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X-RAY MICROSCOPY: PREPARATIONS FOR STUDIES OF FROZEN HYDRATED SPECIMENS

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Abstract

X-ray microscopes provide higher resolution than visible light microscopes. Wet, biological materials with a water thickness of up to about 10 μm can be imaged with good contrast using soft X-rays with wavelengths between the oxygen and carbon absorption edges (at 24 and 43 Å). The Stony Brook group has developed and operates a scanning transmission X-ray microscope (STXM) at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory. The microscope is used for imaging with a current resolution of 50 nm, and for elemental and chemical state mapping.

Radiation damage imposes a significant limitation upon high resolution X-ray microscopy of room temperature wet specimens. Experience from electron microscopy suggests that cryo techniques allow vitrified specimens to be imaged repeatedly. This is due to the increased radiation stability of biological specimens in the frozen hydrated state. Better radiation stability has been shown recently with a cryo transmission X-ray microscope developed by the University of Göttingen, operating at the BESSY storage ring in Berlin, Germany. At Stony Brook, we are developing a cryo scanning transmission X-ray microscope (CryoSTXM) to carry out imaging and spectro-microscopy experiments on frozen hydrated specimens. This article will give an outlook onto the research projects that we plan to perform using the CryoSTXM.

Key Words: X-ray microscopy, soft X-rays, imaging, mapping, water window contrast, hydrated specimens, frozen-hydrated specimens

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Introduction

X-ray microscopes provide intermediate resolution between visible light and electron microscopy. Many biological structures such as the cytoskeleton, synapses and organelles are at or below the resolution limit of the visible light microscope. These structures can be studied in thin sections using electron microscopes or in whole cell mounts using soft X-ray microscopy.

Soft X-rays with energies between 250 and 530 eV (50 to 24 Å) provide "natural" contrast for hydrated organic materials. The basic contrast mechanism involves photoelectric absorption of X-rays in matter. Fig. 1 shows the penetration depth for electrons and X-rays in water. The "carbon edge" and the "oxygen edge" are X-ray *absorption edges*. Absorption edges arise because absorption of X-rays leads to the ejection of electrons and the ionization of atoms. All materials show absorption edges in their X-ray spectrum; for soft X-rays they are mostly at the binding energies for K and L electrons of low-Z elements.

The spectral region between 285 and 530 eV is called the *water window*. Here, water is much more transparent than carbon-based molecules like protein or DNA (see Fig. 1). Soft X-ray microscopes operate chiefly in the water window spectral region:

* It allows biological samples to be imaged in their natural, i.e. wet environment.

* High intrinsic contrast is obtained without staining; however, gold or luminescent labels are used for special studies.

* It allows imaging of samples in up to 10 μm water or ice; therefore, there is no need for sectioning of thick specimens.

Fig. 1 also gives the mean free paths of electrons for elastic and inelastic scattering in protein and water. In the energy range of typical transmission electron microscopes, the mean free path is typically smaller than the penetration distance for soft X-rays used in X-ray microscopes. Longer penetration depth means less absorption of the incident radiation per penetrated sample thickness. Whereas in electron microscopy contrast is generated by the elastically scattered electrons, contrast in X-ray microscopes is due to photoab-

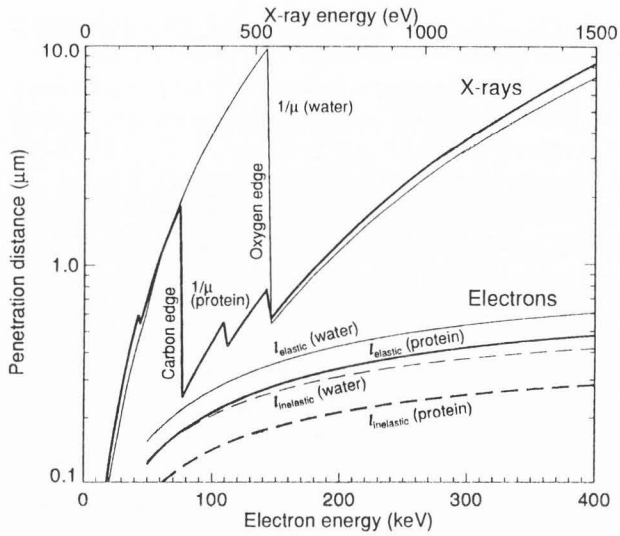

Figure 1. Penetration distance for X-rays and mean free paths l for electrons in water and protein.

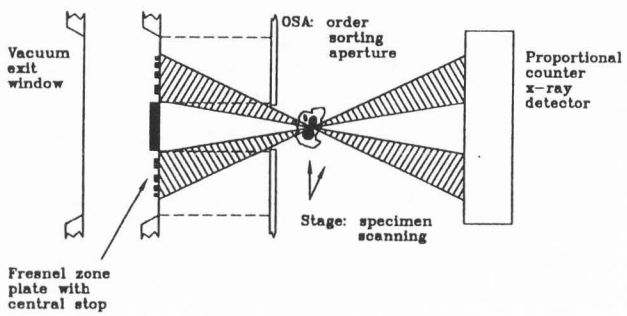

Figure 2. Schematic of the microscope. X-rays are incident from the left.

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sorption of incident X-rays in the sample. Elastic and inelastic scattering is negligible at soft X-ray energies. Minimum radiation doses for sufficient contrast in biological specimens in electron and X-ray microscopes were first calculated and compared by D. Sayre *et al.* [12]. Detailed calculations by Jacobsen and Williams based on [8] and [12] show that frozen samples in a thick waterlayer (>500 nm) can be expected to be imaged with lower dose in X-ray microscopes, even when phase contrast electron microscopy is considered (Jacobsen C, Williams S, Contrast and dose for ice-embedded biological specimens in electron and X-ray microscopy, in preparation). For thin sections, however, electron microscopes provide a lower radiation dose.

The Scanning Transmission X-ray Microscope

Our group at Stony Brook has developed a Scanning Transmission X-ray Microscope (STXM) which operates

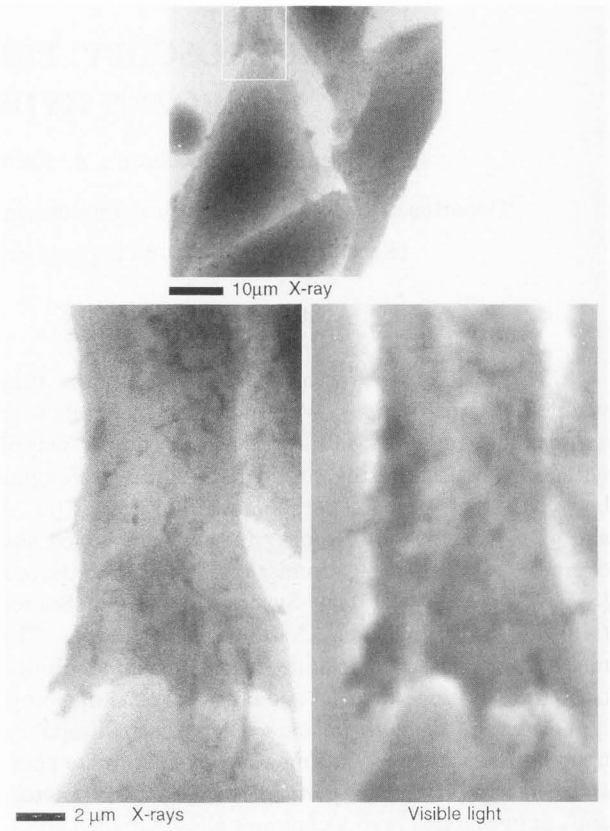

Figure 3. X-ray and optical micrograph of a whole wet cultured Chinese hamster ovarian fibroblast fixed in 1% glutaraldehyde. The optical micrograph was taken at first using a Zeiss 63 x N.A.=1.4 oil immersion objective lens in phase contrast. The cell was then imaged using the X1A Scanning Transmission X-ray Microscope. Figure courtesy J. Fu, C. Jacobsen, A. Osanna (Stony Brook Physics), W. Mangel, W. McGrath (Brookhaven Biology).

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at the X1A beamline of the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory [7, 15, 9, 6]. A spherical grating with a resolving power of up to $\lambda/\Delta\lambda \sim 1800$ is used to select 16 - 50 Å (250 - 750 eV) monochromatic X-rays from an undulator source. The resolving power will be increased to $\lambda/\Delta\lambda \sim 4000$ with a beamline upgrade in 1996. Fig. 2 shows a schematic of the microscope. The monochromatized X-rays are focused by a Fresnel zone plate. The specimen is mounted on a scanning stage and placed in the focal spot by adjusting the position of the zone plate. A transmission image is recorded by scanning the sample through the focal spot. The transmitted radiation is detected with a gas flow proportional counter. The recorded data are used to form the image and are stored on a VAX computer. They can be used directly for

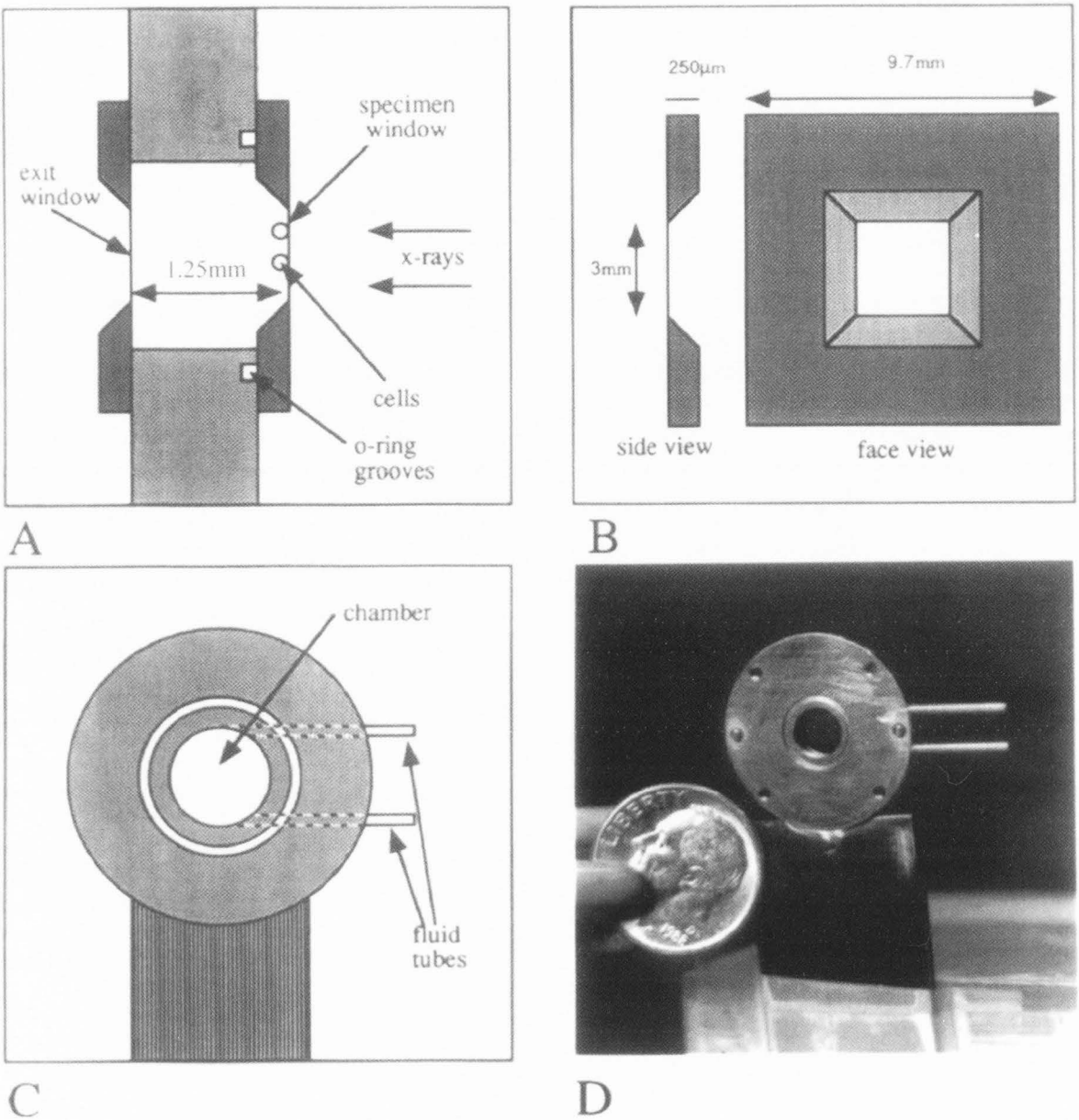

Figure 4. The CalTech wet specimen chamber (from [11]).

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image processing. At present, the microscope operates in an ambient pressure environment. To avoid the strong absorption of X-rays in air, the sample area is flooded with helium.

The resolution of STXM images is primarily determined by the outermost zone width of the zone plate used as a probe-forming optic [4]. The resolution of presently ~ 50 nm is superior to that of a far-field optical microscope (see Fig. 3). 30 nm zone plates have been produced at the University of Göttingen and are also now being fabricated by us with collaborators at Lucent Technologies (Bell Laboratories).

STXM allows two basic modes of operation: the *imaging mode* and the *spectral mode*.

* In the *imaging mode*, the sample is scanned in x and y to obtain a transmission image. The wavelength is kept fixed.

* In the *spectral mode*, the wavelength is changed continuously to acquire an X-ray spectrum of a single point in the specimen. Because zone plates are chromatic optics (i.e., the focal length is determined by the wave length of the X-rays), the zone plate has to be moved

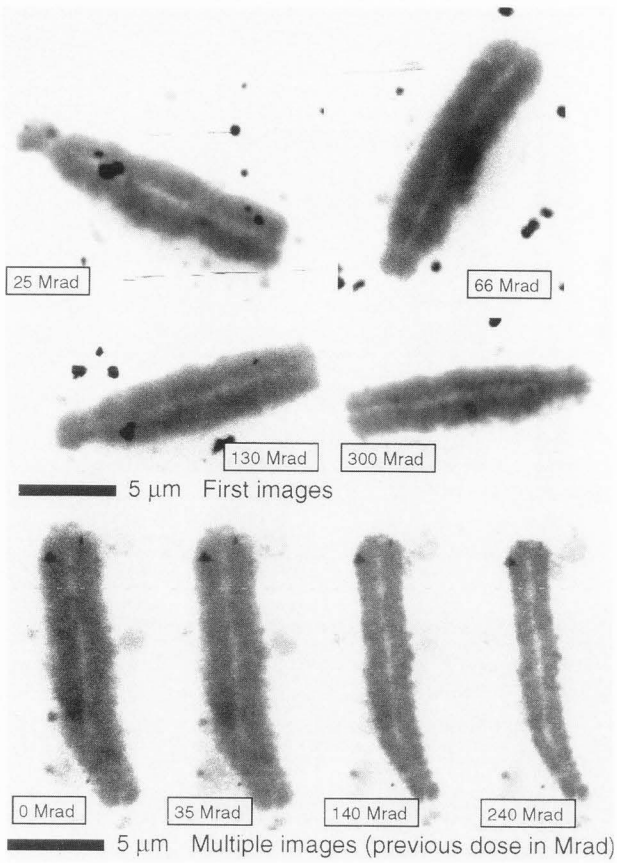

Figure 5. Wet *Vicia faba* chromosomes lightly fixed in 0.2% glutaraldehyde. Multiple images of the same chromosome show degradation due to radiation damage; however first images of previously unexposed chromosomes give accurate mass and diameter measurements up to ~200 MRad. See [14, 16].

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along the beam axis during the scan to keep the sample in focus. By taking several spectra of different sample areas, elemental and chemical properties of the specimen can be determined. In particular, one can combine these two methods and use "chemical contrast" imaging. This makes use of the fact that the spectra of many organic materials show fine structures near an absorption edge. These X-ray Absorption Near-Edge Structures (XANES) are due to e.g. double bonds between C and other atoms. They make it possible to separate different organic constituents like DNA or protein in the specimen by choosing wavelengths for imaging at characteristic peaks. Details about XANES microscopy done at the X1A beamline can be found in [17, 18, 19, 20].

Sample Preparation for X-ray Microscopy

The STXM accommodates three different kinds of sample holders: silicon nitride (SiN) windows, the

Figure 6 on facing page

Figure 6. DNA and protein map for air-dried bull sperm cells. Fig. 6A shows the Carbon XANES spectra for DNA and protamine 1 and 2. The vertical lines indicate the STXM imaging wavelengths. Fig 6B gives the STXM pictures (a, d) and the calculated DNA (c, f) and protein (b, d) distributions for two individual sperm cells. The bar length is 2μm; both pictures have a resolution of 50nm. See [17, 19, 20].

CalTech wet specimen chamber (Fig. 4), and electron microscope grids.

Fig. 4B shows a SiN window. It consists of a silicon frame with a 1000 Å thick SiN membrane. Cells grow very well on SiN windows, and they can be imaged dried or in a wet state after loading them into the wet chamber.

The CalTech wet specimen chamber [11] uses two SiN windows, on one of which the cells have been grown (see fig. 4). The volume inside the chamber is initially filled with a buffer solution that is drained immediately before imaging. Thus, the cells remain in a humid atmosphere. Without exposure to X-rays, the cells can be kept alive for several hours if the chamber is flushed and drained every 20 to 30 minutes.

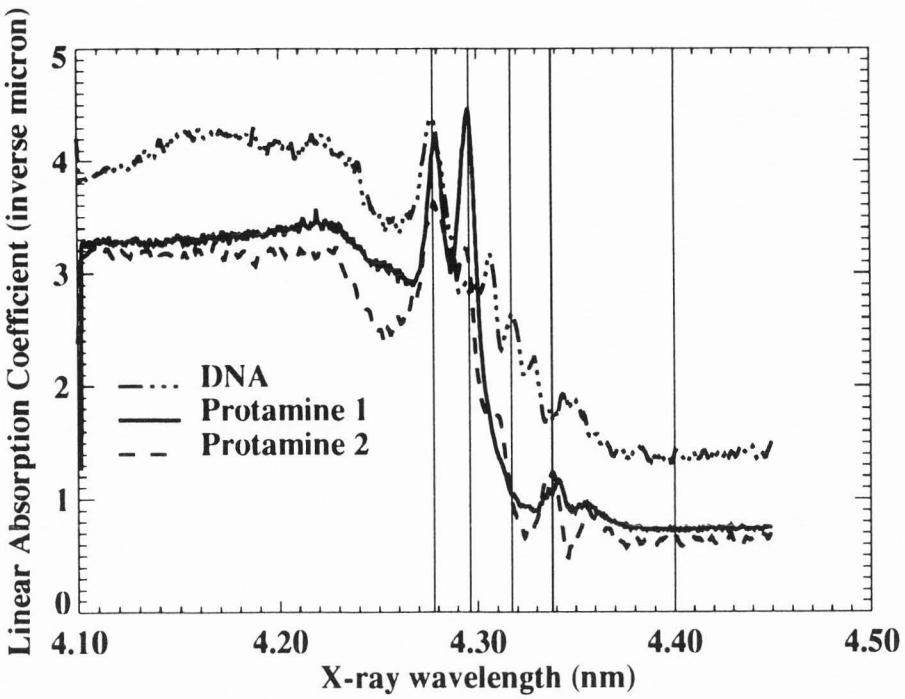
Electron microscope grids are used for dry samples and sections. They allow loading the sample into both a TEM and STXM and thus do comparative imaging.

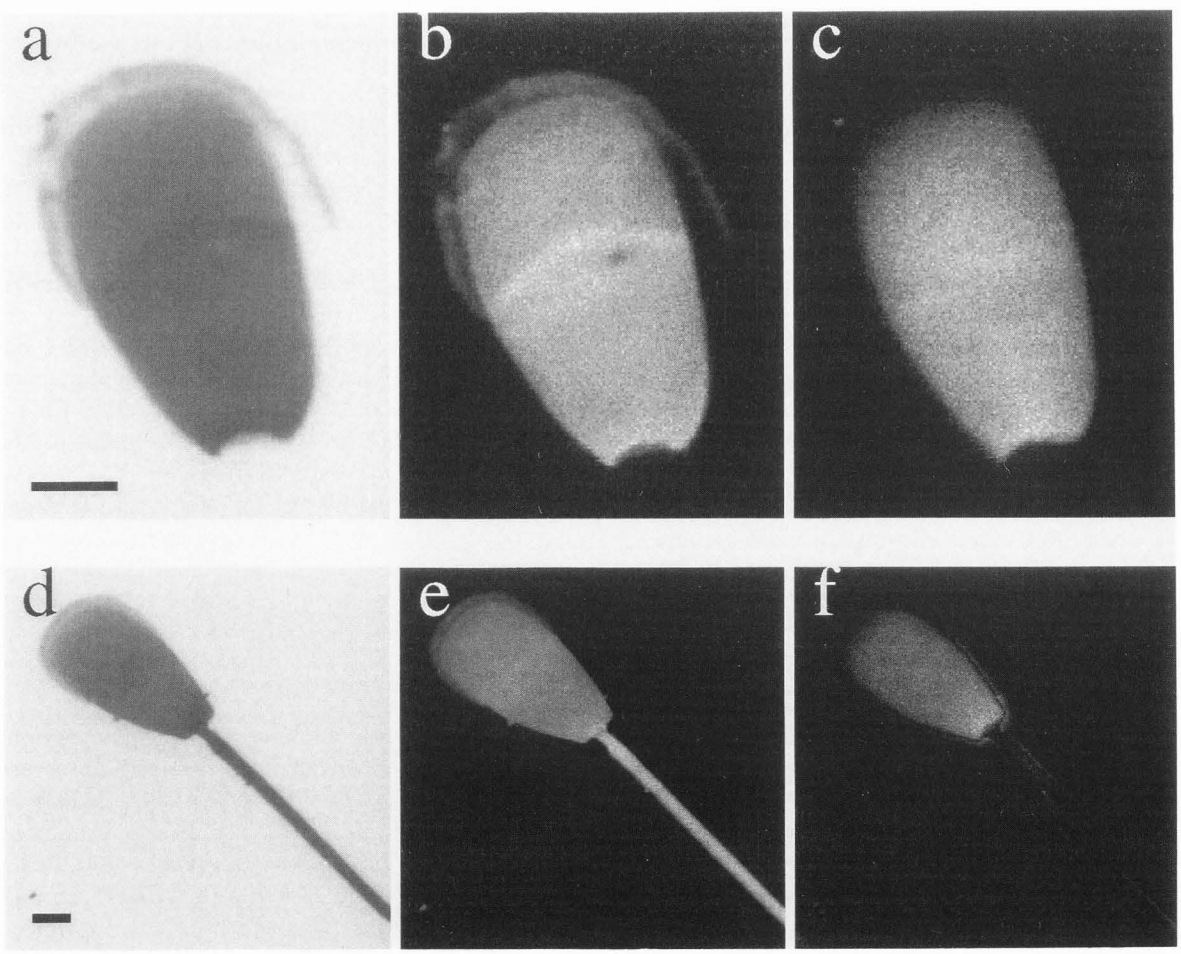
Generally, no additional labeling or staining for contrast is necessary in X-ray microscopes. However, labeling with fluorescent labels like biotinylated terbium is done for luminescence microscopy [10]; gold sphere labeling is used for contrast enhancement in darkfield imaging [1].

Radiation Damage

The dominant interaction of X-rays in matter is the photoelectric absorption. In this process energetic electrons are produced which further ionize atoms and molecules in the specimen. This and the generation of free radicals causes *radiation damage* to the specimen which will ultimately limit the resolution of the microscope in use.

Several groups have investigated radiation damage by X-rays. In Stony Brook, Williams *et al.* have studied radiation damage to metaphase chromosomes as measured by mass loss [16]. First images of previously unexposed, wet, lightly fixed *vicia faba* chromosomes give accurate mass and diameter measurements up to a cumulative dose of about 200 MRad. Multiple images of the same chromosome, however, show degradation already at 35 MRad. (Fig. 5) Unfixed, wet specimens


Linear Absorption Coefficient (inverse micron)
5
4
3
2
1
0
DNA
Protamine 1
Protamine 2
4.10
4.20
4.30
4.40
4.50
X-ray wavelength (nm)


a
b
c
d
e
f

show even more severe radiation damage at comparable doses. The mass loss of wet chromosomes has been determined as a function of the incident radiation dose and the data have be fitted with an exponential mass loss law. It has been found that ionic strength and choice of fixation influence the mass loss under irradiation. Details of the numerical work go beyond the scope of this paper and can be found in [16].

Radiation damage influences the operation of STXM in two different ways. On the one hand, it limits the resolution by not allowing for long exposure times to obtain good photon statistics. On the other hand, one would often like to image a sample repeatedly, for example to get information about the 3D structure (tomography) or to do XANES microscopy.

The Cryo Scanning Transmission X-ray Microscope

To reduce the effects of radiation damage we are planning to image frozen hydrated samples. Cryo methods have been employed in transmission electron microscopy for about 10 years with good results (see for example [2] for an overview). A cryo X-ray microscope has recently been developed by the University of Göttingen. First results [13] show excellent structural preservation of frozen hydrated specimens for radiation doses up to three orders of magnitude higher than what has been possible with room temperature samples.

J. Maser *et al.* at Stony Brook have been developing a Cryo Scanning Transmission X-ray Microscope (CryoSTXM) which is currently being commissioned. A description of the technical features can be found in [9]. CryoSTXM is using an optical arrangement similar to that of STXM (see fig. 2) and will allow imaging and spectral mode operation just like its room-temperature predecessor. To improve thermal insulation and reduce thermal drifts, it has been designed as a vacuum system. It will allow us to use the full water window spectral region for imaging, including the oxygen and nitrogen edge (air leaks in the helium enclosure of STXM have prevented this up to now). Sample preparation will be done by standard cryo methods like plunge-freezing without the necessity of cryo sectioning for whole-cell specimens.

STXM Results and Cryo Plans

While the STXM has been used for studies both of material science and of biological specimens, efforts with cryoSTXM will concentrate on extending the biological studies outlined below. We now describe the current research projects that will be continued with the cryo microscope.

Metaphase chromosome structure

Vicia faba chromosomes have been imaged using water window contrast. Besides the radiation damage studies described above, Williams *et al.* have combined STXM carbon mass measurements with chemical measurements of the total DNA content to find that metaphase chromosomes from four different species all contain 38% DNA by mass [7, 16]. This is in contrast to previous results which gave variable and species-dependent DNA mass fractions. Previous studies have determined the DNA mass through chemical methods which cannot distinguish between different chromosome types in bulk specimens and where the sample is also likely to contain non-chromosome mass. From STXM images Williams *et al.* were able to sort chromosomes by morphology and thus measure the DNA mass from a pure sample. The results of Williams *et al.* suggest a common mechanism for DNA packing at the molecular level, while the differences in mass per unit length and total chromosome length suggest DNA-dependent mechanisms for higher-level chromosome organization. Further studies using chemical state mapping and tomographic imaging are planned with CryoSTXM.

Mapping of protamine and DNA in sperm

Work by Zhang *et al.* has established [17, 19, 20] that the protein-to-DNA ratio is constant among different mammalian sperm cells; however, the protamine-1 and -2 contents vary strongly. Protamine-1 and -2 together constitute 95-99% of the protein present in mammalian sperm. Sperm cells of four different mammalian species have been imaged and the DNA and protein distribution have been determined by imaging at X-ray energies corresponding to distinct peaks of protamine-1 and -2 in their XANES spectra. Fig. 6a shows the spectra for DNA and protein; Fig. 6b shows the results for air-dried bull sperm cells. In human sperm, infertility may be related to protamine-2 deficiency. The protamine-2 content of human sperm has not been determined yet since the dry mass of human sperm heads is too high to view them at the carbon edge; the much larger absorption length of X-rays at the oxygen edge will make this measurement possible in CryoSTXM.

Malaria parasite structure

Malaria is still the largest killer of humans on earth. Searches for a vaccine have been largely unsuccessful so far. Fig. 7 shows the life cycle of *Plasmodium falciparum* in red blood cells. Our studies are aiming at more information about the infection process and the intracellular stages of the parasite. Both light and electron microscopy require some form of staining for contrast and (for electron microscopy) sectioning to reduce the

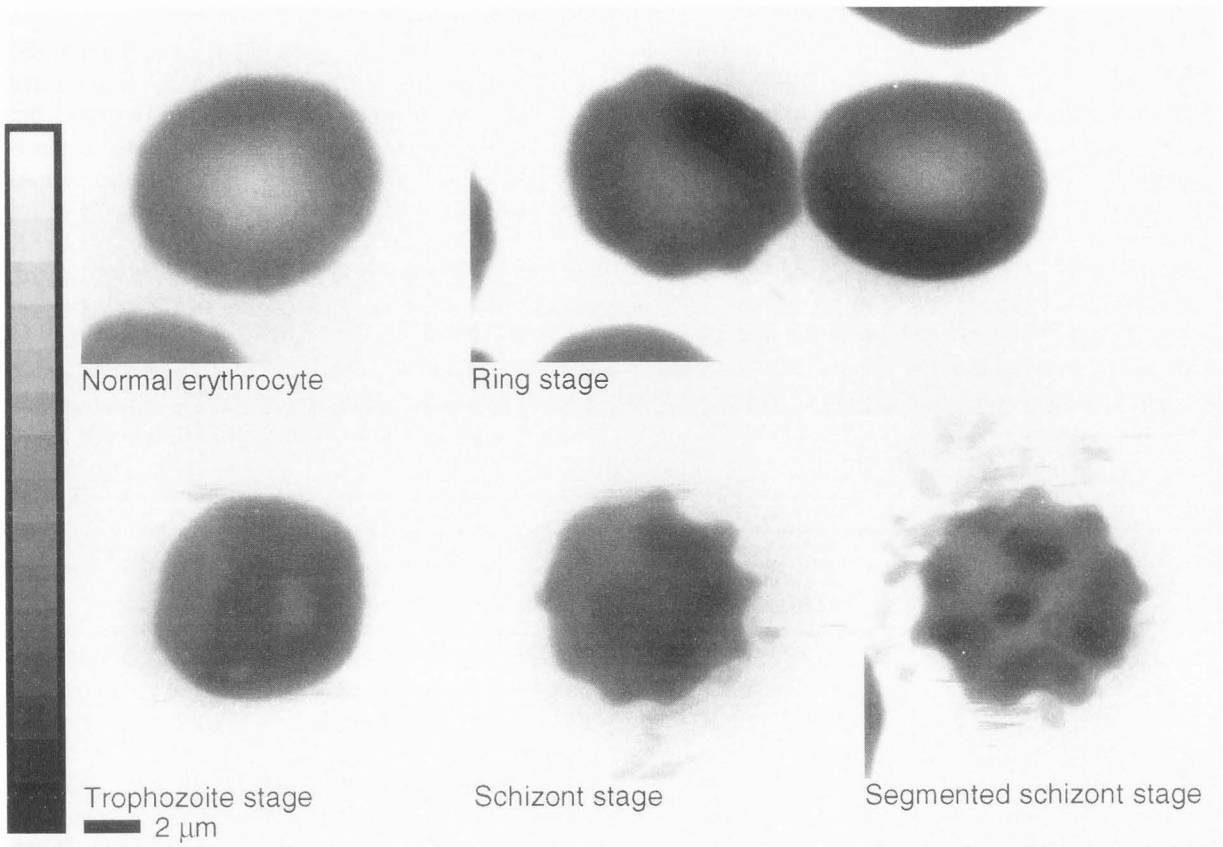


Figure 7. The life cycle of the malaria parasite in human red blood cell as seen in STXM. Figure courtesy W. Mangel, W. McGrath, S. Williams (Brookhaven Biology), C. Jacobsen, J. Kirz, X. Zhang (Stony Brook Physics)

specimen thickness. This has been an issue in the discussion about the evidence for a parasitophorous duct in confocal microscope observations. STXM provides better resolution than confocal light microscopy (which has a resolution of ~200nm) without any sectioning or staining. While we have obtained some images which are suggestive of duct structures, part of the controversy over confocal and electron microscope observations has centered on glutaraldehyde fixation artifacts; it should be possible to avoid such complications with cryoSTXM.

STXM and cryoSTXM both allow for luminescence and darkfield microscopy which are described in [5, 10] (luminescence) and [1] (darkfield). First results on tomography using STXM have been reported in [3].

Conclusion

Soft X-ray microscopy offers new opportunities for quantitative imaging and elemental and chemical state mapping of whole, hydrated cells. Further studies using frozen hydrated specimens will become possible with the development of cryo X-ray microscopes, such as the cryo scanning transmission X-ray microscope of the Stony Brook group. Applications include multiple imaging and tomography of specimens like chromosomes, sperm cells and malaria-infected red blood cells.

Acknowledgements

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Discussion with Reviewers

J. Hainfeld: The X-ray C and O absorption edges are very dramatic as shown in Fig. 1. Have you tried two image differencing, one above the O edge and one below the O edge, for example, as a way to increase contrast even further over "natural" contrast?

Authors: "Two image differencing" is the basic principle of elemental and chemical state mapping that is done routinely in STXM (see section on description of the microscope). Imaging above and below an absorption edge like the carbon edge is the simplest version of this method that usually uses X-ray Absorption Near-Edge Structures (XANES). It is used for example to image samples that are known to contain protein but where a protein standard is not available. While imaging across the carbon edge is easily performed and yields nice results, the method has not yet been used at the oxygen

edge, the reason being residual air in the X-ray path of STXM. As mentioned in the description of our cryo version of STXM, mapping across the oxygen and also the nitrogen edge will be done in the vacuum environment of cryoSTXM. However, for wet specimens the oxygen edge is dominated by water and may be less informative.

J. Hainfeld: In Fig. 3 you compare a STXM and a light microscope cell image, and the light image is obviously of lower quality. However, thick samples, e.g., cells, suffer from out-of-focus information being imaged, resulting in image degradation, whereas the STXM image is produced from a highly collimated source. Could you comment on this effect and would a confocal image be better and perhaps a better comparison?
Authors: The light microscope image shown may indeed suffer from out-of-focus information from the full thickness of the specimen. High depth of focus is in fact an advantage of X-ray microscopy (our STXM has a depth of focus of $\approx 2\mu$m at a spatial resolution of 50nm and a wavelength of 40Å). Confocal microscopy could be used to deliver ~ 200 nm resolution, 500 nm thick optical sections of fluorescently labeled features, which would provide useful complementary information to the X-ray microscope's 50 nm resolution image of total organic mass.

J. Hainfeld: The very high doses sustained by x-radiation in some STXM images result in damage from free radicals. Have you tried to bathe samples in free radical scavengers and does it help?
Authors: We have indeed used free radical scavengers; Williams *et al.* report in [21] that bathing glutaraldehyde-fixed *V. faba* chromosomes in dithiothreitol or ethanol reduces the amount of radiation damage incurred during imaging by a factor of 4 and 10, respectively. Despite this improvement in radiation resistance, we have chosen to concentrate on cryo methods because it offers even more radiation damage resistance and allows one to avoid the artifacts which sometimes accompany glutaraldehyde fixation.

J. Hainfeld: The cryoSTXM has been shown to slow the beam damage of biological specimens. Are these studies at liquid nitrogen temperature and is this where you propose to operate? Would it pay to go to liquid helium temperatures; what is the damage-temperature profile?
Authors: The Göttingen X-ray microscope is operating at liquid nitrogen temperature, and so will the Stony Brook microscope. Even though there may be advantages from operating at liquid helium temperature, we have chosen to begin with using the commercially-developed technologies available for reaching liquid nitrogen temperatures. No damage-temperature profile for cryo X-ray microscopy is available yet.

J. Hainfeld: In reference to the protamine/DNA work, do DNA and protein damage differently in the X-ray beam?
Authors: The mapping of DNA and protein in sperm was done with dried specimens. The studies of Williams *et al.* reported in [16] show no measurable mass loss at doses up to 2.4 Grads. No difference in radiation sensitivity of protein and DNA has been observed.

J.A.N. Zasadzinski: Are there samples that can be analyzed better with the current generation of X-ray microscopes than with currently available electron microscopy techniques - especially when cryo techniques are required?
Authors: In this paper, we have summarized several studies which required the ability to study unsectioned specimens: total mass measurements of chromosomes, protamine mapping in sperm, and a search for a duct structure which extends in three dimensions through malaria-infected erythrocytes. X-ray microscopy is well suited to studying samples for which 0.1 to 0.3 μm thick sections provide incomplete information, or for which chemical state maps of low-Z elements are required.

J.A.N. Zasadzinski: When imaging large samples such as whole cells, what is the practical resolution, and what limits the resolution?
Authors: It is difficult to determine the resolution from images of unknown objects which is what we are looking at with wet samples (they cannot be imaged in a transmission electron microscope before using STXM). Power spectra taken from glutaraldehyde-fixed specimens suggest rendering information close to the 50nm level (for details, see for example [6]). We have not observed any degradation of resolution due to multiple scattering and refractive index variations in large specimens.

J.A.N. Zasadzinski: How many X-ray microscopes do you envision as being necessary for biological research in the US? - One for every university - one for each state or one in the country?
Authors: Due to the fact that X-ray microscopes need a synchrotron to operate, there will not be "one STXM per biology lab". But then, not every laboratory can afford a high voltage-TEM, and yet they are regarded to provide useful information to the community. At the Stony Brook STXM we have demands for much more beamtime than we have to offer. Furthermore, X-ray microscopy is under development at more than a dozen

synchrotron centers worldwide.

G.M. Roomans: Will ice crystals be a problem in obtaining images of frozen-hydrated specimens by X-ray microscopy?
Authors: Cryo electron microscopy indicates that in flash frozen samples there is no sign of ice damage. STXM is insensitive to ice crystals smaller than ~ 10 nm, a requirement that is satisfied by the usual plunge-freezing methods. The Göttingen results indicate that sufficient vitrification of thick samples is possible.

Additional References

[21] Williams S, Jacobsen C, Kirz J, Zhang X, Van't Hof J, Lamm SS (1992). Radiation damage to chromosomes in the scanning transmission X-ray microscope. In: Soft X-ray Microscopy, vol. 1741, Jacobsen C, Trebes J (eds) Society of Photo-Optical Instrumentation Engineers (SPIE), Bellingham, Washington,