping of metabolites via mass spectrometry. MALDI-based mass spectrometric imaging has been used to visualize the specific lipids that change in response to autophagy, in order to correlate with the lipidomics data defined in Tasks 1 and 2. The second component of this effort has focused on optimizing the performance of atmospheric pressure mass spectrometric imaging and applying the imaging technology to spatially map metabolites. These optimizations have reduced the laser spot size to approximately 50 µm, as compared to the 125 µm spot size that was available at the start of the project. Large-scale application studies of key metabolites, such as lipids, involved in autophagy of *Arabidopsis* are under way.

Task 5: Develop computational imaging visualization platform. We have created a visualization platform that makes imaging data accessible to the broader research community in different fields of chemistry, chemical engineering, biochemistry, and biology. Researchers can readily access, comprehend, and explore the data obtained from the combined and diverse analytical chemistries in the context of the biological materials under study. Mass-spectrometry, fluorescent, and optical images can be interactively visualized, with comparisons across molecules and biological conditions. The platform is set to integrate pioneering technologies including photocage visualization.

Systems Biology Based on an Integrated, Mesoscale Imaging and Analysis Framework

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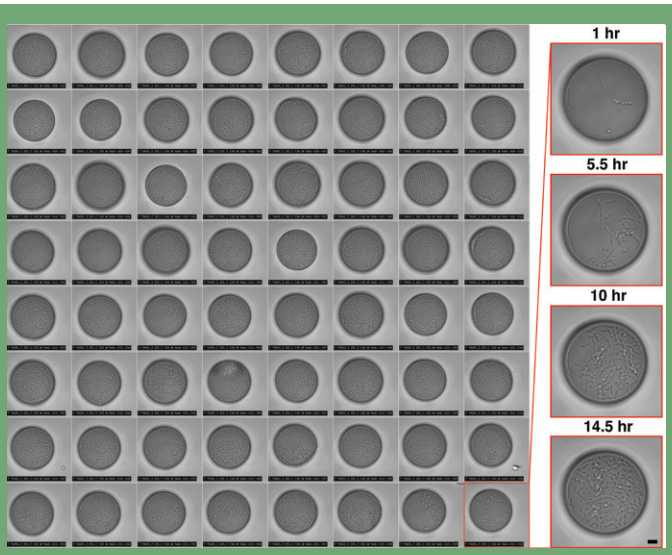
Co-PIs: William Cannon, Ryan Kelly, and Aaron Wright

Research Plans: Our project aims to develop innovative instrumentation, novel chemical probes, and new correlative imaging and modeling technologies to yield an unrivaled view of biosystem dynamics when combined with omics analyses. Specifically, we plan to: (1) construct a transmitted and back-scatter Helium Ion Microscope for simultaneous observation of cell ultrastructure and topology; (2) manufacture new nanofluidic and microfluidic devices to enable higher-throughput and reproducible *in situ* electron and optical microscopy studies; (3) synthesize selective probes for multimodal microscopy applications; (4) perform correlated *in situ*, dynamic, and cryogenic bioimaging and systems biology analysis to gain an all-inclusive view of cellular response to changing environments; and (5) build an iterative modeling and experimental approach to inform and validate biodesign principles.

Accomplishments/Deliverables: This project is driving advancements in (1) nanofluidic devices with 25× larger field-of-view and patterned features for improved window stability and low-dose focusing aides; (2) directed flow 3- and 5-port nanofluidic platforms empowering controlled mixing for dynamic *in situ* electron microscopy; (3) microfluidic devices for (a) filamentous organisms and (b) unicellular organisms in controlled compartmentalized and chemostat modes; (4) multimodal/multifunctional reporter probes for cross-platform bioimaging; (5) metabolic models harnessing a statistical thermodynamic simulation and optimization approach to predict fluxes and metabolite level; (6) new imaging modes and simplified sample preparation for Helium Ion Microscopy; (7) remote cryo-EM data collection and continuous rotation micro-electron diffraction capabilities; (8) a cell-free expression pipeline providing samples for structural biology; and (9) robotic cell loading into micropatterned nanowells for automated time-resolved bioimaging with improved statistics (see figure).



Automated bioimaging in controlled compartmentalized environments. An 8×8 array of 0.5 nanoliter droplets is shown with a time series for one nanowell highlighted to the right. This represents only a fraction of the larger 20×24 array of droplets that are robotically deposited with cells and then imaged automatically with preset delays. In this experiment, yeast cells were imaged every 4.5 hours for over 4 days but only the first 4 frames of nanowell location X15-Y10 are depicted to simplify visualization. Scale bar: 10 microns.

Potential Benefits/Applications: Once realized, the technologies developed in this project will help track cellular responses to environmental perturbation, spatially localize mechanistic pathways, and visualize structural dynamics of macromolecules. The overall portfolio will be extensible to broad biological applications spanning the interrogation of bioenergy feedstock production, complex microbiome architecture, interactions within the rhizosphere, and even biomedicine. In addition to disseminating results through publications, we have also initiated an outreach program to transfer fluidic devices to external collaborators for rapid adoption of these new and maturing technologies. Ultimately, most of the capabilities will be folded into the Environmental Molecular Sciences Laboratory (EMSL) user program including the stand-alone CARS/SRS confocal microscope and cell-free expression pipeline.

Development of Biosensors to Measure the Spatial and Temporal Concentration Profiles of Inorganic Phosphate in Plants During Arbuscular Mycorrhizal Symbiosis

Principal Investigators: Wayne K. Versaw (PI) and Maria J. Harrison (co-PI)
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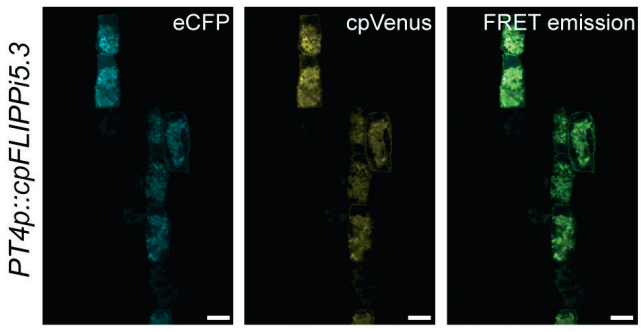
Research Progress: During the current project period, we completed the generation of two sets of inorganic phosphate (Pi) biosensor constructs for expression in a dicot, *Medicago truncatula*, and a monocot, *Brachypodium distachyon*. These include biosensors driven from a constitutive promoter and from arbuscular mycorrhiza (AM)–inducible promoters, which drive expression exclusively in the cells containing arbuscules during AM symbiosis. We initiated analysis of cellular Pi content in both species using confocal ratiometric methods established in the previous project period. For *M. truncatula*, we introduced the constructs into roots via transient transformation and then inoculated the roots with AM spores and allowed the roots to develop AM symbiosis. For *B. distachyon*, transient transformation is not an option so we spent the majority of the project period generating stable transformants and increasing seed stocks to enable experiments. We now have *B. distachyon* plants expressing the cpFLIPPi5.3 biosensor, a null biosensor control, and two single fluorophore controls for the cytosol specifically in colonized cells (AM-inducible promoter) and also a second set that expresses the sensors in all cells (Ubiquitin promoter). We also generated plants with the biosensors and controls targeted to plastids expressed from an AM-inducible promoter. Efforts to develop a Pi biosensor suitable for monitoring the apoplast/periarbuscular space and vacuoles have been unsuccessful. We therefore adopted a new strategy that stems from our discovery of two fluorescent proteins that retain function when targeted to these subcellular compartments. Specifically, we are engineering a population of Pi-binding protein-fluorescent protein chimeras and then screening for individuals that exhibit Pi-dependent changes in fluorescence intensity. Two candidates with >100% change in fluorescence intensity have been identified to date.



Confocal ratiometric imaging of a Pi biosensor, cpFLIPPi-5.3, expressed in *Medicago truncatula* reports relative Pi levels in the cytosol of AM-colonized root cells.

Accomplishments/Deliverables: We confirmed that the imaging and data analysis methods we developed for live monitoring of relative Pi concentrations are applicable to confocal imaging systems with different optics and light detectors. We also confirmed the ability to detect altered Pi levels in a *M. truncatula* Pi transporter mutant in which symbiotic Pi transport is impaired. Some of our bioimaging results are highlighted in two recent review articles in *Current Opinion in Plant Biology*.

Potential Benefits/Applications: The broad utility of our Pi biosensors and associated methodology are exemplified by the fact that they have been adapted by others to explore aspects of Pi and energy metabolism, and to address the efficacy of soil arsenate remediation. Two papers describing these applications are currently in review.

Multiscale Dynamics of Water Regulation by Bacteria in Synthetic Soil Microsystems

Principal Investigators: Leslie M. Shor, Yongku Cho, Daniel J. Gage, and Jessica Furrer
Organizations: University of Connecticut and Benedict College
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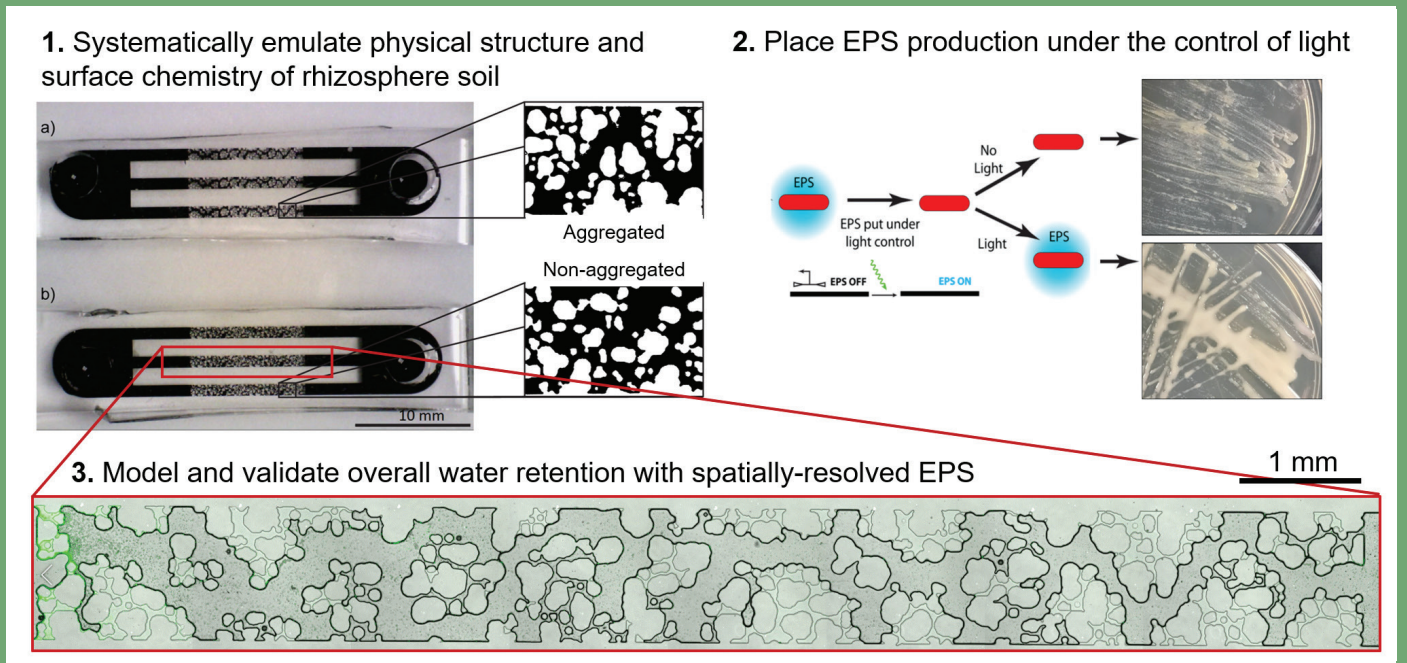
Objective: This project aims to develop a multifunctional imaging platform to link microbial gene expression in the rhizosphere with overall system function. We focus on controlling microbial exopolysaccharide (EPS) production using optogenetic control of the EPS production pathway in *Sinorhizobium meliloti*, and provide the suite of micromodels, molecular tools, and pore-scale modeling to help build systems-level understanding of the water-regulating function of soil microbes.

Accomplishments/Deliverables: We have developed and validated a platform emulated soil micromodel that offers the most physically realistic representation of aggregated and nonaggregated sandy loam soil available. We have developed, validated, and published a lattice Boltzmann model that accurately predicts pore-scale drying behavior within the emulated soil micromodel as a function of relative humidity and surface chemistry, thus enabling prediction of pore-scale water retention behavior under an expanded set of experimental conditions. In addition, we have made significant advances in the development of optogenetic tools needed to place the complex EPS production pathway in *Sinorhizobium meliloti* under the control of light.

Potential Benefits and Impact: We offer an experimental platform linking overall moisture retention function in soils with *in situ* microscale control of gene expression. Prototype imaging platforms are currently in use by other labs around the world to study a range of pore-scale rhizosphere processes. Very little prior work has been done implementing optical control in soil-relevant bacteria. Our molecular work has required extensive development of optical tools that will be valuable in understanding microbially mediated moisture retention in the rhizosphere as well as many other DOE-relevant research questions.



Multifunctional *in situ* platform to link gene expression with overall system function. The platform combines emulated soil micromodels with molecular tools that place a key function of rhizosphere bacteria under the control of light. Shown here are EPS-producing microbes acting with an aggregated soil microstructure to promote an intermediate water content with air-filled macropores and water-filled aggregates that is associated with productive terrestrial ecosystems. This platform and its enabling tools can be applied to a host of BER-relevant research questions.

Development and Refinement of an *In Situ* "Molecular Microscope" Utilizing Ultrahigh-Resolution Mass Spectrometry

Principal Investigator: Gary Stacey
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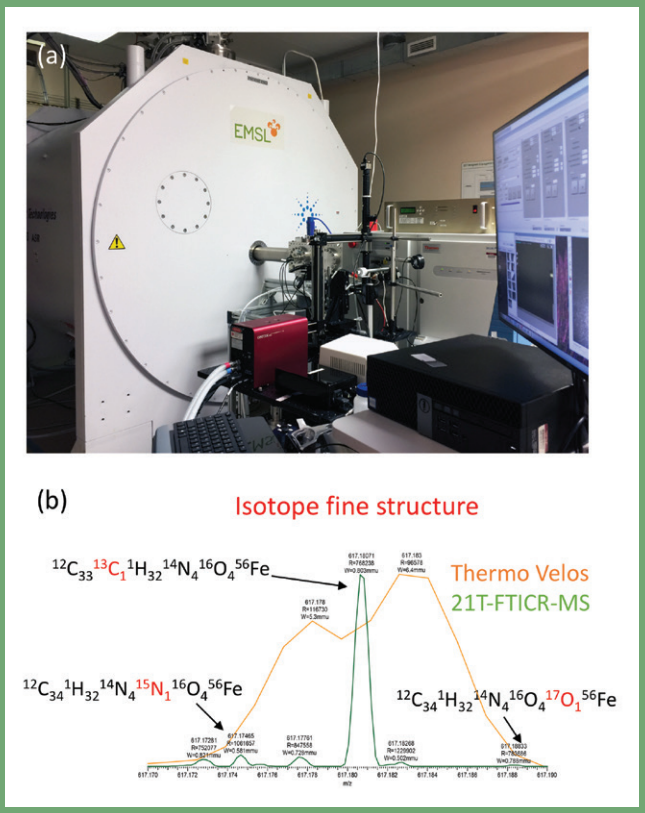
Collaborators: Akos Vertes (George Washington University); Ljiljana Paša-Tolić, Christopher Anderton, and David W. Koppenaal [Environmental Molecular Sciences Laboratory (EMSL), and Pacific Northwest National Laboratory]

Research Plans and Progress: Objectives: (1) Develop an advanced laser ablation electrospray ionization (LAESI) source capable of unlocking a range of biomolecules within a single live cell and (2) couple this source to ultrahigh-resolution mass spectrometry, in an effort to (a) achieve unprecedented levels of molecular information with exceptional spatial detail in complex biological systems and (b) further demonstrate and validate *in situ* analysis using well-characterized plant-microbe interaction systems as models.

Approach and Progress: LAESI is a technology that allows for direct microsampling and molecular imaging of biological tissues and cells in their native site. Two versions of this source are being developed and tested. First, a conventional LAESI source was equipped by multimodal imaging capabilities through a long-distance microscope, which permits simultaneous observation of fluorescence and bright field images to selectively target cells of particular phenotypes. The second LAESI version relies on laser pulse delivery through a sharpened optical fiber, which allows targeting of cells ≥10μm. As a first step, we applied these approaches to the well-characterized model plant-rhizobium system—soybean (*Glycine max*) with a common mutualistic soil bacterium (*Bradyrhizobium japonicum*). Our study illustrated that specific components of the soybean root nodule could be metabolically profiled using the conventional LAESI setup (S. A. Stopka et al., 2017. *Plant J*. **91**, 340). Using molecular tomography with three-dimensional (3D) matrix-assisted laser desorption/ionization (MALDI)–mass spectrometry imaging (MSI), available at EMSL, we confirmed the presence of discriminate metabolites in the anatomical compartments of soybean nodule as measured by LAESI (D. Veličković et al., *ISME*, in revision). We have now interfaced the conventional LAESI source with the high-field (21 Tesla) Fourier transform ion resonance cyclotron mass spectrometer (FTICR-MS) housed at EMSL [see figure, part (a)]. Through a series of systematic studies, we determined that the 21T-LAESI-FTICR-MS is sufficiently sensitive to measure metabolites in a single cell. The unprecedented mass resolving power provided by the 21T-FTICR-MS affords us the ability to attain high-confidence molecular annotations directly from plant tissue, by resolving the isotope fine structure of a molecule [see figure, part (b)].

Current and Anticipated Accomplishments: In addition to those mentioned above, we recently demonstrated single-cell imaging mass spectrometry (IMS) using fiberoptic laser coupled with a Waters Synapt G2-S MS. For each cell, a mass spectrum with ~111 spectral features was obtained, including metaboliltes known from our previous experiments. Project personnel are currently implementing the fiberoptic LAESI system onto the 21T-FTICR-MS, where we will be able to attain single-cell molecular analysis with unmatched spectral resolution.



Implementation of the conventional LAESI source on the 21T-FTICR-MS housed in EMSL. (a) Image of the LAESI source coupled to the ultrahigh-mass resolving power 21T FTICR-MS. (b) Spectra comparison of the +1 Da isotopic peak of *heme* B ($m/z = 617.180 \pm 0.01$) acquired directly from intact soybean nodule tissue using the LAESI coupled to either the Thermo Velos Orbitrap (orange) or the 21TFTICR-MS (green).

Potential Benefits: Our work has generated significant interest from the EMSL User Base, where there are a number of proposals being submitted that aim to utilize this technology to address questions related to a variety of Department of Energy (DOE)–relevant systems. Hence, the system we have developed should see broad application.

The Transparent Soil Microcosm: A Window into the Spatial Distribution and Dynamics of Carbon Utilization and Microbial Interspecies Interactions

Principal Investigators: Elizabeth Shank (PI). Co-PIs: Carol Arnosti, David Berry, and Jeffery Dangl
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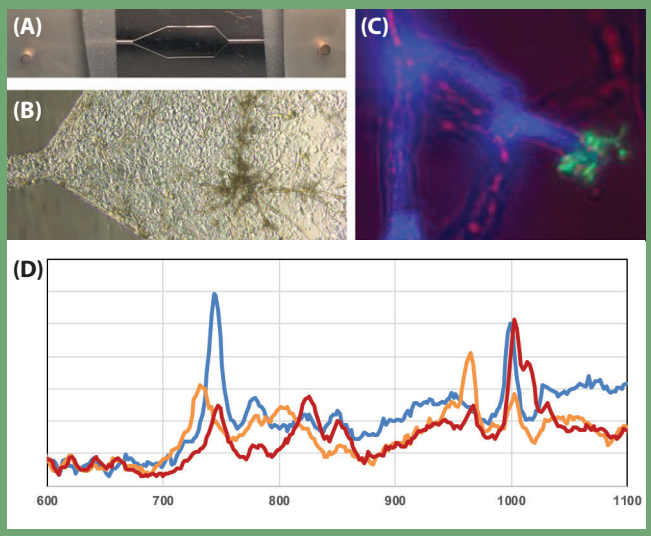
Research Progress and Plans: Our overall objective is to establish a multimodal imaging platform to enable the visualization of the spatio-temporal dynamics of microbial community interactions and carbon flow in soil-like environments. We have made substantial progress towards meeting this goal. We have now fabricated PDMS microfluidic chips that can be used in both static- and continuous-flow incubations and demonstrated that these imaging chambers are compatible with epifluorescence, confocal laser scanning, and Raman microscopy. Specifically, we have grown and then heat-killed ^{13}C-labeled fungi within the chambers, added bacteria that attach to and degrade the dead hyphae, and then, using a combination of fluorescence microscopy and Raman microspectroscopy, monitored the transfer of ^{13}C from the fungal biomass into the bacterial cells (see figure). These data pave the way for establishing these imaging platforms to study a range of questions about microbial activity in soil and soil-like environments. We are testing the use of heavy water (D_2O) labeling as a general metabolic activity marker, and successfully stably coupled "red" fluorophores with xylan, expanding our ability to monitor complex carbon degradation within these microcosms. We have imaged live plant roots over time after colonization by bacteria in a transparent soil–filled chamber, and, through a large-scale analysis of synthetic plant-associated bacterial communities, we have discovered key bacterial species (either indifferent to environmental stresses or that impact plant root–development phenotypes) to serve as model systems in our plant imaging chambers.



(A) An image of the microfluidic chip that enables data to be collected using both confocal fluorescence and Raman microscopy. (B) ^{13}C-labeled fungus (*Mucor fragilis*) growing in a microfluidic microcosm with transparent soil. (C) Fluorescently labeled bacteria (*Bacillus subtilis*, green) form aggregates on a dead fungus (*M. fragilis*, blue) within a microfluidic imaging microcosm; *B. subtilis* degrades the fungus and utilizes it as its sole carbon source. (D) Raman spectrum showing uptake of ^{13}C-carbon from *M. fragilis* by *B. subtilis* (see shift from blue to yellow trace). The addition of free sugar (glycerol, 'gly') suppresses this ^{13}C-carbon uptake (see shift from yellow to red trace). The peak shifts between ^{12}C and ^{13}C thymine and phenylalanine are noted.

Current and Anticipated Accomplishments/Deliverables: A microfluidic imaging chamber enabling the visualization of complex carbon degradation in a bacterial-fungal microcosm using fluorescence and Raman microscopy; an acrylic plant–microbe–transparent soil mesocosm that allows plant colonization by microbes to be visualized over time; stable red fluorescent–labeled xylan, allowing localization of complex carbon within bacteria and imaging chambers.

Potential Benefits/Applications: The design of our microfluidics imaging chamber is simple enough to be cast at the benchtop and loaded using standard pipettes, while our plant–transparent soil microcosms are cut from inexpensive acrylic using a standard laser cutter. These chambers require small reagent quantities while yielding data previously difficult or impossible to obtain. Due to their low manufacturing cost, ease of use, and compatibility with multiple imaging modalities, these platforms are likely to find widespread use among those involved in plant and microbial research, advancing our understanding of microbial community assembly and ecosystem processes within soil habitats.

Development of a Novel High-Precision, High-Resolution SIMS Platform for Elemental and Isotopic Characterization of Microbial Cells at a Systems Level

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This project has developed a new analytical platform for the rapid, high-precision determination of the elemental and stable isotopic composition of microbial cells at sufficiently high spatial resolution to localize and quantitatively map bioessential elements between and within individual cells.

Research Plans and Progress: The research focuses on characterizing three model microbial systems [cyanobacteria, such as *Cyanothece*; purple photosynthetic bacteria (*R. palustris*); and *Methylobacterium*] that are promising for sustainable bioenergy production to validate this platform. At the start of the work, we characterized these systems on the existing state-of-the-art platform, while the instrument upgrades at the heart of our proposal [i.e., new RF Hyperion Oxygen (O) ion source and spatial detector system] were being developed. During this time, we also optimized protocols for preparing microbial cells for analysis by SIMS (e.g., comparing fixed vs. non-fixed cells; liquid deposition vs. embedded and microtomed). During analysis of cyanobacteria we are able not only to resolve individual cells, but also to conduct subcellular elemental localization (such as determining the abundance, size, and distribution of internal polyphosphate bodies). For smaller cells (e.g., *R. palustris*), we are able to obtain precise whole-cell measurements of the incorporation of stable isotope labels (C and N) to quantify rates of microbial C and N fixation—and the variance in these values within microbial populations growing under ostensibly identical conditions [for both *R. palustris* and *Methylobacterium*, as well as the (relatively larger) cyanobacteria investigated].

Current and/or Anticipated Accomplishments/Deliverables for the Project Period: We have developed the protocols and proof-of-concept demonstration to probe the uptake, assimilation, and subcellular localization of key elements, isotopes, and biomolecules associated with important metabolic processes (respiration, nitrogen fixation, light harvesting, and photosynthesis). This also includes development of custom vacuum-compatible, transparent, conductive, and trace metal–free sample-mounting discs, suitable for both microbial analysis via SIMS, as well as optical microscopic inspection; these have now been manufactured, tested, and incorporated into routine microbial imaging work. Both the new detector (RAE imaging system) and the RF plasma Hyperion O ion source have been successfully installed and capabilities demonstrated on the 7f instrument platform. Detailed characterizations of the microbial systems under the new instrument configurations are under way.

Potential Benefits/Applications of DOE-Funded Research: This approach of high-throughput precise analyses over larger fields of view will enable the integration of molecular-scale chemical and isotopic information with biological function to generate a new whole-cell, systems-level understanding of these model systems and an assessment of metabolic heterogeneity within clonal populations. Specifically, the following advances will have wide bioimaging applications: (1) imaging over a relatively large field of view (100 µm × 100 µm), which could include >1000 microbes of about 1 µm, with rapid analysis for isotopic labeling with a relative precision of <1%; (2) relatively rapid acquisition of trace element ion images with micron spatial resolution; and (3) spatially co-registered images of major (e.g., C, N, P) constituent elements paired with corresponding trace element (e.g., Fe, Mo) distribution. This improves our ability to determine rates of uptake for key metabolic processes (e.g., C/N fixation) as well as to understand how these processes may depend on the abundance of essential trace metals and ambient environmental conditions (e.g., pH, light exposure). These results will lead to improved systems-level understanding of a variety of microbial systems that are promising for sustainable bioenergy development. The ability to leverage these results to aid in bioenergy development will have broad benefits to society and the environment by helping to provide safe, renewable, carbon-neutral energy.

Multimodal Nanoscale Chemical Imaging of Intra- and Inter-Cellular Biomolecules Involved in Microbial Interactions

Principal Investigator: Scott Lea
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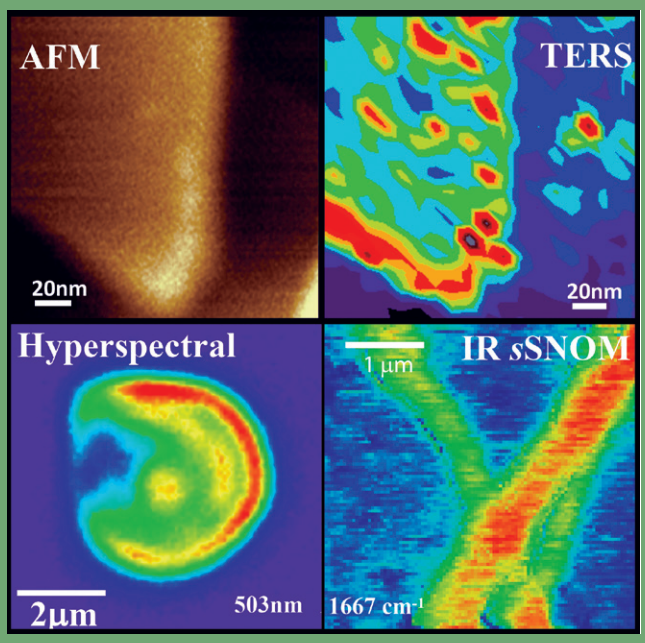
Email: scott.lea@pnnl.gov

Research Objectives: Existing genomic and biochemical methods cannot directly probe the physical connections involved in microbial metabolic processes over the relevant length scales, spanning the nano-meso-micrometer spatial regimes. Determining the location and function of such biomolecules would aid in identifying the mechanisms governing microbial interactions. This project aims to address these technical and conceptual gaps by developing a single multimodal chemical imaging platform that can interrogate biomolecules in living systems using three complementary label-free, nanoscale, ultrasensitive chemical imaging techniques:

- Infrared scattering scanning near-field optical microscopy (IR *s*-SNOM)
- Tip-enhanced Raman nano-spectroscopy (TERS)
- Multimodal hyperspectral optical nano-spectroscopy

Accomplishments: We have built and developed the three different imaging modalities independently prior to integration into a single, multimodal chemical nanoscope. An ambient atomic force microscopy (AFM)–based TERS chemical imaging platform is now operational. As part of our benchmarking experiments, we performed TERS measurements targeting prototypical systems and constructs, and demonstrated single nanometer precision in ambient TERS chemical imaging measurements.[1] We also established an overall broader scope of TERS.[2] Namely, we illustrated that this technique is not restricted to nanoscale chemical imaging, but it can also be used to probe different aspects of local fields confined to a few nanometers. Our new TERS setup is now also equipped with a hyperspectral imager. Using various illumination schemes in our custom setup, hyperspectral fluorescence, optical absorption, dark-field scattering, Raman scattering, and topographic imaging are all possible. Recently, we used this capability to visualize pigments in lipid monolayers and within a single live *T. lutea* cell in solution.[3] For IR *s*-SNOM, we are working with a vendor to obtain an AFM capable of bottom illumination and collection of IR light to support IR *s*-SNOM measurements in solution. The approach would use a ZnSe prism mounted on a piezoelectric scanner to enable evanescent wave illumination and collection of scattered IR light in aqueous environments. We are also benchmarking the IR *s*-SNOM with the TERS and hyperspectral imaging modalities on a number of model biological systems including bacteria, collagen, and cytochromes.

Benefits to Scientific Community: This unique AFM-based instrument could be used to investigate a wide range of biomolecules through their characteristic electronic and vibrational signatures, over the nano-meso-micrometer scales. This platform not only will enable recording of chemical images of single microbial cells with subcellular resolution, but also will enable mapping of entire microbial communities with chemical selectivity. Overall, this capability will afford unmatched levels of joint topographic and chemical characterization of individual live cells and interacting microbial community members. As such, our instrument will enable pursuit of novel research directions within the Department of Energy's Office of Biological and Environmental Research mission portfolio.



The technology being developed will provide *in situ* multimodal chemical imaging, comprising AFM topography and TERS, IR *s*-SNOM, and UV-V is hyperspectral imaging, of biological systems. Shown are images of peptoid sheets, a live *T. lutea* cell, and collagen fibers. Images courtesy Pacific Northwest National Laboratory. Hyperspectral image reprinted from Novikova, I. V., et al., 2017. "Multimodal Hyperspectral Optical Microscopy," *Chemical Physics* 498–499, 25–32. © 2017 with permission from Elsevier.

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2. Bhattarai, A., et al. 2017. "Visualizing Electric Fields at Au(111) Step Edges via Tip-Enhanced Raman Scattering." *Nano Lett.* **17**(11), 7131–7137.
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Far-field Subdiffraction Raman Imaging and *In Situ* Correlative Electron Microscopy for Elucidating Details of Plant Cell Wall Structure and Deconstruction

Principal Investigator: Emily A. Smith
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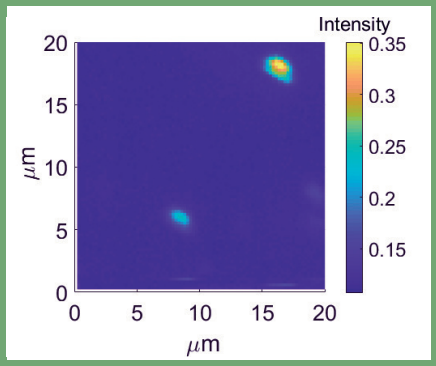
Email: esmith1@ameslab.gov

Research Goals and Progress: We are developing a subdiffraction Raman microscope for imaging plants with 100-nm or better spatial resolution based upon the technique of coherent anti-Stokes Raman spectroscopy (CARS). By achieving saturation in the CARS signal, a subdiffraction point spread function is achieved, which is recovered by signal demodulation. The Raman instrument is being applied to study wild-type and transgenic *Arabidopsis* plants expressing microbial cell wall–modifying enzymes to gain an understanding of cell wall composition and structure. A multimodal approach with *in situ* correlative electron-optical microscopy is extending the interpretation of Raman measurements. Finally, the imaging approaches are being applied to measure cell wall deconstruction mediated by microbial consortia, which will enable the team to optimize the performance of cellulosomes and hemicellulosomes.

The team has assembled the CARS microscope in Ames Laboratory's Sensitive Instrument Facility and obtained preliminary data showing diffraction-limited imaging (see figure). We have obtained and are developing data analysis for correlative electron, fluorescence, and Raman microscopic images of stem cross sections from wild-type and transgenic *Arabidopsis* plants. Two transgenic plant lines have a reduced degree of xylan or pectin acetylation; a third has a reduced amount of ferulic acid esterified polysaccharides. In addition, cell walls were extracted and purified from the same plants to compare the Raman spectra obtained from purified cell walls with the spectra obtained using the intact plant tissues.

Efficient biomass degradation in nature requires synergistic efforts of a team of microbes that secrete or surface-display lignocellulosic enzymes with appropriate ratios and spatial arrangement. To best mimic this natural phenomenon, we are assembling cellulases into mini-cellulosome structures using yeast consortia to study their mechanism of cell wall deconstruction using the developed imaging techniques. As a first step, *Trichoderma reesei* endoglucanase II (EGII) was expressed as a displayed protein on the surface of *Saccharomyces cerevisiae*. We showed that the yeast cells displayed functional EGII carboxylmethyl cellulose degradation.



CARS image of nanodiamond. Courtesy Emily Smith and Avinash Singh, Ames Laboratory.

Current and Anticipated Deliverables: Our current CARS microscope can be applied to study diverse plant systems as we continue to develop its ability to perform subdiffraction imaging. We anticipate the delivery of a fully functional subdiffraction Raman system with imaging capabilities for investigating plant cell wall composition, structure, and deconstruction within the next year. The instrument will have the ability to be used widely by the scientific community to address questions where both chemical and spatial information are needed at the nanoscale.

Project Impact: The imaging capabilities will be developed in the context of biomass and bioenergy production from plant cell wall polymers; the impact of the instrument, however, will extend beyond the constraints of this project since the need for chemically specific imaging with nanoscale spatial resolution applies to many biological processes. Published manuscripts and conference and university presentations will be pursued to disseminate the capability and applicability of the instrument. The instrumentation will enable a better understanding of how plant cell wall properties relate to recalcitrance, and efficient methods for cell wall deconstruction. This will contribute to DOE and BER's mission to enable "more confident redesign of microbes and plants for sustainable biofuel production."

May 2018