

Working Group on Biosciences

Chair: Graham Fleming, University of California, Berkeley

1. Overview of the Biosciences

Research in the biological sciences at the ALS has undergone explosive growth in the past year, with the percentage of users rising from about 4% to about 20% in the period from September 1997 to March 1998. This increase is due in large part to the onset of operation of the Macromolecular Crystallography Facility (MCF), which is using Beamline 5.0.2 at the ALS, as a national user facility for biological crystallography (see Section 2 below), but also to recent scientific breakthroughs using x-ray microscopy to localize subcellular entities at Beamline 6.1.2.

Bioscience research at the ALS is concentrated into three areas: crystallography, microscopy, and spectroscopy. These fields form a set of complementary approaches to study living organisms from the molecular to the cellular levels. Crystallography is used to determine the atomic-resolution, three-dimensional structures of proteins and nucleic acids—the building blocks of life—as well as complexes of these molecules, the interactions of which give rise to biological processes. Microscopy allows us to find the where these biomolecules are localized in the cell and visualize the cell's overall organization. Spectroscopy yields information on the chemistry of these molecules, especially the chemistry of metalloproteins that act as enzymes.

Following the division of bioscience research at the ALS, the Working Group on Biosciences comprised three sub-groups: Protein Crystallography, Soft X-Ray Microscopy, and Biological and Chemical X-Ray Spectroscopy. Here we present separate reports for each of the sub-groups.

2. Protein Crystallography

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2.1 Opportunities in Protein Crystallography

In many respects, protein crystallography is a mature but constantly evolving field. Exciting biology is being done daily by the application of x-ray crystallography to determine macromolecular structures. At the same time, crystallography has its own scientific frontiers. The sub-group on Protein Crystallography pointed to five in particular.

2.1.1 Structure Determination at Very High Resolution

As improvements in molecular biology, purification, and crystallization take place, there has been an increase in the number of protein structures that can be solved to ultrahigh resolution (better than 1.0 Å). Higher resolution results in a more accurate determination of atomic positions, thus leading to an increased understanding of the molecular mechanisms of enzyme catalysis, ligand binding, etc. In some cases, hydrogen atoms, which are important in the many catalytic mechanisms, can be seen. Obtaining higher resolution also means that more experimental data are available by means of the increased number of reflections present, therefore improving the crystallographic-refinement capabilities by increasing the observations-to-parameters ratio. Also, direct methods can be used to solve the phase problem in some cases if adequately high resolution is obtained.

2.1.2 Structure Determination from Microcrystals

The successful routine determination of structures from microcrystals would dramatically increase the number of macromolecules available for crystallographic study. Membrane proteins are a classic example of molecules difficult to crystallize; they are likely to be available (if at all) only as very small crystals. Microcrystals are also the likely first products of proposed robotic systems for protein crystallization. The data from microcrystals can only be collected with the use of synchrotron-radiation sources.

2.1.3 Studies of Large Macromolecular Complexes

Prominent examples of macromolecular complexes include ribosomes and multiprotein or protein/nucleic-acid complexes. Such complexes often represent the most fascinating frontiers of cell biology. Notably, such complexes will not generally be revealed by structural-genomics projects (about which, more later), which will likely deal with individual proteins, separately expressed and crystallized. It is in the specific interactions of biomolecules that biological processes are activated and modulated, necessitating the structure determination of these molecules and their complexes for a complete understanding of these processes at a molecular and cellular level.

2.1.4 Determination of Large Numbers of Structures in Coordinated Projects

Structural-genomics projects propose to characterize a significant fraction of the proteins coded by an entire genome. Such ambitious plans depend absolutely on robotic expression and crystallization, on speedy and accurate data acquisition, and on automated structure determination and refinement. Another example of coordinated projects is iterative structure design, in which rapid feedback of structural information for a

given molecule is used to direct subsequent structural modifications as part of, say, pharmaceutical development, and to influence the priorities with which subsequent structures are examined.

2.1.5 Time-Resolved and Other Mechanistic Studies

Time-resolved studies currently span time scales from minutes to nanoseconds, but efforts are under way to push into the sub-picosecond regime. However, these advances will require substantial ancillary equipment, including fast shutters, laser systems, and single-crystal microspectrophotometers. Other examples of mechanistic studies include coordinated studies of hundreds of protein variants, studies of complexes with hundreds of ligands, investigations at a range of pH values, and studies of freeze-trapped intermediates.

2.2 Current and Future Needs

To address these opportunities, as well as the more routine needs of structure determination, it is natural to ask whether current facilities are adequate. The demand for crystallographic facilities is growing so rapidly that current needs may be an unreliable guide to the scale of facilities required over the next three to five years. As a starting point, however, it is necessary to clearly define the current level of demand. Therefore, the sub-group focused its attention on the West Coast. As indicated in the boxed sidebar, available beam time at West Coast facilities appears to be well matched to the *present* needs of “local” crystallographers.

This conclusion may already be inaccurate, however, as several working group members reported significant “oversubscription” of operating beamlines. This additional demand arises in part from a “latent” group of users. In addition to the needs of established, dedicated crystallographers on the West Coast, demand is increasing among protein biochemists who are now growing crystals in their own laboratories. These users typically possess great insight into medical and scientific issues, and involving them directly in the process of structure determination can promote a highly desirable synergy between biochemistry and structural biology. It will also surely reduce the time that elapses between the identification of an interesting target protein and the use of that structural information to drive new biochemical experiments. Involving protein biochemists early on will also ensure that protein samples are crystallized in the most desirable biochemical states (e.g., phosphorylation state, oxidation state, degree of glycosylation). It is also interesting to note that many of the proteins that are medically most interesting do not pose significant hurdles for structure determination. Many are soluble and fall into the size range that is easily accessible with current techniques.

The number of principle investigators (PIs) who fall in this “latent” category is difficult to estimate. But from an informal survey at an institution represented by a member of the sub-group, the number of protein biochemists interested in protein structure determination by x-ray diffraction was conservatively estimated to be three times that of crystallographers per se. At the same time, the number of projects within these labs is typically limited to one or two at a time. Accounting for the beam time required by this user group would at least double the beam time estimates based on the core of West Coast protein-crystallography groups.

The opportunities presented in Section 2.1 suggest a qualitative change in the way protein crystallography will be carried out in the future. We cite three examples in particular which will require dramatic increases in the resources now available: (1) structural genomics, such as the *Methanococcus jannaschii* project described later in this report, (2) iterative structure-based drug design, and (3) large molecular complexes, such as the ribosome.

Current Demand for Beam Time

About 80 principal investigators pursued macromolecular crystallography in 1997–98 on the West Coast (defined as extending east to the Rocky Mountain states and Texas and including Alberta and British Columbia)—a number that encompasses industry, academia, and government. (We assume that the use of other U.S. facilities by West Coast crystallographers is balanced by the influx of other crystallographers to West Coast facilities.) To project current needs, we made three assumptions:

1. Each PI has an average of five projects active at all times. A range from two to fifteen is likely, with a peak in the range of three to six; only a very few labs have more than eight active projects.
2. Each structural project requires an average of ten data sets (three or four are actually the minimum for *de-novo* structure determinations). Certain structural sub-projects require only one good data set (e.g., a single protein-ligand complex); other projects may require 1000 data sets.
3. Each data set requires an average of 100 images (more if fine λ -slicing is used and if 180° of data are required) or about 12 hours of scheduled beam time. We take 1000 images to be the maximum (e.g., 180° at 0.2° intervals) and about 20 the minimum (e.g., 60° range with 3° oscillations).

The typical facility runs 5000 hours per year—about 220 scheduled user days. Thus, one station can accommodate about 10 PIs, and for the 80 PIs who are assumed to work exclusively at West Coast synchrotrons, eight stations are needed. In fact, seven stations are currently operational (or soon will be): Beamlines 5.0.2 and 5.0.1 (operational within 18 months) at the ALS, and Beamlines I-5, VII-1, IX-1, IX-2, and XI-1 at the Stanford Synchrotron Radiation Laboratory (SSRL). Consequently, there *presently appears* to be a rough balance between station supply and demand, although there is a backlog of interesting projects at all facilities. This conclusion agrees with the 1997–98 BioSync Report (available at www.ornl.gov/hgmis/biosynch).

2.3 The Advanced Light Source: Successes and Opportunities

2.3.1 The Macromolecular Crystallography Facility

Beamline 5.0.2, the first beamline of the ALS Macromolecular Crystallography Facility (MCF), collected its first diffraction patterns from protein crystals on 18 September 1997. Optimized for multiwavelength anomalous dispersion (MAD) experiments, the MCF was initially used to record several data sets from test crystals. These tests, conducted on lysozyme and on a platinum derivative of lysozyme, gave excellent statistics (values of R_{symm} from 3.5% to 4%) to a resolution of 1.6 Å, successfully demonstrating the capabilities to collect and process data accurately.

The subsequent initial user run of the MCF was extremely productive, with users from academic, industrial, and national laboratories successfully collecting MAD data, diffraction data from micro-crystals, and “conventional” diffraction data with extremely rapid throughput. In all, over 60 users

from 18 different groups collected data at the MCF between 15 November 1997 and 31 January 1998. User demand has accelerated. As of 12 April, 1998, the number of users had grown to 94. This figure represents 25 different groups from a strong mix of universities, industry, and national laboratories. Current requests for beam time indicate demand will substantially exceed the time available during the upcoming operational period. MAD results included data from ApoE, a protein important in cholesterol metabolism (to 1.8 Å), and from MJ0577, a hypothetical protein from the genome of *M. jannaschii* (to 1.6 Å).

Data from several microcrystals were also collected. In the most dramatic case, scientists from Roche Biosciences collected an entire data set to 1.8-Å resolution from a frozen microcrystal measuring approximately $40 \times 30 \times 5 \mu\text{m}$. The structure of this drug-design target-inhibitor complex has subsequently been determined by the same group. Both the Scripps Research Institute and Genentech have utilized the high-throughput capability of the MCF for rapid data collection. Also, Barry Stoddard's group at the Fred Hutchinson Cancer Research Center collected 1.6-Å data from Ppo-I, an intron-encoded endonuclease having a base-pair specificity of 20, in less than two hours (see Figure 1).

Initial results from the MCF are thus extremely encouraging, fully justifying its description as a world-class facility. Furthermore, an additional order-of-magnitude improvement in brightness is expected when final optics are installed (replacing the current temporary mirrors) and the beamline is fully optimized.

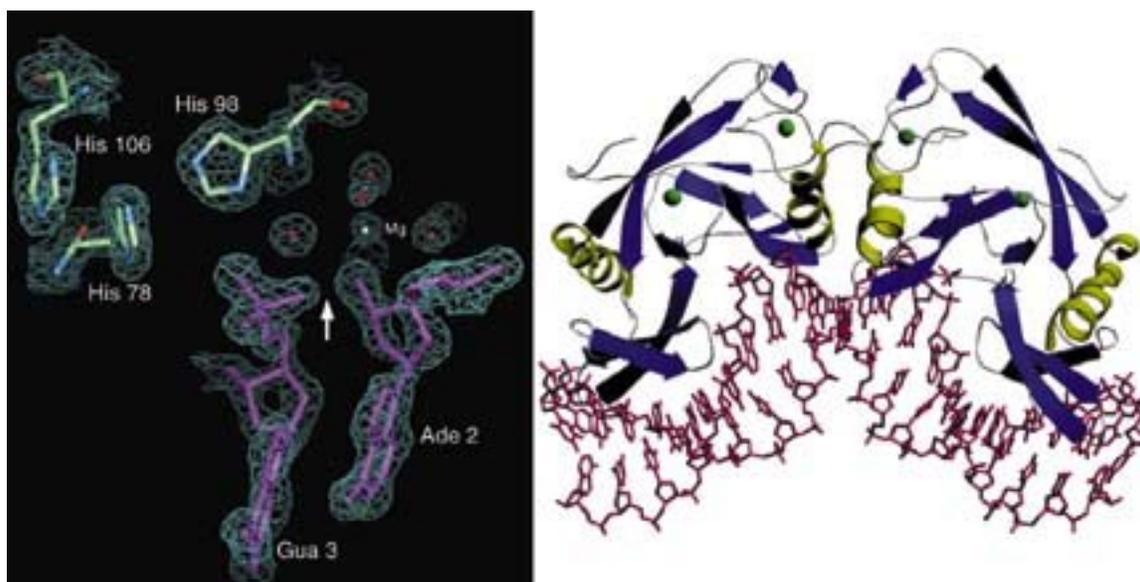


Figure 1. Ppo-I, an intron-encoded endonuclease that recognizes and cleaves 20 base-pair sequences with a high degree of specificity. The structure was solved by Barry Stoddard, Melissa Jurica, and Karen Flick of the Fred Hutchinson Cancer Research Center with data from the Macromolecular Crystallography Facility extending to 1.6-Å resolution. (a) Active site with electron density of Ppo-I. (b) Ppo-I dimer bound to DNA-recognition site. [Figure courtesy of Barry Stoddard, Fred Hutchinson Cancer Center.]

2.3.2 New Opportunities

Within a few short months, the MCF has thus fully confirmed the suitability of the ALS for protein crystallography, raising natural questions about how the most exciting opportunities in the field can be mapped onto the capabilities of this facility. In the course of its discussions, the Protein Crystallography sub-group pointed to six opportunities, each an expression of an exciting opportunity in protein crystallography, judged especially appropriate to the physical capabilities and resident expertise at the ALS.

2.3.2.1 Structural Genomics

In recent years, large-scale sequencing projects have begun to provide gene sequences from a number of organisms and, in some cases, the complete inventory of the organism's genetic information. The first complete sequence was published in 1995, and within two years, ten additional organisms had been completely sequenced. This knowledge will certainly advance our understanding of biology and medicine, especially after the structure and function of the genomic products are determined. To take this next essential step, large-scale structure-determination projects must be combined with the sequencing efforts to look for novel protein-folding patterns, to understand the function of genomic products, and to uncover proteins of previously unknown function. As an important stride in this direction, the fully sequenced microbe, *Methanococcus jannaschii*, is being used in a pilot study for structural-genomics research. Sung-Hou Kim at UC Berkeley and LBNL and his colleagues have chosen several gene products from this organism—some with known homologues and some without—and have begun to determine their structures. Early results from the MCF have already allowed the role of a “hypothetical” protein to be tentatively identified from its structure alone.

2.3.2.2 Iterative Structure-Based Drug Design

The process of drug discovery can be greatly accelerated by knowing the structure of the target molecule, which can allow the design and synthesis of compounds that activate or inhibit the target. This process of rational drug design usually requires an iterative procedure in which trial compounds are designed to fit the active site or ligand-binding region, crystals are obtained of the target-compound complexes, the structures of the complexes are solved and analyzed, and the results then used in the design of the next round of trial compounds. Since many steps and many compounds are needed before yielding a compound with high binding affinity and the needed specificity, several structures need to be solved. This calls for high-throughput crystallography beamlines, which offer both speed and high data quality.

2.3.2.3 Robotic Expression and Crystallization

Expressing, purifying, and crystallizing proteins for crystallography are tremendously demanding of research time and research dollars—far more so than the actual collection and analysis of x-ray data. Crystals are typically grown one at a time, and efforts are made to improve the crystal-growing conditions, one variable at a time. Fortunately, this procedure is ideal for employing the kinds of automated systems recently developed for DNA sequencing, directed evolution, and combinatorial chemistry. By exploiting the high-throughput capabilities of the ALS, an automated approach to protein expression, purification, and especially crystallization has the potential to turn drug design and protein engineering into efficient iterative processes.

2.3.2.4 Membrane Proteins

Although 30% to 40% of the proteins encoded by the genomes of organisms are membrane proteins, fewer than 1% of the structures solved to date fall into this class. There are several reasons for this situation: (1) Membrane proteins exist simultaneously in three distinct environments (extracellular, intracellular, and membrane-embedded), which are difficult to duplicate together in crystallization media; (2) overexpression in heterologous systems rarely produces large quantities of correctly

folded protein, and very few can be purified in adequate quantities from native sources; and (3) once out of the membrane, these proteins are frequently unstable. Further, crystals of membrane proteins usually diffract rather weakly and have large unit-cell parameters. Thus, it is critical for membrane-protein projects to have access to bright synchrotron-radiation beamlines if they are to succeed. During screening, synchrotron radiation is needed to assess the quality of membrane-protein crystals, thus providing guidance in efforts to improve crystallization conditions. And when studying typically small crystals with large unit cells, the ability to achieve a high degree of collimation and a small spot size is necessary for obtaining high-resolution data.

Membrane proteins are responsible for all forms of cell-cell signaling, sensing of external stimuli, and the transport of molecules across the apolar cell membrane. Owing to their biomedical importance, it is critical that synchrotron-radiation resources be dedicated to investigating this challenging family of proteins. The ALS benefits from a large local concentration of researchers with appropriate expertise who might serve as the basis of a scientific program in the structural biology of membrane proteins.

2.3.2.5 Large Molecular Complexes

An accurate and comprehensive understanding of most, if not all, cellular processes requires the atomic-resolution structure determination of the biomolecules involved. Many important biological problems can be understood only in the context of their interactions with other molecules. In this regard, the use of synchrotron radiation has become ubiquitous and vital. The use of synchrotron-based crystallography to determine atomic-resolution structures of large biomolecular complexes—ribosomes, multi-subunit enzymes, transmembrane proteins, DNA-binding assemblies, ligand-receptor complexes—enables an extension of our understanding of molecular and cellular biological processes to the level of atomic resolution. An example is the ribosome, a 2.5 megadalton complex of three RNA molecules and over 50 protein molecules that translates genetic information into the production of proteins. Harry Noller and his colleagues from UC Santa Cruz, working with scientists from the MCF at the ALS, have made progress recently toward solving the structure of this complex. Figure 2 shows the structure of the 70S ribosome at

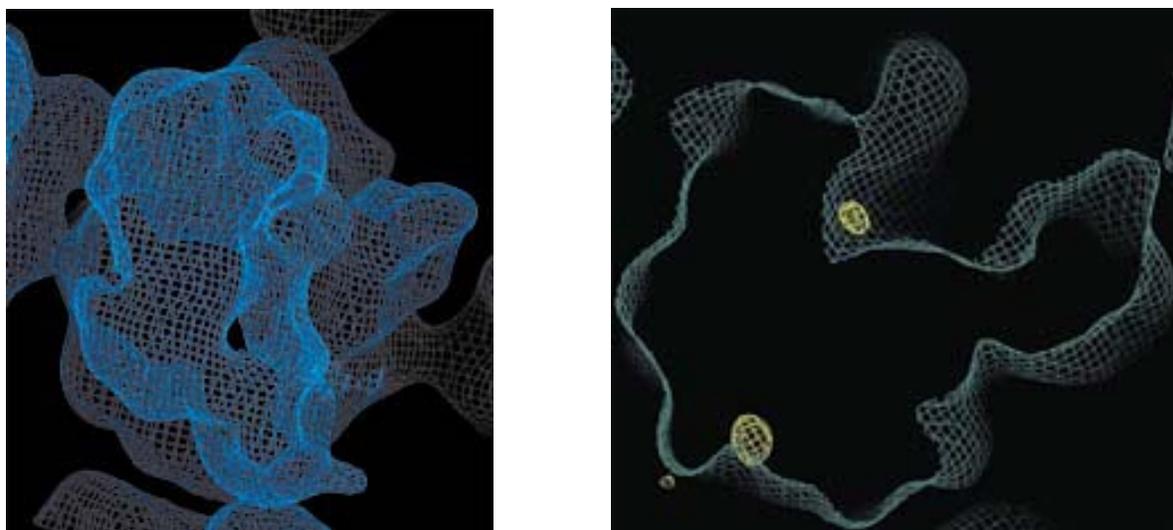


Figure 2. (left) Electron-density map at 30-Å resolution of *T. thermophilus* 70S ribosome contoured at 1 σ . (right) A difference Fourier map contoured at 4 σ showing two sites from a heavy-atom derivative superimposed on the 30-Å map. Phases for both maps are from a molecular-replacement solution using a cryo-EM model of the *E. coli* 70A ribosome. [Figure courtesy of the Henry Noller group at the University of California, Santa Cruz.]

30-Å resolution, obtained using phases derived from electron microscopy. Such projects as this represent unique opportunities for high-brightness synchrotron sources such as the ALS, which are necessary if one is to see diffraction to even moderate resolution, and tuning the energy allows the use of anomalous-diffraction methods for obtaining phasing information.

2.3.2.6 Low-Energy Diffraction

The ALS is especially well suited to utilize x rays in the 2- keV to 8-keV range to collect anomalous data from elements with absorption edges in this region, including uranium, calcium, potassium, and sulfur. The uranium M_V edge would be especially useful, since the anomalous signal at 3.55 keV is large enough to phase large complexes, such as the ribosome. Experiments at these energies require special end-station instrumentation to minimize absorption and to detect the x-rays scattered at large Bragg angles.

2.4 Recommendations

Considering the opportunities for the broad field of protein crystallography, the needs of its practitioners, and the capabilities of the ALS, the Protein Crystallography sub-group identified five specific scientific thrusts for the future development of the facility.

2.4.1 Complete Beamline 5.0.1

A continuing priority for the ALS should be the completion of Beamline 5.0.1, which should roughly double the facility's current capacity for protein crystallography. Beamline 5.0.1 is under construction as a side station from the wiggler, using an asymmetrically cut, curved-crystal monochromator. The wavelengths available will extend from 0.95 Å to 1.6 Å, and the total flux on the sample will be approximately the same as on beamline 5.0.2. This project is jointly funded by LBNL, Amgen, Roche Biosciences, UC Berkeley, and the Lawrence Livermore National Laboratory. The beamline is scheduled to begin operation in August 1999 and will be utilized for monochromatic-crystallography experiments.

2.4.2 Encourage Structural Genomics Research as a Vital Component of the ALS Scientific Program

A detailed time analysis has shown that extraordinary resources will be required to complete the project of solving the structures of all the proteins from a single microbe, such as *Methanococcus jannaschii*. The need to pursue structural genomics projects for a number of such organisms underscores two pressing imperatives: an increase in the beam time available to these projects and enhanced efficiencies in the expression, purification, and crystallization of the genomic products. The following two recommendations address these two issues.

2.4.3 Add Three Superbends

As part of an research and development program for extension of the capabilities of the ALS across the whole energy spectrum, a program was started more than three years ago aimed at producing superconducting bend magnets (superbends) to replace three of the 36 dipole magnets of the ALS. Each magnet can have up to four beamlines. The field at the tangent points is 5 T and therefore increases the critical energy of the superbend synchrotron radiation by a factor of four over a normal bend magnet. The higher critical energy increases the figure of merit for crystallography performance at 12 keV by a factor of 10. For typical crystals (0.3 mm in size), an ALS superbend beamline will give, for example, 2.9 times the useful flux of SSRL wiggler Beamline IX. Not only will this give the

ALS outstanding high-energy performance, it will do so at a fraction of the cost of a high-power wiggler beamline. The proposed ALS superbend sources will deliver flux densities of at least 6×10^{12} photons/(sec·mm²), which is approximately equal to the most intense x-ray field strength tolerable to protein crystals (see Section 2.5 below). By comparison, more powerful radiation sources at third-generation synchrotrons (undulators and wigglers) must be attenuated to the field strength of the superbends to avoid damaging the sample crystals. The ALS superbends, therefore, are an optimal x-ray source for most protein crystallography projects.

Since the ALS superbend sources are substantially less expensive than insertion-device sources, they are a cost-effective solution for the future needs of protein crystallography. Equally important, the superbend designs permit the expansion of scientific capability without compromising the other core scientific programs of the ALS; namely, soft x-ray and vacuum-ultraviolet (VUV) applications. They do not encroach upon the straight sections of the ALS, which are valued for their insertion-device capability. There is apparently no “down side” to the superbend sources, and their implementation at the ALS will permit this facility to achieve a balanced growth of science in many areas, simultaneously.

2.4.4 Develop a Robotic System for Expression and Crystallization

It is highly appropriate to exploit the advantages of robotics at the ALS to reduce the manpower needed for protein expression, purification, and crystallization and to open the door to high-throughput investigations of protein structure. A system currently being developed will use nanoliter amounts of protein sample per trial, instead of the microliter amounts now needed. This modification will save precious protein material, and the microcrystals that will result can be effectively used on beamlines at the MCF. In addition, optical-screening methods will be used to reduce the current effort involved in examining one crystallization trial at a time and recording observations in a notebook. By storing and analyzing recorded images, this automated system will allow protein crystals to be produced far more efficiently. In addition, by using this resource to screen and analyze large arrays of conditions critical in the crystallization of many proteins, for example membrane proteins, we can expect valuable clues to emerge for the crystallization of these difficult proteins.

2.4.5 Promote the Development of Pixel Detectors

There is a clear need for advanced detectors for protein crystallography. Experience during the past decade in this field has shown that every advance in detector design has yielded improvements in both the quantity and the quality of crystallographic structures determined on synchrotron sources. Implementation of image-plate (IP) detectors and their subsequent development and improvement made possible several stages of extraordinary advance over the previously used x-ray film. More recently, CCD area detectors have been implemented on synchrotron sources, with consequent further enhancements in spatial resolution, speed, and data quality.

The development of silicon pixel-array detectors (PADs), now under way at Berkeley (in collaboration with UC San Diego), is very important for this field. These detectors are based on direct photon counting using a high-density array of silicon photodiodes, directly connected to an application-specific integrated circuit (ASIC). The detectors have a photon-energy operating range of 4 keV to 15 keV, a typical array size of 1000 × 1000 pixels, a pixel size of 150 × 150 μm², and an x-ray quantum efficiency of greater than 90% over the total energy range. The pixel detector is capable of full array readout in 10 μs, with a duty cycle (data acquisition to readout) of better than 99%.

Once they are developed, these detectors promise to increase the speed and accuracy of data collection at synchrotron sources relative to CCD systems. In particular, since the LBNL PAD is a digital

counting system, not an analog integrating system, the statistical precision of data collected on the PAD will be limited only by Poisson counting statistics, not by the imprecision and error of signal transfer steps within the instrument. The improved statistical precision of the PADs will have greatest impact on high-resolution data, such as those recorded in studies of charge densities in strongly diffracting crystals, but it will also enhance the quality of data recorded from weakly diffracting crystals. The PAD system also exhibits greater dynamic range than CCD systems. This will enhance the ability of a beamline to elicit high-quality MAD phasing and will improve the ability to acquire, simultaneously, the strong diffraction at low-resolution limits and the weak high-resolution terms.

2.5 Appendix: Flux-Density Limits in Protein Crystallography

Recent observations at the Advanced Photon Source (APS) at Argonne National Laboratory and European Synchrotron Radiation Facility (ESRF) in Grenoble indicate that frozen crystals can tolerate 3×10^{11} to 5×10^{11} photons/sec (12-keV x rays) onto an exposure field defined by a 200- μm square aperture (0.04 mm^2)—equivalent to a flux density of about 1×10^{13} photons/(sec·mm²). Substantially greater flux than this causes the onset of rapid, irreversible damage to the crystal sample and loss of diffracting power. Protein crystals vary, so this limit is not a rigid one, but at twice this flux density, all crystals suffer rapid deterioration in the beam. Below this value, crystals (frozen and maintained at 100 K) survive for hours. The explanation appears to be sample heating. Observations suggest that, at flux densities below about 1×10^{13} photons/(sec·mm²), thermally generated strain on the crystal lattice does not ruin the samples nor is their temperature rise sufficient to increase the mobility of radiation-induced free radicals—the presumed agent of most crystal damage. Rather, the crystals apparently reach an equilibrium state (in which temperature and thermal gradients permit heat deposition and extraction to be balanced) below any threshold of sample damage.

Bend-magnet sources at SSRL and the National Synchrotron Light Source (NSLS) do not deliver flux densities sufficient to damage protein crystals, but new insertion devices can exceed the damage threshold. In particular, the NSLS wigglers on Beamlines X21 and X25 the APS wigglers and undulators, the proposed SPEAR-3 wigglers at SSRL, and the ALS wiggler will destroy most protein-crystal samples unless the beams are attenuated or defocused.

To illustrate this point and to underscore the importance of a source intensity below the damage threshold, Figure 3 illustrates a hypothetical situation for a user acquiring MAD phasing data at various beamlines with crystals of various sizes. The assumptions made in this highly schematic illustration are that the user must spend one hour to prepare the experiment, that reading out each data frame requires 4 sec of dead time, and that the user is collecting four 250-frame data sets (one at each of four energies). We further estimate that an integrated x-ray dose of 1.0×10^{10} photon-mm on the sample is required to provide a “good” data frame (a lower dose will require less time, but at the cost of lower quality data; a higher dose will produce better data, but frames must be integrated for longer times). The shaded area illustrates the limits to flux density—in this region, the flux will damage the crystal sample. In the area below the damage threshold, as the flux increases, it takes progressively less exposure time to record a good data frame until, at about 10^{11} photons/sec, the total exposure time becomes a negligible fraction of the total time of the experiment.

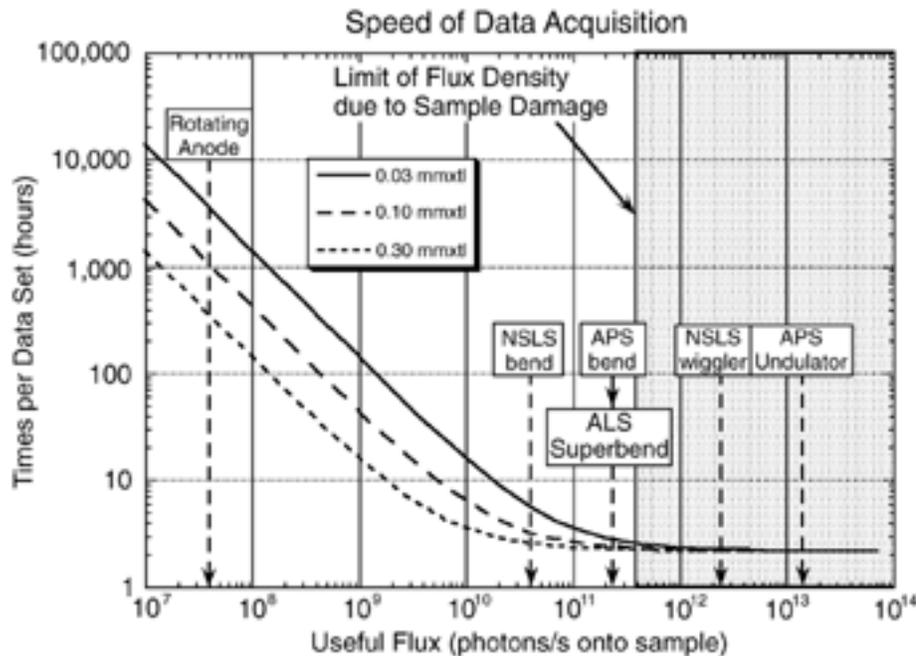


Figure 3. This highly schematic diagram illustrates that, under the assumptions made in the text, there is a limit to the useful flux density due to sample damage, so that there is no benefit to exceeding the flux-density limit. [Figure courtesy of Edwin Westbrook, Argonne National Laboratory.]

3. Soft X-Ray Microscopy: Advances and Opportunities in Cell Biology

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3.1 Introduction

In contrast to protein crystallography, which is a mature scientific field, x-ray microscopy is a rapidly evolving technique still in its infancy. New results suggest a promising role for soft x-ray microscopy. Short-wavelength x-ray imaging provides a spatial resolution more than five times better than that of visible light microscopy (See Table 1). Further, practical techniques have now been demonstrated that will open the door to investigations of keen interest to cell biologists. The Soft X-Ray Microscopy sub-group (1) discussed the exciting new technique for determining protein localizations in cells using soft x-ray microscopy, (2) identified specific biological questions to be addressed using x-ray microscopy, and (3) identified additional technological developments required for the continued advancement of x-ray microscopy in the biological sciences.

The sub-group concluded that the ALS, because of its brightness in the soft x-ray spectral region, is unique in the nation and therefore should be used to promote a program of national leadership and international competitiveness in soft x-ray microscopy.

Table 1. Comparison of features of biological microscopy techniques.

	Visible Light (Confocal) Microscopy	Electron Microscopy	Soft X-Ray Microscopy
Resolution	200 nm	0.1 nm to 0.3 nm (about 1 nm for noncrystalline biological material)	30 nm to 50 nm
Specimen Thickness	50 μm	Standard: 50 nm to 100 nm High Voltage: < 1 μm	10 μm
Hydration State	Hydrated	Dehydrated Hydrated (Cryogenic Only)	Hydrated
Physiology	Live	Dead	Dead

3.2 Challenging Questions in Cell Biology

X-ray microscopy is poised to make a major contribution to the understanding of cellular structure and function. The Soft X-Ray Microscopy sub-group discussed several central topics in cell biology that are ripe for investigation and identified particular ones that should be pursued further. The unique capabilities at the ALS make it essential to pursue these in Berkeley.

3.2.1 Structure/Function Analyses of the Cell Nucleus

Over the past two decades, nuclear architecture has been investigated using complementary microscopy techniques, including transmission electron microscopy (TEM) and, more recently, confocal microscopy. TEM techniques have enabled us to decipher the organization of nuclear domains and higher levels of chromatin arrangement, and confocal microscopy has permitted us to study the dynamics of nuclear components in the entire nucleus. In addition, immunolabeling and *in-situ* hybridization techniques applied using both types of microscopy have underscored the importance of

nuclear organization for nuclear function. The possibility that higher levels of chromatin arrangement and the compartmentalization of nuclear function are related to an underlying structure has been raised. Twenty-four years ago, TEM studies demonstrated that an insoluble, organized structure referred to as the nuclear matrix (NM) was left behind after the removal of soluble proteins, as well as most of the chromatin. Numerous components of this NM fraction, either permanent or transitory, have now been identified. However, the existence of an organized NM that could play a role in nuclear and, ultimately, cellular function is still controversial. This controversy is based on the fact that it is necessary to apply harsh preparation procedures to samples in order to visualize the NM in the electron microscope. The ability to simultaneously image chromatin components and NM components using soft x-ray microscopy in hydrated, non-extracted, and non-sectioned cells (see Figure 4) will bring an essential insight to the study of the interrelationship between nuclear organization and cellular function.

3.2.2 Cell-Extracellular Matrix Interactions and Signaling

Over the last 15 years, studies by LBNL researchers have shown that a reciprocal dialog exists between the mass of fibrous and globular proteins outside the cell (the extracellular matrix or ECM) and the inside of the cell. Recent data indicate that the disruption of this dialog can lead to tumor formation. Restoration of the cell's delicate microenvironment in turn can revert tumors to cell clusters resembling the normal phenotype. Thus a novel strategy to control unchecked cell growth and function has been elucidated. The implications of these discoveries are enormous, both as a tool for the further elucidation of normal cell growth and differentiation and as an assay to screen libraries of compounds of potential therapeutic value against cancer. This is one dramatic example where soft x-ray microscopy can shed additional light on basic molecular mechanisms since the x-ray microscope has the capability to view the cell-cell and cell-ECM interactions at higher resolution than light microscopy.

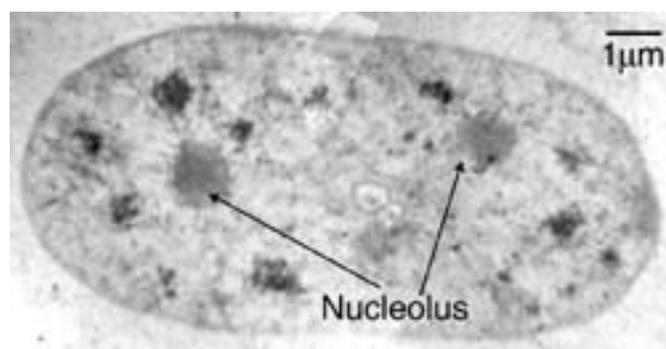


Figure 4. Nucleus of human mammary epithelial cell (S1-50) labeled for splicing factor. This image was obtained from a whole, hydrated cell. Structural details of the nucleus, including nucleoli can clearly be identified in addition to the label, which appears dark. [Figure courtesy of C. Larabell, D. Hamamoto, S. Lelièvre, and W. Meyer-Ilse, LBNL.]

3.2.3 Host-Parasite Interactions

The ability of soft x-ray microscopy to examine thick cells provides a unique opportunity to examine host-parasite interactions. This has been demonstrated at the ALS by the studies of Cathie Magowan, Werner Meyer-Ilse, and colleagues at the LBNL Center for X-ray Optics, who examined the way in which the malaria parasite (*Plasmodium falciparum*) develops in human red blood cells. The ability to examine parasites within the host, without risking the artifacts that accompany embedding and sectioning protocols, provides a powerful tool for understanding these important cell-cell interactions. Unique views of the infected blood cells revealed new information about the contribution of the membranes of red blood cells to parasite development that were not previously detected using other forms of microscopy.

3.2.4 *In-Situ* Hybridization Using X-ray Microscopy

Fluorescent *in-situ* hybridization (FISH) is a widely used method to assay gene expression using light microscopy. The method has been applied in a variety of situations where the tissue distribution of gene transcripts needs to be determined with a reasonable degree of spatial resolution. It has also been used to compare the distribution of gene transcripts in normal and tumor cells. The ability to obtain such information using the increased spatial resolution of x-ray microscopy is of the utmost importance. Development of an *in-situ* hybridization technique for x-ray microscopy (XISH) that is based on silver-enhanced, gold probes is in progress at LBNL by Carolyn Larabell and colleagues and will be a significant breakthrough for the fields of cell and molecular biology.

3.2.5 The Origin of DNA Replication

Visualization of the origin of DNA replication with its associated complex proteins is of great interest for the understanding of basic cell functions. The pathways of these complexes during the replication process have never been seen. The structures of interest are larger than nucleosomes, which are about 11 nm in size, and will be in the reach of soft x-ray microscopy. They would be made up of many proteins and DNA, and new ideas might be needed for “staining” the complexes to take advantage of the x-ray imaging. Antibody-labeled gold markers could be used as a starting point, since antibodies of most of the components of the replication process are available.

3.2.6 Sperm

X-ray microscopy still has a great deal to offer to an analysis of normal sperm development and to characterizing the biochemistry (and different classifications) of male infertility. Chemical imaging by means of near-edge x-ray absorption fine-structure spectroscopy (NEXAFS, also known as XANES) should help identify the fate of some transient parts of proteins that disappear during the course of sperm-cell maturation (leader sequence of protamine 2). The procedure would be to (1) check for the normal progression of this process in infertile samples or identify (and investigate) types of infertility that result from improper ratios of protamine 1 and 2 or improper displacement of histones by protamines and (2) identify cells produced by infertile males that do not contain properly disulfide-bonded protamines. With the ability to measure the mass of the nucleus, once we are able to do three-dimensional imaging, we could use x-ray microscopy to determine the density of packing of chromatin inside the nucleus as an indicator of improper chromatin condensation.

3.3 Environmental Biology

The ability to study hydrated systems with high spatial resolution, combined with the ability to map certain elemental and chemical properties of the sample, makes soft x-ray techniques very valuable for environmental biology. Many species of bacteria can oxidize or reduce transition-metal ions, especially iron and manganese. Imaging with photon energies either above the absorption edges of the transition metal or in the water window for carbon-rich microorganisms provides specific contrast of the metal-containing particles or the organic material. Only soft x rays provide the ability to image these processes with the necessary resolution in the hydrated state.

3.4 Experimental Opportunities with X Rays

Use of shorter wavelength radiation, such as electrons or x rays, provides significantly better spatial resolution than visible-light microscopy. Exploring the utility of chemical or elemental imaging by combining spectroscopy with high spatial resolution will give x-ray microscopy an enormous edge in usefulness. Chemical or elemental imaging cannot be done at the level of whole cells by electron microscopy or other conventional techniques, yet several of the questions discussed above will have chemical/compositional changes associated with it.

The penetration depth of x rays in matter varies significantly throughout the x-ray photon-energy range. We will therefore discuss soft x rays (below about 2.5 keV) and hard x rays (above about 2.5 keV) separately. The transition region between soft and hard x rays is indistinct, and lately also called intermediate x rays.

3.4.1 Soft X-Ray Microscopes Provide High Spatial Resolution

Among all types of electromagnetic radiation, only soft x rays provide a spatial resolution exceeding that of visible-light microscopes for thick samples. Most biological work uses x rays in the water window, that is the range of photon energies between the K-shell absorption edges of carbon (284 eV) and oxygen (543 eV), where organic matter absorbs nearly an order of magnitude more than water. For x-ray energies just below the oxygen edge (e.g., 517 eV, 2.4-nm wavelength), the penetration depth of x rays is also ideally suited to image whole cells with a thickness of a few microns. Contrast in the water window can be either from absorption or phase shift. Microtomography with soft x-ray transmission microscopes has also been demonstrated and provides three-dimensional information with unprecedented spatial resolution.

3.4.2 Elemental and Chemical Mapping

High-resolution absorption spectra from any point on the specimen can be obtained by positioning the specimen to the appropriate spot and scanning the monochromator of a scanning transmission x-ray microscope (STXM). Spectra near the carbon, nitrogen, and oxygen absorption edges are rich in structure (NEXAFS), that provides sensitive information regarding local chemistry. By collecting images at several energies in this region, it is possible to map particular components within the specimen. Xiaodong Zhang of the State University of New York, Stony Brook, et al. examined the distribution of protein and DNA, thereby demonstrating this capability in mammalian sperm nuclei. Figure 5 shows the spectra of different amino acids at the carbon K edge, as obtained with the STXM at the NSLS.

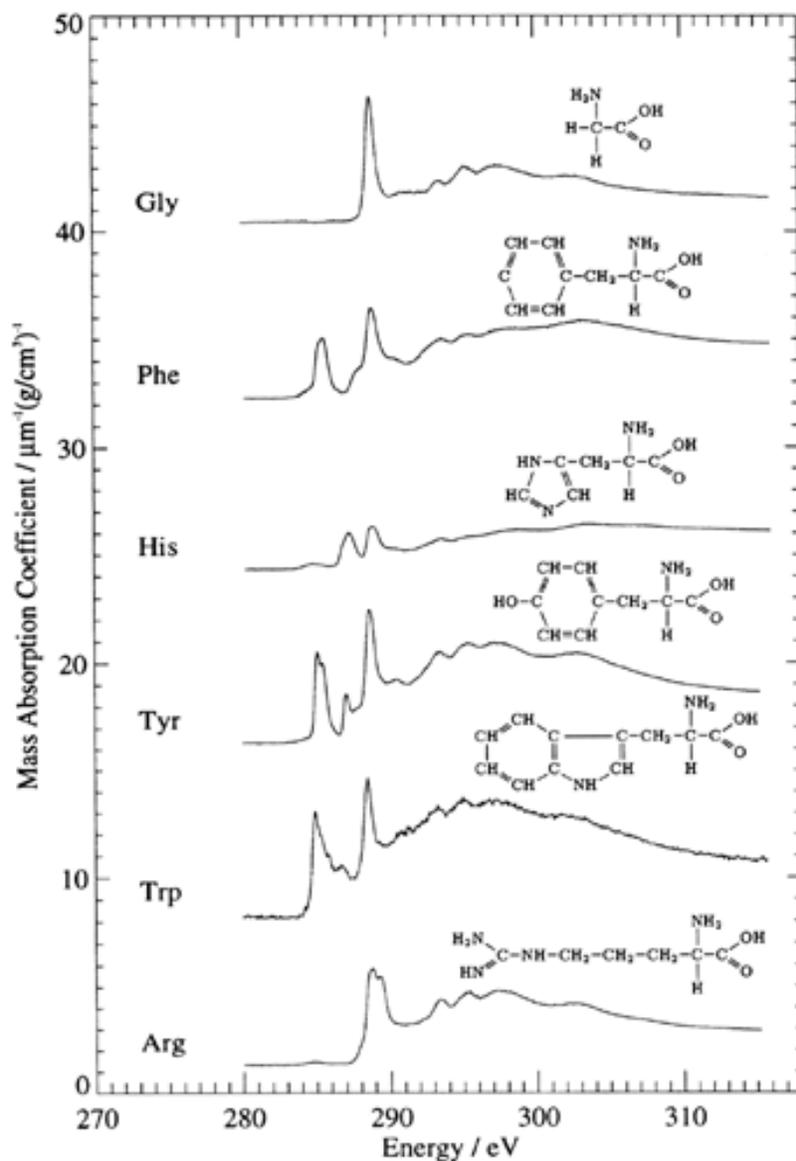


Figure 5. Carbon-edge x-ray absorption near-edge spectra and chemical structure of the six amino acids: glycine (Gly), phenylalanine (Phe), histidine (His), tyrosine (Tyr), tryptophane (Trp) and arginine (Arg). The spectra have been displaced vertically by multiples of eight units. [Figure courtesy of J. Boese, State University of New York, Stony Brook; data from Beamline X-1A at the NSLS.]

3.4.3 Hard and Intermediate X Rays: Spectroscopy, Fluorescence, and Phase Contrast

X-ray fluorescence microscopy and spectromicroscopy with harder x rays have many unique and attractive qualities. In particular, these techniques can provide information on the spatial distribution, oxidation state, chemical environment, and chemical transformations of trace elements.

Although harder x rays do not provide sufficient absorption contrast for high-resolution transmission x-ray microscopy and phase contrast is also reduced when used with harder x rays (but to a lesser degree), the presence of K-shell absorption edges makes harder x rays very useful for spectroscopic methods with spatial resolutions down to about 1 μm . Intermediate-energy NEXAFS at the K-edge

of transition metals provides information about the oxidation state, for example, which is related to solubility and toxicity in plants and microorganisms.

The sensitivity advantage of x rays with respect to electron and proton probes is three to four orders of magnitude for elements with atomic numbers $10 < Z < 40$ and greater than four orders of magnitude for heavier elements. K-edge x-ray spectroscopy is not a new method but its capability is being rapidly extended at the present time because of improvements in available the x-ray source brightness and in the performance of optical schemes for focusing the x-ray probe. With the ALS as the source and the best mirrors and zone plates to form the probe, one can expect parts-per-million sensitivity for the lower Z elements of biological interest (especially sodium, phosphorus, sulfur, chlorine, potassium, and calcium) and parts-per-billion sensitivity for higher Z elements. Mirrors provide the best wavelength tunability and flux (i. e., best sensitivity) at resolutions of $1 \mu\text{m}$ currently, while zone plates offer resolution from $0.25 \mu\text{m}$ to about $0.1 \mu\text{m}$ in the best case to date.

All the methods discussed above use contrast methods that are intrinsic to the sample. As discussed above, specific labeling and gene mapping are also possible with x-ray microscopes.

3.4.4 What Are the Experimental Alternatives to Soft X Rays?

Soft x-ray microscopy is complementary to other methods. The spatial resolution is between that of electron and (confocal) visible-light microscopes (see Table 1). Many efforts are aiming to improve the resolution of visible-light microscopes. Most notable are near-field scanning optical microscopes (NSOM) and two-photon confocal microscopes. NSOM provides very good spatial resolution, but only from a thin layer at the surface of the sample. Although an improvement over conventional confocal microscopes, two-photon microscopes do not reach the resolution provided with present soft x-ray microscopes.

Electron microscopy clearly has a spatial resolution superior to x-ray microscopes. The limited penetration depth of electrons (including that in high-voltage microscopes) as compared to x rays, however, prevents high-resolution imaging of whole cells. Serial sectioning is used to overcome this limitation. However, x-ray microscopy of individual whole cells (about $10 \mu\text{m}$ thick) does not require sectioning.

3.4.5 Further Utilization of High-Resolution Labeling Techniques Is of Utmost Importance

The use of fluorescently labeled antibodies to localize proteins in the light microscope has led to major advances in the understanding of cell structure and function. The amount of information that can be gained from these analyses, however, has been limited by the spatial resolution of the light microscope. To obtain higher-resolution information about the localization of proteins, silver-enhanced, gold-conjugated antibody probes can be used in an x-ray microscope. Henry Chapman and Chris Jacobsen pioneered this technique at Brookhaven National Laboratory using dehydrated cells. Larabell and Meyer-Ilse extended the use of silver-enhanced, gold probes to localize proteins in hydrated cells using the soft x-ray microscope XM-1 at the ALS. This technique, which is a modification of a labeling protocol previously used to localize proteins in the transmission electron microscope, proved to be a powerful approach for examining protein localizations in the x-ray microscope. At the workshop, the Brookhaven group reported using such probes to label microtubules in dehydrated cells that were then examined in the scanning x-ray microscope at the NSLS, and the Berkeley group presented its results based on similar probes to label microtubules in hydrated, whole cells for examination in XM-1. These data demonstrate the proof-of-principle *that x-ray microscopy can be*

used to obtain high-resolution information about the distribution of proteins in whole cells. The Berkeley group extended its studies to obtain high-resolution information about the distribution of proteins in the nuclei of cells. This is an extremely exciting development since nuclear organization, although of the utmost importance, is very poorly understood, owing in large part to the fact that it has been difficult to analyze nuclear structure with standard microscopic techniques. The Berkeley group illustrated the power of the x-ray microscope to visualize the structural composition of whole, hydrated nuclei—a feat not possible with other known types of microscopy. In addition, the group demonstrated the capacity to label proteins in whole, hydrated nuclei using the Ag-enhanced, gold labeling technique. This is a major breakthrough since many of the structure/function studies of the nucleus done to date have been criticized because of the artifacts associated with the elaborate preparation techniques required to visualize the nucleus in the electron microscope. The ability of the soft x-ray microscope to visualize labeled cytoplasmic and nuclear proteins with respect to the underlying cell structures has demonstrated that soft x-ray microscopy can bridge the gap between light and electron microscopic analyses and can obtain information about protein localizations at higher resolution than possible with light microscopy but without the extensive cell preparations required for electron microscopy.

3.5 Instrumental Challenges

X-ray microscopy is a new tool to study cells in a new way and to discover new, even unexpected information because of the high-resolution images it can provide from biological materials. Its usefulness lies in the fact that it compliments other more well-established techniques and at the same time pushes the frontiers of knowledge. State-of-the-art microscopes and support laboratories are needed, to use the x-ray methods. The high resolution of the microscopes is as important as convenient access and the quality of the light source. The utility of the facility is measured by the strength of the weakest link. The instrumental technology has reached a level that already makes routine studies possible, and further investments in instruments will leverage the utility of third-generation light sources like the ALS. Directions for further developments are discussed below.

3.5.1 Improve Zone-Plate Lenses for Soft X Rays

Present zone-plate lenses provide a resolution in the 30-nm to 50-nm range. Future developments of zone-plate lenses are aiming to reach 10-nm to 20-nm resolution. The resolution of “conventional” microscopes, such as XM-1 at the ALS and the University of Göttingen’s x-ray microscope at the Berlin Synchrotron Radiation Facility, (BESSY) is limited by the zone-plate optics only. These instruments will therefore immediately benefit from improvements in zone-plate lenses. Scanning x-ray microscopes will need improved scanning stages in addition.

3.5.2 Cryogenic Sample Preservation

Cryogenic sample preservation is crucial for the application of x-ray microscopy in the biological sciences, since it reduces sample damage at high resolution and when making multiple images. Cryogenic sample stages are therefore either operational or under construction at BESSY, NSLS, and the ALS. Multiple images of hydrated material, as needed for tomography, will definitely require cryogenic preservation. Figure 3 shows the image of an algae imaged at low temperatures with the Göttingen x-ray microscope at BESSY.

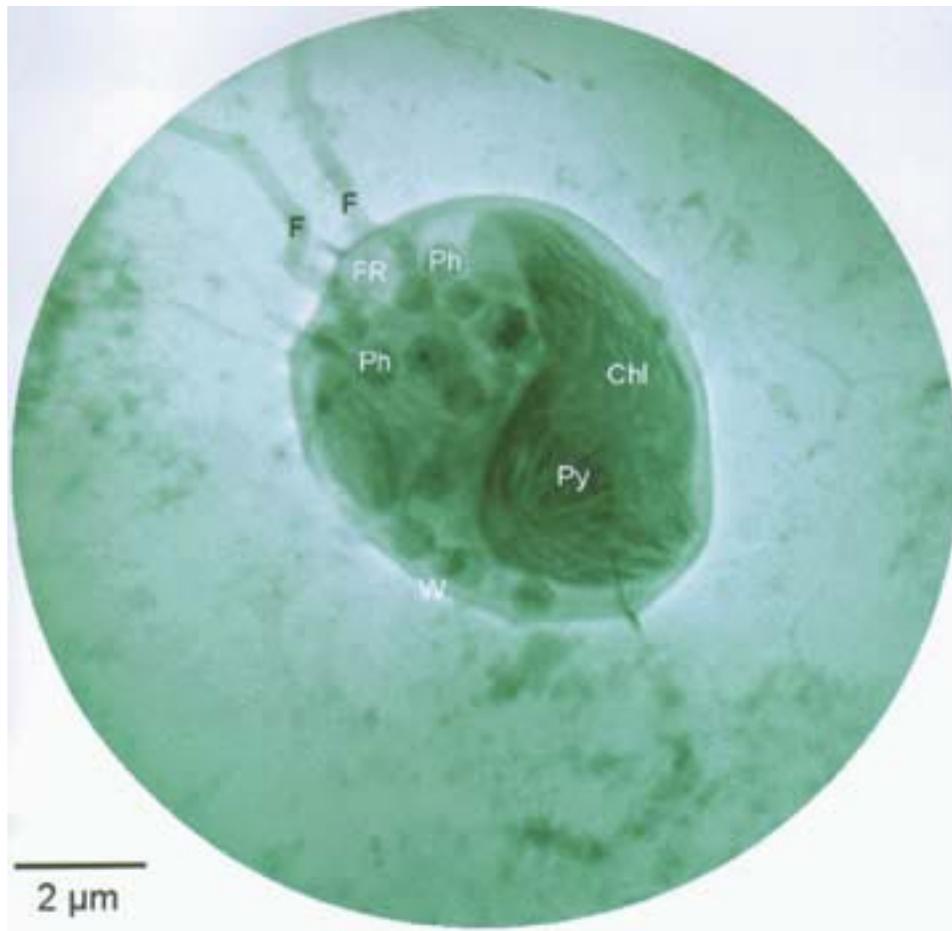


Figure 6. Algae fixed and imaged at low temperatures with the Göttingen x-ray microscope at BESSY in Berlin, Germany. *F* = Flagellum $\varnothing=300$ nm, *FR* = Flagellar root, *Chl* = Chloroplast, *Py* = Pyrenoid, *Ph* = Dense phospholipid vesicles, *W* = Cell Wall. [Figure courtesy of G. Schmahl, University of Göttingen.]

3.5.3 Tomography

Soft x-ray microscopy provides possibilities to obtain three-dimensional information within whole cells. High-resolution tomography has recently been demonstrated using the Göttingen x-ray microscope at BESSY. An early demonstration of tomography with cryogenic samples was made at Beamline X-1A at the NSLS.

3.5.4 Undulator-Based Microscopes

Scanning soft x-ray microscopy offers capabilities complementary to conventional microscopes, such as XM-1 at the ALS and the Göttingen microscope at BESSY. In a scanning x-ray microscope, a microbeam is formed, and the specimen is raster scanned across it. Originally developed by the Stony Brook group at the NSLS, such a system is now also in operation on Beamline 7.0.1 at the ALS. Scanning is also compatible with other schemes, such as dark-field and luminescence micros-

copy. An optimized scanning microscope at the ALS would take about 5 seconds to record a picture with 1000×1000 pixels, with 1000 photons detected per pixel. Such performance with high resolution is highly desired for biological applications, but it requires developmental work for the scanner, beamline, and detector systems.

3.5.5 Precision Instrumentation and Support Laboratories

Soft x-ray microscopy facilities need to be easily accessible for users who might not be familiar with synchrotron-radiation facilities. Precision instrumentation with an easy user interface, software, and data-handling capabilities are crucial, as are support laboratories that are set up for sample handling and preparation.

3.5.6 Hard X-Ray Instrumentation and Other Techniques

Soft x-ray imaging provides high spatial resolution with the best overall utility. Imaging methods that use small pinholes might reach a 10-nm resolution before zone-plate optics with the same resolution are available. The low efficiency of such methods, however, will limit these methods to a very small number of special investigations. Intermediate and harder x rays can also be used if x-ray fluorescence or K-shell spectroscopy are used. Hard x-ray phase zone plates with a spatial resolution of about 0.1 μm and focusing efficiency better than 33% are the present state of the art. Whereas the ALS is the premier source for soft x rays, the activities in the intermediate and hard x-ray ranges should be coordinated with the other synchrotron facilities.

3.6 The Role of the ALS and Berkeley Lab

3.6.1 Undulator-Based Facility

ALS undulators are the brightest sources for soft x rays in the nation. Scanning microscopes depend on brightness because they require spatially coherent radiation. The ALS is therefore the ideal source for these most demanding soft x-ray microscopes. Several proposals to build an undulator-based soft x-ray microscopy facility have been discussed over the past years, the latest one being headed by the environmental-science community (see the report of the Working Group on the Environmental and Earth Sciences). The biological community would benefit from such an instrument and supports an interdisciplinary collaboration to build such a facility.

Since the advantages of high-resolution microscopy not only benefit the biological community but also environmental, materials, chemical, and engineering sciences, special attention has to be put into long-range planning. As demonstrated with existing microscopes, these different applications are both compatible and best served with an instrument that matches the performance of the ALS.

3.6.2 Current Capabilities

A bend-magnet-based x-ray microscope (XM-1) is operational and in use with several biological-research programs. This instrument has begun to attract a growing biological user community. Although the optical layout of XM-1 is identical to the Göttingen microscope at BESSY, its precision instrumentation makes it the most versatile, easily accessible instrument in the world. It is expected to complement future scanning microscopy activities at the ALS. A cryogenic sample stage for XM-1 is under development and has produced first results. Adding tomography capabilities should also further develop this instrument.

The ALS not only offers unique brightness but also necessary support laboratories, and it is also close to local research groups in both biology and technology, thus providing a fruitful environment for successful collaborations.

3.6.3 Relationship to Other Synchrotron Radiation Facilities

X-ray microscopy depends on the quality of the light source. New generations of synchrotron radiation facilities therefore drive new microscope developments. The group at the University of Göttingen, which pioneered soft x-ray microscopy with synchrotron facilities and operates a microscope at BESSY, is planning an x-ray microscopy facility at BESSY II, which is a third-generation machine very similar to the ALS. The microscope at BESSY II will use a beamline with a 4.1-cm-period undulator. An improved conventional microscope with improved spatial resolution, short exposure times, and improved spectral resolution is fully funded and under construction. A scanning microscope is in the planning stages.

Soft x-ray scanning x-ray microscopy was pioneered by the State University of New York at Stony Brook at the NSLS, a second-generation machine. Harder x-ray facilities are located at the APS and the European Synchrotron Radiation Facility (ESRF).

3.7 Recommendations

3.7.1 Fully Support Present Activities in Biological Soft X-Ray Microscopy at the ALS

Biological microscopy research at the ALS presently uses the bend-magnet based microscope XM-1. This instrument should continue to be used for biological research. We emphasize that the newly developed techniques for labeling, cryogenic sample preparations, and future plans for three-dimensional imaging are expected to play an important role in biology.

3.7.2 Construct an Undulator-Based Microscopy Facility

The biological user community supports an undulator-based x-ray microscope facility. A collaboration with other fields, (environmental, materials, chemical sciences) will be beneficial for biology. Both scanning and conventional microscopes would be possible and serve complementary needs. The scanning microscope should be built first to serve the complementary needs with the existing microscope XM-1.

3.7.3 Collaboration with Other Light Sources

The ALS clearly is the premier facility for soft x-ray microscopy and therefore ideally suited for the biological research described here. Other facilities either offer opportunities in the hard x-ray range or have already developed specialized instruments that should be used in collaboration. The unique resources for soft x rays at the ALS need to be utilized with care. Experiments that do not require the ALS might be better done at other facilities.

4. Biological and Chemical X-Ray Spectroscopy

Facilitator: Stephen Cramer, University of California, Davis

Working-group members: Uwe Bergmann, Lawrence Berkeley National Laboratory; Heinz Frei, Lawrence Berkeley National Laboratory; Melissa Grush, University of Tennessee; Keith Hodgson, Stanford University; Brian Kincaid, Lawrence Berkeley National Laboratory; Melvin Klein, Lawrence Berkeley National Laboratory; James Penner-Hahn, University of Michigan; Charles Tarrío, National Institute of Standards and Technology; Vittal Yachandra, Lawrence Berkeley National Laboratory.

4.1 Introduction

The bioinorganic-chemistry community was among the first to adopt synchrotron-based extended x-ray absorption fine-structure spectroscopy (EXAFS) as a routine structural tool. The X-Ray Spectroscopy sub-group discussed the important current issues for biological and inorganic chemistry and tried to define the important science in these areas that can be addressed by x-ray spectroscopy.

Some of the questions discussed by our group included:

- What are the important research areas (now and in the future) in biological and inorganic chemistry that can be addressed by x-ray spectroscopy?
- In what areas should national resources for x-ray spectroscopy be developed?
- What x-ray spectroscopy capabilities are best developed at the ALS?
- What is the expected impact on chemistry and for society?

4.2 Important Research Areas

Metals are important in biology both for their beneficial role in enzyme active sites and structure and for the negative effects of enzyme inhibition or disruption by heavy metals or normally benign metals at unhealthy concentrations. Metalloenzymes play important environmental roles as pivotal agents in the nitrogen, sulfur and carbon cycles, and in the production and consumption of greenhouse gases, such as methane. It is also worth noting that enzymes are a billion-dollar business in the United States alone. Thus, in the three critical areas of (1) human health, (2) environmental impact, and (3) commercial potential, a better understanding of enzymes and related model chemistry could have profound impact. X-ray spectroscopy is an important tool for expanding our knowledge because it can answer the following important questions:

4.2.1 What Are the Molecular, Electronic, and Magnetic Structures of Enzyme Active Sites?

A good example of this kind of problem is the oxygen-evolving complex of photosystem II. Based on their EXAFS work and other information, Melvin Klein at LBNL and co-workers have proposed a model for the structure of this complex (see Figure 7). K-edge EXAFS is valuable for defining the metal-neighbor distances. Investigating the fine structure in the chlorine and manganese K-edge region (NEXAFS) can yield important information about the electronic structure of this complex. Many new experiments such as EXAFS at the manganese L edge and x-ray magnetic circular dichroism (XMCD) spectroscopy of manganese, have been planned for the elliptical-undulator beamline at the ALS.

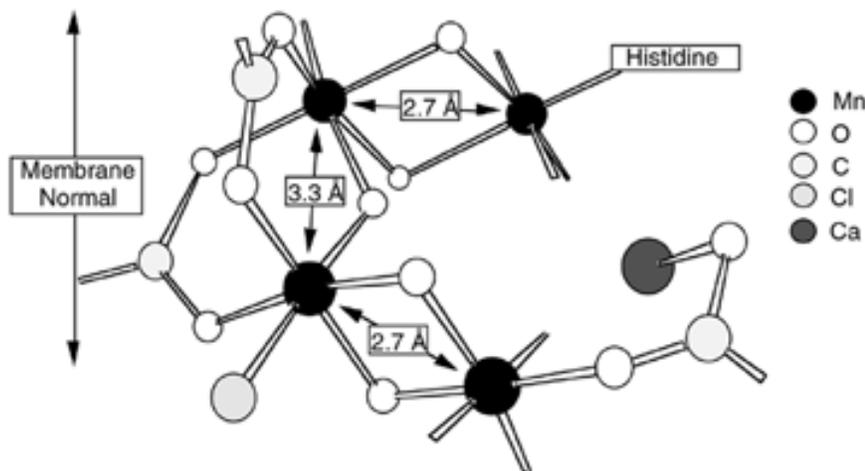


Figure 7. Proposed model for the manganese/calcium/chlorine cluster in the evolution of photosynthetic oxygen. [Figure adapted from Yachandra, DeRose, Latimer, Mukerji, Sauer, and Klein, *Science* **260** (1993) 675.]

4.2.2 How Do the Concentration and Chemical Speciation of Elements Change Across an Organism?

Living systems are not homogeneous, and the gradients of metals and other elements across an organism reveal important information about structure and function. A great deal has already been done by fluorescence microscopy—for example, many beautiful studies of calcium waves in different organisms. However, x-ray spectromicroscopy can discover information inaccessible by other means. For example, James Penner-Hahn of the University of Michigan has used x-ray absorption to study the distribution of zinc in sperm cells. He has seen clear gradients across the cells. Furthermore, the zinc NEXAFS changes at different locations, indicating a spatial variation in the different chemical forms of zinc.

4.2.3 How Does the Chemical Speciation of Elements Change Over Time?

The time dependence of chemical species in an organism is just as important as the spatial variation. X-ray spectroscopy can provide valuable information about species that difficult to observe by other spectroscopies. For example, Klein and coworkers have used sulfur K-edge spectroscopy to monitor changes in the mix of reduced and oxidized sulfur species in the blood before and after drug administration. There are many situations in microbiology where one would like to follow the change in metal speciation after induction of specific enzymes—for example, the change in molybdenum chemistry after induction of the genes for nitrogen fixation.

4.3 Needed National Resources for X-Ray Spectroscopy

4.3.1 Detectors

On one point the sub-group was unanimous—spectroscopy at the moment is just as limited by detectors as it is by beamlines. New detectors need to be developed that are faster and that have higher energy resolution. Since they will be shared by many users, the detectors also need to be “robust” and “supportable.”

4.3.2 High Energy Resolution

There are surprisingly few high-resolution and high-flux beam lines in the country. The best examples are probably Beamlines X-25 and X-27 at NSLS. Many of the beamlines at SSRL are high-flux, but their

resolution is degraded by the pre-crystal focusing optics. For spectroscopic applications, such as NEXAFS, resonance fluorescence, and x-ray Raman spectroscopy, better energy resolution is important.

4.3.3 The 2-keV to 3-keV Region

Another important area that is poorly served at the moment is the 2-keV to 3-keV region, which contains important edges such as the sulfur and chlorine K edges and molybdenum L edges. An ALS beamline should be designed to maximize flux in this region.

4.3.4 A Spectromicroscopy Capability

Although the ALS is pushing the state of the art in soft x-ray microscopy, there are many important hard x-ray experiments where micron-scale resolution would be useful. A beamline capable of moderate resolution spectromicroscopy should be developed.

4.3.5 A Time-Resolved Capability

The ALS should help develop a time-resolved x-ray absorption capability on the millisecond-to-seconds scale. Many biological processes occur on this time scale, and making rapid x-ray absorption routinely available to users would lower the barriers to this kind of work.

4.3.6 Newer Spectroscopies

High resolution x-ray fluorescence and inelastic scattering look promising for site-selective x-ray absorption and better understanding of electronic structure. Secondary monochromators should be available to general users on a beamline to make these experiments possible.

4.3.7 User Issues

There are also many user-related issues that would make the ALS a more productive place for x-ray spectroscopy. Our group noted that other facilities such as ESRF, NSLS, and SSRL provide some of the following:

- Detectors and chambers for spectroscopy users.
- Support staff for spectroscopy science.
- Support for spectroscopy software.
- Support for spectroscopy detector development.
- ESRF and NSLS have mail-order XAFS; SSRL has rapid-turnaround service.

The ALS should consider whether it can do in the soft x-ray region what these facilities do for hard x-ray users.

4.4 Summary of Conclusions

The elliptical undulator beamline should be completed as quickly as possible.

There is a strong need for a high resolution hard x-ray beam line at the ALS to cover the 2 keV to 10 keV region.

Better detectors are needed to take full advantage of both of these beamlines.

Although they are not at the frontiers of technology, x-ray spectromicroscopy on the 1- μm scale and kinetics on the millisecond scale would make very practical contributions to biological spectroscopy.