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Microorganisms have tremendous capability in using small molecules to construct complex and high-value chemicals through orchestrated actions of proteins and enzymes. Inorganic semiconductor nanoparticles (e.g., quantum dots or QDs) are highly stable and can efficiently harvest sun light to produce chemical currency to drive reactions. As such, hybrid inorganic-microbial systems have emerged as a potentially transformative approach to combine the light-harvesting capability of inorganic semiconductors and the ability of microbes to orchestrate complex chemical transformations. Improving and optimizing the performance of such systems requires quantitative understanding of the understanding factors that limit the performance, which is hampered by the difficulty in the needed measurement science because of the large and intrinsic heterogeneity of bacterial cells and inorganic nanoparticles. The objective of the project is to combine quantum materials synthesis, bacterial synthetic biology, and multimodal single-entity imaging to quantitatively study how hybrid QD-bacteria systems convert light to value chemicals at the single-to-sub cell level, with the ultimate goal of gaining insights to guide the engineering of QDs and bacterial genetics for more efficient bioenergy conversion. The project focuses on semiconductor-bacteria hybrids for light-driven carbon dioxide fixation to value chemicals as a model system. The research also uses multidisciplinary approaches that build on collaborative expertise in multimodal single-particle/single-cell imaging, quantum nanomaterials synthesis and engineering, and bacterial genetic engineering, with a central research task on single/sub-cell performance metric imaging in light-to-chemical conversion. The poster will present our recent progress, in measuring sub-cellular level photoelectrochemical currents from a single Ralstonia eutropha cell in contact with a single particle photoanode or a bulk film photoanode, in correlation with the cell’s morphology and cell-particle contact geometry. It will also present our progress in tagging membrane hydrogenase that is essential in mediating electron transfer from semiconductor to downstream CO2 fixation enzymes and its imaging in single Ralstonia cells at the single-molecule level. The research is expected to provide quantitative knowledge to understand the basic materials and biological factors as well as guiding principle to engineer and improve such systems for more efficient energy and chemical conversions, which are central to DOE’s mission.
Spatiotemporal Dynamics of Photosynthetic Metabolism in Single-Cells at Sub-Cellular Resolution

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Metabolism is organized in space and time, even within a single bacterial cell. In cyanobacteria, metabolic processes are highly dynamic and often compartmentalized into discrete regions in the cell. This spatiotemporal regulation of metabolism is critical for oxygenic photosynthesis, \( \text{CO}_2 \)-fixation, and \( \text{N}_2 \)-fixation. However, our understanding of these dynamical systems is limited due to the challenges in observing, measuring, and manipulating processes at the edge and below the diffraction limit of optical imaging techniques. In this collaborative interdisciplinary project, researchers are developing a novel optical imaging system to measure the sub-cellular dynamics of photosynthetic metabolism in actively growing cyanobacterial cells, from primary light-harvesting to production of high-energy molecules. The multifunctional nanoscope will enable a completely new class of experiments for the study of a broad range of biological systems, including cyanobacteria, which will be imaged with high spatial resolution and chemical specificity.

Taking advantage of the expression of several categories of photosynthetic pigment in the cyanobacterium \textit{Synechococcus} sp. PCC 7002 and mutant strains thereof, we utilized this imaging system to demonstrate that the distribution of pigmentation within the cell, and within cell colonies, is regulated by combined directional sensing of incoming light, adhesion to a surface via extracellular matrix, and applied external force, including the normal force of gravity applied to the cell. Cells grown on a substrate orient their thylakoids on the faces of the cell proximal and distal to the substrate and express both chlorophyll and phycobilins in both of these membrane regions. Phycobilins are primarily expressed in the membrane region nearest to the light source, while chlorophyll is preferentially expressed in the region opposite the overall external force applied to the cell. Cells also respond to the presence of nearby cells by producing an overall pigment distribution which follows the same rules as within the cell, but applied to the scale of the overall group. The mechanism for distribution of pigments appears to be regulated by presence of polyphosphate bodies within the cell. Lack of polyphosphate near a given thylakoid region produces a distinct “spotted” pigment distribution wherein pigment is expressed in small patches of membrane. Additionally, removal of polyphosphate completely negates the cell’s ability to sense external forces. Gene expression in bacteria has previously been shown to be affected by near-zero or extremely high gravity. However, no mechanistic basis for gravitropic behavior has been established in bacteria previously. These results suggest that cyanobacteria utilize gravity as a signal to optimize photosynthetic light harvesting during growth on solid substrates, prompting further studies of how they might respond to altered gravitational regimes that could be encountered during spaceflight.
Live-cell, quantum dot-based tracking of plant & microbial extracellular vesicles

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Nearly all organisms tested so far release vesicles from their cellular membranes. These extracellular vesicles (EVs) were originally thought to be either a byproduct of stressed cells or an artefact. EVs from cell-wall bound organisms, like plants and fungi, were some of the last to be studied because it was unclear how EVs were able to make it past the very small pore size of the cell wall. The mode of cell-wall passage remains a mystery, but it is now clear that both plants and fungi generate EVs extensively and they are used as vehicles for intercellular communication. That communication potentially occurs cell-to-cell with in a plant, and also embodies a type of cross-kingdom communication between plants and fungi. Like any form of communication, it is reliant on sending specific signals back and forth. Recent work shows that some of the signals preferentially loaded into plants and fungi EVs are small RNA. This includes canonical micro RNAs and a new class of “tiny” RNAs called tyRNAs. Significant progress has been made in EVs isolated from different organisms and examining their effect when applied to other organisms. However, very little is known about how plant and fungal EVs are delivered and what is the final fate of both the EV and its cargo. The proposed research aims to answer some of the most challenging questions in the new field of plant EVs by developing new Quantum Dot (QD)-enabled technologies for tracking EVs carrying specific small RNA cargo. These new approaches will take advantage of the superior fluorescence characteristics of QDs, including their brightness, photostability, size-tunable emission, and their detectability by both magnetic resonance imaging and electron microscopy. Specific small RNA cargo in EVs will be detected and tracked using QD molecular beacons. Our group has identified a cadmium-free QD and we are in the process of multiplexing our approach to detect different small RNA classes. For more quantitative small RNA detection, a ratiometric sensor using QDs as a scaffold for fluorescent RNA aptamer sensors will be created. All of these new tools will be applied to study the back and forth uptake of EVs from the plant, sorghum, and the fungal pathogen, Colletotrichum sublineola, whose genome we are currently sequencing. EVs from both will be isolated and their small RNA and protein cargo will be identified. Isolation of EVs from sorghum for identification of protein targets has begun. Sorghum and Colletotrichum sublineola knockouts will be made of the genes generating the EV specific small RNAs, proteins, or EV loading machinery. This information will be used to design specific small RNA sensors, which will allow to answer several fundamental questions regarding plant and fungal extracellular vesicles, including how diverse extracellular vesicles are, how extracellular vesicles are taken up by recipient cells, and how do they move systemically within a plant. Answering these questions will enable new approaches to developing crop plant varieties, including sorghum, that are more resilient to disease, and other stresses.
Tracking Lignocellulosic Breakdown by Anaerobic Fungi and Fungal Cellulosomes

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Anaerobic fungi degrade plant biomass through invasive, filamentous growth, and the secretion of multi-protein biomass-degrading complexes called fungal cellulosomes. Despite their potential for lignocellulosic bioprocessing, no non-destructive, real-time imaging tools exist to probe anaerobic fungi or the action of their cellulosomes across spatial and temporal scales. We will develop new bioimaging approaches to learn how anaerobic fungi orchestrate lignocellulose degradation through their unique multi-protein cellulosomes – and how these fungi are able to access carbohydrate biopolymers encased in lignin. Multiplexed imaging tools based on the synthesis of novel quantum dot (QD) nanobody fusions targeted at different components of fungal cellulosomes will reveal where cellulosome components are localized within the fungal ultrastructure and at the interface with lignocellulose. Our approach benefits from a suite of new genomic, transcriptomic, and proteomic data obtained for three strains of anaerobic fungi, which enables the synthesis of custom quantum dot and nanobody probes to localize cellulosomes and track their dynamics without suffering photobleaching effects. We will also leverage a cell-free production pipeline to reconstitute quantum dot-tagged fungal cellulosomes in vitro and characterize enzyme rearrangement, kinetics, substrate breakdown, and high-resolution structure via Cryo-EM. As a complementary approach, we will advance genetic tools for the anaerobic fungi to conjugate quantum dot probes onto cellulosome components in vivo, which further enables hypothesis testing of protein function in genetically recalcitrant anaerobic systems. Overall, this project will establish new state-of-the-art bioimaging capabilities to observe cellulosome dynamics and localization in situ with label-based and label-free approaches and will reveal critical attributes of fungal cellulosomes that can be engineered and exploited for bio-based fuel and chemical production.
The objective of this project is to develop innovative and improved imaging instrumentation that can enable visualization and quantitative characterization of molecular and genomic biomarkers and their dynamic role in carrying out cellular processes and function in plant systems related to bioenergy development. We will develop an advanced Multimodal Optical Sensing And Imaging Combinatory (MOSAIC) system based on plasmonics-enhanced optical techniques. Plasmonics is derived from the word plasmon that refers to oscillations of large numbers of electrons, which produces strong electromagnetic fields on metallic nanostructures. MOSAIC integrates various techniques including surface-enhanced Raman scattering (SERS) and plasmonics-enhanced two-photon luminescence (PE-TPL) imaging for use in visualizing and tracking important biomarkers such as nucleic acid targets for bioenergy applications. A multimodal SERS-TPL combined with X-ray fluorescence imaging system will also be developed for spatial co-registration of nanoprobe in living plant systems.

One promising alternative energy to fossil fuels is the next generation biofuels made from nonfood biomass, such as lignocellulose (woody parts) in plant wastes or hydrocarbons produced by photosynthesis (e.g., terpenes and fatty acids) in plants and certain microbes. However, current production of cellulosic and hydrocarbon biofuels is far from optimal, and requires further research to improve efficiency and reduce costs. In order to design a new strategy to increase production, it is important to elucidate the regulation of the terpene synthesis pathway. The advanced MOSAIC system will provide the much needed tools for elucidating the regulation of the terpene synthesis pathway in order to synthesize terpenes more efficiently for biofuel production, and for tracking pathways of carbon fixation in plant systems of interest to the DOE bioenergy program.
A long-standing goal towards advanced understanding of plant and microbial systems for bioenergy applications involves characterization and quantification of multiple complex biological processes \textit{in vivo}. Such quantification includes determining the specific copy number and function of enzymes, tracking metabolic pathways, and specifically activating the selected pathways with desired flux. While conducting these studies, it is also desirable to monitor the associated transport of materials (metabolites) within cells or across cellular membranes, and microbe-microbe interactions. Such an ambitious and expansive effort in uncovering molecular biology of interest using \textit{in vivo} high-throughput, non-destructive, real-time tracking of subcellular components in living cells; requires multiple simultaneous modes (multimodal) of imaging using quantum probes and sensors.

Here we are developing design rules for creating targeted atomic and molecular clusters to study, and even control cell biology, at a single-molecule level. There are three paradigms/functionalities we have developed/are developing: 1) active atomic cluster and passive molecules on the surface (to ensure efficient uptake); 2) passive atomic cluster (to aid transport/imaging) and designed sequence of biomolecules to track and modulate cell biology; and 3) active atomic and molecular components leading to creation of new nano-organism (nanorgs) with desired biological function and tunable material functionality. We prepared a new quantum dot tool (QD) toolkit to study these three regimes, using 50-60 different colored QDs, through multicolor or hyperspectral imaging, and use 5 different simultaneous imaging modalities (super-resolution, hyperspectral, Foerster resonance energy transfer (FRET), cryogenic transmission electron microscopy, and pump-probe visible-terahertz) to achieve the desired high imaging resolution. The proposed high-resolution includes high spatial (Angstrom- to nanometer-scale), spectral (≈1-5 nanometers), and temporal (few millisecond) resolution, using the extensive QD toolkit with designed atomic and molecular clusters. The QD probes with unique chemical structures are developed for selective targeting of subcellular components with high precision to uncover their structure, function, and location. Imaging studies of these subcellular components such as macromolecules that catalyze metabolic and/or transport reactions, and specific biochemical substrates and metabolites will provide a better understanding of the metabolic processes (anabolic and catabolic) in biological systems. The proposed work represents a paradigm shift in bioimaging, uncovering metabolic pathways, and mapping the spatiotemporal dynamics in living biological systems in real-time. The novel aspects of the work include: 1) QD toolkit functionalized with genetically encoded peptide nucleic acids; 2) high-resolution imaging modalities, and 3) optically manipulating and tracking the physiology of single-cells using quantum state matched QD-enzyme biohybrids. These QD-based imaging and manipulation methods will be tested in two model organisms: cyanobacteria where auto-fluorescence is a limitation for conventional fluorescent proteins by spectral overlap; and thermophilic anaerobes where the maturation times of fluorescent proteins are extremely long. The proposed QD toolkit and multimodal imaging developed under this project will have applications in the study of other microbial and plant systems for bioenergy production.
Expanding the utility and range of quantum and polymer dots for multiplexed super resolution fluorescence imaging in plants

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The emission spectra of plant pigments currently limit the number of fluorescent colors that can be imaged simultaneously and, therefore, the number of cellular components that can be imaged within a single experiment. Hence, developing fluorescent tags and a microscope system that can image beyond the visible range or Near Infrared (NIR) would greatly enhance plant imaging capabilities. Extending imaging into the NIR would support the interrogation of multiple proteins/molecules simultaneously, but would inevitably decrease spatial resolution due to the increase of the diffraction limit of light. To address this need, we are developing a super resolution fluorescence imaging (SRFI) platform that would allow imaging of NIR-emitting molecules, as well as quantum dots (QDs) and polymer dots (Pdots). The advantage of QDs over organic dyes is their broad absorption and narrow emission peaks, which supports multiplexed imaging with a single excitation wavelength. QDs also show higher photostability and photoblink-both required traits for single dot-based SRFI. The longest emission wavelength of commercially available semiconductor QDs is 800 nm. Advances in inorganic NIR-emitting QDs will be realized by forming stable shells around the QD cores and modifying their surface with passivating ligands to improve their oxidation stability in biological solutions. The QDs will be functionalized to support their use for antibody tagging, as well as for use in biological suspensions. To further increase availability of NIR-emitting dots, we are also developing luminescent polymer dots (Pdots), which are not available commercially. Pdots are carbon-based nanoparticles made of hydrophobic semiconducting polymers, with synthetically controlled diameters of 5-100 nm. Pdots have minimal cytotoxicity and are, thus, more suitable for biological applications than inorganic QDs. In order to advance biology and to fully demonstrate the utility of the resources developed from this project, we are focusing on the challenging effort to identify and visualize plant receptors (proteins) within nanodomains on the plasma membrane (PM). These experiments seek to examine the dynamic nature of receptor movement and degradation at a single molecule level as a result of environmental perturbations. Practical use of bioenergy crops will require plants with high yield and tolerance to a variety of biotic and abiotic stresses. Plants are rooted in place and, hence, have evolved mechanisms to recognize environmental threats and respond. Environmental sensing is largely localized to the PM, where a plethora of receptors and associated proteins form complex and dynamic interactions in response to specific environmental stimuli. Paraphrasing the FOA, the innovative approaches we are using include “QD-based-imaging approaches and complementary optical imaging instrumentation for observation and characterization of multiple complex biological processes, including development of probes functionalized with specific, active molecules to bind with specific cellular targets that will enable dynamic localization and imaging to validate hypotheses related to cellular signaling while dramatically enhancing our ability to measure processes in and among living cells.”
Cellulose is the most abundant renewable carbon energy source on earth. Efficient conversion of cellulose to a liquid fuel is hampered by its high crystallinity (where water cannot even enter the matrix). In addition, it is encased by both lignin and hemicellulose. The initial step for this process is pretreatment and is followed by enzymatic hydrolysis of the cellulose by cellulases. The mechanism of cellulases have been difficult to study due to it being insoluble. Kinetic steps such as processive hydrolysis cannot be studied by tradition biochemical methods. The enzyme action is initiated by the binding of cellulase to cellulase, followed by processive hydrolysis that includes cyclic rounds of cellobiose release, and termination. Furthermore, not much is known on how lignin and hemicellulose impact this mechanism.

The overall goal of this project is to develop methods for elucidating the kinetic and chemical factors affecting cellulase action on lignocellulose \textit{in vitro}, and in the future \textit{in situ}. This will be achieved by advancing the technology of time-resolved 3D multi-resolution imaging techniques. The 3D capability is essential because, in real samples, the cell wall / lignocellulose morphology is complex and extends in all three dimensions. The technology continuously tracks the 3D position of a single quantum-dot tagged cellulase with 10-microsecond ($\mu$s, $10^{-6}$ s) time resolution and $\sim$10-nanometer (nm, $10^{-9}$ m) XYZ 3D localization precision. At the same time, two-photon laser-scanning fluorescence lifetime imaging microscopy reports on the microscopic details of the cellulase’s microenvironment and activity.

Merging our newly developed time-resolved 3D multi-resolution imaging technology with quantum-confined multi-color nanoscale emitters will allow us to visualize and detect enzyme initiation, processive turnover, possible diffusion in a random-walk manner, and finally termination. These kinetic steps will be observed with pure cellulose and in the presence of lignin and hemicellulose. Our approach will be able to unambiguously follow these mechanism-defining actions of the enzyme due to its nanometer-precision and microsecond 3D tracking. Thus, our research will obtain kinetic parameters and define the rate-limiting step in cellulase-mediated hydrolysis of cellulose.

We plan to achieve the project goal through two aims: (1) To develop a new time-resolved 3D multi-resolution microscope that simultaneously visualizes the dynamics of different scales \textit{in situ}—the molecular / nanometer scale and the cellular / lignocellulose scale—continuously from $\mu$s to minutes and even hours. (2) To critically evaluate the platform by quantitatively resolving cellulase dynamics, one molecule at a time. The technologies will be generalizable to biofuel research areas beyond fungi, extending to the basic science of heterogeneous enzymatic catalysis. Empowered by these new technologies, the community will begin to address those knowledge-gap bridging questions, which will lead to a more predictive understanding of microorganism-enzyme-metabolite synergy.

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