X-ray microscopy with synchrotron radiation

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X-ray microscopes, using synchrotron radiation sources, are allowing high resolution studies into the structure and chemistry of whole hydrated single cells.

What would be the ideal probe for studying the morphology and composition of a single eucaryotic cell? One would like the probe to have a short wavelength (for obtaining high resolution) and to have the ability to allow imaging of organic matter at high contrast relative to water. Multiple scattering effects would be minimal, yet one would have appreciable contrast from small structures. Ideally the probe would be non-ionizing (to prevent radiation damage) and would be available in copious amounts. Finally, this reagent would provide a means to understand composition based on contrast due to differences in the interaction between the probe and different components of the specimen and also to employ site-specific labeling methods.

No single probe can provide everything simultaneously. Visible light, electrons, X-rays and neutrons all provide various combinations of the above (although neutron microscopy is limited by the lack of sufficiently bright sources). X-rays would provide all, but for the ionizing nature of the radiation. In recent years the technology of X-ray microscopes has improved considerably, especially through the use of synchrotron sources. Now, whole-mount, frozen hydrated specimens can be imaged at resolutions approaching 30 nm with <1 keV X-rays and three-dimensional images at 0.1 micron (or better) resolution can be obtained using tomography, the technique of CAT scans. Trace element distributions can be mapped with unprecedented sensitivity using >4 keV X-ray microprobes. Furthermore, both soft and hard X-ray microscopes can make use of resonant structure in the spectrum near absorption edges to obtain information on the identity and chemical bonding of many elements. The introduction of cryogenic methods is mitigating many of the prob-

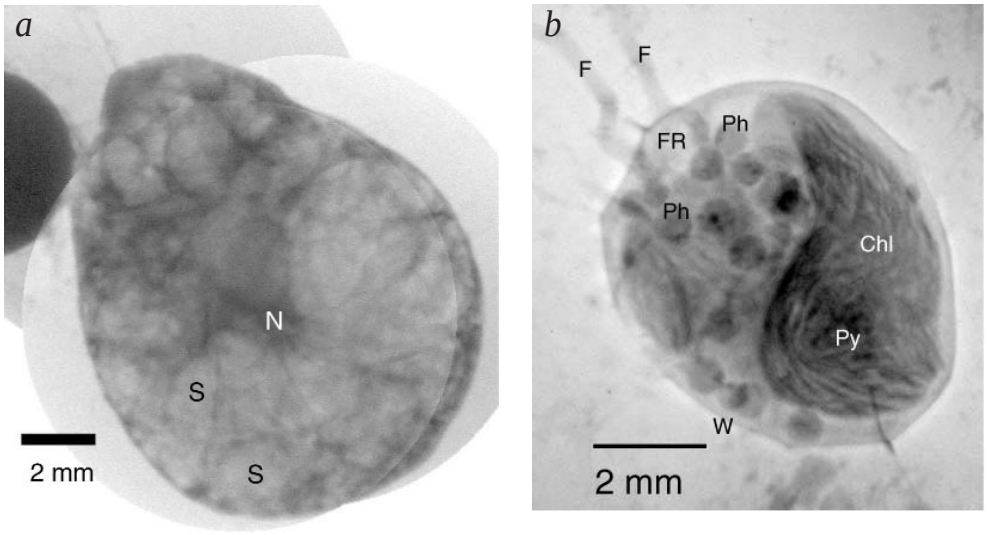

Fig. 1 High resolution X-ray micrographs of fresh water organisms allow the visualization of structures less than 100 nm in size in hydrated, unsectioned specimens using 516 eV X-rays. ***a***, *Chilomonas paramecium* imaged using the ISA TXM in Denmark (scale bar is 2 μm). The cell was exposed to a 10^{-4} M Al salt solution for 12 h as part of a study of the toxic effects of metal ions on freshwater protozoa. Compared to control specimens (not shown), flagella are lost, the nucleus (N) is relocated, and the distribution of starch grains spaces (S) is altered. The starch grains show up as transparent spaces in this specimen due to fixation with 2.5% glutaraldehyde in phosphate buffer prior to imaging at room temperature. From J. Abraham and R. Medenwaldt. ***b***, The alga *Chlamydomonas rheinhardtii* imaged in the frozen hydrated state using the Göttingen TXM at BESSY in Berlin. Flagella (F) of ~300 nm diameter emerge from flagellar roots (FR), and the chloroplast (Chl) with the pyrenoid (Py) can be identified inside the cell wall (W). Since no chemical fixatives were used in this case, phospholipid vesicles (Ph) remain well preserved (compare to (*a*)); in addition, no changes are seen following exposure to cumulative doses as high as about 10^{12} rads (dose for the above image: 4×10^8 rads)[8].

lems of radiation damage, and new methods for labeling sites of biological interests are under development. Here, we describe a few of these recent developments for biological imaging using synchrotron radiation. Further details are available elsewhere[1,2].

X-ray interactions and radiation damage

Below 10 keV, X-ray photons are primarily absorbed or phase shifted as they pass through matter. This means that X-ray microscopes offer unique opportunities for quantitative imaging without inelastic or multiple elastic scattering effects. Furthermore, the penetration of X-rays in hydrated biological materials can be large: at energies between the carbon and oxygen absorption edges at 284 and 534 eV respectively (the so-called 'water window'), water layers of up to 10 microns thick are easily penetrated while thin cellular structures provide sufficient contrast[3,4]. In spite of this high contrast, one must still illuminate the specimen with sufficient X-rays per pixel, and the resulting radiation dose to the specimen is ~10^8 rads for imaging mammalian cells at 50 nm resolution (the dose can be reduced by a factor of 5–10 if phase contrast is used[5]). This dose is not modest in view of the fact that < 1,000 rads is lethal in most organisms. Although dry specimens tend to be quite stable, the required dose is high enough so that the more sensitive wet specimens, even if chemically fixed, can be imaged only once before significant radiation damage is observed[6]. If higher resolution is desired, the signal must come from a smaller volume, and therefore the dose (energy deposited per unit mass) increases dramatically. It remains, nevertheless, significantly less than what would be needed for penetrating micrometer thick water layers with electrons even if the technology of energy filtered electron microscopy can be extended to 400 keV[7]. Thus, radiation dose has, in the past, been a serious limitation to biological studies with X-ray microscopes; however, independent efforts by Gerd Schneider in the laboratory of Günter Schmahl at Universität Göttingen in Germany[8], and by Jörg Maser and others in our laboratory at Stony Brook[9], have led to the introduction of cryogenic methods for studying frozen hydrated specimens. For a number of reasons (primarily the immobilization of radiolytical products in the ice matrix), such frozen biological specimens can tolerate a radiation dose of up to 10^{12} rads with little or no apparent negative effects. This development makes it possible to exploit the capabilities of X-ray microscopy on specimens which have not been modified by sectioning or chemical fixation (Fig. 1).

Instrumentation and biological applications

A number of approaches have been used to construct successful synchrotron X-ray microscopes. Here, we concentrate on those which use zone plates as focusing optics to obtain high magnification images in transmission X-ray microscopes (TXMs), or produce finely focused spots through which the specimen is rastered in scanning transmission X-ray microscopes (STXMs). The spatial resolution of these microscopes is ultimately limited by the width of the outermost and finest ring shaped zones of the zone plate, which can be < 30 nm using electron beam fabrication methods[10,11]. To focus harder X-rays, the thickness of the zone plate must be increased, a particularly difficult fabrication task for the finest zones, which would have to be tall and narrow. It is presently easier to achieve 30–50 nm resolution with soft X-rays (<1 keV), although probe sizes of 200 nm and smaller have been made with 8 keV X-rays[12].

Soft X-ray TXMs have been pioneered by the Göttingen group at the Berlin synchrotron radiation laboratory (BESSY); microscopes of this type are also in operation at, for example, the Advanced Light Source (ALS) in Berkeley, California, the Instrument Center for Synchrotron Radiation Aarhus (ISA) in Denmark, the Photon Factory in Japan, and nearing operation at the European Synchrotron Radiation Faciclity (ESRF) in Grenoble. These microscopes are able to deliver high resolution images with exposure times of seconds. This offers unique opportunities

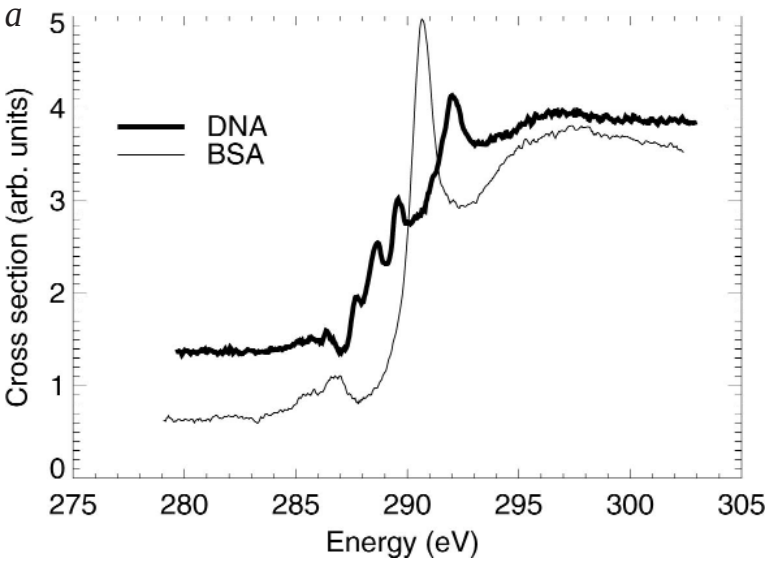

Fig. 2 Soft X-ray microscopes can be used to map compositional difference. ***a***, The near-edge absorption spectra of DNA and bovine serum albumin, showing significant differences caused by different bonding states of carbon in DNA and protein. ***b***, A number of images of dehydrated bull sperm were collected at different absorption resonances; one is shown here. From these images, quantitative maps of protein and DNA were obtained by Zhang *et al*.[15] which were then used to rule out certain models of how protamines bind chromatin in sperm. By using the X-ray microscope, immature and abnormal sperm could be excluded from the measurements.

for studies of subcellular structure in unsectioned, hydrated specimens (Fig. 1). For example, by studying cells of the tasmanian rat kangaroo, *Potorus tridactylis apicalis* (ptK2 cells) fixed at various points in the cell cycle using the Göttingen TXM, Methe *et al.* observed the attachment of microtubules to chromosomal centromeres, and their results are significantly different from details seen in electron micrographs of post-fixation stained specimens[13]. The absence of sharp, highly contrasted intracellular structures is attributed to the hydrated state of the specimen and the lack of heavy metal staining in their work. Also, using the ALS TXM, Magowan *et al.* examined erythrocytes infected by the malarial parasite *Plasmodium falciparum* and they observed a redistribution of mass associated with the morphological changes of the infected cells — including those treated with cysteine protease inhibitors[14], which is important for understanding how these chemicals inhibit hemoglobin metabolism and for developing new therapies against malaria.

Research on soft X-ray STXMs has been pioneered by the Stony Brook group at the National Synchrotron Light Source (NSLS), and additional zone-plate-based STXMs are in operation at APS and ALS, and are planned or being commissioned at ESRF, BESSY II (which will replace BESSY), and elsewhere. (Zone plate STXMs for photoelectron microscopy studies of surfaces are also in operation at Elettra in Trieste, the Synchrotron Radiation Research Center (SSRC) in Taiwan, and ALS in Berkeley). These microscopes require spatially coherent illumination for maximum resolution, and are therefore well matched to high brightness undulator sources. Even so, exposure times of minutes are common, considerably longer than with TXMs. Whereas TXMs tend to use condensor zone plates as monochromators with an energy resolving power E/ΔE of ~300, STXMs tend to use reflective grating monochromators with resolving power of 3,000 or more. The higher energy resolution makes STXMs especially well suited to studies that exploit near-absorption-edge resonances to image the distribution of the chemical bonding states of major constituents in a sample. For example, the locations of protein and DNA in sperm from a variety of species have been mapped, showing that, in species where protamine 2 is present, it partially replaces protamine 1 as the structural element in packing DNA[15]. This conclusion was reached by measuring the overall protein:DNA ratio using STXM and noting that it is independent of the protamine 2: protamine 1 ratio, which is known from other measurements to vary dramatically among the species. Furthermore, Buckley *et al.* have used similar resonances for examining calcified tissue[16]. STXMs are also capable of imag-

ing the overall structure of a cell with bright field microscopy and unambiguously locating gold labels at antibody tagging sites using dark field microscopy[17]. Other labeling techniques with visible light emitting probes are also under development[18,19].

Emerging developments

Compared to electron microprobes, which have element-specific fluorescent lines that appear on a large and continuous X-ray background, X-ray microprobes offer about a 1,000-fold increase in sensitivity for trace element mapping. Successful microprobes have been operated with 5–15 keV X-rays for a number of years at the NSLS, Hamburger Synchrotronstrahlungslabor (HASYLAB), and elsewhere. As a result of recent developments in zone plates and Kirkpatrick-Baez mirror pairs, instruments of this sort are now able to work at sub-micron resolution. This has allowed Yun *et al.* (pers. comm.) to study trace element distribution at the APS by X-ray fluorescence, and manganese oxidation states by near edge spectroscopy, in plant root hairs.

One virtue of X-ray microscopy is its ability to study single cells in their entirety, rather than be limited to thicknesses of ~400 nm, as is the case in electron tomography of frozen hydrated specimens[20]. To exploit this capability, a full three-dimensional image must be created to deal with what would appear as complex overlapping structures on a single view. Because of their low numerical aperture optics, X-ray microscopes allow one to treat high resolution images as simple projections through a specimen. This has been used to demonstrate tomography on microfabricated test patterns[21] and mineralized bacteria sheaths[22] at better than 100 nm axial resolution. More recently, Y. Wang in our group has obtained a three-dimensonal reconstruction of a frozen hydrated fibroblast in a cryo-STXM (unpublished data). Presently, experiments are aimed at coupling tomography at improved resolution with the spectromicroscopy capabilities illustrated in Fig. 2, except on hydrated frozen specimens, rather than dry specimens.

X-ray microscopes add a unique set of capabilities to biological research. They can be used for three-dimensional imaging of intact cells, and to obtain information on the chemical and trace element makeup of unlabeled cells, which cannot be done using other methods. Improvements in spatial resolution through zone plate development, and in radiation dose tolerance using cryomicroscopy, are increasing the range of studies carried out at synchrotron radiation sources.

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