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Soft X-ray microscope: A new biological tool

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Soft X-ray microscopy is a technique which has tremendous application potential for biological imaging in the native aqueous environment. It offers a structural resolution of ~ 50 nm, which is between that of an electron microscope and the conventional optical microscope. The main advantage of this technique, however, is that it facilitates imaging of relatively thick (1–10 µm) samples at high resolution in the wet condition without the necessity of external contrast-enhancing agents. Although this technique has been proved to be highly useful for biological imaging, it is still not widely used because of the technological difficulties. This paper deals with the technological issues of soft X-ray microscopy vis-à-vis the recent developments in these areas which will widen the application potential of this technique.

Soft X-ray microscopy

The optical and electron microscopy techniques are being used successfully for the characterization and design of materials in various applications. These techniques, however, are not extensively used for characterization in biological sciences. The optical microscopy technique is simple in nature, offers flexible conditions for observation

and the radiation used for imaging is benign to the cells. However, the maximum resolution that can be achieved and the depth of focus are poor, which limit the usage of this technique. The recently developed confocal optical microscope facilitates 3D imaging, which is not possible with the conventional optical microscope. However, the point-to-point resolution is only ~ 0.2 µm, an order of magnitude lower than that offered by soft X-ray microscopy. Electron microscopy, on the other hand, is a technique capable of resolving features down to subnanometer sizes. This technique, however, is not widely used for imaging biological materials because of the following reasons: (i) The specimen thickness should be < 100 nm to realize resolutions of the order of ≤ 5 nm. This means careful and complex specimen preparation is required which can cause irreversible damage to the sample; (ii) The high energy electron beam used for imaging can also lead to an irreversible damage to the sample; (iii) The weak dependence of scattering cross-section for electrons on atomic number makes artificial staining a necessity for contrast enhancement; (iv) The samples can be observed only in dried state which means imaging in the native aqueous environment is not possible.

The soft X-ray microscopy technique is ideally suited for imaging biological materials as it bridges the

resolution gap between the optical and electron microscopies. The soft X-rays are generally in the wavelength range 1.0–12.0 nm and hence offer the possibility of imaging at sub-optical resolutions. Since the attenuation length for soft X-rays in typical biological molecules is several micrometers, thick samples (as compared to electron microscopy) can be imaged directly without compromising the resolution. This is shown in Figure 1 where the variation of linear absorption coefficient μ with soft X-ray wavelength is shown for carbohydrates, proteins, lipids, nucleic acids and water. An important point to be noted in Figure 1 is the absorption coefficient variation for the different biological molecules and water in the wavelength range 2.34 nm to 4.38 nm (between the K absorption edges of oxygen and carbon). The attenuation length in water is higher by about an order of magnitude compared to the biological molecules and this facilitates imaging of thick, wet and unstained samples. Hence this wavelength region is often referred to as the 'Water Window' region. Some typical examples of biological imaging performed using soft X-ray microscopy are mentioned in-brief below^{1,2}.

The structure of fibroblast cells, 2 μm thick, was recently studied by scanning transmission X-ray microscopy. It was found that high resolution, 32 nm per pixel, images of 'wet' fibroblasts could be obtained without causing any structural damage to the cells (Figure 2)³. Buckley *et al.*⁴ demonstrated the possibility of elemental mapping of biological tissues using X-ray microscopy. The variation in Ca-concentration in human articular cartilage section was determined by X-ray absorption difference imaging (Figure 3). The structure of DNA in the nucleus of a sperm cell was studied by Da Silva *et al.*⁵. They found that about 1/3 of the

nuclear volume is occupied by water which is retained even after 20 min holding at low pressures. The 3-D structure of human spermatozoa in aqueous suspension was studied by X-ray stereomicroscopy. An area of increased density in the posterior half of the head was observed which was not seen earlier by electron microscopy⁶.

These examples and the many others reported in ref. 7 clearly show the high application potential of soft X-ray microscopy for biological sciences.

The three main components of an X-ray microscope are: (i) Source of high brilliance soft X-ray beam; (ii) Optical elements for treating the pre- and post-sample soft X-ray beam; (iii) Soft X-ray detectors.

Soft X-ray sources

Different types of soft X-ray sources are available for X-ray microscopy. The primary criteria that define the nature of X-ray beam irrespective of the type of source are:

Brightness. Brightness is defined as the radiated photon flux per unit area per unit solid angle. A related quantity which is more often used to describe an X-ray source more precisely is the 'spectral brightness' B given by the relation.

$$B = F/[(\Delta A)(\Delta \Omega)(BW)]$$

where F is the photon flux (photons per second) per unit area ΔA per unit solid angle $\Delta \Omega$ of relative spectral bandwidth BW . The relative bandwidth $\Delta \lambda / \lambda$ is often expressed as a percentage.

Coherence. The maximum resolution that can be achieved depends on the spatial and temporal coherence length of the X-ray beam which is a function of the source size d , wavelength λ and the angular divergence of the beam θ . The volume in 'phase space', $d \cdot \theta$ should be a minimum for realizing very high resolutions.

Synchrotron radiation source

The synchrotron radiation is close to an ideal source for X-ray microscopy because of its brightness and coherence. This source is ideally suited for all types of X-ray microscopy such as transmission and scanning transmission. The major synchrotron radiation sources where X-ray microscopy is being investigated are: BESSY in Berlin, Germany⁸; National Synchrotron Light Source (NSLS) in Brookhaven, USA^{6,9}; Synchrotron Radiation Source (SRS) in Daresbury, UK¹⁰; Photon Factory (PF) in Tsukuba, Japan¹¹; and BEPC in Beijing, China¹². The brightness of the beam from synchrotron radiation sources is typically in the range 10^{13} to 10^{18}

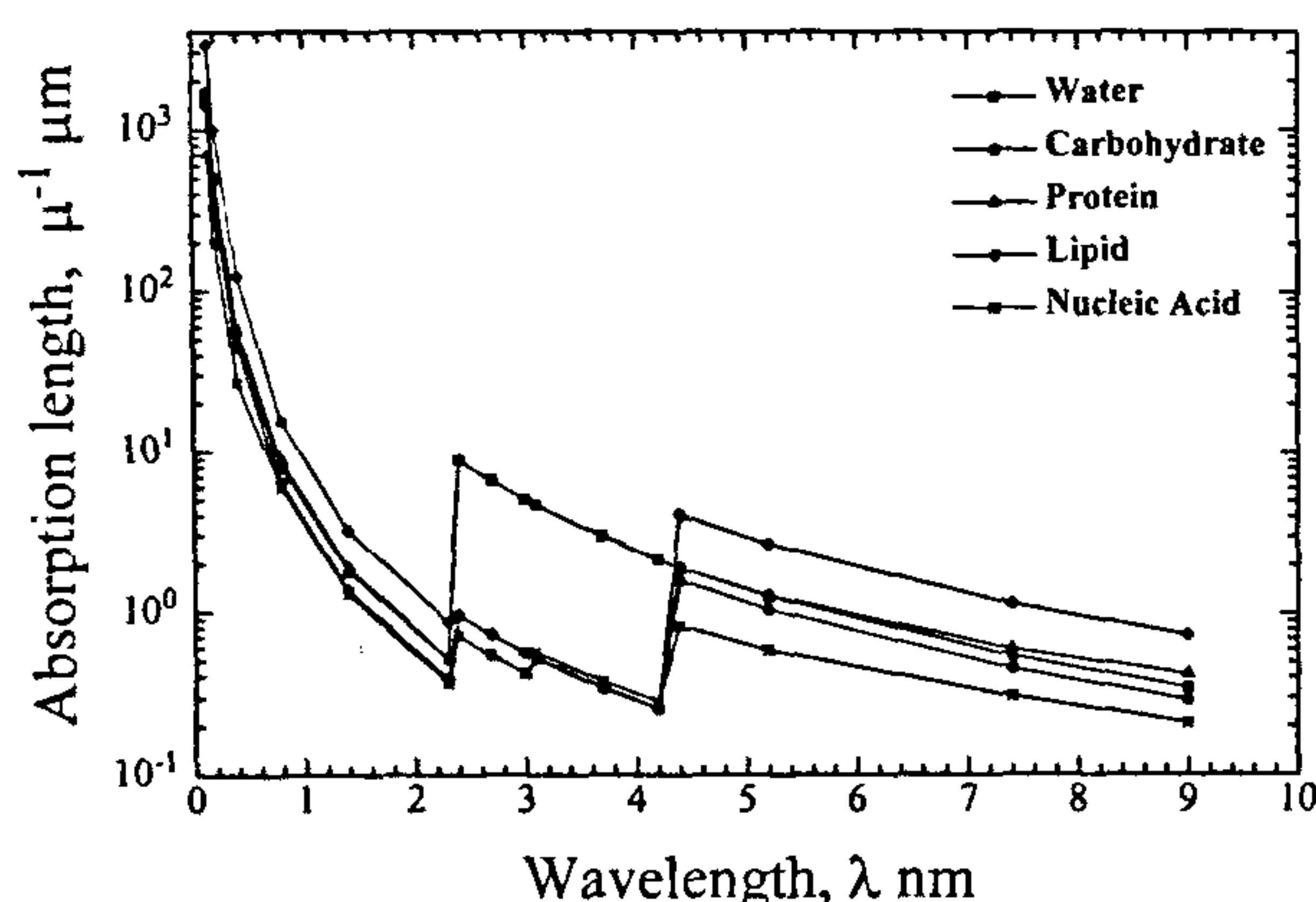


Figure 1. The absorption length μ^{-1} where μ is the linear absorption coefficient, variation as a function of radiation wavelength λ for different biological molecules *vis-à-vis* water indicates a wavelength window region ideally suited for maximum contrast. This window, λ from 2.34 nm to 4.38 nm, is often referred to as the 'water window' region suitable for X-ray microscopy.

photons $\text{s}^{-1} \text{mm}^{-2} \text{mrad}^{-2} (0.1\% \text{ BW})^{-1}$ depending on the type of device used to accelerate the electrons in the ring. The major disadvantage of synchrotron radiation source which offsets the quality of X-ray beam it can provide is its limited accessibility. Also, from the point of view of X-ray microscopy alone the synchrotron radiation sources are too uneconomical.

Laser-produced plasma source

The limited accessibility of synchrotron radiation sources has been the primary reason in developing alternative soft X-ray sources for microscopy. One such development is the production of soft X-rays from hot plasmas which are generated by bombarding a metallic target material with high power pulsed lasers. The X-ray emission from such hot plasmas comes in very intense pulses of short duration (order of nanoseconds) with enough flux to form the image of wet, live specimens. The time period is sufficiently short that neither specimen motion nor radiation damage can blur the image.

Lasers with a wide range of parameters have been used to generate plasmas. The typical wavelengths are in the range 193 nm (ArF excimer laser) to 1.064 μm (Nd:YAG laser) with pulse energies of a few mJ up to several tens of kJ. The pulse widths in all the cases, however, are \leq nanoseconds giving a power density of $> 10^{15} \text{Wm}^{-2}$. Different types of target materials ranging from low atomic number element such as carbon to high atomic number element such as gold have been used depending on the type of soft X-ray radiation required. Medium to high atomic number targets give a broad band spectrum which requires monochromation to be used in a scanning X-ray microscope. On the

other hand, a low atomic number target gives a quasi-monochromatic line spectrum which does not need monochromation.

The typical brightness value of the X-ray beam produced from laser-plasma sources is in the range 10^{10} – 10^{13} photons $\text{s}^{-1} \text{mm}^{-2} \text{mrad}^{-2} (0.1\% \text{ BW})^{-1}$, comparable to the synchrotron sources.

The feasibility of microscopy using laser-plasma generated soft X-ray beams has been demonstrated in refs 13–16. The main advantages of laser-plasma produced X-ray source are:

- It allows 'flash' imaging which can be used to study time-dependent dynamic changes in wet samples,
- It has wider accessibility compared to synchrotron radiation sources,
- It has compatibility for table top X-ray microscopy, and
- It is highly economical relative to a synchrotron.

Soft X-ray optics

The design of optical elements in general can be based on one of the three basic principles: refraction, reflection and diffraction. The optical elements for visible light microscopy are based on refraction by glass lenses. However, such refractive lenses cannot be used for soft X-rays as most materials at these wavelengths have a refractive index close to 1. This leads to lenses with very large focal lengths which preclude their usage. Also, the absorption cross-section of materials for soft X-rays is very high. Hence the normal refractive lenses cannot be used to focus soft X-rays but instead they have to be based on either reflection or diffraction.

Reflecting mirror optics

The reflectivity of most of the materials depends on the complex index of refraction n and the angle of incidence θ_i . For any given material the reflectivity R decreases with increasing angle of incidence. However for $\theta_i < \theta_c$, the critical angle, total external reflection of the incident beam takes place increasing the value of R to 1. This configuration is generally termed as grazing incidence reflection. In order to achieve beam focusing with grazing incidence reflection, the surfaces must be curved and this introduces aberrations which distort the image. The two major sources of aberration for the case of spherical reflecting mirrors are: astigmatism and spherical aberration. Both these aberrations depend on the angle of incidence θ_i and they increase with decreasing angle of incidence.

It can be seen from the above discussion that the magnitude of the aberrations increases with decreasing angle of incidence while the reflectivity increases with decreasing θ_i . To overcome the aberrations at grazing

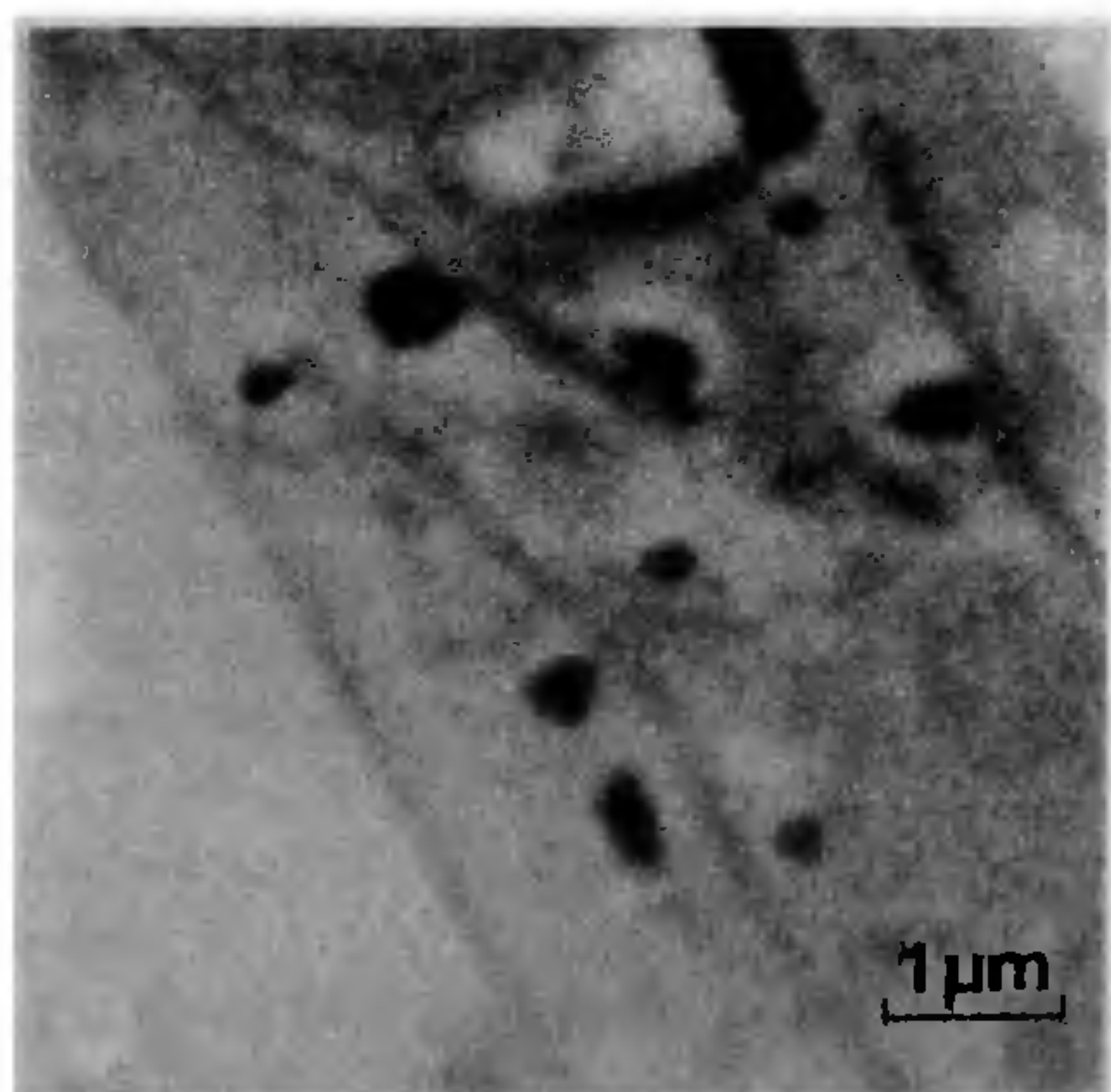


Figure 2. A scanning transmission X-ray microscopy image of a fibroblast taken at high resolution using 3.41 nm wavelength soft X-rays. Features of size $\approx 1.0 \mu\text{m}$ and less can be easily resolved (ref. 3).

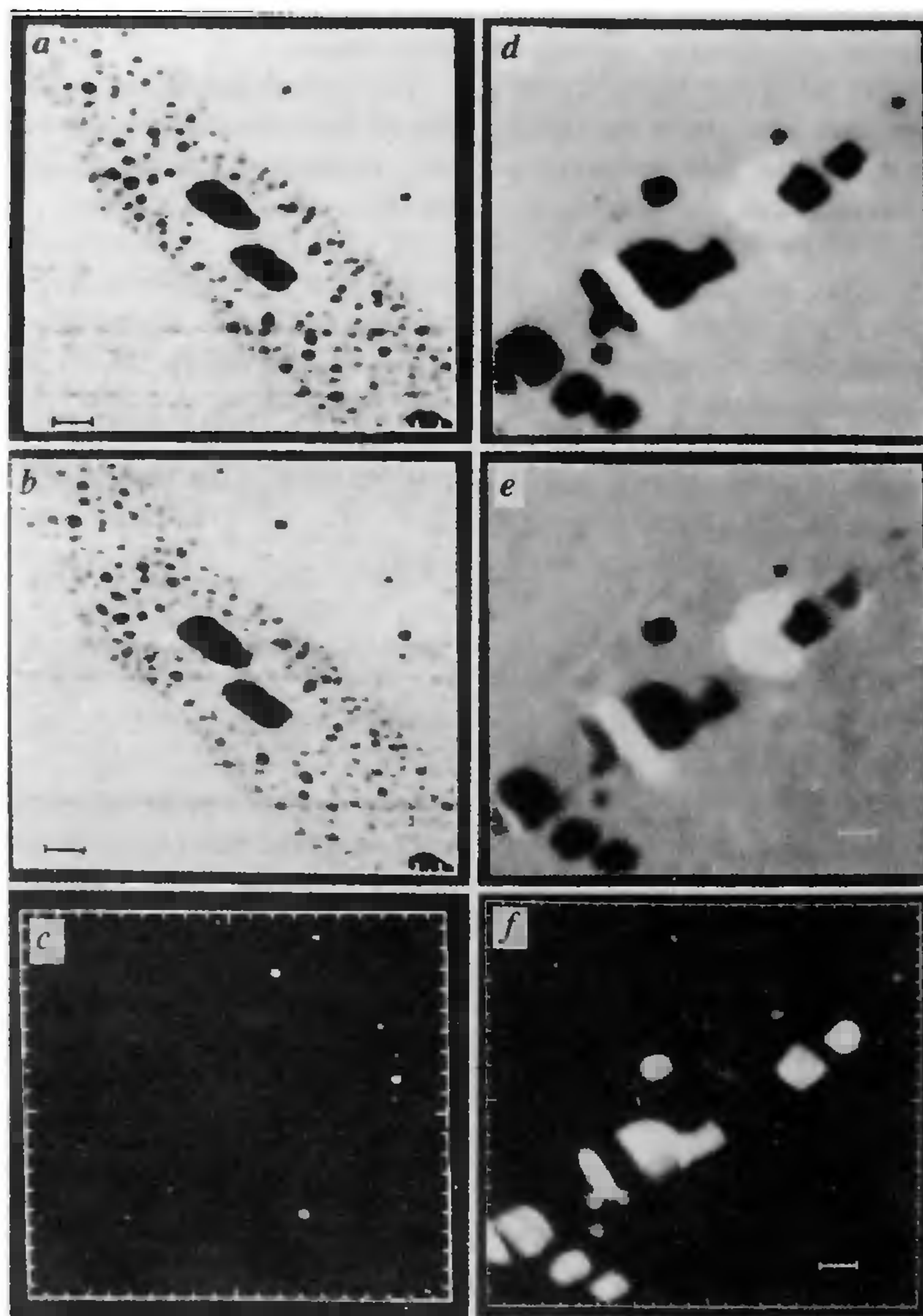


Figure 3. Scanning transmission X-ray images of human articular cartilage section taken around the calcium edge at 3.53 nm. (a) and (b) show the images taken before and after the edge while (c) is the calcium difference map made from (a) and (b). (d) and (e) are images of calcium phosphate crystals found in the cartilage with (f) showing the calcium distribution map. The level of calcium in the maps is indicated by the degree of brightness (ref. 4).

angles of incidence either a combination of spherical mirrors or aspherical mirrors such as Kirkpatrick-Baez and Wolter type optics are generally used. Anderson *et al.*¹⁷ and Ohsuka *et al.*¹⁸ have used Wolter type optics for X-ray microscopy. However the main disadvantages of grazing incidence reflection optics are: (i) The mirrors are not wavelength selective and hence the beam needs to be monochromatized; (ii) The grazing angle of incidence requires a very large mirror surface to collect a fraction of the incident beam, resulting in low overall reflected intensities; and (iii) Practical difficulties involved in the making of large and complex mirror surfaces with extremely low tolerances.

An alternative to grazing incidence reflection optics is normal incidence reflection optics. The aberrations

which are severe at grazing angles of incidence vanish completely for $\theta_i \sim 90^\circ$. However, the reflectivity of the mirror surface at normal incidence, given by the relation $R = [(1 - n)/(1 + n)]^2$, is only 10^{-5} to 10^{-6} . In order to overcome the problem of reflectivity at normal incidence, artificially structured multilayer reflection mirrors are currently being developed.

Multilayer reflection mirrors

In its simplest form a multilayer mirror consists of alternating layers of low and high electron density materials, generally referred to as spacer and absorber respectively. The enhanced reflecting properties of the

multilayer at or near normal incidence stem from the coherent addition of weak reflections from many interfaces. Since there are no naturally occurring crystals with such properties, artificially structured multilayers offer an attractive alternative. The main advantages of multilayer reflecting mirrors are as follows: (i) Operation at near normal angles of incidence leads to a considerable reduction in the mirror aberrations; (ii) Since the multilayers are wavelength-selective they serve as both monochromators and focusing elements when made on curved surfaces; (iii) The overall reflectivity of a multilayer mirror will be much higher compared to single surface mirrors operating under identical conditions.

Several multilayer mirror systems with high reflectance values have been made¹⁹⁻²¹. The main drawback of these multilayer reflectors is that they are not designed for operation in the 'water window' region of X-radiation. For soft X-ray microscopy in this region the wavelength of interest is 2.3 to 4.4 nm and this corresponds to a period of 1.2 to 2.2 nm, assuming normal incidence conditions. This means the individual layers in the multilayers, absorber and spacer, are only a few monolayers in thickness. Preparation of such thin layers with a relatively high interface quality demands the selection of correct materials on the basis of optical constants, and most important, the chemical and structural compatibility of the component materials. The chemical compatibility-mutual solubility and the compound formation tendency at the interface can be judged from the equilibrium phase diagram. Recently it has been shown that multilayers with a period of 2.4 nm can indeed be made for use in the water window wavelength region²². However, their reflectance values are quite low to be used in a microscope. These studies indicate that considerable progress still needs to be made for the complete realization of optical elements based on multilayers.

Zone plate optics

Zone plates based on diffraction of radiation have been the optical elements of choice for X-ray microscopy. These are used in transmission mode because of the very small normal incidence reflectivity of materials. The zone plates can be regarded as circular diffraction gratings with radially increasing line densities. The line density is so arranged that the radiation diffracted from different zone boundaries is brought to focus along the axis of the zone plate. The spatial resolution in the images obtained depends on the outermost zone width, d_N and is given as $1.22 d_N$ for the first order diffraction. This relation shows that the resolution limit increases as the outer zone width decreases. The examples discussed under 'Soft X-ray microscopy' used zone plate objectives for X-ray imaging. Although the capability

of zone plate objectives for X-ray microscopy has been amply demonstrated, there are several limitations (mentioned below) which need to be overcome for their large scale usage.

- The present day technology used for the preparation of microelectronic devices does not allow fabrication of zones < 50 nm wide on a regular basis. This limits the attainable resolution to about 60 nm and above.
- The first order diffraction efficiency of absorber zone plates is only $\approx 10\%$. When such zone plates are used as objective lenses, either the exposure time to radiation or the radiation intensity has to be increased considerably to generate high resolution images. This can lead to radiation damage of the sample.
- The background intensity caused by the zeroth order and out-of-focus higher orders leads to significant loss of contrast for extended objects.
- In the absence of a monochromator, the zone plates suffer from severe chromatic aberration since the focal length is inversely proportional to λ , the radiation wavelength.

Soft X-ray detectors

A wide variety of detectors ranging from the more conventional X-ray film to the more recent charge coupled devices (CCD) are being used for the detection of soft X-rays. The topic of detectors is treated exhaustively by Fraser²³ and Delaney and Finch²⁴. Hence these are not discussed here in detail and for the purpose of giving a completeness to the subject, soft X-ray microscopy, only the criteria that needs consideration during selection of a suitable detector are mentioned.

The distinguishing criteria for classifying detectors are: (i) *Spatial resolution*; (ii) *Sensitivity* – ability to achieve efficient detection of single photons; (iii) *Transfer function stability* between input and output in order to achieve high photometric accuracies; (iv) *Broad spectral response* coupled with energy resolution for single photons over the entire spectral range of interest.

It should, however, be mentioned here that there is no single detector available which satisfies all the above conditions. X-ray photographic films, although extensively used, suffer from very poor energy resolution and a narrow spectral response. They, however, have by far the best spatial resolution, $\approx 1.0 \mu\text{m}$ among the various types of detectors.

Radiation damage

The topic of radiation-induced damage in soft X-ray microscopy had not attracted much attention so far because of the technological problems involved in making

the microscope itself. However, the recent rapid developments that have taken place in the soft X-ray sources and optical elements preparation have brought to focus the topic of radiation-induced damage. Hence this is discussed below together with some recent experimental results on radiation-induced damage.

When an X-ray photon interacts with the material, photoelectric absorption which results in amplitude change and a phase shift of the incident radiation occurs. Either of these changes in the incident beam, amplitude and phase, or both together can be used to generate contrast in imaging. In order to generate a detectable contrast level (above the background noise) the incident radiation should have sufficient brilliance. Such high brilliance radiation can lead to damage in the sample, specially when collecting high resolution images from thick, wet and unstained samples. In order to quantify the possible damage, a factor known as 'dosage' D , defined as the energy absorbed by unit mass of the absorbing material is used. It has been found both theoretically and experimentally that dosages in the range of 10^7 Gy (10^9 rad) are needed to realize resolutions of the order of 10 nm in samples 5–10 μ m thick in the water window region. At these dosage levels the energy absorbed will be sufficiently high to lead to severe damage.

Goodhead *et al.*²⁵ found that the surviving fraction of mammalian cells when irradiated with 278 eV carbon K X-rays at dosages of the order of 10 Gy was negligible. Also, it was observed that within the DNA molecules the radiation-induced damage leads to strands breaking, base and sugar damage and cross linking which are highly deleterious effects in rendering the mammalian cells inactive²⁶. Gilbert *et al.*³ observed a severe visible damage in the case of fibroblast cells 3 h after exposure to 10^4 Gy radiation of 3.41 nm wavelength in a scanning transmission X-ray microscope (STXM). The contractile organelles of muscle (myofibrils) were studied by STXM using 3.2 nm wavelength radiation²⁷. It was observed that 2.5×10^4 Gy radiation was sufficient to cause serious impairment of the contraction mechanism.

It is clear from the above examples that radiation-induced damage in soft X-ray microscopy cannot be avoided but only reduced. The radiation damage can be considerably reduced if the observation is finished before structural changes begin. The damage mechanism can be envisaged to proceed as follows; the absorption of X-ray photons results in the formation of ions and secondary electrons. These ions and electrons cause the formation of free radicals which are responsible for bond breaking²⁸. The lifetime of such free radicals is found to be $\sim 10^{-3}$ s, which sets the upper limit for the time of observation. Thus, using a pulsed radiation with sufficient brilliance the damage can be minimized or even completely avoided by completing the process of

image collection before structural damage begins. This indeed is made possible with the development of laser plasma produced soft X-ray sources. The X-ray beam from such sources is inherently pulsed with pulse widths typically in the range of 10^{-9} s. If 10^3 exposures are needed to gather complete information from a region of the sample, it amounts to a total exposure time of only 10^{-6} s which is 3 orders of magnitude lower than the time for critical damage of the biological material.

Summary

The X-ray microscopy technique has a definite potential to bridge the gap between optical microscopy and electron microscopy for studying biological materials. The main advantage of X-ray microscopy is that it facilitates imaging thick, wet, unstained samples when performed in the 2.34 nm to 4.38 nm 'water window' region. The source of soft X-rays, synchrotrons, although ideal in terms of the quality of the beam they offer, are not ideal for large-scale usage. However, the development of laser plasma-produced soft X-ray sources has considerably changed this scenario. The availability of high brilliance beams either in monochromatic form or wide band form depending on the type of target used to create the plasma, has made possible the realization of table top soft X-ray microscopy. The other main advantage of laser plasma produced soft X-ray beams is that they are inherently pulsed. The use of these ultra short-pulsed beams reduces the extent of radiation-induced damage in the sample by several orders of magnitude.

The non-availability of high yield optical elements is one of the main factors limiting the widespread use of soft X-ray microscopy. The diffraction zone plates when used as condensers and objectives in the microscope require very high brilliance beams for imaging as they have very low efficiencies. This in turn leads to radiation-induced damage of the sample. Apart from this, the zone plates are difficult to make reliably with the currently available technologies. As for multilayer-based reflective optical elements, ideally they have distinct advantages over zone plates both in terms of resolution and efficiency. However they are extremely difficult to make because the individual layers in the multilayer are extremely thin, few monolayers thick, to make with zero defects. The development of optical elements is a major issue which will ultimately decide the widespread usage of the soft X-ray microscopy technique in biological sciences.

The technological developments in soft X-ray microscopy have far outpaced the studies on understanding the radiation-induced damage mechanism. It is now known experimentally that the cell mortality is extremely high when exposed to low brilliance soft X-rays. The

exact reasons for this cell inactivity, however, are not clearly known. Hence carefully planned, detailed studies are required to understand the basic mechanism of cell damage. This becomes even more relevant because the nature and extent of damage is dependent on the composition and structure of the absorbing material.

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