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2.6 Visualizing Sub-cellular Organization Using Soft X-ray Tomography

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<td>ALS</td>
<td>advanced light source</td>
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<tr>
<td>CT</td>
<td>computed tomography</td>
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<td>TEM</td>
<td>transmission electron microscopy</td>
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Glossary

**Advanced Light Source**  A third-generation synchrotron located in Berkeley, California.

**Alignment**  Prior to the calculation of a tomographic reconstruction, all projection images are placed on a common rotation axis to take account of unavoidable non-axial displacement of the specimen position during tomographic data acquisition.

**Amorphous ice**  An amorphous, noncrystalline, solid form of water produced by rapidly cooling liquid water.

**Bend magnet**  A dipole magnet in the magnetic lattice of a synchrotron storage ring that bends the trajectory of the stored electron beam, causing the emission of synchrotron X-ray radiation.

**Condenser zone plate**  A Fresnel optic used as condenser lens in an X-ray microscope.

**Cryogenic imaging**  Imaging close to liquid nitrogen temperature (~196 °C).

**Cryorotation stage (cryostage)**  Computer-controlled stage that maintains the specimen at a cryogenic temperature.
temperature during data collection; it also allows rotation or tilting of the specimen, enabling the collection of a tomographic data set.

**Fiducial** A small, high-contrast object that is added to the specimen during imaging and is used as a point of reference for the alignment of projection images in a tomographic data set.

**Micro zone plate** Fresnel optic used as an objective lens in an X-ray microscope.

**Normalization** Division of the image of a specimen by a specimen-free image of the illumination; this eliminates systematic errors in the data due to non-uniform illumination.

**Projection image** A single two-dimensional image from a tomographic data set.

**Reconstruction** The process of computationally recombining aligned projection images to form a virtual, three-dimensional representation of a specimen.

**Soft X-rays** X-rays with energies less than 1.8 keV.

**Synchrotron** A charged particle accelerator that can be used as an intense source of X-rays.

<table>
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<tr>
<th><strong>Through-focus series</strong></th>
<th>A sequence of images generated by moving a specimen in successive steps through the focus point of a microscope. One image is collected at each step.</th>
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<tr>
<td><strong>Tomography</strong></td>
<td>A method for producing a three-dimensional image of the internal structure of an object.</td>
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<tr>
<td><strong>Undulator</strong></td>
<td>A synchrotron-based magnetic insertion device used to generate narrow band X-ray radiation with a brightness that is several orders of magnitude greater than that generated by a bend magnet.</td>
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<tr>
<td><strong>Water window</strong></td>
<td>A region of the electromagnetic spectrum where water is weakly absorbing. Photons with energies in the X-ray water window are easily transmitted through water but strongly absorbed by organic material composed of atoms of carbon and nitrogen.</td>
</tr>
<tr>
<td><strong>Wiggler</strong></td>
<td>A synchrotron-based magnetic insertion device used to generate X-ray radiation with a brightness that is several orders of magnitude greater than that generated by a bend magnet. The X-rays produced have a broader spectrum and less coherence than radiation from an undulator.</td>
</tr>
<tr>
<td><strong>Zone plate</strong></td>
<td>A Fresnel optic used to focus X-rays in a soft X-ray microscope.</td>
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### 2.6.1 Introduction

Richard Feynman said, "It is very easy to answer many of these fundamental biological questions; you just look at the thing! Make the microscope one hundred times more powerful and many problems of biology would be made very much easier." Of course, Feynman went on to say this statement was an exaggeration and a gross simplification of the issues. However, it is clear that imaging has been, and will continue to be, one of the most informative biophysical techniques available for biological and biomedical research, and that the development of new microscopy techniques usually leads to scientific progress and new discoveries. Since the invention of the light microscope more than 400 years ago, there has been sustained progress in the development of increasingly more powerful microscopes. During this time, light- and electron-based techniques have been prevalent in terms of imaging cells. However, soft X-ray microscopy has started to play an increasingly important role. Bright field soft X-ray microscopy occupies a unique niche in cell imaging and is highly complementary to existing transmission imaging modalities that employ visible light and electrons for specimen illumination. The comparatively short wavelength of soft X-rays allows for higher spatial resolution than can be readily achieved using light, and because soft X-rays can penetrate thick biological materials, soft X-ray microscopes can be used to image whole, hydrated eukaryotic cells without the need to cut the cell into thin sections (as would be the case using an electron microscope).

Irrespective of whether a microscope uses light, electrons, or X-rays for imaging, it can be used to generate a two-dimensional (2-D) representation of a 3-D object. In the case of soft X-ray microscopy, such 2-D images can usually be considered as simple projections of the specimen X-ray absorption coefficient. In a 2-D soft X-ray microscope projection image of a eukaryotic cell, the complex internal cellular structure becomes confusingly superimposed, making it difficult to obtain meaningful information. Fortunately, by using tomography, it is relatively easy to generate a 3-D volume from an appropriate set of 2-D projection images. If a sufficient number of images are collected over a total range of rotation angles equal to or exceeding 180°, the resultant tomographic reconstruction of the specimen will have an isotropic spatial resolution (i.e., free from ‘missing wedge artifacts’).

In clinical computed tomography (CT), X-rays with very short wavelength are used to image the internal structure of a patient’s body with exquisite detail, typically at a spatial resolution of a millimeter or better. The challenge in developing soft X-ray tomography as a modality for imaging sub-cellular organization has been applying the CT concept at a much smaller scale using much lower energy X-rays. Now that this challenge has been met, soft X-ray tomography is growing in stature. Soft X-ray tomography is widely applicable, and it can be used to image specimens ranging from relatively small, simple prokaryotes to large, highly complex eukaryotic cells or even communities of cells, such as those in a biofilm or a tissue section.

Biological soft X-ray tomography is in its infancy, but the data collected to date demonstrate it is a powerful, unique tool for cellular imaging and analysis. During approximately the next decade, there will be rapid and significant improvements in spatial resolution, contrast, and image processing methods resulting from ongoing developments in X-ray sources, instrument design, optics, and data analysis. This chapter provides an overview of the current state of soft X-ray tomography, highlighting both basic principles and active areas of development. Many image processing methods used for soft X-ray tomography are heavily leveraged on decades of development in transmission electron microscopy (TEM); therefore, we limit our discussion in this chapter on their application to soft X-ray microscopy.
2.6.2 Soft X-Ray Microscopy

2.6.2.1 History and Evolution of X-Ray Microscopy

The development of X-ray microscopy began in the 1940s. The first microscopes, developed by Paul Kirkpatrick and Albert Baez, used grazing-incidence reflective optics to focus X-rays onto the specimen. In concept, these instruments resemble a conventional bright field light microscope. In other words, a ‘condenser’ lens focuses X-rays onto the specimen, and an ‘objective’ lens forms an image of the exit wave on a detector. There was some initial expectation that this new imaging modality could be applied to biological specimens, including the misguided notion that it could be used to image live cell dynamics. However, these early microscopes were almost exclusively applied to materials research and did not produce any noteworthy biological images. The potential of soft X-ray microscopy for biological imaging did not begin to be realized until the mid-1990s. At this point, Fresnel zone plate optical systems had replaced grazing-incidence optics, and soft X-ray microscopes began to be built at synchrotron light sources. By operating in the ‘water window’ region of the X-ray spectrum, zone plate transmission soft X-ray microscopes began to produce high-resolution 2-D projection images of biological specimens. Unlike electron microscopy, where the limited penetration depth of electrons in biological material means cells must be ‘sectioned’ to less than 500 nm, soft X-rays can penetrate biological specimens up to 15 μm thick. This allowed even relatively large eukaryotic cells to be imaged intact and fully hydrated. The inherent contrast produced by the differential absorption of soft X-rays also allowed cells to be imaged without chemical fixation or staining.

The previously mentioned advances showed that biological imaging using soft X-ray microscopy was an attractive, viable proposition. However, to be useful required the further development of tomographic methods for the production of 3-D reconstructions of the specimen. In principle, this is simply a matter of imaging the specimen from a number of different angles. However, all biological materials are sensitive to damage by intense light, whether it is the ultraviolet illumination of a fluorescence microscope or the photons in a soft X-ray microscope. Ideally, tomographically imaging a cell requires collection of projection images sequentially over a rotation range of 180°. Because radiation damage is, for the most part, cumulative, repeatedly exposing the cell to X-rays opens up the possibility of delicate cellular structures being damaged, giving rise to artifacts. Fortunately, cooling the specimen can reduce or delay the observable effects of radiation damage. Consequently, carrying out data collection for tomographic reconstruction requires the specimen be cooled and remain cold during the collection of all projection images. This is achieved using a full rotation cryostage or a cryo-tilt stage, depending on the amount of specimen rotation permitted (generally, either type of stage is commonly referred to simply as a cryo-stage). If soft X-ray tomographic data are collected from specimens held at liquid nitrogen temperature, the effects of radiation damage are minimized to the point that there are no discernible differences between the first image and an image of the same field of view taken after several hundred other high-contrast images have been collected from the specimen.

Currently, there are two operational synchrotron-based, full-field soft X-ray tomography beamlines, with several more being planned or under construction. One of these microscopes, known as XM-2, is located at the Advanced Light Source in Berkeley, California (http://www-als.lbl.gov), and the other is located at U41 of the Helmholtz Zentrum Berlin, Germany (http://www.helmholtz-berlin.de). Conceptually, both of these microscopes are similar. However, because XM-2 was specifically designed for cellular imaging (as opposed to being designed as a multipurpose instrument), the following discussion focuses primarily on this instrument as the model for the technique.

2.6.2.2 X-Ray Sources

To overcome inherent and unavoidable instabilities in microscope instrumentation, it is important that tomographic data be collected as rapidly as possible. This minimizes specimen drift, which degrades the quality and clarity of the resultant projection images, and therefore the quality of final 3-D tomographic reconstructions. As a result, soft X-ray tomography is best carried out using an intense stable source of X-rays because this ensures images with good signal-to-noise can be collected using a short exposure time. Synchrotron-based electron storage rings are currently the optimal source of X-rays for this purpose. Synchrotrons are typically large national or international user facilities (see http://lightsources.org for a list of synchrotrons and their locations worldwide). Each synchrotron storage ring is capable of simultaneously supplying synchrotron radiation to 30 or more experimental end stations.

A synchrotron-based electron storage ring is a type of accelerator in which high-energy electrons are maintained in a cyclical orbit in an ultra-high vacuum vessel. Under such conditions, electrons emit photons and therefore lose energy. By balancing this energy loss with energy input from a radio frequency electric field, it is possible to maintain electrons at constant energy in a stable orbit circulating around the synchrotron storage ring. The general layout of a synchrotron is shown in Figure 1. Electrons are fired from an electron gun into a linear accelerator (where they reach an energy of approximately 100 MeV). The accelerated electrons then enter a ‘booster ring’ and are further accelerated to a relativistic energy of a few gigaelectron volts. Finally, the electrons are diverted into the ‘storage ring’ where they are maintained in a tightly focused stable orbit by specialized dipole and multipole magnets. As the electrons experience the acceleration required to maintain a closed orbit, they lose energy due to the emission of synchrotron radiation; this energy loss is compensated for by the application of a synchronized radio frequency electric field. A typical storage ring is not circular; rather, it is a collection of long ‘straight sections’ connected by regions where the electron beam is deflected by dipole magnets termed bend magnets (Figure 1). In the straight sections, the path of the electron can also be perturbed by applied magnetic fields. By carefully controlling such orbital perturbations, X-ray beams with specific spectral, temporal, and spatial characteristics can be generated by so-called linear insertion devices termed undulators and wigglers (both are composed of a linear array of magnets). Insertion devices are the brightest and most versatile sources of X-rays, but they are expensive and require a
complex optical system to effectively couple the radiation into the condenser optic of a full-field soft X-ray microscope. Less expensive bend magnets – which are the most abundant X-ray source at a synchrotron facility – do not require complex optical systems to couple to a full-field X-ray microscope. XM-2 at the Advanced Light Source (ALS) utilizes bend magnet radiation, whereas U41 relies on an undulator source.

### 2.6.2.3 The Advanced Light Source

XM-2 is a new beamline sited at sector 2 of the ALS. The X-ray photons used by this microscope originate from a 1.3-T, dipole bend magnet (one of 36 bend magnets integral to the ALS magnetic lattice, each bend magnet deflects the electron orbit by \(10^\circ\)). A dipole bend magnet is an ideal photon source for biological soft X-ray microscopy due to the high source brightness in the important water window energy range. Another important property of the bend magnet source is its low spatial coherence; highly coherent or 'laser-like' sources produce speckle artifacts if they are used for specimen illumination in a transmission X-ray microscope. This is analogous to using a laser instead of a halogen lamp for the illumination source in a conventional bright field light microscope. For the ALS, the stored electron beam is distributed in 328 individual storage rings, where additional acceleration occurs. Once the electrons achieve relativistic energies, they are injected into the storage ring, where they are held in a stable, continuous orbit. The trajectories of electrons in the storage ring are perturbed either by a bend magnet or by a magnetic insertion device such as an undulator or wigglers. Such perturbations generate an intense beam of electromagnetic radiation. This radiation exits the storage ring and is piped to the experimental stations for use in a wide range of experiments. Radiation generated in this manner covers the electromagnetic spectrum from the infrared (\(<100\) meV) to hard X-rays (photons \(>100\) KeV). Copyright © EPSIM 3D/JF Santarelli, Synchrotron Soleil and distributed under Wikimedia Commons License. Copyright by Public Domain.

![Diagram of a typical synchrotron light source](image1)

**Figure 1** (a) Diagram showing the layout of a typical synchrotron light source. Electrons are accelerated first along a linear accelerator and then around a 'booster ring,' where additional acceleration occurs. The trajectories of electrons in the storage ring are perturbed by a bend magnet or by a magnetic insertion device such as an undulator or wigglers. Such perturbations generate an intense beam of electromagnetic radiation. This radiation exits the storage ring and is piped to the experimental stations for use in a wide range of experiments. Radiation generated in this manner covers the electromagnetic spectrum from the infrared (<100 meV) to hard X-rays (>100 KeV). Copyright © EPSIM 3D/JF Santarelli, Synchrotron Soleil and distributed under Wikimedia Commons License. Copyright by Public Domain. (b) Simplified schematic of one kind of soft X-ray microscope used at a synchrotron radiation facility. Polychromatic X-rays from the bend magnet source are reflected from a plane mirror and then focused on the object by the condenser zone plate lens. A magnified image of the sample is formed by the objective zone plate lens on a back-thinned, directly illuminated CCD detector. McDermott, G.; Le Gros, M. A.; Knoechel, C. G.; Uchida, M.; Larabell, C. A. Soft X-ray tomography and cryogenic light microscopy: The cool combination in cellular imaging. *Trends. Cell. Biol.* **2009**, *19*, 587–595.
bunches. This produces an intense pulsating X-ray flux. The pulse duration is of order 2 ps, and the pulse repetition frequency is of order 500 MHz, which is rapid compared to the image exposure time. The ALS storage ring operates in 'top off mode' with electron beam energy of 1.9 GeV and a constant average beam current of 500 mA. This operating mode increases the stability of the microscope because it ensures that the optical components that experience a high X-ray flux are subject to the same amount of heating throughout an experiment. The alternative operation mode is one in which the intensity of the electron beam in the storage ring decays over time due to electron-electron scattering and interaction of the beam with residual gas in the storage ring vacuum chamber. In this mode, the beam is refreshed every few hours by injecting additional packets of high-energy electrons; because of this, the heat load on the beamline optics varies significantly between injections. On XM-2, the acquisition of a complete set of projection images takes only a few minutes, with a typical exposure time of 100 ms. The source stability is such that background images for image 'flat fielding' and 'normalization' need only be collected once prior to acquiring the projection image series needed for tomography.

2.6.2.4 X-Ray Microscope Optical Systems: Fresnel Zone Plates

Typically, X-ray reflective optics operate at shallow angles and are usually termed grazing incidence optics. This is due to the strong absorption and low X-ray refractive index of most materials (the refractive index is close to unity for most materials, irrespective of the X-ray energy). A soft X-ray microscope employing grazing incidence mirrors for the imaging optics can only operate at a very low numerical aperture (NA); thus, such an instrument will have a correspondingly low spatial resolution imaging capability. Refractive lenses, similar to those used in light microscopy, cannot be used for soft X-rays due to the strong absorption of soft X-rays by most materials. Fortunately, a solution exists, and soft X-ray microscopes, such as XM-2, use Fresnel zone plate lenses to focus X-rays (Figure 1(b)).

An X-ray zone plate lens consists of a number of concentric nanostructured metal rings, or zones, structured on a thin X-ray transmissive silicon nitride membrane (see Attwood for a detailed description of zone plate structure and theory). To focus the X-rays into a single spot, which is required for high spatial resolution imaging, the zones decrease in width as they move outward from the center toward the edge (Figure 2). In XM-2, the condenser zone plate lens has an overall diameter of 1 cm and an outer zone width of 60 nm. The objective zone plate lens has a diameter of 60 mm and an outer zone width of 50 nm. This produces a numerical aperture of 0.02 and a resolution below 50 nm at 2.4-nm imaging wavelength.

The resolution of an X-ray microscope is mostly dependent on the quality and NA of the objective zone plate. Zone plates are one of the most precise large-scale, noncrystalline, man-made 3-D structures that can be manufactured; fortunately, modern nanofabrication methods have made them readily available. For example, the zone plate foundry operated at Lawrence Berkeley National Laboratory can easily produce more than 100 high-quality zone plate objective lenses in 24 man-hours.

Figure 2 Scanning electron microscope image of one quadrant of an objective zone plate lens. The magnified view of the outermost zones is shown in the inset. Image courtesy of Dr. W. Chao, CXRO, LBNL.
In addition to spatial resolution, depth of field is also an important characteristic of a high-resolution microscope. Depth of field is the distance along the optical axis close to the focal point of the microscope objective lens over which an object remains 'in focus.' For tomographic imaging, the optimum situation is an infinite depth of field with the maximum possible lateral resolution. In reality, the depth of field varies with the resolution, with the depth of field decreasing as the resolution increases. Weiss and colleagues showed that the illumination produced by a zone plate condenser lens has a finite bandwidth, and this wavelength spread has a significant effect on the depth of field.\(^9\) A careful analysis demonstrated that for the bandwidth used in practice in a soft X-ray microscope, the images can be regarded as simple projections for any reasonable value of specimen thickness at spatial resolutions of approximately 50 nm. This greatly simplifies both data collection and data processing for tomography. Rather than needing to take measures such as collecting a through-focus series at each angle, a single image can be collected. However, fully exploiting the highest resolution zone plates available—currently better than 15 nm\(^2\)\(^3\)\(^24\)—in thick (i.e., up to 10 μm) specimens will require developing data acquisition strategies that involve a combination of through-focus deconvolution and tomography. This is an important future direction in this field.

### 2.6.2.5 X-Ray Interactions and Contrast Mechanisms: The Water Window

Soft X-ray microscopes typically use photons with energies within the range 250 eV (5 nm) to 1800 eV (0.7 nm). For biological imaging, the most important region of this energy range is between the K shell absorption edges of carbon (284 eV, \(\lambda = 4.4\) nm) and oxygen (543 eV, \(\lambda = 2.3\) nm). X-rays with energies between these absorption edges are absorbed in an order of magnitude more strongly by carbon- and nitrogen-containing organic material than by water (Figure 3). The relative transparency of water to X-ray photons results in this region being termed the water window.\(^13\)\(^–\)\(^17\) In the context of biological materials, imaging with water window photons produces images with a strong quantifiable natural contrast; this eliminates the need for contrast enhancement procedures to visualize sub-cellular structures.\(^4\)\(^,\)\(^17\)

For X-ray photons of energy less than 10 KeV, photoelectric absorption processes dominate the interaction with matter. Inelastic mechanisms such as Compton scattering are weak at low photon energies. Thus, soft X-ray photons tend to be elastically scattered (i.e., diffracted) or completely absorbed; very few inelastically scattered photons reach the detector. This is a major difference from many of the processes that generate contrast in TEM. In water window soft X-ray tomography, the X-rays are strongly absorbed by carbon; consequently, it is possible to differentiate cellular components based on their linear X-ray absorption coefficient. For example, lipids are more highly absorbing than other cellular constituents and, therefore, readily distinguished.\(^4\)\(^,\)\(^5\)\(^,\)\(^9\)\(^,\)\(^17\) As a result, it is possible to obtain images with a high signal-to-noise ratio from a thick biological specimen.

![Figure 3](image-url) Transmission of X-rays through hydrated organic material. The attenuation length for X-rays in water increases markedly for wavelengths greater than 2.4 nm because these X-rays have insufficient energy to excite the oxygen K edge; however, at these wavelengths, protein and other organic material are still strongly absorbing. This is the physical origin of the soft X-ray ‘water window.’

### 2.6.3 Soft X-Ray Tomography: Theory

#### 2.6.3.1 Introduction to Tomography

If a specimen is imaged from a number of different well-chosen positions, then the data contained in this 2-D image set can be computationally inverted to produce a 3-D volumetric representation of the specimen. In principle, these different positions can be achieved either by changing the focal position of the specimen or by changing the angle from which the specimen is imaged. The former method is used for optical sectioning-based 3-D reconstructions, whereas the latter is used for tomographic 3-D reconstructions. The tomographic approach is well suited to 3-D biological imaging using the soft X-ray microscope XM-2.\(^2\)\(^,\)\(^9\) In a typical experiment, a number of projection images are collected from different angles. This set of projection images is traditionally referred to as a tilt series,\(^25\) and this familiar term is adopted here.

The number of images in a tilt series needed for a 3-D reconstruction increases with specimen thickness and spatial resolution.\(^26\) To obtain 3-D isotropic resolution, one should record \(N\) evenly spaced projections over 180°, with \(N = πD/d\), where \(D\) is the object size (thickness), and \(d\) is periodicity.\(^27\) Reconstruction of a 10-μm object at 100-nm full isotropic resolution (200-nm periodicity) requires approximately 150 images according to this formula, and at 50-nm resolution, approximately 300 images would be required. However, it is not always necessary to collect as many images as the formula indicates in order to resolve features at a given resolution. For experiments at XM-2, 90 images spanning a range of 180° is typically sufficient for a specimen a few micrometers thick.\(^2\)

The radiation dose accrued by the specimen during collection of a tomographic data set necessary for a 30-nm reconstruction is approximately 10\(^5\) Gy. The maximum dose that a thick, frozen-hydrated specimen can tolerate is poorly understood. With electron irradiation (as in TEM), thin specimens begin to show severe bubbling and morphological
An X-ray microscope image is a measurement of the intensity of X-rays incident on the detector after passing through the specimen. To correct for inherent inhomogeneities in the specimen illumination, the images in the tilt series are normalized using 'specimen-free' images called 'flat field images,' which are collected without a specimen in the field of view. These are, like the data images, corrected for dark current. A few flat field images are averaged before dividing each image in the rotation series – pixel by pixel – by the average flat field. This procedure results in a normalized projection image data set. In addition to correcting for any inhomogeneity in the illumination, the tomographic reconstruction of a normalized image data set gives rise to a quantitative 3-D map of the X-ray linear absorption coefficient of the specimen. Figure 5 shows a raw projection image and the same image after being corrected and normalized.

The raw images in a rotation series also contain artifacts caused by bad or debris-covered pixels in the CCD detector. In practice, the normalization process described previously does not correct these artifacts. If such ‘bad pixel’ artifacts cover only a few pixels at a time, they can be corrected by replacing the value of the bad pixel with a value based on an interpolation from surrounding pixels.

2.6.3.2.2 Image alignment
Prior to calculating a volumetric reconstruction, the images in a corrected and normalized tilt series must be aligned to a common rotation axis. This is necessary because the sphere of confusion of the goniometric specimen stage is much larger than the resolution of the microscope. The alignment problem can be parameterized in a number of ways. Our description focuses on just one of these, in which the deviations from a perfect rotation of the specimen during collection of a tomographic projection image data set are mathematically described by various translation and rotation matrices.

Each image of the data set represents a projection of the specimen onto a plane. A projection matrix $A_i$ can be defined, which projects a coordinate, $r_j = (x_j, y_j, z_j)$, of the untitled specimen in the microscope reference frame onto a coordinate, $p_{ij} = (u_{ij}, v_{ij})$, of the projection image plane. $p_{ij}$ is given by

$$p_{ij} = A_i r_j + d_i$$

where $d_i = (\Delta u_i, \Delta v_i)$ represents translation after projection in the $u$, $v$ plane. $A_i$ can be further decomposed into a product of matrices, including a matrix $R_i$ representing rotation about the microscope $z$ axis followed by projection onto the $(u, v)$ plane; a matrix $Y_i$ representing rotation about the microscope $y$ axis and projection onto the $(u, v)$ plane; a matrix $X_i$ describing rotation about the microscope $x$ axis; and a matrix $D_i$ that includes overall scaling, skew, and differential scaling of the axes. In total, $A_i$ is given by

$$A_i = R_i Y_i X_i D_i$$

$D_i$ is important in electron tomographic measurements of plastic embedded specimens to take into account shrinkage of the specimen due to radiation damage during data collection. For the case of soft X-ray microscopy applied to cryoimmobilized specimens, such shrinkage has not been detected to
date in the measurements, \textsuperscript{9} therefore, the parameters describing specimen shrinkage are not used in the alignment of a soft X-ray tilt series data set.

Aligning the tilt series consists of determining the matrix elements for the projection matrix $A_i$ and the translation vector $d_i$ for each projection image. A number of different methods can be used to determine these parameters, including fiducial marker alignment, cross-correlation alignment, and iterative reconstruction combined with alignment.

\subsection{2.6.3.2.2.1 Fiducial alignment}

Fiducials are high-contrast objects, visible in a number of adjacent images in the tilt series that are used to determine image alignment.\textsuperscript{32,33} Fiducials can either be added to the specimen or be naturally occurring features. However, in practice, it has been more common to use exogenous fiducial markers. In both soft X-ray and electron tomography, fiducials are typically generated by the addition of gold nanoparticles to the specimen. In most soft X-ray work to date, 100-nm gold particles have proven to be excellent fiducial markers. They are sufficiently X-ray absorbing to appear as high-contrast objects in an image, and this, in conjunction with their spherical shape, means that they can be localized accurately in each image of the tilt series (Figure 6).

The first step in fiducial alignment is to identify individual markers on each image in the tilt series. This can be done manually or semiautomatically using software packages such as IMOD (developed by the Boulder Lab for 3-D Fine Structure of Cells and Tissues).\textsuperscript{34} For the case of manual alignment, a graphical interface allows a user of this software to click on each point in each image of the tilt series where a fiducial marker is located. In semiautomatic mode, this is done by marking all of the fiducials on a single image of the tilt series, following which the software tracks the fiducials onto the remaining images. Based on the fiducials identified on the images in the tilt series, a 3-D fiducial marker model $(u_{ij}', v_{ij}')$ is calculated and iteratively optimized by minimizing the sum of the squared differences between the measured fiducial positions $(u_{ij}, v_{ij})$ and the corresponding positions reprojected from the

$$E = \sum_i \sum_j ((u_{ij}' - u_{ij})^2 + (v_{ij}' - v_{ij})^2)$$

The main advantage of using fiducial markers for alignment is that this method produces a level of accurate, globally consistent alignment that other methods have typically not yet achieved.\textsuperscript{31,35} However, there are two main disadvantages to fiducial markers. First, it can be difficult to evenly distribute markers across the specimen. These difficulties have been overcome in many instances by using a standardized procedure to add the fiducial markers as a coating on the outside of the sample holder. This procedure distributes the markers evenly and without aggregation, and it avoids problems caused by the interaction of fiducial markers with the specimen. A second problem is that it is common that automatic bead tracking is not fully successful, and the manual correction of the resulting errors or the requirement of locating fiducials in every image is very time-consuming. The process of fiducial alignment can take from a few minutes (for high-quality data sets with well-separated fiducial markers that can be automatically identified and tracked) to many hours (for data sets where markers are difficult to distinguish or where specimen characteristics are used as fiducials). In some cases, alignment becomes the rate-limiting step in data processing throughput. Typically, the fiducial markers in soft X-ray microscopy images have lower contrast compared to their surroundings than do fiducial markers observed in images produced by electron tomography measurements. This is related to both the higher natural contrast of the biological specimens being imaged with X-rays and the significantly larger specimen thicknesses examined by X-ray tomography. This lower fiducial marker contrast is the main cause of the failure of standard automatic tracking procedures.

\subsection{2.6.3.2.2.2 Cross-correlation alignment}

By cross-correlating successive images in a rotation series, the relative shift between each pair of images can be found.\textsuperscript{25,36} By measuring the relative spatial correlation of successive image...
pairs, the complete rotation series can be transformed into a common frame of reference, and a perfect rotation axis can be defined for the image tilt series. There are a number of additional steps that, in practice, significantly improve the success of this approach. For example, to reduce the method's sensitivity to noise, low-pass filtering can be used; in addition, a high-pass filter might be used to keep the algorithm from being misled by large-scale features. To avoid wraparound effects (which occur when correlation is performed using Fourier transform methods), a suitable window function can be used, or images can be extended using single value padding. The major advantage of cross-correlation is that it is fast and does not require significant user input to achieve an excellent first approximation to the required aligned projection image data set.

2.6.3.2.3 Iterative reconstruction and alignment

A third approach is to perform iterative combined reconstruction and alignment; this is often called the 3-D model-based method.\textsuperscript{35,37} In this method, an initial reconstruction is performed using images that have been aligned by cross-correlation. Based on this reconstructed volume, sets of reference projections are calculated. The original images are compared with these reference projections to determine the new alignment parameters for each original projection image. The volume is reconstructed with the newly aligned images, and the process is repeated iteratively until convergence. A drawback of this approach is that it does not guarantee a solution that is the true global minimum, and it can depend strongly on the initial alignment input. In addition, it is computationally intensive and in practice requires a computer cluster to produce results in a reasonable amount of time. The development of automatic alignment methods is critical to the advancement of high-throughput soft X-ray tomography; therefore, such methods are an important computational research topic for the field.

2.6.3.2.3 Reconstruction techniques

Tomographic reconstruction is the process of using the aligned 2-D images from a tilt series to calculate a 3-D representation of the specimen.\textsuperscript{10} If the tilt series contained an infinite number of noiseless perfectly aligned images, the choice of a reconstruction algorithm would be irrelevant because all common reconstruction algorithms would produce the same tomographic volume. However, experimental tilt series contain a finite number of images with both noise and imperfections in alignment.\textsuperscript{38} A variety of algorithms have been developed, each with unique strengths and weaknesses when applied to real experimental data; this means that the algorithm used to reconstruct a given data set must be carefully chosen to obtain the best possible reconstruction. Reconstruction techniques can be classified into two main categories: Fourier methods and real space methods, which are also known as iterative reconstruction methods.\textsuperscript{9,38} These two classes of algorithm are briefly described, and the relative advantages and disadvantages of each approach when applied to soft X-ray data are discussed.

2.6.3.2.3.1 Fourier methods

Fourier methods rely on the central slice theorem, which is briefly described here for the case of a 2-D object, \( f(x,y) \).\textsuperscript{38} A projection of this object, \( P_\theta(t) \), consists of a series of line integrals, each taken in the direction \( \theta \) at a displacement \( t \) from the origin:

\[
P_\theta(t) = \int_{\theta,t} \int_{\text{line}} f(x,y) \text{d}s = \int_{-\infty}^{\infty} f(x,y) \delta(x \cos \theta + y \sin \theta - t) \text{d}x \text{d}y
\]

Figure 6  Soft X-ray microscope image of a capillary tube containing yeast cells and gold fiducial markers of 100-nm diameter; some markers are shown bordered by red squares.
where $\delta$ is the delta function. $P_\theta(t)$ is known as the radon transform of $f(x,y)$. The left panel of Figure 7 illustrates this idea. In this figure, the equation for the line $AB$ is

$$x \cos \theta + y \sin \theta = t$$

The Fourier transform of $f(x,y)$ is given by

$$F(u,v) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} f(x,y)e^{-2\pi i (ux+vy)} \, dx \, dy$$

and the Fourier transform of $P_\theta(t)$ is given by

$$S_\theta(w) = \int_{-\infty}^{\infty} P_\theta(t)e^{-2\pi iw t} \, dt$$

It can be shown that

$$S_\theta(w) = F(w \cos \theta, w \sin \theta)$$

which is the central slice theorem. In words, the value of the Fourier transform of an object along a line through the origin (a central slice or section) at a given angle is the same as the Fourier transform of the projection profile collected at that angle. This correspondence is shown in Figure 7. The left panel represents the object $f(x,y)$ in real space, whereas the right panel represents the object in Fourier space. In the figure, the Fourier transform of the projection data collected at angle $\theta$ gives the Fourier space data along the line CD. After Fourier transforming a number of projection images taken at different angles, values of the object throughout Fourier space can be determined. The representation of the object in real space can then, in principle, be obtained by (in this example) the 2-D inverse Fourier transformation of the Fourier space object.

In practice, a method commonly used to obtain a 3-D reconstruction of the object from the Fourier transformed 2-D projection images is known as R-weighted back-projection. In this method, the inverse Fourier transform for each of the weighted, Fourier transformed projections is taken individually, and the various inverse transforms are summed in real space. This process is known as back-projection because the result of

Figure 7  Illustration of the Fourier slice theorem for a 2-D object. The Fourier transform of a projection image of the object taken at an angle $\theta$ is equal to the Fourier transform of the object along a radial line passing through the origin of Fourier space. The complete 2-D Fourier transform of the object can be assembled from a number of 1-D Fourier transforms of successive 1-D projection images of the object.

Figure 8  Reconstruction quality as a function of the number of projections. Soft X-ray tomographic reconstruction of liposomes in a capillary tube using (a) 2, (b) 4, (c) 9, and (d) 90 projection images. The fidelity of the reconstruction improves as more independent projection images of the object are used for the tomographic reconstruction.
The inverse transform of each projection is to smear it out, or back-project it, across the real space object being reconstructed. This is illustrated in Figure 8, which shows the results of the back-projection of 2, 4, 9, and 90 projections. It is clear in the case of 2 and 4 projections that the back-projection smears the information from each projection across the reconstruction in real space. By summing the information from many projections, a true representation of the specimen is revealed.

A major advantage of Fourier techniques is that they are much faster than alternative methods. In addition, they yield an analytical result: There is no need for determining stopping criteria or other parameters as is the case for real space methods described next.

2.6.3.2.3.2 Real space methods

In real space methods, values of the voxels in the reconstructed volume are seen as variables that must be modified so that they are in agreement with the projection image data. Such methods can be formulated as a set of linear equations:

\[ \sum_{j=1}^{N} w_{ij} f_j = p_i \quad i = 1, 2, \ldots, M \]

Figure 9 aids in explaining these equations. \( f_j \) represents the value of voxel \( j \) in the reconstructed volume, \( p_i \) represents a pixel in a projection image, and \( w_{ij} \) represents the weight of voxel \( f_j \) to projection pixel \( p_i \). Reconstructing the volume then becomes a problem of solving this set of equations. Another way to write the problem is as an optimization problem:

\[ \min_{f} \sum_{j=1}^{N} \| P_j f - p_j \|^2 \]

where \( f \) represents all of the voxels in the object; \( p_i \) represents all pixels in the projection image set, which consists of \( N \) independent images; and \( P_j \) is a projection operator. In this formulation, the sum over \( N \) projections must be minimized by varying \( f \). Because of the extremely large number of variables involved (a cube with 512 pixels along each dimension has more than \( 10^8 \) voxels), the problem must be solved using iterative methods. A number of formulas can be used to

![Figure 9](image_url) In real space methods, the absorption along each ray \( P \) is calculated by summing the contributions from all of the voxels \( f \) through which the ray is passing. The contributions of a given voxel to a given ray are determined by a weighing factor, \( w \) (depicted by the blue and red areas for two different voxels). \( w \) is both ray and voxel dependent.

![Figure 10](image_url) (a) Diagram of the two most commonly used specimen holders for soft X-ray tomography. Flat silicon nitride membrane designed for extended specimens larger than 10 \( \mu m \) (left) and capillary specimen holders ideally suited to image cells that grow in suspension, such as yeast, blood cells, or prokaryotic cells (right); scale bar = 1 \( mm \). (b) Images of the holders shown in panel a. (c) Capillary specimen holder loaded with yeast cells; scale bar = 5 \( \mu m \).

![Figure 11](image_url) CAD model of the specimen stage and goniometer assembly for the cryostage used on the NCXT soft X-ray microscope XM-2 at the Advanced Light Source synchrotron at LBNL. The goniometer has a slip ring assembly that allows a 360° specimen rotation in addition to motorized tip tilt eucentric alignment of the specimen.
determine how to update voxel values during each iteration. The weight $w_{ij}$ can be determined as shown in Figure 9. This can also be achieved by any one of a number of simple interpolation methods. There are a number of alternative methods for implementing the iterative minimization. In addition, there are a number of ways of defining what constitutes an iteration; for example, multiple projections can be used to calculate the new voxel value without updating the value, and then the average of these can be used as the new value. Iterative methods that are commonly used include the algebraic reconstruction technique (ART), the simultaneous iterative reconstruction technique (SIRT), and penalized likelihood.39–41

An advantage of the iterative methods is that it is easy to incorporate additional information into the algorithm; for example, prior knowledge about volume constraints and positivity can be used to guide the reconstruction process. In cases in which the number of projections collected corresponds to an underdetermination of the 3-D volume or in the case of noisy projection images, iterative reconstruction methods have been found to produce reconstructions with less noise at a given resolution than Fourier methods.41–43 However, iterative methods require a significant amount of computing power. Additional issues are that different parts of the image can converge at different rates, the volumes may have a non-uniform spatial resolution, and there are parameters that control the iterations and the stopping point that must be chosen and optimized.

**2.6.3.2.3 Regularization**

It is only possible to measure a finite number of experimental projection images; as such, a large number of possible reconstructions are consistent with the projection data set (i.e., the inverse problem is ill posed). This means that one issue that must be confronted during the reconstruction process is how to 'regularize' the data. For the case of isotropic 'full rotation' tomographic data, the effect of undersampling shows up as uncertainty in the high spatial frequency components of the reconstruction. In this case, a low pass band limit can be imposed, and a unique reconstruction can be obtained.

In Fourier methods, enforcement of this band limit is generally implemented using a low pass filter on the projection images during the back-projection process, or the final reconstructed volume can be filtered; in the absence of such filtering, the reconstruction appears very noisy.38 In iterative methods, regularization can be similarly implemented, although other types of regularization are also useful. One option is to add a penalty term to the optimization equation given previously.45 A penalty function is added to the equation; the parameter $\beta$ controls the overall importance of this addition on the reconstruction. The added term is chosen to penalize differences between pixels in a specified neighborhood, thus enforcing a lower noise level:

$$\min_{f} \sum_{j=1}^{N} \|Pf - p_j\|^2 + \beta \cdot (\text{penalty})$$

For some iterative methods (including SIRT and ART), the regularization is controlled by stopping rules that modify the number of iterations used to obtain a reconstruction.36,41–42

**2.6.3.2.4 Visualization**

The most basic visualization of a 3-D data set is the display of a one-voxel-thick orthoslice through the volume, either in gray scale or with an adjustable color map. Volume rendering is a second type of visualization, which can be used to view the entire 3-D volume. A third form of visualization is to show the surfaces, or boundaries, of objects of interest. This can be done automatically by displaying an 'iso-surface' connecting all the
points in a volume corresponding to a chosen threshold value. Alternatively, objects of interest in the image can be segmented and the resulting boundaries can be displayed as surfaces.

The software package Amira, from Visage Imaging (www.amira.com), is one of the primary commercial software packages for visualizing volumes. The Amira orthoslice module allows visualization of selected slices from a volume reconstruction from any orientation, whereas other modules allow volume-rendering visualization with a variety of options. Additional modules can be used to create, edit, and display surfaces, and the program has a segmentation editor for creating and modifying volume segmentations. Also, the Amira suite has the capability to generate sophisticated animations of volume visualizations for use in presentations and publications.

### 2.6.3.2.5 Segmentation and analysis

Segmentation is the process of partitioning an image or a 3-D volume into regions of voxels, such as determining the voxels corresponding to a particular organelle of interest. One approach is to manually segment slice by slice (i.e., a slice through the volume is displayed and an image manipulation tool is used to select a particular region in that slice). This is repeated for each slice (or at intervals in a range of slices) in order to select an entire region in 3-D space. Because biological specimens usually contain a large number of complex structures, this kind of segmentation is frequently needed for segmentation of at least some of the structures in each specimen. In other cases, this approach is used to ‘clean up’ problems in a segmentation that was carried out automatically.

In most cases, some sort of automation is possible in the segmentation process. One option is to carry out segmentation based on thresholds, where voxels are selected based on whether their values fall in a selected region (where the voxel values correspond to X-ray absorption values). A threshold can be applied to a full image or as part of a ‘region growing algorithm,’ where voxels adjacent to a seed point are added as long as they fall in a selected value range. Segmentation based on a threshold or a region-growing algorithm can often be refined by morphological operations (e.g., opening, closing, filling, erosion, and dilation) to yield a segmentation that is a close match to the object boundaries. Amira includes a segmentation editor that incorporates a large number of both manual and automatic segmentation options, including those mentioned here.

### 2.6.4 Soft X-Ray Tomography: Practice

Good imaging begins with good specimen handling to avoid causing damage to delicate sub-cellular structures of the specimen. This is particularly important when the specimen is imaged using a high-resolution technique such as soft X-ray tomography. Artifacts generated by specimen preparation protocols cannot be mitigated or eliminated by imaging techniques; they can only be accounted for. Fundamentally, for soft X-ray tomography, little or no specimen preparation is

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**Figure 13** A projection image of cryoimmobilized NIH 3T3 cell obtained by stitching together multiple fields of views. In soft X-ray microscopy, the contrast is generated primarily by carbon-rich material absorbing X-rays more strongly than water (i.e., water window imaging). In this image, mitochondria, the nucleus, and the nucleolus are clearly seen without the use of metal stains. Consequently, the information obtained by this method is both quantitative and representative of the specimen in a near-native state. Scale bar = 2.5 μm. Reproduced with permission from Figure 1a in Meyer-Illse, W.; Hamamoto, D.; Nair, A.; Lelievre, S. A.; Denbeaux, G.; Johnson, L.; Pearson, A. L.; Yager, D.; Legros, M. A.; Larabell, C. A. High resolution protein localization using soft X-ray microscopy. *J. Microsc.* 2001, 201, 395–403.
needed in advance of data collection. In most cases, cells can be taken directly from their growth chamber and immediately mounted for cryofixation and imaging. There are two principal systems for mounting specimens: capillary tubes for cells in suspension (including prokaryotes, yeast, and blood cells) and flat silicon nitride membranes for larger cells, such as adherent eukaryotic cells or sections of tissue.

2.6.4.1 Specimen Mounting

2.6.4.1.1 Cells mounted in glass capillaries

Capillaries are considered to be the optimal specimen holder because they allow full rotation through 360°. Thin glass microcapillaries that taper very quickly to an extended thin region are created using a micropipette puller. After they have been pulled, the capillaries have a cross section ranging from 5 to 12 μm in diameter for at least 1 mm along the length of the tube. This configuration is the ideal combination of mechanical stability while providing an extended area along which cells can be imaged (Figure 10). Cells are loaded with suitable medium into the capillary through the broad end using a pipette fitted with a microloder tip. Typically, the capillaries are loaded with 1 μl of a suspension that contains between 10 000 and 100 000 cells per milliliter. For most cell types, capillary force is sufficiently strong that the cells are drawn into the tapered region. In some instances – for example, if the cells are particularly ‘sticky’ – it is necessary to use centrifugal force to pull the cells into the fine bore. To minimize damage to the cell, this is carried out using the minimum force possible. Fiducial markers for alignment of the projection images are generated using 50- to 100-nm-diameter gold nanoparticles attached to the outside of the capillaries.

Figure 14  Three different fields of view containing Caulobacter crescentus cells mounted in a capillary tube. (a and d) Orthoslices through the reconstructions; (b and e) volume rendered view; (c) 3-D model of the data shown in panels a and b; (f) 3-D model of a third field of view – the cells highlighted in the box are shown in a cross section view in the inset. Scale bar = 1.0 μm (a) and 1.5 μm (d). The field of view shown in panels a–c contained approximately 200 cells, each of which was simultaneously reconstructed. This highlights the level of throughput that can be achieved using soft X-ray tomography; the data necessary to tomographically reconstruct this field of view took less than 3 min to collect.
2.6.4.1.2 Cells mounted on flat membranes
To prepare specimens such as adherent cells for X-ray microscopes, the following procedures were developed using 100-nm-thick silicon nitride membranes with silicon frame supports (Figure 10). The holder is first sterilized by immersing in 70% ethanol and then rinsed with distilled water. The membrane is coated with poly-L-lysine before plating. Cells can then be directly cultured on the holder by suspending it in the growth flask. After reaching a desired confluence, the cells are washed with phosphate buffered saline. Prior to freezing, excess liquid is removed using filter paper. Only a thin layer of liquid is left on the membrane. Before fiducial markers can be

Figure 15 Soft X-ray tomography of Escherichia coli cells overexpressing yellow fluorescent protein (YFP). (a) A representative E. coli cell after segmentation showing YFP inclusion bodies (yellow) and nucleoid (purple). (b) The reconstructed volumes of 40 segmented E. coli cells expressing YFP; scale bar = 1.0 μm.
added, they must be incubated overnight with an excess of bis(p-sulfonatophenyl) phenylphosphine. This prevents the salts in cell culture media from causing the nanoparticles to precipitate.

### 2.6.4.2 Cryocooling

Specimens must be rapidly frozen prior to imaging using one of several available methods, including plunge freezing, propane jet freezing, and high-pressure freezing. Cells suspended in capillaries for imaging at XM-2 are typically frozen using a flash cooling technique. The specimen is rapidly cooled to liquid nitrogen temperature by a stream of cold helium gas or by a cryogenic liquid-like propane. This method freezes the specimen in the tip of the capillary very quickly, leading in large extent to amorphous ice, meaning that the structure of the ice is essentially the same as that of the liquid before freezing. For larger cells and tissues, high-pressure freezing followed by trimming and sectioning of the cryogenic specimen can be done.

### 2.6.4.3 Cryorotation Stages

Cryofixation techniques are well-known from cryo-electron microscopy and have been implemented in electron microscopes for a number of years. However, the implementation in high-throughput soft X-ray tomography has only recently been achieved. The first cryogenic specimen stages designed for soft X-ray microscopy did not permit the specimen to be rotated around an axis; however, these instruments produced data that showed that cryocooling made tomography feasible. Two cryogenic stages capable of rotation were developed independently (one by the Schmahl group at the BESSY 1 synchrotron in Berlin and the other by Larabell and Le Gros at the National Center for X-ray Tomography (NCXT) at the ALS). The first tomographic images of cells produced by these two

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**Figure 16** Segmented volumes of the three phenotypes displayed by Candida albicans: (a) yeast-like (b) germ-tube, and (c) hyphal cells. The hyphae shown in panel c is 47 μm long. To image this cell, which is significantly larger than the 15-μm microscope field of view, projection series were collected from sequentially overlapping fields of view along the length of the cell. These projection series were individually reconstructed and then computationally stitched together to form a single, seamless tomographic reconstruction. Scale bar = 1.0 μm. From McDermott, G.; Le Gros, M. A.; Knoechel, C. G.; Uchida, M.; Larabell, C. A. Soft X-ray tomography and cryogenic light microscopy: The cool combination in cellular imaging. Trends Cell Biol. 2009, 19, 587–595.

**Figure 17** Diploid Saccharomyces cerevisiae cells imaged at each phase of the cell cycle using soft X-ray tomography. Organelles are color coded as follows: blue, nucleus; orange, nucleolus; gray, mitochondria; ivory, vacuoles; and green, lipid bodies. Scale bar = 1 μm. From Uchida, M.; Sun, Y.; McDermott, G.; Knoechel, C.; Le Gros, M. A.; Parkinson, D.; Drubin, D. G.; Larabell, C. A. Quantitative analysis of yeast internal architecture using soft X-ray tomography. Yeast 2011, 28(3), 227–236. Copyright by Wiley.
instruments energized the community with the promise of what can be achieved. Of the two designs, the cryorotation stage developed at the NCXT turned out to be the more robust and reliable in terms of day-to-day use. This cryorotation stage design has progressed to the next phase of development and is now a key component in XM-2. To date, this cryorotation stage has produced several thousand tomographic reconstructions of cells. A major advantage of this particular design is that the specimen is not held in vacuum (as is the case with an adapted EM cryostage developed for the new Berlin X-ray microscope). Instead, the specimen is kept at atmospheric pressure and is cooled by an adjustable flow of cryogenic gas (helium cooled to liquid nitrogen temperature). This new instrument is based on many of the ideas and techniques used in X-ray crystallography. The specimen is mounted on an x-y-z-θ stage, and motorized stages are used to center the region of interest in the specimen on the rotation axis (Figure 11). After centering, a specific cell is well aligned.

Figure 18 Invagination of the plasma membrane in a *Saccharomyces cerevisiae* endocytic mutant. FM4-64–labeled plasma membrane in wild-type (a) and endocytic mutant, Δsj1Δsj2 (b) cells. Comparison of plasma membrane structure in wild-type (c) and endocytic mutant (i.e., synaptojanin double mutant, Δsj1Δsj2) (d) cells. Deep membrane invaginations are seen in mutant cells (right). Scale bar = 2.0 μm. Sample provided by Prof. D. Drubin, University of California at Berkeley.

Figure 19 Two different types of membrane invaginations were seen in the yeast shown in Figure 18 based on their linear X-ray absorption coefficients (LAC). From the differences in LAC values (i.e., dark, 0.67 μm⁻¹; light, 0.46 μm⁻¹), it is clear that the internal compositions of these two membrane-bound structures are different.
to the rotation axis of the cryostage, and a complete tomographic data set can be collected without any refocusing of the specimen. To ensure that the minimum flow of gas is used to maintain an adequately low temperature of the specimen without introducing mechanical instabilities, an intermediate heat shield cooled by thermal connection to a nitrogen gas primary cooling loop is used. This heat shield provides most of the cooling power required to keep components that are close to the specimen cold. The primary loop also cools a dual heat exchanger system that is utilized to produce the low-temperature helium gas used for direct specimen cooling (Figure 12). This arrangement greatly reduces the flow of helium gas required to keep the specimen cold. The slow flow of cold helium gas is efficient and remains stable throughout data collection, and it prevents the ingress of moisture and atmospheric gasses.

The cryo-specimen stage at XM-2 can be used for single axis tomographic measurements on both flat and cylindrical specimens. For specimens with a cylindrical geometry, particularly those that can be loaded into thin-wall glass tubes, full 360° tomographic data sets can be collected. For extended flat specimens, full rotation is not possible due to both mechanical interference between the specimen and microscope components and the increasing thickness of a flat specimen imaged at high angle. The system is designed to allow up to ±75° tomography on such flat specimens.

The design of this atmospheric pressure cryostage allows the incorporation of other imaging modalities, such as visible light and fluorescence microscopy. As a result, the XM-2 cryostage now incorporates a state-of-the-art cryogenic light microscope for correlated imaging.

2.6.5 Application of Soft X-Ray Tomography in Cell Biology

The first reported soft X-ray tomographic reconstruction was of the algae *Chlamydomonas reinhardtii*.\(^9\) This was a landmark publication in the field, and even today, it stands as an exceptional example of what can be achieved with soft X-ray tomography. Subsequent work was carried out to image both fission and budding yeasts.\(^2,4,8,54,55\) This work was performed using a first-generation, prototype cryorotation stage mounted on XM-1, a general-purpose soft X-ray microscope at the ALS. Because XM-1 was primarily used for magnetism and materials research, it did not have the optimal configuration for biological imaging. However, these images were highly informative and further demonstrated the utility of the technique.

In addition to an optical system optimized for biological imaging, XM-2 is fitted with a second-generation cryorotation stage. The combination of these two factors greatly improved image quality and fidelity. This is particularly the case when the specimen is mounted in a capillary. In this configuration, the specimen can be freely rotated, thus avoiding the so-called
‘missing wedge’ or ‘cone’ of data inherent in electron tomography. As a representative example of the capability of this relatively new technique, some work imaging bacteria, yeast, and mammalian cells is demonstrated in Figures 13–23. It is clear that soft X-ray tomography is an exciting new modality for quantitatively imaging cell morphology and sub-cellular organization at high spatial resolution.

2.6.6 Localizing Molecules Using Correlated Light and X-Ray Microscopy

It is possible to localize molecules directly in a soft X-ray tomographic reconstruction by immunolabeling them with electron-dense tags. This type of labeling has been very well developed for electron and light microscopy. However, there are disadvantages to immunolabeling. This method requires the specimen to be chemically fixed and treated with membrane-permeabilizing agents (e.g., detergents and organic solvents) to allow relatively large antibody molecules ingress to the cell. Clearly, disrupting the integrity of membranes could result in a significant leakage of proteins and lipids out of the cell and, as a result, potentially cause serious damage to delicate sub-cellular structures. Once they are inside the cell, antibodies may have very limited access to the target epitope-containing molecule. It is therefore preferable to localize molecules using fluorescence microscopy and correlate this with soft X-ray tomography. This technique has been developed and the necessary instrumentation described. Le Gros and colleagues outlined the development of a cryogenic high numerical-aperture light microscope and its integration with soft X-ray tomography to allow a specimen to be imaged with visible light and soft X-ray microscopy (Figure 24). In the case of XM-2, correlation of the data from these two imaging modalities proved to be straightforward; both imaging techniques can be carried out on the same instrument by a simple translation of the specimen.
There is an inherent significant benefit to using the cryogenic high numerical-aperture light microscope for fluorescence imaging. It is well known that cryocooling significantly enhances the fluorophore lifetimes. This has been reported to be a factor of 30 or more increase in the fluorescent lifetime of yellow fluorescence protein expressed in *Escherichia coli* cells.

2.6.7 Summary and Future Prospects

Imaging techniques have played an extremely important role in biological research since the invention of the microscope, with recent emphasis on high-resolution quantitative imaging methods. This chapter introduced two new imaging techniques that are uniquely suited to quantitative imaging at better than 50-nm resolution. The first technique, soft X-ray tomography, is capable of producing unprecedented insights into the structure of cells and tissue specimens in a near-native state. The high spatial resolution and quantitative contrast mechanism of soft X-ray tomography reveal subtle sub-cellular features in cryoimmobilized cells, making it an excellent nondestructive biological imaging tool. The second technique, cryofluorescence microscopy, makes it possible to collect high-resolution 3-D data on the location of specific molecules in unprocessed cells. Combining cryofluorescence and X-ray imaging, we can now determine the position of fluorescently tagged molecules in the context of a high-resolution reconstruction of unstained cell architecture. These new technologies enable novel structure-function analyses that are not possible with other imaging modalities. Future developments in these areas promise greatly expanded application of these imaging technologies to a wide range of biological research.

References


Figure 24

Correlated images of *Schizosaccharomyces pombe*. (a) Cryoimmobilized, high-aperture fluorescence image of *S. pombe*. The vacuoles were stained with CMFDA (5-chloromethyl fluorescein diacetate). (b) Soft X-ray tomography of the same cell shown in an orthoslice from the tomographic reconstruction. (c) Segmented vacuoles were overlaid on the orthoslice. (d) The completely segmented cell. Nucleus, blue; nucleolus, orange; mitochondria, gray; vacuoles, white; and lipid-rich vesicles, green. Scale bar = 1.0 μm.


