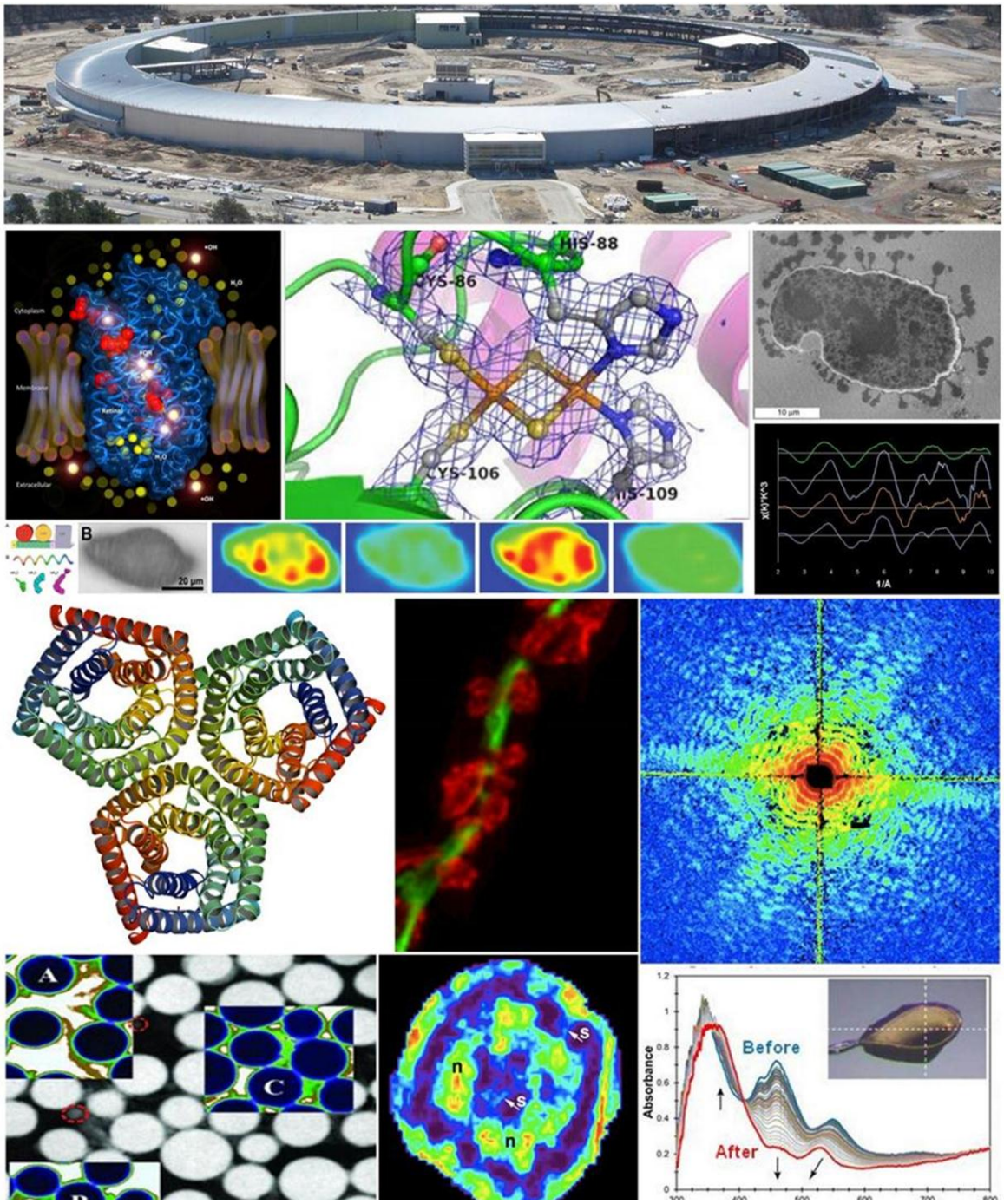
OPPORTUNITIES FOR BIOLOGICAL SCIENCES AT NSLS-II, PART B



BROOKHAVEN
NATIONAL LABORATORY

Significant New Biological Research at NSLS-II

A primary mission of BER is to provide research opportunities and scientific user facilities that advance our understanding of complex biological and environmental systems. Today, BER's scientific user facilities span a wide range of capabilities; however, the challenges associated with biological complexity create a need for new and innovative tools. The National Synchrotron Light Source-II (NSLS-II) will provide such tools. NSLS-II will provide a range of new and/or improved techniques that will enable multi-scale exploration: (1) at the molecular level, to understand how genes determine biological structure and function, (2) at the cellular level, to understand how molecular processes are coordinated to execute cell function, and (3) at the level of microbial communities and higher organisms to understand how cells interact and respond to their environment.

In this document, we present examples of significant new experiments with potentially high impact in biological research that will be enabled by NSLS-II. Specifically, we provide examples how the unique characteristics of the NSLS-II facility will:

- enable the determination of **atomic resolution structures of macromolecular complexes** involved in a wide range of areas, including those relevant to BER missions such as biofuel production, light harnessing, and contaminant cleanup
- facilitate **structural and chemical dynamics** measurements with the time resolution necessary to fully characterize new enzymes and complex assemblies in order to understand their functions as "macromolecular machines"
- provide a range of **nanometer-resolution probes** to image the complex structures and molecular chemistry of biological materials, including lignocellulosic biomass, microbes and microbial communities, and microbe-plant interfaces
- allow **in vivo chemical imaging and dynamics** of heterogeneous systems in their natural environments
- make available **high-throughput technologies** and a **multi-technique approach** for correlating genomics with structural and functional information

Atomic resolution structures of complex macromolecular systems

Macromolecular crystallography (MX) is the preeminent method for structural biology worldwide. Its intrinsic value is recognized by Nobel Prize selection committees with five Nobel Prizes in Chemistry in recent years: Walker (1997), MacKinnon (2003), Kornberg (2006), Tsien (2008), and Ramakrishnan, Steitz, and Yonath (2009) all heavily relied upon synchrotron radiation for their research.

The frontier challenges in structural biology now include large macromolecular systems and viruses, membrane proteins, protein-protein complexes, and protein-nucleic acid complexes. However, these systems are very difficult to crystallize; and, oftentimes, crystals that are obtained measure only a few microns along an edge. We anticipate that NSLS-II will provide an ideal opportunity to use microcrystals for diffraction studies due to its small electron beam profile within the storage ring, and consequently its world-leading X-ray brightness and flux density. And for complex biological systems that are not compatible with conventional macromolecular crystallography methods, new experimental approaches are under development that will take advantage of the unique characteristics of NSLS-II.

Multi-protein complexes: To date, the most common approach for determining structures of multi-protein complexes is by isolating the individual components, solving their structures separately, and re-assembling the complex based on the structures of the individual components. However, it is often not possible to stably isolate the individual proteins for crystallization. Moreover, the complex may not be a simple sum of the individual units; protein-protein interactions can cause structural alterations during assembly. Thus, crystallization of the entire protein complex is a much more desirable and accurate approach. On the other hand, diffraction from large multi-protein complexes is generally weak and crystals, if obtained, are often small.

For example, microbial enzymes, including cellulases and hemicellulases, have been shown to efficiently degrade the microcrystalline cellulose structure as multi-protein complexes. *Calidcellulosirupor* is a group of extreme thermophilic bacteria that are known to ferment polymeric carbohydrates at high rates. Integrated genome and proteome datasets identified the presence of highly active cellulolytic enzymes and revealed their unusual thermostability. Thus, *Calidcellulosirupor* is a promising anaerobic bacterium for improving high-temperature biomass conversion [1]. To date, mass spectrometric analyses have identified numerous multi-protein complexes involved in the fermentation, but there are no structural details. Successful crystallographic analysis of these complexes will require the brightness from a source that can be focused efficiently into micron-sized X-ray beams. Indeed, one key advantage that NSLS-II brings to the global MX community will be the ability to determine structures from microcrystalline samples, such as those enzymes that catalyze the critical transformation steps in lignocellulose breakdown.

Membrane protein structures: Membrane proteins represent approximately one-third of the known gene sequences and they are involved in numerous biological processes that take place at cell and organelle membranes, such as cell recognition, signal transduction, chemical sensing, and transportation. Despite their importance, structural determination of membrane proteins remains a grand challenge in structural biology. The key issue is that these proteins usually have to be extracted out of their native environment of cellular and organelle membranes and reconstituted into detergent micelles for structural studies. In addition to potential loss of biological activity during solubilization, the presence of detergents also makes it difficult to crystallize these proteins and impossible to extract protein-only information from the solution scattering data.

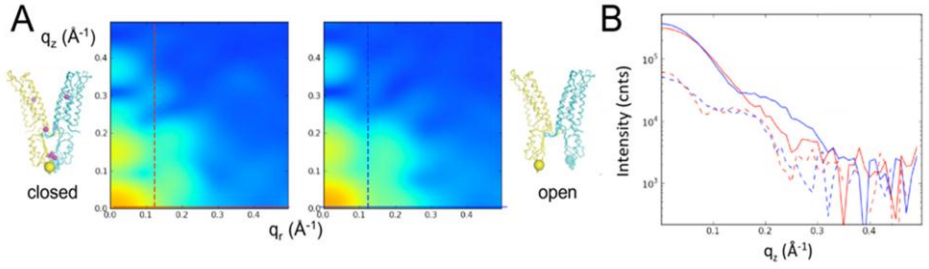


Figure 1. Simulated of X-ray scattering data from zinc transporter YiiP in a planar lipid bilayer. (A) The calculated scattering intensity of the closed and open conformations of YiiP show qualitative differences. (B) These difference should be detectable as shown in line profiles corresponding to q_r=0.12 Å^{-1} (dashed lines) and q_z=0 (solid lines). Note that noise has been simulated for scattering from a 1cm^2 bilayer sample at the expected X-ray intensity at NSLS-II.

Embedding membrane proteins in two-dimensional planar lipid bilayers that resemble their native environment in a liquid-like state represents a promising alternative for examining the structures of membrane proteins and how they interact with the lipid membrane. This approach has already had some success in cryo-EM studies of membrane protein 2D crystals [2]. It has also been adapted for grazing incidence X-ray scattering and diffraction measurements, analogous to the transmission solution scattering and crystallography methods for soluble proteins. In these structures, the X-ray

scattering signal is determined by the electron density contrast of the protein against the constant background of the bilayer membrane structure. The contrast not only depends on the protein structure itself, but it also varies as a function of protein orientation in the membrane and it is even sensitive to deformation of the membrane from hydrophobic mismatching.

For example, YiiP is a membrane transporter that catalyzes Zn^{2+}/H^{+} exchange across the inner membrane of *E. coli*. YiiP, with its Y-shaped architecture, dramatically differs from most cylindrically shaped membrane proteins [3]. The incorporation of YiiP into the membrane necessitates reorganization of lipids surrounding the irregular trans-membrane domain surface to minimize hydrophobic mismatch. The ensuing bilayer deformation would directly impact the active site for zinc transport. The molecular responses to hydrophobic mismatch may have great functional significance. Emerging evidence suggests that a large collection of lipophilic channel modulators act through deforming the bilayer, and by directly binding to the protein-lipid interface. X-ray scattering data from YiiP in planar lipid membranes (Figure 1) can reveal how protein conformation changes depending on the presence of zinc ions. More importantly, due to the sensitivity of the scattering data to the deformation in the lipid membrane structure, modeling of the scattering data may reveal the YiiP protein-lipid interface to help understand how lipids may reshape the active site for zinc transport.

To date, there have been reports of X-ray diffraction studies on 2D crystals of membrane-associated proteins [4, 5] as well as X-ray scattering studies of plant viruses adsorbed to a lipid membrane resembling a 2D solution [6]. However, routine application of these methods in structural biology will necessarily entail exceedingly small scattering volumes (e.g. ~5 nm x 1 cm x 1 cm). Such studies will require continued technical development and, more importantly, a bright source like NSLS-II to produce enough protein scattering signal above the water scattering background.

Correlated atomic and electronic structure of metalloenzymes: Recent bioinformatics surveys show that approximately 47% of all enzymes with known three-dimensional structures contain metal ions or complex centers [7]. Therefore, understanding the relationship between atomic and electronic structure is crucial for obtaining fundamental mechanistic insights into biology. This includes macromolecules critical to DOE missions such as photosynthesis and H_2O oxidation, N_2 fixation and NO_x metabolism, C_1 metabolism (methane and CO_2), H_2 metabolism, O_2 activation, and the sulfur cycle (S_0 and SO_x metabolism). For example, H_2 has enormous potential to serve as a renewable and environmentally friendly energy alternative to fossil fuels. Nature has produced enzymes (hydrogenases) that drive the hydrogen cycle in the biosphere. H_2 is also produced as a by-product of N_2 fixation by nitrogenase enzymes. In all microbial systems studied to date, the enzymes that catalyze the production of H_2 from $2H^{+}$ and two electrons are metalloenzymes, which are among the most efficient H_2-forming and consuming catalysts known.

The bio-production of H_2 using microbes must overcome several hurdles before it is commercially viable. For example, hydrogenase enzymes are rapidly inactivated in aerobic solution. Thus, targeted bioprospecting is necessary to identify and to characterize hydrogenases that are particularly active/stable and that can be readily adapted for use in bio-solar hydrogen production. One example is the *Anabaena sp. strain 7120*, where the enzyme is localized to the microaerobic environment. In an effort to increase H_2 production by this strain, site-directed mutagenesis near the Fe-Mo cofactor resulted in several variants that significantly increased their rates of H_2 production [8]. Single-crystal spectroscopy correlated with X-ray diffraction provides a way to promote, trap and study reaction intermediates within the macromolecular crystal. This will provide strategies to improve H_2 production and to further engineer microbial strains for enhanced photobiological production of H_2 in an aerobic, nitrogen-containing environment.

Synthetic biology: A major difficulty in converting biomass into biofuels lies in its recalcitrance to breakdown. Sequencing efforts and biochemistry have demonstrated that microbial enzymes are rather slow on their own, but nature has greatly enhanced their reactivity by linking them together in large multienzyme collections known as "cellulosomes". However, efforts to reproduce these effects *in vitro* using purified components have moved slowly [9]. As a result, such large multicatalytic complexes have not yet materialized in large-scale bioreactors.

Efforts in synthetic biology are underway for creating artificial cellulosomes of very high stability, defined composition, and with tailored reactivity for specific substrates. However, structural characterization of these multienzyme complexes is extremely challenging. MX has been very successful at determining the structure of individual cellulosome components, but a complete cellulosome has not yet been solved. Despite high-resolution structures of all of the individual cellulosome modules, the mechanism of enzymatic synergy remains poorly understood. Alternatively, solution X-ray scattering can provide direct shape information on artificial cellulosomes that are too large for MX. The high brilliance and high-throughput formats being developed at NSLS-II are ideally suited to the combinatorial nature of artificial cellulosome synthesis (Figure 2). With automated high-throughput methods, structural information can be obtained rapidly from thousands of different designed cellulases. This has the potential not only to screen the constructs for activity (by enzyme assays) but also to read out shape complementarity directly and tune reactivity to complex substrates.

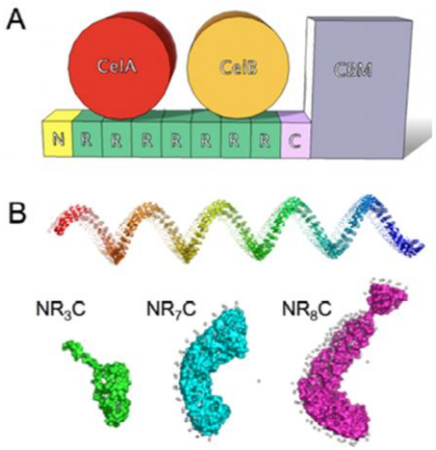


Figure 2. (A) Schematic diagram of a short artificial cellulosome unit. (B) Large cellulosomes with well-defined structure can be constructed using repeated ankyrin domain templates. Solution scattering measurements on fragments of the template have confirmed the expected curved structure.

Structural and Chemical Dynamics with Fast Time Resolution

In order to fully understand the function of multi-domain proteins and multi-protein complexes, it is necessary to understand the dynamics of protein folding and protein-protein interactions. While this is difficult with protein crystals, X-ray scattering, footprinting, and spectroscopy can be used to study these complex systems in solution.

Transmembrane protein signaling: Membrane proteins play critical roles in cellular signaling processes and ion (or water) transport across the biological membrane. Many transmembrane proteins contain hydrophobic cores with strategically placed, charged residues that appear critical for their proper function. Observation of structural dynamics surrounding these residues as well as more global conformational changes in the macromolecule on biologically relevant timescales can provide crucial information for understanding the mechanisms by which these proteins function.

X-ray footprinting of macromolecules is a unique approach for analyzing the solvent accessibility of individual residues in proteins as a function of protein conformation. X-rays generate hydroxyl radicals that covalently label solvent-exposed sites, which can subsequently be detected by mass spectrometry. By using the high X-ray flux from synchrotron radiation, microsecond time resolution can be achieved, providing a time-resolved high resolution structural picture of solvent-accessible amino acid residues and "bound" water molecules.

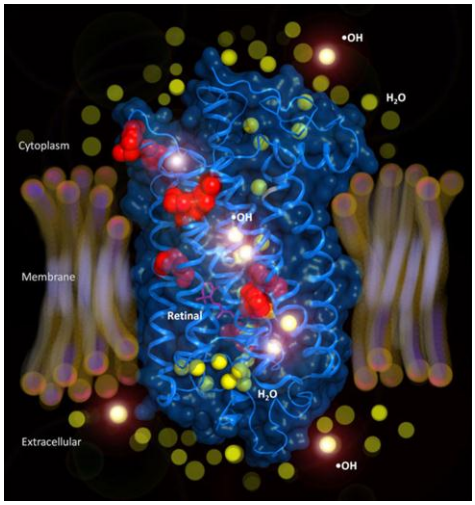


Figure 3. Radiolytic footprinting of membrane-bound rhodopsin demonstrates the structural activation of bound waters as a function of receptor signaling status. X-rays ionize water molecules inside and outside the membrane protein to radicals (•OH, glowing spheres) that react with adjacent amino acid side chains. As the protein changes its structure during signaling, the pattern of reactivity of water within the protein changes reflecting the transmission of the signal through the membrane [10].

As an example, rhodopsin is a member of a group of proteins called the G-protein coupled receptor (GPCR) superfamily, which exhibits a conformational change when turned "on," leading to interactions with other proteins and sending information across cell membranes to regulate many important molecular pathways (Figure 3). Synchrotron X-ray footprinting has been used to study these different conformational states, providing a molecular map pinpointing where water molecules sit inside the protein when it is off and on [10]. Results indicate that the water molecules rearrange in response to the protein being turned on and interact with key areas necessary for the protein's function. Understanding how these proteins and associated signaling pathways are controlled can have a large impact.

The major barrier to providing high signal-to-noise footprinting data for the exploration of protein dynamics on shorter timescales is related to achieving higher X-ray flux density. The damping wiggler insertion device at NSLS-II is expected to provide world-leading flux in the energy range of interest and would represent an entirely unique international resource.

Combinatorial enzyme kinetics: The development of pretreatment chemistries with the associated enzymes for converting plant biomass to fermentable sugars for the cellulosic biofuels industry is a multi-faceted effort. Work involves identification of enzymes expressed in bacteria and fungi; extraction, characterization, and modification of the resulting proteins; and production and testing in a realistic biochemical assay. New methods are desired for all of these characterization steps to better understand deconstruction mechanisms.

High enzyme loading is a major economic bottleneck for the commercial processing of pretreated lignocellulosic biomass to produce fermentable sugars [11]. Optimizing the enzyme cocktail for specific types of pretreated biomass allows for a significant reduction in enzyme loading without sacrificing hydrolysis yield. One approach to enzyme mixture development uses liquid robotics to explore full-factorial combinations of multiple (> 10) enzymes, which can be efficiently performed in multiwell plates. This approach is suited to complementary high-throughput synchrotron structural measurements of the plant materials during and after enzyme action. MX may be used to determine enzyme structures and hence help to elucidate function. SAXS/WAXS measurements can be conducted through aqueous medium and can quantify the breakdown of crystalline cellulose, hemicelluloses, and other tissue structures. X-ray footprinting can probe tertiary structures in physiologically relevant solutions on a millisecond time scales. And synchrotron spectroscopy techniques are available for probing different aspects of chemical and physical order in samples.

Electron transport: Flavin-dependent proteins catalyze a wide range of biochemical reactions including aerobic and anaerobic metabolism, light emission, photosynthesis, DNA repair, plant phototropism, and the activation of oxygen for hydroxylation and oxidation reactions. While flavin adducts are critical intermediates in many flavoenzyme reactions, they are rarely detected even by rapid transient kinetics methods because of high reactivity. Thus, electron transport in these highly

reactive systems requires a combination of structural and spectrophotometric analysis for characterization of the transient intermediates.

As an example, choline oxidase from *Arthrobacter globiformis* catalyzes the four-electron oxidation of choline to glycine betaine via two sequential, FAD-dependent reactions with betaine aldehyde as an obligatory enzyme-bound intermediate. The crystal structure of choline oxidase revealed the presence of a C4a-oxygen adduct. The structure of the trapped flavin adduct derives from aerobic crystals of choline oxidase at cryogenic temperatures. Correlated single-crystal microspectrophotometry showed that the adduct forms rapidly *in situ* at 100 K. The high reduction potential of the enzyme-bound flavin is proposed to promote FAD reduction in the X-ray beam. This also facilitates C4a-O(OH) adduct formation, but an insufficient proton inventory at cryogenic temperatures prevents the decay of the complex. Thus, the use of complementary methods with time-resolved spectroscopic data and cryogenic temperatures provides a powerful strategy to study reactive intermediates in enzyme catalysis.

Nanometer Resolution Probes for Structural and Chemical Imaging

In addition to understanding the molecular-level structure of enzyme systems and how they relate to function, the next step in a systems-biology approach is to understand the location and interactions of these molecules at the cellular level.

Lignocellulose ultrastructure, chemistry, and recalcitrance: Currently we have a limited understanding of the structural properties of plant cell walls that impart strength and resistance to degradation, hindering development of better strategies for biomass deconstruction. For example, lignin greatly reduces the availability of cellulose for conversion into fermentable simple sugars, which therefore lowers the efficiency of biomass conversion to ethanol or other biofuels. Lignocellulose structure is heterogeneous on the nanoscale, challenging structural visualization.

Existing nanoscale-imaging techniques, such as electron microscopy, have proven difficult for artifact-free imaging of biomass materials, due to possibly invasive sample preparation in order to meet the electron imaging condition of "thin sample". Thus, new nanoscale imaging approaches are needed to visualize the lignocellulosic network in order to improve its breakdown efficiency.

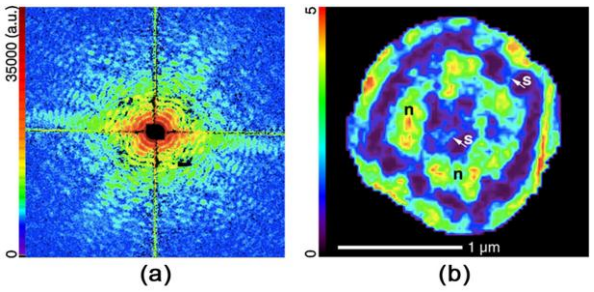


Figure 4. (a) An assembled diffraction pattern of a frozen-hydrated *D. radiodurans*. The total exposure time is 7 min using focused 8 keV x rays. The measured diffraction signals at the edge of the array extend to 30 nm spatial halfperiod in the sample. (b) 2D reconstruction of D. radiodurans. Arrows at ''s'' highlight a diagonal structure that may be the septum; areas labeled with ''n'' indicate possible nucleoid regions [15].

A newly developed X-ray technique, X-ray diffraction microscopy (XDM) is a strong candidate for imaging samples of tens of micron in size and thickness close to their natural state (Figure 4). It relies on the high coherence of advanced synchrotron sources like NSLS-II to image objects at a spatial resolution approaching the diffraction limit of the X-ray photons. The expected resolution with biological samples is 5 to 10 nanometers. In the past, XDM has shown the nanoscale structures of yeast [12-14], bacteria [15] and chromosomes [16]. More recent development of scanning XDM provides unlimited field of view. Thus, the technique can be applied to samples in more natural en-

vironments [17, 18]. With the high brightness of NSLS-II, XDM can provide high resolution imaging technique in structure investigations of biological materials, such as crystalline cellulose within plant cell walls, which are not easily accessible by other existing methods.

It is also important to understand the chemistry behind the recalcitrance of lignocellulose. Enzymatic degradation offers one solution to the challenges of economically separating cellulose from lignin, which motivates the need to study enzymatic degradation *in situ*. Scanning X-ray transmission microscopy (STXM) can provide chemically-specific images of the organic composition of plant cell walls and can be used for assessing the chemical effects of enzymatic degradation [19]. With the high brightness and broadband nature of NSLS-II, X-ray beams can be focused efficiently to sub-30 nm and one can visualize the specific locations within the cell wall where cellulosic enzymes are most active, and determine the nature of the chemical byproducts of the reaction.

Microbial biofilm ultrastructure, composition, and function: Microbial communities are known to significantly alter both physical and geochemical conditions in the subsurface. One biofilm characteristic that is continuously debated is its physical structure or morphology, which varies with the microbial species and other factors. Naturally, different types of biofilm structure can lead to very different biofilm function such as CO_2 sequestration and bioremediation capacity. Much of the research on microbial biofilm structure, both from the mathematical modeling and observational perspectives, has been carried out for microorganisms growing on flat substrates in the absence of a porous medium. Two-dimensional studies have provided very important insight, but biofilm structures adhering in channel-and-cluster geometry, for example, cannot be described well in two dimensions. Likewise, mass transfer will be very different in 3D vs. 2D.

Synchrotron-based imaging provides a unique methodology for quantifying microbial structure in three-dimensional porous media. Conventional X-ray micro-CT provides a spatial resolution of 5 – 10 microns, insufficient to visualize individual microbes in the porous media [20]. However, synchrotron nano-CT and fluorescence tomography are capable of imaging objects in 3D with a spatial resolution approaching 100 nm [21]. Imaging can be performed by attaching a suitable X-ray contrast agent to the biophase, either directly or via metal-labeled antibodies to the microorganisms. Ultimately, this approach will allow visualization of a variety of microbial biofilms in natural soils as well as key contaminants readily imaged by synchrotron X-rays (e.g. U, Tc-99, Sr-90, Cs-137, I-129).

Symbiosis at the plant-microbe interface: Recent studies have shown that endophytic bacteria in the environment of the plant root can enhance the rate of biomass production [22]. However, very little is known about the mechanism of plant growth promotion or about the symbiotic relationship between the plant root and the bacteria. Complex interactions between plant roots, microbes, and soil minerals occur in the rhizosphere – an intricate microenvironment consisting of the few millimeters of soil in the immediate vicinity of plant roots, which is directly influenced by root activity and by the associated microbes. A detailed understanding of the biogeochemical interactions occurring in the rhizosphere has been limited by difficulties in observing these micro- and nano-scale processes *in situ* and in real time. Nanoscale X-ray imaging provides a nondestructive means to study the structure and transport of materials at the plant-microbe interface [23].

One particular issue for biofuels feedstock production on marginal lands (i.e. poor soil fertility) is to understand how endophytic bacteria assist plants in coping with nutrient-limited soil conditions (e.g. Fe deficiency). Iron is needed for photosynthesis; however, it is practically insoluble in most soils and natural waters and is often the "limiting" micronutrient for plant growth [24]. In studying the role of endophytic bacteria in Fe acquisition for plants grown on marginal soils, one needs to investigate the heterogeneity in Fe distribution and speciation *in situ* within the rhizosphere (Figure 5).

X-ray fluorescence microscopy (XFM) and fluorescence computed nanotomography (fCNT) are unique and powerful methods to study the 2D and 3D distributions and elemental associations on the (sub)micron scale. In addition, nanobeam X-ray absorption spectroscopy (XAS) can be used to determine the molecular speciation of an element (e.g. Fe) at each pixel of an X-ray fluorescence image. With NSLS-II, biogeochemical processes will be observable for the first time at the sub-cellular level, providing new information on the growth mechanisms, and rapid data rates with advanced X-ray detectors a wide range of root-microbe systems can be examined quickly.

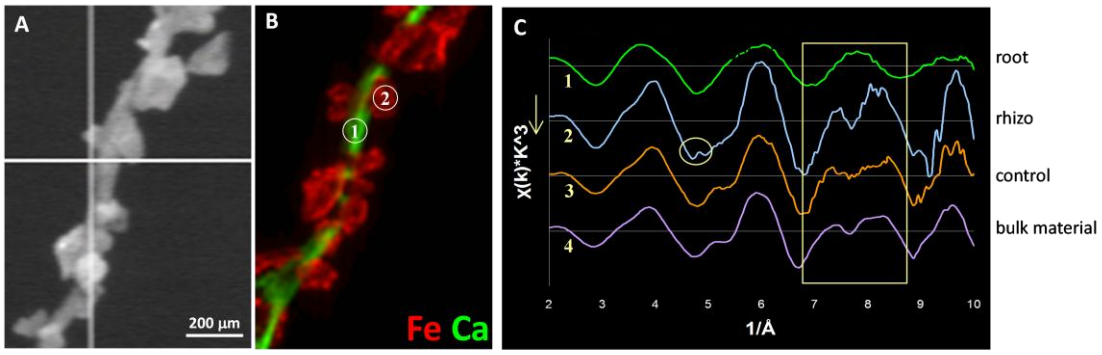


Figure 5. (A) optical image of root and rhizosheath, (B) XFM image of Fe and Ca distribution, and (C) Fe micro-EXAFS data collected from 1) a plant root, 2) associated rhizosheath (adhering soil components), 3) an unplanted control, and 4) the bulk soil material. These spectra show that root activity altered the solid phase speciation in the rhizosphere and roots absorbed aqueous Fe released from the weathering of the 'non-labile' solid phase. Unplanted controls were nearly identical to bulk soil material, indicating natural microbiota did not significantly alter Fe speciation.

Microbe-mineral interface interactions: A great challenge in biogeochemistry is to understand the processes at the microbe-mineral interface [25]. Although the ramifications of microbe-mineral interactions can be seen on a macroscopic scale, to understand the underlying processes, a microscopic approach is necessary. For this, a nanoprobe instrument that can image the interface with very high spatial resolution and concurrently characterize it chemically is extremely helpful [26]. NSLS-II with its unmatched small emittance is uniquely suited for high resolution STXM experiments and will clearly improve our knowledge about these processes. As an example, interactions at the microbe-mineral interface in soils and sediments can influence contaminant behavior. The DOE's Genomics Science program has identified a number of microbial systems that have the potential to remediate environmental contaminants. For example, *Shewanella* and *Geobacter* – two GTL model systems – can enzymatically transform U (VI), which is soluble and moves in groundwater, to U (IV),which is insoluble and precipitates out of the groundwater as abiologically unavailable solid [27]. Conversely, recent studies have indicated that microorganisms capable of biooxidation of U(IV) inhabit uranium-contaminated and uncontaminated DOE sites. These organisms, including strain *TPSY* and *C. millennium*, can utilize U(IV) as the sole electron donor which has the potential to produce mobile U in anoxic environments. In order to predictably model remediation efforts based on U(VI) reduction, it is essential to understand the microorganisms and the physiology of anaerobic, U(IV) biooxidation and the impact of this microbial metabolism on the long term sequestration of U in the environment.

Genetic Control of Metal Ion Uptake, Transport, and Storage: Opportunities to examine how genetic variations in organisms affect their interactions with the environment are driven by the rapidly increasing quantity of nucleotide sequence data provided by DOE's Genomics Science Program. In the future, reliance on plants for energy as well as food will place a strain on the already scarce prime agricultural land, shifting plant production to nutrient-limited soils, and will

require growing crops with fewer inputs of primary macronutrients. Optimizing nutrient acquisition and use will rely on information about the genetic control of metal ion uptake, transport and storage. Functions of the ion transport and storage genes need to be known. Identifying the organelle in which a particular element accumulates will allow us to rapidly target the genes responsible.

Our ability to characterize the function of genes involved in elemental homeostasis requires instrumentation that provides suitably high detection sensitivity at sub-micrometer spatial resolutions. Additionally, a fundamental requirement for ionomic instrumentation is the ability to implement high-throughput analysis to achieve the required functional analysis of the genes and gene networks that directly control the ionome and to integrate results with bioinformatic tools. Synchrotron-based X-ray fluorescence imaging techniques are extremely well suited to studies of these interactions at the cellular and subcellular level, potentially *in vivo*. Focused-beam X-ray fluorescence beamlines at NSLS-II will be able to achieve dynamically variable spatial resolutions from just under 1 μm to tens of nm's while simultaneously providing world-leading photon flux to enable high-throughput analysis with an elemental detection sensitivity at the attogram level. Ultimately the relevant data can be obtained using whole-plant, high resolution X-ray fluorescence computed microtomography (fCMT). Three-dimensional fCMT gives users a non-invasive, spatially resolved and multi-elemental analysis technique that images the metal concentration of specific cell layers and organelles in plants as close to their natural state as possible.

In Vivo Imaging of Cellular Chemistry

Following the tradition of NSLS, NSLS-II will provide the broadest spectrum of synchrotron light available to scientists in the USA. The low energy infrared beams, which are non-ionizing, are ideally suited for *in vivo* imaging without the complications of radiation damage. And even with intense X-rays, techniques like X-ray footprinting will take advantage of the radiolytic cleavage of water to probe structural and functional biology in living cells.

Real-time chemical imaging of biomass conversion: In addition to understanding the structural and functional genomics of cellulolytic bacteria, it is also necessary to elucidate how these processes occur in real time on real-world substrates such as lignocellulose. New, non-invasive chemical imaging methods are needed to address spatially heterogeneous environments.

Synchrotron-based Fourier transform infrared imaging (FTIRI) provides a unique opportunity to image chemical changes in a heterogeneous system at high (micron) spatial resolution as a function of time. For example, the cell wall architecture of *Zinnia elegans* was examined upon acidified chlorite treatment and shown to exhibit a breakdown of lignin and loss of polysaccharides [28]. To date, real-time synchrotron-based FTIRI has only been performed with single-pixel infrared detectors, which generate images in a raster-scan fashion, i.e. one pixel at a time. This approach is exceedingly slow, limiting the time resolution of the experiments to a time scale of hours. However with NSLS-II, infrared light from multiple dipole sources will be used to illuminate a 128x128 pixel array detector, enabling millisecond (or better) time resolution. In addition, pixel oversampling can be employed to improve the spatial resolution by a factor of ten, i.e. to < 1 micron. Lastly, the stability of the electron beam at NSLS-II will provide a 100-fold improvement in signal-to-noise of the data, which will also be necessary to achieve fast time resolution.

Structural dynamics in living cells: Despite the value of *in vitro* experiments, questions often remain as to whether the observations from these experiments truly represent events occurring

within living organisms. *In vivo* observation of biomolecular structure, dynamics and intermolecular interactions is thus a daunting but extremely desirable goal for understanding the natural function of biological molecules. Synchrotron X-ray footprinting is a promising technique for studying structural dynamics in living cells by providing a rapid generation of hydroxyl radicals with short pulses of intense X-rays. As proof of principle, X-ray footprinting was used to probe the structure of ribosomal RNA in frozen *E. coli* cells [29], providing tertiary structural information as well as allowing observation of protein-RNA contacts with nucleotide resolution in living cells.

Living cell systems contain significant amounts of hydroxyl radical scavengers and thus require exposure times of hundreds of milliseconds with the current technology. Such long exposure times may produce radiation-induced alterations that perturb the cellular machinery under investigation, resulting in poor data quality as well as limiting time resolution for observation of dynamics. Short exposure times are essential to drive this technology forward and allow *in vivo* X-ray footprinting of more complex systems and on physiologically relevant timescales. To reach these shorter exposure times, the high flux density of a NSLS-II damping wiggler source will enable the use of transient submillisecond or single-digit millisecond pulses, revolutionizing *in vivo* X-ray footprinting.

Multi-Modal and High-Throughput Technologies

It is becoming increasingly clear that understanding the complexity of biological systems requires multi-modal exploration. Thus, the next generation of scientific user facilities will need to provide integrated approaches that accommodate a wide range of complementary techniques. Thus, it is important to emphasize that the wide range of techniques afforded by NSLS-II will enable multi-technique integration and cross-disciplinary approaches to doing science at the facility. Specifically, NSLS-II is planning on achieving this mode of research through a "biology village" environment, which will strategically locate beamlines for scientific interaction and take advantage of programmatic synergies through shared equipment, technology, and human resources. By jumpstarting today, NSLS beamlines are working together approach several angles of a scientific problem by using complementary techniques on the same sample. To mention a few, MX and SAXS have been combined to understanding the structure and function of a zinc peptidase [30]. SAXS and X-ray footprinting were used together to study the structure and folding of the tetrahymena ribozyme [31]. MX and XAS have been combined to demonstrate the photoreduction of ferric myoglobin nitrate [32]. STXM and FTIR spectromicroscopy have been combined to study the organic composition and distribution of interplanetary dust particles [33]. FTIR and XFM have been used in tandem to examine bacterial reduction of chromium [34] and XFM and XRD together have shown the distribution and speciation of iron in the rhizosphere of *Alyssum murale* [35].

In addition to a multi-technique approach to characterizing complex biological systems, NSLS-II will also make available high-throughput technologies for rapidly correlating genomics with structural and functional information. As one can imagine, the tremendous amount of genetic information that has been obtained through the Genomics Sciences Program has generated a huge number of variants to be screened and characterized. Without advanced photon sources, detectors, computers, robotics, and data collection/analysis routines, a high-throughput approach would not be possible. Today, high throughput technologies are becoming widely used in MX [36], SAXS [37], and XAS [38]. At NSLS-II, it is expected that these approaches will also be extended to the imaging beamlines, where the high brightness of the source will permit, for the first time, rapid tomographic (3D) imaging at the nanoscale.

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