

# Technology Development and Use

## Imaging, Molecular, and Cellular Analysis

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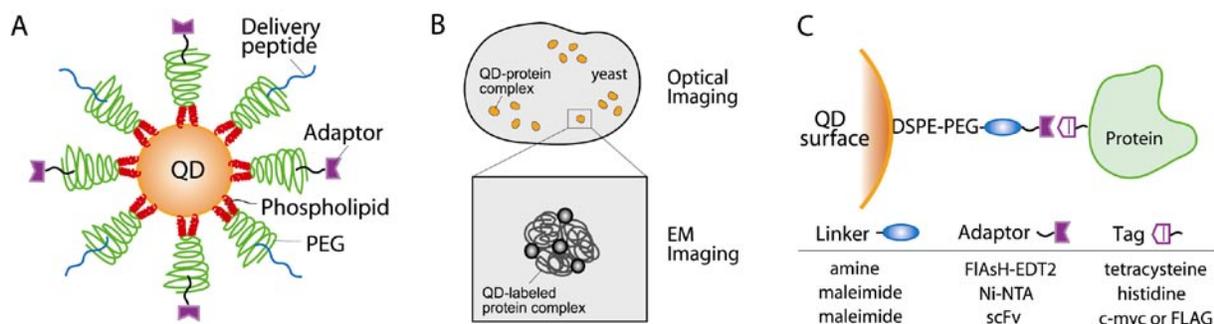
### Probing Single Microbial Proteins and Multi-Protein Complexes with Bioconjugated Quantum Dots

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We have been developing quantum-dot (QD) based strategies for imaging and identification of individual proteins and protein complexes in microbial cells. Currently, there is a lack of novel labeling reagents for visualizing and tracking the assembly and disassembly of multi-protein molecular machines. There is no existing method to study simultaneous co-localization and dynamics of different intra-cellular processes with high spatial resolution. As shown in Figure 1, the multifunctional quantum-dot bioconjugates we develop consisting of a quantum dot of 2-6 nm in size encapsulated in a phospholipid micelle, with delivery peptides and protein targeting ligands (adaptors) conjugated to the surface of the QD through a biocompatible polymer. After internalization into microbial cells, the adaptor molecules on the surface of QD bioconjugates bind to specific target proteins or protein complexes that are genetically tagged. Optical imaging is used to visualize the localization, trafficking and interaction of the proteins, resulting in a dynamic picture but with a limited spatial resolution (~200 nm). The same cells is imaged by EM to determine their detailed structures and localize the target proteins to ~4 nm resolution. For each protein or protein complex, selected tags are tested to optimize the specificity and signal-to-noise ratios of protein detection and localization. This innovative molecular imaging approach integrates peptide-based cellular delivery, protein targeting/tagging, light microscopy and electron microscopy.

Figure 1. (A) Schematic illustration of a multifunctional quantum dot bioconjugate consisting of encapsulated QD with targeting adaptor and delivery peptide on its surface; (B) correlated optical and EM imaging of the same cell gives both temporal and spatial information on a protein complex; (C) possible conjugation and tagging strategies for optimizing detection specificity and sensitivity. Note that molecules are not drawn to the exact scale.



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To achieve the goals of this DoE GTL project, we have developed quantum-dot bioconjugates with QDs encapsulated in a micelle. Phospholipids conjugated to monomethoxy PEG was used to form micelles in which the hydrophobic core of a DSPE-PEG micelle provides a cavity to encapsulate individual QDs, while the dense PEG polymer layer on the outer surface facilitates conjugation of linker molecules and delivery peptides. To facilitate bioconjugation for attaching adaptor molecules and delivery peptide, different functionalized PEG-lipid derivatives, such as DSPE-PEG-maleimide and DSPE-PEG-amine were used. To generate site-specificity, two tagging strategies were examined. The first used FIAsh-EDT<sub>2</sub> as the adaptor molecular on the QD surface and tetracysteine (Cys-Cys-Xaa-Xaa-Cys-Cys) as the tag engineered on the target protein. In the second approach, Ni-NTA was conjugated to coated QDs and the specific protein was modified to have a histidine tag. The specific targeting was demonstrated using a model system.

We performed a preliminary study of peptide-based delivery of dye molecules and quantum dot bioconjugates into yeast and *E. coli* using specifically three different peptides, TAT, polyArg, and a peptide (*ArgSerAsnAsnProPheArgAlaArg*) that has been used for delivering GFP into yeast *S. cerevisiae*. Two yeast strains, ACY 193, a wild-type yeast strain, and ACY651, a permeable yeast strain were used. We found that the polyArg peptide was the most efficient one for yeast delivery.

As part of our effort to develop QD-based technologies to identify and track individual protein complexes in microbial cells, we are advancing electron tomography as a promising new tool to image such complexes both *in vitro* and *in vivo* within small microbial cells. A new helium-cooled, 300kV, FEG, “G2 Polara” FEI TEM at Caltech was used to image purified protein complexes, viruses, and whole bacterial cells. The Polara has allowed us to record automated tilt series of a single sample cooled with either liquid nitrogen (~90K) or liquid helium (~10K). Specifically, we have recorded tilt series of purified hemocyanin and reconstructed hundreds of individual particles at various doses at each temperature, all from different “holes” of the same grid square in a single data collection session to minimize confounding variables. Surprisingly, the contrast from proteins gradually fades when they are cooled by liquid helium and iteratively imaged. Thus liquid nitrogen cooling is preferred. Using a prediction-based tracking software, we performed an automatic tilt series collection without any extra tracking or focusing images, allowing a robust data collection and reducing the time required to record a large amount of data. Further, through delivery of FEI’s first “flip-flop” cryo-rotation stage, we have begun recording dual-axis tilt series of frozen-hydrated samples routinely. This has the advantages in improved point-spread-function; however, we found that our software for merging the two tilt series is not optimal, and we are presently working to improve that. These technological advances have allowed us to visualize directly cytoskeletal elements within small microbial cells and the domain structure of purified multienzyme complexes, both are key imaging goals of the Genomics:GTL program.

As a model system to study protein localization, we have been investigating the migration of *Dictyostelium discoideum* under defined extracellular stimuli. This organism responds quickly to changes in the direction of cyclic nucleotide, adenosine 3', 5'-cyclic monophosphate (cAMP) gradient. These highly polarized amoebas are characterized by continuous protrusion and retraction of pseudopodial extensions. Various localized structural responses occur in polarized *D. discoideum*. These include the localization of the  $\beta$  subunit of the heterotrimeric guanine nucleotide-binding proteins (G proteins) in a shallow anterior-posterior gradient, as well as the biased distribution of actin. Furthermore, the orientation of aggregating cells is dominated by the chemoattractant-induced polymerization of actin.

We have utilized custom-fabricated microfluidic devices to stimulate a cell in local domains both with two-dimensional and three-dimensional control while simultaneously visualizing its response with fluorescent microscopy using quantum dots. Both peptide-based delivery and electroporation

were used to internalize QD-probes into the *Dictyostelium*. The dynamics of the actin cytoskeleton and the localization of  $\beta$  subunit of G protein in response to extracellular stimuli were studied using quantum-dot probes. Further, double labeling strategies were developed to localize extracellular cAMP receptors. This technique will be combined with high-resolution electron microscopy imaging to visualize individual proteins and protein complexes.

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### Single-Molecule Imaging of Macromolecular Dynamics in a Cell

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In order to monitor macromolecular dynamics optically in living cells and to relate these observations to cellular functions two sets of tools will be essential. It will be necessary to have fluorescent probes that can be used for site-specific labeling *in vivo* and non-bleachable probes that are biocompatible with the cellular environment. The first year of this joint research project focused on the development of these two enabling technologies.

To address the need for non-bleachable and biocompatible probes, we have synthesized and characterized biocompatible gold nanoparticles, or nanotags. Nanotags will allow long-term (hours) monitoring of molecular dynamics in cells. To allow direct optical observation, the size of nanoparticles has to be greater than 10 nm. However, the surface chemistry of nanoparticles in this size range has been known to be challenging due to their propensity to aggregate. We have developed surface passivation protocols that allow stabilization of large nanoparticles and yet retain reactivity for further functionalization that is required for biological tagging.

An important aspect of these nanotags is their biocompatibility. We have developed protocols to coat the stabilized nanoparticles with various passivation agents. Biocompatibility has been stringently tested against both biochemical and biological criteria. For the former, enzymes tethered to nanotags were found to retain their reactivity, whereas for the latter, mammalian cells that contain nanotags were able to survive and propagate for several generations without aggregation of nanotags. We have also developed nanotags that will allow investigation of macromolecule rotation dynamics, as well as those that will allow multiplexing.

Finally, progress towards specific labeling of proteins with FRET donor and acceptor pairs that can be used in living cells and the status on instrumentation will be presented.

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## Developing a High Resolution Method for Protein Localization in Whole Bacterium

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Bacteria lack intracellular membranes, yet the distribution and localization of many bacterial proteins are precisely controlled during cell cycle. Light microscopy has been used with great success to map the fluorescently labeled proteins, although the ~ 200 nm resolution is much to be desired, especially considering the extremely small size of the bacterial cells.

Electron tomography reaches much higher resolution than light microscopy, to ~ 10 nm, thus can be used in principle for protein localization. However one serious problem needs to be addressed. This has to do with the currently achievable resolution. Although an order of magnitude better than light microscopy, it is still short of resolving most of the proteins in the tomograms of bacteria embedded in vitreous ice. One may either strive to improve the tomogram resolution, which is the route taken by other groups, or as we decided, to specifically tag the proteins for their identification with electron dense labels, such as nanogold.

We have synthesized 3 nm diameter nanogold particles with the functionalized chemical group Ni-NTA, to be used for labeling 6X-His tagged proteins. This is being tested and optimized on expressed proteins with and without the His tag to improve specificity and efficiency of labeling.

The bacteria we choose to study, *Ralstonia metallidurans*, is 0.3-0.5  $\mu\text{m}$  in thickness. To improve the contrast of the small gold label in the relatively thick transmission electron microscopy (TEM) tomograms, we will record additional scanning transmission electron microscopy (STEM) tomograms, which is known to provide enhanced contrast for high atomic number elements, such as the nanogold particles. In order to make our Jeol 2010F FasTEM/STEM microscope capable of performing tomography in both TEM and STEM modes, we have replaced the existing objective lens pole piece with a large gap one that has no limit on tilt angle. The microscope operating software FasTEM has been patched to work with the new large gap pole piece in STEM mode. We have also acquired and successfully installed a Gatan Digiscan for recording digital STEM images. The automatic tomography procedure has been implemented in TEM mode, and we are in the process of developing a Gatan Digital Micrograph-based script for automatic tomography in STEM mode. STEM tomography has been demonstrated previously in manual operation mode with plastic or inorganic samples. It is essential to record STEM tomographic tilt series in an automatic mode to minimize the radiation damage for frozen hydrated bacterial cells. We expect this to be done within a few months, and we will then proceed to image the labeled bacterial cells.

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## Novel Vibrational Nanoprobes for Microbiology at the Single Cell Level

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The measurement of intracellular chemical concentrations and molecular fluxes provides essential information for systems-biological models of cells. This information, however, is difficult to obtain at the single cell level – especially in living cells where chemical levels can change rapidly in response to external or internal events.

We are studying individual microbes by a combination of optical spectroscopy techniques to obtain dynamic chemical profiles at the single cell level. Raman spectroscopy in combination with optical tweezers is used to non-destructively capture individual microbes in their native environment and assess their chemical composition within seconds. We have used this technique to dynamically monitor changes in the total protein concentration of individual cells due to increased expression after external stimulation. By focusing on changes in particular Raman peaks of a microbe we can follow trends in the overall intensity of specific peaks on an even faster timescale – down to *milliseconds* – without the need for exogenous probes. We will present examples and applications of these powerful vibrational spectroscopy techniques.

To monitor chemicals at low concentrations or chemicals that cannot typically be measured by Raman spectroscopy we also present the development of nanoscale sensors based on functionalized metal nanoparticles and surface-enhanced Raman scattering (SERS). As an example, the SERS spectrum from individual silver nanoparticle (50-80 nm in diameter) clusters functionalized with 4-mercaptobenzoic acid (4-MBA) is shown to exhibit a characteristic response to the pH of the surrounding solution, and is sensitive to pH changes in the range of 6 to 8. Measurements from nanoparticles incorporated into individual cells demonstrate that these nanoparticle sensors retain their robust signal and sensitivity to pH when incorporated into a cell. These sensors can be probed almost entirely background-free and their signals do not suffer from photobleaching, which makes them attractive long-term probes for chemical concentrations that cannot be probed by conventional Raman spectroscopy.

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**Instrumented Cell for Characterization of Mammalian and Microbial Cells**

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Scientists at Lawrence Livermore National Laboratory are developing novel methodologies for high throughput cell and bacterial analysis through an internally funded Laboratory Directed Research and Development project, Instrumented Cell. The project parallels work to be conducted in the Facility for Analysis and Modeling of Cellular Systems, the final step of which requires the ability to measure and predict dynamic events within individual cells in order to achieve a comprehensive understanding of living systems. Like the Cellular Systems Facility, work focuses on experimental capabilities for the integrated observation, measurement, and analysis of the spatial and temporal variations in cellular and microbial systems.

There is a growing interest in the prospects for a new “quantitative biology”, in which experimental data is integrated into predictive models of biological systems that can in turn be used to design and evaluate new experiments. There are many challenges to realizing this vision of quantitative biology; perhaps the most significant being the need to get precise data on the concentration and distribution of cellular components that will be required to develop such models. There are many analytical capabilities at LLNL that show promise for enabling quantitative measurements of cellular processes in individual cells. Instrumented Cell is developing and applying technical capabilities to measure and manipulate biochemical concentrations at the single-cell level with the ultimate goal of developing quantitative models of cellular processes. With the LLNL Microfabrication Facility, microstructures are being developed to isolate cells and make single cell measurements with the analytical tools possessed by the Chemistry and Energy and Environment directorates.

The first year of Instrumented Cell focused on production of tools capable of manipulating the environment of individual cells, and production of analytical measurements of the cells' reaction to stimuli. These capabilities provide quantitative data on an individual cell's responses to stimuli, such as characterization of uptake and effect of the new siHybrid gene silencing technology as well as the uptake of nanoparticle sensors.

Cultivation and maintenance of microbes and microbial communities under controlled conditions, including the ability to interrogate the function of individual microbial cells in the context of a characterized physicochemical environment, is another key technology needed by GTL. We are developing highly controlled systems for growing and maintaining microbial populations and communities via chemical and microfabrication-based platforms, with an emphasis on development of robust protocols that are adaptable to different strains of bacteria. We have made considerable progress in isolation of discreet bacterial communities, which will assist in high throughput analysis. Our projected plan consists of on-chip analysis of gene silencing via fluorescence experiments and off-chip cloning and mass spectrometry experiments.

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## Chemical Imaging of Biological Materials by NanoSIMS

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The NanoSIMS 50 represents the state-of-the-art for *in situ* microanalysis for secondary ion mass spectrometry (SIMS), combining unprecedented spatial resolution (as good as 50 nm) with ultra-high sensitivity (minimum detection limit of ~200 atoms). The NanoSIMS incorporates an array of detectors, enabling simultaneous collection of 5 species originating from the same sputtered volume of a sample. The primary ion beam (Cs<sup>+</sup> or O<sup>-</sup>) can be scanned across the sample to produce quantitative secondary ion images. This capability for multiple isotope imaging with high spatial resolution is unique to the NanoSIMS and provides a novel new approach to the study of biological materials. Studies can be made of sub-regions of tissues, mammalian cells, and bacteria. An example of the detail afforded by NanoSIMS imaging is depicted in Fig. 1, showing the distributions of N and P in individual cancer cells. Major, minor and trace element distributions can be mapped on a submicron scale, growth and metabolism can be tracked using stable isotope labels, and biogenic origin can be determined based on composition. We have applied this technique extensively to mammalian cells (Fig. 1) and bacterial spores (Fig. 2), and we are initiating a study of growth and metabolism of bacteria. Results from these studies will be discussed.

Fig. 1. NanoSIMS secondary ion images showing the distributions of N (measured as CN) and P in sectioned cancer cells.

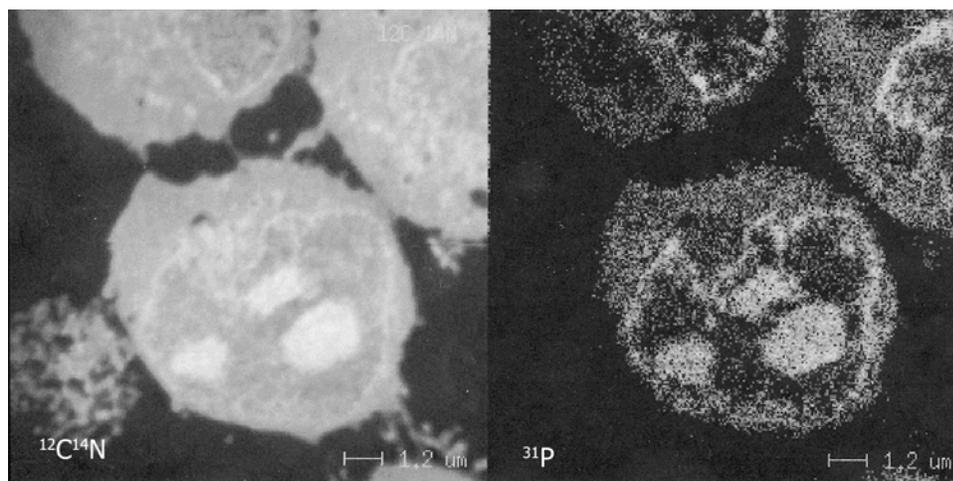
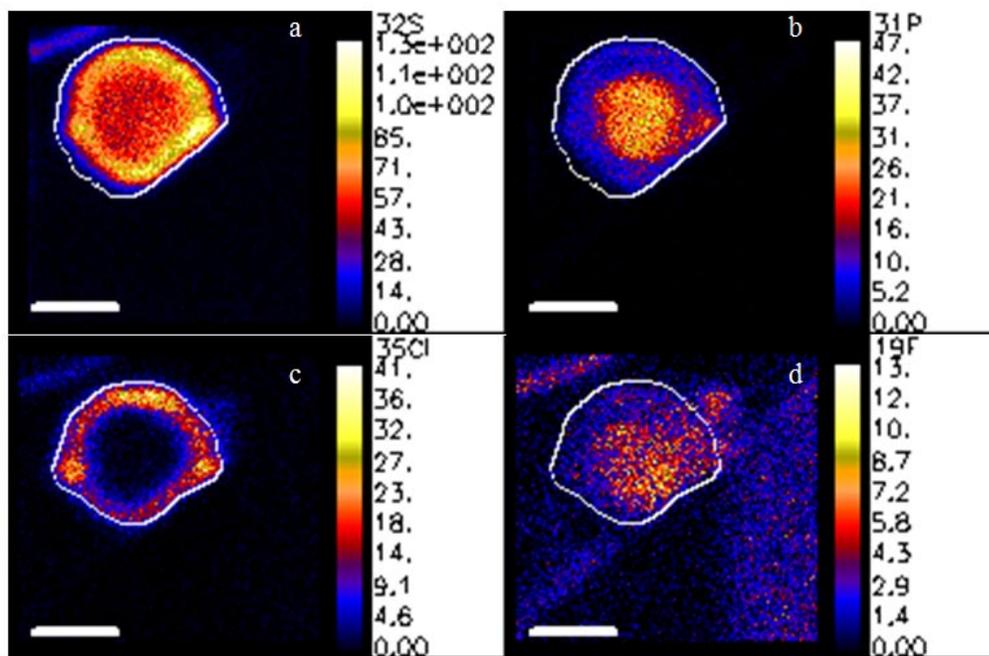


Fig. 2. NanoSIMS (a) sulfur, (b) phosphorus, (c) chlorine and (d) fluorine images of a sectioned *Bacillus thuringiensis israelensis* spore showing chemical zonation. The S and P images were collected simultaneously, and then the Cl and F images. The color bars show the total counts collected for each species, and the scale bar is 500 nm.



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## Direct Determination of Affinity in Individual Protein-Protein Complexes in Mono and Multivalent Configurations Using Dynamic Force Spectroscopy

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Our laboratory at LLNL has been developing techniques for direct determination of the energy landscapes for biological molecule interactions. Interactions between proteins drive a vast variety of cellular events, and direct determination of the strength of these interactions is important to the efforts in understanding cellular metabolism and high-throughput characterization of protein complexes. Recent advances in single biological molecule manipulation and measurement have enabled direct measurements of interaction forces between individual biological molecules. We have been using atomic force microscopy (AFM) to determine energy barriers and kinetic parameters for the dissociation of individual protein-protein complexes.

We used the atomic force microscope (AFM) to measure the binding forces between single molecule mucin1 (Muc1) protein and an antibody screened against Muc1. Muc1 is overexpressed on cell surfaces in a number of human cancers. Our collaborators at the UC Davis Cancer Center use antibodies to Muc1 as the targeting mechanism for delivery of radioimmunotherapeutic drugs, which consist of several such antibodies tethered to a common radioactive payload. Direct determination of binding affinities for mono and multivalent configurations of such drugs is critical for their optimization.

Our measurements utilized the proteins linked to the surfaces of the AFM tip and sample by flexible tethers (Figure 1). This is a versatile and general approach that spatially separates specific interactions and allows quick rejection of non-specific binding events. We have confirmed measurement of specific interactions by blocking it in a competition assay. Moreover, we were able to identify and discriminate between single and multiple rupture events by monitoring the interaction force and the nature of the tether stretch.

Measurements of the binding strength as the function of the bond loading rate (*dynamic force spectra*) allowed us to determine energy barriers, thermodynamic off-rates and the distance to the transition state for simultaneous dissociation of one, two, and three protein-protein pairs (Figure 2). Remarkably, the dynamic force spectra for single and multiple bonds show very similar slopes corresponding to the bond width for individual protein complex. These experimental observations confirm the theoretical prediction for unbinding of molecular bonds in parallel configuration. We also show that although our measured bond strength scales linearly with the number of molecule pairs, multivalent configuration leads to a precipitous decrease in the thermodynamic off-rates for the complex dissociation. Finally, we will discuss approaches for performing these measurements in high-throughput manner for potential end-line characterization of protein complexes and affinity tags.

This work was funded by the LLNL LDRD program.

Figure 1: Schematic of the measurement setup. (A) gold coated tip, (B) thiol surfactant, (C,E) PEG tethers, (D) Muc1 antibody and Muc1 peptide complex.

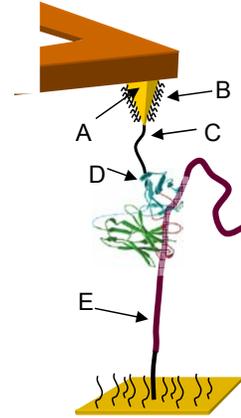
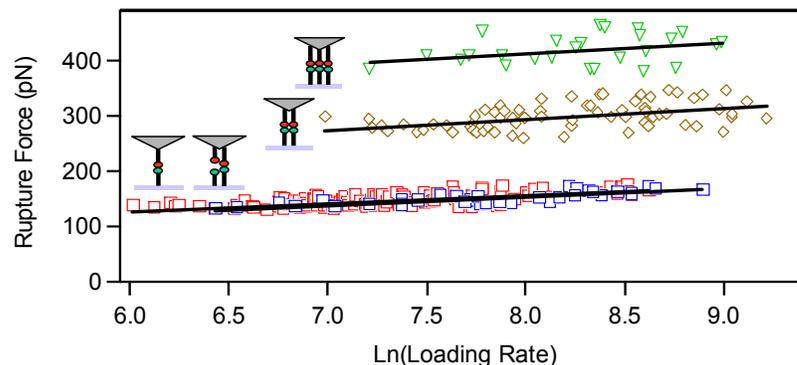


Figure 2: A dynamic force spectrum showing rupture events for one ( $\square, \square$ ), two ( $\diamond$ ), and three ( $\Delta$ ) bonds. The blue square points ( $\square$ ) correspond to stepwise ruptures of single bonds in quick succession, and red square points ( $\square$ ) correspond to individual single bond rupture events. The least squares line fits predict the thermodynamic off-rates of  $7 \cdot 10^{-3} \text{ s}^{-1}$ ,  $7 \cdot 10^{-5} \text{ s}^{-1}$ , and  $4 \cdot 10^{-9} \text{ s}^{-1}$  for the rupture of one, two, and three bonds respectively.



## Electron Tomography of Intact and Sectioned Microbial Cells

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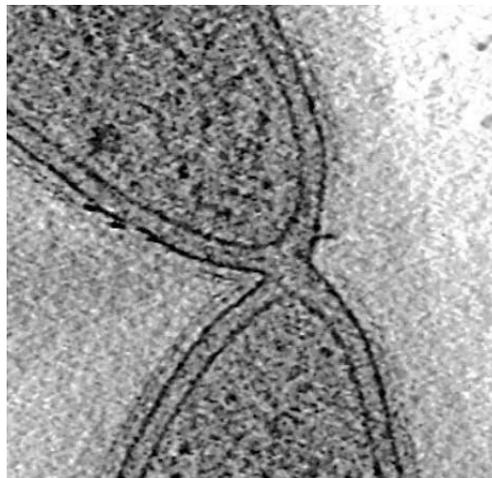
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Electron tomography is an effective tool for the study of subcellular structure at a range of resolutions. In many labs tomography is being used to understand the overall structure and interplay of sub-cellular organelles of eukaryotic cells. Such work is generally carried out on plastic-embedded, stained and sectioned samples. The resolution can be high enough to identify individual molecular complexes and even to understand conformational changes associated with their functions. When cells are suitably thin, they can also be examined without sectioning. The best preservation of intact cells is obtained by rapid freezing, which forms a vitreous embedding medium that maintains the cell in a lifelike state. Although this type of preparation generally precludes the use of contrast agents, the resolution is, in principal, sufficient to identify many of the major macromolecular complexes within the cell. Such information can give insights on localization and distribution of protein complexes and will be essential for the ultimate goals of understanding and building complete computational models of the microbes.

We have been studying several microbial cells, including *Caulobacter crescentus*, *Magnetospirillum* and *Deinococcus radiodurans*, in frozen-hydrated preparations. The work with *Caulobacter* provides a particularly interesting example of the type of information one can obtain by straightforward interpretation of the 3-D data in a tomogram. The cell membranes are very well resolved in thin slices extracted from the reconstructed volume, as shown in fig. 1. Using manual or automated procedures one can segment the volume to more clearly represent features such as the membranes, as shown in fig. 2. These features could not be unambiguously interpreted from simple projection images of the cells without having seen the structure in three dimensions. This work involved examining cells close to the time at which they completed division and revealed the differential closure of the inner and outer membranes.

Cell membranes are among the easiest features to identify in these reconstructions because they are continuous, extended structures in three dimensions. Our goal of identifying the major macromolecular complexes will require more sophisticated template matching tools as well as the best resolution we can achieve. As a test of the achievable resolution in such work, we have been investigating the structure of a large rhabdovirus, sonchus yellow net virus. Tomographic reconstructions show

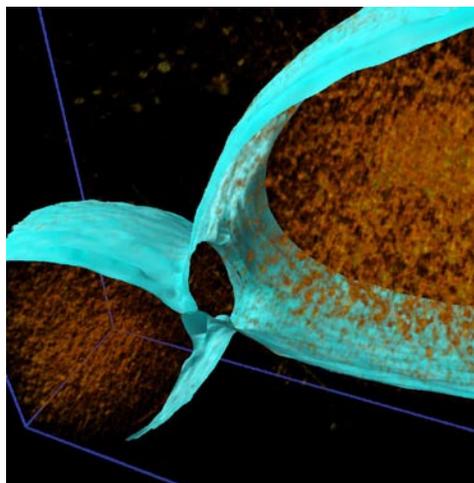
Figure 1. A slice 1 nm thick through the 3-D tomographic reconstruction of a *Caulobacter* cell that has almost finished dividing. The membranes are well resolved, along with parts of the periodic S-layer and subcellular densities that correspond to large protein complexes.



very clearly the 5-nm period structure of the coiled nucleoprotein core and even the trimeric structure of the ~70 kD glycoprotein that studs the surface of the virus. This work, along with results from a number of other labs, support the projection that we will be able to identify complexes within bacteria that have a molecular weight with a lower limit of 500 – 750 kD.

In other work, we have been using conventionally embedded microbial cells to monitor changes following a change in culture conditions. *Desulfovibrio vulgaris* cells, exposed to oxygen stress, were monitored by FTIR spectroscopy. At times where the IR spectra showed interpretable changes, cells were prepared for microscopy. Changes in the 3-D structure of the cells can be correlated with apparent metabolic changes indicated by spectroscopy.

Figure 2. Surface representation of the outer membrane of a different *Caulobacter* cell in a tomographic reconstruction, also showing some of the cell contents.



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## Probing the High-Resolution Architecture and Environmental Dynamics of Microbial Surfaces by *in vitro* Atomic Force Microscopy

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The capability to image single microbial cell surfaces at nanometer scale under native conditions would profoundly impact our understanding of specific cellular processes, environmental response and bioremediation. Even though complete genome sequences are available for various microbes, the relationships between the organization and function of protein complexes within bacterial membranes and how these protein complexes respond to the change in the environment and chemical stimulants are not understood.

We have recently demonstrated that *in vitro* atomic force microscopy (AFM) can address spatially explicit bacterial spore coat protein interactions and their structural consequences at near-molecular resolution under physiological conditions. The direct visualization of the environmental response of individual *B. atrophaeus* spores revealed that upon dehydration, spore dimensions decreased by ~12%, followed by a nearly complete recovery in size upon rehydration. The observed decrease in the size of bacterial spores and concomitant change in spore coat surface morphology following dehydration are due to the contraction of the internal spore core and/or cortex. These studies establish that the dormant spore is a dynamic physical structure and provide an experimental platform for the elucidation of molecular scale bacterial spore processes, including germination, under native conditions. For the first time, species-specific high-resolution native structures of bacterial endospores including the exo-

sporium and crystalline layers of the spore coat of four *Bacillus* species were visualized in their natural environment, namely air and fluid. We found that strikingly different species-dependent structures of the spore coat appear to be a consequence of nucleation and crystallization mechanisms that regulate the assembly of the outer spore coat and proposed a unifying mechanism for outer spore coat surface self-assembly.

These studies establish *in vitro* AFM as a powerful tool capable of providing a direct insight into molecular architecture and structural variability of microbial surfaces as a function of spatial, temporal, developmental and environmental organizational scales. We are currently developing approaches to utilize AFM for probing of the ultra-structure and environmental dynamics of several bacteria including *Arthrobacter oxydans* and *Thiobacillus denitrificans*.

1. M. Plomp, T.J. Leighton, K.E. Wheeler and A.J. Malkin (2005), *Biophysical J.*, 88.

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### Real-Time Gene Expression Profiling of Single Live Cells of *Shewanella oneidensis*

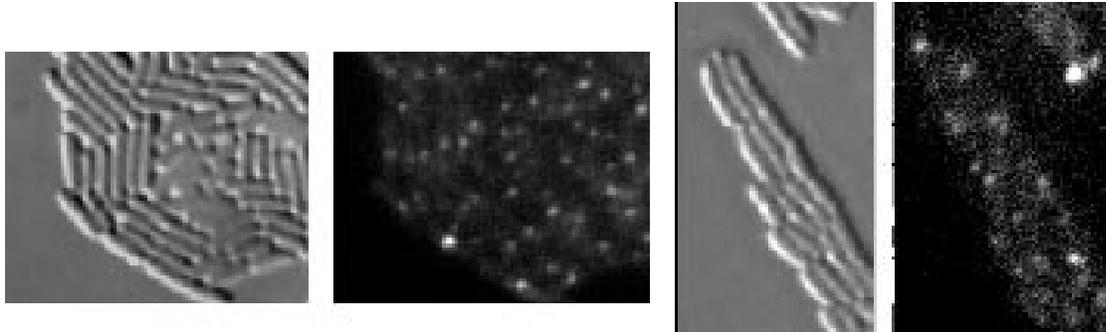
X. Sunney Xie\*, Jie Xiao, Ji Yu, Long Cai, Paul Choi\*, Nir Friedman, Xiajia Ren, and Luying Xun\*

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Our objective is to make real-time observations of gene expression in live *Shewanella oneidensis* MR1 cells with high sensitivity and high throughput. Available technology is sufficient for the detection of gene expression at high levels; whereas, new techniques need to be developed to study expression that produces only a few protein molecules. Our efforts are divided into three areas: Developing sensitive protein reporters, adapting and fine-tuning a practical cloning strategy for library construction, and testing automation techniques for high throughput measurements with single-molecule microscopes.

Modified  $\beta$ -Galactosidase and green fluorescence protein (GFP) are used for reporters. Each  $\beta$ -galactosidase molecule generates hundreds of fluorescent molecules per second, and the product formation can be monitored in real-time. On the flipside, it is hard to observe single GFP molecules in live cells. Among the available GFP reporters, Venus GFP was chosen because of its enhanced fluorescence and short maturation time. We have detected single Venus GFP in bacterial cells after the reporter protein is attached to relatively immobile cellular components (Fig. 1). This is a major step, enabling us to follow gene expression at low signal levels. N-Terminal fusion with ubiquitin (an eukaryotic tag and degradation system) or C-terminal fusion with SsrA (a bacterial tag and degradation system) have been constructed to shorten the cellular lifetime of reporter proteins, so that cell cycle related gene expression can be monitored with single cells.

Fig. 1. Differential interface contrast (left) and fluorescence (right) images of *E. coli* cells generating Venus GFP. The fusion genes are expressed at very low levels. Upon cellular immobilization, single GFP molecules are detectable as individual dots above the cell auto-fluorescence background.



A powerful cloning method that uses  $\lambda$  phage integrase was adapted for the construction of reporter libraries for *Shewanella oneidensis* MR-1. Cloning genes into entry vector is essentially the same as described by the commercial Gateway cloning method; however, the subsequent transfer of the cloned genes to destination vector with desired reporters is done by conjugation, an economic *in vivo* approach in *Escherichia coli*. The library is then transferred into *S. oneidensis* by conjugation. Since the destination vector cannot replicate in *S. oneidensis*, the plasmids integrate into the genome by homologous recombination between the cloned gene and the original gene on the chromosome. This creates two copies of the same gene on the chromosome separated by the reporter and vector DNA. Thus, every *S. oneidensis* gene can be tagged by a reporter to monitor gene expression. We have begun library construction of *S. oneidensis* into the entry vector. The clones can be transferred into different destination reporter vectors before being conjugated into *S. oneidensis*.

To conduct global studies with reporter libraries, we are combining microfluidics with single-molecule microscopy. Microfluidic chambers, including channels and valves, have been fabricated and tested with single cells carrying  $\beta$ -galactosidase. When fluorogenic substrate DDAO-gal is provided, the cells release the fluorescent DDAO into the chamber. Due to the fast catalysis of  $\beta$ -galactosidase, even a single copy of the enzyme in a cell can generate enough fluorescent signals for detection. Experiments are in progress to improve the use of microfluidics for microscopy studies and automation.

Though preliminary, our first-year results are encouraging. Complementary to DNA microarrays and mass spectrometry, our experiments will allow continuous measurements of gene expression profiling in live cells. Pushing the detection limit will enable the observation of gene expression at low signal levels, providing a complete picture of global gene expression.

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## High Throughput Fermentation and Cell Culture Device

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The Genomics:GTL (GTL) program requires that multiple microorganisms be grown with high throughput under a variety of carefully controlled-state conditions. Additionally, recombinant clones will also require culture under controlled conditions at high levels of expression, high throughput and fast production turn-around. These endeavors require technology to a) grow specific biomass under well-characterized states b) rapidly identify optimal culture conditions for expression of tagged proteins and complexes, c) rapid scale-up to obtain necessary protein samples, d) express intact protein complexes, and e) grow microbial cells in nonstandard conditions. Towards the creation of high throughput, controlled-environment instrumentation to meet these challenges, we will present the design and function of a microreactor system with parametric controls comparable to stirred vessel bioreactors. We will further demonstrate that this type of system can be used to enhance the throughput in complex, but routine, workflows.

The system designed is a bench-top, computer-controlled microreactor system. The microreactor uses a disposable cassette (SBS standard) system with 24 individually controlled 10 ml reactors. Each reactor has independent control of temperature, pH, dissolved oxygen. The current configuration covers the following range of operation:

- Temperature (control range of 20 C to 45 C, 0.1 C resolution)
- pH (acidifying and alkalizing from pH 5 to pH 9; 0.01 resolution)
- Dissolved oxygen (0 to over 100 % air saturation, 0.1 to 10% resolution)
- Cassette agitation (0-500 RPM, 2 mm integrated orbital shaker)

Figure 1: Microreactor Prototype



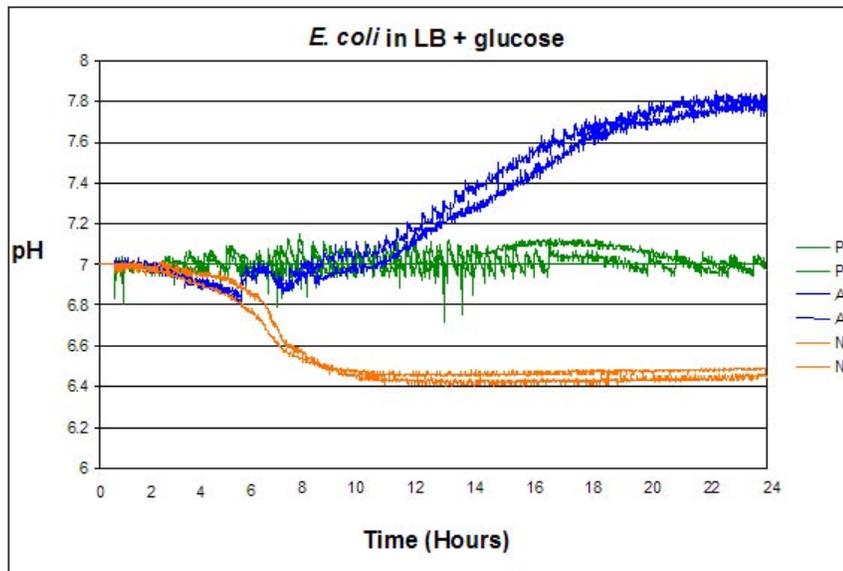
The development phase of the project has been completed. During this phase the following techniques have been developed and refined:

- Fabrication and testing of cassettes with printed sensor dots and gas permeable membranes.
- Manufacture of a 24 reactor instrumentation block with integrated heaters, gas supply manifold, and pH/DO measurement system
- Validation and calibration of the system with buffers and model system

Significant data on the use of the instrument with several biological systems of interest to GtL has been collected using the microreactor system. We will present our results on the following:

- Fine tuning of parametric control using *E. coli*, *B. subtilis* and *S. oneidensis*.
- Demonstration of a parametric control experiment in an organism of DOE interest; The growth and the state of the metal reduction pathway in *Shewanella oneidensis* MR-1 is mapped as a function of pH, temperature, and dissolved oxygen.
- Determination of success or failure criteria on the basis of the sensor data and quality of replicates generated in the demonstration study.

Figure 2: Sample Data showing *E. coli* growth in LB with glucose. The lines marked N (orange) have neither pH control nor are supplied oxygen and thus become anaerobic and acidic. The lines marked A (blue) have oxygen control, but no pH control and thus remain aerobic and become basic. The lines marked P (green) are anaerobic, but have  $\text{NH}_3$  based pH control enabled with a setpoint of pH 7 and so maintain a constant pH.



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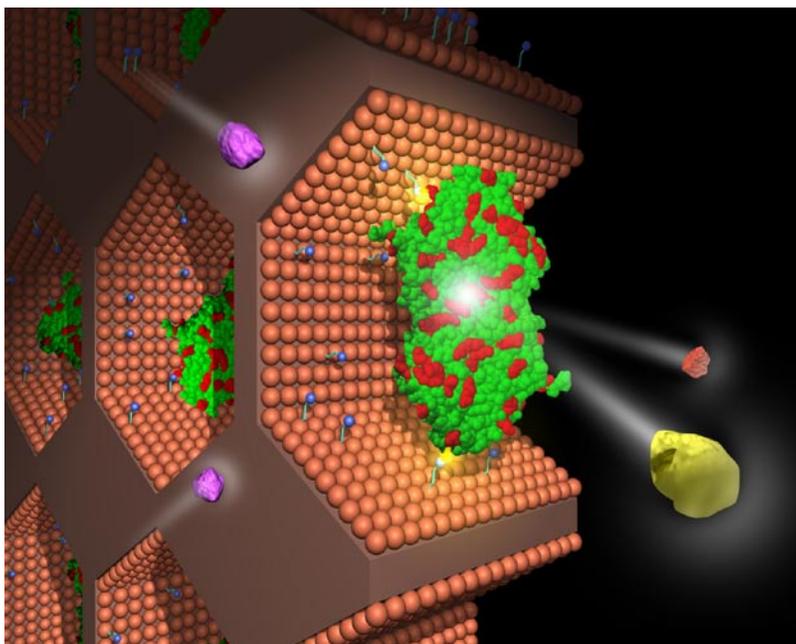
## Immobilized Enzymes in Nanoporous Materials Exhibit Enhanced Stability and Activity

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Enzymes (proteins) are the nano-machines of cells. In cells, molecular crowding provides enhanced protein stability and can induce order-of-magnitude enhancements in catalytic reaction rates compared to enzymes in solution. We recently demonstrated that enzymes can be artificially crowded through immobilization on surfaces to thereby increase their reaction rates and stability. Combining appropriately functionalized, nanoporous silica (FMS) with enzymes result in immobilizations at high enzyme concentrations that exhibit enhanced stability and activity compared to enzymes in the same solution. To date we have used either carboxylethyl- or aminopropyl- FMS to either entrap or covalently immobilize three different enzymes: glucose oxidase (GOD), glucose isomerase (GI), and organophosphorus hydrolase (OPH). The working buffer and its ionic strength affected the efficiency of protein entrapment. The data is consistent with electrostatic charges contributing an important parameter governing immobilization efficiency. Net negatively charged enzymes preferred entrapment in positively-charged FMS and vice versa. The optimal percent functionalization and pore sizes must be determined empirically. In general, pore sizes slightly larger than the enzymes appear optimal. Approaches that utilize spontaneously entrapping or covalently linking with heterobifunctional crosslinking agents produce immobilized enzymes that exhibit comparable  $K_m$  and  $V_{max}$  to the free enzyme in solution. The combination of FMS and proteins offers an excellent platform for biological reaction engineering. Our approach could be used to make more sensitive sensors, for decontamination, to develop advanced separations based on the high specificity of protein-mediated interactions, and

Figure 1: Depicts OPH immobilized in nanoporous material reacting with substrate molecules.



to generate energy enzymatically provided that suitable enzymes and proteins could be identified and produced. A potential advantage of this approach is that non-living, yet efficient enzymatic chemical reactors could be deployed in environment-friendly and environment-compatible materials (e.g. silica) without the need to maintain complex biological communities or recombinantly-engineered microbes.