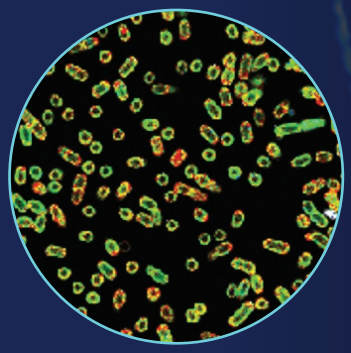
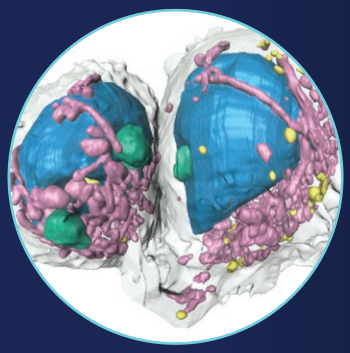
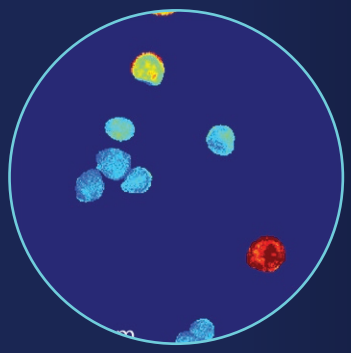
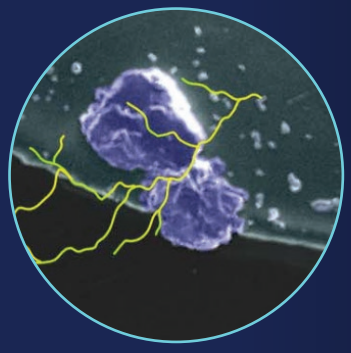
Biomolecular Characterization and Imaging Science Program

2023 Principal Investigator Meeting Proceedings









U.S. DEPARTMENT OF ENERGY | Office of Science

Biological and Environmental Research Program

Biomolecular and Characterization Imaging Science Program

2023 Principal Investigator Meeting

April 17–19, 2023
Bethesda, MD

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About BER

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

Cover Images

Background Image: Interferometric image of live *Medicago truncatula* roots. Pseudocolor represents optical phase (in radians), thus providing insight about the biosystem's dry-density or dry-mass, see p. 72; **Circle 1:** Fluorescence lifetime imaging microscopy showing single-cell resting membrane potential variables, see p. 15; **Circle 2:** 3D rendering of U2OS cells, see p. 36; **Circle 3:** Molecular crowding in the cytoplasm of yeast cells, see p. 49; **Circle 4:** Extraction of K^+ and NA^+ from minerals through mycelia, see p. 76.

Suggested Citation

U.S. DOE. 2024. Biomolecular Characterization and Imaging Science Program 2023 Principal Investigator Meeting Proceedings, U.S. Department of Energy Office of Science. https://doi.org/10.2172/2202464.

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Published January 2024

U.S. DEPARTMENT OF ENERGY | Office of Science

Biological and Environmental Research Program

Prepared for the
U.S. Department of Energy
Office of Science
Biological and Environmental Research Program
Germantown, MD 20874-1290

Prepared by
Biological and Environmental Research Information System
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Managed by UT-Battelle, LLC
For the U.S. Department of Energy
Under contract DE-AC05-00OR22725

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Preface

The 2023 U.S. Department of Energy (DOE) Biological and Environmental Research (BER) program's Biomolecular Characterization and Imaging Science (BCIS) Principal Investigator Meeting expanded in scope from previous Bioimaging Science Program (BSP) meetings to include BER Structural Biology and Imaging Resources, which are located largely at DOE Office of Science national laboratories. The BCIS meeting was part of BER's Biological Systems Science Division (BSSD) annual PI meeting, which was held April 17–19, 2023, and featured parallel meetings of the BCIS and Genomic Science programs (GSP). The meetings were held together to encourage networking and idea exchange across technologies and biological application areas, forging new multidisciplinary collaborations among researchers from adjacent BSSD programmatic areas.

Two joint BCIS-GSP sessions were held: "BCIS Technologies for Investigating the Rhizosphere" and "Joint Emerging Topics and Technologies." The rhizosphere session focused on scientific findings from BCIS and GSP PIs, including national laboratory collaborations. The intent was to identify new opportunities to measure and understand the complex community of microbes, roots, and soils that support plant growth under challenging environmental conditions. The emerging technologies session highlighted forward-looking approaches and tools to tackle challenges within the scope of BSSD research on investigating and modifying genomic and molecular function. A final interactive discussion of the BCIS program was led by plenary session chairs.

> *"...to understand the translation of genomic information of plants and supporting microbes into the mechanisms that power living cells, communities of cells, and whole organisms for the benefit of bioenergy and the U.S. bioeconomy."*
>
> **2023 Meeting Objective**

BSSD Biomolecular Characterization and Imaging Science

BSSD's overarching goal is to provide necessary fundamental science to understand, predict, manipulate, and design biological systems that underpin innovations for bioenergy and bioproduct production and enhance our understanding of natural, DOE-relevant environmental processes (DOE BER Biological Systems Science Division Strategic Plan, 2021).

Within BSSD, the goal of BCIS is to provide access to imaging and measurement technologies and to develop new ones to visualize the spatial and temporal relationships of key metabolic processes governing phenotypic expression in plants and microbes. The extended goal of dynamic imaging is to functionally connect cellular components and interdependent organisms. Information on the time and place of chemical reactions *in situ* can identify causal relationships between biological activators and downstream effectors. Therefore, an objective of the joint GSP-BCIS PI meeting is to understand the translation of genomic information of plants and supporting microbes into the mechanisms that power living cells, communities of cells, and whole organisms for the benefit of bioenergy and the U.S. bioeconomy.

Development of use-inspired technologies is supported to advance hypothesis-driven biological research by measuring and modeling key metabolic processes in microbial cells, multicellular plant tissues, and communities. BCIS supports fundamental imaging research for proof-of-concept studies of novel untested methods and devices and development of biologically validated working prototypes. Also supported are innovative imaging technologies for investigating biological systems with light or particle irradiation. Both early scientific research and mid-stage technology development are included within BCIS core funding efforts, while technology transfer and commercial development are supported by Small Business Innovation Research (SBIR) and Small Business Technology Transfer (STTR) programs and occasionally other funding opportunities.

BCIS supports established and evolving technologies for structural biology and imaging research via unique crystallography, scattering, spectroscopy, imaging, cryogenic electron microscopy (cryo-EM), and tomography capabilities available at national user facilities. BCIS-supported resources support evaluation of critical structures

and functions in biology that occur across a wide range of distances (subnanometer to centimeter) and times (subpicosecond to minutes).

The overall objectives (DOE BER Biological Systems Science Division Strategic Plan, 2021) of BSSD's biomolecular characterization and imaging science portfolio are to:

- Enhance the accessibility of bioimaging and structural biology infrastructure within the research community and at DOE user facilities.
- Develop and enhance tools for sample handling and transfer, optimizing the samples for multiple imaging modalities and approaches.
- Develop fast and sensitive detectors with extremely high rates of data collection and the necessary computational tools to handle large, real-time, noisy, multimodal, and multiscale data.
- Develop multifunctional, *in situ*, and nondestructive observation technologies for repetitive sample analyses for systems biology research.
- Visualize the spatial and temporal dynamics of expressed biomolecules within or between living plant or microbial cells and their communities.
- Explore quantum science concepts for optical imaging and sensing of cellular processes.
- Incorporate newly developed technologies into DOE user facilities or provide opportunities for commercial development through DOE programs for SBIR-STTR.

BER also supports efforts at academic institutions and DOE national laboratories to develop quantum sensing and imaging approaches for circumventing fundamental limitations of classical optical techniques. BER sponsored a 2021 National Academy of Sciences workshop and proceedings on Quantum Science Concepts in Enhancing Sensing and Imaging Technologies: Applications for Biology to identify both promising quantum-measurement methods and quantum-based biological processes. A market research study was prepared on Transitioning Quantum Imaging and Sensing Technologies to Bioimaging Markets. DOE supports five large-scale Quantum Information Science (QIS) centers across the Office of Science to develop materials, sensors, computation, and communication network capabilities.

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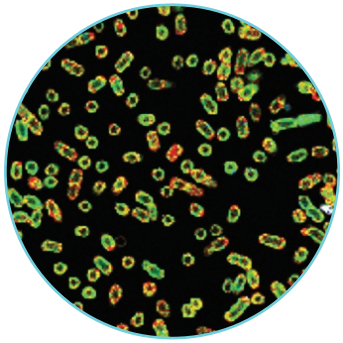

Chapter 1
Quantum Imaging and Sensing

The Biological Systems Science Division (BSSD) within DOE's Biological and Environmental Research program (BER) supports research on new quantum-enabled imaging and sensing approaches at DOE national laboratories and universities across the country. These new approaches could dramatically enhance the ability to measure biological processes in and among living cells and enable dynamic localization and imaging of cellular processes, advancing DOE missions in bioenergy and the environment.

National Laboratory Quantum Imaging Approaches–2020 Projects

Fundamental quantum science–enabled research on imaging probes, detectors, and sensors overcome current challenges related to suboptimal stability and photobleaching, enabling prolonged imaging studies. For example, quantum-entangled pairs of single-photon-emitting probes can potentially enable subdiffraction-limited functional imaging in living tissue. Five national laboratory projects are developing novel bioimaging and characterization technologies to dynamically image, measure, and model key microbial and plant metabolic processes *in situ*, nondestructively, and in real time.

University Quantum Sensing Approaches–2022 Projects

Five university projects are conducting fundamental research on new concepts or developing use-inspired prototype technologies for biomolecular sensing approaches that exploit quantum phenomena or incorporate quantum science concepts. These approaches offer an advantage over conventional methods by enhancing spatial and temporal resolution, measurement speed, long-term sample stability, or bioimaging technology sensitivity.

University Quantum Imaging Approaches–2022 Projects

Six university projects are conducting fundamental research on new concepts or developing use-inspired prototype technologies for imaging approaches that exploit quantum phenomena or incorporate quantum science concepts. Compared to conventional methods, these approaches enhance spatial and temporal resolution, measurement speed, long-term sample stability, or bioimaging technology sensitivity. Projects include development of light sources that use entangled photons, squeezed light amplitude, or single-photon detectors. New imaging systems are employing infrared, visible, and X-ray light sources, and researchers are developing instrumentation and methods for cellular imaging and molecular spectroscopy.

Probing Photoreception with New Quantum-Enabled Imaging

Kevin Crampton[1], Samantha Powell[1], Jory Brookreson[1], Nick Black[2], Saleem Iqbal[2], Patrick El-Khoury[1], Robert Boyd[2], **James E. Evans[1*]** (james.evans@pnnl.gov, PI)

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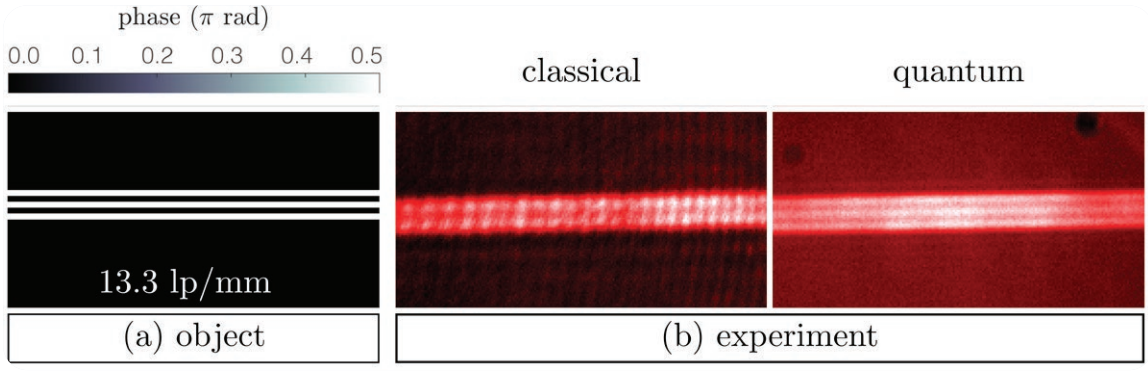
[2]University of Rochester, Rochester, NY

Project Goals: This project will develop new hybrid quantum-enabled imaging platforms that combine advances in adaptive optics, quantum entanglement, coincidence detection, ghost imaging, quantum phase-contrast microscopy, and multidimensional nonlinear coherent spectromicroscopy to characterize photoreception. The approach has three main aims that 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.

During the current project period, the team installed Leica and Olympus optical microscopes with fluorescence, coherent anti-Stokes Raman scattering (CARS), stimulated Raman scattering (SRS), and multi-passed (MP) imaging modes. Researchers also installed a Picoemerald OPO and two Coherent lasers to be used in aims 1 to 3. The team initiated work on both ghost imaging and 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. For aim 2, the team demonstrated the ability to perform quantum-enhanced phase imaging without coincidence counting. For aim 3 researchers have detected 1.2 picosecond quantum beats with time-resolved coherent Raman scattering. Finally, the team has expressed multiple proteins involved with photoreception and have begun their structural and spectroscopic characterization. Researchers have cultured all cell types that will be imaged as part of the testing and commissioning phases. Looking ahead to the next project period, the team plans to finish development of two-color entangled quantum ghost imaging as well as begin applying aims 1 to 3 to quantum-enabled imaging and probing of biological samples.

Funding Statement: Pacific Northwest National Laboratory is operated by Battelle for the U.S. Department of Energy (DOE) under Contract DE-AC05-76RL01830. This program is supported by the DOE Office of Science through the Genomic Science Program of the Biological and Environmental Research Program under FWP 76295. The work was performed at the Environmental Molecular Sciences Laboratory (grid.436923.9), a DOE Office of Science user facility sponsored by the BER program.



A Comparison of Resolution Between Classical Phase-Shifting Holography and Quantum Phase-Shifting Holography. (a) A series of three horizontal bars with a maximum phase shift of π/2 were used to measure resolution with a spatial frequency of the bars at 13.3 lp/mm. **(b)** Experimental results (interferograms) indicate that only the quantum phase–shifting holography scheme can resolve the bars at this spatial frequency. [Adapted from A. N. Black et al. "Quantum-Enhanced Phase Imaging Without Coincidence Counting." In Press.]

*Presenting Author

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

Ted A. Laurence[1] (laurence2@llnl.gov, PI), Tiziana Bond[1], Chuck Boley[1], Greg Bude[1], Claudio Bruschini[2], Kevin Cash[3], Edoardo Charbon[2], Dominique Davenport[1], Matthew A. Horsley[1], Shervin Kiannejad*[1], Paul Mos[2], Erin Nuccio[1], Mike Rushford[1], Sam Saccomano[3], Ty Samo[1], Michael Wayne[2], Peter K. Weber[1]

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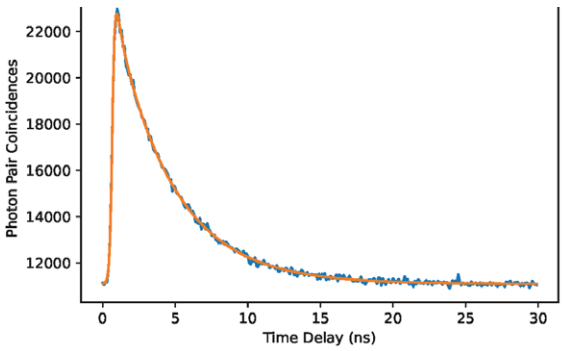
[3]Colorado School of Mines, Golden, CO

For technical objective 1, the team has performed fluorescence lifetime measurements using visible quantum-entangled photons from the Type-I SPDC source developed last year based on a 266 nm laser, which completed technical objective 2. The timing correlations of the entangled photon pairs can be exploited to measure the fluorescence lifetime of the sample under study. In such an experiment, the signal photon serves as the probe, exciting the sample, causing the emission of a fluorescence photon, which is then measured by a photon counting detector. Although entanglement is broken once absorption occurs, a time correlation between the idler and fluorescence photon remains, merely delayed by the time for the transition to the excited state during absorption and the subsequent vibrational relaxation before fluorescent emission occurs. Thus, a delayed time correlation exists between the idler and fluorescence photons, and coincidences can be measured between them with the coincidence spike containing within its decay the fluorescence lifetime of the molecule. While this was theorized about a decade ago, to the best of the team's knowledge, this is the first time it has been realized experimentally. Additionally, researchers are testing resolution in standard ghost imaging and in 3DQ imaging with the Phase I single photon–counting avalanche photodiode (SPAD) array detectors delivered from EPFL and a TimePix3 detector on loan. These will be compared with modeling of the imaging performed under this project. For technical objective 3, the team is integrating and testing the 2D SPAD arrays delivered by EPFL, and EPFL is now developing the Phase 2 SPAD arrays with much greater pixel count.

Technical objective 4 is focused on characterizing resource exchanges and services that define mutualistic interactions, which improve the ability to meaningfully leverage microbiomes that enhance host health. The team plans to apply the 3DQ to visualize how microbial interactions and mutualisms impact oxygen respiration and depletion in three dimensions. Currently, team members are developing oxygen-sensitive nanoparticle systems that can be used with the 3DQ or confocal microscopes to assay oxygen gradients in microhabitats. Recent work focuses on two bioenergy systems: quantifying microscale oxygen gradients in the phycosphere zone surrounding the diatom *Phaeodactylum tricornutum* and detecting real-time oxygen dynamics in the rhizosphere and detritosphere zones surrounding bioenergy switchgrass roots and nearby decomposing material (respectively). In the algal system, researchers show that organic particles from diatom extracellular polymeric substances generated robust oxygen signals, with oxygen concentration approaching 0 mg L^{-1} near particle surfaces and 5 mg L^{-1} approximately 10 µm away, likely driven by intense respiration of attached and embedded *Marinobacter* sp. 3-2. Meanwhile, bacterial filaments putatively identified as *Haliscomenobacter* sp. were closely associated with the diatoms and consistently exhibited oxygen drawdown of 2 mg L^{-1}. In the soil system, the team shows that the nanosensors are compatible with certain types of transparent soils, which allows researchers to assay respiration in microbial habitats in a solid matrix. Imaging live bacteria directly in soil is a grand challenge, and this work will enable visual connection of oxygen levels with microbial activity and specific microhabitats in real time.



Fluorescence Lifetime of R6G Measured Using Entangled Photons. In this graph, fluorescence lifetime curves generated by graphing coincidence counts as a function of time delay for Rhodamine 6G at 100 µM in ethanol using a 1.1 NA objective. A 266-nm pump beam generates Type 1 SPDC in a BBO crystal. The resulting cone of entangled photons is separated using a dichroic mirror. The higher-energy photons, the "signal" photons, excite the fluorescence in the Rhodamine 6G sample, and the resulting fluorescence photons are timed. The lower-energy photons, the "idler" photons, are timed and correlated with the fluorescence photons excited by the signal photons. The resulting fluorescence lifetime curve is fitted to a model accounting for the instrument response of the detectors. The lifetime of 4.00±0.03 ns matched values from the literature. This is an important milestone in demonstrating the 3DQ methodology. [Courtesy Lawrence Livermore National Laboratory]

*Presenting Author

A Quantum Enhanced X-Ray Microscope

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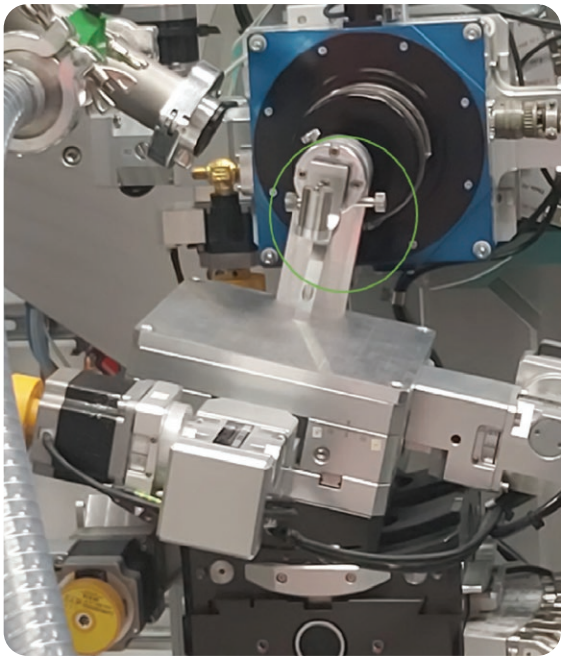
High-accuracy measurements require producing images with a high signal-to-noise ratio. Typically, this is achieved using high-input flux, but for living cells, a high-incident dose complicates the image by the inducing of radiation damage leading to unwanted artifacts. The use of the quantum properties of light, in this case X-rays, offers a new opportunity for imaging, in that the use of quantum correlations of the two-photon system allows retrieval of the image with minimal dose requirements. This approach has powerful implications in applications for biological and environmental science where the sample would normally be damaged by the X-rays during imaging. Exploiting the correlation of photons inherent to ghost imaging, a sample could be illuminated by less intense beams and thus remain unmodified during the experiment. Further, the quantum nature of the imaging process may allow higher signal-to-noise and better spatial resolution of thick samples.

The delivery of an X-ray quantum microscope is built upon four pillars: Experimental methods, nonlinear media for generating entangled photons, biological systems, and data analysis.

Experimental Methods: The team has created an X-ray optical system for measurement of ghost imaging. Several configurations of optic scheme have been deployed and tested. Data collection protocols have been integrated with the analysis tools to allow close to real time data reconstruction. A preliminary configuration has been developed to allow for imaging using entangled X-ray photons generated by parametric down conversion.

Nonlinear Media for Generation of Entangled Photons: In the X-ray region, available media have very low cross sections for the conversion. However, measurements using high quality diamond single crystals have confirmed the ability to produce entangled X-rays. Further refinement of protocols and X-ray configurations has allowed augmentation of flux. Researchers continue to look to improve the generation of entangled X-rays using novel nonlinear media.

Biological Systems: As the model system to verify this project's imaging, *Medicago truncatula* is the model legume species that will be studied in its symbiotic interactions with *Sinorhizobium medicae* and *Sinorhizobium meliloti*.



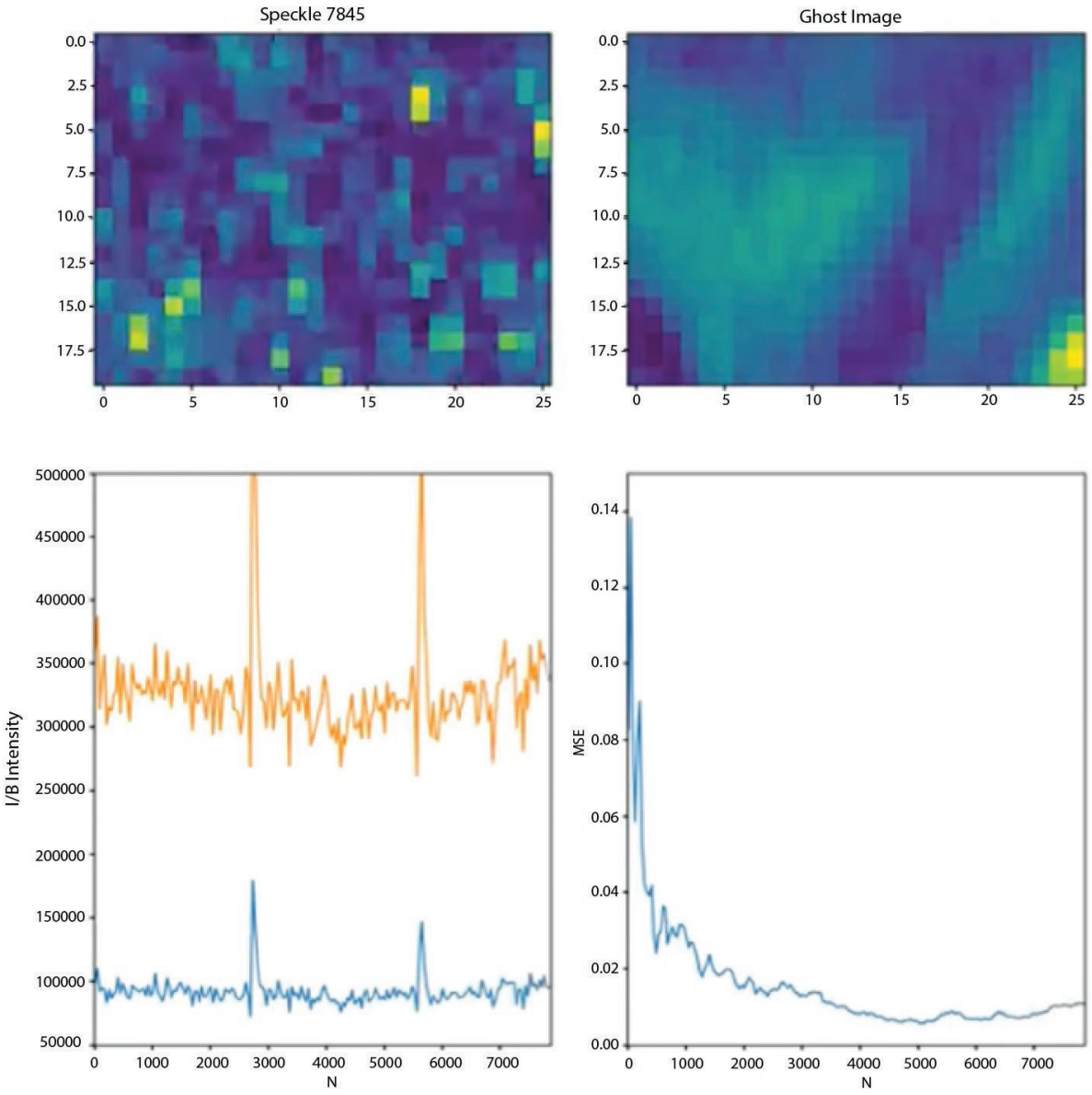
Nonlinear Media for Generating Entangled Photons. Diamond crystal on goniometer (circle), aligned for parametric down conversion measurements at incident energy 15 keV, producing two 7.5 keV correlated photons. [Courtesy Brookhaven National Laboratory]

Experiments have started to prioritize investigation. Once the prototype system is validated, adaptation of the growth chambers to the beamline will be needed. The team has started characterization of these systems using more traditional X-ray imaging techniques with the expectation of more impactful imaging being possible once the ghost imaging program is fully functional.

Data Analysis: A major effort has been placed on refactoring and developing code for as close to real-time processing as possible. This task has meant that researchers must integrate the data acquisition and analysis code into the X-ray beamline control system. Team members have continued improvement of the ghost imaging reconstruction algorithms. As use of parametric down conversion continues, researchers have developed code to identify and validate correlated photon pairs of the current energy.

The ultimate goal in this project is to achieve the potential of ghost imaging for X-ray image reconstruction of *in vivo* thick samples, or otherwise optically opaque biological samples. Results to date are being prepared for publication in peer-reviewed journals.

*Presenting Author



Exploiting the Correlation of Photons Inherent to Ghost Imaging. **Top left:** Speckle pattern from X-ray source. **Top right:** Ghost image reconstruction segment. **Bottom left:** Intensity correlation between successive images. **Bottom right:** Estimated error between frames. [Courtesy Brookhaven National Laboratory]

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

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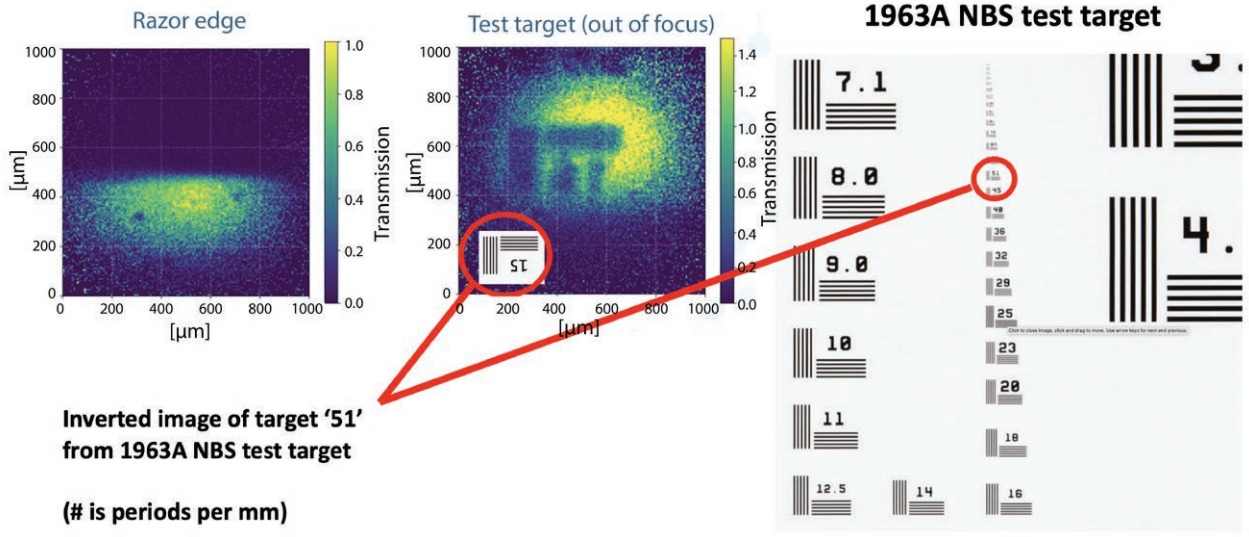
Project Goals: This project is exploiting quantum-entangled light to visualize important plant components (water, lignocellulose, and lipids) in bioenergy crops in a highly noninvasive and nonperturbative manner. In this quantum ghost imaging approach, plants will be probed with near-infrared photons (NIR), but the image of these plants will be generated with visible photons that never interacted with the sample. By using two detectors (a single element bucket detector for the probing photons) and a time-resolved (or fast-gated) imaging detector for image formation with the visible entangled photon, image formation noise can be greatly reduced. As such, images can be formed under extremely low light conditions—over an order of magnitude less photon flux than from starlight. The research team is advancing this promising quantum-enabled imaging method to its full potential by using a unique Los Alamos–developed detector technology. In particular, researchers are exploiting a unique time-resolved single-photon-counting imaging detector (Nocturnal Camera or Ncam), which enables measuring coincidence photon events with an order of magnitude better timing resolution (~100 ps) over the current state of the art (several nanoseconds). This new imaging approach is being tested on two plant species that demonstrate different mechanisms and pathways for carbon storage: a grass (sorghum) and a dicot (*Camelina*). Initial focus is on measuring an important and largely abundant plant constituent with a large mid-infrared absorption: water. However, the research team aims to mature this technology towards nearly simultaneous measures of plant water, lipid, and lignocellulose content over the course of the proposed research, ultimately leading to more informative measurements of plant environmental responses.

Progress: The team has made substantial progress towards many of the program goals. In particular, researchers have created nondegenerate entangled photon pairs, via spontaneous parametric down conversion (SPDC), at wavelengths (signal, ~540 nm, idler ~1550 nm) that overlap a water absorption band. The team has characterized the entangled photon spectral output and generation rate as a function of laser pump power and crystal temperature. Researchers also measured the temporal correlation of the entangled photon pairs using two single-element optical detectors: a silicon single photon–counting avalanche photodiode (SPAD) for the visible signal photon and an InGaAs SPAD for the infrared (IR) photon. Following this dual single-element detector measurement, researchers sought to duplicate this measurement using Ncam with a single-element InGaAs SPAD as the



Imaging Simple Test Targets by Quantum Imaging Methods Using Ncam as the Image Detector. Experimental interferometric image of root tissue highlighting the increased refractive index of the cell wall. [Courtesy Los Alamos National Laboratory]

*Presenting Author

bucket detector. Team members encountered some technical difficulties synchronizing photon streams on the InGaAs bucket detector SPAD and the Ncam imaging sensor. However, researchers overcame these difficulties and now have correlated entangled photon counts recorded between Ncam and the single-element bucket detector.

More importantly, the team has begun to image simple test targets by quantum ghost imaging methods using Ncam as the imaging detector. The top left part of the figure on p. 6 shows the first test target (a razor blade) that blocks half of the idler beam in the IR (1550 nm), with the image formed from visible light impinging upon the Ncam imaging sensor. The sharpness of this edge provides a determination of the current spatial resolution (~30 microns). The figure also shows another test target: a National Bureau of Standards 1963A high-resolution test target imaged at a slightly out-of-focus position. Researchers are currently working towards automating the focusing procedure, imaging more biologically relevant plant samples, and expanding the entangled photon wavelength palette to provide image contrast at wavelengths more sensitive for lignocellulose and lipid content.

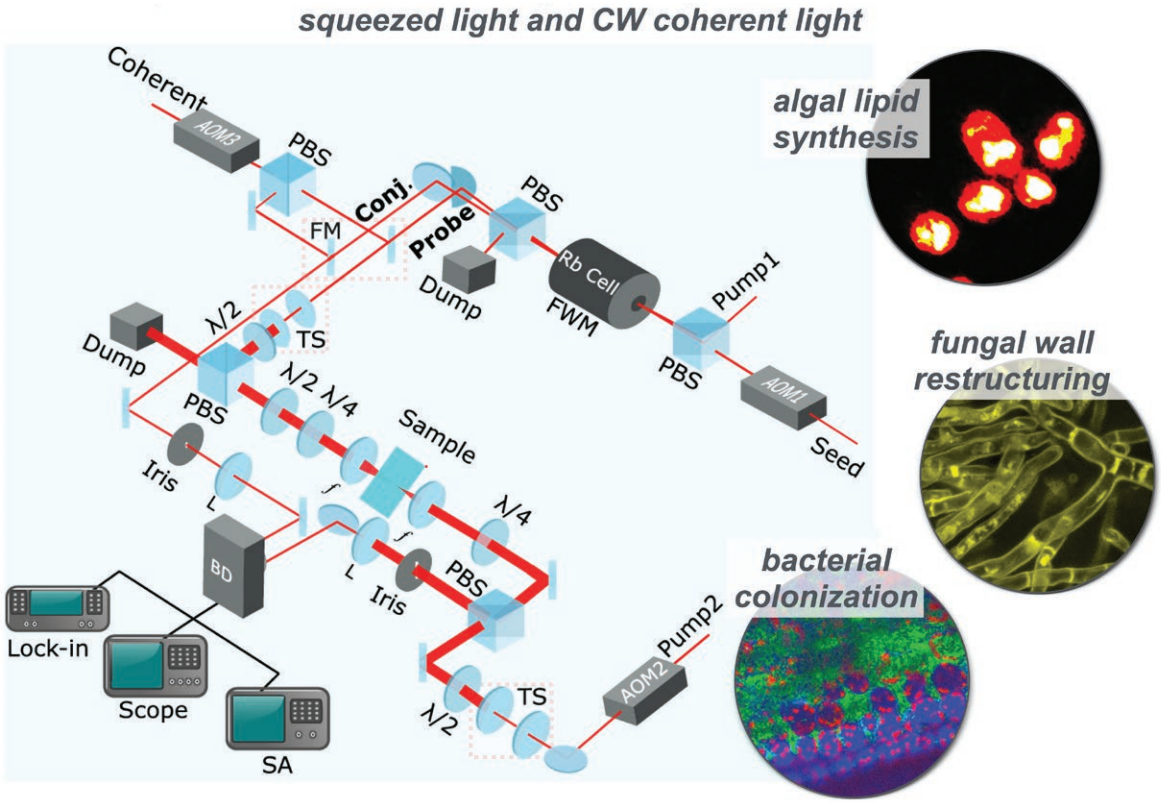
Funding Statement: Los Alamos National Laboratory is managed by Triad, LLC for the U.S. Department of Energy under contract no. 89233218CNA000001. The Center for Integrated Nanotechnologies is supported under contract no. 89233218CNA000001. This research was supported by the DOE Office of Science, Biological and Environmental Research Program grant no. 0000253585.

Next-Generation Stimulated Raman Scattering Microscopy to Overcome Photodamage and Resolution Limitations for Real-Time Tracking of Lipid/Carbohydrate Interfaces in Plants, Algae, and Fungi

Bryon Donohoe* (PI)

National Renewable Energy Laboratory, Golden, CO

A prototype for a next-generation stimulated Raman scattering (SRS) microscope is currently being designed, assembled, and integrated. This microscope will use squeezed light and structured illumination to provide extended observation and chemical probing of biological events without jeopardizing the system's structural integrity or dynamics. The signal-to-noise ratio of SRS will be enhanced by the squeezed light source. As a result, the range of chemical imaging investigations will be expanded, and the risk of photodamage will be reduced to accommodate large regions of interest and long-term image acquisition. Furthermore, the spatial resolution of SRS will be enhanced through structured illumination of squeezed light. This multi-institutional interdisciplinary team of biologists, chemists, physicists, microscopists, and spectroscopists is developing squeezed light and super-resolution microscopy advancements, applying the new capability to specific biological questions and challenges and validating the improvements in sensitivity and resolution with correlative microscopic and spectroscopic techniques. The impetus for this project stems from a long-standing desire to visualize dynamic metabolic reactions in living plants, algae, and fungi in response to environmental stressors in live systems and over long enough periods to visualize complex reactions and the restructuring of lipid/carbohydrate interfaces.



Quantum-Enhanced SRS Layout Using Two-Mode Squeezed Light and Standing-Wave Coherent Light and Examples of the Three Main Biological Systems Under Investigation. [Adapted with permission from Tian, L., et al. 2022. "Quantum-Enhanced Stimulated Brillouin Scattering Spectroscopy and Imaging," *Optica Publishing Group* **9**(8), 959–64. DOI:10.1364/OPTICA.467635. ©2022 Optica Publishing Group.]

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Deployable Quantum Sensors for High Spatial Resolution Nuclear Magnetic Resonance

Ashok Ajoy* (PI)

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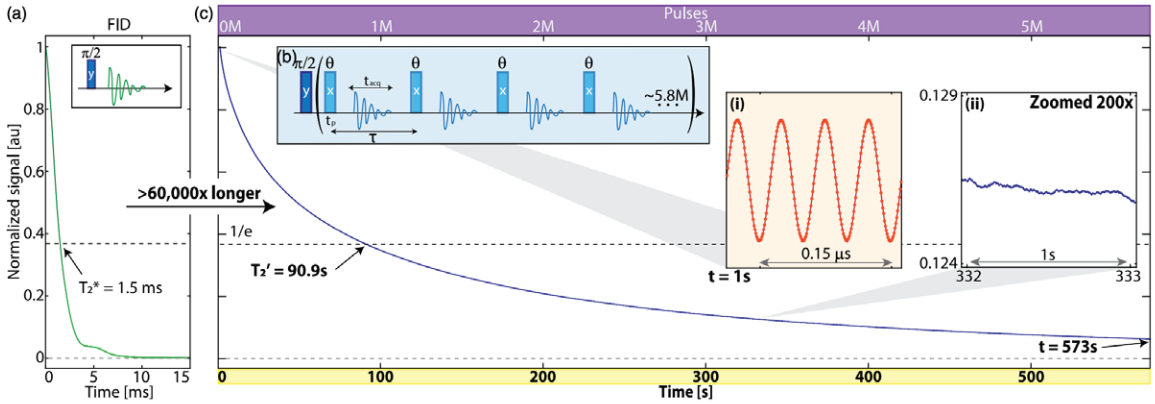
Project Goals: New nuclear magnetic resonance (NMR) quantum sensors based on deployable nanoparticles for high chemical specificity exudate analysis in a fabricated ecosystem (EcoFAB).

The team reports on new experiments that demonstrate the potential of hyperpolarized nuclear spins to serve as sensitive magnetometers and chemical sensors of their local environment. Goals are to leverage this to create new forms of submicron-scale NMR detectors using hyperpolarized nanoparticles. This will enable sensors with high spatial resolution and high chemical specificity that can unravel spatial information of metabolite and microbe interactions in EcoFAB devices.

Here the team reports on protocol development for this form of quantum sensing with ^{13}C nuclei in diamond nanoparticles. The ^{13}C nuclei are optically hyperpolarized through interactions with lattice nitrogen-vacancy (NV) centers.

The methodology leverages two important features: (1) the ability of sensor nuclei to be rapidly hyperpolarized to >3% levels through optical pumping with an array of lasers, which enables over 100-fold faster hyperpolarization than traditional methods (Sarkar et al. 2021), and (2) their ability to be put into long-lived Floquet prethermal states that can be rendered highly sensitive to external magnetic fields. Researchers leverage the exquisite transverse spin lifetimes possible in ^{13}C nuclei under RF driving, wherein scientists observe lifetimes in excess of $T'_2 \approx 90$ s at room temperature (Beatrez et al. 2021; see figure). These long-lived states constitute an extension of >60,000 fold over conventional free induction decay (FID) times of $T^*_2 \approx 1.5$ ms in this system. Simultaneously, when exposed to a time-varying (AC) magnetic field, the nuclei undergo secondary precessions



Floquet Driving and Lifetime Extension. (a) Conventional ^{13}C free induction decay with $T^*_2 \approx 1.5$ ms. **(b)** Floquet drive consists of a train of θ-pulses applied spin-locked with the ^{13}C nuclei. Spins are interrogated in t_{acq} windows between the pulses (blue lines), and the nuclear precession is sampled every 1 ns. Pulse repetition rate $\omega = \tau^{-1}$, and sequence not drawn to scale. **(c)** Minutes-long lifetimes of the transverse state result from the Floquet sequence ($\theta \approx \pi/2$). Data (blue points) show single-shot measurement of survival probability in the state ρI, and the line is a fit to a sum of five exponentials. Here t_{acq}=2 μs, t_p=40 μs and τ=99.28 μs, and the 573 s period corresponds to ≈5.8 M pulses (upper axis). The first 100 ms is neglected here for clarity. **Inset (i):** Raw data showing measurement of the ^{13}C spin precession, here at 1 s into the decay. **Inset (ii):** Data zoomed 200x in a 1 s window. Using a 1/e-proxy yields $T_2 \approx 90.9$ s. This corresponds to a >60,000-fold extension compared to the free induction decay. [Reprinted with permission from Beatrez, W., et al. 2021. "Floquet Prethermalization with Lifetime Exceeding 90 s in a Bulk Hyperpolarized Solid," *Physical Review Letters* **127**(17), 70603. DOI:10.1103/PhysRevLett.127.170603. ©2020 American Physical Society.]

*Presenting Author

that carry an imprint of their frequency and amplitude; this forms the basis of their use as sensors (Sahin et al. 2022; Beatrez et al. 2023). Hyperpolarization and continuous spin readout enable significant gains in sensitivity and resolution. Use of correlation techniques allow enormous gains in signal-to-noise. The team demonstrated a Fourier-limited spectral resolution better than 100 mHz and single-shot sensitivity better than 70pT at a bias field of 7T, among the best reported for any high-field magnetic field sensor.

These experiments suggest interesting new opportunities for deployable spin sensors for use with EcoFABs to enable NMR measurements that identify and quantify rhizosphere metabolites *in situ* and nondestructively, with micrometer spatial resolution and subsecond temporal resolution.

Funding Statement: This work was supported in part by the DOE Office of Science, Biological and Environmental Research Program grant no. DE-SC0012345.

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Noninvasive Imaging of Nitrogen Assimilation in the Rhizosphere via Quantum-Entangled Hyperpolarized Spin States

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Nitrogen fertilizer synthesis for agriculture sustains about half of the human population (Erisman et al. 2008). Recent studies show that nitrogen input from nitrogen fertilizer synthesis and river runoff will pose a serious and growing problem with intensifying climate change (Sinha et al. 2017; Steffen et al. 2015). Excessive fertilization also leads to the release of considerable amounts of nitrous oxide into the atmosphere posing additional aggravating challenges to the bioenergy balance of modern society (Reay et al. 2012). To address these major societal challenges, improvements to today's agricultural strategies are necessary. This team aims to develop a new, noninvasive quantum sensing approach to directly observe metabolic transformation in the rhizosphere to acquire currently inaccessible knowledge.

The research team is developing quantum spin technology to image biochemical pathways in the rhizosphere with unprecedented chemical detail and sensitivity. Specifically, the proposed technology transfers the quantum entangled nuclear spin order of hydrogen gas to metabolites (Hövener et al. 2018), including nitrate, amino acids, and pyruvate (TomHon et al. 2022), to enable molecular imaging of their metabolic transformations without any penetration depth limitations (Bagnall et al. 2020) such that molecular turnover and metabolism can be observed directly in soil. The team is working towards proof-of-concept demonstrations of its quantum sensing approach to study nitrogen assimilation of barrel medic (*Medicago truncatula*). *M. truncatula* is a model legume for biological nitrogen fixation mediated by rhizobia and symbiosis with arbuscular mycorrhizal (AM) fungi, which transport nutrients, including nitrate, to the plant. In return, rhizobia and AM fungi receive fixed carbon from the plant (Garcia et al. 2016; Ossler et al. 2015; Bonfante and Genre 2010). However, there is no technology to date that can track individual metabolic events noninvasively in unperturbed soil to answer some of the most important questions about which metabolic pathways the molecules actually follow. After establishing this new quantum sensing approach to molecular imaging on roots, it can be applied to any plant of interest to study their molecular machinery.

Current technology designed to noninvasively monitor the metabolic turnover of naturally occurring biomolecules in plants, roots, or smaller model systems faces major obstacles. The obstacles for optical techniques include penetration depth limitations and chemical specificity. In contrast to optical techniques, nuclear magnetic resonance (NMR) and magnetic resonance imaging (MRI) are well-established noninvasive techniques that easily identify small changes in chemical structure and perform noninvasive imaging. However, NMR, and especially MRI, are notoriously insensitive and require large samples and high concentrations. Therefore, the study of low-concentration metabolites remains out of reach. With quantum-entangled sources of nuclear spin order (Fisher and Radzihovsky 2018), parahydrogen in particular, the research team has overcome current technological shortcomings and achieved sensitivity gains of up to seven orders of magnitude (Shchepin et al. 2014; Theis et al. 2015), which promise tracking of low-concentration metabolites and their chemical transformations (Cavallari et al. 2018) deep inside soil.

This project has three central objectives. The award was initiated October 2022, and work on this award has been running for 5 months:

Objective 1. Hyperpolarization of Metabolites Central to Nitrogen Assimilation: This objective entails the transfer of nuclear spin polarization from quantum-entangled parahydrogen to pyruvate, alpha-ketoglutarate (also called 2-oxoglutarate), nitrate, glutamate, and nitrogen gas. This task entails the design and modeling of novel polarization transfer catalysts and optimization of sample composition, temperature, magnetic fields, and applied waveforms. Thus far the polarization of pyruvate and alpha-ketoglutarate has been established and optimized. New waveforms for polarization transfer have been developed. Work on nitrate, glutamate, and nitrogen gas is outstanding.

Objective 2. Phantom Imaging of Hyperpolarized Metabolites: The team has been working towards imaging the spin-polarized metabolites and their biochemical transformations in phantoms (glass vials). This task entails injection of the nuclear spin-polarized metabolites into phantoms where molecular transformations are observed. Molecular transformations will be induced chemically in phantoms or biochemically in cultures of the free-living nitrogen-fixing bacterium *Azotobacter vinelandii*. Thus far, researchers have established biochemical transformations in phantoms and phantom-imaging of hyperpolarized pyruvate.

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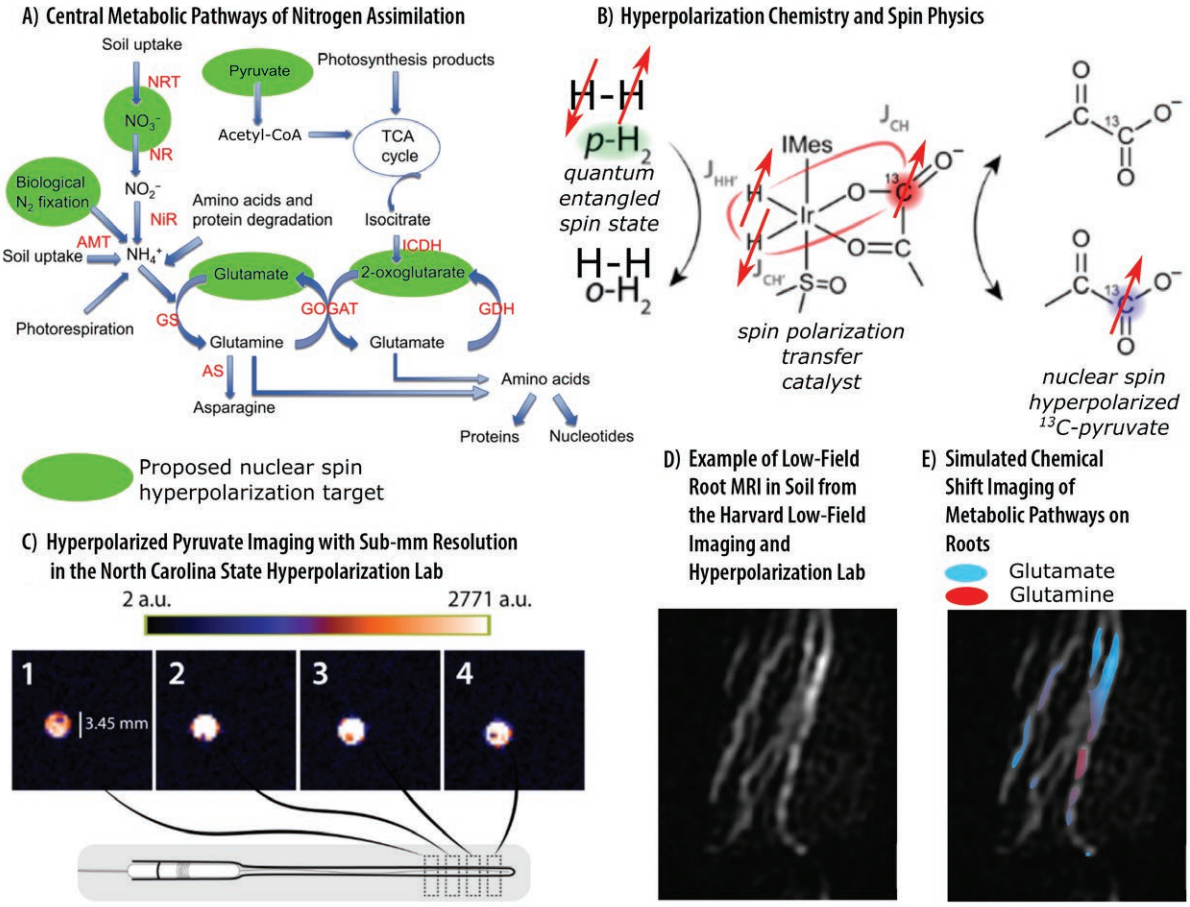

Using Quantum Entangled Nuclear Spin States for Noninvasive Magnetic Resonance Imaging of Nitrogen Assimilation in the Rhizosphere. (A) Metabolic conversions of relevance in nitrogen assimilation; (**B**) Hyperpolarization chemistry: the entangled nuclear spin state of hydrogen is used to induce spin alignment on a metabolite (pyruvate) mediated by a polarization transfer catalyst; **(C)** Recent results demonstrating pyruvate imaging at a low mm concentration with sub-mm resolution in the Theis Lab; **(D)** Low-field root MRI images in soil by Rosen Lab; (**E**) Simulated chemical shift images that report on metabolism and molecular turnover from glutamate to glutamine. [(A) Adapted and reused under a Creative Commons license (CC by 4.0) from Lu, J., et. al. 2016. "Expression of a Constitutively Active Nitrate Reductase Variant in Tobacco Reduces Tobacco-Specific Nitrosamine Accumulation in Cured Leaves and Cigarette Smoke," *Plant Biotechnology Journal* **14**, 1500–10. (B) Courtesy North Carolina State University. (C) Adapted with permission from TomHon, P., et al. 2022. "Temperature Cycling Enables Efficient ^{13}C SABRE-SHEATH Hyperpolarization and Imaging of [1-13C]-Pyruvate," *Journal of the American Chemical Society* **144**(1), 282–87. DOI:10.1021/jacs.1c09581. (D, E) Reused under a Creative Commons license (CC BY-NC-ND 4.0) from Bagnall, C., et al. 2020. "Low-Field Magnetic Resonance Imaging of Roots in Intact Clayey and Silty Soils," *Geoderma* **370**, 114356. DOI:10.1016/j.geoderma.2020.114356.]

Objective 3. Structural and Hyperpolarized Metabolic Imaging of Colonized vs. Uncolonized Roots: This is a comparative study, imaging of roots and their metabolism in water and in soil. This task is split in two: first, imaging the roots structure in growing media and in soil, followed by imaging of root metabolism under varying colonization conditions with rhizobia, AM fungi, both (rhizobia and AM fungi), vs. uncolonized. Thus far, the plant models for imaging have been established.

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FLIM- and Antibunching-Based Optical Recordings of Bacterial Resting Membrane Potentials

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Project Goals:

1. To develop a calibrated resting membrane potential (RMP) using the VoltageFluors recording approach, which is capable of measuring small changes of bacterial RMPs in a high-throughput manner [using Phasor–fluorescence lifetime imaging microscopy (FLIM) analysis].
2. To develop widefield FLIM imaging modalities using time-gated and time-resolved "single-photon counting" (TCSPC) single-photon avalanche diode (SPAD) arrays capable of measuring RMP changes within bacterial biofilms using classical and nonclassical light.

Precise and calibrated measurements of steady-state RMPs and small changes in RMPs are of crucial importance for energy generation, metabolism, and stress response as well as cell-to-cell communication and coordination among bacteria in a biofilm. However, conventional electrode-based methods are not suitable for recording RMPs from tiny bacterial cells. Optical electrophysiological techniques that utilize fluorescence intensity changes also require rigorous calibration for meaningful quantification of RMP changes. Accurate and precise recordings of minute RMP changes require noise-immune optical tools. Here the team has developed a calibrated RMP recording approach that is capable of measuring small changes of bacterial RMPs. The approach relies on two components: (1) a novel optical transducer that utilizes an intrinsic photoinduced electron transfer (PeT) mechanism to sense RMP changes by fluorescence lifetime changes and (2) a quantitative phasor analysis of the recorded confocal FLIM data using a home-written code (AlliGator). This code provides high-throughput quantification of pixel-wise lifetime information. Using this approach, team members have estimated RMPs for *Bacillus subtilis* under normal culture conditions and RMP changes under perturbing chemical conditions (using RMP modulating ionophores). To improve throughput, researchers are developing and incorporating a time-gated SPAD array imager (SwissSPAD3) and (a TCSPC) SPAD array imager (Piccolo), which will be tested with nonclassical (quantum) and classical light. The use of nonclassical light in excitation via entangled two-photon absorption and in emission via antibunching [$g(2)(\tau)$] will be tested and benchmarked for membrane potential imaging.

Funding Statement: This research was supported by the DOE Office of Science, Biological and Environmental Research Program, grant no. DE-SC0020338, DE-SC0023184.

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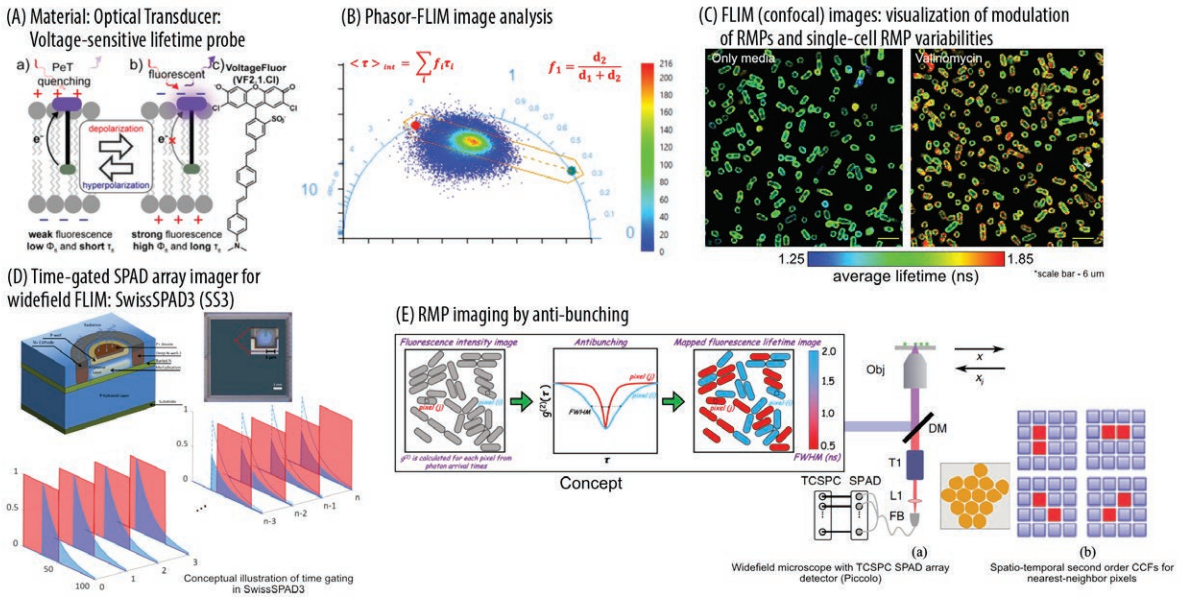

A Calibrated Bacterial Resting Membrane Potential (RMP) Approach. (A) Voltage-sensitive fluorophores VF2.1Cl detecting MP change via photoinduced-electron transfer (PeT). **(Aa)** When the RMP gets hyperpolarized, PeT is accelerated and quenches fluorescence. **(Ab)** When the RMP is depolarized, PeT is restricted and fluorescence is enhanced. **(Ac)** Chemical molecular structure of VF2.1Cl. **(B)** Phasor analysis of an acquired fluorescence lifetime imaging microscopy (FLIM) dataset. **(C)** FLIM images for visualization of modulation of RMPs and single-cell RMP variabilities. RMP changes are displayed as fluorescence lifetime contrast. **(D)** Time-gated single-photon avalanche diodes (SPAD) array imager (SwissSPAD3) for widefield FLIM. Cross section of the p-i-n SPAD and camera module (top panel) is displayed. **(E)** The principles of RMP sensing via antibunching [g(2) (τ)] imaging. The full-width half-maxima of the antibunching dip is extracted for each pixel and used as a proxy for the excited-state lifetime of the voltage probe, which in turn reports on the MP. The intensity image is then false-colored to represent different lifetimes, and hence MP values, in the image. [(A–C) Courtesy University of California–Los Angeles; (D) Reprinted with permission from Ulku, A. C., et al. 2019. "A 512 × 512 SPAD Image Sensor with Integrated Gating for Widefield FLIM," *Institute of Electrical and Electronic Engineers* **25**(1), 1–12. ©2019 Optica Publishing Group; (Ea) Reprinted with permission from Tenne, R., et al. 2019. "Super-Resolution Enhancement by Quantum Image Scanning Microscopy," *Nature Photonics* **13**, 116–22. ©Springer Nature. (Eb) Reprinted with permission from Schwartz, O., et al. 2013. "Super-Resolution Microscopy with Quantum Emitters," *ACS Publications* **13**(12), 5832–36. ©2013. American Chemical Society.]

Cutting-Edge Imaging Technologies to Empower Bioenergy Discoveries

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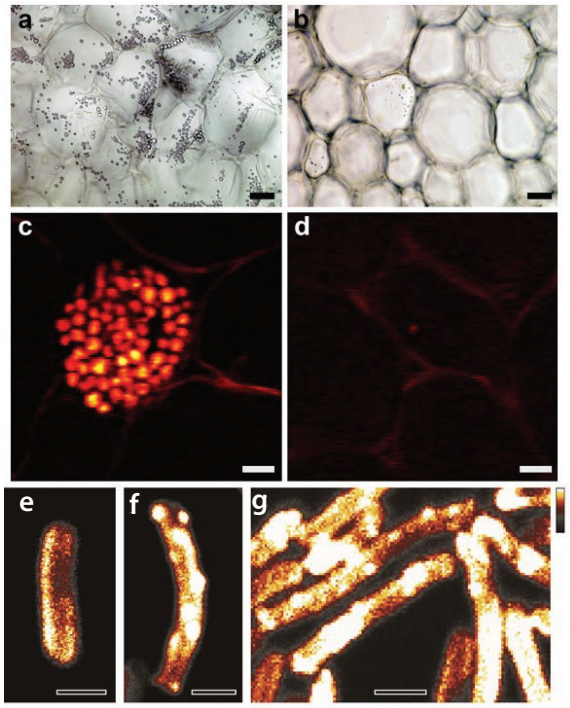
Plants and microbes are major organisms engineered to produce renewable fuels and chemicals to replace petroleum. However, the development of high-efficient organisms is greatly limited by *in vivo* quantification capability for the target molecules due primarily to two challenges. First, 3D, *in vivo*, and real-time quantification of chemical species inside microbes represents a major challenge for classic light microscopy. Second, visualization of chemical species during dark reactions is difficult because the light intensity used in classic light microscopy will interfere with photosynthesis reactions. State-of-the-art stimulated Raman scattering (SRS) microscopy offers solutions for 3D, quantitative, *in vivo*, and real-time imaging. The team first utilized SRS to thoroughly analyze an engineered subcellular organelle for enhanced terpene production. Photosynthetic terpene production represents one of the most energy- and carbon-efficient biological routes for reducing carbon dioxide to hydrocarbons. Researchers carried out the integrated pathway and organelle engineering to improve squalene accumulation in tobacco. First, they modified the animal oleosin protein and engineered it into tobacco leaves to produce subcellular oil droplets to enhance squalene production. The SRS imaging revealed the clear formation of droplets in plant leaves. Second, the chemical species identification indicated that the droplets contain squalene. The imaging results correlate well with gas chromatography mass spectrometry (GC/MS) results, highlighting a fourfold increase in squalene production in the engineered plants with squalene droplets. Third, the team has engineered cyanobacteria to produce limonene at high productivity. Using SRS, researchers identified the hydrophobic interaction among the cells caused by limonene, which further leads to the development of auto-sediment-based low-cost harvesting technology. Using this technology, team members developed a semicontinuous cultivation to achieve the highest reported outdoor algal productivity. Those well-engineered organisms provide a robust and versatile toolbox for the development of cutting-edge imaging technologies for 3D, *in vivo*, and real-time quantification of chemical species. Lastly, researchers are applying quantum imaging with undetected photons (QIUP) and ghost imaging to visualize the changes of the molecular process using a very low dose of probing light. These new imaging capabilities are ideal for probing biological processes during the dark-to-light transition, particularly since light-sensitive processes cannot be viewed with traditional technologies. In addition to QIUP and ghost imaging, researchers are including infrared probes to prevent light interference of the process. Overall, cutting-edge imaging technology has substantially improved the fundamental understanding and technology development to empower bioenergy discoveries.



***In Vitro* Droplet Composition Analysis by Stimulated Raman Scattering Microscopy (SRS).** **(a, b)** Leaf vein section under light microscope for FPS-SQS-HPG and FPS-SQS lines, respectively. **(c, d)** Droplets imaging in leaf vein section for these lines under SRS microscopy at frequency 2900 cm^{-1}. Scale bar is 10 μm. **(e–g)** Limonene production enables cell aggregation in UTEX 2973. SRS chemical imaging identifies chemical compositions in the droplets. A significantly higher limonene signal is found in L524 **(e)** compared to wild-type **(f)**. This observation is more evident at the L524 cell surface, where limonene droplets appear to attach to the outer cell surface. In L524 cell aggregation, the surface-attaching limonene appears to form inter-cell junctions bridging cells **(g)**. [(a–d) Reprinted with permission from Zhao, C., et al. 2018. "Co-Compartmentation of Terpene Biosynthesis and Storage via Synthetic Droplet," *Cell-Free Synthetic Biology* **7**(3), 774–81. DOI:10.1021/acssynbio.7b00368 © 2018 American Chemical Society. (e–g) Reused under a Creative Commons license (CC By 4.0 International) from Long, B., et al. 2022. "Machine Learning-Informed and Synthetic Biology-Enabled Semi-Continuous Algal Cultivation to Unleash Renewable Fuel Productivity," *Nature Communications* **13**, 541.]

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Quantum Optical Microscopy of Biomolecules Near Interfaces and Surfaces

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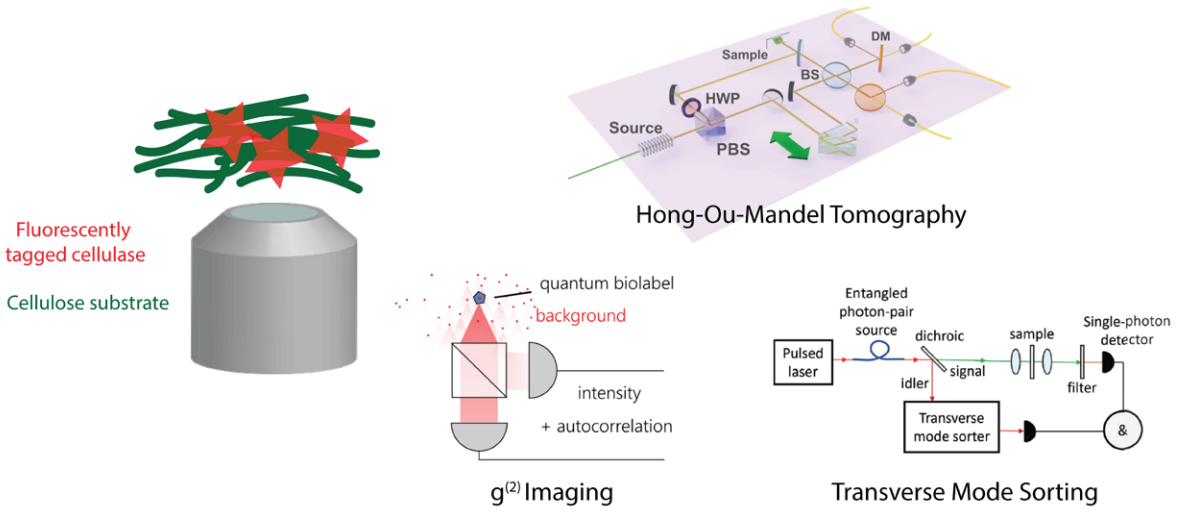
Since the publication of Hooke's *Micrographia* in 1665, the scientific disciplines of light microscopy and (sub)cellular biology have progressed in lockstep with one another. Advances in the spatial and temporal resolution, specificity, and sensitivity of optical methods have continually led to new capabilities and insights in biological imaging. The pace of this evolution has quickened in the past century, as a mastery of the physics of light according to Maxwell's equations has been wielded to more fully exploit classical effects like interference and diffraction. As the classical limits of light microscopy near saturation, however, sustained improvement in bioimaging technology is ultimately untenable without a more fundamental shift in research direction. Just as the field of quantum computing has gained prominence in anticipation of the inevitable breakdown of Moore's Law, quantum-enabled light microscopy will likely provide the path forward for (sub)cellular biological imaging.

This project aims to help lead this effort by developing three complementary quantum microscopy modalities that each address a different challenge inherent to (sub)cellular microscopy:

1. Hong-Ou-Mandel Interference Microscopy to enable loss- and noise-tolerant depth imaging with exquisite resolution;
2. $g^{(2)}$ Microscopy to facilitate orders-of-magnitude sensitivity improvement in focusing and tracking single-quantum emitters atop oppressive classical backgrounds at reduced excitation powers; and
3. Transverse Mode Sorting Microscopy to enable super-resolution microscopy at low excitation powers and high temporal resolution.

Preliminary project results demonstrating progress in developing these constituent techniques. The team will ultimately incorporate them into a common imaging platform that can provide access to the many scales of interest in energy-relevant plant and microbial biology. The combined technique, quantum optical microscopy of biomolecules near interfaces and surfaces (QuOMBIS), will be especially powerful for tracking and imaging individual and few fluorescently labeled biomolecules in the context of nearby biological interfaces and surfaces. Upon development of the methods, researchers will apply the platform to unravel and harness the enzymatic conversion of biomass into renewable fuels.



Quantum Optical Microscopy of Biomolecules Near Interfaces and Surfaces (QuOMBIS). The QuOMBIS platform marries quantum optical techniques, which take advantage of photon-photon correlations in three distinct ways in order to improve signal-to-background and resolution without requiring high excitation doses. The integrated apparatus will be applied to image and track single and few fluorescently labeled cellulases acting on cellulose substrates in an effort to uncover fundamental processes in the production of biofuel. [Courtesy University of Illinois–Urbana-Champaign]

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University Quantum Imaging Approaches–2022 Projects

Squeezed-Light Multimodal Nonlinear Optical Imaging of Microbes

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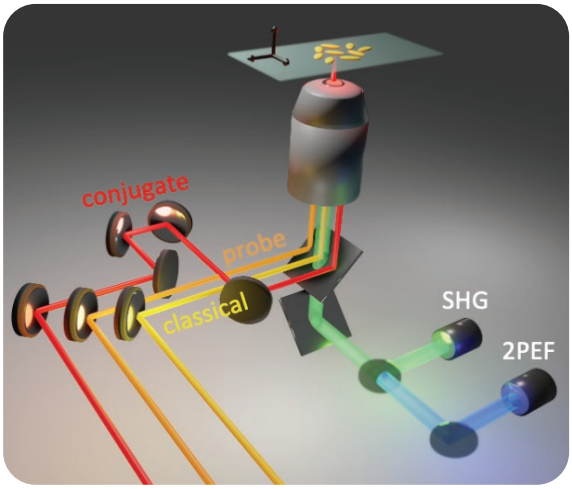
Project Goals: The overarching goal is to develop multimodal quantum nonlinear optical imaging based on a squeezed-light source for co-registered, steady-state two-photon excited fluorescence, two-photon-excited fluorescence lifetime imaging, and second-harmonic generation microscopies. To validate the capabilities and advantages of these squeezed-light-based quantum light imaging modalities against the corresponding classical microscopies at the cellular level, researchers will image the growth and dynamics of a phenazine-producing bacterium in a synthetic microbial community or during plant colonization. The team will also evaluate the feasibility of using bacterial phenazines as intrinsic biomarkers for live cell imaging of *Pseudomonas* strains isolated from the rhizosphere.

Biological processes in plant-microbe communities involve the movement and transformation of various molecular species that exist in complex chemical environments, often in low abundance and nonuniformly distributed within the biosystem. A comprehensive understanding of these complex processes requires the use of imaging techniques to probe the localization of molecular species with ever-increasing fidelity. Advanced nonlinear optical (NLO) imaging technologies have offered a variety of unique contrast mechanisms for direct *in vivo* visualization of biological processes taking place over diverse time and length scales. However, the high level of light intensity that is commonly used for such NLO imaging microscopies can often cause photobleaching and photodamage, severely disturbing biological processes and functions and compromising viability. This phototoxicity is particularly problematic for gaining a comprehensive understanding of biological function, which often necessitates prolonged imaging measurements. The quantum nature of light provides promising opportunities to enable noninvasive and nondestructive bioimaging.

The goal of this project is to develop multimodal quantum NLO imaging modalities based on a squeezed-light source for co-registered steady-state two-photon-excited fluorescence, two-photon-excited fluorescence lifetime, and second-harmonic generation microscopies. The high degree of temporal correlation between the photons in the squeezed light can result in not only a favorable linear intensity-scaling of the two-photon interaction rate compared to the quadratic dependence for classical light but also enhanced NLO responses. These unique properties provide a revolutionary route towards performing NLO microscopy with substantially lower excitation power than is currently feasible, and thus it can be leveraged as a fundamental mechanism for solving the long-standing challenge of photodamage and phototoxicity in bioimaging.



Multimodal Quantum Nonlinear Optical Microscope. Squeezed light for imaging will be provided by probe and conjugate beams generated by four-wave-mixing in ^{87}Rb vapor. Two-photon excited fluorescence (2PEF) and second harmonic generation (SHG) signals will be spectrally separated and measured with photon-counting detectors, and frequency domain fluorescence lifetime imaging (FD-FLIM) will be measured with a separate, analog photomultiplier tube. [Courtesy Oak Ridge National Laboratory]

To accomplish this overarching goal, the team's research encompasses the following three primary objectives. In objective 1, researchers will focus on developing squeezed-light sources and methods for measuring molecular cross sections, establish protocols for reliably quantifying the advantage of quantum imaging modalities over their corresponding classical equivalents and develop squeezed-light frequency-domain fluorescence lifetime spectroscopy. Objective 2 will be centered on developing the co-registered

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multimodal quantum NLO microscope with steady-state, two-photon-excited fluorescence and second harmonic generation modalities by coupling a squeezed-light source and detection system implemented in JILA to an existing multimodal imaging platform at ORNL. Two-photon-excited fluorescence lifetime imaging will also be developed following the implementation and optimization of the corresponding spectroscopic approach at JILA using model molecular species and intrinsic biomarkers selected based on their two-photon absorption cross sections. In objective 3, researchers will validate the unique capabilities and advantages of these quantum light microscopies against the corresponding classical imaging modalities by visualizing the growth and dynamics of a phenazine-producing bacterium, such as *Pseudomonas* sp. GM17. This visualization is done in a synthetic microbial community and during plant colonization to examine the feasibility of using phenazine as an intrinsic probe to identify and track specific bacterial strains, especially how the presence of a phenazine-producing microbe influences the spatial organization and composition of the community.

Funding Statement: This research is supported by the DOE Office of Science, Biological and Environmental Research Program, grant no. DE-SC0023538. Oak Ridge National Laboratory is managed by UT-Battelle, LLC for the U.S. Department of Energy under contract no. DE-AC05-00OR22725. This program is supported by the DOE Office of Science through the Genomic Science Program of the Biological and Environmental Research Program under FWP ERKPA62.

Development of Classically Entangled Light for Depth-Resolved Quantum Mimicry Bioimaging

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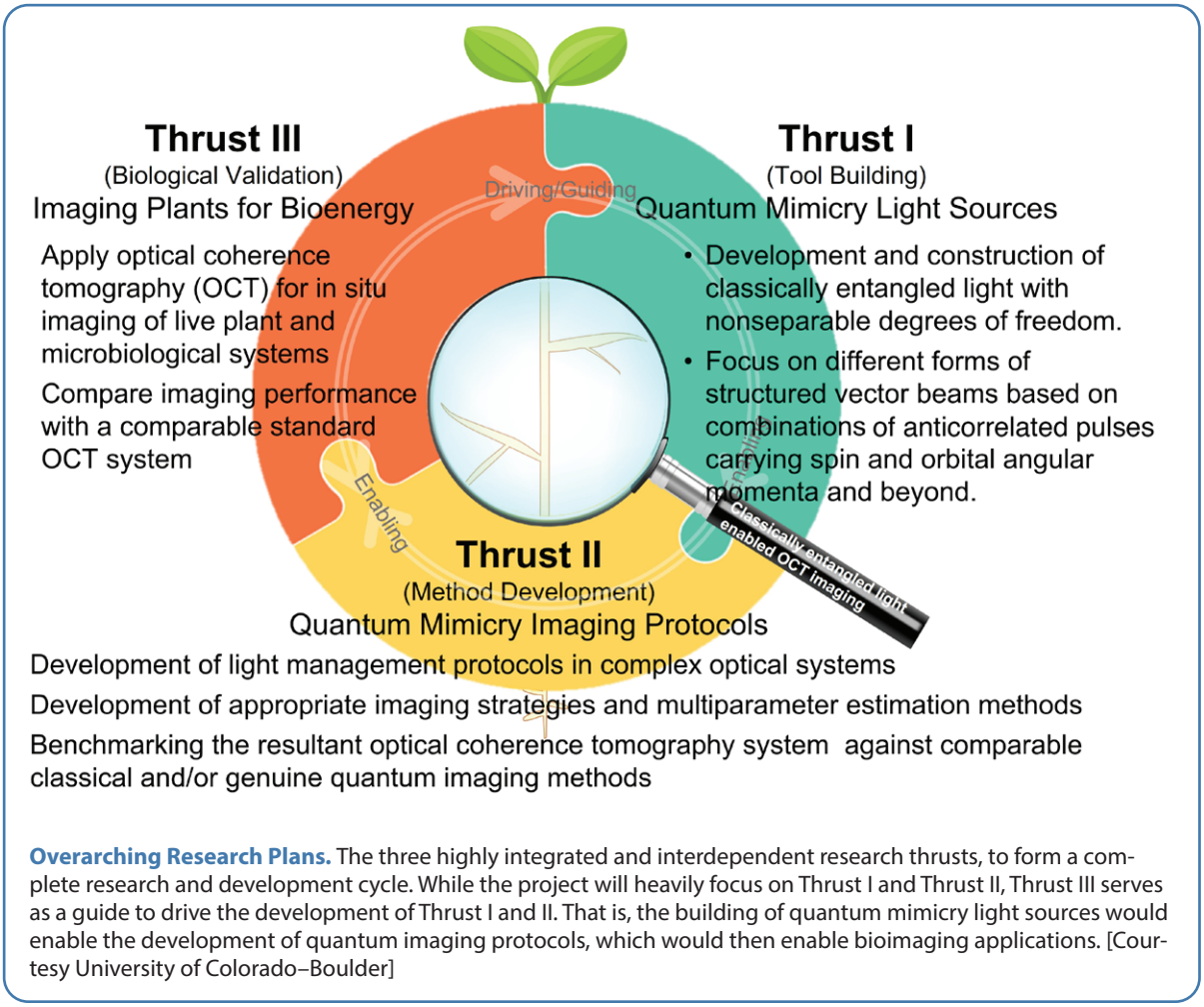
Project Goals: This project aims to build upon the principles of classical entanglement of light and develop new and untested (1) classes of anticorrelated light sources and (2) quantum-inspired imaging protocols that fit into the theoretical framework for recapitulating desirable super-performing imaging traits (e.g., the performance that surpasses those set by classical limits). More specifically, efforts will be focused on testing the quantum-like characteristics of newly developed light pulses and applying them to enhance the performance of optical coherence tomography, a label-free cross-sectional imaging method that is suited for *in situ* probing of plant biology.

Quantum imaging has attracted growing interest over the past three decades, motivated by successful demonstrations that it could outperform its classical counterpart in several aspects. However, challenges associated with the low brightness of entangled photons and reliance on photon-sparse imaging protocols have stalled attempts at translating those technologies to practical biological field use. Those issues have also necessitated long data acquisition times and make imaging of dynamical biological processes challenging. Surprisingly, several phenomena once thought to be exclusive to quantum-entangled photons had been successfully replicated with classical light carrying anticorrelations or nonseparable degrees of freedom (e.g., spin and orbital angular momenta, wavelengths, spatial, and temporal modes). These discoveries



Overarching Research Plans. The three highly integrated and interdependent research thrusts, to form a complete research and development cycle. While the project will heavily focus on Thrust I and Thrust II, Thrust III serves as a guide to drive the development of Thrust I and II. That is, the building of quantum mimicry light sources would enable the development of quantum imaging protocols, which would then enable bioimaging applications. [Courtesy University of Colorado–Boulder]

*Presenting Author

gave rise to an emerging field known as classical entanglement or mode-entanglement of light, such as those involving arbitrarily tailored vector beams. The ability to perform quantum mimicry using special forms of classical light has far-reaching implications, both in the potential of overcoming inherent shortcomings of quantum light sources and in the practical considerations of translating those advantages for robust imaging applications. The project will perform research on the underpinning principles for optical wavefront and field control of structured vector beams, such that the knowledge can be applied to the design and construction of light sources for quantum mimicry imaging. The team will subsequently develop interferometric systems and image reconstruction protocols for optical coherence tomographic bioimaging based on the considerations for those classically entangled beams. Characterization of the imaging instrument and validation of its performance will be carried out to benchmark its performance against comparable technologies without classical entanglement. The expected outcomes could potentially lead to quantum-like imaging advantages without sacrificing optical brightness. The quantum-like advantages or enhancement pursued in this project include low-noise, high-sensitivity imaging through turbid and scattering media. These enhanced capabilities could benefit plant research on multiple fronts, from imaging dynamically evolving bioevents with high precision to probing photosensitive biosystems with the lowest dose possible. By collaborating with experts in plants and microbiological systems at a later phase of the project, the developed imaging technology will be designed to be applicable for future *in situ* imaging of plant biological systems relevant to biomass and bioenergy investigations.

Funding Statement: This research is supported by the DOE Office of Science, Biological and Environmental Research Program grant no. DE-SC0023314.

Establish X-Ray Quantum Imaging for Subcellular Structures

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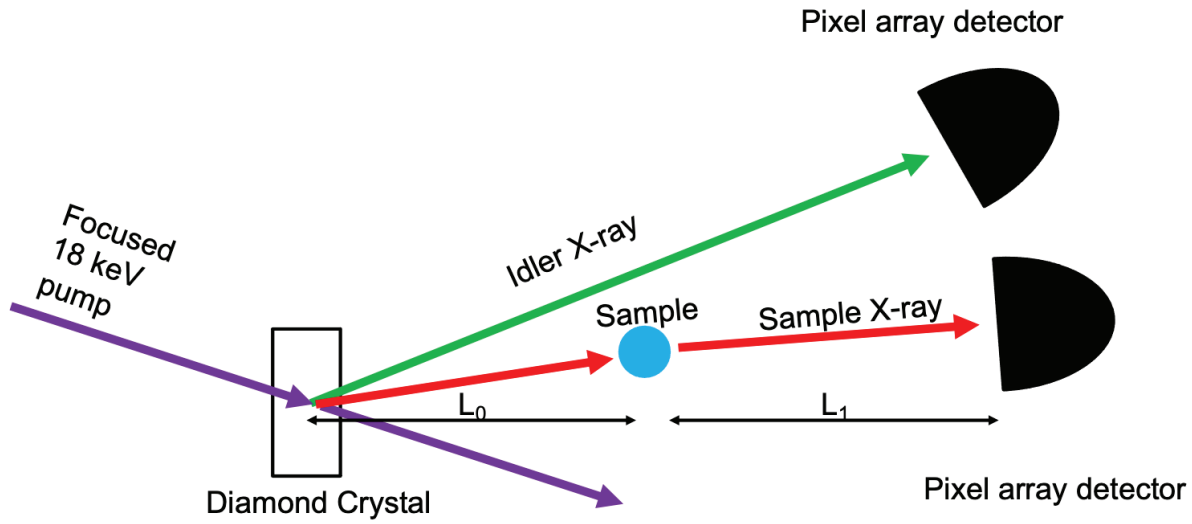
X-ray quantum imaging (XQI) has the potential to revolutionize imaging of sensitive biological objects such as subcellular structures. XQI exploits the nonclassical behavior of higher-order correlations of quantum-entangled photons. This allows users to overcome the limits of classical imaging with X-rays and substantially reduces the required dose to image-transparent, weakly absorbing biological objects. It is anticipated that XQI could bridge nanometer-sized molecular structure with micron-sized cellular structure and thus provide new insights on how energy flow is regulated by macromolecular assemblies at the mesoscale.

XQI takes full advantage of the unique high-repetition rate hard X-ray capabilities of the Linac Coherent Light Source-II and the subsequent High Energy upgrade. This allows for unprecedented generation of quantum-entangled photons and facilitates quantum imaging with X-rays in a fashion not possible with any other X-ray source. Compared to classical X-ray imaging methods, XQI can significantly reduce the dose, improve the signal-to-noise ratio, and give better spatial resolution due to decreased radiation damage. Consequently, cryopreservation of the sample may no longer be required to reach nanometer resolution, eventually enabling *in vivo* imaging at ambient temperatures.



Schematic Layout of the Setup for X-Ray Quantum Imaging (XQI). Pump X-rays of energy 18 keV are focused on a diamond crystal, where two entangled streams of biphotons are produced by parametric down conversion of X-rays. One of the streams (red) interacts with the sample, whereas the other stream (green) remains unchanged. [Courtesy University of Wisconsin–Milwaukee]

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Mid-Infrared Single-Photon-Counting Photodetectors for Quantum Biosensing

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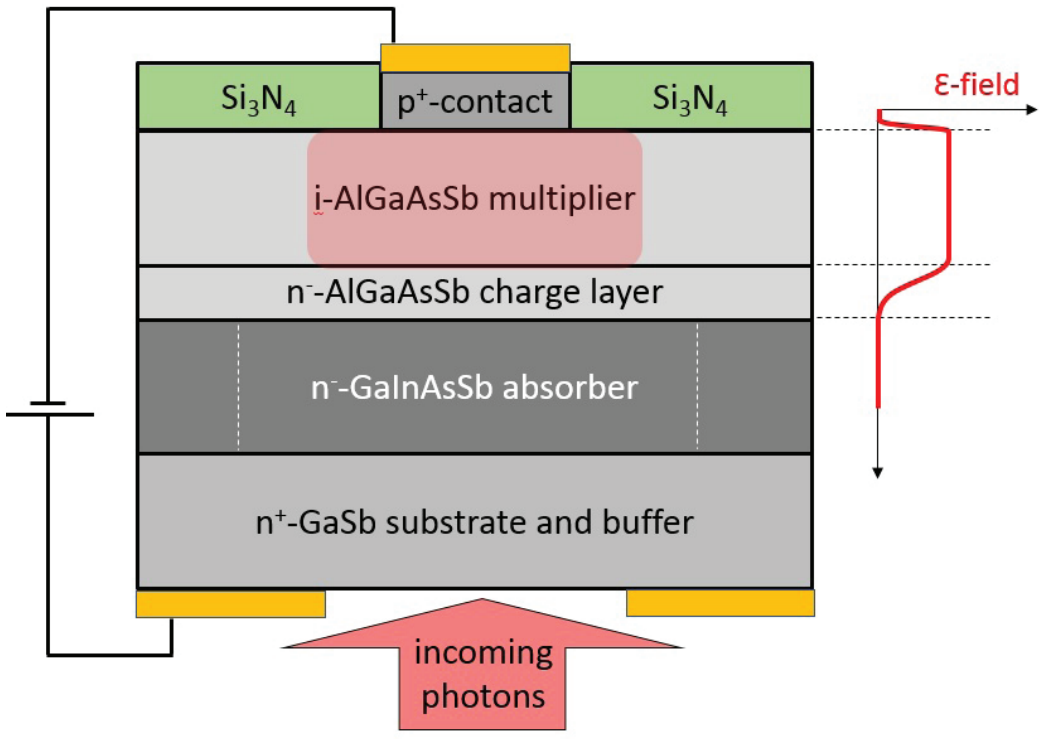
Project Goals: The project goal is design and development of the single-photon-counting avalanche photodiodes operating in mid-infrared region with cutoff wavelength above 3 μm. The devices are based on epitaxially grown III-V-Sb separate absorption and multiplication heterostructures optimized for hole-initiated impact ionization. Application of the novel devices as a bucket detector in mid-infrared quantum ghost imaging of biological tissue is envisioned.

Compact and efficient single-photon-counting photodetectors operating in spectral region near and above 3 μm are in demand for noninvasive biosensing and bioimaging. In the near-infrared regions of spectrum up to 2 μm-single-photon detection sensitivity adequate for quantum light experiments has been demonstrated in InP- or GaSb-based semiconductor avalanche photodetectors (APD) operating in Geiger mode, (i.e., biased above the avalanche breakdown threshold and equipped with quenching electronics; Donnelly et al. 2006; Duerr et al. 2007). Fundamental increase of thermal generation/recombination noise and an increased probability of tunnel breakdown in narrow bandgap semiconductors necessitates the use of separate absorption, charge, and multiplication (SACM) APD device architecture to evade high electric field in the narrow bandgap materials (Campbell 2016). In SACM APDs, optical absorption occurs in a narrow bandgap but carrier multiplication takes place within wide bandgap layers. The properties of the heterointerface between narrow bandgap absorber and wide bandgap multiplier play an important role. Transport of the photogenerated carriers from absorber to multiplier through the charge-control layer should not be obstructed by band discontinuities. This is necessary to avoid the presence of electric fields and corresponding depletion regions in a narrow bandgap semiconductor. The benefits associated with the elimination of the depletion region in narrow bandgap absorbers were demonstrated in barrier infrared photodetectors (Maimon and Wicks 2006). The corresponding reduction of the thermal generation current and suppression of the surface leakage currents in barrier photodetectors ensure the device provides low noise and high sensitivity (Klipstein et al. 2011). In III-V-Sb barrier photodetector



Schematic Structure of the Hole-Injected GaSb-Based Single-Photon Avalanche Diode. [Courtesy State University of New York at Stony Brook]

*Presenting Author

heterostructures the valence band discontinuity between InAsSb "absorber" and AlGaAsSb "barrier" can be minimized to enable efficient hole transport without penetration of electric field into the narrow bandgap material. The GaSb-based mid-infrared barrier photodetector technology can be further enhanced by adding internal gain. The internal gain will come from the hole-initiated impact ionization in the AlGaAsSb wide bandgap layer. Due to its specific band structure character, the hole impact ionization coefficient in AlGaAsSb is predicted to be atypically high compared to other semiconductors (Collins et al. 2018). Operation of the corresponding SACM APDs in Geiger mode can yield single-photon-counting functionality in the mid-infrared region of spectrum.

The development of the mid-infrared SACM APDs demands technological capability to grow heterostructures containing: (1) a layer made of an alloy with bandgap below 400 meV and long carrier lifetime; (2) a layer made of an alloy with bandgap above 1.5 eV with high impact ionization coefficients and low band-to-band tunneling probabilities; and (3) a transition layer providing unimpeded transport of photogenerated carriers from absorber to multiplier regions. Development of comprehensive technological solutions, understanding of the fundamental material limitations, and demonstration of the mid-infrared III-V-Sb SACM hole-APDs are the goals of the first-year efforts.

The team has designed and grown: (1) heterostructures for carrier lifetime characterization in InAsSb and GaInAsSb narrow bandgap alloys; (2) heterostructures for studies of the impact ionization coefficients of electrons and holes in wide bandgap AlGaAsSb alloys; (3) test barrier photodetector heterostructures; and (4) test avalanche photodetector heterostructures. The figure on p. 23 illustrates one of the test barrier photodetector heterostructures, which demonstrates required optical sensitivity above 3 μm in a wide temperature range.

The fabrication and characterization of the test avalanche photodetectors as well as carrier lifetime and impact ionization studies are in progress.

Funding Statement: This research is supported by the DOE Office of Science, Biological and Environmental Research Program grant no. DE-SC0023165.

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Entanglement Enhanced Quantum Stimulated Raman Spectroscopy Imaging Lab-on-a-Chip

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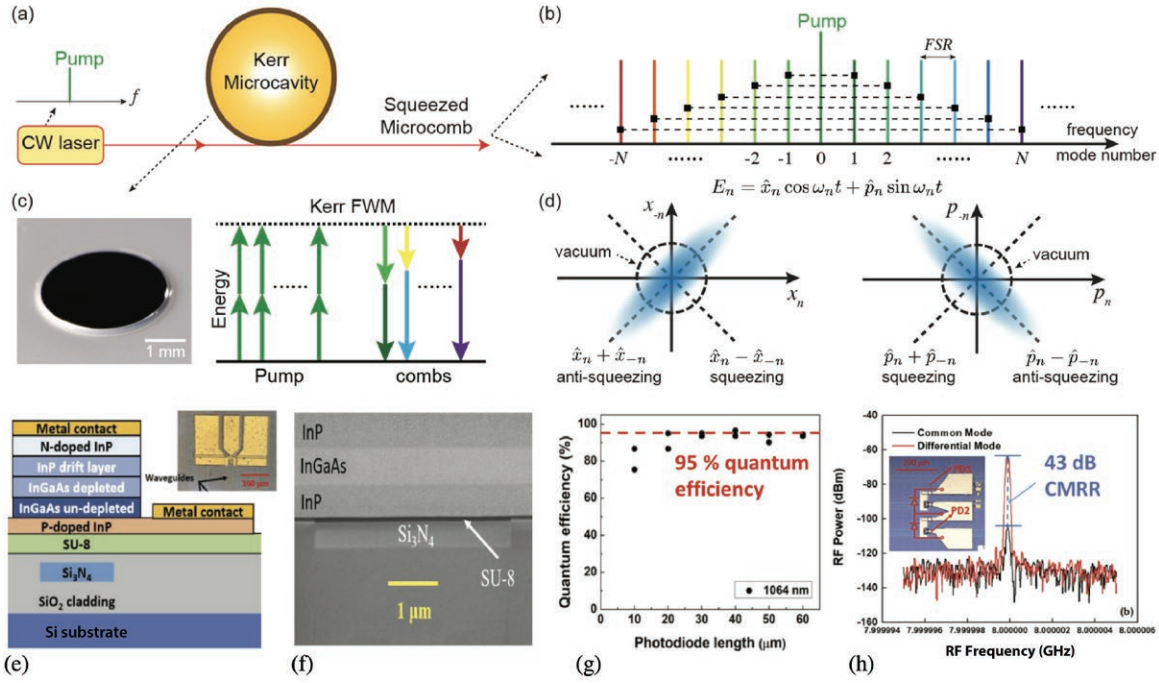
Stimulated Raman spectroscopy imaging is used in numerous biological imaging applications for its ability to resolve dense or overlapping vibrational spectral bands as well as spatially differentiate the molecular distribution. However, both spectral and spatial resolutions of classical nondestructive stimulated Raman bioimaging are constrained by the input optical power to avoid photodamaging of biological samples. Integrating quantum enhancement into nonlinear bioimaging will allow imaging of biomolecular distributions at resolutions not reachable by classical techniques. To obtain practical quantum sensors that fully intersect with biological imaging application space, this project is pursuing fully integrated sources of entanglement and detection, attempting a step forward towards the quantum biology lab-on-a-chip. The team has performed on-chip generation of continuous-variable quantum entanglement and recently developed integrated high quantum-efficiency photodiodes will be discussed. To create a framework for quantitative comparison and validation of the quantum enhancement, researchers are also preparing suitable biomass specimens and conducting classical bioimaging.



Integrated Photonics for Squeezed Quantum Frequency Comb Generation and Detection. (a) A continuous-wave (CW) pump laser is coupled to a microresonator, which has thousands of longitude resonance modes with their frequencies separated by the resonator free-spectral-range (FSR), as shown in **(b)**. **(c)** The Kerr nonlinearity in the microresonator creates broadband parametric gain as the pump photon pairs (green) can be converted into signal and idler photons at lower and higher frequency modes. **(d)** This nonclassical correlation creates two-mode vacuum squeezing and thus unconditional EPR entanglement of the optical quadrature fields between frequency modes n and –n, which are connected by dashed black lines in the optical spectrum. **(e)** Cross-sectional illustrations of photodiodes (PDs) on SiN waveguide. **(f)** SEM picture of the PDs cross section. **(g)** Measurement of photodiode quantum efficiency. **(h)** Common mode rejection ratio measurement for integrated balanced photodiodes. [Courtesy University of Virginia]

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Development of a Quantum-Optimal Bioimaging System for Plant-Microbiome Interactions

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Project Goals:

- Quantum-information optimal multi-passed (MP) microscope design and development.
- Demonstrate MP-stimulated Raman scattering microscopy (MP-SRS) for high-sensitivity, label-free chemical imaging.
- Develop technologies for quantum-optimal quantitative phase imaging and extend this approach towards interaction-free measurements.
- Apply artificial intelligence/machine learning methods to establish Raman signatures of different bacterial and fungal species in different environmental conditions.
- Apply the Raman MP microscope to follow plant-bacteria interactions during infections of isolated plant cells and tissues.
- Use the Raman MP microscope to elucidate how individual bacterial species interact with each other under a changing biofilm environment.
- Develop microfluidic devices coupled with Raman MP microscopy for efficient separation and concentration of soil bacteria into single species.
- Single-cell geno- and phenotyping and cryo-electron tomography of purified cells.

The team presents progress towards the development of quantum-information optimal MP imaging technologies based on reimaging optical systems (Juffmann et al. 2016). An MP microscope interrogates a sample multiple times in a programmable and deterministic fashion. This leads to a metrological advantage for imaging weak scatterers. This enhanced sensitivity can yield a significant reduction in sample damage or can reduce image acquisition time. The approach can enter a quantum nondestructive regime where the photon interaction with the image target is fully coherent and the imaging process becomes quantum nondestructive when conditioned upon the detection of single photons. Recent theoretical analysis has shown that this imaging approach saturates quantum information bounds and compares favorably with bounds obtained using squeezed and other entangled probe states, but it avoids the technical complexity associated with the production of such states (Koppell and Kasevich 2022).

As proof-of-concept experiments, the team will use these protocols for the study of microbe-microbe and microbe-plant interactions in MP-SRS configurations. These configurations will enable volumetric, chemically specific imaging of thick samples. Furthermore, building on the demonstration of continuous-wave MP flow cytometry (Israel et al. 2022), the Raman MP microscope will be integrated into extremely efficient, label-free microfluidic separators for isolating single species of soil bacteria. The team plans to design these quantum imaging technologies into compact and robust systems for shared use among the BER science community.

Funding Statement: This research is supported by the DOE Office of Science, Biological and Environmental Research Program grant no. DE-SC0023076.

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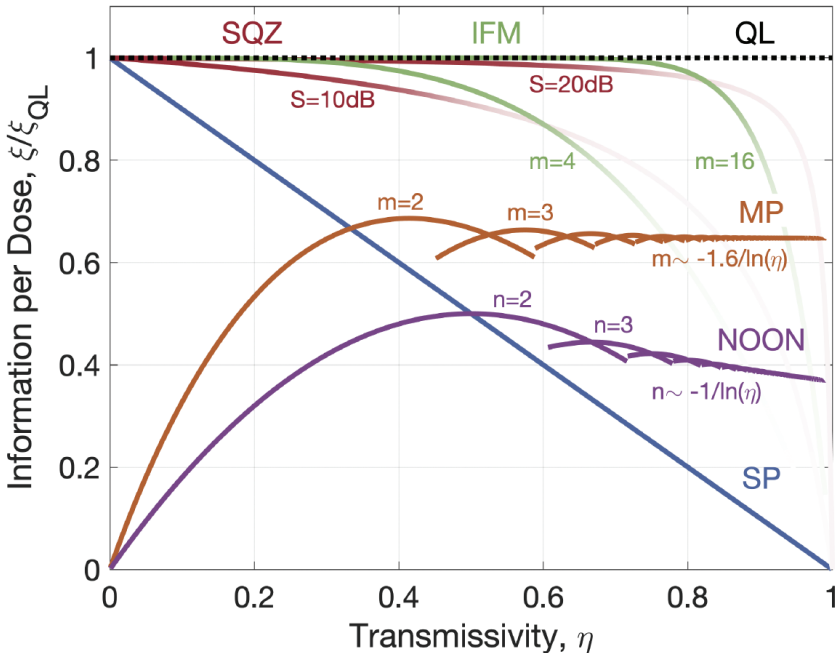
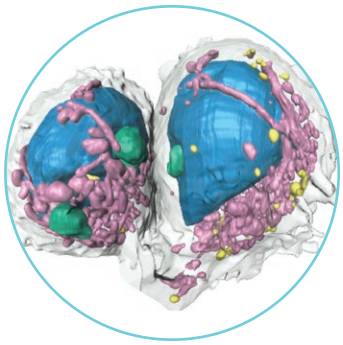


Quantum Fisher Information Per Dose as a Function of Sample Transmissivity for Single Pass (SP), Entangled NOON, Multi-Pass (MP), Squeezed (SQZ), and Interaction Free (IFM) Configurations. Information of each protocol is normalized to the quantum limit (QL). The performance of the MP protocols competes favorably with entangled SQZ and NOON protocols for transmissive samples. The MP protocol does not require exotic quantum states. [Reprinted with permission from Koppell, S., et al. 2022. "Transmission Electron Microscopy at the Quantum Limit," *Applied Physics Letters* **120**(19), 190502. DOI:10.1063/5.0086148.]



Chapter 2
Structural Biomolecular Characterization User Resources

Functional insights into genomic and environmental systems can be illuminated through structural and morphological information obtained using the BER-supported structural biology and imaging user resources. The following resource descriptions outline strategic approaches and BER science advances enabled through measurements made with these capabilities. New developments and uses of the capabilities also are described, along with the enhanced potential of these distinctive techniques through cross-disciplinary collaboration with other facilities.

Rapid Characterization of Macromolecular Solution Structures for BER Projects

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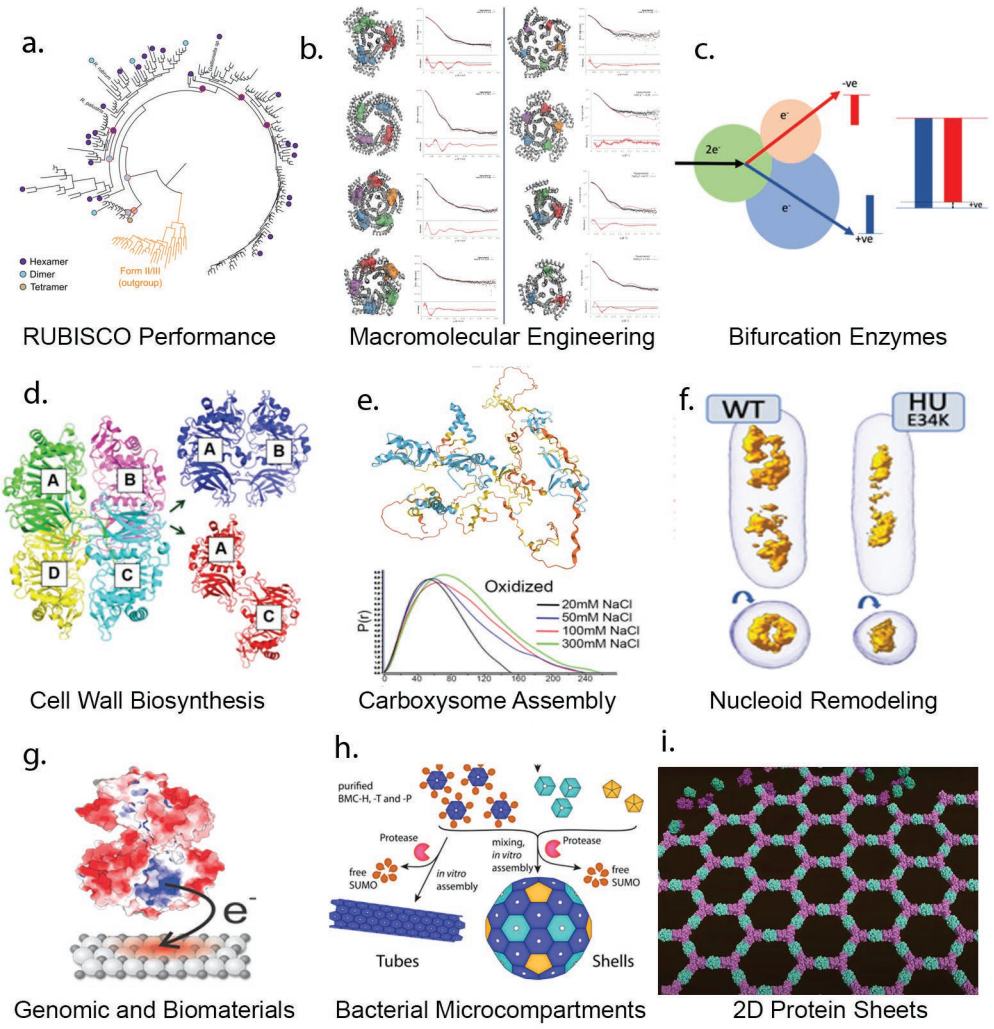
Lawrence Berkeley National Laboratory (LBNL), Berkeley, CA

A major gap in genomic characterization is a full understanding of most gene product functions and mechanisms. To fill this gap, the Integrated Diffraction Analysis Technologies (IDAT) project applied unique X-ray technologies to DOE BER programs. Funded by BER, IDAT has aided in the elucidation of plant and microbial metabolic pathways and particularly those involved in carbon cycling. IDAT will help develop new capabilities for DOE Joint Genome Institute (JGI), Molecular Foundry (MF), and Joint BioEnergy Institute (JBEI) studies. IDAT will support users during the Advanced Photon Source shutdown. IDAT will open new avenues for DOE BER investigators to engineer macromolecules and biomaterials.

The team's unique strength is in bridging small angle X-ray scattering (SAXS) at the SIBYLS synchrotron beamline to other methods by applying computational tools. SAXS has intermediate resolution and can be collected in high throughput in almost any solution condition. SAXS not only bridges spatial scales but also *in vitro* to *in vivo* aspects of crystallography and cellular tomography. The LBNL SIBYLS synchrotron beamline applies two primary SAXS technologies: high-throughput SAXS (HT-SAXS) and size-exclusion coupled SAXS (SEC-SAXS). Microliter and microgram quantities collected at a rate of 100 samples per hour have been very impactful for many DOE BER applications. SAXS has been particularly empowered by emergent structure prediction algorithms that provide subnanometer local accuracy. Together, structure prediction with SAXS can inform conformation, dynamics, and mechanism in high throughput.

A deep and broad understanding of biological macromolecular structure, conformation, and mechanism is fundamental to DOE BER missions for a predictive understanding of complex biological systems. The SIBYLS beamline is a national resource for the collection and integration of SAXS data. Application of IDAT technologies to DOE BER-related projects drives further development. IDAT collaborators gain an understanding of how the chemospatial structure of multicomponent biomolecules enables the dynamic regulation of these processes.



Example Complex Biological System Studies Supported by IDAT. Project staff work with BER researchers to characterize cellular and biological materials including lipids, cell walls, cellulose, and more. [(a) Courtesy Liu, A., et al. 2022. "Structural Plasticity Enables Evolution and Innovation of RuBisCO assemblies," *Science Advances* **8**, eadc9440. DOI:10.1126/sciadv.adc9440 licensed under Creative Commons Attribution 4.0 International. (b) Revised under a Creative Commons license (CC By 4.0 International) from Gerben, S., et al. 2023. "Design of Diverse Asymmetric Pockets in *De Novo* Homo-oligomeric Proteins," *Biochemistry* **62**(2), 358–68 published by American Chemical Society. (c) Courtesy University of Georgia. (d) Reprinted with permission from Prabhakar, P. K., et al. 2023. "Structural and Biochemical Insight into a Modular β-1,4-Galactan Synthase in Plants," *Nature Plants* **9**, 486–500. DOI:10.1038/s41477-023-01358-4. (e) Courtesy Lawrence Berkeley National Laboratory. (f) Revised under a Creative Commons license (CC By 4.0 International) from Remesh, S.G., et al. 2020. "Nucleoid Remodeling During Environmental Adaptation is Regulated by HU-Dependent DNA Bundling," *Nature Communications* **11**, 2905. DOI:10.1038/s41467-020-16724-5. (g) Reprinted with permission from Fukushima, T., et al. 2017. "The Molecular Basis for Binding of an Electron Transfer Protein to a Metal Oxide Surface," *Journal of the American Chemical Society* **139**(36), 12647–54. DOI:10.1021/jacs.7b06560 ©2017 American Chemical Society. (h) Reprinted with permission from Hagan, A., et al. 2018. "*In Vitro* Assembly of Diverse Bacterial Microcompartment Shell Architectures," *Nano Letters* **18**(11), 7030–37 ©American Chemical Society. (i) Courtesy University of Washington]

Understanding Metal Binding by Metallochaperones Using Synchrotron X-Ray Spectroscopy Methods: Two Interlaboratory Collaboration Stories

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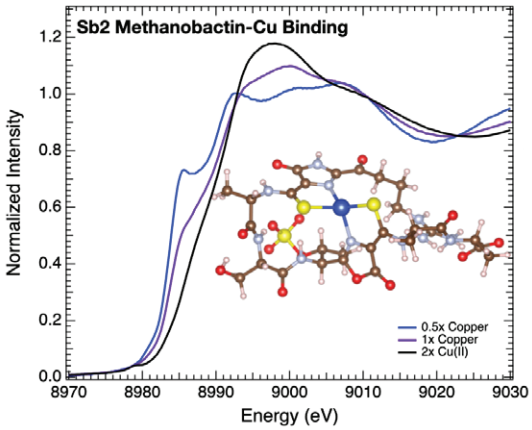
Methanotrophic bacteria metabolize methane as their source of carbon and energy. Methane conversion to energy involves a copper (Cu)-containing enzyme, pMMO. To facilitate Cu uptake from the environment, methanotrophic bacteria secrete a Cu-binding peptide called methanobactin. Methanobactin binding to Cu(II) leads to its spontaneous reduction to Cu(I). However, the mechanism of reduction and the resulting structural changes are unknown. In a collaboration with the ORNL science focus area (SFA), "Biogeochemical Transformation at Critical Interfaces," researchers have applied Cu and S K-edge X-ray absorption spectroscopy (XAS) methods combined with electron paramagnetic resonance (EPR) and density functional theory (DFT) methods to elucidate the molecular basis of methanobactin-Cu binding.

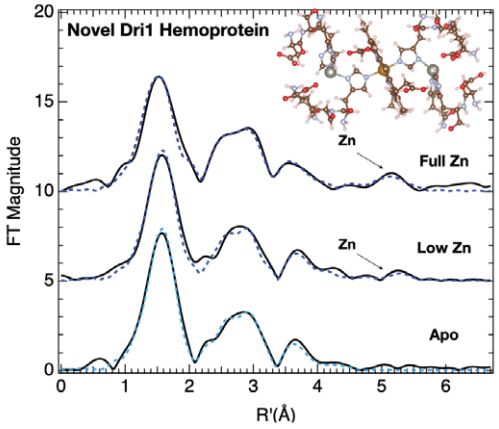
Hemoproteins play critical roles in the chemical framework of the cell as an essential protein cofactor and signaling molecule that controls diverse processes and molecular interactions. In another collaboration, researchers at the "Quantitative Plant Science Initiative" SFA at Brookhaven National Laboratory have identified a family of dimeric hemoproteins of previously unknown function. The identified heme iron is uniquely axially coordinated by two zinc-bound histidine residues, forming an unusual twofold symmetric zinc-histidine-iron-histidine-zinc site. The team has applied iron K-edge XAS and extended X-ray absorption fine structure coupled with DFT calculations to understand the role of zinc in stabilizing the bound heme.

Study results highlight the potential of interlaboratory collaborations for a multitechnique approach to BER science enabled by a dedicated BER-supported outreach program at SSRL.

Funding Statement: The SSRL Structural Molecular Biology Resource supports the development of advanced methodologies and research, collaborative research, service, training, and dissemination in structural molecular biology using synchrotron radiation at SSRL. The integrated program is supported by the DOE Biological and Environmental Research Program and by the National Institutes of Health, National Institute of General Medical Sciences (P30GM133894).



Elucidating the Molecular Basis of Methanobactin-Cu Binding. Normalized Cu K-edge XAS data illustrating reduction of Cu(II) with increasing methanobactin (Sb2). **Inset:** Structure of Sb2-bound Cu (blue sphere). [Courtesy SLAC National Accelerator Laboratory.]



Understanding the Role of Zinc in Stabilizing the Bound Heme. Fourier transforms of the Fe K-edge EXAFS data of the novel Dri1 hemoprotein, showing the appearance of Zn signal with increased Zn loading. **Inset:** The crystal structure of the Dri1 protein with the heme Fe (bronze) flanked by two axial Zn (grey) ions bridged via histidine ligands. [Courtesy SLAC National Accelerator Laboratory]

*Presenting Author

Laboratory for BioMolecular Structure: A DOE-Funded National Cryo-EM Center

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https://www.bnl.gov/cryo-em/

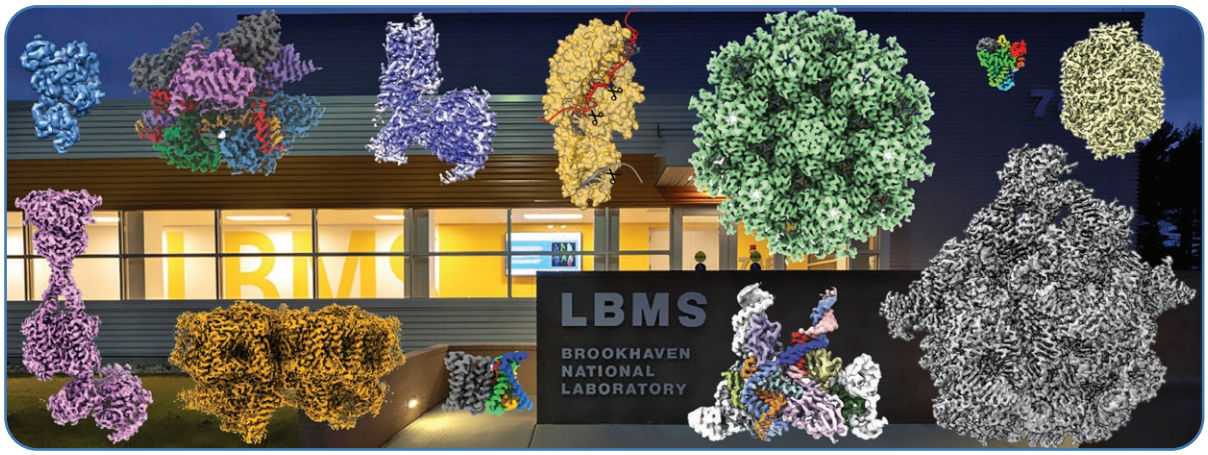
Project Goals: The Laboratory for BioMolecular Structure (LBMS) at Brookhaven National Laboratory (BNL) provides peer-reviewed research access, support, and training for the use of cryo-electron microscopy (cryo-EM). Cryo-EM has been widely employed to determine biomolecular structures, and the establishment of LBMS has made it possible for the research community to advance foundational knowledge of the biological complexity of plant and microbial metabolism and their interfaces.

A key LBMS goal is to attract DOE BER–sponsored researchers to take advantage of LBMS' cryo-EM and help them in all phases including project initiation, sample preparation, data collection, processing, and interpretation. Three tiers of trainings are offered to educate new users so that they can conduct independent research later and to ensure experienced users get the best data possible.

LBMS also establishes a culture of innovation to extend the state-of-the-art through exploring new methods of sample preparation, data collection and analysis, and automation leading to an improvement in throughput and accuracy of the structures determined.

To advance understanding of biological processes and complexity, cryo-EM has become a preferred method for studying structures of biological macromolecules and high-order machinery. However, for many institutions and research groups, acquiring and operating a state-of-the-art cryo-EM facility remain cost prohibitive. With the joint support from DOE and the state of New York, LBMS at Brookhaven National Laboratory, a national cryo-EM center, provides cutting-edge instruments and operations for imaging biological structures and processes. LBMS provides merit-based no-cost access to nonproprietary users as well as cost-recovery access to proprietary users.

The mission of LBMS is to accelerate fundamental understanding about the biological building blocks and their functions in all living organisms. LBMS strives to foster expeditious developments in biotechnology and medicine to meet the nation's urgent needs in biofuels and healthcare. LBMS fulfills its mission by offering training and access to



Gallery of Structures Determined Using the Laboratory for Biomolecular Structure (LBMS) Facility in 2022. [Reused under Creative Commons 4.0 International Licenses from Chai, J., et al. 2023. "Structural Basis for Enzymatic Terminal C–H Bond Functionalization of Alkanes," *Nature Structural & Molecular Biology* **30**, 521–26. Chai, J., et al. 2021. "Structural Basis for SARS-CoV-2 Envelope Protein Recognition of Human Cell Junction Protein PALS1," *Nature Communications* **12**(1), 3433. Goswami, H. N., et al. 2022. "Molecular Mechanism of Active Cas7-11 in Processing CRISPR RNA and Interfering Target RNA," *Elife* **11**, e81678. Pang, C., et al. 2023. "Structural Mechanism of Intracellular Autoregulation of Zinc Uptake in ZIP Transporters," *Nature Communications* **14**, 3404. Schuler, G., et al. 2022. "Structural Basis for RNA-Guided DNA Cleavage by IscB-wRNA and Mechanistic Comparison with Cas9," *Science* **376**(6600) 1476–81. Das, A., et al. In press. "Coupled Catalytic States and the Role of Metal Coordination in Cas9," *Nature Catalysis*. Flesher, D. A., et al. In preparation. "Structure of a Mutated Photosystem II Complex Reveals Perturbation of the Oxygen-Evolving Complex."]

*Presenting Author

highly advanced cryo-EM capabilities to the broad research community.

In 2022, LBMS supported 92 users to collect 809,032 images, and 71 structures were determined. Ten papers were accepted/published, and 14 manuscripts are under review. LBMS also offers trainings to current and potential users. In addition to in-person trainings for sample preparation and screening, and remote training for data collection on the high-resolution EM, researchers hosted the second annual cryo-EM course, which was attended by approximately 400 attendees from 24 countries. For current and potential LBMS users, the team organized four quarterly cryo-EM workshops, which focused on practical aspects of cryo-EM techniques and involved intensive interactions among instructors and attendees. The average rating of the workshops was 4.5/5.0, and 100% of the attendees would recommend the workshops to others.

To support users to perform their best achievable research, LBMS explores and optimizes emerging methods of instrumentation, sample preparation (negative staining, vitrification, cryo-focused ion beam milling), data collection (single-particle analysis, cryo-electron tomography), data analysis, and automation. LBMS is a user-centric facility with excellence through user training, user support, instrument operation, and facility development.

Funding Statement: Brookhaven National Laboratory is managed by Brookhaven Science Associates for the U.S. Department of Energy under contract no. DE-SC0012704. This program is supported by the U. S. Department of Energy, Office of Science, through the Genomic Science Program of the Biological and Environmental Research Program under FWP KP1607011.

A Disordered Plant Microtubule-Associated Protein Reorganizes Microtubules

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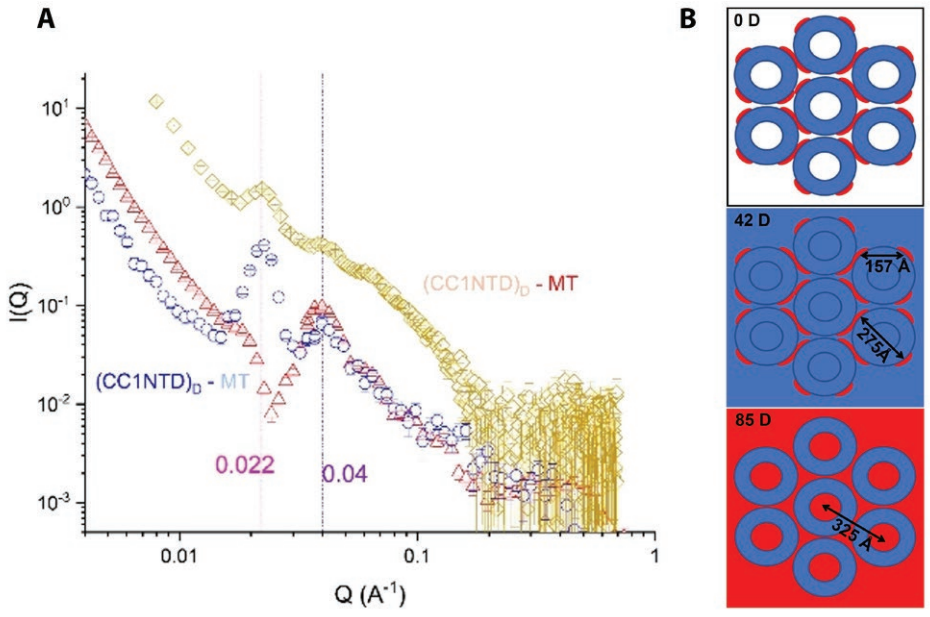
https://www.ornl.gov/facility/csmb

Project Goals: The Center for Structural Molecular Biology (CSMB) at Oak Ridge National Laboratory (ORNL) is a national user facility funded to support and develop the user access and science research program of the Biological Small-Angle Neutron Scattering (Bio-SANS) instrument at the High Flux Isotope Reactor (HFIR). Bio-SANS is dedicated to the analysis of the structure, function, and dynamics of complex biological systems. The CSMB also operates a Bio-Deuteration Laboratory for the expression and purification of deuterium-labeled biomacromolecules and for the synthesis of small molecules and ligands in support of the biology neutron scattering program. This resource complements capabilities at other DOE BER facilities for structural biology. The CSMB supports a vibrant biological research community from academia, industry, and government laboratories.

The inherent conformational flexibility of intrinsically disordered proteins (IDPs) and intrinsically disordered regions (IDRs) is recognized as being functionally important for both inter- and intra-interactions between proteins in a wide variety of metabolic processes. In particular, disordered regions of multidomain proteins are thought to play crucial roles in the regulation of different cellular processes.

Here, the team reports on Companion of Cellulose Synthase 1 (CC1), which has been recently identified to maintain cellulose synthesis during salt stress in *Arabidopsis* (Endler et al. 2015). Based on bioinformatics and structural modeling using Alphafold2, CC1 is predicted to be a multidomain protein with an N-terminal cytosolic disordered region (CC1NTD), a transmembrane region, and a C-terminal apoplastic region. Previously, the disordered region of CC1 was found to interact with cortical microtubules in a similar fashion to human Tau protein, but the structural basis for the interaction is not known (Kesten et al. 2019). In this work, researchers investigated the solution



Small-Angle Scattering Characterization of Microtubule-CC1NTD Complexes. (A) SAXS of CC1NTD–microtubule complex (gold), SANS of deuterated CC1NTD–microtubule in 42% D_2O buffer (blue) and 85% D_2O buffer (red). **(B)** Schematic illustration of how SANS contrast highlights different parts of the microtubule–CC1NTD complex. Microtubules are represented by hollow cylinders (blue), and CC1 is represented by lines (red). The structural parameters calculated from SANS are indicated. [Courtesy Oak Ridge National Laboratory]

*Presenting Author

structure of CC1NTD and its interaction with microtubules using small-angle X-ray and neutron scattering (SAXS and SANS). These techniques are ideally suited to studies of flexible and disordered proteins and dynamic complexes. In addition, the specific labeling that is observable with neutrons and enabled by deuteration enhances the visibility of specific parts of complex assemblies, thus providing unique structural information that is unattainable by other means. Size exclusion chromatography-SAXS analysis revealed that CC1NTD exists as a redox-dependent equilibrium mixture of monomers and dimers. SAXS also showed that there is a structural rearrangement of microtubules in the presence of CC1NTD that supports bundling of microtubules. Contrast variation SANS was used to study the structure of deuterium-labeled CC1NTD when associated with microtubules. Analytical fits of SAXS and SANS allowed determination of the structural parameters of the microtubule-CC1NTD complex. SANS showed that CC1NTD has a regular distribution on the microtubules and likely forms links between the microtubules that result in their hexagonal organization that was determined from SAXS. Computational modeling showed that CC1NTD assumes an extended conformation when associated with microtubules and that an arrangement of extended CC1NTD cross-bridges between microtubules to form a hexagonal assembly that best fits the experimental data. In summary, this preferential arrangement of CC1NTD along microtubules supports the cross-bridging model of a microtubule associated protein bundling microtubules. The extended configuration of CC1NTD favors the formation of a microtubule assembly similar to cortical microtubule arrays and, at the same time, maintains a microtubule arrangement that may facilitate cellulose synthesis during salt stress.

Funding Statement: This work is supported by project ERKPA14, funded by the Biological and Environmental Research Program in the Department of Energy (DOE) Office of Science. Neutron scattering experiments on Bio-SANS were supported by the Center for Structural Molecular Biology funded by DOE BER project ERKP291. Oak Ridge National Laboratory is managed by UT-Battelle, LLC for the U.S. Department of Energy under contract no. DE-AC05-00OR22725.

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Kesten, C., et al. 2019. "The Companion of Cellulose Synthase 1 Confers Salt Tolerance Through a Tau-Like Mechanism in Plants," *Nature Communications* **10**(1), 857.

Structural Biology Center at Sector 19 of the Advanced Photon Source

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[2]University of Chicago, Chicago, IL

https://sbc.aps.anl.gov

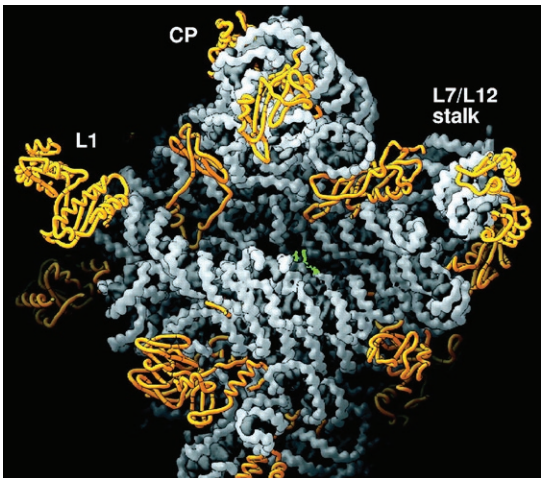
Project Goals:

1. Provide an integrated X-ray macromolecular structural biology platform and advanced user support at the Advanced Photon Source (APS) for BER and general users.
2. Develop and implement new methods and applications in macromolecular crystallography.
3. Support diverse user outreach and training activities.

The Structural Biology Center (SBC) at Argonne National Laboratory has operated insertion device (ID) and bending magnet (BM) beamlines at Sector 19 of the APS as a user facility for macromolecular crystallography since 1997. The facility was funded by DOE BER. The 19ID undulator beamline was designed and built to take full advantage of the high flux, brightness, and quality of X-ray beams delivered by the APS and was the first macromolecular crystallography facility open to users. 19BM was added to user program in 1999. These two beamlines delivered small, very low angular divergent X-ray beams onto micrometer-size crystal samples, thereby permitting studies of large and complex molecular systems at atomic resolution. The high flexibility, inherent to the optics design, coupled with a kappa-geometry goniometer and beamline control software enabled development of optimal strategies for protein crystallographic experiments, thus maximizing the chances of their success. Large-area detectors allowed high-quality diffraction data to be measured rapidly to the crystal diffraction limits.

Users collected data on site or remotely. Data were collected and processed, and structures were determined with advanced software in near real time. Many users had limited synchrotron/crystallography expertise, and SBC staff provided extensive training and support. The facility offered a flexible schedule on one of the most efficient data collection and structure determination platforms for protein crystallography.

The SBC promoted scientific and technological innovation in support of the DOE mission by providing world-class macromolecular crystallography to the BER and biology research community. The SBC exploited major advances in macromolecular X-ray crystallography and addressed the most challenging structural biology problems to expand scientific knowledge. The SBC was an important component in structural biology innovation, structural genomics, metagenomics, proteomics, and genomics research, with a major focus on systems biology, bio-nanomachines, medicine and biocatalysis. These fields are highly relevant to bioenergy resources, health, national security, and a clean, sustainable environment. More recently, the SBC has contributed to serial crystallography, data analysis, the high-performance computing pipeline, and SARS-CoV-2 research.



The *Haloarcula marismortui* Large Ribosomal Subunit in the Rotated Crown View. The L7/L12 stalk is to the right, the L1 stalk is to the left, and the central protuberance (CP) is at the top. In this view, the surface of the subunit that interacts with the small subunit faces the reader. RNA is shown in gray in a pseudo-space-filling rendering. The backbones of the proteins visible are rendered in gold. The Yarus inhibitor bound to the peptidyl transferase site of the subunit is indicated in green (64). The particle is approximately 250 Å across. [Reprinted with permission from Ban, N. et al. 2000. "The Complete Atomic Structure of the Large Ribosomal Subunit at 2.4 Å Resolution," *Science* **289**(5481), 905–20. DOI:10.1126/science.289.5481.905.]

Funding Statement: The SBC program was supported by the U. S. Department of Energy, Office of Science, through the Genomic Science Program, Biological and Environmental Research Program, under FWP PRJ1000333 and was operated for the DOE Office of Science at the Advanced Photon Source by Argonne National Laboratory under Contract No. DE-AC02- 06CH11357.

*Presenting Author

Soft X-Ray Tomography at the Advanced Light Source

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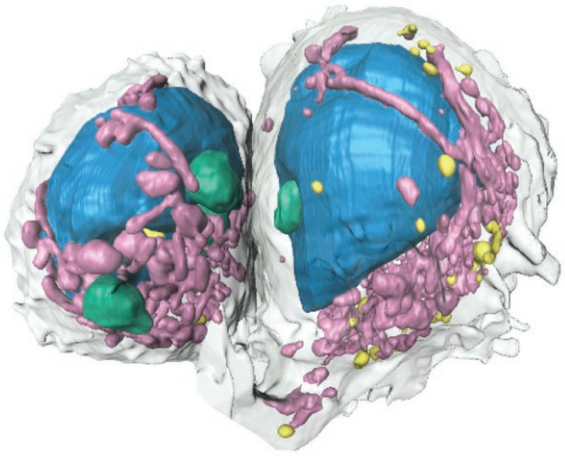
Soft X-ray tomography (SXT) is an imaging technique capable of characterizing and quantifying the structural phenotype of cells. In particular, SXT is used to visualize the internal architecture of fully hydrated, intact eukaryotic and prokaryotic cells at high spatial resolution (50 nm or better). Image contrast in SXT is derived from the biochemical composition of the cell and obtained without the need to use potentially damaging contrast-enhancing agents (e.g., heavy metals). The cells are simply cryopreserved prior to imaging and are therefore imaged in a near-native state. As a complement to structural imaging by SXT, the same specimen can also be imaged by correlated cryo-light microscopy. By combining data from these two modalities specific molecules can be localized directly within the framework of a high-resolution, 3D reconstruction of the cell. This combination of data types allows sophisticated analyses to be carried out on the impact of environmental and genetic factors on cell phenotypes.



Extension of Soft X-Ray Tomography (SXT) Imaging Volume. 3D rendering of the segmentation of cells shown in SXT volume of 17.8 µm × 17.8 µm × 13.0 µm contains two organelle-rich U2OS cells, ice, and surrounding dense glass capillary. [Reused under a under Creative Commons license (CC By 4.0 International) from Ekman, A., et al. 2023. "Extending Imaging Volume in Soft X-Ray Tomography," *Advanced Photonics Research* **4**(5), 2200142. DOI:10.1002/adpr.202200142.]

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The Center for BioMolecular Structure: CBMS at National Synchrotron Light Source II

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The Center for BioMolecular Structures (CBMS) studies life at its smallest and largest scales, from examining the behavior of its molecular building blocks to their influence on Earth's environment. The CBMS offers an environment that allows visiting scientists, partners, and collaborators to succeed in their hypothesis-driven research by combining staff expertise with a numerous advanced research tools. CBMS addresses these questions through operating a suite of experimental facilities. The center works closely with its partner facility, the Laboratory for BioMolecular Structure, to support research in all biology-related or environmental studies with a major focus in the following:

Biophysical, Chemical, and Medical Sciences: Understanding the function of biological molecules requires knowledge of their molecular structure. This knowledge has a wide impact on understanding of mechanisms and the engineering of new functions. CBMS focuses on both animal and plant kingdoms in this line of research.

Plant Science: Plants are part of our daily lives, serving as climate and environmental regulators as well as sources of food and energy. CBMS seeks to understand the fundamental pathways operating within plants and their cellular structure, use of plants as bioremediators, and the origin of various plant disease.

Cell and Tissue Analysis: The analysis of cells and tissues provides a wide variety of information ranging from tumor development to functions of subcellular organelles. Understanding fundamental functions impacts mankind's ability to develop targeted responses for infections and diseases in the animal or plant kingdoms.

Environmental Chemistry: Atmospheric science focuses on the interactions of Earth's atmosphere, the role in climate change, and the effect on geological formations. Understanding changes throughout time enhances the ability to predict their social, economic, and environmental impact.

To support this diverse portfolio of environmental and life science studies, CBMS has created and continues to develop a suite of advanced research capabilities. The research tools are supported by expert staff who cover many research fields for multidisciplinary investigations.

To study the elemental distribution and the complex chemistry within biological and environmental samples, staff help develop and operate a suite of experimental stations with the National Synchrotron Light Source II (NSLS-II) Imaging

CBMS Bioimaging Training Workshop, August 2022. Participants and staff at various points of the experiment, including beamline preparation (top left); data collection and preliminary online assessment of images acquired (top right, bottom left); selection and preparation of materials for examination on the beamline instruments. [Courtesy Brookhaven National Laboratory]

*Presenting Author

and Microscopy Program. The team develops and operates a dedicated suite of macromolecular crystallography (MX) beamlines at NSLS-II for cutting-edge research on the molecular structure and function of complex biomolecules using extremely small crystals. CBMS offers a dedicated X-ray scattering beamline to address fundamental questions in structural biology. This beamline enables visiting researchers to study biological molecules in their native environment and to image cells and tissues.

The CBMS offers a wide range of education and training opportunities for all levels of synchrotron expertise. The training and education programs focus on the state-of-the-art tools available through the CBMS-operated beamlines. By offering these opportunities, the center strives to foster the education of the future scientific workforce to understand the potential of and exploit the cutting-edge techniques and methods for hypothesis-driven research. The center offers training courses on a quarterly basis for structural biology with an annual in-depth school on the use of the light source for imaging biological and environmental sciences. To advertise its users' science, the center runs the open CBMS seminar series monthly, which draws an audience from across the globe.

Funding Statement: CBMS is primarily supported by the National Institutes of Health, National Institute of General Medical Sciences (NIGMS) through a Center Core P30 Grant (P30GM133893), and by the DOE Biological and Environmental Research Program (KP1607011). As part of NSLS-II, a national user facility at Brookhaven National Laboratory, work performed at the CBMS is supported in part by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences Program under contract number and DE-SC0012704.

Advances to Accelerate Biosystems Characterization at the BSISB Imaging Program

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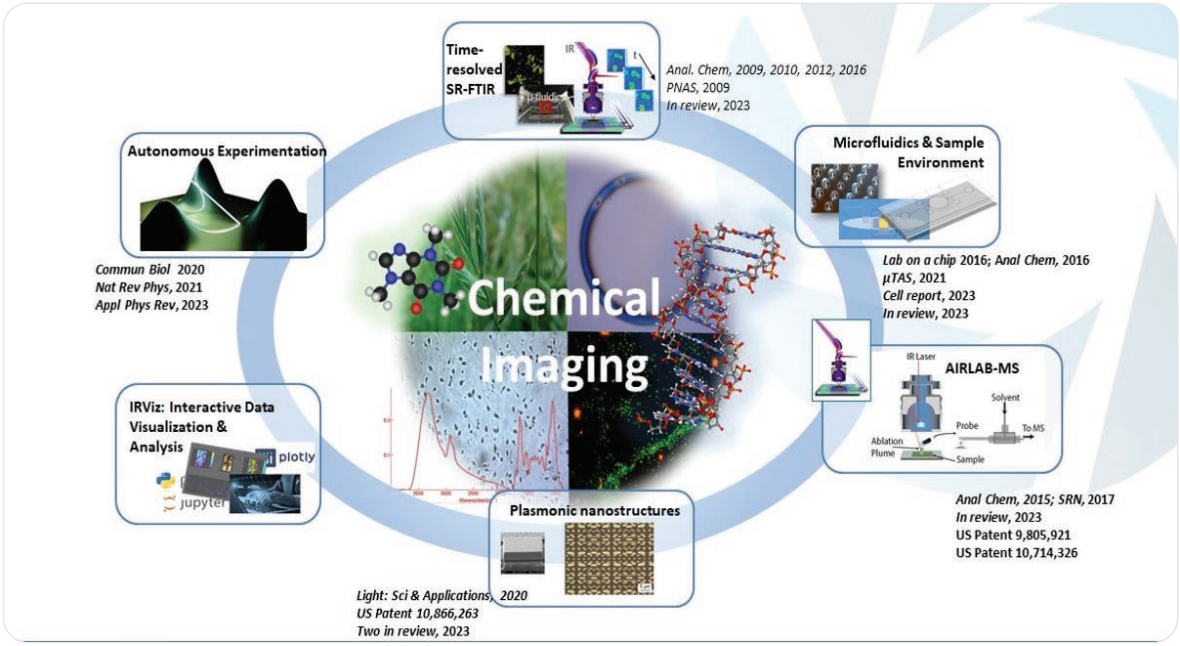
https://bsisb.lbl.gov

Infrared (IR) spectroscopy is a powerful biomolecular sensing tool that exploits the distinct vibrational modes of the covalent bonds present in virtually all organic molecules. Unlike visible light, mid-IR photon energies can only induce vibrational excitations of covalently bonded atoms or groups of atoms in organic molecules. A highly beneficial feature is that absorption frequencies are a characteristic of each functional group, permitting immediate information about the molecular composition of a biological sample from its mid-IR absorption spectrum even without a priori knowledge. Another advantage of mid-IR spectroscopy is its excellent sensitivity to hydrogen bonding. Changes in hydrogen bond lengths or bond angles of as little as 0.01 Å or 1°, respectively, can provide clear differences among vibrational spectra, in addition to frequency shifts and changes in absorption intensity. The specificity and sensitivity of IR spectroscopy make it an excellent tool for studying the structure and intra- and intermolecular interactions of biological macromolecules, which both affect and are affected by their immediate hydrogen bonding environment. However, the specificity and sensitivity of IR spectroscopy for sensing and analyzing tiny amounts of biomaterials are fundamentally limited by the low absorption cross section of biomolecules ($\sigma_{abs} \approx 10^{-20}$ cm^2) in the mid-IR range, or by the strong absorption of liquid water—a common medium for aqueous samples of biological interest, or by both. The BSISB imaging program has developed new methods to overcome these limitations, with examples from microbiology and bioenergy sciences.



Imaging and Sensor Capabilities for Label-Free Chemical and Structural Characterization of Biosystems. These capabilities are available to research communities at the BER-sponsored BSISB imaging program. [Courtesy Lawrence Berkeley National Laboratory]

*Presenting Author

Opportunities Linking Omics and Structural Biology at Pacific Northwest National Laboratory: Excelling at Cryo-EM

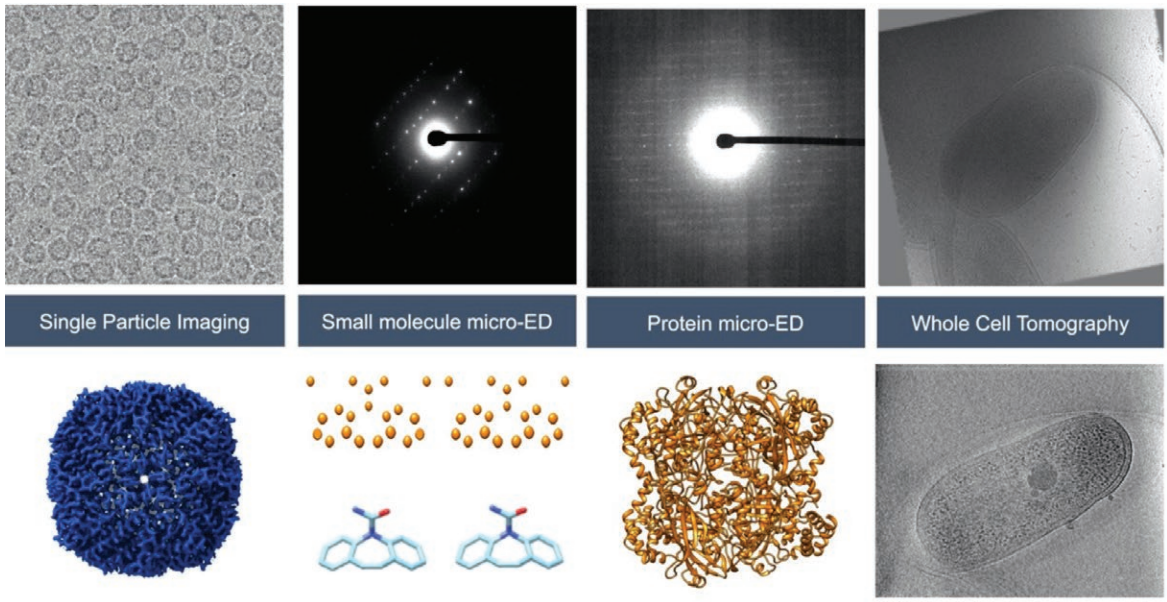
Trevor Moser, Irina Novikova, Amar Parvate, Samantha Powell, **James E. Evans*** (PI)

Pacific Northwest National Laboratory (PNNL), Richland, WA

Project Goals: This project is focused on the operation of a new state-of-the-art cryogenic transmission electron microscope (Krios G3i) at the Environmental Molecular Sciences Laboratory (EMSL) at PNNL to advance DOE BER user research in protein/small molecule structural biology and whole cell ultrastructure. The operation of the Krios G3i instrument is a joint funding venture between EMSL and BER. The microscope is available to the general EMSL user community and BER researchers in a 50/50 split allocation.

This project was designed to rejuvenate cryo-EM research at EMSL with a new microscope and new semi-automated or automated workflows. EMSL users can access this instrument free of charge via the normal EMSL user proposal calls, which permit combining cryo-EM with other EMSL capabilities such as mass spectrometry or super-resolution fluorescence microscopy. Access is offered free of charge for BER users, with PNNL staff time funded by this current project. The BER access mechanism allows for an expedited submission and review process for cryo-EM–only projects.

The Krios G3i is fully operational and has been applied to multiple EMSL and BER users projects. The microscope has complete screening, data collection, and image processing workflows for (1) microelectron diffraction of small molecule or protein crystals, (2) single-particle analysis of soluble and membrane protein complexes, and (3) electron tomography of whole cells or isolated organelles. It is equipped with a K3 direct electron detector, Ceta-D camera, phase plate, and Bioquantum energy filter. In addition to semiautomated data collection, the facility has installed automated image processing workflows for real-time monitoring feedback of session quality and full 3D reconstruction of all workflows. To date, the facility has demonstrated sub-Å resolution microelectron diffraction, sub-2 Å resolution from 3D single-particle protein structure determination, and subnanometer resolution for whole-cell tomography. While the facility provides rapid access for samples that arrive prefrozen on clipped and prescreened grids, users can also begin with samples that arrive in buffer and require all steps of the cryo-EM workflow. In a subset of cases, users can also start from a provided gene of interest and employ the cell-free expression system to produce enough protein for structural characterization. Highlighted are several recent user results as well as an example of going from receiving



Benchmarking Examples of Each of the Cryo-EM Sample Workflows Available to Users on the New Krios G3i Microscope at EMSL. Row 1: Raw data. **Row 2:** Reconstructed volumes from samples ranging from small molecules through whole cells. [Courtesy Pacific Northwest National Laboratory]

*Presenting Author

a gene clone through cell-free expression and cryo-EM structure determination in less than 9 days. The facility will also showcase the new user friendly AutoMicroED software that was developed, which permits quick and direct determination of small-molecule structure even from heterogeneous datasets to accelerate science discovery with micro-electron diffraction.

Funding Statement: Pacific Northwest National Laboratory is operated by Battelle for the U.S. Department of Energy under Contract DE-AC05-76RL01830. This program is supported by the U. S. Department of Energy, Office of Science, through the Genomic Science Program, Biological and Environmental Research Program, under FWPs 74194 and 74195. The work was performed at EMSL (grid.436923.9), a DOE Office of Science User Facility sponsored by the BER program.

Breaking the Size Barrier for Cryo-EM of Small Proteins

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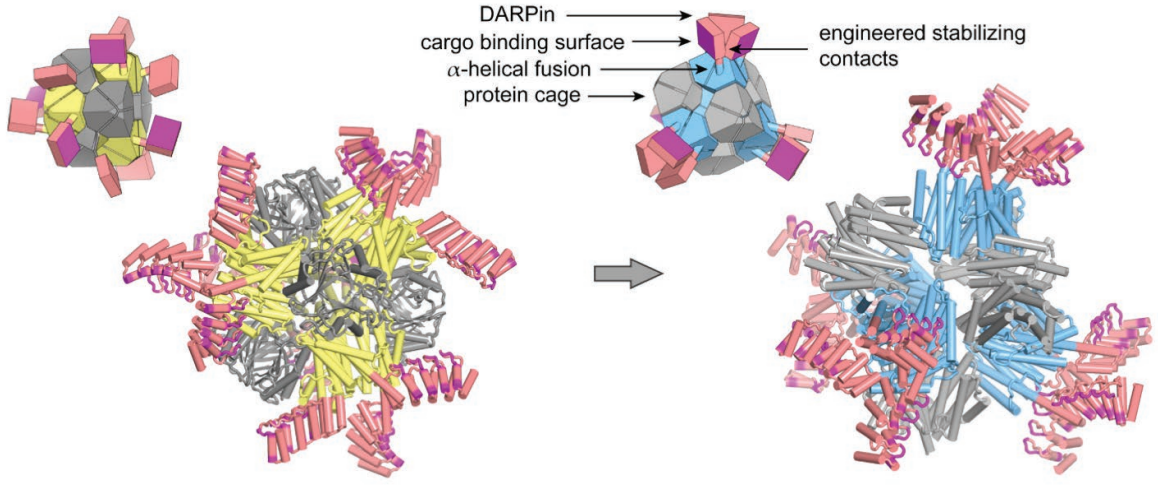
Project Goals: One of the goals in the UCLA-DOE Institute for Genomics and Proteomics is to develop cryo-electron microscopy (cryo-EM) imaging technologies that break through the size barrier for small proteins, to allow both atomic imaging and *in situ* localization of proteins.

Cryo-EM has become a leading method for atomic detail structure determination for large protein and nucleic acid complexes. It is, however, limited by the size of the macromolecule that can be imaged. A molecular mass of around 50 kDa is the current practical lower bound. This leaves a large fraction of important cellular proteins out of reach. Scaffolding approaches have been pursued for some time as a potential solution that might make cryo-EM a near-universal solution to the problem of protein structure determination. The idea is to bind the small cargo protein of interest to a larger protein carrier or scaffold, which can be imaged readily. The key challenges have been how to make the mode of binding or attachment sufficiently rigid and how to create a system that is modular or plug-and-play. In recent work, researchers have demonstrated a general solution, creating a scaffold system that is modular and rigid enough to reach better than 3 Å resolution structures for small proteins, in one case down to 19 kDa (Liu et al. 2018; Liu et al. 2019; Castells-Graells 2022).

The scaffold is based on a designed protein cage, which provides a large size (200 Å diameter and 600 kDa mass) and advantageous symmetry for image processing. Rather than antibody-based proteins, modular adapters are based on DARPin proteins (see figure). DARPin proteins are highly helical, providing certain engineering advantages, and have been developed by others as a diversifiable platform for selecting sequence variations (in a series of loop regions) that bind to essentially any protein of interest. The team has demonstrated success on a series of small proteins, including GFP as a test case, as well as KRAS as a model for protein therapeutic research. Ongoing efforts are to expand the workflow to proteins and enzymes of major interest in energy science, including microbial and plant enzymes.

The next major advance will be to adapt scaffold systems of this type to the problem of identifying and imaging diverse proteins in their cellular environments, *in situ*. A major gap in understanding cellular structure and function comes from the difficulty of identifying specific proteins within cells on the basis of cryo-electron tomography (cryo-ET). There is a tremendous need for a facile system for labeling any protein



A Scheme for Rigidifying a Modular Cryo-EM Imaging Scaffold. Protein engineering to create a rigid and modular scaffold for imaging small proteins by cryo-EM. [Reused under a Creative Commons license (CC By ND-NC 4.0 International) from Castells-Graells, R., et al. 2022. "Rigidified Scaffolds for 3 Angstrom Resolution Cryo-EM of Small Therapeutic Protein Targets," *bioRxiv*. DOI:10.1101/2022.09.18.508009.]

*Presenting Author

of interest in a way that translates to cryo-ET imaging. Scaffolds based on designed protein cages offer prospects for identifying the location of specific proteins within cells based on their identifiable geometric features (e.g., cube shapes) and their ability to be repurposed to bind any desired protein. Preliminary cryo-ET studies are in progress, beginning with *Escherichia coli* minicells, to determine what size range of designed scaffolds will be amenable for this new line of imaging technologies.

Funding Statement: This research was supported by the DOE Office of Science, Biological and Environmental Research Program grant no. DE-FC02-02ER63421 and by NIH grant R01GM12985.

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Biomass Pretreatment and Structural Changes Shown from Scattering and Modeling

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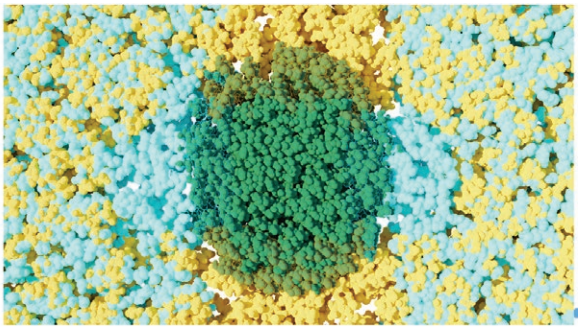
[2]University of Tennessee, Knoxville, TN

https://sfa-biofuels.ornl.gov

Project Goals: The Solvent Disruption of Biomass and Biomembranes Science Focus Area (SFA) provides fundamental knowledge about how solvents alter the structures of plant cell walls and microbial membranes. The overarching hypothesis is that knowledge of partitioning or binding of the solvent from the bulk phase to biomass or biomembranes can help predict maximal or minimal disruption. Solvents disrupt biological structures comprising amphiphilic molecules and polymers (e.g., membranes and biomass).

Determining common biophysical principles of solvent disruption will lead to new understandings of how solvents affect the relevant structures. This information will help determine the ultimate microbial limits in tolerating specific solvents as well as the eventual design of cosolvents best suited for pretreatment. The team will integrate the power of world-class neutron scattering capabilities and leadership-class supercomputing facilities available at ORNL. These capabilities are complemented by expertise in biodeuteration and biomembranes at ORNL, plant cell wall chemistry at the University of Tennessee, and interpreting small-angle neutron scattering (SANS) data at the University of Cincinnati.

A sustainable bioeconomy will undoubtably rely on the efficient production of lignocellulosic biofuels that can be combusted directly in automobile engines or catalytically upgraded to long-chain hydrocarbons for use as diesel and aviation fuels. Plant cell wall structure of biomass is an intricate design of several carbohydrate polymers encased in the hydrophobic lignin polymer to protect against degradation. The recalcitrance to deconstruction of lignocellulosic biomass due to the complex physicochemical structure of plant cell walls is a challenge in biological-based biorefinery systems due to the complex physicochemical structure of plant cell walls. Pretreatment and genetic modification are two approaches in biomass conversion that have succeeded in modifying the structure of lignocellulose to enable better enzymatic deconstruction. However, the structural differences among pretreatment-solubilized biomass biopolymers have not been extensively investigated. This SFA's goal is to understand the molecular-level mechanism that drives efficient biomass deconstruction. ORNL scientists have reported direct experimental and computational evidence of physical chemical principles underlying pretreatment. Discussed below is the SFA's use of molecular dynamics (MD) simulations, experimental pretreatments with acids and with acidified solvents combine the scattering measurement to elucidate structural changes in the three key biomass polymers (cellulose, hemicellulose, and lignin). This will be illustrated by several examples.



Solvent Disruption for Biomass Deconstruction. Local demixing of water (blue) and tetradydrofuran (yellow) on the surfaces of cellulose (green). Water and tetrahydrofuran are mixed in the bulk away from the cellulose. [Courtesy Oak Ridge National Laboratory]

The team determined that solvent mixtures with both hydrophilic and hydrophobic interactions are key for efficient deconstruction of biomass as revealed by neutron scattering and molecular simulation. Researchers elucidated the effect of tetrahydrofuran (THF)-water pretreatment on the nanoscale architecture of biomass and the role the co-solvents play in solubilizing lignin and cellulose (Pingali et al. 2020). *In situ* SANS determined temperature-dependent changes in biomass morphology: Whereas lignin dissociates over a wide temperature range (>25 °C), cellulose disruption occurs only above 150 °C. SANS with contrast variation and MD simulations provided direct evidence for the formation of THF-rich nanoclusters (~0.5 nm) on the nonpolar cellulose surfaces and on hydrophobic lignin, and equivalent water-rich nanoclusters on polar cellulose surfaces.

In another example, three organosolv pretreatment systems—ethanol (EtOH)—THF, and γ-valerolactone (GVL)—in dilute acidic aqueous, were used on wild-type (WT) and two transgenic switchgrasses with altered lignin. All organosolv pretreatments caused a significant reduction in the molecular weights of lignins, particularly, and up to ~90% decrease was

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observed in EtOH-pretreated lignin compared to untreated lignin. A correspondence was found between the molecular weight reduction of lignin molecules in the experiments and the number of hydrogen bonds between lignin and the organic solvents as calculated in the MD simulation, suggesting a connection between the depolymerization of lignin and its ability to hydrogen bond with the organic solvents.

To understand the role of noncellulosic switchgrass polymers on the overall efficiency of pretreatment, the structural evolution of the noncellulosic polymers of the plant cell wall was investigated during dilute acid pretreatment by employing *in situ* SANS on various polymer fractions from switchgrass (Yang 2021). In this study, researchers observed real-time structural changes not possible to observe by any other technique. These interpretations were consistent with the MD simulations. These results suggest that not only lignin but also hemicellulose can form aggregate particles within plant cell walls during pretreatment. These concepts can be employed to tune pretreatment technologies that maximize deconstruction of biomass and facilitate the separation of its components for upgrading to energy and materials.

Funding Statement: This research is supported by the U.S. Department of Energy (DOE), Office of Science, through the Genomic Science Program, Biological and Environmental Research Program, under FWP ERKP752. Oak Ridge National Laboratory (ORNL) is managed by UT-Battelle, LLC for U.S. DOE under contract no. DE-AC05-00OR22725. It used neutron scattering resources at the High Flux Isotope Reactor, a DOE Office of Science, Scientific User Facility operated by ORNL. SAXS measurements were performed at the LiX beamline of the National Synchrotron Light Source II, a U.S. DOE Office of Science User Facility operated by Brookhaven National Laboratory under Contract No. DE-SC0012704.

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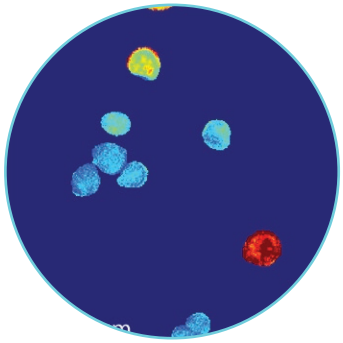

Chapter 3
Bioimaging

The goal of the Bioimaging Science program 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 supports fundamental research or use-inspired technologies of new bioimaging or sensing approaches for bioenergy and the environment at both DOE national laboratories and universities.

National Laboratory Bioimaging—2022 Projects

Technology research demonstrates an advantage over state-of-the-art techniques or identifies biological characteristics that cannot currently be measured. With these aims, national laboratory projects are imaging within native environments (e.g., opaque soil and anaerobic media), detecting novel targets relevant to biological function, and expanding capacity for evaluating established hypotheses of cellular function. In addition, new methods can potentially inspire unanticipated biological hypotheses based on novel information.

One current challenge is understanding how metabolic pathways are organized within topological constraints at the subcellular scale in complex living systems. Techniques are needed to understand dynamic organismal function and the location of metabolites and macromolecules, which will enable researchers to evaluate the spatiotemporal dependence of metabolic processes within thick living plant tissue and among microbial communities in the rhizosphere.

University Imaging Instrumentation and Approaches—2021 Projects

University projects are creating a versatile toolbox of new instrumentation capabilities for imaging biological processes within and among cells in living plants and microorganisms. These multimodal, multiphoton imaging capabilities can better resolve cellular structures nondestructively, thereby advancing biological understanding underpinning DOE energy and environmental missions.

National Laboratory Bioimaging–2022 Projects

Developing a High-Throughput Functional Bioimaging Capability for Rhizosphere Interactions Utilizing Sensor Cells, Microfluidics, Automation, and AI-Guided Analyses

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https://wordpress.cels.anl.gov/bioimaging

Project Goals: The complex dynamics of root-microbe interactions in the rhizosphere drives recognizable spatial structures. However, knowledge of the specific factors that lead to their development and sustain them for plant health and productivity is sparse.

This project aims to develop a unique functional imaging technique that exploits native sense-and-respond circuits of plant growth–promoting rhizobacteria (PGPR) to monitor chemical exchange between the plant root and microbe during the different phases of colonization. Several native PGPRs will be turned into biosensor cells, and root colonization will be evaluated with *Arabidopsis* and *Camelina*. Genetic variants of *Arabidopsis* with gain or loss of function will provide drastically altered local environments, resulting in colonization patterns that differ from those observed previously. An orthogonal X-ray imaging approach will provide high-resolution elemental analysis of the local environment, and imaging throughput in general will be accelerated by automation and artificial intelligence (AI)-driven analysis. In addition, the team aims to advance with automation the throughput of current bioimaging capabilities that leverage imaging chips developed with BER funding and to develop an AI-guided image analysis strategy. This combined high-throughput AI bioimaging capability, along with advanced analytical techniques offered by the Advanced Photon Source (APS) and the Environmental Molecular Sciences Laboratory, will capture the dynamic chemical shifts and colonization patterns in the rhizosphere.

Research Plans and Progress: To identify key signals orchestrating root-microbe interactions, researchers will develop a unique imaging technique that takes advantage of native sense-and-respond circuits of PGPR to monitor chemical exchange between the plant root and microbe during the different phases of colonization. These efforts entail three aims:

- Develop and apply a library of microbial sensor cells.
- Perform high-throughput imaging and biological validation.
- Implement automation and AI-guided analysis.

During the previous "Small Worlds" bioimaging project (PIs: Kenneth Kemner and Mark Hereld), researchers successfully used sensor cells in microfluidic chips (RMI-chip) to detect xylose exudate from aspen roots and to identify reactive oxidative species (ROS) in the rhizosphere microenvironment. The team is integrating these sensors into selected PGPRs (*Pseudomonas simiae* WCS417, *Pseudomonas fluorescens* SBW25, *Pseudomonas migulae* 8R6). The repertoire of biosensors will be drastically increased by a method based on recent work from Co-PI Yoshikuni's group. They employed a massively parallel approach, a chassis-independent recombinase-assisted genome engineering (CRAGE)–enabled randomly barcoded promoter sequencing, to identify a large library of promoters in *P. simiae* WCS417 that altered transcription of a reporter upon root colonization. The method will be applied to the other target PGPRs.

Root colonization assays will be performed in agar plates in Jean Greenberg's laboratory. Greenberg has developed several *Arabidopsis* mutants with drastic changes in morphometric parameters of the plants (changes in root morphology, shoot, and overall size). She has demonstrated the plant growth–promoting effect of *P. simiae* WCS417 on *Arabidopsis*. Colonization on the agar plates will be assessed by plating assays and microscopy of bacteria labeled with fluorescent proteins and biosensors, providing a baseline understanding of colonization patterns and activation in wild type and mutant plants with altered morphometric responses. Root colonization behavior in fabricated ecosystems (EcoFAB) and the RMI-chip will be compared with those observed on agar plates. Frozen samples will be generated using flash freezing followed by slicing with an (ultra-)microtome at a cryogenic temperature and transferred to the Bionanoprobe (BNP) beamline at APS for analysis. The BNP is dedicated to performing elemental and ultrastructure analysis of soft materials with 20 to 100 nm spatial resolution. Other APS capabilities will be provided by the eBERlight program. AI-based methods will be used to optimize image collection, image

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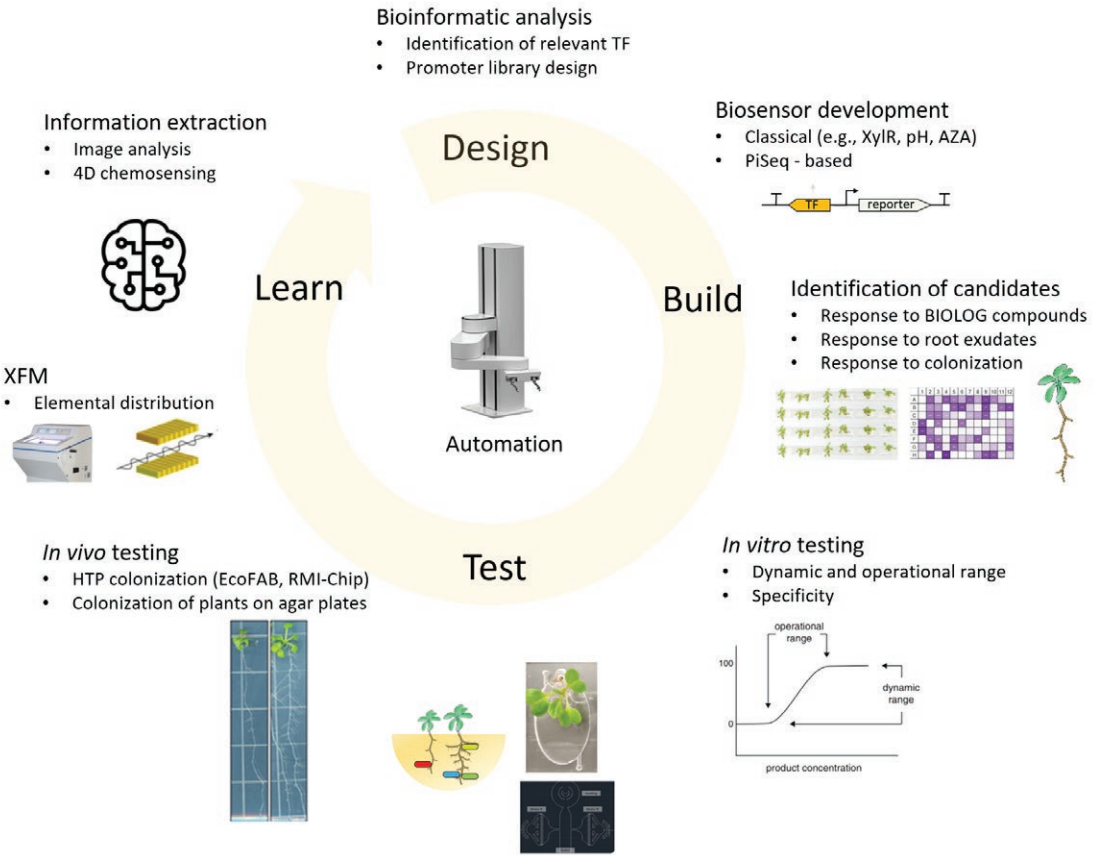
processing, and guide automation where applicable. The images obtained from the different imaging modalities, the spatial and chemical signals will be combined, and AI-based image analysis tools will be used to extract maximum information. Researchers plan to achieve the automated extraction of quantitative phenotypic data and computationally identify temporal changes and spatial locations of interest.

Funding Statement: Argonne National Laboratory is managed by UChicago Argonne, LLC for DOE under contract number DE-AC02-06CH11357. This program is supported by the U. S. Department of Energy Office of Science through the Biomolecular Characterization and Imaging Science Program, Biological and Environmental Research Program under FWP 39156.



High-Throughput Functional Bioimaging for Rhizosphere Interactions. The team plans to develop and apply a library of microbial sensor cells and use them for chemosensing during root colonization of *Arabodopsis* and *Camelina*. A subset of samples will be imaged using X-ray fluorescence microscopy. Computational methods will be used to combine the data from the different imaging modalities and will drive follow-up experiments as part of the design-build-test-learn cycle. [Courtesy Argonne National Laboratory]

Visualizing Spatial and Temporal Responses of Plant Cells to the Environment

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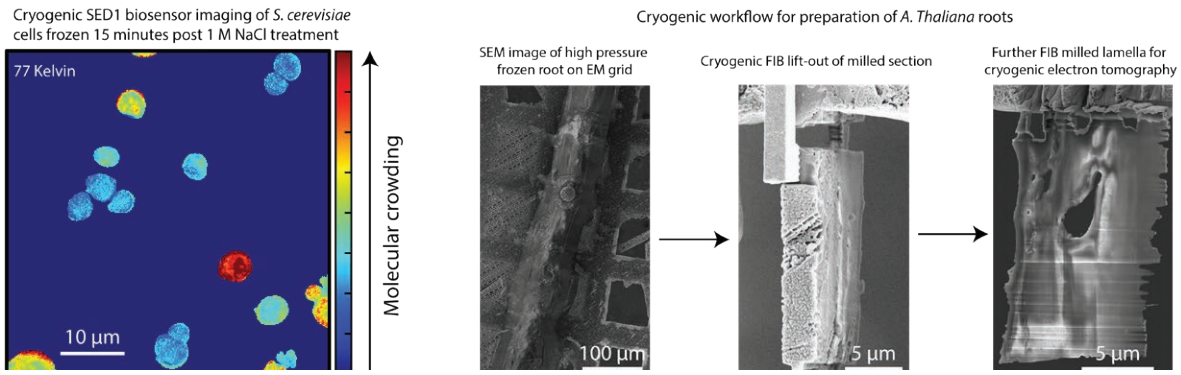
Project Goals: Cryogenic electron tomography (cryo-ET) is a powerful approach to observe subcellular architecture; it can even achieve near-atomic resolution when specific complexes can be computationally identified, aligned, and averaged. Advances in this area have led to a situation where biological insight is often not limited by resolution, but rather a lack of context with which to interpret the observed structures. This research focuses on the development of advanced cryogenic fluorescence microscopy methods, which when correlated with state-of-the-art cryo-ET methods, can provide this much-needed context.

This approach is known broadly as cryogenic correlative light and electron microscopy (CryoCLEM). Using fluorescent biosensors calibrated for cryogenic conditions, the team can provide physiological context, and super-resolution methods can provide the locations of specific proteins of interest too small to be identified by cryo-ET alone. The eventual goal of these correlative methods is to apply them to the study of the plant plasma membrane–cell wall interface.

Perception of, and response to, biotic effectors (e.g., microbes and pathogens) and abiotic effectors (e.g., salinity, drought, and nutrients) result in both the deployment of molecular defense responses close to the plasma membrane–cell wall interface and in the dynamic remodeling of the primary plant cell wall. The team's aim is to observe the response of this critical interface to various stressors with nanometer-scale spatial resolution and with physiological context and specific molecular labeling provided by CryoCLEM. First milestones toward this goal include the cryogenic calibration of a molecular-crowding sensor for CryoCLEM and the team's advanced workflow for preparing plant tissue samples for cryo-ET.



Cryogenic Correlative Light and Electron Microscopy of Biologically Relevant Tissues Fluorescent Using Biosensors. Left: Map indicates the degree of molecular crowding in the cytoplasm of yeast cells osmotically shocked prior to plunge freezing. The crowding was determined at cryogenic temperatures using the FRET-based SED1 fluorescent biosensor expressed cytosolically. The ability to readout this biosensor under cryogenic conditions demonstrates the compatibility of the SED1 biosensor, and sensors like SED1, for cryogenic correlative light and electron microscopy. **Right**: Cryogenic scanning electron micrographs of *Arabidopsis thaliana* roots at various stages of the focused-ion-beam milling process used to thin the tissue for subsequent cryogenic electron tomography. [Courtesy Stanford University]

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Novel Multimodal Chemical Nano-Imaging Technology to Visualize and Identify Small Biomolecules Exchanged in Microbial Communities

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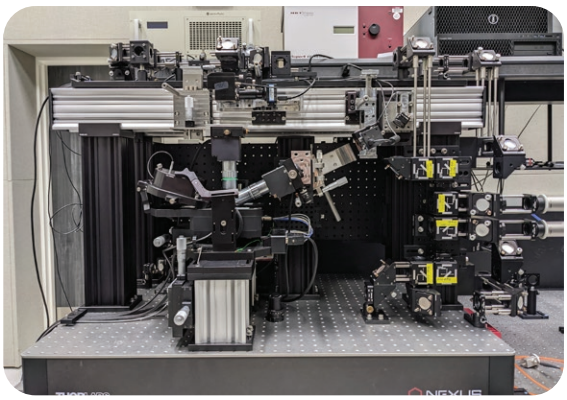
The goal of this project is to develop disruptive bioimaging technologies that will significantly advance understanding of microbial metabolism and communication in real space. This is an outstanding challenge because existing approaches do not have the required spatial resolution needed to obtain a molecular-level understanding of intercellular communications. This project will combine nano-optics tools with multimodal nonlinear optical spectroscopy to identify and image small biomolecules with nanometer spatial resolution under physiological conditions. Specifically, the team will enhance the spatial resolution in optical extinction, Raman/fluorescence, and coherent Raman/two-photon fluorescence/second-harmonic generation spectroscopy down to approximately 1 to 2 nm under ambient conditions to visualize metabolites involved in a wide range of microbial and plant processes using these different contrast mechanisms. This technology will provide significant advantages over current approaches to metabolic mapping, simultaneously achieving ultrahigh spatial resolution, ultrahigh detection sensitivity, fast acquisition speeds, and ease of switching between the various nanospectroscopy and nanoimaging modalities.

Even though linear nano-optical measurements have been demonstrated, nonlinear nano-optical measurements comprise a novel high-risk high-reward aspect of this project. Nonlinear nano-optical measurements, while providing improved signal-to-noise and improved signal-to-background ratios, are challenging when chemical imaging and identification of small biomolecules are the goals, as this requires spectrally resolved (i.e., hyperspectral) detection schemes (e.g., in coherent Raman-based vibrational nanoimaging and nanospectroscopy).

These coherent nano-optical measurements, however, typically require long collection times that generally restrict their usefulness in a point scanning hyperspectral nanoimaging scheme where full-time traces must be recorded at every spatial position. The team plans to overcome this difficulty by decreasing collection times by multiple orders of magnitude using time-series analysis and machine learning.

With these nonlinear nano-optical measurements, a new set of nanoscopic selection rules is expected compared to classical micro- and macroscopic measurements. Researchers will therefore develop a theoretical framework to assign experimental observables—primarily the linear and nonlinear optical signatures of biomolecules involved in microbial communications—by coupling *ab initio* molecular dynamics–computed optical spectra to classical finite difference time-domain simulations to fully reproduce experimental plasmon-enhanced spectral nanoimages. Much like the time-domain Raman measurements, these simulations are time consuming. Researchers will apply machine learning and tools for time-series analysis to dramatically accelerate these simulations.



The BIGTUNA Multimodal Optical Imaging Capability. In its early stages of development, BIGTUNA can accommodate top, bottom, and/or side illumination and collection under ambient conditions for several near-field linear and nonlinear optical imaging modalities, including tip-enhanced Raman, tip-enhanced photoluminescence (fluorescence), and tip-enhanced two-photon photoluminescence. [Courtesy Pacific Northwest National Laboratory]

To optimize and validate the technical performance of technology for bioimaging applications, the research team will benchmark the system using environmental consortia of anaerobic methane-oxidizing archaea and syntrophic partner sulfate-reducing bacteria. Using these consortia, this technology will allow the researchers to spatially resolve the electronic and vibrational signatures of large multiheme cytochromes embedded in the extracellular matrix, thereby providing the first direct evidence that these proteins predicted in the genomes are exported into the extracellular space between the cells.

While current effort is devoted to constructing and optimizing the new multimodal nanoimaging technology (see figure), the team is also taking the opportunity to refine measurements

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for several imaging modalities, including demonstration of sub-5-nm spatial resolution for tip-enhanced two-photon fluorescence (Wang et al. 2023) and furthering the interpretation of complex spectra obtained through tip-enhanced Raman scattering (Mantilla et al. 2022).

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Optical and X-Ray Multimodal-Hybrid Microscope Systems for Imaging of Plant Stress Response and Microbial Interactions

Dara Dowlatshahi[1,2], Adam Bowman[2], Rose Knight[1,2], James Safranek[1,1] Johanna Weker[1], Jacob Summers[2], Mary Beth Mudgett[2], Federica Brandizzi[3], Mark Kasevich[2], **Soichi Wakatsuki[1,2]*** (soichi@slac.stanford.edu, PI)

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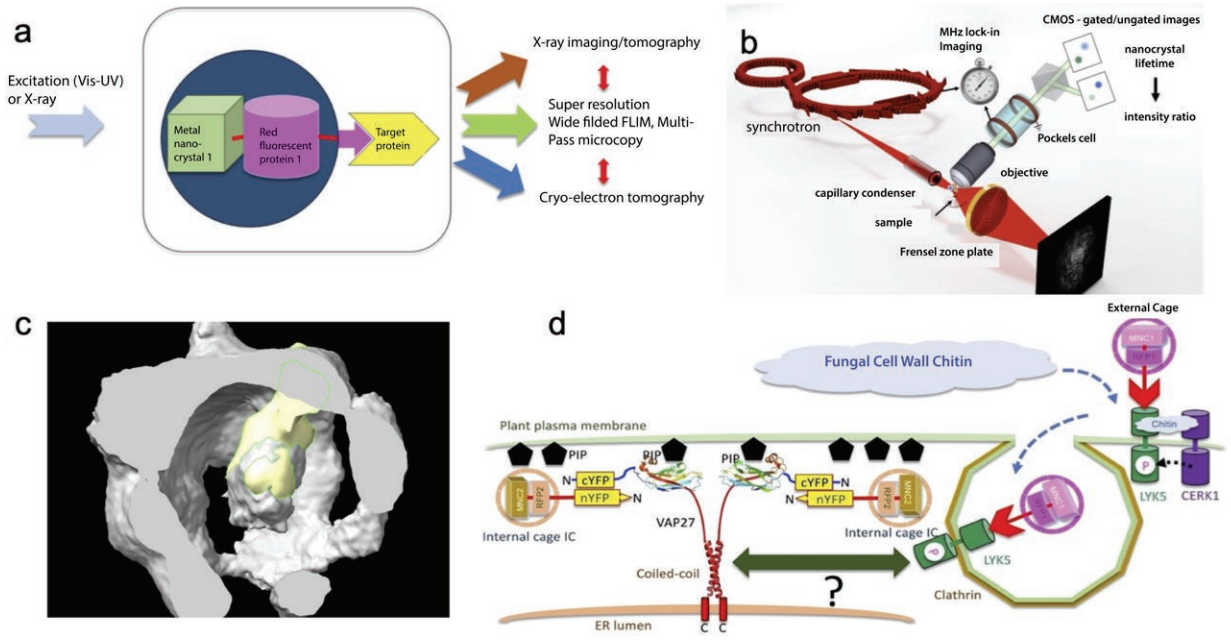
Project Goals: Development of next-generation correlative X-ray, light, and electron tomography by incorporating caged fluorescent protein and metal nanocrystals as both tracers for X-ray imaging/microscopy, electro-optical fluorescence lifetime imaging microscopy (EO-FLIM), and fiducial markers for cryogenic electron tomography (cryo-ET; see figure). The initial application will be to study plant-bacterial pathogen interaction at the plant-cell surface and transport of vesicles. In this early phase of development, the team is highlighting plans and progress for a high-frequency EO-FLIM setup at the Stanford Synchrotron Radiation Lightsource (SSRL), caging of fluorescent fusion proteins for conjugation to metal nanocrystals with cage protein, and establishment of plant systems to monitor membrane trafficking and transport.

X-Ray-FLIM Coupling. The current EO-FLIM configuration uses 40 and 80 MHz resonant gating (Bowman et al. 2019; Bowman and Kasevich 2021). The plan is to double this to 158.8 MHz in wide-field mode at SSRL X-ray imaging beamline BL 6-2. Some of the accomplishments in this area include: (1) sourcing of components for a new FLIM microscope to be integrated in X-ray microscopy beamlines for hybrid, X-ray pulse locked FLIM (see figure panel b) or used as a stand-alone instrument; (2) testing the timing patterns at 158.72 MHz with uniform fill (124 bunches) on the SSRL synchrotron on machine safety and electron stability; (3) developing alternative approaches to produce X-ray excitation single pulses using a novel X-ray chopper crystal monochromator to select individual X-ray pulses from 476.3 MHz using a spinning silicon crystal at 75,000 rpm.

Novel Biomarkers for Multimodal Imaging. The team's previous work demonstrated the fusion of small proteins to the surface of a protein cage-like structure for cryo-EM structural determination of small biomolecules, which are referred to as the double-shell system (Zhang et al. 2022). Modeling



Imaging Plant Stress Response and Microbial Interactions. (a) Concept of novel combinatorial biomarker design for multimodal, multiscale bioimaging. **(b)** Hybrid imaging of plant-pathogen interactions using lock-in synchronization between X-ray photoluminescence, X-ray microscopy, and fluorescence lifetime imaging microscopy. **(c)** Preliminary cryo-EM image of a protein cage with GFP at the center. **(d)** Design of combinatorial biomarkers for studying fungal-pathogen plant interaction through endocytosis and endoplasmic membrane–plasma membrane contact site. [Courtesy Stanford University]

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work supported the feasibility to modify the existing system to encapsulate cargo having, for example, an external affibody and encapsulate a fluorescent protein within the protein cage that leaves headroom for conjugation of metal-based nanocrystals (MNCs) approximately 3 to 5 nm in size that emit X-ray photoluminescence with compatible short lifetimes (see figure panel a). Some key accomplishments in this area: refining modeling work in alpha-fold, designing protein expression constructions, developing purification strategies, and performing cryo-EM analysis of first-generation caged fluorescent proteins (see figure panel c).

The Brandizzi group used *Arabidopsis thaliana* as model plant species to show that members of the VAMP-associated proteins (VAPs) family, VAP27-1 and VAP27-3, play a critical role in determining the topology of endocytosis in plant cells (Stefano and Brandizzi 2018). The goal is to analyze these processes using protein cages (see figure panel d) for hybrid multimodal X-ray imaging, cryo-ET, and super-resolution imaging approaches. While the team develops these systems, here are details of progress on establishing plant systems: (1) Vap27-YFP reporter strains grown and maintained for EO-FLIM measurements; (2) Design and generation of constructs for expression and purification of VAP27 proteins and mutants for *in vitro* characterization supporting future *in vivo* experiments.

Funding Statement: This program is supported by the U.S. Department of Energy Office of Science through the Biomolecular Characterization and Imaging Science program of the Biological and Environmental Research Program under BCIS FWP 100878.

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

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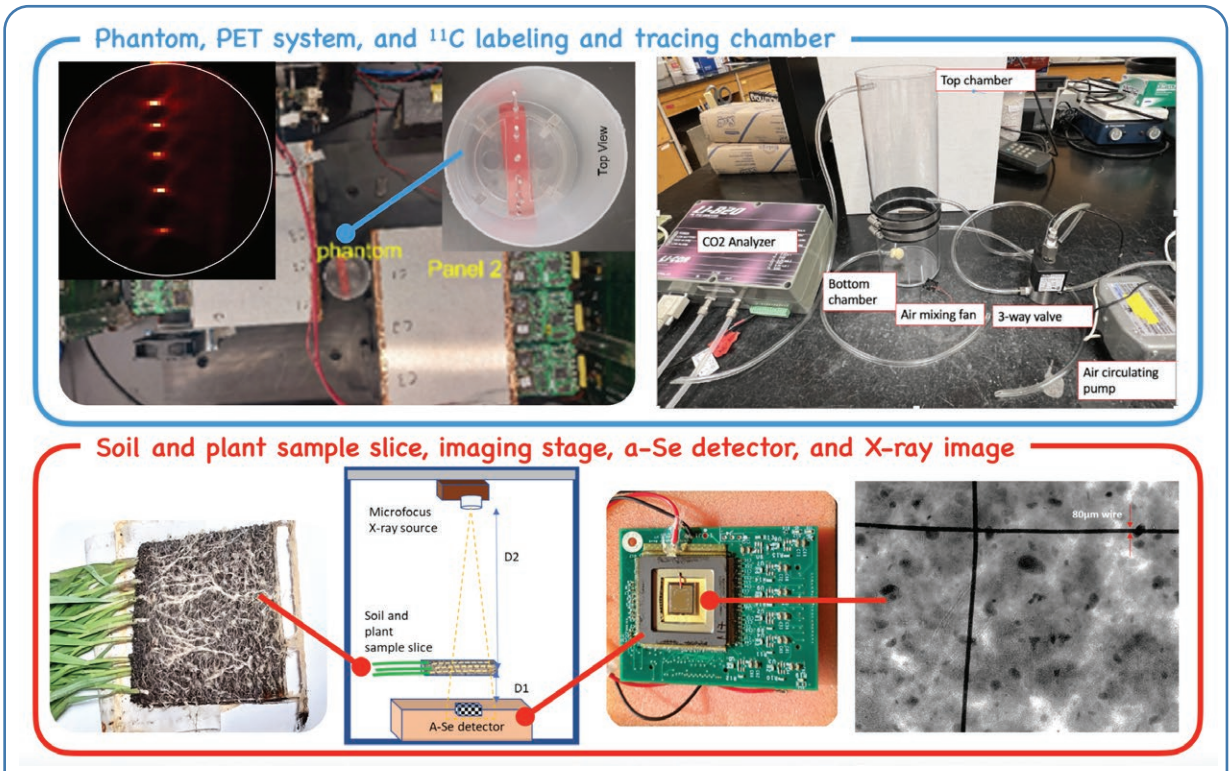
Research Plans and Progress: Soil structure, described as either the status of aggregation or distribution and networks of pore spaces, controls Earth system functions. Due to the inadequacy of soil structure data from analysis of intact soils, soil structure has not been successfully incorporated into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

The project was started in August 2021. Main goals for this year are to:

- Test the functionality of the prototype PET scanner.
- Assemble and test radiotracer infrastructure for ^{11}C radioisotope labeling.
- Test image capability of the X-ray detector.

Anticipated Accomplishments and Deliverables: A custom-built cylindrical-shaped phantom was developed



A System for Quantitative *In Situ* 3D Images of Dynamic Rhizosphere Phenomena. Top: Custom-built phantom and plant chamber for dual-panel CZT-PET image performance analysis. **Bottom:** 5-mm slice of soil with live grass plant and an X-ray image of plant root location utilizing a high-resolution (~ 8 μm) selenium detector. [Courtesy University of California–Santa Cruz]

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for initial characterization of the system for plant study (see figure). Two basics experiments were conducted: (1) 5-Capillary tubes filled with approximately 20-25 μCi of fluorodeoxy-glucose (FDG) per tube were placed in the center of the phantom using capillary tube holder, and data were collected for 3 hours, and (2) a uniform slab-shaped phantom was filled 200 μCi and acquired data for 5 hours for the purpose of system normalization. Researchers are conducting the experiment by introducing soil into the phantom to study the attenuation interference and effect of soil on the reconstructed image.

The custom-designed ^{11}C-labeling and tracing chamber was tested at the Stanford Cyclotron and Radiochemistry Facility (see figure). For achieving plant photosynthesis, researchers utilize multiple LED lighting boards. Currently, the team is transferring the PET system and ^{11}C-labeling chamber at the dedicated exhaust fume hood located at cyclotron facility.

For the X-ray detector, researchers evaluate the benefits of using advanced small pixel (7.8μm) direct X-ray detector and microfocus X-ray source technologies to produce significantly higher spatial sampling resolution compared to existing X-ray computed tomography (XCT) methods commonly used. Due to the density and high X-ray absorption of the soil and relative low absorption of the root material and to ensure that adequate contrast signal-to-noise was achieved in the images, 5-mm-thick slices of soil and biomatter were created. 2D images of two different soil and biomass samples were acquired and demonstrated that 10μm to 15μm features were visible with adequate contrast signal-to-noise for further analysis (see figure). Currently, researchers are designing a rotation system to be able to create CT images.

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

Deep Chemical Imaging of the Rhizosphere

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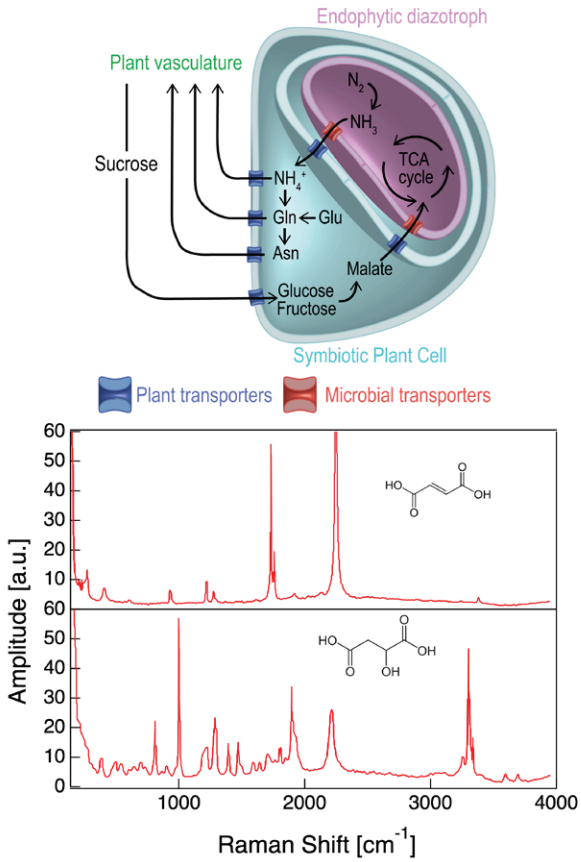
Beneficial diazotrophic microbes promote plant growth and productivity by consuming sugars and other compounds exuded by roots and, in turn, provide fixed nitrogen to the plant. Although a large fraction of the carbon fixed by plants during photosynthesis is secreted through roots to sustain the root microbiome, this metabolic exchange is not well understood. Understanding the nitrogen-carbon nexus will help to develop transformative biofertilization technologies that require a smaller carbon commitment from plants to the nitrogen-fixing microbes. This team is building a label-free microscope to image metabolic activity and chemical exchange between plants and bacteria deep within thick living plant roots and their associated rhizosphere microbial communities.

The project's second-year goals called for establishing reference plant systems and validating the instrumentation. The team will report on instrument development and depth-of-imaging benchmarks for the broadband coherent Raman microscope and on correlating organelle-resolved dynamic signatures of plant-bacterial systems of interest using dynamic quantitative phase microscopy. For initial testing of the instrumentation, researchers have selected the root nodules of *Medicago truncatula*, an extensively characterized model system for symbiotic nitrogen fixation, as the plant-bacteria reference for validation. Wild-type *M. truncatula* plants and four Tnt1 retrotransposon insertion mutant lines with a knockout of either MtSWEET11 or MtSWEET1 genes were obtained. These sugar transporters have been implicated in the interaction of *Medicago* with nodule-forming nitrogen-fixing bacteria. The nitrogen-fixing bacterium *Sinorhizobium meliloti* was shown to form nodules in *Medicago* in the laboratory. The team has now established a range of symbiotic and free-living diazotrophic bacterial cultures growing on nitrogen-free media. Researchers are currently verifying nitrogen fixation by these strains in the laboratory and then will image the strains *in planta* and in solid media.

Over the next year, the team plans to transition from imaging the model nodulating plant-symbiont system (*Medicago–Sinorhizobium*) to bioenergy crop plants that do not have nodules and are colonized by free-living diazotrophs. Along these lines, the team has begun collaborating with Oak Ridge National Laboratory's (ORNL) Plant-Microbe Interfaces (PMI) group. PMI researchers isolated a diazotrophic *Rahnella* strain that was shown to colonize the roots of tree species *Populus deltoides* (poplar). In addition, a knockout mutant of *Rahnella* for nitrogenase was produced by PMI. These strains were obtained from ORNL and shown to grow on nitrogen-free media. The team is now testing them for nitrogen fixation.



Towards Imaging Metabolic Activity and Chemical Exchange in the Rhizosphere. Top: Metabolite exchange in nodules of bacterially infected plants. **Bottom:** Raman spectra of fumaric and malic acids. [Courtesy Georgia Institute of Technology]

*Presenting Author

Understanding Plant Signaling via Innovations in Probe Delivery and Imaging

No-cost extension project

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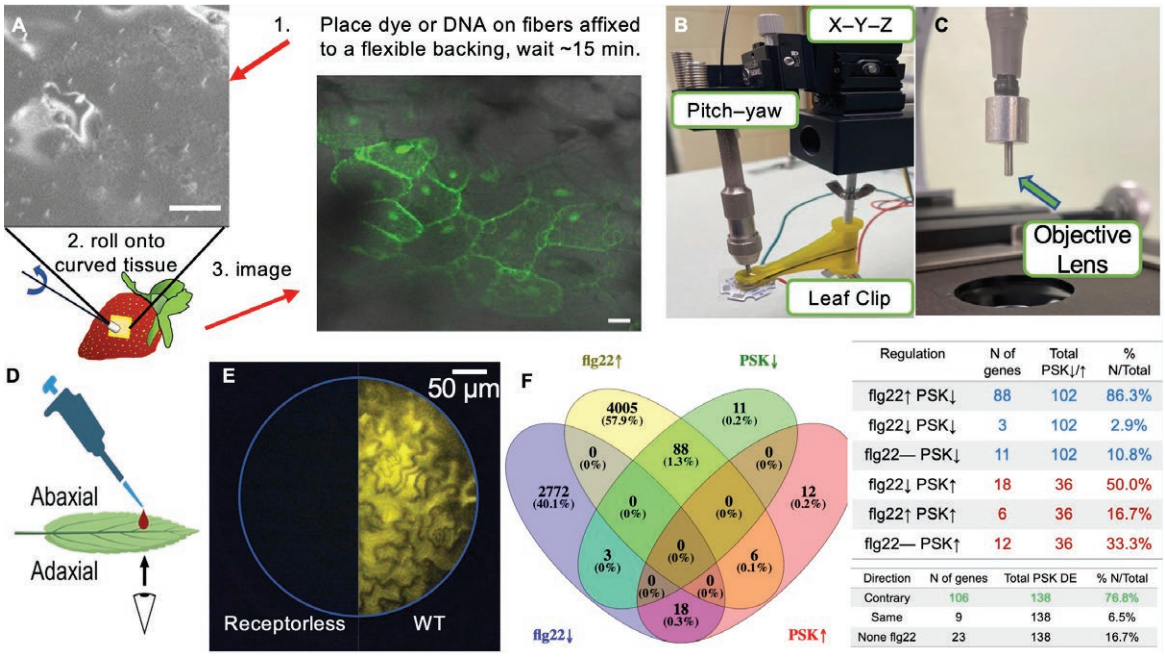
Goals: The team aimed to optimize nanofibers to deliver DNA expression constructs to plant cells and to develop and use a custom-built fiberoptic microscope and image analysis platform that enables iterative, nondestructive measurements of plant tissues over time. These tools were developed together with research aimed at understanding receptor-mediated trafficking of the growth-promoting PSK peptide and responses. The goals are (a) to use the microscope to image the trafficking of a fluorescent bioactive peptide and its receptor; (b) to improve and test different nanofiber designs for delivering probes to plants, and (c) to further discover and validate the transcriptional changes due to PSK-induced signaling.

Microscope: The team upgraded the fiberoptic microscope to optimize plant stabilization and imaging. The microscope includes two LED light sources plus a new white LED for brightfield imaging and interchangeable fiberoptic lenses with different magnifications. The team created a mount to allow upright imaging, fabricated a 3D-printed leaf clip, and mounted the fiber on an extensible arm with 5-axis control (X-Y-Z plus pitch and yaw) for precise sample manipulation and fine focus to obtain high-resolution, iterative micrographs using live plants. With these refinements, the team observed receptor-dependent trafficking of the bioactive peptide PSK-TAMRA from one side of a leaf to the other.



Regulation	N of genes	Total PSK↓/↑	% N/Total
flg22↑ PSK↓	88	102	86.3%
flg22↓ PSK↓	3	102	2.9%
flg22— PSK↓	11	102	10.8%
flg22↓ PSK↑	18	36	50.0%
flg22↑ PSK↑	6	36	16.7%
flg22— PSK↑	12	36	33.3%

Direction	N of genes	Total PSK DE	% N/Total
Contrary	106	138	76.8%
Same	9	138	6.5%
None flg22	23	138	16.7%

A Fiberoptic Microscope and Imaging Analysis Platform to Understand Plant Signaling. (A) Schematic of using vertically aligned carbon nanofiber arrays (visualized by scanning electron microscopy after transfer to flexible substrate (SU-8) to apply dye or DNA to a curved plant surface that is later imaged by confocal microscopy. Bars are 20 µm. **(B)** Fiberoptic objective mounted in a free-floating leaf clip. Imager can also be used with a robotic arm to image different parts of a plant. **(C)** Fiberoptic objective mounted in an upright microscope body. **(D)** Assay of transverse movement of labeled peptide from one side of a leaf to the other. **(E)** Composite TAMRA fluorescence observed from the adaxial leaf surface 12 hours after application of 10 µM PSK peptide–TAMRA to the abaxial face in the indicated plants. **(F)** Venn diagram indicating the number of overlapping PSK-and flg22-peptide regulated genes that are differentially expressed. Table indicates that a majority of the shared gene targets are regulated with opposing (contrary) directionality; these are mainly regulated by one family of transcription factors. PSK down-/flg22 upregulated genes are associated with plant defenses. [Courtesy University of Chicago and Argonne National Laboratory]

*Presenting Author

Nanofibers: The team reported success in using vertically aligned carbon nanofiber arrays (VANCFs) to deliver and get expression of DNA constructs to various plant tissues (Morgan et al. 2022). Through a user proposal at the Center for Nanophase Material Sciences, the team designed and implemented a strategy to transfer VACNFs from a rigid silicon substrate to SU-8, a flexible substrate. Importantly, this permits use of the fibers to deliver reagents to curved plant structures. To overcome the hydrophobicity of SU-8, fibers in the flexible film were coated with a 2- to 3-nm layer of silicon oxide. Using a rolling motion to drive fibers through plant cells, the team succeeded in delivering DNA and dye to various curved plant organs. The team has been invited to submit a manuscript for *JoVE Journal* that includes the recently achieved innovations.

Biological Materials/Deliverables: The team conducted a time-series transcriptomic analysis of root and shoot responses to PSK and revealed tissue-specific and time-dependent plant responses to this peptide hormone. The study also included a comprehensive analysis of PSK effects on whole seedlings during early development. The team found that PSK down-regulates the expression of a specific transcription factor family that regulates plant defense genes. This family is also the most enriched transcription factor in PSK down-regulated genes that associated with plant immunity. By comparing the transcriptome data with publicly available data, the team found that PSK has the opposite regulatory effects on that transcription factor family and defense-related genes compared with the responses triggered by the microbe-associated molecular pattern peptide flg22. This observation may explain the antagonism between these two peptide ligands (PSK and flg22). Researchers are currently testing predictions from the RNA-seq experiments using qPCR, along with physiological and biochemical read-outs and are preparing a manuscript based on the findings.

DOE Funded Research Benefits for Dissemination and Deployment of Bioimaging Technology:

1. A major advance is the iterative, nondestructive fluorescence imaging of bioactive peptides, their receptors, and output signaling responses in intact 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.

2. Nanofibers for introducing nonpermeable probes and biomolecules into plant cells accelerates the discovery of plant signaling response components in many species in response to many stimuli/environmental. Fibers serve the dual goal of providing fiducial markers for the iterative imaging developed. Finally, the approach can also be used for genome editing.

Funding Statement: This research was supported by the DOE Office of Science's Biological and Environmental Research Program, grant no. DE-SC0019104.

Reference

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

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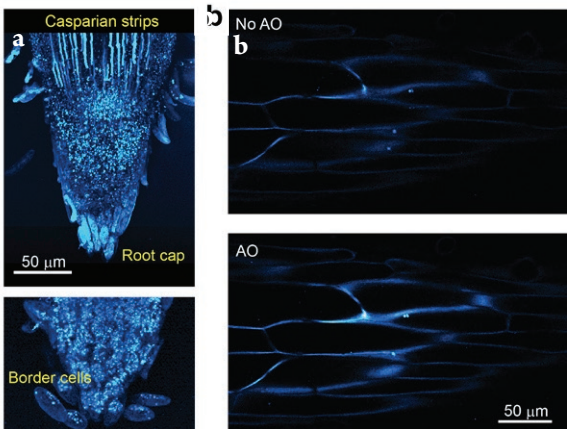
Visualizing root morphology at cellular and subcellular resolution is critical for understanding plant growth and accompanying structural changes, as well as their interactions with microbes in the rhizosphere. Optical microscopy based on second harmonic generation (SHG) and third harmonic generation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

The Northen and Vogel laboratories have successfully manufactured EcoFABs and cultured root-microbe systems to study their interactions. The Ji lab has finished building a SHG homodyne mixing setup and is very close to getting *in vivo* data. The team is waiting for parts to arrive for constructing the THG homodyne mixing setup. With the non-homodyne THG system, the team has (1) further extended the ability to image various structures of live *Brachypodium distachyon* roots (including border-like cells next to root caps), (2) characterized the location of Casparian strips, and (3) mapped starch granules and p granules (see figure).

The team has combined adaptive optical microscopy with SHG and THG microscopy for plant roots. Researchers have succeeded in improving the image brightness of root cells (Fig. 1b). Using THG and three-photon fluorescence imaging, they have imaged the interaction between a live *Arabidopsis thaliana* root and two bacterial strains of *Pseudomonas simiae* (wildtype and IAA-producing strains). The team has also succeeded in imaging fungal interaction with *B. distachyon* roots. A next step is to add bacteria to the root-fungus system to observe their interactions.



Third-Harmonic Generation Images of *Brachypodium distachyon* Roots. (a) Volume rendering of root caps, with Casparian strips within and border-like cells surrounding root caps. **(b)** Images of cells in the mature zone acquired without and with adaptive optical (AO) correction. [Courtesy University of California, Berkeley]

*Presenting Author

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

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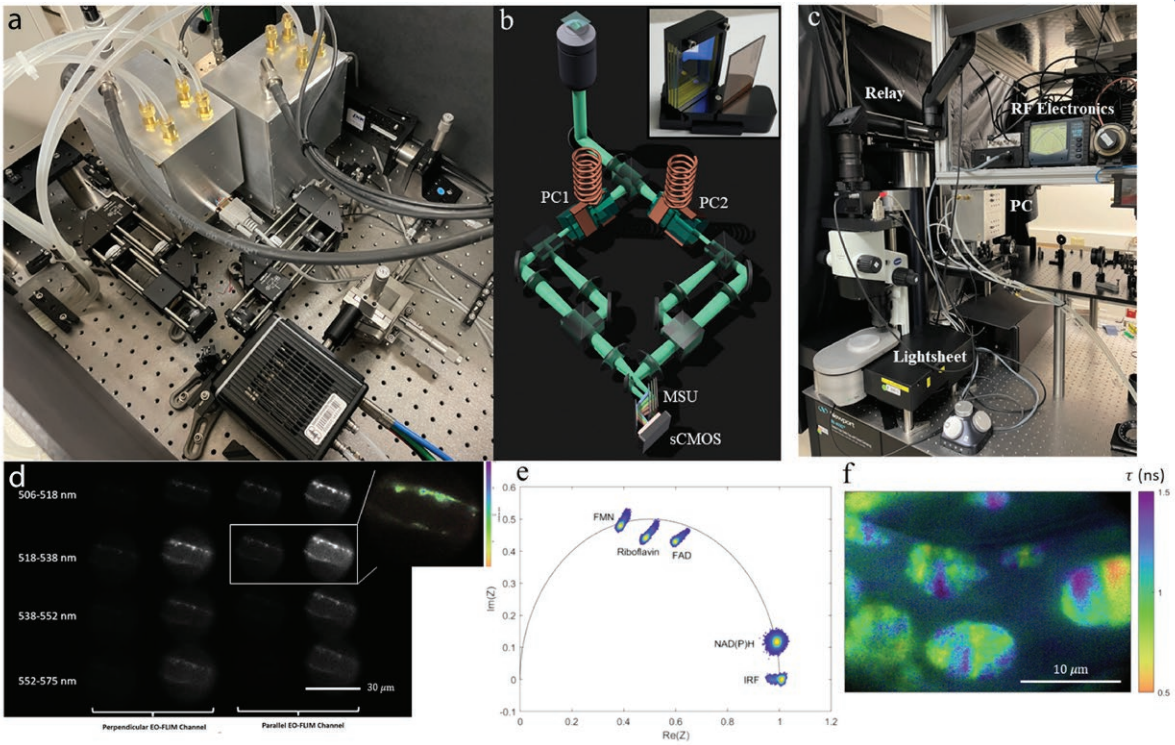
Project Goal: The goal is to realize a high-speed lifetime imaging platform for light-sheet microscopy of metabolic pathways and plant-microbe interactions using electro-optic fluorescence lifetime microscopy (EO-FLIM; Bowman and Kasevich 2021; Bowman 2019). Wide-field optical modulators allow efficient lifetime capture combined with low noise readout on standard scientific cameras. This system will find broad applications in plant imaging and will provide lifetime contrast using both fluorescent labels and endogenous autofluorescence. Multidimensional imaging optics enable lifetime multiplexing and unmixing of autofluorescent signatures. These optics allow simultaneous acquisition of space, polarization, nanosecond time, and wavelength.

Research Plans and Progress: The team has completed the design and construction of two custom microscopes for EO-FLIM imaging. The first microscope (see figure panels a, b) allows multidimensional wide-field FLIM and will be expanded in the future for 2-photon light-sheet excitation. Its design includes two 40 MHz resonant Pockels cells and a compact multispectral unit (MSU) for simultaneous FLIM imaging in several spectral bands. The MSU is broadly applicable to any microscope, and in this system, it enables ongoing experiments with multilabel and autofluorescence unmixing. The microscope is equipped with a



EO-FLIM Optics and Data. (a,b) Multidimensional EO-FLIM optics using two resonant Pockels cells. **(b, inset)** Multispectral unit (MSU) uses a stack of dichroic filters to create four spectrally separated bands on an sCMOS camera sensor as shown in **(d)**. **(c)** EO-FLIM optics developed for lightsheet microscopy with wide field of view, including an 80-MHz resonant large-aperture Pockels cell **(d)** 4x4 array of images output from multidimensional optics encoding four spectral channels as rows and both lifetime and polarization information as columns. Shown here is imaging of vesicle transport in an *Arabidopsis* root hair. **(inset)** Lifetime image calculated from the ratio of one pair of output images in the array. **(e)** Phasor plot showing the signatures of various autofluorescence species excited with ultraviolet light. **(f)** Lifetime image of structures in a plant leaf generated using phasor analysis. [Courtesy Stanford University]

*Presenting Author

supercontinuum laser source for multiband excitation and also a doubled Ti:Sapphire laser and pulse-picker for ultraviolet excitation. The second microscope (see figure panel c, p. 60) is a light-sheet platform for large field-of-view FLIM imaging. The resonant Pockels cell for this system is driven at 80 MHz and provides a 17 mm aperture for imaging. Imaging optics have been optimized, and initial volume acquisitions are underway.

Critical to both systems is an efficient data analysis pipeline. The team's previous work has primarily focused on rapid single-frame lifetime estimation using a single camera exposure. By combining multiple exposures taken at different Pockels cell drive phases, extraction of multiexponential information is possible. Multiphase EO-FLIM data is well-suited to phasor analysis to study multiexponential decays without fitting or histogram binning. The team is now implementing phasor analysis on EO-FLIM datasets to allow real-time display of lifetime data as it is acquired (see figure panels e, f, p. 60). Phasor analysis will also enable multilabel unmixing and visualization of lifetime shifts from autofluorescent species upon binding to different substrates.

Several biological samples have been developed for FLIM imaging. A collection of engineered *Pseudomonas putida* cells, each with a different fluorescent protein of choice, has been generated. This collection covers a large spectral range and will be used for unmixing FLIM signals from a population of bacterial cells containing different fluorescent proteins. The same strains are being used for phasor analysis of outer membrane vesicles. eGFP fused with tetraspanin Tet-8 serves as a marker of vesicles in *Arabidopsis* plants for live FLIM imaging of the root hair cells (see figure panel d). Two carbon cycling enzymes, 4-hydroxybutyl-CoA dehydrogenase (4HBD) and enoyl-CoA reductase/carboxylase (ECR), as well as glucose-6-phosphate dehydrogenase (G6PD), have been expressed and purified with and without autofluorescent molecules, NADPH and/or FAD. These will help researchers establish FLIM and phasor signatures (see figure panel e), which will be used for unmixing multidimensional live imaging of cells expressing these enzymes.

The team has also applied EO-FLIM optics to kilohertz-rate, high-speed FLIM imaging of a FRET-based genetically encoded voltage sensor in *Drosophila*, enabling lifetime detection of action potentials *in vivo*. Lifetime readout significantly improves the signal-to-noise and stability of voltage recordings (Bowman et al. 2023). This work enables future directions for imaging dynamic signals throughout plants.

Funding Statement: This research was supported by the DOE Office of Science, through the Biomolecular Characterization and Imaging Science Program, Biological and Environmental Research Program, grant no. DE- SC0021976.

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

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Project Goals: The research team is developing new approaches for live cell imaging deep within the wood-forming tissues of trees to understand developmental and physiological processes underlying wood formation and function directly in woody bioenergy feedstocks. Two types of miniaturized, minimally invasive implantable imaging probes are being used: miniscopes and cannula. These microscopy approaches are being tested by applying them to three challenges relevant to bioenergy feedstock development in poplar: (1) analysis of lignification and impacts of altered cell wall polysaccharides on lignin formation, (2) vessel element differentiation and the impact of abscisic acid levels on vessel cell properties, and (3) fiber development in both tension-wood-inducing and normal growth conditions.

Wood formation is the biological basis of lignocellulosic biomass production for bioenergy applications. Wood is also the water-conducting tissue of woody stems and affects how woody plants mitigate salinity and water stress under marginal growth conditions. The lack of appropriate tools currently limits the team's ability to image progenitor cells, the procambium, because they arise deep beneath the bark.

Researchers report progress using embedded optical probes to do live cell imaging in poplar stems. The first approach uses miniaturized epifluorescence microscopes (miniscopes) fitted with implantable gradient index (GRIN) lenses that provide access to internal tissues and minimize tissue damage (0.5 or 1 mm diameter). This approach has the advantage of directly rendering images and video in real time and can be used in combination with fluorescent probes and dyes compatible with a range of different excitation and emission filters. The second approach uses computational cannula microscopy with an individual cannula or an optrode array. A fluorescence microscope focused on the back of the cannula provides a range of excitation and emission frequencies including hyperspectral imaging. This approach has the advantages of smaller diameter probes (0.22 mm) and larger fields of view (0.20 mm; Guo et al. 2023). Thus, the team anticipates reduced tissue damage with the cannula/optrode array approach. However, in contrast to the miniscope, machine learning–based image processing algorithms are necessary to convert spatially scrambled fluorescent signals into images.

The team reports important steps toward optimizing technical approaches needed to achieve higher-resolution live cell imaging of cells within woody stems. For the miniscope approach, imaging of tissue within exposed regions of stems have been achieved, including contrasting images with and without GRIN lenses, and determining the effect of GRIN lens positioning on image resolution, field of view, and depth of field. For the cannula approach, machine-learning algorithms were developed that can render high-quality images of tissue sections from cannula probes. This approach was extended to enable large field-of-view imaging using optrode arrays (Guo et al. 2023). Currently, researchers are moving from proof-of-concept experiments to developing techniques and technology and toward biology-based experiments. Goals include imaging of biological processes central to bioenergy applications including: (1) analysis of lignification and impacts of altered cell wall polysaccharide components on lignin formation, (2) vessel element differentiation and the impact of abscisic acid levels on vessel cell properties, and (3) fiber development in both tension wood and under normal conditions.

Funding Statement: This research was supported by the U.S. Department of Energy, Office of Science, Biological and Environmental Research program award number DE- SC0021996, and Interagency Agreement Number 89243021SSC000074 to AG.

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*Presenting Author

Integrative Imaging of Plant Roots During Symbiosis with Mycorrhizal Fungi

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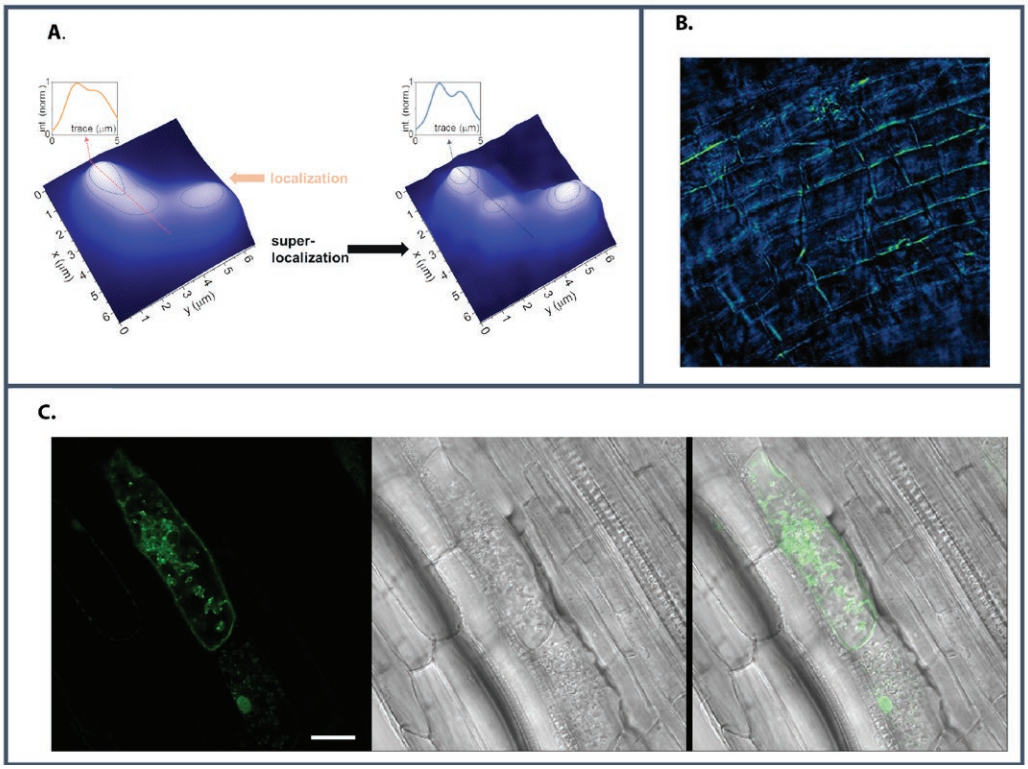
Research Plans: To improve understanding of how plants and mycorrhizal fungi interact, the research team is designing and assembling an optical imaging system that can quantify the development of each symbiont, determine nutrient exchange rates, and localize key metabolic pathways. To accomplish this, researchers are integrating quantitative-phase (or interferometric) imaging with light-sheet Raman and fluorescence microscopy to perform such multimodal imaging deep into the multiply scattering root tissue. In this context, the team is combining tailored optical beams and photon-sparse detection that overcome the illumination's spatiotemporal broadening deep in the root tissue. These hardware advances are accelerated by theoretical analyses, deep learning for image reconstruction, and the development of tailored gene-encoded biomarkers.

Current and Anticipated Accomplishments: Since its beginning in September 2021, this project has focused on four aspects. Researchers first assessed theoretically and experimentally how optical beams propagate in a simulated root-tissue environment. In this context, preliminary results indicate that the accelerating Airy beam can self-heal upon scattering, enabling deeper penetration than conventional Gaussian beams. These results are now being validated against Bessel beams and further-engineered Airy beams. Second, researchers leveraged their recent investigations on



Imaging the Rhizosphere. (A) Sparse-photon superlocalization improving magnification by two-fold with no field-of-view penalty. **(B)** Experimental interferometric image of root tissue highlighting the increased refractive index of the cell wall. **(C)** Live tracking of the location and accumulation of the Vapyrin protein in *M. truncatula* root cells colonized with an AM fungus. Vapyrin is tagged with cpVenus, and it accumulates in the cytoplasm but also in punctate accumulations close to the fungal hyphae. [(A) Reused under a Creative Commons license (CC BY NC-ND 4.0 International) from Dunn, L., et al. 2022. "Video-Rate Raman-Based Metabolic Imaging by Airy Light-Sheet Illumination and Photon-Sparse Detection," *Proceedings of the National Academy of Sciences USA* **120**(9), e2210037120. (B) Courtesy University of Idaho. (C) Courtesy Boyce Thompson Institute.]

*Presenting Author

photon-sparse and Airy light-sheet microscopy to demonstrate video-rate Raman bioimaging exhibiting 1000-fold lower irradiance than coherent Raman approaches (see figure panel A; Dunn et al. 2023). In relation to this project, researchers also introduced a photon superlocalization strategy that improves magnification without penalizing the field-of-view (see figure panel B; Dunn et al. 2023). This strategy overcomes a persistent limitation in light-sheet microscopy, where the need of long-working-distance objectives limits magnification. Third, the team is completing an interferometric microscope that quantifies dry-density and dry-mass of entities residing within a multiply scattering environment. This approach combines phase-shifting interferometry with computation-free image reconstruction by backpropagation. Fourth, researchers are expanding their existing palette of gene-encoded biomarkers to track cellular alterations that underlie host cell accommodation of symbionts (see figure panel C). Immediate next steps in this project are to: (1) improve and apply the self-healing properties of accelerating beams in root imaging; (2) complete the integration of a light-sheet microscope with time-resolved photon-sparse detection dedicated to plant root imaging; and (3) reconstruct photon-sparse images by adopting denoising algorithms typically applied in quantum imaging and astronomy.

Benefits and Applications: Bioenergy crops can form mutualistic associations with mycorrhizal fungi. These associations supply crops with nutrients that are limited in most arable lands and accelerates atmospheric CO_2 sequestration. The optical imaging system developed in this project is expected to offer quantitative understanding of the plant root interactions with mycorrhizal fungi and to contribute to a long-standing DOE goal in energy prosperity: to attain a predictive biosystems understanding through transformative instrumentation solutions. Further, this optical imaging system is designed on commercially available hardware, thus improving its accessibility to the broader scientific community.

Funding Statement: This research was supported by the DOE Office of Science, Biological and Environmental Research program, grant no. DE-SC0022282.

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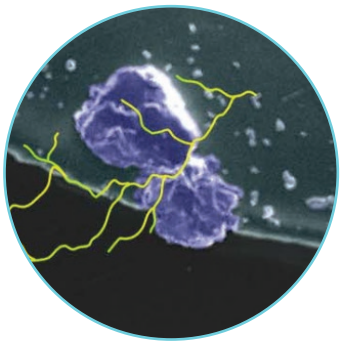

Chapter 4
Emerging Technologies and Approaches for BER Research

Technologies for Investigating the Rhizosphere

BSSD-supported scientists are conducting research that demonstrates the impact of technologies on rhizosphere components and communication. The focus on how enabling technologies can reveal both rhizosphere structure and function supports BER's larger objective of understanding the fundamental biology underlying solutions for bioenergy and the bioeconomy.

This research includes the use of advanced imaging capabilities, sensing techniques, and spatial methods for mapping chemical composition within the rhizosphere. Researchers also are evaluating species interactions and metabolic cooperation in synthetic and real soils by defining system components with chambers for co-cultures of bacteria, fungi, and plant roots. Additional multiscale, multimodal investigations are providing a deeper understanding of the interrelationships of roots, microbes, and metabolites.

Emerging Topics and Technologies

These presentations highlight forward-looking approaches and tools to tackle challenges within the scope of BSSD research on investigating and modifying genomic and molecular function. Topics included how to advance or expedite ongoing research or pave the path for entirely new insights.

Technologies for Investigating the Rhizosphere

A Reproducible and Tunable Synthetic Rhizosphere Microbial Community Enables Quantitative Plant-Microbe Studies

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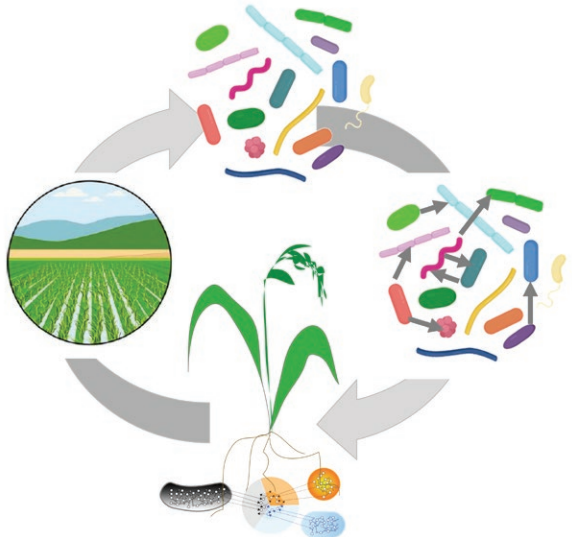
Plants form intimate intertwined relationships with the microbial communities in and around their roots. These rhizosphere communities promote plant-microbe interactions to advance sustainable agriculture, thus making them an important field in agricultural research. However, investigation in this field is often hindered by the complexity of these communities and reproducibility of data. Therefore, the development of model microbial community systems that aid mechanistic studies under highly controlled conditions would enable new insights into complex plant-microbe systems. Here, the team developed a model synthetic community (SynCom) of 16 microorganisms commonly found in the rhizosphere of diverse grass species.

The team addresses two key challenges in developing standardized model communities that maintain community diversity over time and are reproducible: storing and resuscitating these communities after cryopreservation for dissemination of the SynCom. The model rhizosphere community grows reproducibly between replicates and experiments, with a high community alpha-diversity achieved through growth in low-nutrient media and through the adjustment of the starting composition ratios for the growth of individual organisms.

The research team successfully cryopreserved the SynCom and archived subsequent resuscitation, allowing for easy replication and dissemination. The 16-member SynCom grows reproducibly in fabricated ecosystem devices (EcoFABs), demonstrating the application of this community to an *in vitro* plant-microbe system. EcoFABs allow reproducible research in model plant systems, offering the precise control of environmental conditions and the easy measurement of plant microbe metrics, from multiomics data generation to advanced imaging. Combined with quantitative microbiome methods that (1) account for total microbial load, (2) determine absolute abundance, and (3) discriminate between dead and live cells, this SynCom enables detailed mechanistic studies and is critical for replicating plant-microbe ecosystem processes, properties, and dynamics in reproducible laboratory environments.



Model Microbial Community Systems. A synthetic microbial community (SynCom) obtained by multi-omics-guided isolation is utilized to unravel microbe-microbe interactions and interactions with the host in reproducible model ecosystems (EcoFABs). This iterative approach aids predictive modeling of the rhizosphere and provides mechanistic insights into plant-microbe interactions in the rhizosphere. [Courtesy University of California–San Diego]

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Finding Clarity Through Imaging: Why the Answer May Lie in the Roots

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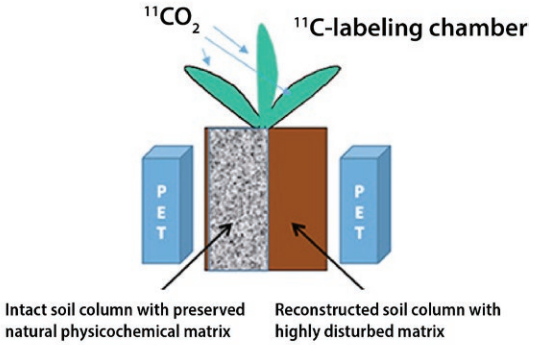
The team is developing a dedicated positron emission tomography/computed tomography (PET/CT) imaging system with high spatial resolution. This technology utilizes recent advances made in room-temperature, wide-bandgap semiconductor detector technology for high sensitivity and high spatial resolution PET/CT imaging of plants. Utilizing cadmium zinc telluride (CZT) in the proposed PET system, team members can image the dynamics of short half-life tracers (e.g., $^{11}CO_2$). Utilizing amorphous selenium (a-Se) in micro-CT, researchers can image the soil-root interface's structural features with high contrast.

The application of PET/CT imaging in studying soil structure and dynamic rhizosphere phenomena provides valuable insights into plant-soil interactions and offers numerous biological user applications. By integrating a noninvasive imaging technique with existing soil and plant analysis, researchers can enhance understanding of complex biological systems and contribute to the development of sustainable agriculture and environmental practices. The approach enables visualizing and quantifying nutrient uptake efficiency, helping optimize fertilizer application strategies and improve crop productivity. It enables the assessment of root-system architecture and the study of soil microbial communities and their interactions with plant roots, shedding light on symbiotic relationships and potential biocontrol strategies. PET/CT can be employed in assessing soil contamination and the effectiveness of bioremediation approaches.



High-Contrast Rhizosphere Imaging. A new imaging system for plants and soil using positron emission tomography (PET) will enable scientists to study the effects of the soil matrix on biological activity in the rhizosphere. [Courtesy University of California–Santa Cruz]

*Presenting Author

RhizoGrid Root Cartography Spatially Maps Plant-Microbe Interactions in the Rhizosphere

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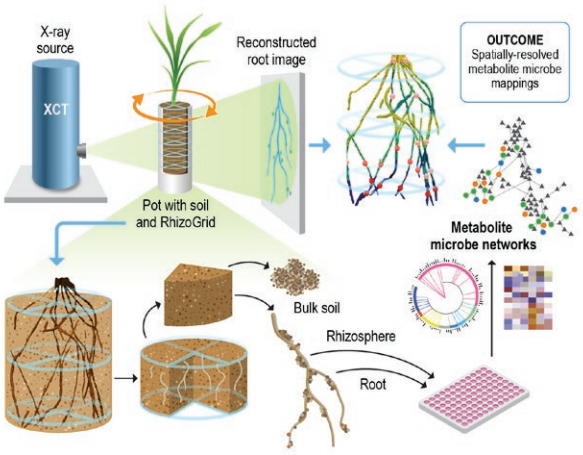
Research Plans and Progress: The rhizosphere, one of the most dynamically regulated soil environments, is influenced by plant roots and exudated root metabolites that favor the recruitment of beneficial microorganisms. The plant microbiome has a direct effect on plant health and acclimation to extreme environments. However, plant-microbe interactions in the rhizosphere have proven complex and difficult to study. The plant root itself is functionally heterogeneous, but current technologies require homogenization, incurring a loss of spatial resolution and preventing a holistic view of rhizosphere plant-microbe interactions. Determining the chemical signals involved in recruiting and maintaining specific root microbiomes under changing climates is a major challenge in rhizosphere biology. By understanding fundamental principles governing the spatial recruitment of beneficial microbiomes in the root-associated rhizosphere, researchers can engineer synthetic rhizosphere microbiomes to sustainably promote highly productive and stress-tolerant biomass cropping systems.

The RhizoGrid, an integrated imaging and spatial multiomics platform, maps metabolomic and metagenomic measurements to root structures. By integrating molecular and taxonomic information with 3D root images from plants grown in soil, RhizoGrid enables unprecedented access to investigate the heterogeneity and complexity of root exudate–microbial interactions in the rhizosphere. This platform sensitively identifies microenvironments within the plant's hidden half: the root.

Potential Benefits and Applications:

- A major challenge with conventional molecular imaging technologies at the whole-plant scale is the lack of spatiotemporal resolution. By linking the molecular and taxonomic measurements to specific locations of a 3D root system, the team can begin to understand microenvironment-specific effects.
- The RhizoGrid workflow enables identification of spatially distributed metabolic niches along the roots and the microbial population co-enrichments with specific root-exudated metabolites.



Integrated Imaging and Spatial Multiomics. The RhizoGrid 3D root cartography workflow generates spatially resolved rhizosphere-relevant molecular information on plant-microbe interactions at a resolution meaningful for synthetic biology–enabled system optimizations. [Reused under a Creative Commons license (CC BY 4.0 International) from Handakumbura, P., et al. 2021. "Visualizing the Hidden Half: Plant-Microbe Interactions in the Rhizosphere," *mSystems* **6**(5), e00765–21.]

- Understanding fundamental principles that govern the spatial recruitment of beneficial microbiomes within a root-associated rhizosphere facilitates the engineering and environmental control of synthetic rhizosphere microbiomes that promote crop resilience.

Reference

Handakumbura, P. P., et al. 2021. "Visualizing the Hidden Half: Plant-Microbe Interactions in the Rhizosphere," *mSystems* **6**(5). DOI:10.1128/mSystems.00765-21.

Project Team: Pubudu Handakumbura*, Ryan McClure, Albert Rivas Ubach, Anil Battu, Kate Schultz, Tamas Varga, Kylee Tate, Yuliya Farris, Christer Jansson, Janet Jansson, Rob Egbert.

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Biological Imaging Using Entangled Photons

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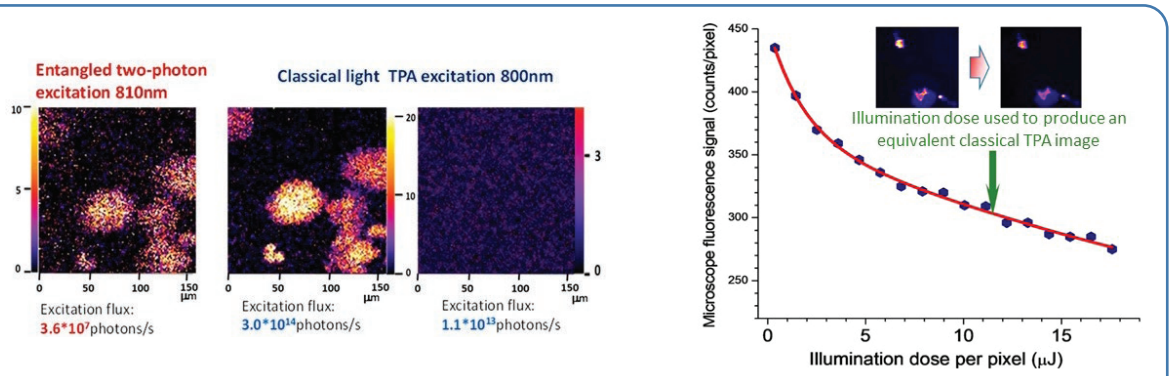
Research Plans and Progress: The ability to provide improved *in vivo* capabilities for direct visualization of biological processes is the focus of this effort. The team seeks to develop truly noninvasive and noncontact imaging opportunities over numerous time and length scales. Addressing the major challenge of photodegradation and phototoxicity in the acquisition of a comprehensive understanding of biological function often necessitates prolonged imaging measurements. Specifically, in this investigation, the team seeks to provide the full theoretical potential of entangled two-photon fluorescence microscopy such that its capabilities not only compare to or surpass those of corresponding classical modalities but also leverage extremely low excitation intensity.

Studies are underway to provide (1) a unique combination of novel technical innovations between the University of Michigan (UM) and Oak Ridge National Laboratory (ORNL), (2) fundamental understanding and theoretical predictions between the university participants, and (3) validation of the novel imaging capabilities through probing complex biosystems in the rhizosphere primarily carried out at ORNL. Advances have been made on entangled two-photon fluorescence microscopy for ultralow light bioimaging to protect against photobleaching in biological systems. Recent technical work is underway at both UM and ORNL to upgrade the entangled photon source as well as aspects of the entangled two-photon microscope. Collaboration between Northwestern University (NU) and UM has established spectral characteristics important to the entangled process, which may be used in future microscope experiments in the rhizosphere-plant biological media. The teams have collaborated on unique poplar plant samples provided by ORNL. Preliminary measurements on these samples have been carried out. The next goal is to provide comparisons between the classical and entangled images of root hairs of the poplar plants. Additionally, the ORNL team has investigated various pulse-shaping approaches, and related theory has been developed at NU. Initial development has been focused on adaptive pulse shaping for the pump laser for entangled photon generation, as ideal spectral and/or temporal characteristics of the pump laser for entangled two-photon spectroscopic and microscopic remain unexplored. A free space entangled photon generation and detection system has been built by introducing a tracer beam for the alignment and optimization. An understanding gained through this effort will enable the team to further extend such a pulse-shaping approach to optimize and control the entangled photon pairs for optimized response.



Classical vs Entangled Microscopy Images. Left: Cancer cells can be imaged using an ultralow flux of entangled photons. Producing an equivalent image with classical two-photon absorption (TPA) required a flux that was approximately seven orders of magnitude greater than that used in the entangled TPA image. Cancer cells imaged via an ultralow flux of entangled photons do not show any detectable photodamage, while significant photobleaching occurs under the conditions required to produce an equivalent image using classical photons. **Right:** To achieve equivalent entangled TPA and classical TPA images, total illumination doses of 2.8pJ/pixel and 11.5 μJ/pixel were required, respectively. The above plot of counts/pixel shows that significant photodamage occurs at the dosage used for the equivalent classical TPA image. [Reprinted with permission from Varnavski, O., et al. 2022. "Quantum Light-Enhanced Two-Photon Imaging of Breast Cancer Cells," *The Journal of Physical Chemistry Letters* **13**(12), 2772–81. DOI:10.1021/acs.jpclett.2c00695. ©2022 American Chemical Society.]

*Presenting Author

Biocompatible Surface Functionalization Architecture for a Diamond Quantum Sensor

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https://q-next.org/

Project Goals: Diamond-based quantum sensing enables nanoscale measurements of biological systems with unprecedented sensitivity. Potential applications of this emerging technology range from the investigation of fundamental biological processes to the development of next-generation medical diagnostics devices. One of the main challenges faced by bioquantum sensing is the need to interface quantum sensors with biological target systems. Specifically, such an interface needs to maintain the highly fragile quantum states of the sensor and, at the same time, be able to extract intact biomolecules from solution and immobilize them on the quantum sensor surface. This work overcomes these challenges by combining tools from quantum engineering, single-molecule biophysics, and material processing.

Recent developments in quantum engineering and diamond processing have brought us considerably closer to performing nanoscale nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) spectroscopy of small ensembles and even individual biomolecules. Notably, these advances have enabled the detection of a single ubiquitin protein and the probing of the EPR spectrum of an individual paramagnetic spin label conjugated to a protein or DNA molecule. More recently, lock-in detection and signal reconstruction techniques have enabled one- and multi-dimensional NMR spectroscopy with spectral resolution comparable to conventional NMR spectrometers. More advanced control sequences at cryogenic temperatures have further enabled mapping the precise location of up to 27 ^{13}C nuclear spins inside of diamond. Yet, biologically meaningful spectroscopy on intact biomolecules remains elusive. One of the main outstanding challenges, which is required to perform nanoscale magnetic resonance spectroscopy of biomolecules, is the need to immobilize the target molecules within the 10–30 nm sensing range of a highly coherent nitrogen vacancy (NV) qubit sensor. Immobilization is necessary because an untethered molecule would otherwise diffuse out of the detection volume within a few tens of microseconds.

Various avenues to the functionalization of high-quality single crystalline diamond chips have been pursued over the last decade. However, none of the currently known approaches has led to the desired results of interfacing a coherent quantum sensor with target biomolecules. For example, hydrogen-terminated diamond surfaces can readily be chemically modified and form biologically stable surfaces; but near-surface NV centers are generally charge-unstable under hydrogen termination, posing open challenges for NV sensing. On the other hand, oxygen-terminated diamond surfaces have been used to create charge-stable NV^- centers with exceptional coherence times within 10 nm from the diamond surface. However, perfectly arranged ether-terminated diamond surfaces generally lack chemically functionalizable surface groups (such as carboxyl or hydroxyl groups), making it difficult to control immobilization density and surface passivation. Other platforms such as diamond nanocrystals can generally be functionalized due to their heterogeneous surface chemistry, but they do not possess the coherence times needed for NMR spectroscopy.

The team's approach overcomes these limitations by utilizing a 2-nm-thick Al_2O_3 layer deposited onto an oxygen-terminated diamond surface by atomic layer deposition (ALD). This Al_2O_3 adhesion layer is silanized to create an amine-terminated surface, which in turn is then grafted with a monolayer of heterobifunctional polyethylene glycol (PEG) via an NHS reaction, a process also referred as PEGylation. The PEG layer serves two purposes. First, it passivates the diamond surface to prevent nonspecific adsorption of biomolecules. Second, by adjusting the density of PEG molecules with functional groups (e.g., biotin or azide), it allows the researcher to control the immobilization density of proteins or DNA target molecules on the diamond surface. Furthermore, the small persistence length of the PEG linker allows the immobilized biomolecules to undergo rotational diffusion. This tumbling motion is the basis for motional averaging of the NMR spectra and helps to prevent immobilization of molecules in biologically inactive orientations. The density of binding sites can be controlled by adjusting the stoichiometric ratio of methyl-terminated PEG and functional PEG groups, for example, biotin-terminated PEG for biotin-streptavidin binding or azide-terminated PEG for click chemistry. Using fluorescent single-molecule microscopy, researchers then investigated the adsorption density of proteins on the surface of the diamond quantum sensor. The number of binding events shows a clear dependence on the density of functional PEG molecules. Using this approach, researchers were able to control the protein-binding density by several orders of magnitude.

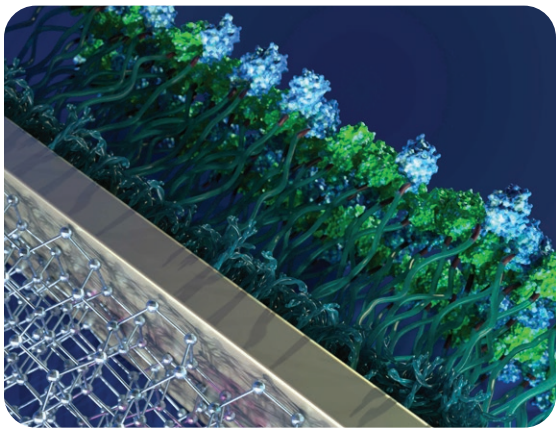
*Presenting Author

In parallel, team members studied the impact of functionalization architecture on the spin coherence (T_2) of near-surface NV centers. Long coherence times are essential to NV-based quantum sensing because the sensitivity is generally proportional to ~$\sqrt{T_2}$. Specifically, researchers showed that the coherence time of a NV center under dynamical decoupling is minimally impacted by the team's biocompatible surface modification technique. Furthermore, researchers did not register any sizable reduction in spin lattice relaxation after the surface modification. Based on these qubit coherence times and sensor-target distances, the team can predict that the NMR signal of an individual ^{13}C nuclear spin can be detected with integration times as short as 100 seconds.

Combining existing NV sensing techniques with the team's molecular pulldown platform will enable NMR and EPR spectroscopy of intact biomolecules in a relevant biological environment. Existing microfluidics platforms can readily be combined with the team's diamond passivation and functionalization method. This will pave the way to label-free high-throughput biosensing with applications in quality management in the pharmaceutical industry; target screening for drug discovery; single-cell screening for metabolomics and proteomics; and detection of cancer markers. Furthermore, positioning individual biomolecules within the 10-nm sensing range of a single NV center brings the community closer to performing EPR and NMR spectroscopy on individual intact biomolecules. When combined with nanowire-assisted delivery platforms, such a technology could enable single-molecule magnetic resonance spectroscopy within the context of a cell.

Magnetic resonance spectroscopy with single-molecule sensitivity could provide insights into receptor-ligand binding events, post-translational protein modification (e.g., phosphorylation processes), and the detection of subtle protein conformational changes in living cells, which can enhance understanding of complex signaling pathways that are not accessible by current technologies.

Funding Statement: The biochemical modification of the diamond surface and the investigation of NV coherence was supported by NSF Grant No. OMA-1936118. The conjugation of DNA molecules to the diamond surface was supported by NSF Grant No. OIA-2040520. The study of the biochemical stability of the developed interface was supported by NSF QuBBE QLCI (NSF OMA-2121044). The development of a setup for probing NV coherence was supported by the Swiss National Science Foundation (SNSF) Grant No. 176875. The investigation of surface spins and their impact on NV coherence is based upon work supported by Q-NEXT (Grant No. DOE 1F-60579), one of the U.S. Department of Energy Office of Science National Quantum Information Science Research Centers. The growth of ultrathin Al_2O_3 layers was funded by SNSF Grant No. 183717. The spectroscopic investigation of surface termination acknowledges support from the U.S. Department of Defense through the National Defense Science and Engineering Graduate Fellowship Program and DMR1752047. We furthermore acknowledge the use of the Pritzker Nanofabrication Facility at the University of Chicago (NSF ECCS-2025633), the University of Chicago Materials Research Science and Engineering Center (DMR-2011854), as well as the Imaging and Analysis Center at Princeton University (DMR-2011750).



Diamond-Based Quantum Sensing. Interfacing a coherent qubit sensor with fragile biological systems is an outstanding challenge in quantum sensing. The image shows an artistic visualization of a biocompatible surface functionalization architecture for diamond-based quantum sensors recently developed by the Maurer Lab at the University of Chicago. For more details see Xie, M., et al. 2022. "Biocompatible Surface Functionalization Architecture for a Diamond Quantum Sensor," *Proceedings of the National Academy of Sciences USA* **119**(8), e2114186119. [Courtesy University of Chicago and Argonne National Laboratory]

Reference

Xie, M., et al. 2022. "Biocompatible Surface Functionalization Architecture for a Diamond Quantum Sensor," *Proceedings of the National Academy of Sciences USA* **119**(8), e2114186119. DOI:10.1073/pnas.2114186119.

Plant Root Imaging During Symbiosis with Mycorrhizal Fungi

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Project Goals: This project develops an optical microscope that quantifies the metabolic interactions between soil microorganisms and plant roots. This development is aided by optics theory, image processing by deep learning, and the creation of tailored gene-encoded biomarkers. To develop this platform, the team is integrating fluorescence, Raman, and quantitative-phase imaging modalities that can specifically image plant roots with subcellular resolution that are otherwise opaque due to scattering. Subsequently, the team will apply this platform to improve fundamental understanding of the symbiosis between plants and mycorrhizal fungi by independently quantifying the development of each symbiont, determining the underlying nutrient exchange rates, and localizing key metabolic pathways.

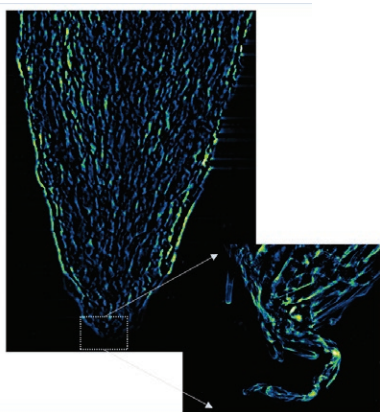
Since its beginning in September 2021, this project has focused on translating the team's previous work in single-cell imaging (Vasdekis et al. 2019) to multiply scattering *Medicago truncatula* root segments. In this context, the team has adapted quantitative-phase imaging, a method that quantifies the dry mass of single cells (see figure panel A) in a label-free fashion to plant root imaging (see figure panel B). Researchers are also developing light-sheet microscopy that integrates tailored optical beams that can penetrate deep into tissue with image acquisition and reconstruction that is congruent with the particle nature of light. Using the latter, the team has accelerated Raman bioimaging by more than 1000-fold beyond state-of-the-art approaches, enabling them to quantify the metabolic activity of individual cells at video rates (Dunn et al. 2023). Here, researchers will detail these imaging approaches, how they have enabled unmasking key insights into cellular growth and metabolism (Nemati et al. 2022), and how the team plans to apply them to quantify the mutualistic association between mycorrhizal fungi and bioenergy crops. While these associations are known to supply crops with nutrients and accelerate atmospheric CO_2 sequestration, the optical microscope developed in this project is expected to offer quantitative understanding of these associations.

Funding Statement: This research was supported by the DOE Office of Science, Biological and Environmental Research program, grant no. DE-SC0022282.

A.

Imaging the Rhizosphere with Subcellular Resolution. Quantitative-phase imaging of a growing *Escherichia coli* microcolony that started from a single cell; cells grow strictly in 1D as uniquely enabled by an invisible microfluidic array. [Reused under a Creative Commons license (CC By 4.0) from Nemati, S., et al. 2022. "Density Fluctuations, Homeostasis, and Reproduction Effects in Bacteria," *Communications Biology* **5**, 397.]

B.



Label-Free Quantitative-Phase Imaging For Plant Roots. Preliminary interferometric image of a *Medicago truncatula* root tip; inset displays a higher magnification of the root tip enabling root hair visualization. [Courtesy University of Idaho]

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*Presenting Author

Imaging the Rhizosphere Using Synchrotron Techniques

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Plant-microbe interactions in the soil shape the chemical, physical, and biological characteristics of the rhizosphere. The efficient exchange of carbon and nutrients between plants and microbial partners in the rhizosphere fuels the development and health of entire ecosystems and has large-scale impacts on nutrient cycling and climate. Studying nutrient partitioning *in situ* has been challenging due to the complex nature of the rhizosphere and the difficulty to quantify nutrient abundance and speciation at multiple length scales. Modern synchrotrons, like the National Synchrotron Light Source II, offer a suite of multiscale and multimodal imaging techniques that can be combined to investigate heterogeneous natural systems. Synchrotron-based techniques including X-ray fluorescence microscopy and Fourier transform infrared microspectroscopy are well-suited for spatially resolved analysis of nutrients in plants, microbes, and the rhizosphere. The team is developing and using synchrotron imaging tools to study carbon/nitrogen (Victor et al. 2017) and trace element (Zhang et al. 2021) cycling in the rhizosphere of mycorrhizal systems.

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*Presenting Author

Revealing the Molecular Universe of Environmental Microbiomes Using Mass Spectrometry Imaging

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Christopher R. Anderton[1]* (PI)

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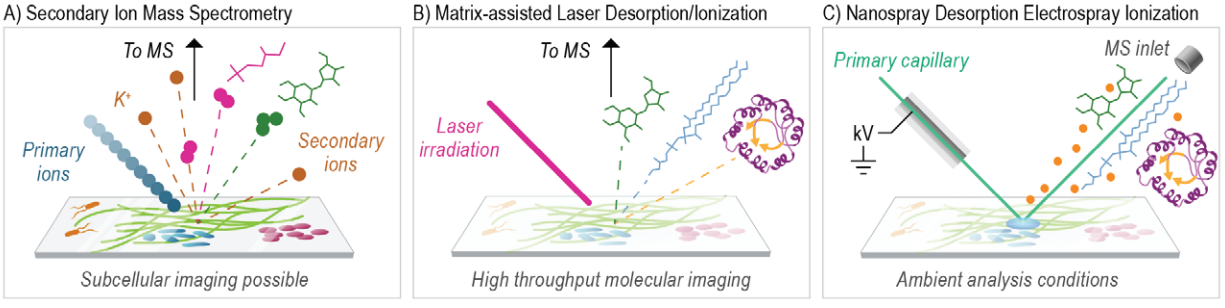
Terrestrial systems and environmental microbiomes represent complex mixtures of interacting species with diverse physiologies and phylogenetic origins, and their functional outcomes are critical to biogeochemical cycles. Little is known about the molecular niches within these systems and exchanges that occur within multi-kingdom systems, where, for example, measuring the molecular transactions among interacting species is a major technical challenge. Within the last decade, the team at the Environmental Molecular Sciences Laboratory has developed novel instrumentation and methods to explore the spatial metallome, metabolome, lipidome, and N-glycome of environmental samples ranging from plant tissue to microbial communities. Research included elucidating their interkingdom interactions, helping reveal molecular processes responsible for regulating global biogeochemical cycles.

Specifically, researchers developed and utilized several spatially resolved mass spectrometry (MS) and mass spectrometry imaging (MSI) approaches, including secondary ion mass spectrometry (SIMS), matrix-assisted laser desorption/ionization (MALDI), laser-ablation electrospray ionization (LAESI), nanospray desorption electrospray ionization (nano-DESI), and liquid extraction surface analysis (LESA) to explore a variety of plant and microbial systems. In some cases, these ionization sources were coupled to unique ultrahigh mass-resolution mass spectrometers (e.g., 21 Tesla Fourier-transform ion cyclotron mass spectrometer) for high-confidence molecular formula annotations. In other cases, researchers utilized an ultrahigh-resolution pre-mass analysis ion mobility mass spectrometer for confident identification and localization of isomeric compounds. In many examples, the team used optical microscopy methods and correlative analysis with spatial mass spectrometry and MSI approaches to link molecular information with sample localization and identify the cellular origin of detected molecules.

Using MALDI-MSI, the team determined the molecular location of key metabolites and lipids within multiple plant-based interkingdom consortia and microbial microbiomes. For example, researchers demonstrated how metabolic asymmetry exists within specialized soybean root organs (i.e., nodules) as a function of the plant's symbiosis with soil bacteria capable of fixing nitrogen. Recently, the team developed a protocol to explore the spatial N-glycome of soybean root nodules using enzyme-assisted MALDI-MSI, where researchers observed changes in the N-glycome as a function of altered biological nitrogen fixation ability. Coupling MALDI with LESA, researchers were able to measure the changing disaccharide profiles within a *Sphagnum* (peat moss) microbiome obtained from the DOE Spruce and Peatlands Under Changing Environments (SPRUCE) site. The team also used this approach to explore the molecular heterogeneity of *Bacillus* biofilms in 3D. Using SIMS, researchers were able to determine how key micronutrients are acquired from environmental sinks and biotically redistributed across soil and microbial microenvironments. The use of ambient



Imaging the Molecular Universe. Mass spectrometry imaging approaches developed and employed within the Environmental Molecular Sciences Laboratory user program to explore the spatial metallome, metabolome, lipidome, and N-glycome of environmental samples ranging from plant tissue to microbial communities. [Courtesy Pacific Northwest National Laboratory]

*Presenting Author

ionization liquid extraction-based MSI methods (i.e., LESA, nano-DESI) afforded the ability to measure agar-based microbial interactions under native conditions. LAESI-MSI allowed researchers to molecularly profile native plant tissue, and this technique has shown promise in high-throughput spatial metabolomics of living plants down to the single-cell level. The team is currently developing new sampling methods to temporally map the metabolome and lipidome of rhizospheres and soil microbiomes in field-like settings with MSI.

Reduced Complexity Synthetic Soil Habitats Facilitate Multimodal Imaging of Soil Ecosystem Processes

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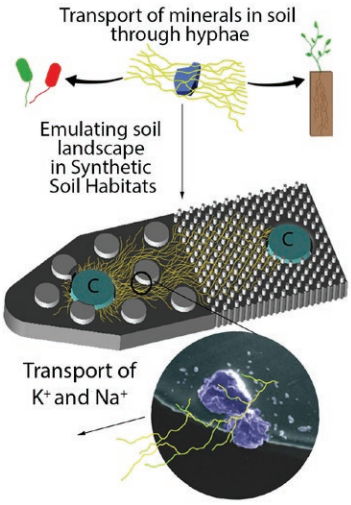
[1]Pacific Northwest National Laboratory, Richland, WA; [2]Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, Menlo Park, CA

Microbial community and plant development in soils depends on access to spatially disconnected organic and inorganic nutrients. Understanding the spatial relationship between soil nutrient hot spots and microbial and plant growth requires methods that can spatially visualize these processes. However, soils are highly heterogeneous and opaque and thus a challenging medium for spatial and *in situ* characterization. Researchers have developed synthetic soil habitats that replicate specific physical and chemical properties of soils, reducing the complexity of analysis for soil-driven processes. These habitats support plant and microbial growth while enabling the use of mass spectrometry and spectroscopic imaging methods to characterize processes in a soil-like environment.

The synthetic habitats are manufactured from a UV-curable resin with indium tin oxide–coated glass backing using a combination of Bosch etching and soft lithography techniques. The soil habitats can be custom built to reproduce the heterogeneous pore size distribution of soil columns, including replication of soil pores and soil microaggregates. The team can amend the soil habitats with soil minerals such as potassium (K)-feldspar, hematite, and kaolinite to study the effects of mineralogy on microbial and plant growth. Researchers have demonstrated compatibility of these polymer-glass-based habitat platforms with mass spectrometry imaging techniques, X-ray photoelectron spectroscopy, scanning electron microscopy/energy dispersive X-ray analysis, and synchrotron-based techniques such as X-ray fluorescence (XRF), and X-ray absorption near-edge structure spectroscopy (XANES).

Here, K-feldspar–amended habitats were fabricated for observing fungal-driven weathering and extraction of K from K-feldspar minerals. K is a macronutrient essential for mitigating drought in plants. The team observed that the soil fungus, *Fusarium Sp. DS 682,* weathered K-feldspar minerals to extract and transport K through fungal hyphae in a drought-like environment within a habitat platform. Fungi grown within the K-feldspar–amended, water-stressed habitats bridged carbon hot spots only in the presence of K-felspar mineral. This observation was based on the combination of optical microscopy used for optimization of fungal growth and biomass distribution with chemical imaging of fungal exudates. Using mass spectrometry imaging, researchers observed that the fungi exuded a range of organic acids for K weathering and extraction from K-feldspar, such as citric, malic, tartaric, and fumaric acid. While the exuded acids demonstrate a distance-dependent exudation from the carbon-rich nutrient source, the distribution of the K-chelated acids within the hyphae was uniform. For example, XRF imaging and XANES spectra of fungal biomass demonstrated K complexed with tartaric acid even in the regions where no tartaric acid exudation was observed. These results suggest that the K complexed with the organic acids and was transported through hyphae, perhaps being stored for future use. Uptake of mineral-derived K is an important biogeochemical process that influences microbial and plant growth in soil. These reduced-complexity synthetic soil habitats facilitate investigation of similar complex soil processes such as nutrient cycling and organic matter-mineral interaction through multimodal imaging technologies.



Synthetic Soil Habitats. Schematic of a soil micromodel, which emulates natural soil pore spacing and mineralogy. Fungal growth in a water- and nutrient-limited environment is enhanced in mineral-doped micromodels due to the extraction of K^+ and Na^+ from minerals through mycelia. The PDA plugs—C1 and C2—are the only nutrient sources present in the micromodel, and the fungus was inoculated onto C1. [Reused under a Creative Commons license (CC By 4.0 International) from Bhattacharjee, A., et al. 2002. "A Mineral-Doped Micromodel Platform Demonstrates Fungal Bridging of Carbon Hot Spots and Hyphal Transport of Mineral-Derived Nutrients," *mSystems* **7**(6), e0091322.]

*Presenting Author

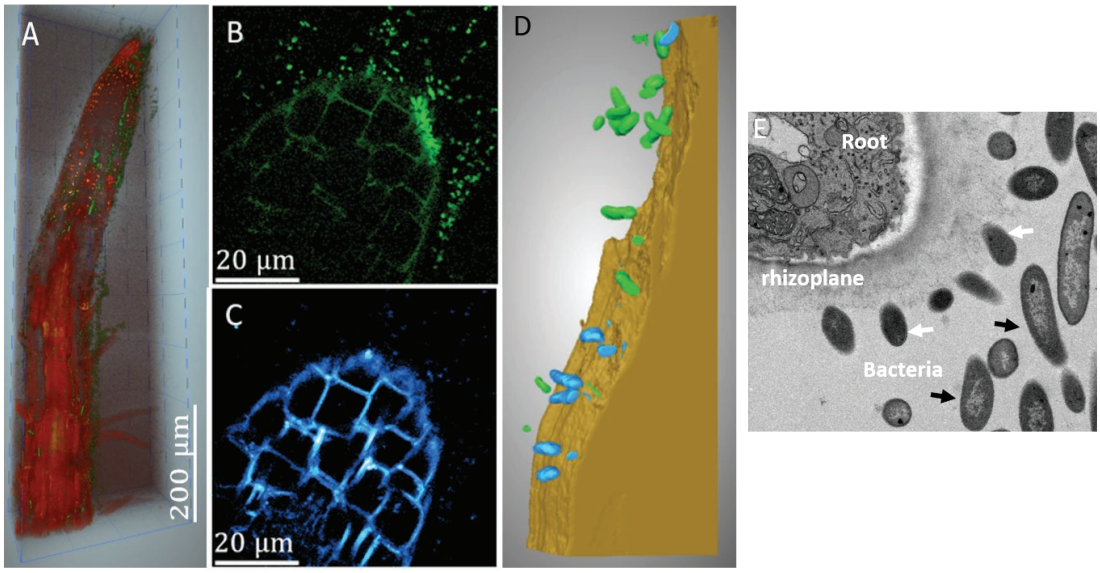
Multimodal Imaging for Root-Microbe Visualization

Nathalie Elisabeth* (PI)

Lawrence Berkeley National Laboratory, Berkeley, CA

The transition from fossil fuel to biofuel requires improved feedstock productivity. Plant-microbe interactions, specifically those occurring in and around the root system (i.e., the rhizosphere) are essential for plant health, as some beneficial soil microbes can supply plants with growth-promoting substances and help mitigate biotic or abiotic stress.

The use of engineered rhizobacteria is a promising tool for sustainable agriculture of food and biofuel crops. Therefore, exploring the mechanisms by which plant growth-promoting bacteria (PGPB) interact with their host plants is essential to understanding and controlling soil microbial communities. Imaging these interactions across scales is key to resolving the interplay across its entire organization. Using *Arabidopsis thaliana* and a mutant strain of the rhizobacteria *Pseudomonas simiae* expressing indole-3-acetic acid (IAA) phytohormone as well as a wild-type control, the team developed a novel multimodal imaging workflow for mapping engineered microbes in the root environment, from the overall root architecture down to the single-cell level. Confocal laser scanning microscopy (CLSM), 3-photon fluorescence (3P), and third harmonic generation (THG) were used to visualize the distribution of *P. simiae* mutants in the rhizosphere of *A. thaliana* and pinpoint regions of interest. Targeted areas with clonal and mixed bacterial populations were identified and biopsied for ultrastructural analyses using focus ion beam scanning electron microscopy (FIB-SEM) as well as transmission electron microscopy (TEM). Hard X-ray tomography (HXT) was used as an intermediate step for easier correlation between light and electron microscopy. For the first time, CLSM, 3P, THG, HXT, FIB-SEM and TEM were successfully used together to precisely document the distribution of engineered rhizobacteria in the *Arabidopsis* root system. 2D and 3D structural and ultrastructure analyses showed successful root colonization and provided data on mutant bacteria location. Engineered microbes under the influence of root exudates were swimming and dividing. They eventually settled on the rhizoplane, establishing tight interactions through physical contact with the root. The correlative bioimaging workflow presented here will help advance understanding of root-microbe interactions, driving progress toward a well-managed use of PGPB as food and biofuel crop inoculants.



***Arabidopsis* Root Colonized by GFP-Labeled *Pseudomonas simiae*. (A)** 3D rendered z-stack images of a root colonized by fluorescent bacteria (green) imaged with CLSM. **(B,C)** Multiphoton microscopy showing root autofluorescence surrounded by fluorescent bacteria (B, green) Power = 1.5 MW. Third Harmonic Generation showing plant cell wall and organelles (C, cyan). **(D)** 3D rendering of segmented bacteria from FIB-SEM volume showing bacteria physically attached (blue) and not attached (green) to the root (brown); **(E)** TEM image of root cells surrounded by bacteria with direct contact to root mucilage (white arrows) and more distant bacteria (black arrows). [Courtesy University of California–Berkeley]

*Presenting Author

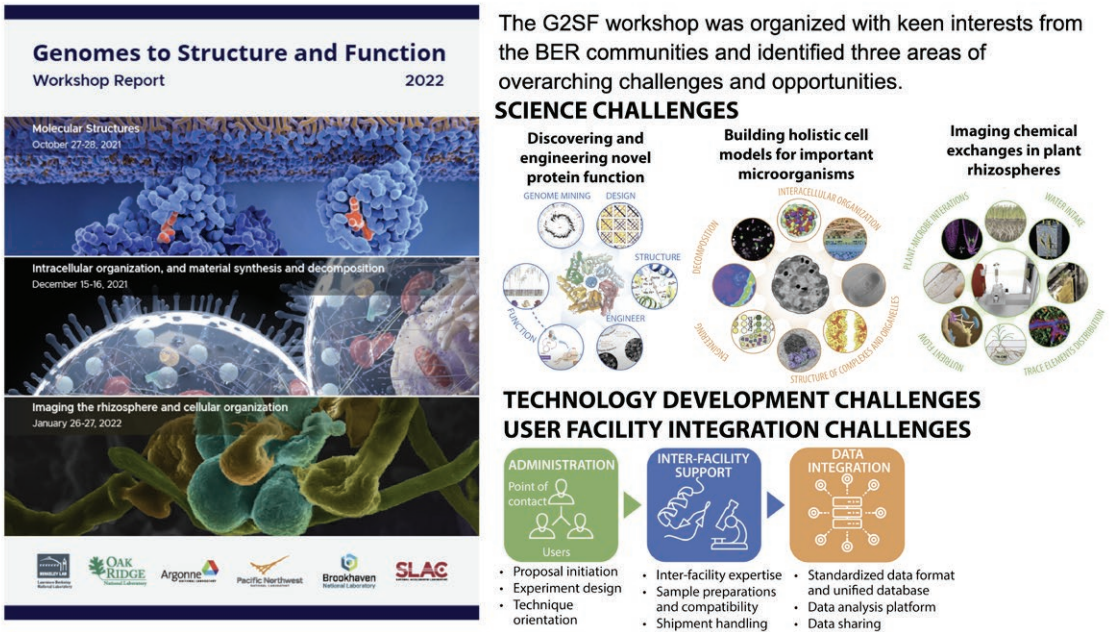
Emerging Topics and Technologies

Interfacility Collaboration: Overarching Challenges and Opportunities Identified Through the "Genomes to Structure and Function" Virtual Workshop

Yasuo Yoshikuni* (PI)

U.S. DOE Joint Genome Institute, Lawrence Berkeley National Laboratory, Berkeley, CA

The goal of BER is to achieve a predictive understanding of complex biological, Earth, and environmental systems with the aim of advancing the nation's energy and infrastructure security. To pursue this goal, collaborations among experts in diverse research areas that lead to multidisciplinary projects are indispensable. The roles of DOE's user facilities, which offer unique and powerful resources for such research projects, are evolving, and expectations for the facilities are increasing. To respond to users' needs, the Joint Genome Institute (JGI) and Environmental Molecular Sciences Laboratory (EMSL) initiated the Facilities Integrating Collaborations for User Science (FICUS) program in 2014. This collaboration has grown into a successful program, advancing more than 100 multidisciplinary projects to date. Similarly, the new interfacility collaborations among the JGI, EMSL, and user resources for BER structural biology and imaging at the DOE Basic Energy Sciences program's synchrotron and neutron facilities are becoming essential for cutting-edge transdisciplinary science. To explore the need for the BER research community to combine genomic, functional, and structural approaches to advance their research, the team hosted the Genomes to Structure and Function virtual workshop, focusing on molecular structures, intracellular organization, material synthesis and decomposition, rhizosphere imaging, and cellular organization. This workshop identified three major overarching challenges and opportunities: science, technology development, and interfacility integration. A report summarizing these findings was recently completed: wp.me/pf6Jq0-4XH.



Genomes to Structure and Function. To further explore the need for the BER research community to combine genomic, functional, and structural approaches to advance their research, a committee representing seven DOE national laboratory user facilities held a workshop to identify challenges and opportunities. [Courtesy Lawrence Berkeley National Laboratory]

*Presenting Author

eBERlight—A User Program for Biological and Environmental Research at the Advanced Photon Source

Zou Finfrock[1], Alex Lavens[1], Krzysztof Lazarski[1], Changsoo Chang[1], Jerzy Osipiuk[1], Gyorgy Babnigg[1], Youngchang Kim[1], Kenneth Kemner[1], Andrzej Joachimiak[1,2], **Karolina Michalska[1]*** (kmichalska@anl.gov, PI)

[1]Argonne National Laboratory, Lemont, IL; [2]University of Chicago, Chicago, IL

Light sources provide a wide range of X-ray–based tools for research pertinent to the DOE Office of Science BER program's mission. Over the past 25 years, the Advanced Photon Source (APS) at Argonne National Laboratory has been at the forefront of research in biological, geological, geochemical and environmental sciences. The ongoing generational upgrade of the APS facility will offer transformative opportunities for the BER community to address scientific challenges. After completion in 2024, the APS Upgrade (APS-U) will become the nation's brightest high-energy, storage ring–based X-ray source, delivering X-rays that will be 500 times brighter than they are today. The APS-U will allow researchers to study samples at higher resolutions and unprecedented spatial and temporal scales. The combination of macromolecular crystallography, X-ray fluorescence microscopy, tomography, absorption spectroscopy, and small/wide angle X-ray scattering will enable visualization of biological and environmental samples at scales ranging from angstroms to centimeters and timescales from picoseconds to seconds. With the X-ray source's high brightness, investigation of the dynamics of biological processes will be achievable. In addition to the extraordinary spatial resolution across a large field of view, high-throughput and multimodal data collection will provide unprecedented statistical analysis of complex biological and environmental systems, allowing scientists to address their enormous heterogeneity. To maximize APS-U impact on BER science, the eBERlight program is being developed to specifically support the user community pursuing research within the BER mission. eBERlight is expected to allocate beamtime, facilitate and coordinate access, and support its users along their entire interaction with the APS—helping with the project and proposal development, design of the experimental workflow, sample preparation, data collection, and analysis. To ensure an optimal infrastructure for the one-stop portal, the program will also leverage additional Argonne National Laboratory resources for sample preparation and data analysis.

Funding Statement: Argonne National Laboratory is operated by UChicago Argonne, LLC for the U.S. Department of Energy under Contract No. DE-AC02-06CH11357.

eBERlight User Program. eBERlight will develop an integrated research platform and comprehensive user support for BER-relevant research at the DOE Advanced Photon Source. [Courtesy Argonne National Laboratory]

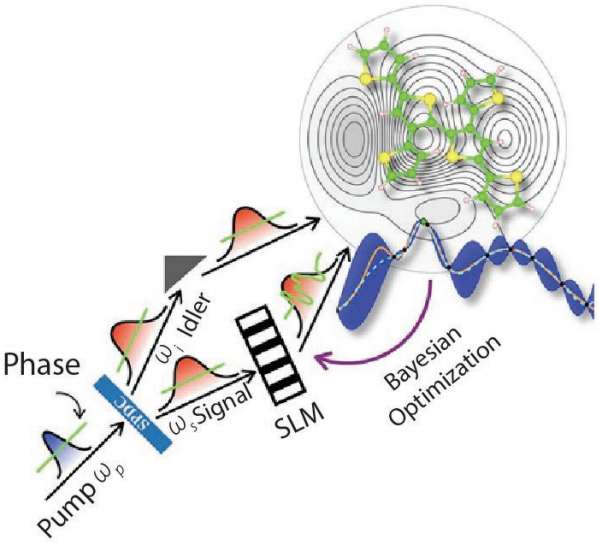
*Presenting Author

Emerging Technologies: Quantum Imaging

Theodore Goodson III* (tgoodson@umich.edu, PI)

University of Michigan, Ann Arbor, MI

The organization and function of microbial communities in the plant rhizosphere are influenced by interactions among organisms within a complex and dynamic physical and chemical environment. These environments, which include growing plants and microbial communities, complex metabolites, and nutrient gradients, cannot be measured in a noninvasive and nondestructive manner using current bioimaging technologies. Imaging with quantum light offers the opportunity to realize long-term nondestructive imaging investigations of new species in the plant-microbial system. Both optical and magnetic quantum sensing approaches can potentially be used to study rhizosphere systems. From ultrasensitive magnetometers to entangled photon microscopy, researchers are using quantum approaches to explore environmental biology-relevant science questions and examining potential paths forward for these technologies.



Transition amplitude to the two-photon state

$$T_f = \sum_m D_m \int_{-\infty}^{\infty} dt_2 \int_{-\infty}^{t_2} dt_1 e^{i\omega_{fm}t_2} e^{i\omega_{mg}t_1} \langle 0,0|\hat{E}_1(t_1)\hat{E}_2(t_2)|\Phi\rangle + (1 \leftrightarrow 2)$$

$$\hat{E}_1(t_1) = \sqrt{\frac{1}{cA}} \int_0^{\infty} d\omega_1 \sqrt{\omega_1}\ \hat{a}_1(\omega_1) e^{-i[\omega_1 t_1 + \phi(\omega_1)]}$$

$$\hat{E}_2(t_2) = \sqrt{\frac{1}{cA}} \int_0^{\infty} d\omega_2 \sqrt{\omega_2}\ \hat{a}_2(\omega_2) e^{-i\omega_2 t_2}$$

Spectral Phase Modeling for Two-Photon Absorption of Molecules. Scheme for optimizing phase properties of pulse shaper for entangled two-photon absorption using a Bayesian algorithm. [Reprinted with permission from Giri, S. K., and G. Schatz. 2022. "Manipulating Two-Photon Absorption of Molecules Through Efficient Optimization of Entangled Light," *The Journal of Physical Chemistry Letters* **13**(43), 10140–46. ©2022 American Chemical Society.]

*Presenting Author

Single-Cell and Spatial Omics of BER-Relevant Systems Using a Nanodroplet Processing and Advanced Mass Spectrometry Approaches

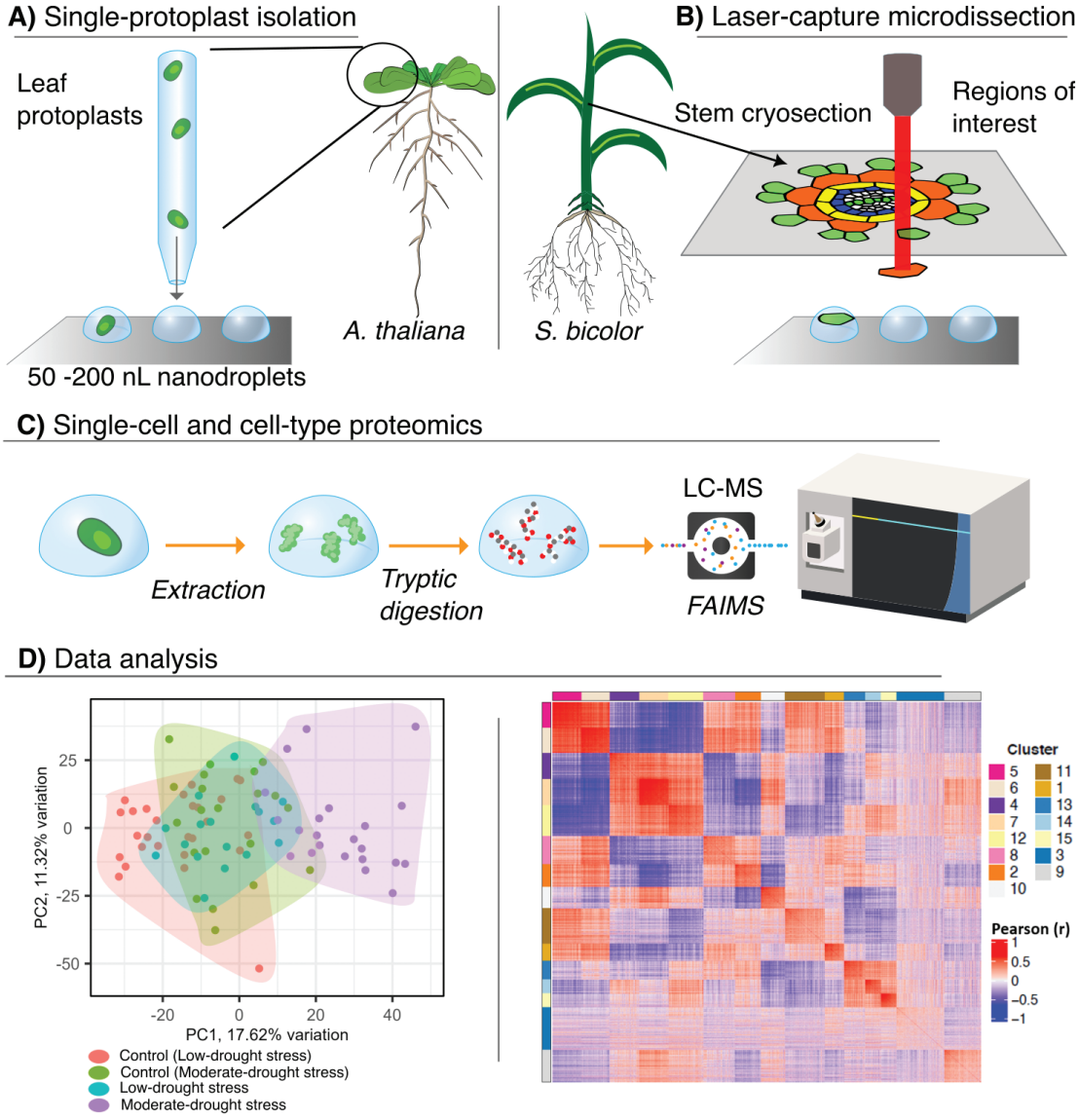
James M. Fulcher[1], Sarah M. Williams[1], William Chrisler[1], Liyu Andrey[1], Lye Meng Markille[1], Vimal Kumar Balasubramanian[1], Jaeho Song[2], Gary Stacey[2], Amy Marshall-Colon[3], John Mullet[4], Amirhossein H. Ahkami[1], Ying Zhu[5], **Ljiljana Paša-Tolić[1]*** (PI)

[1]Pacific Northwest National Laboratory, Richland, WA; [2]University of Missouri, Columbia, MO; [3]University of Illinois, Urbana, IL; [4]Texas A&M University, College Station, TX; [5]Genentech Inc., South San Francisco, CA

Single-cell and spatial molecular profiling (omics) provides invaluable information on structural organization and cell-to-cell interactions in native tissues, organs, and communities. While sequencing-based technologies have revolutionized understanding of single-cell heterogeneity, they only provide a partial view of a cell's phenotype. Proteins are of particular interest in establishing cellular identities because they are the primary effectors of biological function, and their modification state and abundance cannot be inferred from mRNA measurements. Yet, measuring proteins in small tissue regions or single cells remains a major challenge and key analytical objective. Furthermore, current approaches for probing spatial distribution of the proteome typically rely on antibodies, which limit multiplexing and require *a priori* knowledge of protein targets. To address these challenges, the team developed nanodroplet processing in one pot for trace samples (nanoPOTS), integrated it with laser-capture microdissection and/or single-cell sorting, and applied it to region-specific and single-cell plant proteomics. Researchers demonstrate the approach in studying developmental and tissue-region changes in *Sorghum bicolor* and rhizobial-legume symbiote processes, as well as drought-induced impacts on single protoplasts from *Arabidopsis*. This approach was further extended to global profiling of the transcriptome and proteome from same single cells. Multimodal measurements were enabled by a microfluidics workflow, (nanoSPLITS: nanodroplet splitting for linked multiomic investigations of trace samples), which allows for dividing nanodroplets containing single-cell lysate onto two arrays for downstream transcriptomics and proteomics. The team identified expected drought-stress-related proteins in single *Arabidopsis* protoplasts, providing validation of this novel single-cell multiomics approach.

Notably, researchers could also characterize clusters of covarying proteins, some of which were related to drought response while others have not yet been described. These highly correlated protein clusters provided new insights into potential protein complexes or interactors, and several were validated at the mRNA level using the Klepikova Atlas, which contains high-resolution spatiotemporal sequencing data for *Arabidopsis*. Together, these novel spatial and single-cell omics technologies provide new insights into the systems biology of plants and microbes, facilitating their use for achieving energy independence and clean energy.

*Presenting Author



Nanodroplet Processing in One Pot for Trace Samples (nanoPOTS) Platform. **(A)** Isolation of single protoplasts from an *Arabidopsis* leaf to study drought impacts at single-cellular resolution. **(B)** Cryosectioning and laser-capture microdissection of *Sorghum* stem for cell-type-specific proteomics. **(C)** Steps performed with the nanoPOTS platform including protein extraction and tryptic digestion followed by liquid chromatography-mass spectrometry analysis of the tryptic peptides. **(D)** Data analysis of single-cell and cell-type-specific proteomes. PCA analysis of drought-impacted single leaf protoplasts (left panel) and protein covariation correlation matrix across *Sorghum* cell types (right panel). [Courtesy Pacific Northwest National Laboratory]

Appendix A: Detailed Agenda

Biomolecular Characterization and Imaging Science (BCIS) Principal Investigators Meeting

April 17–19, 2023

Bethesda, MD

This meeting expanded on past Bioimaging Science Program Principal Investigators meetings to encompass the U.S. Department of Energy (DOE) Biological and Environmental Research Program's (BER) Structural Biology Program, which supports access to DOE user facilities for studies in structural biology. This meeting highlighted new multidisciplinary collaborations among researchers from adjacent Biological Systems Science Division programmatic areas.

Plenary Session 1: National Laboratory Quantum Imaging 2020 Projects

During this plenary session, several advancements in quantum-enabled bioimaging and sensing were presented, all of which shared the common goal of overcoming challenges related to signal sensitivity or photobleaching damage.

Probing Photoreception with New Quantum-Enabled Imaging

- *James Evans (Pacific Northwest National Laboratory)*

This project focuses on the development of quantum entanglement, coincidence detection, ghost imaging, quantum phase-contrast microscopy, and multidimensional nonlinear coherent microscopy techniques. These developments will aid in achieving super-resolution analysis with less energy hitting the sample, thus empowering better probing of light-triggered biology.

The 3DQ Microscope

- *Shervin Kiannejad, representing Ted Laurence (Lawrence Livermore National Laboratory)*

This novel system uses quantum entangled light and correlation measurements to develop three-dimensional quantum fluorescence lifetime-based imaging for volumetric fluorescence and scattering analysis of bioenergy specimens.

A Quantum Enhanced X-Ray Microscope

- *Sean McSweeney (Brookhaven National Laboratory)*

This project aims to significantly advance the field of X-ray microscopy ghost imaging based at the National Synchrotron Light Source II. This research offers potential groundbreaking implications for studying the elemental distribution and complex chemistry within biological and environmental samples with minimal dose.

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

- *James Werner (Los Alamos National Laboratory)*

This project focuses on using quantum techniques to monitor plant health with a unique time-resolved single-photon-counting imaging detector that enables measuring coincidence photon events with an order of magnitude better timing resolution.

Next-Generation Stimulated Raman Scattering Microscopy

- *Bryon Donohoe (National Renewable Energy Laboratory)*

This technology aims to overcome current limitations in photodamage and resolution for label-free imaging by using squeezed light to enhance real-time tracking of lipid/carbohydrate interfaces in plants, algae, and fungi.

Plenary Session 2: University Quantum Sensing Approaches for Bioenergy 2022 Projects

Five university projects explore innovative quantum sensing approaches for biomolecular sensing in bioenergy.

Quantum Diamond EcoFAB Microscope or *In Situ* Nuclear Magnetic Resonance (NMR) Root Exudate Molecules

- *Ashok Ajoy (University of California–Berkeley)*

This project explores novel approaches to studying root exudates using advanced quantum sensing with NMR and ^{13}C diamond nanoparticles.

Noninvasive Imaging of Nitrogen Assimilation in the Rhizosphere via Quantum-Entangled Hyperpolarized Spin States

- *Thomas Theis (North Carolina State University)*

This noninvasive research could provide new insights into currently undetectable metabolic transformation occurring in the rhizosphere.

Quantum-Enabled Membrane Potential Imaging of DOE-Relevant Bacterial Communities

- *Debjit Roy, representing Shimon Weiss (University of California–Los Angeles)*

This project aims to create a better understanding of bacterial behavior and interactions in energy-related contexts.

Quantum Imaging for Metabolic Pathways and Hydrocarbon Production *In Planta*

- *Joshua Yuan (Washington University in St. Louis)*

This project aims to revolutionize understanding of metabolic pathways and hydrocarbon production in plants, which is critical for bioenergy development.

Quantum Optical Microscopy of Biomolecules Near Interfaces and Surfaces

- *Mikael Backlund (University of Illinois–Urbana-Champaign)*

This project focuses on adapting quantum optical techniques to track and image individual, and few, fluorescently labeled biomolecules in action at biological surfaces, such as what occurs during cellulosic breakdown by cellulases.

Plenary Session 3: 2023 Structural Biology and Imaging User Resources I

This session highlighted significant advancements and tools in the fields of biomolecular characterization, structural biology, and imaging available at DOE user facilities. The resources are primarily focused on understanding the structure and function of biological macromolecules alone and within their native cellular environment.

Rapid Characterization of Macromolecular Solution Structure for BER Projects

- *Greg L. Hura (Lawrence Berkeley National Laboratory)*

This X-ray scattering technique contributes significantly to understanding macromolecular structures in solution. Project staff work with BER researchers to characterize cellular and biological materials including lipids, cell walls, and cellulose.

Understanding Metal Binding by Metallochaperones Using Synchrotron X-Ray Spectroscopy Methods

- *Ritimutka Sarangi (SLAC National Accelerator Laboratory)*

This capability allows for the exploration of the intricate mechanisms of metal binding in biological systems, which could have wide-reaching implications for bioenergy and environmental research.

A Disordered Plant Microtubule-Associated Protein Reorganizes Microtubules

- *Hugh O'Neill (Oak Ridge National Laboratory)*

This underlying neutron-based capability empowers visualizing structure and dynamics of deuterium-labeled biomacromolecules and can offer new insights into plant biology and bioenergy.

Laboratory for BioMolecular Structure Cryo-EM Facility

- *Liguo Wang (Brookhaven National Laboratory)*

This facility provides access to cryo-electron microscopy capabilities for DOE researchers. Located adjacent to the National Synchrotron Light Source II, the facility provides direct synergy and multimodal access to X-ray synchrotron instrumentation for structural biology applications.

Structural Biology Center (SBC) at Sector 19 of the Advanced Photon Source

- *Andrzej Joachimiak (Argonne National Laboratory)*

For nearly 27 years, this user resource provided protein expression and purification for X-ray macromolecular crystallography to elucidate atomic-level, three-dimensional structures. SBC research focused on proteins relevant to BER missions, especially plant, fungal, and bacterial proteins, and on improving hardware and software for data collection at synchrotron beamlines. It also served the broader biological community with impressive productivity. SBC has closed operations to make way for a new resource called eBERlight, which will be a virtual collaborative access team for the BER community.

Plenary Session 4: 2023 Structural Biology and Imaging User Resources II

This session continued the focus on structural biology and imaging resources at user facilities.

Soft X-Ray Tomography at the Advanced Light Source

- *Carolyn Larabell (Lawrence Berkeley National Laboratory)*

This soft X-ray tomography beamline is key to understanding cellular morphology and internal architecture of intact biosystems at ~30 nm spatial resolution and in a near-native state. It permits direct annotation of visualized features based on calibrated linear absorption coefficients, which enables comparison of morphologies between different strains, environmental conditions, or time-points to identify and track morphological dynamics.

Imaging Across Scales: News from the Center for BioMolecular Structure at NSLS-II

- *Sean McSweeney (Brookhaven National Laboratory)*

This talk highlighted the advanced imaging capabilities at NSLS-II and its many applications to biological research including biophysical, chemical, and medical sciences; cell and tissue analysis; plant science; and environmental chemistry.

Advances to Accelerate Biosystems Characterization at the Berkeley Synchrotron Infrared Structural Biology (BSISB) Imaging Program

- *Hoi-Ying N. Holman (Lawrence Berkeley National Laboratory)*

BSISB infrared spectroscopy capabilities empower studying the structure and intra- and intermolecular interactions of biological macromolecules in a label-free and nondestructive manner.

Opportunities Linking Omics and Structural Biology at Pacific Northwest National Laboratory: Excelling at Cryo-EM

- *James Evans (Pacific Northwest National Laboratory)*

This cryo-EM capability with workflows for small molecule, protein, and whole-cell structural analysis is available either via any of the current Environmental Molecular Sciences Laboratory user calls, or through a separate DOE BER allocation. Users can link cryo-EM with cell-free expression and other analysis modalities, including native mass spectrometry, to yield more holistic analysis of their samples.

Breaking the Size Barrier for Cryo-EM of Small Proteins

• *Todd O. Yeates (University of California–Los Angeles)*

Although not within a national laboratory–based user facility, this project aims to extend the near-atomic resolution capabilities of cryo-EM to smaller proteins through the development of scaffolding approaches. This work could open new avenues in structural biology at UCLA and would be extensible to user research occurring at the Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Brookhaven National Laboratory, SLAC National Accelerator Laboratory, or other user facilities with cryo-EM.

Biomass Pretreatment and Structural Changes Shown from Scattering and Modeling

• *Brian Davison (Oak Ridge National Laboratory)*

This Science Focus Area work uses neutron scattering to help understand how solvents alter the structures of plant cell walls and microbial membranes. The goal of the research is to enhance the efficiency of lignocellulosic biofuels production by understanding the molecular-level mechanisms of biomass deconstruction.

Plenary Session 5: National Laboratory Bioimaging 2022 Projects

There were four national laboratory bioimaging projects that started in 2022. These cutting-edge projects entailed new approaches combining multimodal correlative methods, the incorporation of novel probes to obtain functional imaging, and/or application of emerging imaging modalities to *in situ* measurements to follow metabolic processes occurring in plant and microbial communities.

Developing a High-Throughput Functional Bioimaging Capability for Rhizosphere Interactions

• *Gyorgy Babnigg (Argonne National Laboratory)*

This project focuses on using advanced imaging technologies to study and accelerate characterization of plant-microbe interactions utilizing sensor cells, microfluidics, automation, and AI-guided analyses. By utilizing the sense-and-respond capabilities of plant growth-promoting rhizobacteria, chemical exchanges between microbes and plant roots can be tracked and combined with orthogonal X-ray based elemental analysis to study the complex dynamic interactions and spatial patterns that contribute to plant health and productivity.

Visualizing Spatial and Temporal Responses of Plant Cells to the Environment

• *Peter Dahlberg (SLAC National Accelerator Laboratory)*

The aim of this project is to develop new bioimaging tools to understand how plant cells respond to environmental factors, which is vital for optimizing plant growth for bioenergy.

Novel Multimodal Chemical Nano-Imaging Approach

• *Scott Lea, of Pacific Northwest National Laboratory*

This project is developing multimodal linear and nonlinear optical imaging modalities at the nanoscale using near-field approaches for high-resolution *in situ* chemical imaging of metabolites exchanged between microbes in a symbiotic consortium. This nano-optical microscopy would advance understanding of microbial metabolism and communication.

Optical and X-Ray Multimodal-Hybrid Microscope Systems for Imaging of Plant Stress Response and Microbial Interactions

• *Soichi Wakatsuki (SLAC National Accelerator Laboratory)*

This project seeks to combine optical and X-ray imaging to study membrane trafficking and transport, microbial interactions, and plant stress responses including plant–bacterial pathogen interactions at the plant-cell surface.

Plenary Session 6: University Quantum Imaging Approaches 2022 Projects

This session featured the second set of presentations on cutting-edge quantum imaging technologies for bioenergy research led by universities.

Squeezed-Light Multimodal Nonlinear Optical Imaging of Microbes

- *Ralph Jimenez (University of Colorado–Boulder)*

This project aims to develop quantum-enhanced imaging techniques for studying microbial communities, which could revolutionize understanding of microbial processes relevant to bioenergy.

Quantum Mimicry Optical Coherence Tomography for Advancing Plant Science for Bioenergy

- *Chen-Ting Liao (University of Colorado–Boulder)*

This project explores the use of a quantum-inspired, label-free, cross-sectional imaging method that is suited for *in situ* probing of plant biology with low noise and high sensitivity through turbid media.

Establish X-Ray Quantum Imaging for Subcellular Structures

- *Peter Schwander (University of Wisconsin–Milwaukee)*

This project focuses on developing quantum imaging techniques for studying subcellular structures with X-rays, which could provide new insights into cellular processes important for bioenergy.

Mid-Infrared Single-Photon-Counting Photodetectors for Quantum Biosensing

- *Leon Shterengas (State University of New York at Stony Brook)*

This research aims to design and develop new single-photon-counting avalanche photodiodes operating in mid-infrared regions for biosensing applications.

Entanglement Enhanced Quantum Stimulated Raman Spectroscopy Imaging Lab on a Chip

- *Xu Yi (University of Virginia)*

This project aims to integrate quantum enhancement with Raman spectroscopy to unlock new biological details at the molecular level.

Development of a Quantum-Optimal Bioimaging System for Plant-Microbiome Interactions

- *Shaun Burd, representing Mark Kasevich (Stanford University)*

This project focuses on developing quantum-optimal multiphoton-stimulated Raman scattering microscopy for volumetric and chemically specific imaging of microbe-microbe and microbe-plant interactions.

Plenary Session 7: University Imaging Instrumentation and Approaches 2021 Projects

This research focuses on creating a versatile toolbox of new bioimaging instrumentation capabilities. These tools are intended for imaging biological processes within and among cells in living plants and microorganisms. Multimodal multiphoton imaging capabilities featured prominently, enabling deeper penetration to nondestructively resolve cellular structures.

Non-Destructive Three-Dimensional Imaging of Processes in the Rhizosphere Utilizing High Energy Photons

- *Shiva Abbaszadeh (University of California–Santa Cruz)*

This project aims to advance the understanding of the rhizosphere's dynamic processes through innovative imaging techniques.

Deep Chemical Imaging of the Rhizosphere

- *Marcus T. Cicerone (Georgia Institute of Technology)*

This project focuses on exploring chemical processes and interactions in the rhizosphere with advanced imaging methods.

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

- *Na Ji (University of California–Berkeley)*

This research emphasizes enhancing the understanding of plant-microbe interactions through cutting-edge optical microscopy.

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

- *Adam Bowman, representing Mark Kasevich (Stanford University)*

This research explores novel methodologies for studying metabolic pathways.

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

- *Leslie Sieburth (University of Utah)*

This project aims to illuminate metabolic and cellular processes in woody tissues.

Integrative Imaging of Plant Roots During Symbiosis with Mycorrhizal Fungi

- *Andreas E. Vasdekis (University of Idaho)*

This project focuses on the symbiotic relationships between plants and fungi.

Plenary Session 8: Discussion of the BCIS Program Direction

In a roundtable discussion led by plenary session chairs and moderated by James Evans (Pacific Northwest National Laboratory), attendees focused on the scientific opportunities for principal investigators from the BCIS program. The discussion focused on supporting bioenergy and environmental research within BER and identified key biological questions and novel methods for exploring bioenergy and environmental research.

What new opportunities could be developed to promote awareness and collaboration with BER genomics, biology, and environmental research?

- There is a need to increase awareness and collaboration between BCIS and BER genomics, biology, and ecological research.
- More joint sessions between BCIS and Genomic Science program (GSP) PI meetings have been suggested to improve alignment and communication, along with increasing electronic accessibility or hosting multiple BCIS and GSP overview meetings throughout the year for meaningful communication.
- Empower broader invitations to participants from outside BCIS (and GSP) programs to infuse fresh perspectives and insights that could be leveraged outside of DOE to accelerate innovation and technology/method adaptation by a larger community.
- Overcoming communication barriers is essential, especially considering the different focuses of the groups (science-driven versus tool/method-driven). Bridging this gap requires making BCIS capabilities understandable to a broader audience.
- Establish a graduate student fellowship program and a faculty fellowship program to encourage collaboration; various implementation routes were discussed.
- The creation of "SciAlog" bioimaging workshops encouraging GSP and BCIS participants to mix and match their skills and expertise.
- A BCIS project portal, such as BERstructuralbioportal.org, which would serve as a comprehensive resource hub to foster collaboration and increasing visibility and impact.

How can individual investigators in the quantum and bioimaging portfolio leverage resources at the national laboratories and vice versa?

- Collaborative relationships between researchers at user facilities, national laboratories, and academia are vital for individual investigators in quantum and bioimaging to maximizing their resources.
- Organizing summary presentations and matchmaking sessions between university and laboratory PIs can enhance collaborative efforts.
- Encourage collaborative projects by drawing inspiration from large topic-specific models, introducing similar collaborative opportunities, or offering supplements.
- Expanding the Facilities Integrating Collaborations for User Science (FICUS) user program, currently centered at the DOE Joint Genome Institute (JGI) and the Environmental Molecular Sciences Laboratory (EMSL), to also include BCIS-funded facilities that could encourage cross-disciplinary collaboration and draw BCIS experts into GSP symposia.

What is the most effective balance of novel concepts, prototype development, biological validation, and technology translation?

- The BCIS portfolio is extensive in science but generally narrow in technology translation stage. The balance between novel concepts, prototype development, biological validation, and technology translation is crucial.
- The inception of the bioimaging program can reveal the trajectory from concepts to practical applications, thus a retrospective analysis of the BCIS portfolio is needed.
- Funding and time challenges in transitioning new capabilities to broader user applications are acknowledged. Collaborations and continuous funding are essential for technology maturation and wider application.
- Not all projects will make it to the finish line, and it is highlighted that without continued funding for adaptation or tool and method maturation, projects within BCIS may struggle to reach a larger audience.
- Project usefulness may not be readily apparent in many cases at the proof-of-principle stage. Proof-of-principle projects, if still aligned with DOE mission areas, could be renewed with an emphasis on collaborating with a target GSP team where the litmus test for a useful technological advancement is its capability to address a problem that intrigues biologists.
- The importance of technology delivering novel insights in biology is highlighted, and that might not have been possible without unique approaches. Achieving novel biology could be addressed with projects at the intersection of physics and biology.

How do we measure program value to BER biology and BCIS investigators?

- Assessing the value of the BCIS program is challenging due to its multidimensionality and the breadth of science covered.
- Different views exist on what constitutes program value. Some examples include: demonstrating new scientific principles, establishing new technical capabilities, demonstrating tangible biological breakthroughs, commercialization potential, economic impact, and developing work force capabilities.
- There is consensus that tracking the citation history of developed technology and the number of unique users is considered one tangible metric of success. Citation histories of critical advances often include precedents that are far afield than were published years beforehand.
- Considering the human aspect of the program's value is also important, including the development of interdisciplinary expertise and the career opportunities that are created.

Are there scientific gaps or under-represented capabilities in the current portfolio?

- The necessity for integrated software development and establishing standards for evaluating instrument amenability to various biosystems relevant to BER is emphasized.
- Identifying standard samples, strains, and environmental conditions relevant to BER and GSP researchers will promote faster imaging innovations.

- There is a call for robust computational capabilities, not just through supercomputers but through separate clusters accessible to both BCIS and GSP researchers.
- The scarcity of comprehensive databases for images and data relevant to BER biology hinders algorithm and code development. Establishing such databases, particularly for machine learning and AI, is beneficial.
- The maturation of structural biology over the last 30 years provides an illustrative example. The field thrives because it is bolstered by clear standards, consistent integration, and a set of rules that ensure coherence and progress including peer-reviewed journal enforcement of depositing data in public repositories (e.g., Protein Data Bank, Electron Microscopy Data Bank). Consider the marvel of AlphaFold—such a leap would have been inconceivable without the foundational support of the Protein Data Bank, and this was well beyond a single-investigator-level project.

Breakout Session 1: BCIS Technologies for Investigating the Rhizosphere

The goal of this joint BCIS and GSP breakout session was to provide insights into the structure and function of the rhizosphere and its significance in understanding the basic biology of bioenergy and a bioeconomy. The session began with a brief introduction and a high-level overview of how BCIS investigators are developing and employing technologies to evaluate the physical structure, component organisms, and intra- and interspecies communication within the rhizosphere. The overview was followed by presentations highlighting current research for mapping the spatial organization and chemical composition of the rhizosphere with advanced imaging and sensing techniques. These techniques use defined microbial communities of bacteria, fungi, and plant roots along with transparent ecosystem devices or other microfluidic chambers for controlled, quantitative measurement of species interaction and metabolic cooperation in synthetic soils. The presentations also described ongoing investigations that employ multiple correlated approaches to evaluate the interrelationships of roots, microbes, and metabolites in real soils. Additionally, the presentations outlined how advances in quantum microscopy can broaden opportunities into the functional relationship of soil microbiome and supported plants.

A Reproducible and Tunable Synthetic Rhizosphere Microbial Community Enables Quantitative Plant-Microbe Studies

- *Karsten Zengler (University of California–San Diego)*

Native rhizosphere microbial communities are complex and difficult to study reproducibly. This project is focused on the development of a model synthetic community (SynCom) of microorganisms for quantitative studies. The goal is to extend this to the study of microorganisms commonly found in the rhizosphere of grass species to illuminate hard to decipher plant-microbe interactions.

Finding Clarity Through Imaging: Why the Answer May Lie in the Root, Not the Branches

- *Shiva Abbaszadeh (University of California–Santa Cruz)*

The team's goal focuses on developing advanced imaging techniques to understand plant-soil interactions by investigating intact 3D soil samples with a positron emission tomography and computed tomography (e.g., PET and CT) imaging system at high spatial resolution. New detectors being employed can image dynamics of short half-life tracers ($^{11}CO_2$) in conjunction with architectural analysis of the soil-root interface with micro-CT visualization and quantification of nutrient uptake efficiency and soil contamination in intact soils.

Visualizing the Hidden Half: Plant-Microbe Interactions in the Rhizosphere

- *Pubudu P. Handakumbura (Pacific Northwest National Laboratory)*

This work is empowered by RhizoGrid, which is an integrated imaging and spatial multiomics platform to map metabolomic and metagenomic measurements of root structures in thin slices of root and soil samples labeled with ^{11}C and imaged with microfocus X-rays followed by spot sampling of metabolic and microbe niches along root systems.

Biological Imaging using Entangled Photons

• *Theodore Goodson (University of Michigan)*

This project introduces a novel approach to imaging in biological research. Fluorescence microscopy typically requires illumination levels that can produce sample photodamage or fluorophore photobleaching. Quantum approaches with pairs of entangled photons provide a way to irradiate the sample with ultralow intensity to avoid damage while coupled photons are measured without direct interaction with the sample. Upgrades to the entangled photon source as well as aspects of the entangled two-photon microscope will enable observation of genetically encoded fluorescent proteins to investigate the long-term stress response of microbial variants without the prospect of light-induced damage.

Biocompatible Surface Functionalization Architecture for a Diamond Quantum Sensor

• *Peter C. Maurer (University of Chicago)*

This project seeks to engineer quantum sensors to address the challenge of quantum state stability and selectivity for biomolecules. Biochemical selectivity with multilayered coatings and functionalized molecular receptors will enable magnetic resonance spectroscopy with single-molecule sensitivity of receptor-ligand binding events, post-translational protein modification, and the detection of subtle protein conformational changes within living cells.

Plant Root Imaging During Symbiosis with Mycorrhizal Fungi

• *Andreas E. Vasdekis (University of Idaho)*

This project focuses on the symbiotic relationships in plant roots. Advanced optical microscopy techniques are being developed to investigate the root microbe environment at subcellular resolution with deeper tissue penetration. Airy optical beams are less subject to deep-tissue scattering than typical Gaussian beam cross-sections and have been used for video-rate Raman chemical imaging. Combined with genetically encoded biomarkers of host accommodation of symbionts, these methods attain a predictive understanding of biosystems for accelerating atmospheric carbon dioxide sequestration.

Imaging the Rhizosphere Using Synchrotron Techniques

• *Tiffany Victor-Lovelace (Brookhaven National Laboratory)*

Modern synchrotrons offer multiscale and multimodal imaging techniques that can be combined to investigate heterogenous natural systems. Synchrotron-based techniques including X-ray fluorescence microscopy and Fourier transform infrared microspectroscopy are well-suited for spatially resolved analysis of nutrients. This project adapts these capabilities toward the elemental analysis of pine-ectomycorrhizal fungi samples in soil to reveal the redistribution of iron from sand to fungal sheaths and the structural reorganization of surrounding soils.

Revealing the Molecular Universe of Environmental Microbiomes Using Mass Spectrometry Imaging

• *Arunima Bhattacharjee (Pacific Northwest National Laboratory)*

This project explores enabling technology that permits modeling of the chemical and physical characteristics of different soil habitats using transparent resin particles that reproduce pore-size and microaggregate structure. The modeled soil can be doped with minerals such as K-feldspar, hematite, and kaolinite to investigate the effects of mineralogy on microbial and plant growth including visualizing K^+ ion-tartaric acid flux through hyphae. The polymer-glass habitats are also compatible with mass spectrometry imaging techniques, X-ray spectroscopy, energy dispersive X-ray analysis, scanning electron microscopy, and synchrotron-based techniques such as X-ray fluorescence and X-ray absorption.

Reduced-Complexity Synthetic Soil Habitats Facilitate Multimodal Imaging of Soil Ecosystem Processes

• *Christopher R. Anderton (Pacific Northwest National Laboratory)*

To identify the specific molecular niche exchanges within complex terrestrial systems and multikingdom environmental microbiomes, this research develops chemical-specific imaging methods to map the spatial metallome, metabolome, lipidome, and N-glycome of environmental samples ranging from plant tissue to microbial communities. This approach uses spatially resolved mass spectrometry and mass spectrometry imaging instrumentation available at PNNL/EMSL and has been used to measure metabolic and glycomic asymmetry in nitrogen-fixing root nodules.

Multimodal Imaging for Root-Microbe Visualization

• *Nathalie Elisabeth (Lawrence Berkeley National Laboratory)*

Plant growth can be influenced by genomic engineering of rhizobacteria that express phytohormones. This project documents plant interactions with labeled rhizobacteria using multimodal visualization with high resolution optical microscopy followed by biopsy for ultrastructural analyses using focus ion beam scanning electron microscopy as well as transmission electron microscopy and hard X-ray tomography. The multiscale visualization reveals how engineered microbes move along roots and the physical details of final colonization and attachment.

Breakout 2: 2023 Joint Emerging Topics and Technologies

This session concluded the GSP and BCIS PI meeting and focused on forward-focused approaches and tools to address challenges in BER genomic science and molecular function research. It aimed at advancing ongoing research and paving the way for new insights and broadly available tools and capabilities at user facilities.

Inter-Facility collaboration: Genomes to Structure and Function

• *Yasuo Yoshikuni (Lawrence Berkeley National Laboratory)*

This project highlights how challenging research questions are being addressed using advanced tools available through the BER user facilities and resources including JGI, EMSL, experimental stations at the light and neutron beamlines, and cryo-EM facilities. The most powerful access mechanism enables combining multiple techniques through a coordinated arrangement such as the FICUS program, wherein a single user application provides access to multiple user facilities. Additional needs to further optimize such opportunities include increasing throughput with automation that includes robotics and the incorporation of AI and ML, improving administrative aspects, and developing cross-facility standards.

eBERlight: A User Program for Biological and Environmental Research at the Advanced Photon Source

• *Karolina Michalska (Argonne National Laboratory)*

This is an emerging resource at the Advanced Photon Source, which will offer a suite of X-ray techniques to probe targets across BER's programs to characterize phenotypes, 3D structures, and dynamics that can be correlated with omics and biophysical data.

Natural and Synthetic Mobile Genetic Elements in Bacteria*

• *Joe Schoeniger (Sandia National Laboratories)*

Pivoting from describing facilities available to researchers, this presentation details an approach to identify mobile genetic islands and enhance the secure encoding of new elements in bacterial systems that naturally rearrange their genetic material.

Visualizing Rhizosphere Systems with Quantum Light

• *Theodore Goodson (University of Michigan)*

This talk delves into quantum-enabled imaging approaches for imaging and sensing biological systems and emphasized the opportunity to dramatically reduce noise, increase imaging potential, and decrease sample damage by using squeezed light or entangled photons for ghost imaging of biological samples.

Single Cell and Spatial Omics of BER-Relevant Systems Using Nanodroplet Processing and Advanced Mass Spectrometry Approaches

• *Ljiljana Paša-Tolić (Pacific Northwest National Laboratory)*

This presentation provides insight into the value of single-cell omics techniques, which empower spatial profiling of the proteome and transcriptome in a global manner. They are available through the EMSL user program.

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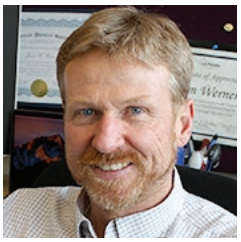

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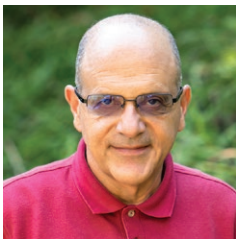

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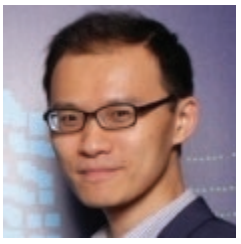

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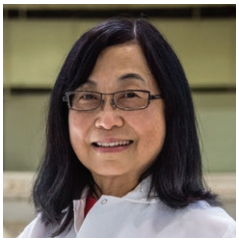

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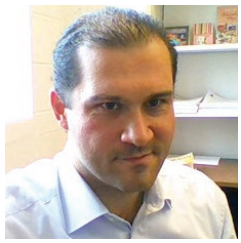

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Emerging Technologies and Approaches for BER Research — Technologies for Investigating the Rhizosphere

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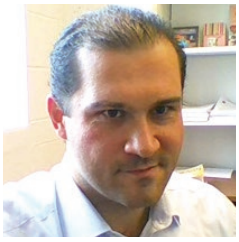

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