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Soft X-ray Tomography and Cryogenic Light Microscopy: The Cool Combination in Cellular Imaging

Gerry McDermott¹, Mark A. Le Gros², Christian G. Knoechel¹, Maho Uchida¹, and Carolyn A. Larabell^{1,2}

¹Department of Anatomy, University of California San Francisco, Ca

²Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, Ca

Abstract

Soft x-ray tomography (SXT) is ideally suited to imaging sub-cellular architecture and organization, particularly in eukaryotic cells. SXT is similar in concept to the well-established medical diagnostic technique computed axial tomography (CAT), except SXT is capable of imaging with a spatial resolution of 50 nm, or better. In soft x-ray tomography (SXT) cells are imaged using photons from a region of the spectrum known as the 'water window'. This results in quantitative, high-contrast images of intact, fully hydrated cells without the need to use contrast-enhancing agents. Cells are therefore visualized very close to their native, fully functional state. The utility of SXT has recently been enhanced by the development of high numerical aperture cryogenic light microscopy for correlated imaging. Taking this multi-modal approach now allows labeled molecules to be localized in the context of a high-resolution 3-dimensional tomographic reconstruction of the cell.

Introduction

Our understanding of cell structure and behavior has been particularly dependent on imaging. As a consequence, the emergence of new imaging modalities potentially leads to new insights and discoveries in cell biology. In this review we will examine soft x-ray tomography (SXT), an emerging technique for quantitatively imaging whole, hydrated cells in 3-dimensions. SXT imaging is both unique and complementary to existing imaging techniques, such as light and electron microscopy. The soft x-ray illuminating photons used in SXT penetrate biological materials much more easily than electrons. This allows specimens up to 10 μm thick to be imaged. Unlike electron microscopy this means there is no need to section eukaryotic cells with an ultramicrotome prior to being imaged by SXT. Since contrast in SXT is produced directly by the differential absorption of x-rays there is no need to dehydrate or stain specimens. Consequently, SXT produces high-resolution views of specimens in a near-native state. Recently, SXT was made even more powerful by the development of high numerical aperture cryogenic light microscopy for correlated imaging. This combination of light and x-ray modalities allows tagged molecules to be localized in the context of a high-resolution, 3-dimensional image of a cell. This meets a long-standing

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Corresponding author: Larabell, C.A. (Carolyn.Larabell@UCSF.edu).

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need in cell research, and as such has enormous transformational potential in academic, industrial and clinical research.

We will begin by reviewing the development of soft x-ray microscopy for biological imaging, and then describe the extension of this technique to 3-dimensional tomographic imaging.

Soft X-ray Microscopy

X-ray microscopes have been in use for materials science research since the 1940s¹. However, it wasn't until the 1990s that the technique became capable of satisfactorily imaging biological specimens²⁻⁵. At this point, soft x-ray microscopes operating in the 'water window' region of the spectrum began to be equipped with high-efficiency CCD cameras. The advances resulted in the production of projection images with stunning clarity and contrast from a range of cells and tissue specimens⁶⁻⁸.

Soft x-ray microscopes have three clear advantages over light and electron microscopes. Firstly, the short wavelength means they can image cells at significantly higher spatial resolution than is possible with light. Secondly, fully hydrated specimens up to 10 μm thick can be imaged without being sectioned⁹⁻¹³, and finally, image contrast is obtained directly from the absorption of x-rays by the specimen. The 'water window' is the region of the spectrum that lies between the K shell absorption edges of carbon (284 eV, $\lambda=4.4$ nm) and oxygen (543 eV, $\lambda=2.3$ nm). Photons within this energy range are absorbed an order of magnitude more strongly by carbon- and nitrogen-containing organic material than by water⁹. This absorption adheres to Beer-Lambert's Law and is therefore linear with thickness and concentration⁵. Consequently, images produced by soft x-ray microscopes are quantitative, with each biochemical component having a signature x-ray Linear Absorption Coefficient (LAC)^{9, 12}. As a result, cell structures are visualized directly, with image contrast being derived from differences in biochemical composition and density. For example, dense lipid-rich structures exhibit significantly greater x-ray absorption than organelles such as vacuoles that have significant water content.

The optical arrangement of a soft x-ray microscope is relatively simple, and similar in concept to that of a simple bright-field microscope. A condenser lens illuminates the specimen and an objective focuses the transmitted light onto a detector. This optical configuration is shown in simplified form in Box 1, Figure I. Due to the physical characteristics of soft x-rays conventional glass lenses can't be used. Instead, soft x-ray microscopes rely on Fresnel zone plates or x-ray focusing capillaries (see Box 1, Figure II)^{5, 14, 15}.

Prior to being imaged in a soft x-ray microscope cells must be mounted in a suitable holder. This is typically a thin-walled glass capillary tube or a flat silicon nitride membrane. Capillary holders have an advantage of permitting the specimen to be rotated through 360°. Depending on the magnification, the field of view in a soft x-ray microscope is up to 15 μm , allowing multiple cells to be imaged per data set. In the case of bacteria this can be tens or even hundreds of cells per experiment. With larger, higher order cells, such as yeast, each tomographic data set typically contains three to seven cells. This compares very favorably with electron tomography, where cells must first be cut into sections that are a maximum of 200-500 nm thick, either using a microtome, or by ion milling¹⁶. This is an onerous task and in a recent review was identified as being the 'Achilles heel of electron tomography' and the underlying reason why so few cells have been imaged in 3-dimensions using this technique¹³. Consequently, SXT represents a significant leap forward in terms of our ability to visualize cells.

Soft X-ray Tomography

As with light and electron microscopes, a soft x-ray microscope can only produce 2-dimensional representations of a 3-dimensional specimen. Such images can, of course, be very informative (see Figure 1) and in the past they have provided insights on the general structure and organization of various types of cells^{5, 6, 8, 17}. However, for most biological specimens 2-dimensional imaging results in structural features being confusingly superimposed, rendering high-resolution projection images of complex eukaryotic cells virtually meaningless^{9, 11, 18, 19}. However, if a sufficient number of 2-dimensional images are recorded at incremental angles around a rotation or 'tilt' axis a 3-dimensional tomographic reconstruction of the specimen can be calculated^{13, 18, 20, 21}. The principles of tomography are described briefly in Box 2.

Evolution of the Cryogenic Rotation Stage for Tomography

All biological materials eventually get damaged when they are exposed to intense sources of radiation, irrespective of whether this is the ultra violet illumination in a fluorescence microscope, electrons in an electron microscope, or the photons in an x-ray microscope. Tomographic imaging requires a number of projection images to be collected from the same specimen. Therefore, steps must be taken to mitigate the effects of radiation damage. The most efficient way to do this is to mount the specimen in a cryogenic stage and image it after vitrification. The first cryogenic specimen stages designed for soft x-ray microscopy did not permit the specimen to be rotated around an axis. However, it was possible to use these instruments to collect enough images from a specimen to show that cryo-cooling does indeed reduce radiation damage to the point that tomography is feasible^{6, 22, 23}.

More recently, two cryogenic stages capable of rotation were developed independently (one by the Schmahl group at the BESSY 1 synchrotron in Berlin, the other by Larabell and Le Gros at the National Center for X-ray Tomography (NCXT) at the Advanced Light Source (ALS), Berkeley). The first tomographic images of cells produced by these instruments energized the community by providing glimpses of what could be achieved with the technique^{10, 12, 24, 25}. The cryo-rotation stage developed at the NCXT has the advantage of holding the specimen at atmospheric pressure, rather than retaining it in a vacuum (as is the case with the adapted EM cryo-stage used in²⁶). By bathing the specimen in cryogenic helium heat is transferred efficiently, thus keeping the specimen vitrified at a stable temperature throughout data collection. In addition, this configuration of cryo-rotation stage allows the ready incorporation of other imaging modalities. In the past year the NCXT cryo-rotation stage has proven to be a reliable workhorse that has produced the data for several thousand tomographic reconstructions of cells. A representative of these reconstructions is shown in Figure 2, together with light microscopy images of similar cells for comparison.

Soft X-ray Tomography: Applications

The beauty of SXT is that it can be applied to virtually any imaging problem in cell biology, from imaging simple bacteria, to visualizing the internal structure of high-order eukaryotic cells and even tissue specimens. The first reported soft x-ray tomographic reconstruction was of the alga *Chlamydomonas reinhardtii*^{12, 25}. This was a landmark publication for the development of the field, and showed that cryogenically cooling sufficiently mitigated radiation damage to allow biological cells to be tomographically imaged with high fidelity. Even to this day, this work by Weiss and colleagues stands as an exceptional example of what can be achieved with SXT.

In later work, Larabell and colleagues carried out a number of studies on both fission and budding yeasts^{24, 27, 28}. Data were collected for these publications using a prototype cryo-

rotation stage mounted on XM-1, a general-purpose soft x-ray microscope at the ALS. Since XM-1 was primarily used for magnetism and materials research, it was not optimally configured for biological imaging. The images produced in these studies were, however, highly informative and helped guide specimen handling protocols and data collection strategies used in later work.

After the commissioning of a XM-2 in 2008, the earlier work of Larabell and colleagues was quickly surpassed. In addition to having an optical system optimized for biological imaging. Incorporated into the new microscope is a second-generation cryo-rotation stage that contains a large number of improvements over the prototype. For example, it has much greater mechanical and thermal stability. The combination of new microscope and cryostage has lead to improved image quality and fidelity. As a representative example of the capability of this new facility some recent work imaging T-cells is shown in Figure 3 and the morphological differences in the yeast *Candida albicans* are shown in Figure 4. Of particular note is the capability to tomographically image cells that exceed the field of view, such as the *Candida* hyphal cell shown in Figure 4. This cell measured 47 μm lengthwise (and 2 to 3 μm in width along the hyphal tube). Since the cell was mounted in a thin-walled glass capillary, it was a straightforward procedure to collect five successive fields of view, and thus image the entire cell. In this study, each field of view was reconstructed individually before being ‘stitched’ together *in silico* to form a composite image of the entire cell. Imaging a cell this size using EM tomography is an impossible challenge, since it would require imaging approximately 200 sections of the same hyphal cell. Using soft x-ray microscopy the projection images necessary to complete this enormous reconstruction were collected in less than 20 minutes.

In a soft x-ray microscope, such as XM-2, the observed spatial resolution is determined by the zone plate optical elements (Box 1, Figure II). In microscopes that utilize alternate condensers the factors that spatial resolution are more complicated and out with the scope of this review (however, it should be noted that microscopes with capillary condensers, such as the one in BESSY, currently have potential to image with a spatial resolution of better than 20 nm). Most SXT reports in the literature have used zone plates that produce images with a spatial resolution of 50 to 60 nm. As with all imaging modalities there is much debate in the community regarding spatial resolution. To the point one might assume that the resolution limit is the ultimate criterion of quality in any imaging technique. In a recent review Leis and colleagues directly questioned this assumption¹³. In their opinion measurement of the absolute resolution limit is a rather academic exercise and of limited importance compared to determining how much useful information can be obtained from an imaging technique, or how well this correlates with ‘reality’. In other words, the most important considerations are “can the imaging technique under consideration adequately answer the questions being posed?” and “are the images produced by it a true representation of the live, fully functional specimen?” Leis and colleagues also proposed that ‘direct’ imaging techniques are significantly more informative and produce more accurate representation of the specimen than ‘indirect’ imaging of specimens. In this context, ‘direct’ imaging methods are those that rely on intrinsic properties of the specimen, such as the absorption of x-rays according to biochemical composition or density to form the image. Whereas ‘indirect’ methods are those where specimens are stained prior to imaging, in which case it is actually the stain that is imaged rather than the specimen *per se*. How accurately this represents the specimen is a function of factors such as how well the individual components in a cell bind the stain. This is obviously an unknown. On the other hand, SXT is a ‘direct’ imaging method with minimal specimen preparation requirements, and therefore maximal retention of specimen integrity.

Another positive attribute of SXT over more established cellular imaging methods is that it produces isotropic data. This is particularly true when the specimen is mounted in a capillary that can be freely rotated without angular limitation. Cells that are too large to fit in capillaries are mounted on flat silicon nitride specimen holders. Due to instrumental limitations these holders do not have completely unlimited angular rotation, however this limitation is minimal and does not have a significant impact on the fidelity of the reconstruction. In contrast, in EM tomography tilting the specimen causes it to become 'thicker' than the depth through which the electrons can penetrate. This places rather restrictive limitation on the maximum tilt resulting in a significant wedge of missing data. This reduces the signal-to-noise in EM tomographic reconstructions, and makes techniques such as automatic segmentation significantly more difficult to apply.

The excellent signal-to-noise in a SXT tomogram makes it relatively straightforward to segment a reconstructed cell into organelles and other sub-cellular structures based on their LACs (Box 3). Table 1 lists the measured, average LACs for organelles in the segmented *Candida albicans* cells shown in Figure 4. It should be clear from these data that, for example, mitochondria are readily distinguishable from lipids or vacuoles.

Whilst informative, the LAC values alone cannot give information on the location of specific molecules, this requires the molecules be tagged and visualized directly with SXT, or by the use of a correlated microscopy method.

Localizing Molecules using Correlated Cryogenic Light Microscopy

Determining the location of specific molecules and complexes in a cell is one of the keystones of modern cell biology. However, the combination of localization data with structural imaging is even more informative than either technique alone. SXT has the potential to play a major role in this regard. Molecules can be localized directly in a tomographic reconstruction of a cell by immuno-labeling them with electron dense tags⁶. This type of labeling has been very well developed for use in electron and light microscopies. However, there are distinct disadvantages to immuno-labeling. The specimen typically chemically fixed and then treated with membrane permeabilizing agents, such as detergents or organic solvents to allow the relatively large antibodies to enter the interior of the cell. Obviously, disrupting the integrity of membranes to allow large molecules to enter the cell also results in a significant leakage of proteins and other molecules out of the cell, potentially causing serious damage to the specimen. Furthermore, once inside the cell the antibodies may have limited, or no access to the target epitope-containing molecules.

A less damaging method for localizing molecules is to use genetically encoded labels, such as a Fluorescent Protein (FP), to localize molecules of interest using fluorescence microscopy, and correlate this data with SXT. In a recent paper, Le Gros and colleagues describe the development of cryogenic high numerical aperture light microscopy, and its integration with SXT²⁹. This microscope has now been integrated into the NCXT cryo-rotation stage to allow correlated light and x-ray imaging to be carried out on the same specimen.

Future Directions

Soft x-ray tomography is now living up to long-held expectations¹³ as a high-resolution, 3-dimensional imaging technique capable of visualizing intact eukaryotic cells in a close-to-native state. Furthermore, using the latest cryo-rotation stages a large number of cells can be quantifiably imaged in a very short space of time, making SXT ideally suited to generating statistically significant quantities of phenotypic data.

Several new SXT instruments are either under construction or being considered for construction at synchrotrons around the world^a. Rapid progress is now also being made on the development of 'table-top' x-ray sources with characteristics that make them amenable for use as sources for soft x-ray microscopy^{30, 31}.

As discussed above, the absolute spatial resolution achieved using an imaging technique such as SXT is not the major consideration. That said, there are clearly some particular situations where SXT imaging can benefit from increased spatial resolution. Fortunately, advances in nanofabrication technologies can be applied to manufacturing zone plates with incrementally higher spatial resolution. The current generation zone plates have a maximum spatial resolution of 15 nm³². These enhanced resolution optics are easily incorporated into existing soft x-ray microscopes. However, concomitant with an increase in resolution there is a decrease in the depth of field. With zone plates with a resolution better than 25 nm the depth of field is smaller than the thickness of most eukaryotic cells. Overcoming this limitation requires the development of computational methods and the use of modified data collection strategies. The most obvious solution is to use established methods such as deconvolution to overcome the depth of field limitations. This combination of deconvolution and tomography (termed DETOMO) is computationally straightforward and relies on adaptation of existing code, rather than the development of completely new algorithms (Le Gros *et al.*, unpublished observation).

Similar to macromolecular crystallography SXT is also highly amenable to automation and remote operation. Currently, SXT facilities, such as the NCXT, are in the process installing robots capable of mounting specimens, and implementing software to automatically collect, process and archive data. There are a number of advantages to automation, besides making life easier for the experimenter. Most biological experiments are time and/or cell cycle dependent. Consequently, it is essential that specimens can be collected, cryo-fixed and stored until they can be imaged. Much work is also being directed towards automating segmentation, one of the current bottlenecks in both EM tomography and SXT. In general, software packages developed for EM tomography can be used on SXT data without modification, however, since SXT produces isotropic data with excellent signal-to-noise there is a need for software optimized for SXT to take full advantage of these unique characteristics.

Perhaps the most exciting recent developments in SXT imaging is the recent development of correlated x-ray and cryo-light microscopies²⁹. Using this combination of modalities it is possible to determine the location of labeled proteins using fluorescence, and then place this information directly into a high-resolution tomographic reconstruction of the cell (Box 4). This represents an enormous leap forward in our ability to image cells, and cellular processes.

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MAL and CAL are the inventors on patents for the cryogenic immersion microscope (WO/2006/113916) and the cryo-tomography x-ray microscope stage (US-2009-0129543-A1).

^aSXT microscopes are either under construction or being considered at the following synchrotron light sources: Elettra (<http://www.elettra.trieste.it/>), Diamond (<http://www.diamond.ac.uk/>), ALBA (<http://www.cells.es/>), and SOLEIL (<http://www.synchrotron-soleil.fr/>).

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Glossary

ART	Algebraic Reconstruction Technique. An iterative algorithm used to reconstruct 3-dimensional volumes from 2-dimensional projection images.
Alignment	The adjustment of projection images in a rotation (tilt) series to account for physical shifts in the specimen holder during the collection of a rotation series; due to imperfect instrumentation.
ALS	The Advanced Light Source, Berkeley, California. A third generation synchrotron, and the world's brightest source of soft x-rays (http://als.lbl.gov).
BESSY	The Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung. A synchrotron x-ray light source in Berlin, Germany (http://bessy.de) Note, BESSY has been renamed as Helmholtz-Zentrum Berlin fuer Materialien und Energie GmbH.
Cryo-fixation	Cooling the specimen by methods that prevent water from turning into crystalline ice.
Filtered back-projection	Technique for tomographic reconstruction where the projection rays are 'back-projected' through Fourier space to form the 3-dimensional volume.

LAC	Linear Absorption Coefficient. The fraction of a beam of radiation absorbed per unit thickness of absorber, as defined by the Beer-Lambert law.
Orthoslice	An orthoslice is an extraction of absorption values along a plane perpendicular to one of the coordinate axis (in other words, the generation of a 2-D representation from a 3-D volume). The complete reconstructed volume of the specimen consists of a number of such planes.
NCXT	The National Center for X-ray Tomography (http://ncxt.lbl.gov).
Projection image	An individual image from a rotation (tilt) series.
SXT	Soft x-ray tomography.
Soft x-rays	Photons with wavelengths of one to several nanometers, and energies up to 1 keV.
Rotation series	A number of projection images that have been collected around a rotation axis.
Tilt series	Synonymous with rotation series, more commonly used in electron tomography.
Water window	The region of the electromagnetic spectrum lies between the K shell absorption edges of carbon (284 eV, $\lambda=4.4$ nm) and oxygen (543 eV, $\lambda=2.3$ nm).
Tomographic reconstruction	Computationally combining aligned projection images from a rotation (tilt) series to form a 3-dimensional volumetric reconstruction of the specimen.
Zone plate	Diffraction lenses used in soft x-ray microscopes (see Box 1).

Box 1 The optical arrangement of a soft x-ray microscope

Optical layout of a soft x-ray microscope

The optical configuration of XM-2, a soft x-ray microscope for biological and biomedical tomography at the National Center for X-ray Tomography⁹ is shown in Figure I. Polychromatic x-rays from a synchrotron 'bend magnet' are steered to the condenser by a mirror. In this configuration a condenser lens both focuses the x-rays onto the specimen and functions as a monochromator. The objective lens generates a magnified image of the specimen on the detector. In this instrument the condenser and objective optics are Fresnel zone plates. In other instruments 'undulators' are used in place of 'bend magnets' as the x-ray source³³, and 'rotating condensers'³⁴ or 'capillaries'²⁶ are employed in place of zone plates as the condenser.

Lenses for soft x-ray microscopy

Soft x-ray microscopes use Fresnel zone plates either as both condenser and objective lenses, or just as the objective. Unlike a conventional lens a zone plate relies on diffraction rather than refraction to focus the light⁵. Zone plates consist of radially symmetric rings, known as Fresnel zones, which alternate between opaque and transparent (see Figure II). Currently, the highest resolution zone plates available have an outer width of 15 nm^{32,35}. In a soft x-ray microscope such as XM-2 the spatial resolution obtained is determined by the outer width of the objective zone plate⁵.

Box 1, Figure I.

Schematic of the main components in XM-2, a new soft x-ray microscope purpose designed for cellular imaging.

Box 1, Figure II.

Scanning electron micrograph of a Fresnel zone plate⁵. The inset shows the outer most zones in close-up.

Box 2 The basic principles behind tomographic reconstruction

In Figure I, projection images of two yeast cells mounted in a glass capillary tube are taken at angular increments around a rotation axis. For clarity, projection images are shown at 30 deg intervals; in practice, images are collected at much smaller angular increments, typically every 1 or 2 degrees over a total range of 180°. The projection images are then used to calculate a 3-dimensional reconstruction of the specimen either by 'Filtered Back Projection' ³⁶ or Algebraic Reconstruction Techniques (ART) ³⁷. In this figure, the two yeast cells are colored teal and purple and the glass capillary in red. Tomographic data are grayscale in general. The central reconstruction shows the same two cells, complete with the surrounding capillary specimen holders.

Box 2, Figure I.

A 3-dimensional reconstruction of two yeast cells in a glass capillary (top center) calculated from 2-dimensional projection collected sequentially around a rotation axis (shown at 30° increments).

Box 3 Segmentation

Segmentation is the process of computationally isolating, visualizing and quantifying specific cellular components, such as the cytoskeleton or organelles, in a tomographic reconstruction. Each voxel in a soft x-ray tomographic reconstruction is a direct measurement of the X-ray Linear Absorption Coefficient (LAC) at the corresponding location in the cell. Since biological materials absorb soft x-rays according to Beer's Law, the LAC values for similarly sized voxels depends solely on the concentration and composition of biomolecules present, with water having an order of magnitude lower LAC than molecules such as lipids and proteins. LAC values for homogeneous solutions of isolated biomolecules can be readily measured or calculated. For example, pure water in the form of ice was calculated to have a LAC of $0.109 \mu\text{m}^{-1}$ whereas a model protein with the chemical composition $\text{C}_{94}\text{H}_{139}\text{N}_{24}\text{O}_{31}\text{S}$ was calculated to have a theoretical LAC of $1.35 \mu\text{m}^{-1}$ ¹². In practice, most of the voxels in a 50 nm resolution soft x-ray reconstruction of a cell will contain a heterogeneous mixture of biomolecules. Using a single wavelength measurement it is not possible to unambiguously distinguish the chemical composition of a heterogeneous mixture. However, most organelles and cellular structures are sufficiently similar in their biochemical composition to allow them to be identified and differentiated from the surrounding cell contents. For example, the relatively high water content in vacuoles makes them readily distinguishable from organelles more dense in biomolecules, such as nuclei and mitochondria. Even relatively small variations in organelle LAC can be distinguished, for example the boundaries between nuclei and nucleoli are very clear.

Segmentation can be carried out manually or semi-automatically. Manual segmentation is done interactively by outlining regions of interest based on a threshold of the absorption value or by visual inspection. Alternatively a cell can be completely segmented into sub-volumes by more automatic methods. The software tools used for this task use absorption values and/or the magnitude of gradients between regions in the reconstruction. A variety of standard image manipulation approaches such as smoothing and edge detection techniques are routinely used. Starting with a user defined threshold of the absorption values, or some interactively defined seed points, and using region growing algorithms regions of interest can be defined and delineated. If necessary watershed or level set methods can be used to further define the borders of specific sub-cellular components. For further information see, for example, the itk Insight Toolkit at <http://www.itk.org>.

Once the cell has been segmented, *a priori* knowledge such as size, position inside of a cell, boundary conditions, and so on can also be used to aid identification of the segmented volumes. With recently developed instrumentation it is now possible to carry out correlated high-resolution light and x-ray tomographic imaging on the same specimen. This will permit unambiguous assignment of additional organelle identities to segmented volumes²⁹.

Box 4. Localizing molecules in a soft x-ray reconstruction of a cell

Molecules can be tagged and localized in a soft x-ray reconstruction by two distinct methods; either directly in the tomogram using electron-dense, metal tags or by tagging the molecule with a fluorescent moiety and using correlated light microscopy.

Direct

The direct method is the least favored, since this generally requires the cell to be fixed and the membranes permeabilized to allow a tag, which is normally conjugated to an antibody, ingress inside the cell. An advantage to this method is that it examines the position of native proteins and uses modified protocols and commercially available reagents previously developed for other imaging modalities (such as those described in ³⁸). For example, these electron-dense tags have been generated using 1.4 nm gold-conjugated secondary antibodies. Followed by enhancement with silver to produce metal tags that were approximately 50 nm in diameter ⁶. More recently, nano-particles, such as TiO₂, have been used as tags³⁹. These tags have the advantage of having an x-ray absorption edge in the region of the 'water window'. This allows two tomographic data sets to be collected, one at an energy corresponding to an x-ray absorption maximum of titanium, the other at lower energy. Calculation of a difference tomogram allows the position of the labeled molecules to be accurately determined in the cell.

Correlated

High numerical aperture correlated light microscopy is a very recent development ²⁹. In this technique the specimen is cryo-fixed and then imaged using both fluorescence and soft x-ray methods. The latter visualized the cellular architecture into which the fluorescently tagged molecules can be localized. There are a number of very significant advantages to this method. Firstly, tagging with fluorescence molecules is a mainstay of biology that can be readily applied to most cell types, with the capability of tagging multiple molecules in the same experiment ^{40, 41}. Secondly, the specimen does not have to be processed in any way, eliminating the possibility of structural damage. Thirdly, imaging the same specimen using two different modalities eliminates ambiguities during interpretation. Finally, the fluorescence signal can be monitored prior to cryo-fixation using live cell imaging until the appropriate state has been reached, or to permit selection of a specimen with specific characteristics.

Figure 1.

Soft x-ray projection image of cryo-fixed NIH 3T3 cells. In this image the contrast is generated by absorption of 'water window' x-ray photons by the carbon and nitrogen in the nuclear and cytoplasmic organelles, and not by the use of metal stains. Consequently, the information obtained by this method is quantitative and represents the specimen in a close-to-native state. Scale bar=5 μm . (from ⁶).

Figure 2.

Comparative images of the fission yeast *Schizosaccharomyces pombe* using light and soft x-rays **a**) Conventional fluorescence microscopy (vacuoles labeled with CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) from Invitrogen) **b**) Differential Interference Contrast (DIC) light microscopy **c**) Orthoslices (see glossary) through a soft x-ray tomographic reconstruction **d**) The reconstruction in **c** after segmentation. Organelles are identified by their characteristic shape, and measured x-ray Linear Absorption Coefficients (LACs). Key: nucleus, blue; nucleolus, orange; mitochondria, grey; vacuoles, white; lipid-rich vesicles, green. (X-ray tomographic reconstructions are an update of the work outlined in ^{27,28}). Scale bar=1 μ m.

Figure 3.

X-ray tomography of a lymphocyte (T-cell). (a-d) Orthoslices from the tomographic reconstruction of a cryo-fixed T-cell imaged in a capillary. (e-h) Segmented volumes. (e) Cell surface with numerous filopodial extensions. (f-h) Cut-away views showing the typical highly folded large nucleus (cyan) surrounded by a small rim of cytoplasm (purple). Multiple highly absorbing vesicles (yellow) surround the nucleus (cyan), along with less absorbing structures, including mitochondria (magenta) in the cytoplasm. Chromosomes (salmon) and nuclear bodies (green) are seen in the nucleus. Cell is 8 μm in diameter. Scale bars=1 μm .

Figure 4. Tomographic imaging of the three phenotypes displayed by the yeast *Candida albicans*. **a)** Yeast-like cells, **b)** pseudo-hypha, and **c)** hypha. Images after segmentation of the major organelles are shown. The measured Linear Absorption Coefficients for each segmented organelle are given in Table 1. The hyphal cell, shown in **c** has a total length of 47 μm . Five tomographic data sets were collected sequentially along the length of the cell. These were ‘stitched’ together after reconstruction using the software package Amira from Visage Imaging Inc. Key: nucleus, blue; nucleolus, purple; mitochondria, orange; vacuoles, yellow, lipid drops, dark green. Scale bar=1 μm . After figures in (M. Uchida *et al.*, submitted).

Table 1

Measured, average LACs for organelles in the segmented *Candida albicans* cells

AVG LACs (μm^{-1})	
Lipid droplets	0.75 ± 0.09
Mitochondria	0.45 ± 0.03
Nuclei	0.28 ± 0.03
Nucleoli	0.37 ± 0.04
Vacuoles	0.20 ± 0.03