Visualizing and quantifying cell phenotype using soft X-ray tomography

*Gerry McDermott[1)], Douglas M. Fox[2)], Lindsay Epperly[1)], Modi Wetzler[3)], Annelise E. Barron[3)], Mark A. Le Gros[4)] and Carolyn A. Larabell[1)4)]**

Soft X-ray tomography (SXT) is an imaging technique capable of characterizing and quantifying the structural phenotype of cells. In particular, SXT is used to visualize the internal architecture of fully hydrated, intact eukaryotic and prokaryotic cells at high spatial resolution (50 nm or better). Image contrast in SXT is derived from the biochemical composition of the cell, and obtained without the need to use potentially damaging contrast-enhancing agents, such as heavy metals. The cells are simply cryopreserved prior to imaging, and are therefore imaged in a near-native state. As a complement to structural imaging by SXT, the same specimen can now be imaged by correlated cryo-light microscopy. By combining data from these two modalities specific molecules can be localized directly within the framework of a high-resolution, three-dimensional reconstruction of the cell. This combination of data types allows sophisticated analyses to be carried out on the impact of environmental and/or genetic factors on cell phenotypes.

Keywords:
cellular; correlated; cryo-light; morphology; multi-modal; three-dimensional

DOI 10.1002/bies.201100125

[1)] Department of Anatomy, University of California, San Francisco, CA, USA
[2)] Department of Chemistry, University of California, Berkeley, CA, USA
[3)] Department of Bioengineering, Stanford University, Stanford, CA, USA
[4)] Lawrence Berkeley National Laboratory, Berkeley, CA, USA

***Corresponding author:**
Carolyn A. Larabell
E-mail: Carolyn.Larabell@ucsf.edu

Abbreviations:
LAC, linear absorbance coefficient; **SXT,** soft X-ray tomography.

Introduction

Soft X-ray tomography (SXT) is a relatively recent addition to the suite of imaging tools used by biologists [1]. Once firmly in the realm of physicists and material scientists, SXT has been developed specifically for biological imaging, and is now used by a growing community of researchers to quantitatively image cells, including eukaryotic cells [2]. In this essay we aim to familiarize the reader with the characteristics of SXT imaging. In particular, we will describe the necessary instrumentation, outline the types of specimen amenable to this type of imaging, and describe how SXT can be used to characterize and quantify cell phenotypes. In addition, we will summarize recent progress on the development of cryogenic light microscopy for correlated imaging. By combining data from light- and X-ray-based modalities, fluorescently tagged molecules can be localized directly in a three-dimensional, tomographic reconstruction of the cell. Combining protein localization with cell structure this way meets a long-standing need in biology. We will begin by describing the driving force behind the development of SXT and the particular niche this modality occupies in the biological imaging arena.

Why image cells with soft X-rays?

Cell biology occurs over an enormous range of scale, with critical events taking place at the atomic, molecular, and cellular levels [3]. Ideally, we would like to visualize all of the events that take place in a cell, since this would give the most complete picture of cell function, and the complex networks of interactions that give rise to the observed phenotype and cellular behaviors. Naturally, we would like to do all of the required imaging using a single imaging modality, but, clearly, this is not even close to feasible [1]. Each imaging technique operates optimally within a fairly narrow set of parameters (such as specimen size and maximum spatial resolution). Consequently, imaging over a range of scale can only be achieved by using combinations of techniques, with each one imaging over a specific regime [1].

© 2012 WILEY Periodicals, Inc.

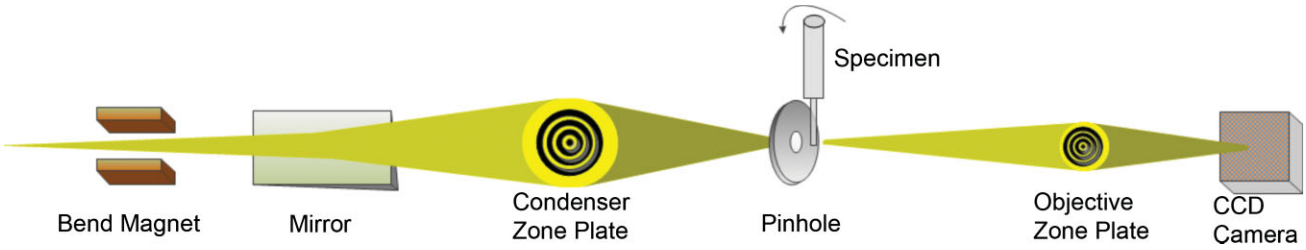

Figure 1. Optical layout of XM-2, a soft X-ray microscope located at the Advanced Light Source, Lawrence Berkeley National Laboratory, Berkeley, California. Soft X-rays are derived from a bend magnet in the synchrotron lattice. The condenser zone plate focuses X-rays onto the specimen, which is mounted on a rotating cryogenic stage. X-rays transmitted through the specimen are focused onto the detector (a CCD camera) by the objective zone plate. An order sorting pinhole is placed between the condenser and the specimen.

Imaging modalities are primarily classified by the physical characteristics of their specimen illumination [1, 4]. This is a sensible approach; in broad terms, the specimen illumination dictates factors such as the maximum spatial resolution and the size range of specimens that can be imaged [5]. For example, bright-field light microscopy – using relatively long wavelength visible light – is best suited to imaging specimens that are a few microns or larger in size, and used to answer questions where the required spatial resolution is hundreds of nanometers [5]. On the other hand, if higher spatial resolution views of the specimen are needed, then shorter wavelength sources of illumination – such as electrons or X-rays – are generally used [1, 2, 6–9] (a notable exception to this trend is the so-called ''super resolution'' fluorescence methods that will be discussed below).

The obvious question becomes ''where does SXT fit in the context of the other imaging modalities used in biology?'' Clearly, to address this we must first look at the characteristics of the specimen illumination. SXT data is collected using a soft X-ray microscope, typically using specimen illumination that falls within a spectral region known as the ''water window'' (i.e. approximately 517 eV or 2.4 nm) [10]. X-rays in this region have an important and unique property; biomolecules attenuate the transmission of this light an order of magnitude more strongly than does water [11, 12]. This attenuation follows the Beer-Lambert Law, and is therefore linear with thickness and a function of the bio-molecular species present at each point in the specimen [13]. This latter factor is responsible for producing contrast in soft X-ray microscopy of biological specimens, and allows cells to be imaged without the need to use heavy metal stains, or any other form of contrast enhancing agent [10, 11, 13]. Soft X-ray, water-window photons are relatively penetrating compared to electrons in terms of transmission through biological specimens, i.e. 15 μm compared to 500 nm, respectively [1]. Consequently, thick biological specimens, such as intact eukaryotic cells can be imaged by SXT without being cut into ''sections'' that are between 100 and 500 nm thick prior to imaging [13].

In summary, SXT is capable of imaging intact, hydrated ''thick'' cells and the image contrast is derived directly from the specimen's biochemical composition. SXT images the full field, rather than specific, labeled molecules only, as is the case with fluorescence microscopy. Consequently, even though ''super resolution'' fluorescence techniques can achieve similar, or even better levels of spatial resolution they are not really comparable with SXT. Cellular soft X-ray imaging fills a niche by operating in a specimen and resolution regime that cannot be readily accessed by any other imaging techniques. There is one further characteristic of SXT that makes it well suited to cellular imaging; data can be collected rapidly. In practice cells can be taken from their growth chamber and directly mounted in a suitable holder. Data collection only takes a few minutes once the cells have been mounted. We will now describe the basic instruments used to collect SXT data.

Soft X-ray microscopy

X-ray microscopy is not a new technique. Indeed, Kirkpatrick and Baez [14] developed the first X-ray microscopes back in the late 1940s. These microscopes used grazing-incidence reflective optics (eponymously termed K-B optics) to focus the X-rays onto the specimen. Initially these microscopes appeared amenable to imaging biological specimens, but unfortunately this optimism was never fulfilled. Using X-ray microscopy to image cells is a more recent success story.

The focus of this article is soft X-ray imaging, however we should note that hard X-ray microscopes have recently been used to image cells (see ref. [15] for work comparable to that discussed here). We will not discuss this work here, due to space limitations, however, the interested reader should be aware that progress is being made using phase contrast methods to image cells in hard X-ray microscopes.

Returning to SXT imaging. The real potential of using soft X-ray microscopes for imaging cells only became apparent when these instruments began to be installed at third generation synchrotron light sources [10, 16]. Almost as soon as they came into operation in the 1990s, this generation of soft X-ray microscopes began to produce high-fidelity images of biological specimens [10, 17]. For the first time, the structural details were present in soft X-ray microscope images that could be correlated to corresponding data from light- and electron-based microscopes [12, 18, 19]. These exciting results catalyzed and renewed efforts to develop soft X-ray microscopy specifically for imaging biological specimens, particularly for tomographic imaging. This was not a major task, but one that required some further technological breakthroughs.

Soft X-ray microscopes share similar design principles to simple light microscopes (Fig. 1). In the latter, a condenser

lens focuses light onto the specimen, and an objective lens refocuses unabsorbed light into an eyepiece or onto a detector [10, 11, 20]. Light microscopes rely on glass lenses to perform these functions. However, in terms of soft X-rays, most materials have a very low refractive index. Moreover, glass lenses such as those used in a light microscope would totally absorb the soft X-ray illumination before it even got to the specimen. Consequently, glass lenses cannot be used in soft X-ray microscopes, instead these instruments rely on nanofabricated lenses called Fresnel zone plates [11].

Fresnel zone plates contain radially symmetric ''rings'', known as Fresnel zones, that alternate between being opaque and transparent to X-ray photons [10, 21]. Soft X-rays diffract around the opaque zones [10]. To function as a lens, the Fresnel zones are spaced such that the diffracted X-rays constructively interfere at a desired focal point. The condenser zone plate focal point would typically be the specimen. In a zone plate lens the zones get thinner and more closely packed moving outwards from the center point towards the outermost ''zone'' [21]. In the simplest case, the maximum spatial resolution obtainable from that particular microscope's optical system is determined by the spacing between adjacent zones in the outermost rings in the objective zone plate [22]. To date, SXT imaging of cells has typically been carried out using objective zone plates with a spatial resolution of 25–50 nm [2]. Zone plates capable of imaging at a spatial resolution better than 15 nm are now being manufactured routinely [23]. Installing ultra high-resolution optics in an existing soft X-ray microscope is straightforward. However, as the spatial resolution of the lens increases, the depth of field decreases [24]. Consequently, there comes a point where the depth of field is less than the thickness of the specimen. Overcoming this shortfall requires the development of techniques such as a combination of through-focus deconvolution with tomography [24].

Typically, soft X-ray microscopes are equipped with a Fresnel zone plate condenser and objective. Alternatively, a glass capillary waveguide can be used for the condenser [25]. Both types of condenser have inherent advantages and disadvantages. However, both microscopes produce images with equal fidelity. For the sake of simplicity, we will limit our discussion to soft X-ray microscopes that employ Fresnel zone plate condensers, but stress that these are not better than capillary waveguides in terms of image formation.

Soft X-ray tomographic data collection

In addition to a soft X-ray microscope, carrying out three-dimensional tomography on biological specimens required an additional piece of technology: a specimen stage that allows the specimen to be rotated [12, 18]. Imaging a typical cell by SXT requires collection of projection images at angular increments over a 180° range (usually, 180 projection images collected at 1° rotational increments around a central axis) [24]. This presents two clear challenges. First, biological specimens are damaged when they are exposed to intense beams of photons in a soft X-ray microscope. Collecting tomographic data has the potential to generate a cumulative radiation dose that could lead to radiation damage and experimentally induce artifacts. Fortunately, cooling the specimen to cryogenic temperatures whilst it is being imaged can mitigate radiation damage [24]. The second challenge is to prevent structures from moving or changing inside the specimen during the time it takes to collect a number of exposures. With a high spatial resolution technique, such as soft X-ray microscopy, even relatively small movements inside the cell during data collection have a profound impact on the fidelity of the calculated tomogram. To avoid this, the specimen must be fixed prior to being imaged. There are two possible methods for doing this, either using chemical fixatives or cryogenic immobilization [12]. Chemical fixation is potentially very damaging to cellular structures [1] and has been seen, in high resolution electron microscopy studies, to cause the collapse of highly solvated structures, such as vacuoles, leading to concerns about other possible damage to fine structures [1]. Artifacts generated by specimen preparation protocols cannot be mitigated or eliminated postfacto; they can only be noted and accounted for in the interpretation of the images.

Cryogenic fixation is considered to be the better option for retaining the integrity of cellular structure [1]. This method is virtually instantaneous and has been shown to retain fine structural details inside the cell. Accordingly, cryofixation is the principle method used in SXT to minimize artifacts due to specimen preparation and to maximize the final quality and fidelity of biological images [12, 24, 26–32]. Typically, the specimen is rapidly cooled to liquid nitrogen temperature by mounting it in a holder and plunging this into a stream of cold helium gas, or into a cryogen, such as liquid propane [2]. Both methods cool the specimen quickly enough to minimize the formation of crystalline ice. Instead, the specimen is encapsulated in amorphous ice. In other words the structure of the ice inside the cell remains similar to that of the liquid before freezing, thus minimizing damage due to the formation of crystalline ice. This approach was first used for X-ray imaging by Weiss and colleagues at the Helmholtz Zentrum synchrotron light source in Berlin with their report that the alga *Chlamydomonas reinhardtii* had been imaged in exquisite detail using SXT [12]. This work provided a key proof-of-concept in the field and even today it remains a good example of the capabilities of SXT. Unfortunately, the cryo-rotation stage used in this work was difficult to operate and labor intensive. As a result, during the lifetime of that particular microscope there were no further reports of SXT imaging of biological specimens. The most recent generation of SXT-specific cryo-rotation stages were developed by the National Center for X-ray Tomography at the Advanced Light Source, Berkeley, California. These stages are highly automated, and both robust and simple to use [24]. Other soft X-ray microscopes have been fitted with modified electron tomography cryorotation stages [33]. These instruments have two inherent disadvantages. Firstly, they must operate in a vacuum, and secondly, they are restricted in the maximum rotation (tilt) angle. The latter leads to incompleteness in the tomographic data due to the fact that certain orientations of the specimen cannot be imaged. This missing information leads to the possibility of artifacts in the final tomographic reconstruction of the specimen.

Preparing specimens for SXT data collection

In general, specimen preparation for SXT imaging is minimal, and can be as trivial a procedure as taking cells from their growth chamber and transferring them directly into a suitable specimen holder [24]. This takes a matter of seconds, and requires no prior experience beyond knowing how to use a pipette.

Soft X-ray tomography can be used to image virtually any cell type of interest to biologists, from simple bacteria, to yeast, algae, and higher order eukaryotes. Using a 50 nm optic, specimens up to a maximum thickness of ~15 μm can be imaged readily [22]. The exact limit on specimen thickness correlates with biochemical composition and the optical system installed in the microscope [23]. For example, very densely packed specimens with a low water content will be limited to a maximum thickness less than for a specimen with low density of biomolecules, and a high water content. The limit on specimen thickness also depends on the spatial resolution of the optics. As the resolution increases the depth of focus decreases. At some point the depth of focus will become shallower than the thickness of the specimen [23]. Consequently, small cells have the potential to be readily imaged at higher spatial resolution more easily than very large cells. Even so, a particular strength of SXT compared to other high resolution cellular imaging techniques is the capacity to image intact, fully hydrated eukaryotic cells [2].

Data collection for tomography

As with all microscopes, a soft X-ray microscope can only produce a two-dimensional representation of the specimen [12]. For very simple specimens that have little structural organization a projection image such as this may be perfectly adequate. However, in the case of biological cells the internal structure is highly complex [24]. In a projection image these structures become superimposed on top of each other, making interpretation difficult or even impossible [13]. In clinical medicine this principle is the reason why the three-dimensional views from computer tomography (CT) are so powerful compared to standard two-dimensional X-ray images.

In practice, collecting tomographic data on a soft X-ray microscope, such as XM-2 at the National Center for X-ray Tomography, is akin to using a light microscope, in terms of difficulty. The specimen holder is placed in the cryorotation stage and then aligned with respect to the illumination and the center of rotation. The microscope is then set up to record projection images at set increments around the rotation axis. On XM-2, each image requires an exposure of 100 milliseconds or less, followed by a few seconds of lag while the detector reads out, and the specimen is rotated to its new position. In general, there is no need to realign the specimen during the collection of data. In total, it only takes a few minutes to collect the series of projection images necessary for the calculation of a tomographic reconstruction. The total radiation dose received by the specimen is in the region of 10^8 Gy. Provided the specimen remains at cryogenic temperatures this dose does not appear to cause visible damage when imaged at a resolution of 50 nm. Indeed, it is possible to collect a number of tomographic data sets from the same specimen with no appreciable change in the specimen (Le Gros, unpublished observation).

Data processing and analysis

Tomography is very commonplace in research, industry, and clinical settings. Consequently, the developers of SXT have benefited enormously from a wealth of readily available, sophisticated software packages for data processing and analysis. A discussion of these is beyond the scope of this article, however it is important for the reader to be aware that there is a rich library of algorithms available for reconstruction of three-dimensional volumes from two-dimensional soft X-ray microscope projection series, based on a number of different algorithms (such as back-projection and algebraic reconstruction techniques (ART) [34, 35]). Once reconstructed these volumes can be analyzed and viewed using an equally wide array of software and image handling packages. Metrics such as the volume, surface area, and surface-to-volume ratio of either the entire organism or the particular feature of interest can be extracted literally with a single click once the tomogram has been calculated. The linear absorption coefficient (LAC), a measure of the attenuation of soft X-rays by structures inside the cell, is calculated and used to identify, differentiate, and isolate structures within a cell [2]. Each organelle type has a characteristic average LAC value. For example, the nucleus has a different average LAC value than the nucleolus, which is in turn different from the mitochondria [2]. Surprisingly, these characteristic values not only hold between cells of the same type, but also frequently are seen to hold between cells from different species [31].

Regions within a cell that have similar LAC values can be isolated from the other cell contents. This process is generally referred to as segmentation. For some questions only one organelle – perhaps the nuclei, or mitochondria – will be segmented, and quantified. For other studies every organelle inside the cell will be segmented and characterized (e.g. organelle volumes, densities, and proximity to other organelles). Comparisons can then be made between species, cells that differ in ploidy, or have been exposed to specific environmental factors [31]. In other words, quantifying the structural phenotype of a cell as a function of a variable.

Recent examples of soft X-ray tomographic data

Cellular phenotype is the sum of a cell's observable characteristics or traits, such as morphology and/or biological activity, and is a manifestation of gene expression and environmental factors, and the interactions that occur between the two. These characteristics define and constrain the overall capabilities and function of a cell [36]. Fortuitously, many phenotypic traits of a cell are readily observable by microscopy. This is one of the principle reasons why imaging is ubiquitous in cell biology, and microscopes are considered essential instruments in most research laboratories. As stated

above, imaging cells requires a multi-modal approach in which the instrument and methods selected are optimally suited to the particular specimen or information needed [1, 3].

The range of organisms imaged using SXT has expanded rapidly in the past few years. A partial list of organisms (and structural features) studied to date includes *Candida albicans* (phenotypic switching in response to antifungal peptoids) [30], the nuclear structure in lymphocytes (T-cells) [2], *Schizosaccharomyces pombe* (cytokinetic contractile ring, actin during cell division, and vacuoles) [28], the structure of the malaria parasite, *Plasmodium falciparum*, in the interior of red-blood cells [32], mouse adenocarcinoma cells [27, 29], and *Saccharomyces cerevisiae* (quantification of organelles as function of cell cycle, ploidy, and species) [31].

In Figures 2 and 3 we present a snapshot view of the type of phenotypic changes that can be readily imaged using SXT. Figure 2 shows growth-phase dependent lipid body formation and mobilization in *Mycobacterium smegmatis* and illustrates the qualitative and quantitative information that can be obtained from the LAC values. The highly variable extent of lipid body formation between individual bacteria, especially during log phase growth, is immediately apparent, as is the transition to larger structures during stationary phase. Qualitative impressions can be quantified rigorously and reproducibly. Data can be analyzed semi-automatically for all the organisms within a single field of view or, in cases where this is not possible (e.g. if the LAC value of the surrounding cell contents is very close to the LAC value of the object of interest) by a slower, manual process.

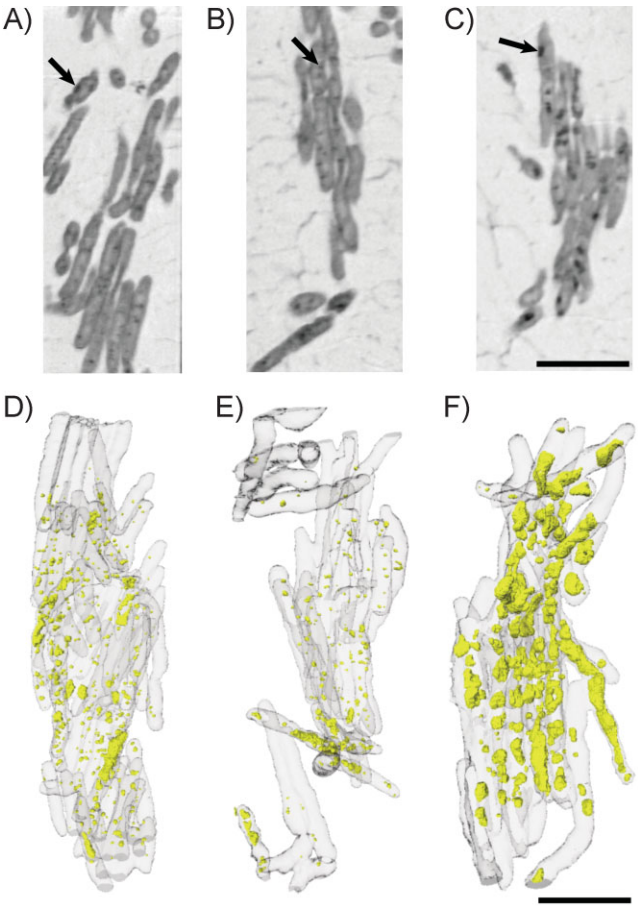

Figure 2. Orthoslices from three-dimensional tomographic reconstructions (**A**–**C**) and segmented volumes (**D**–**F**) of *Mycobacterium smegmatis* during early log phase growth (A, D), late log phase growth (B, E), and stationary phase (C, F). The lipid bodies (arrows a–c), which are highly absorbing (linear absorption coefficient, LAC, 0.71 μm^{-1}) are easily distinguished from the surrounding organic material (LAC, 0.38 μm^{-1}). Lipid bodies were segmented and color-coded yellow (D–F) to show the changes in relative concentration under different conditions. Scale bars (A–C) = 3 μm, (D–F) = 2 μm.

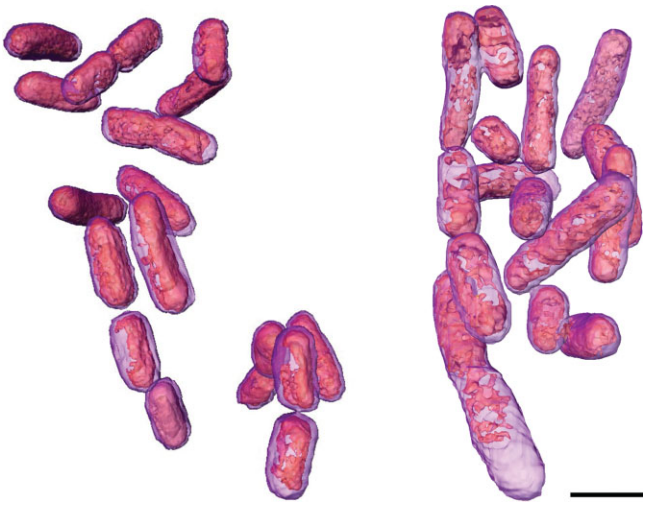

Figure 3. Segmented volumes from three-dimensional tomographic reconstructions of *E. coli* before (left) and after (right) treatment with antimicrobial agent. Regions color-coded magenta are more dense (LAC = 0.49 μm^{-1}) than surrounding areas (LAC = 0.35 μm^{-1}). Cell surface is color-coded lilac. Scale bar = 1 μm.

Figures 3 and 4 shows how differences in cellular phenotype can be visualized using SXT. In this example, cells (bacterial and yeast) were treated with an antimicrobial agent prior to being imaged, and then compared with images obtained from untreated, control cells. In this relatively simple example, we can see that SXT is capable of visualizing relatively subtle changes in phenotype, such as changes in the surface of yeast cells, or the shifts in density inside bacterial cells as a consequence of exposure to this particular antimicrobial molecule. These examples are given to highlight the type of phenotypic changes that can be seen using SXT, and are far from exhaustive in terms of the capabilities of the technique.

Correlated imaging

An exciting area of on-going development related to SXT is the development of cryogenic high numerical aperture light microscopy [24]. This is highly relevant since it opens up the opportunity for correlated imaging studies to be carried out on the same specimen. In other words, the specimen is subjected to sequential imaging using two complementary

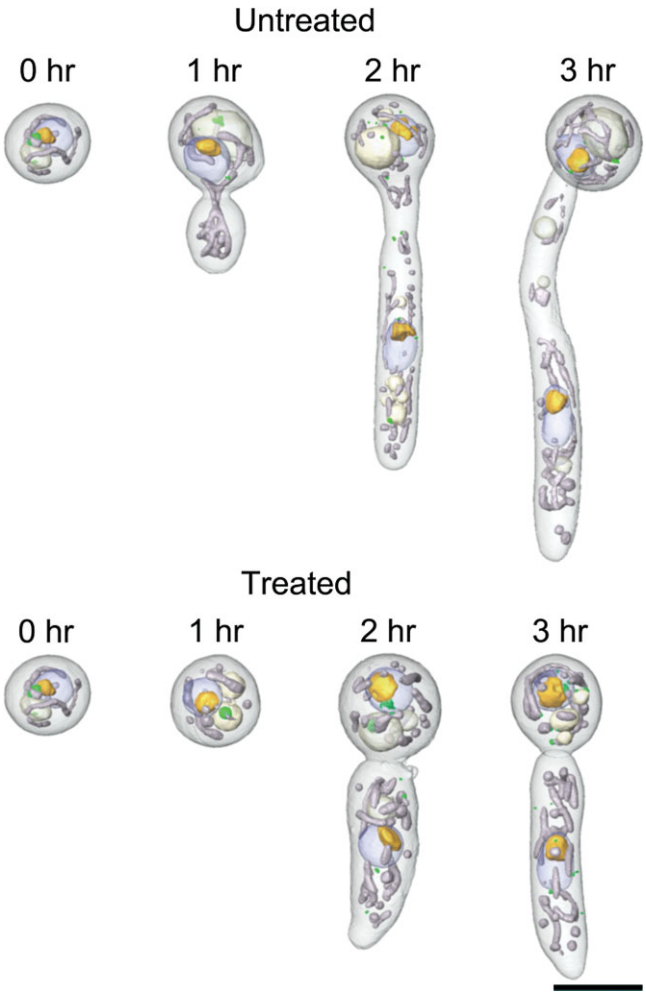

Figure 4. Segmented volumes from three-dimensional tomographic reconstructions of the yeast, *C. albicans*, before and after treatment with an antifungal agent. Segmented organelles are color-coded as follows: Nucleus, lilac; nucleolus, orange; mitochondria, gray; lipid droplets, green; vacuoles, ecru. Scale bar = 5 μm.

modalities. This approach is one that has been explored for a number of years. However, it only recently became possible to collect cryogenic fluorescence images from biological specimens using a microscope that allows use of an immersion fluid with a refractive index closely matched to the specimen, and, therefore the use of high numerical aperture lenses [37]. The soft X-ray microscope XM-2 is now fitted with an integrated high-numerical aperture cryo-light microscope [24]. Specimens can be imaged with cryo-light microscopy, and then translated into position to allow collection of SXT data. The data from the two modalities can be processed and analyzed individually, and then combined to form a composite image [24, 37]. In this way the location of fluorescently tagged molecules can be mapped into the sub-cellular structures in the cell and visualized in an SXT reconstruction [2, 24, 37].

In general, microscope images are limited by diffraction to a spatial resolution approximately half the wavelength of the illuminating light [38]. For light microscopes, with illumination of 500–600 nm this means the resolution is limited to ~250 nm at best. However, a number of "super-resolution" imaging techniques have been developed that extend beyond this resolution [39–47]. All of these methods are capable of localizing fluorescently tagged molecules in a cell specimen at a spatial resolution significantly better than the optical diffraction limit. For this type of imaging the working lifetime of the tag is critically important, since accurate localization depends heavily on fluorescence intensities. The efficiency of photon-detection in a microscope is estimated to be in the region of 1%. Therefore, the number of photons produced by a fluorophore before bleaching directly impacts the signal-to-noise ratio; the longer the working life of the fluorophore the better. At room temperature, fluorophores typically produce 10^5–10^6 photons before photo-bleaching [48, 49]. Thompson et al. [49] calculated this number of photons theoretically allows collection of 100 images with a signal sufficient to be localized with a precision of 65 nm, or only ten images that allow a precision of 20 nm. Consequently, super-resolution methods would benefit greatly from extending the photoactive lifetime of a fluorophore. In general, virtually any fluorescence microscopy experiment would be enhanced if the fluorophores output a consistent signal over the duration of the experiment. The solution to this problem is to collect fluorescence data at cryogenic temperatures, where the lifetime of the fluorophore is significantly increased (usually a 30–50 times increase compared to room temperature). Given the ready availability of cells containing proteins tagged with genetically encoded molecules, such as green fluorescent protein (GFP), or the ease with which many vital fluorescent dyes can be internalized by a cell it is now assumed that almost any organelle, molecular complex, or individual protein can be fluorescently tagged. Consequently, there is enormous scope for carrying out correlated light and SXT studies. Virtually any cell that would be interesting to image by SXT will either have existing fluorescently tagged molecules, or it would be easy to generate cells with specific elements labeled for fluorescence imaging.

Conclusions and prospects

Soft X-ray tomography has emerged as a powerful new technique for imaging the structural phenotype of a cell. The use of soft X-rays to illuminate the specimen offers a number of advantages, such as the ability to image unstained, fully hydrated eukaryotic cells at high spatial resolution. This is a new way of looking at cells and as a result SXT is now generating new insights into the structural phenotypes of cells, and how this changes in response to factors, such as progression through the cell cycle, genetics, or environmental variables.

In the near future, ultra high-resolution zone plates will allow cells to be imaged with a significantly greater level of detail. Correlated cryo-fluorescence and X-ray imaging will become standard practice. This will allow fluorescently tagged molecules to be localized directly in a high-resolution three-dimensional reconstruction of a cell. This promises to have a significant impact in fields ranging from basic cell biology to biotechnology and biomedical research.

In closing, SXT has come of age and has emerged as a mainstream technique with wide applicability. The future for

the technique looks very bright, especially in terms of the further evolution of correlated imaging methods. At the moment, SXT is limited to microscopes located at synchrotron light sources. However, rapid progress is being made on the development of ''table top'' X-ray sources [50–53]. These will allow soft X-ray microscopes to become more widely available, and open up the possibility of the technique becoming as commonplace as electron microscopy, or, in the most optimistic case, confocal light microscopy.

Acknowledgments

We thank Drs. Maho Uchida and Christian Knoechel for their help creating figures. This work was funded by the US Department of Energy, Office of Biological and Environmental Research (DE-AC02-05CH11231), the National Center for Research Resources of the National Institutes of Health (P41RR019664), and the National Institutes of General Medicine of the National Institutes of Health (GM63948). Douglas M. Fox was supported by a National Institutes of Health NRSA Training Grant (1 T32 GMO66698). The Advanced Light Source is supported by the US Department of Energy, Office of Science.

References

1. **Leis A**, **Rockel B**, **Andrees L**, **Baumeister W.** 2009. Visualizing cells at the nanoscale. *Trends Biochem Sci* **34**: 60–70.
2. **Larabell CA**, **Nugent KA.** 2010. Imaging cellular architecture with X-rays. *Curr Opin Struct Biol* **20**: 623–31.
3. **Subramaniam S.** 2005. Bridging the imaging gap: visualizing subcellular architecture with electron tomography. *Curr Opin Microbiol* **8**: 316–22.
4. **Hosking CR**, **Schwartz JL.** 2009. The future's bright: imaging cell biology in the 21st century. *Trends Cell Biol* **19**: 553–4.
5. **Cowhig J.** 1974. *The World under the Microscope*. New York: Bounty Books.
6. **Lucic V**, **Leis A**, **Baumeister W.** 2008. Cryo-electron tomography of cells: connecting structure and function. *Histochem Cell Biol* **130**: 185–96.
7. **Luçic V**, **Forster F**, **Baumeister W.** 2005. Structural studies by electron tomography: from cells to molecules. *Annu Rev Biochem* **74**: 833–65.
8. **Baumeister W**, **Vanhecke D**, **Asano S**, **Kochovski Z**, et al. 2011. Cryo-electron tomography: methodology, developments and biological applications. *J Microsc* **242**: 221–7.
9. **Baumeister W.** 2004. Mapping molecular landscapes inside cells. *Biol Chem* **385**: 865–72.
10. **Attwood DT.** 1999. *Soft X-rays and Extreme Ultraviolet Radiation: Principles and Applications*. Cambridge, New York: Cambridge University Press. p. 470.
11. **Kirz J**, **Jacobsen C**, **Howells M.** 1995. Soft X-ray microscopes and their biological applications. *Q Rev Biophys* **28**: 33–130.
12. **Weiss D.** 2000. *Computed Tomography Based on Cryo X-ray Microscopic Images of Unsectioned Biological Specimens*. Göttingen: Georg-August University of Göttingen.
13. **Le Gros MA**, **McDermott G**, **Larabell CA.** 2005. X-ray tomography of whole cells. *Curr Opin Struct Biol* **15**: 593–600.
14. **Kirkpatrick P**, **Baez AV.** 1948. Formation of optical images by X-rays. *J Opt Soc Am* **38**: 766–74.
15. **Zheng T**, **Li W**, **Guan Y**, **Song X**, et al. 2011. Quantitative 3D imaging of yeast by hard X-ray tomography. *Microsc Res Tech*, in press. DOI: 10.1002/jemt.21108.
16. **Schmahl G**, **Rudolph D**, **Schneider G**, **Thieme J**, et al. 1996. Diffraction optics for X-ray imaging. *Microelectron Eng* **32**: 351–67.
17. **Schmahl G.** 2007. Personal view: the development of X-ray microscopy with synchrotron radiation during the last two decades. *Synchrotron Radiat News* **20**: 27–8.
18. **Larabell CA**, **Le Gros MA.** 2004. X-ray tomography generates 3-D reconstructions of the yeast, *Saccharomyces cerevisiae*, at 60-nm resolution. *Mol Biol Cell* **15**: 957–62.
19. **Meyer-Ilse W**, **Hamamoto D**, **Nair A**, **Lelievre SA**, et al. 2001. High resolution protein localization using soft X-ray microscopy. *J Microsc* **201**: 395–403.
20. **Falcone R**, **Jacobsen C**, **Kirz J**, **Marchesini S**, et al. 2011. New directions in X-ray microscopy. *Contemp Phys* **52**: 293–318.
21. **Andersen EH**, **Harteneck B**, **Olynick D**, **Meyer-Ilse W**, et al. 1999. Nanofabrication of X-ray Zone Plates with the Nanowriter Electron-Beam Lithography System. X-ray Microscopy VI. Berkeley, CA: American Institute of Physics.
22. **Denbeaux G**, **Anderson E**, **Chao W**, **Eimuller T**, et al. 2001. Soft X-ray microscopy to 25 nm with applications to biology and magnetic materials. *Nucl Instrum Methods A* **467**: 841–4.
23. **Chao W**, **Harteneck BD**, **Liddle JA**, **Anderson EH**, et al. 2005. Soft X-ray microscopy at a spatial resolution better than 15 nm. *Nature* **435**: 1210–3.
24. **McDermott G**, **Le Gros MA**, **Knoechel CG**, **Uchida M**, et al. 2009. Soft X-ray tomography and cryogenic light microscopy: the cool combination in cellular imaging. *Trends Cell Biol* **19**: 587–95.
25. **Guttmann P**, **Zeng X**, **Feser M**, **Heim S**, et al. 2009. Ellipsoidal capillary as condenser for the BESSY full-field X-ray microscope. *J Phys: Conf Ser* **186**: 012064.
26. **Gu WW**, **Etkin LD**, **Le Gros M**, **Larabell C.** 2007. X-ray tomography of *Schizosaccharomyces pombe*. *Differentiation* **75**: 529–35.
27. **Muller WG**, **Bernard Heymann J**, **Nagashima K**, **Guttmann P**, et al. 2012. Towards an atlas of mammalian cell ultrastructure by cryo soft X-ray tomography. *J Struct Biol*, in press. DOI: 10.1016/j.jsb.2011.11.025.
28. **Parkinson DY**, **McDermott G**, **Etkin LD**, **Le Gros MA**, et al. 2008. Quantitative 3-D imaging of eukaryotic cells using soft X-ray tomography. *J Struct Biol* **162**: 380–6.
29. **Schneider G**, **Guttmann P**, **Heim S**, **Rehbein S**, et al. 2010. Three-dimensional cellular ultrastructure resolved by X-ray microscopy. *Nat Methods* **7**: 985–7.
30. **Uchida M**, **McDermott G**, **Wetzler M**, **Le Gros MA**, et al. 2009. Soft X-ray tomography of phenotypic switching and the cellular response to antifungal peptoids in *Candida albicans*. *Proc Natl Acad Sci USA* **106**: 19375–80.
31. **Uchida M**, **Sun Y**, **McDermott G**, **Knoechel C**, et al. 2011. Quantitative analysis of yeast internal architecture using soft X-ray tomography. *Yeast* **28**: 227–36.
32. **Hanssen E**, **Knoechel C**, **Klonis N**, **Abu-Bakar N**, et al. 2011. Cryo transmission X-ray imaging of the malaria parasite, *P. falciparum*. *J Struct Biol* **173**: 161–8.
33. **Schneider G**, **Heim S**, **Guttmann P**, **Rehbein S**, et al. 2005. Novel X-ray microscopes for 3-D and fs-Imaging at BESSY. *Proc 8th Int Conf X-ray Microsc* **7**: 349–52.
34. **Herman GT.** 1980. *Image Reconstruction from Projections*. New York: Academic Press.
35. **Marabini R**, **Herman GT**, **Carazo JM.** 1998. 3D reconstruction in electron microscopy using ART with smooth spherically symmetric volume elements (blobs). *Ultramicroscopy* **72**: 53–65.
36. **Alberts B**, **Johnson A**, **Lewis J**, **Raff M**, et al. 2008. *Molecular Biology of the Cell*. New York: Garland Science. p. 1392.
37. **Le Gros MA**, **McDermott G**, **Uchida M**, **Knoechel CG**, et al. 2009. High-aperture cryogenic light microscopy. *J Microsc* **235**: 1–8.
38. **Abbe E.** 1873. Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung. *Arch Mikrosk Anat* **9**: 413–68.
39. **Bates M**, **Huang B**, **Dempsey GT**, **Zhuang X.** 2007. Multicolor super-resolution imaging with photo-switchable fluorescent probes. *Science* **317**: 1749–53.
40. **Betzig E**, **Patterson GH**, **Sougrat R**, **Lindwasser OW**, et al. 2006. Imaging intracellular fluorescent proteins at nanometer resolution. *Science* **313**: 1642–5.
41. **Friedenberger M**, **Bode M**, **Krusche A**, **Schubert W.** 2007. Fluorescence detection of protein clusters in individual cells and tissue sections by using toponome imaging system: sample preparation and measuring procedures. *Nat Protoc* **2**: 2285–94.
42. **Hell SW**, **Dyba M**, **Jakobs S.** 2004. Concepts for nanoscale resolution in fluorescence microscopy. *Curr Opin Neurobiol* **14**: 599–609.
43. **Manley S**, **Gillette JM**, **Patterson GH**, **Shroff H**, et al. 2008. High-density mapping of single-molecule trajectories with photoactivated localization microscopy. *Nat Methods* **5**: 155–7.
44. **Punge A**, **Rizzoli SO**, **Jahn R**, **Wildanger JD**, et al. 2008. 3D reconstruction of high-resolution STED microscope images. *Microsc Res Tech* **71**: 644–50.

45. **Rust MJ**, **Bates M**, **Zhuang X.** 2006. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat Methods* **3**: 793–5.
46. **Schermelleh L**, **Carlton PM**, **Haase S**, **Shao L**, et al. 2008. Subdiffraction multicolor imaging of the nuclear periphery with 3D structured illumination microscopy. *Science* **320**: 1332–6.
47. **Willig KI**, **Harke B**, **Medda R**, **Hell SW.** 2007. STED microscopy with continuous wave beams. *Nat Methods* **4**: 915–8.
48. **Moerner WE**, **Orrit M.** 1999. Illuminating single molecules in condensed matter. *Science* **283**: 1670–6.
49. **Thompson RE**, **Larson DR**, **Webb WW.** 2002. Precise nanometer localization analysis for individual fluorescent probes. *Biophys J* **82**: 2775–83.
50. **Bertilson M**, **von Hofsten O**, **Vogt U**, **Holmberg A**, et al. 2011. Laboratory soft-X-ray microscope for cryotomography of biological specimens. *Opt Lett* **36**: 2728–30.
51. **Bertilson M**, **von Hofsten O**, **Hertz HM**, **Vogt U.** 2011. Numerical model for tomographic image formation in transmission X-ray microscopy. *Opt Express* **19**: 11578–83.
52. **Bertilson M**, **Von Hofsten O**, **Lindblom M**, **Wilhein T**, et al. 2008. Compact high-resolution differential interference contrast soft X-ray microscopy. *Appl Phys Lett* **92**: 064104.
53. **Takman PAC**, **Stollberg H**, **Johansson GA**, **Holmberg A**, et al. 2007. High-resolution compact X-ray microscopy. *J Microsc* **226**: 175–81.