CONTACT X-RAY MICROSCOPY

A New Technique for Imaging Cellular Fine Structure

LORENA BEESE, RALPH FEDER, AND DAVID SAYRE
IBM Thomas J. Watson Research Center, Yorktown Heights, New York 10598

ABSTRACT Contact x-ray microscopy potentially allows living, wet cells to be visualized at a resolution of up to 100 Å. Furthermore, differential absorption by specific elements permits the study of the distribution of those elements in biological specimens. In contact x-ray microscopy, soft x-rays (10 Å to 100 Å) pass through a biological sample and expose an underlying x-ray sensitive polymer (resist), producing an image that reflects the photon absorbance within the specimen. The high penetrating power of soft x-rays enables images to be obtained from specimens up to several microns thick. In this paper, the technique is described, some of the areas currently under study are considered, and biological examples of the use of contact x-ray microscopy are given.

INTRODUCTION

Imaging biological specimens using soft x-rays (10 Å–100 Å) provides a bridge between light and electron microscopy. In contact x-ray microscopy, soft x-rays pass through a biological sample and expose an underlying x-ray sensitive polymer (resist), producing an image that reflects the photon absorbance within the specimen. The technique is capable of resolution of the order of 100 Å, beyond that of light microscopy, and without many of the constraints of electron microscopy. Staining of biological specimens is unnecessary, as adequate contrast is produced by the differential absorption of photons. Because this absorbance is a function of the energy of the incident x-rays, tunable synchrotron radiation can be used to vary the contrast in the x-ray images, accentuating regions of different elemental composition. Another feature of x-ray microscopy is that the high penetrating power of x-rays enables images to be obtained from specimens up to several microns thick. Perhaps the most exciting possibilities arise from the fact that soft x-rays lend themselves to the study of wet specimens at atmospheric pressures, in essence high-resolution in vivo imaging.

Contact x-ray microscopy is not a new idea. Indeed, recording an X-ray image of a specimen in contact with photographic film (radiography) came into being almost simultaneously with the discovery of X-rays themselves and this technique was refined by Goby (1913) who recorded images on photographic emulsions and used the optical microscope to enlarge the radiographic image, thus creating the technique of microradiography. The advantages of using soft x-rays to obtain contrast in biological specimens were first recognized by Lamarque (1936). Other major contributors to the development of the technique included Engstrom (1946), and Cosslet and Nixon (1952; 1960). In 1956 Ladd et al. showed that virtually grainless x-ray–sensitive materials could be used instead of silver-halide photographic film, and the image viewed in the electron microscope.

The technique assumed its present form in the mid-1970s when the close connection between contact microscopy and x-ray lithography was recognized (Feder et al., 1976; Spiller et al., 1976; Spiller and Feder, 1977). The use of high-resolution resists and accompanying silicon technologies originally developed for lithography opened the way for high-resolution contact x-ray microscopy.

Most recently, technical developments in x-ray sources such as a flash unit (Bailet et al., 1982; Feder et al., 1985) capable of producing a 100 ns pulse of soft x-rays, and tunable synchrotron radiation, have allowed new types of experiment of particular interest to biologists. It is now possible to image living wet cells and potentially to investigate dynamic processes occurring in less than a millisecond.

In many respects contact x-ray microscopy is still in its infancy. While highly detailed images of cell structures have been obtained, to interpret these images fully it is necessary to work backwards from these images, accurately reconstructing the x-ray absorption and thus the specimen giving rise to it. In this paper we will describe the technique, outline some of the areas currently under study, and discuss the types of biological problems where it may be of use.

TECHNIQUES OF CONTACT X-RAY MICROSCOPY

Contact x-ray microscopy is conceptually simple. The specimen is placed in contact with a thin film of an x-ray–sensitive polymer (resist) and is exposed to a parallel beam of x-rays, generally 20 Å–50 Å in wavelength, roughly normal to the resist surface. The photons that are not absorbed by the specimen enter the resist, breaking

bonds of the resist polymer. Damage to the resist is proportional to the number of photons received; areas of greatest damage correspond to regions where the specimen absorbed least. The exposed resist is etched, or developed, by dissolving the damaged material in an appropriate solvent such that the dissolution rate is indirectly a function of the number of incident photons. Variations in the developed resist profile reflect a specimen's absorption; high areas on the resist correspond to regions in which the specimen absorbed the incident radiation relatively strongly. The X-ray image is then read out by examining the resist topography using either scanning or transmission electron microscopy.

X-ray Detection

The x-ray detector used most extensively in contact microscopy is the high-resolution resist, polymethyl methacrylate (PMMA). PMMA is a high-molecular weight, long-chain organic polymer which can be made to form an amorphous thin film. Because the long polymer chains are randomly tangled, the resist dissolves very slowly in solvents such as methyl isobutyl ketone (MIBK). During an x-ray exposure, photons are absorbed by the resist. These absorbed photons produce secondary electrons which, depending upon their energy, break chemical bonds within the polymer, creating in chain scissions. In the regions where photons were absorbed, the polymer molecules, in effect, have a lower molecular weight, which causes an increase in the solubility of the resist in that region.

The resolution of the resist is determined by the mean free path of the secondary electrons. For PMMA the mean free path was found experimentally to be ~50 Å when the incident photons were 43.6 Å and the dosage to the resist was $\sim 10^4$ J/g (Spiller and Feder, 1977). At shorter wavelengths (higher energy photons) the resolution deteriorates, becoming ~200 Å when exposed to 20 Å photons.

A detailed description of PMMA resist preparation has been given by Feder in the paper by Kirz and Sayre (1980). Briefly, PMMA is dissolved in chlorobenzene and a few drops of this solution are spun onto the desired substrate. The concentration of the solution and the speed of spinning determine the thickness of the resulting film. The spinning process produces films of constant thickness to within 20 Å. The film is dried and cured for 1 h at 160°C. Typically, detectors consist of a 5,000-Å film of PMMA using either a silicon wafer or a 1,000-Å window of silicon nitride (Feder and Sayre, 1980) for a substrate. The detectors are roughly the same size and shape as an electron microscope grid.

After exposure to x-rays, the resist is developed by immersion in 1:1 or 1:2 mixtures of MIBK in isopropyl alcohol. The dissolution rate of the resist material in this solvent is a function of the x-ray exposure (Ranby and Rabak, 1975). Development is stopped with pure isopropyl alcohol, and the surface dried with nitrogen. At present, development is monitored using an optical microscope with interference contrast optics.

Readout

The simplest way to examine the developed resist topography without compromising spatial resolution is to use a scanning electron microscope (SEM). To prevent charging, a thin (100 Å) coating of 40% palladium-60% gold is evaporated onto the resist surface. From SEM images the developed resist profile is easily visualized, although it is difficult to obtain quantitative height information this way.

The transmission electron microscope (TEM) can also be used to visualize the resist profile of certain samples. Variations in optical density of the electron micrograph image correspond to variations in thickness (or height) of the resist. (The density fluctuations within the resist and its substrate are assumed to be negligible.) To permit TEM examination the resist must be on a very thin substrate, such as the silicon nitride windows described in the previous section. Unfortunately, these windows are quite fragile and, at present, cannot be used for all experiments, particularly those using plasma X-ray sources.

Using TEM, images of the developed resist can be compared directly with TEM images of the original sample since both reflect internal density fluctuations as well as thickness. TEM images also lend themselves to digitization, one of the prerequisites for any quantitative technique.

Preliminary studies are in progress to determine whether the scanning tunnelling microscope (Binnig et al., 1982) could be used to obtain high-resolution readout of the resist heights. This could provide somewhat more accurate measurement of the resist topography than is currently possible using TEM.

Experimental Design: Soft X-ray Sources and Types of Experiment

The soft x-ray source is selected to match the type of experiment; at present, no one source is perfect for all applications of contact x-ray microscopy. Sources differ widely in their intensity, some requiring as little as several nanoseconds to expose the resist while others necessitate exposures of over 10 h. Rapid exposures are essential for imaging living cells in an aqueous environment. Tunability and control over spectral bandwidth are other important considerations and are essential for elemental mapping studies. In essence, the x-ray source defines the type of experiment and also dictates the requirements for specimen preparation.

Stationary Target Source. Historically, the stationary target source has been the workhorse for contact microscopy. Results have appeared in numerous studies (Feder et al., 1976, 1981, 1984; Manuelidis et al., 1980;

Panessa et al., 1980). X-rays are produced by accelerating electrons into a solid target. The emitted radiation is the strong characteristic radiation of the target superimposed on a broad band of white radiation whose magnitude and energy depends upon the accelerating voltage of the incident electrons. A electron gun source with a carbon target (C K_α 44.8 Å radiation) is used routinely for contact x-ray microscopy (Feder et al., 1981). The advantages of this system are that it is small, reliable, relatively inexpensive, and thus can be part of any laboratory. Unfortunately, the system generates a relatively low x-ray flux which results in long exposure times (typically >10 h).

Specimen preparation is similar to that required for electron microscopy, although staining is unnecessary. Specimens are exposed under vacuum and consequently the effect of dehydration on the structural integrity of a sample is a concern, as it is in electron microscopy. The best images have been obtained from relatively thin (<1μm) specimens. Samples are prepared on standard electron microscope grids held in contact with the photoresist in a spring-loaded holder.

Samples can be exposed more than once without significant loss of structural detail at a resolution of 200 Å. This enables stereo pairs to be made. After exposure, the sample can be examined by electron microscopy and compared with its x-ray image. The need for long exposures makes living, wet cell imaging prohibitive on this unit. Because the wavelength is limited by the choice of target materials, experiments that require the source to be tunable are also not possible on the stationary target source.

Synchrotron Radiation. Synchrotron radiation has many advantages over stationary target radiation. The high intensity of synchrotron radiation enables exposures to be made in minutes. Because of their tunability, synchrotrons are by far the most suitable sources for elemental mapping. The experiments described in this paper were done on beam line U15 of the ultraviolet ring at the Brookhaven National Synchrotron Light Source. The U15 beam line is built around a torodial grating monochromator with a 1-mrad acceptance tunable in the 15–50 Å wavelength range (Kenney et al., 1983; Kirz and Rarback, 1985).

At present, specimen preparation and constraints are the same as for the stationary target source because the samples are subjected to a vacuum. In general, experiments that could be done using the stationary source can be done more rapidly using synchrotron radiation. In addition, by changing the x-ray wavelength the contrast in the x-ray images can be varied. This is because it is possible to take images above and below an absorption edge of an element. Absorption coefficients of several biologically important elements as a function of wavelenth are shown in Fig. 1.

The major limitation is that synchrotrons are not always available and must be shared with other users. Currently,

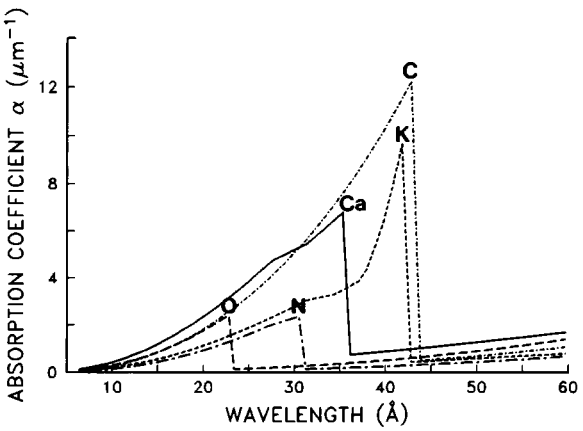

FIGURE 1 Linear absorption coefficients per micron of material plotted as a function of wavelength. This plot was derived from data compiled by Henke et al. (1981) assuming densities of 2.26 gm/ml for C, 0.86 for K, 0.81 for N, 1.14 for O, and 1.55 for Ca.

synchrotron radiation is not sufficiently intense to permit wet cell imaging, although the proposed installation of undulators (Bienenstock and Winick, 1983) is expected to increase the intensity to a point where wet cell imaging may be feasible.

Pulsed Plasma X-ray Sources. Pulsed plasma sources produce a single high intensity burst of x-rays that can be used to image living wet cells. The design described in this paper is available commercially (Maxwell Laboratories, San Diego, CA) and is referred to as a flash, *Z*-pinch or puff x-ray source. The flash source uses a high energy electric discharge to produce a plasma (high density ionized gasses) that radiates a 100-ns burst of x-rays (Pearlman and Riordan, 1981). Plasmas can also be produced using a laser in place of the high energy discharge. Such lasers have recently been used to produce x-rays for contact microscopy (Rosser et al., 1985); these x-rays (~27–100 Å; Feder et al., 1985) are more strongly absorbed by protein than by water. The absorption curves of water and protein are shown in Fig. 2. The difference in absorption enables high-contrast images to be obtained from living wet cells.

An environmental chamber (Fig. 3) maintains the sample in a hydrated state and at atmospheric pressure. Specimens and the surrounding layer of buffer should be <4 μm thick, although imaging thicker specimens may be possible with a reduction in resolution. The rapid exposure allows study of processes occuring in milliseconds.

At present the flash sources have several limitations. The most serious limitation is that the sample is destroyed by the exposure. Experiments that would require more that one exposure of a given sample are not possible. A related problem is that the readout of the resist is limited to SEM because the fragile silicon nitride windows break during the exposure. The flash sources are also not monochromatic or highly tunable. Current research is directed toward overcoming these problems.

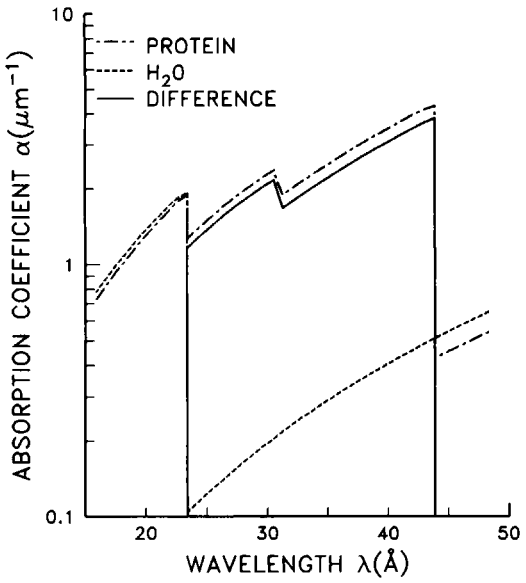

FIGURE 2 Linear absorption coefficients per micron of material plotted as a function of wavelength. The solid line represents the difference between the absorption of protein (dotted line) and water (dashed line). Note the x-ray "window" between 24 Å and 44 Å. The plot was derived as for Fig. 1, assuming a density for protein of 1.35 g/ml, and estimating the mass fractions in protein to be 0.53 C, 0.16 N, and 0.23 O.

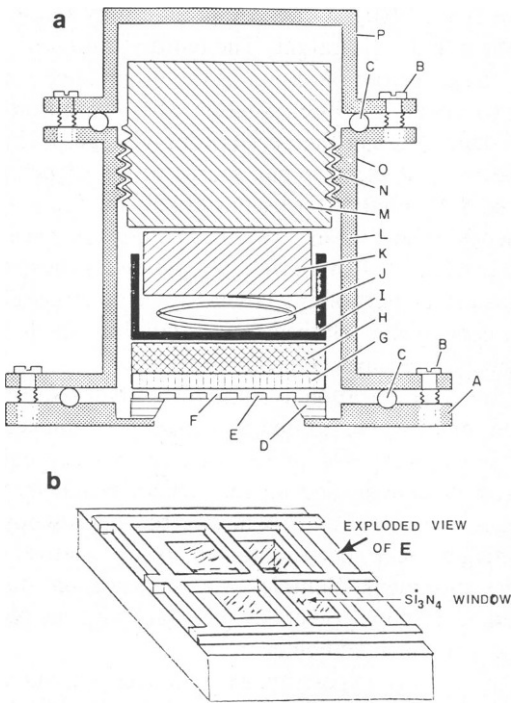

FIGURE 3 (*a*) Diagram of the environmental chamber, showing (*A*) substrate holder, (*D*) substrate for silicon nitride window, (*E*) resist honeycomb, (*F*) specimen area, (*G*) resist, (*H–K*) spring mechanism to hold resist in close contact with the specimen, (*B*, *C*, and *L*) body of the chamber with O-ring seals, (*M* and *N*) threaded spring compressor, and (*P*) top of the chamber. (*b*) Honeycomb specimen subchamber (enlarged view of *E*).

INTERPRETATION OF X-RAY IMAGES

Qualitatively, one can interpret the images arising from contact x-ray microscopy as two-dimensional projections of the three-dimensional absorbance function of a specimen. The topography of the developed photo-resist contains information about a specimen's absorbance; high areas on the resist correspond to regions in which the specimen absorbed the incident radiation relatively strongly. Ideally, one would like to know the precise relationship between resist topography and specimen absorption. Quantifying contact x-ray microscopy becomes important not only for putting x-ray images on a standardized absorption scale, but also for qualitatively interpreting these images. At present, conclusions about specimen absorption directly from the resist topography should be drawn with caution because the ratios of heights, and even the projected shapes of certain surface features, are functions of the development of the resist. Ideally, one would like to "correct" the resist topography, transforming it into a true absorption map, and then interpret the x-ray images. Nevertheless, with experience and with knowledge of the development history of a particular resist, it is possible qualitatively to interpret the topography, and thus the x-ray image. Selected examples of images are given in the next section.

The resolution or sharpness of the x-ray image is somewhat diminished by penumbral blurring arising from the finite source size. The geometric resolving distance in the plane of the specimen, P, is given by the equation $P = s(d/D)$ where s is the specimen-resist distance, d is the source size, and D is the source-specimen distance. The equation is derived by requiring that the penumbrae of two features in the specimen be separated in the resist (Kirz and Sayre, 1980). If the resist is in direct contact with the specimen, then the maximum value of s is the specimen thickness. For a typical experiment with a 1-μm thick sample, a source size of 1 mm, and a specimen-source distance of 20 cm, the penumbral blurring, P is only 50 Å. Although the requirement of complete penumbra separation is not necessarily an accurate reflection of detectable image separation, it is clear that any reasonable requirement will give a value for P of this order of magnitude. Penumbral blurring is minimal in most cases, but this calculation demonstrates the importance of maintaining close contact between the specimen and the resist surface for achieving sharp images.

The assumption that the x-ray image arises solely from absorption by the specimen is only an approximation. More precisely, contact x-ray microscopy records the sum of the incident and diffraction fields generated by the object. Because the detector is placed in the extreme near field, this is a good approximation to the absorbance. The approximation deteriorates with an increase in specimen thickness, where the near field approximations become invalid. The diffraction resolving distance, R is $R \simeq \sqrt{(\lambda s)}$ where λ is the wavelength and s is the sample thickness. This relationship is derived by requiring that the image of a

feature fall outside the first fringe of the Fresnel diffraction pattern of a neighboring feature (Kirz and Sayre, 1980). With 30-Å wavelength x-rays and a 1-μm thick sample, the resolution is limited to ~550 Å. Features in a thin layer near the bottom of the specimen would appear distinct, while features lying higher in the specimen would appear slightly blurred. Diffraction effects are particularly apparent at edges of an x-ray image (Fig. 4).

Making use of the diffraction information may, however, be the way of the future for contact x-ray microscopy. Several interesting schemes have been proposed which at the very least could enable the x-ray images to be sharpened and could potentially produce three-dimensional images. One approach involves considering contact x-ray microscopy as a special form of Gabor holography. If the incident radiation is sufficiently coherent, the diffracted and transmitted x-rays forming the contact image might be recombined, although the reconstruction would be rather complicated because the reference signal (in this case the transmitted beam) would reflect the absorption of the specimen. Another possibility that has been proposed (Sayre and Feder, 1981) is recording the near-field diffraction three-dimensionally using a stack of thin x-ray resist detectors. These patterns might then be phased and reconstructed, perhaps using methods analogous to those of crystallography.

CONTACT X-RAY IMAGES

Contact x-ray images have been made of a wide variety of biological samples (Feder et al., 1981; 1984; Cheng et al., 1984). Perhaps of greater significance, several papers have reported new structural observations that were aided by the special characteristics of contact x-ray imaging. For example, Manuelidis et al. (1980) found evidence that in interphase human tissue culture nuclei, discrete chromosomes are in specific three-dimensional positions (Fig. 5). Although earlier studies by electron microscopy had suggested this ordered structure, the x-ray images revealed the alignment and ordering with less ambiguity. This study illustrates how x-ray microscopy can contribute by being able to image internal structure in thick electron dense specimens. The technique was also important in a study suggesting that proteoglycan may exist as an interdigitating network formed through mucopolysaccharide side chains (Panessa et al., 1980). Use of contact x-ray microscopy enabled the proteoglycan sample to be imaged unstained, unfixed, and hydrated.

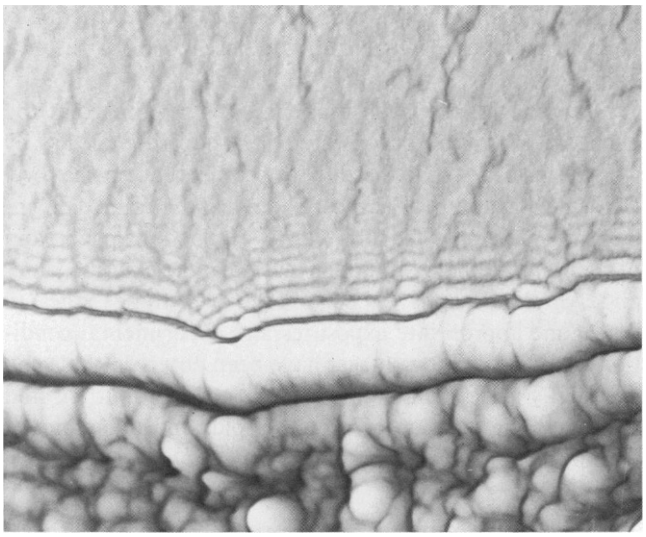

FIGURE 4 Diffraction fringes in an x-ray image of a 1 μm section of rabbit psoas muscle (sample courtesy of R. Hexter) made using 36 Å monochromatic x-rays. Over 10 fringes are visible at the edge of the muscle (8,570 ×).

Imaging Living Cells

The most important contribution of contact x-ray microscopy may lie in its application to living, wet specimens. The

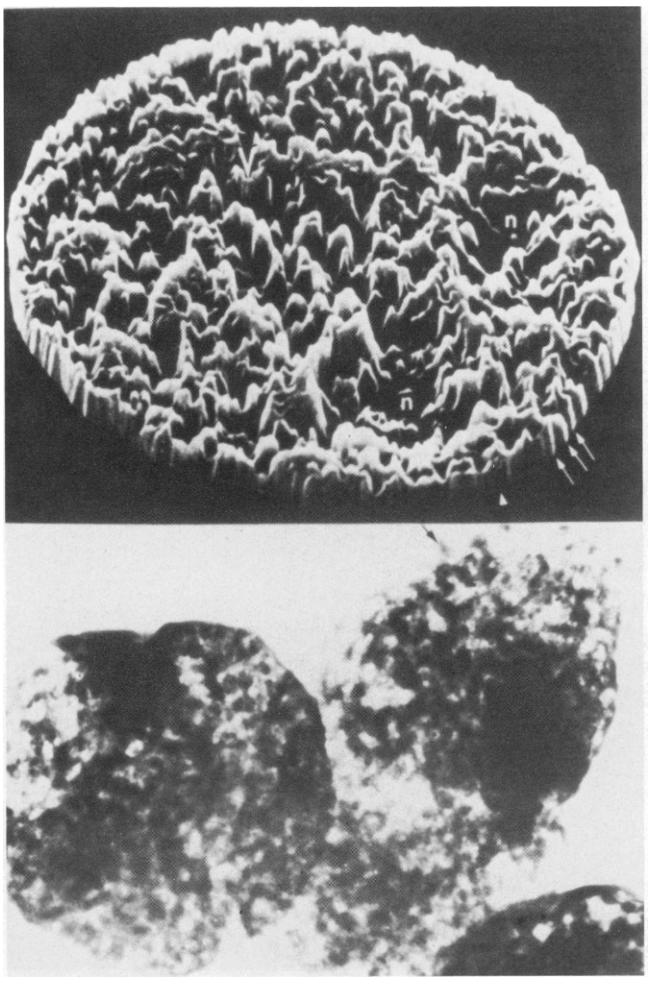

FIGURE 5 (*Top*) SEM image of x-ray resist of a nucleus from human neuroectodermal tumor tissue culture line TC526 (membrane removed with urea). Arrays of vertical features are thought to represent ordered arrays of chromosomes. (*Bottom*) High voltage electron micrograph of similar nuclei (15,000 ×). Further details are given by Manuelidis et al. (1980).

first images of this type have already been obtained using a flash x-ray source (Feder et al., 1985). The contact image (Fig. 6) captures a human blood platelet hydrated and presumably still alive at the time of the exposure. The image is quite detailed; features <100 Å diam are visible. Contrast in the image arises largely from the differential absorption by protein and water; water absorbs x-rays with wavelengths between 23 and 43 Å only weakly while protein is a strong absorber in this spectral region (Fig. 2).

X-ray images of both living and air-dried (Feder et al., 1981) activated platelets suggest that pseudopods contain a central core of photon-absorbing material that continues into the cell interior. Detailed filamentous structures had previously been observed in platelet pseudopods using electron microscopy, but their continuation into the cytoplasm of the cell had been uncertain.

With a flash source contact microscopy currently offers stop motion imaging suitable for qualitative investigation of dynamic processes occurring over sub-millisecond times. The technique may be particularly useful when applied to the study of pseudopod formation, axonal transport, or cell motility, where direct visualization of cell morphology at a resolution higher than that possible by optical microscopy could contribute to problems that are currently difficult to address by other techniques.

Elemental Mapping

Preliminary experiments have been made to assess the feasibility of using contact x-ray microscopy to determine the distribution of elements (differential absorption microanalysis) within biological samples. As discovered by Engstrom (1946), differences between images of samples exposed to wavelengths slightly above and slightly below the absorption edge of an element could, in principle, be used to indicate the distribution and concentration of the element.

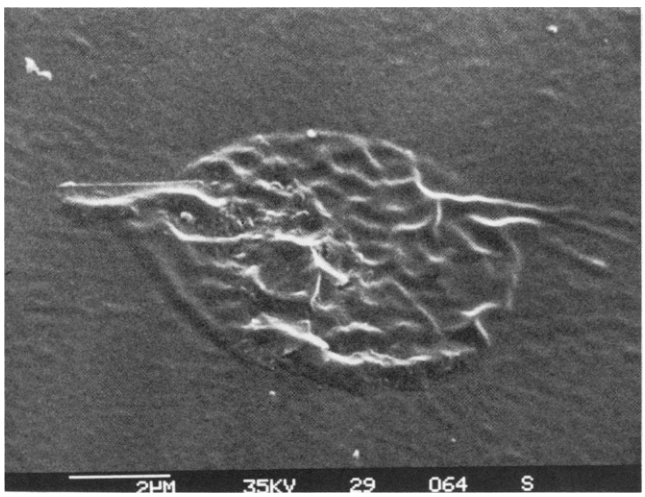

FIGURE 6 X-ray image of a wet, presumably still-living human platelet at an early stage of pseudopod formation. This is an SEM image of exposed resist, a copolymer of PMMA–methacrylic acid. The developed resist is coated with a 100 Å film of gold palladium. The exposure was made over a period of 100 ns using the flash x-ray source produced by Maxwell Laboratories.

The inorganic calcium phosphate phase of bone is being studied in the region of the calcium L absorption edge, making use of the absorption spectrum determined by Kenney et al. (1985). These experiments are being done in collaboration with Dr. F. Cinotti at Brookhaven National Laboratory and are aimed at the elucidation of the pattern of calcium deposition along collagen in bone. Bone was also selected as a test specimen because the concentration of calcium is quite high and so differences in absorption should be dectable, and its structure has been widely studied. Bone samples were prepared from human skull tissue which had been fixed in glutaraldehyde, embedded in epoxy resin, and cut into 0.2 μm sections (Kenney et al., 1985). To facilitate comparison, samples received the same incident x-ray dosage and the resists were developed under identical conditions.

A pair of x-ray images of bone exposed above the calcium absorption edge (at 35.2), where calcium absorbs strongly, and below this edge (at 35.5 Å), where calcium absorption is significantly less, is shown in Fig. 7. Regions of high x-ray absorption appear dark in these TEM images of the x-ray resists. Since these wavelengths are separated by only 0.3 Å, or ~1%, the expected change in the absorption coefficients of elements other than calcium is ~3%. By comparison, the change in calcium absorption is nearly an order of magnitude (Kenney et al., 1985).

The images taken above and below the absorption edges have a somewhat different appearance. Below the edge (Fig. 7 *b*), the absorption pattern is relatively weak and indistinct, while above the edge (Fig. 7 *a*) the pattern is strong and highlighted by features ~100 Å thick. Ideally, one would digitize and subtract these images, producing a map of calcium absorption. In practice, the subtraction is not yet possible because of difficulties in scaling the two images.

Differential absorption microanalysis requires that the elements be quite concentrated (of the order of 1% by weight) in order that the differences be above noise level (Kirz, 1980*a* and *b*). For most biological material, elemental analysis would be limited to carbon, nitrogen, and oxygen. Preliminary studies of muscle, platelets, ribosomes and lymphocytes have been made over these edges to determine appropriate exposures and experimental conditions. Differences in contrast have been observed that may eventually be correlated with elemental distibution, and thus with the location of protein, lipid, and other phases within the biological sample.

DISCUSSION

The next step in the development of contact x-ray microscopy requires quantitation of the x-ray images. There are several important problems that must be solved before

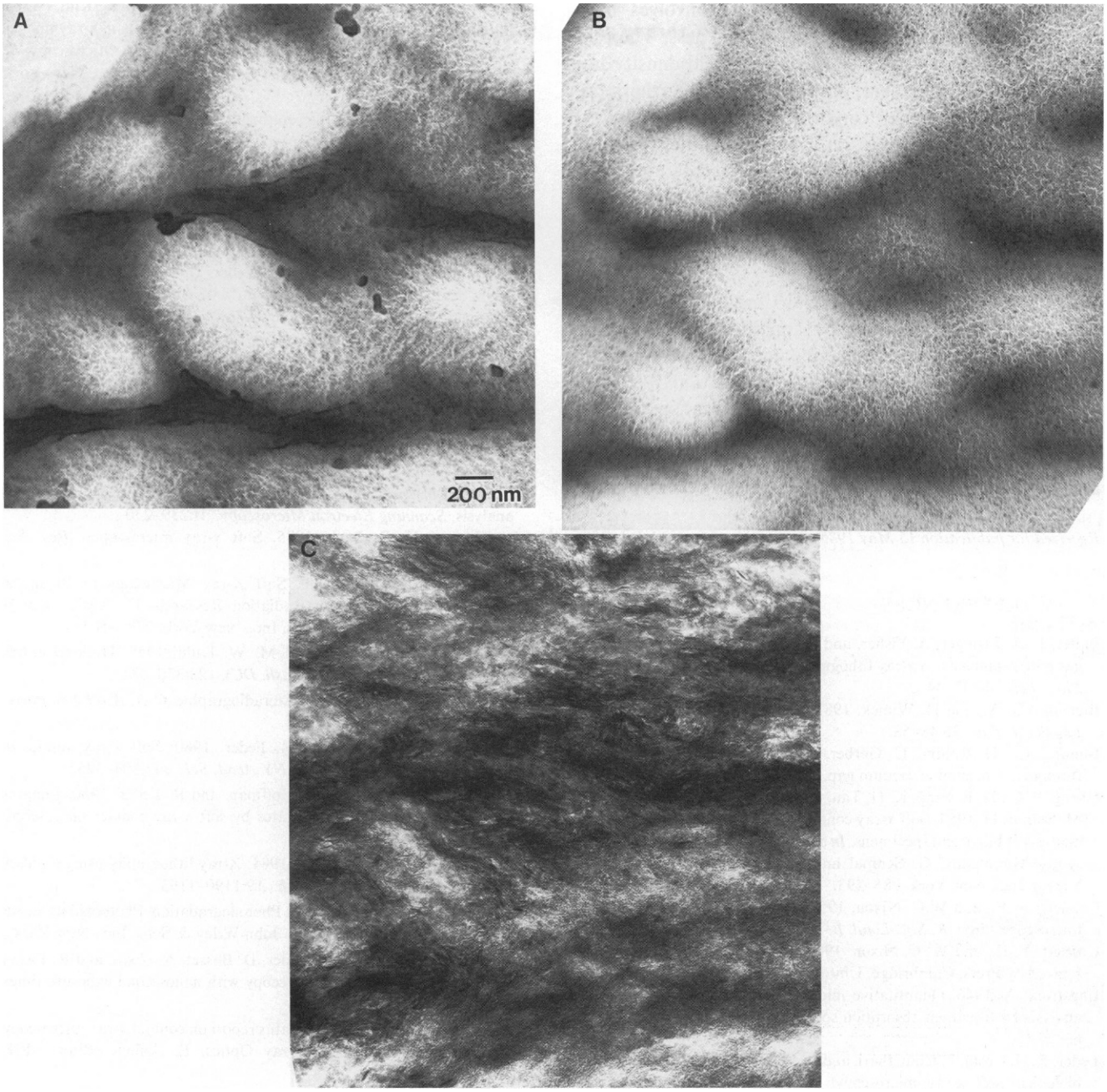

FIGURE 7 (*A*) X-ray image of a 0.2 μm section of human skull tissue on a marker electron microscope grid exposed to 35.2 Å monochromatic x-rays from a synchrotron source. Calcium absorbs strongly at this wavelength. (*B*) X-ray image on a second resist of the same bone sample exposed to 35.5 Å x-rays, which are only weakly absorbed by calcium. (*C*) Direct TEM image of the region of the bone sample shown in *A* and *B* after x-ray exposures (55,000 ×).

contact microscopy will achieve its potential. The first involves standardization of the resist development so that a function relating the dissolution rate of the resist material to x-ray dosage (for a given wavelength) can be determined. Knowing the variations in resist thickness and the length of development, this function could be used to derive the incident x-ray dose on a point of the resist. The problem is complicated by the fact that resist development is isotropic; sideways development causes rounding and ultimately the loss of features on the resist. This can be minimized experimentally by restricting the depth of the development to roughly five times the minimum resolvable distance desired (Sayre and Feder, 1981; Kirz and Sayre, 1980). To a limited extent it may be possible to correct for sideways development by constructing an algorithm that identifies height gradients in the resist and operates similarly to the edge enhancement algorithms used to sharpen images.

The second problem to be overcome involves high-resolution determination of the resist thickness. The relationship between resist thickness and optical density in a TEM image is, at present, only qualitative. Furthermore, resists developed under conditions that are optimal for retaining high-resolution information, namely shallow etching, present considerable difficulties in obtaining TEM images. Thickness readout may be achieved using a scanning tunneling microscope or, if the substrate could be made much thinner, using scanning transmission electron microscopy (STEM).

Transforming x-ray images into maps of the photons transmitted by a specimen by applying the appropriate dissolution and readout functions may become as routine as, in crystallography, converting diffraction intensities recorded on photographic film into reciprocal space structure amplitudes. When this is possible then experiments that require the combination of images such as elemental mapping or three-dimensional imaging can become reality.

Received for publication 15 May 1985 and in revised form 11 July 1985.

REFERENCES

Bailet, J., A. Ettinger, A. Fisher, and R. Feder. 1982. Evaluation of the gas puff Z-pinch as an x-ray lithography and microscopy source. *Appl. Phys. Lett.* 40:33–44.

Bienenstock, A., and H. Winick. 1983. Synchrotron radiation research. *Physics Today.* 36:48–58.

Binning, G., H. Rohrer, C. Gerber, and E. Weibel. 1982. Tunneling through a controllable vacuum gap. *Appl. Phys. Lett.* 40:178–179.

Cheng, P. C., H. B. Peng, K. H. Tan, J. W. McGowan, R. Feder, and D. M. Shinozaki. 1984. Soft x-ray contact microscopy and microchemical analysis of biological specimens. *In* Springer Series in Optical Sciences: X-ray Microscopy. G. Schmal and D. Rudolph, editors. Springer-Verlag, Inc., New York. 285–293.

Cosslett, V. E., and W.C. Nixon. 1952. An experimental x-ray shadow microscope. *Proc. R. Soc. Lond. B.* 140:422.

Cosslett, V. E., and W. C. Nixon. 1960. X-ray Microscopy. Cambridge University Press, Cambridge, United Kingdom.

Engstrom, A. 1946. Quantitative micro- and histochemical elementary analysis by Roentgen absorption spectrography. *Acta Radiol. Suppl.* 63.

Feder, R., J. Costa, P. Chaudhari, and D. Sayre. 1981. Improved detail in biological soft x-ray microscopy: study of blood platelets. *Science (Wash., DC).* 212:1398–1400.

Feder, R., D. Sayre, E. Spiller, J. Topalian, and J. Kirz. 1976. Specimen replication for electron microscopy using x-rays and x-ray resist. *J. Appl. Phys.* 47:1192–1193.

Feder, R., and D. Sayre. 1980. Recent developments in x-ray contact microscopy. *Ann. NY Acad. Sci.* 342:213–234.

Feder, R., V. Mayne-Banton, D. Sayre, J. Costa, B. K. Kim, M. G. Baldini, and P. C. Cheng. 1984. Recent developments in x-ray microscopy. *In* Springer Series in Optical Sciences: X-ray Microscopy. G. Schmal and D. Rudolph, editors. Springer-Verlag, Inc., New York. 279–284.

Goby, P. 1913. X-rays in microradiography. *C. R. Acad. Sci. Paris.* 156:686–688.

Henke, B. L., P. Lee, T. J. Tanaka, R. L. Shimabukuro, and B. K. Fujikuwa. 1981. The atomic scattering factor, $f_1 + if_2$, for 94 elements for the 100 to 2000 eV photon energy region. *In* AIP Conference Proceedings: Low Energy X-ray Diagnostics. D. T. Attwood and B. L. Henke, editors. Institute of Physics, New York. 340–388.

Kenney, J., J. Kirz, H. Rarback, R. Feder, D. Sayre, and M. Howells. 1983. Scanning soft x-ray microscopy with a Fresnel zoneplate at the National Synchrotron Light Source. *In* Science with Soft X-rays. F. J. Himpsel and R. W. Klaffky, editors. SPIE. 447:158–163.

Kenney, J., C. Jacobsen, J. Kirz, H. Rarback, F. Cinotti, W. Tomlinson, R. Rosser, and G. Schidlovsky. 1985. Absorption microanalysis with a scanning soft x-ray microscope mapping the distribution of calcium in bone. *J. Microscopy.* In Press.

Kirz, J. 1980*a*. Mapping the distribution of particular atomic species. *Ann. NY Acad. Sci.* 342:273–287.

Kirz, J. 1980*b*. Specimen damage considerations in biological microprobe analysis. *Scanning Electron Microscopy.* II:239–250.

Kirz, J., and H. Rarback. 1985. Soft x-ray microscopes. *Rev. Sci. Instrum.* 56:1–13.

Kirz, J., and D. Sayre. 1980. Soft X-ray Microscopy of Biological Specimens. *In* Synchroton Radiation Research. H. Winick and S. Doniach, editors. Plenum Press, Inc., New York. 277–318.

Ladd, W. A., W. M. Hess, and M. W. Ladd. 1956. High-resolution microradiography. *Science (Wash. DC).* 123:370–371.

Lamarque, P. 1936. Histologie-historadiographie. *C. R. Acad. Sci. Paris.* 202:684–685.

Manuelidis, L., J. Sedat, and R. Feder. 1980. Soft x-ray studies of interphase chromosomes. *Ann. NY Acad. Sci.* 342:304–325.

Panessa, B. J., J. B. Waren, P. Hoffman, and R. Feder. 1980. Imaging unstained proteoglycan aggregates by soft x-ray contact microscopy. *Ultramicroscopy.* 5:267–274.

Pearlman, J., and J. C. Riordan. 1981. X-ray lithography using a pulsed plasma source. *J. Vac. Sci. Tech.* 19:1190–1193.

Ranby, B., and J. F. Rabak. 1975. Photodegradation, Photooxidation, and Photostabilization of Polymers. John Wiley & Sons, Inc., New York.

Rosser, R. J., K. Baldwin, R. Feder, D. Busset, A. Coles, and R. Eason. 1985. Soft x-ray contact microscopy with nanosecond exposure times. *J. Microscopy.* In press.

Sayre, D., and R. Feder. 1981. Status report on contact x-ray microscopy. *In* High Resolution Soft X-ray Optics. E. Spiller, editor. SPIE. 316:56–61.

Spiller, E., R. Feder, J. Topalian, D. Eastman, W. Gudat, and D. Sayre. 1976. X-ray microscopy of biological objects with carbon K and synchrotron radiation. *Science (Wash. DC).* 191:1172–1174.

Spiller, E., and R. Feder. 1977. X-ray lithography. *In* Topics in Applied Physics. H.-J. Queisser, editor. Springer-Verlag, Inc., New York. 22:35–92.

DISCUSSION

Session Chairman: Benno Schoenborn

Scribes: Eric Betzig and Katherine Crumley

SCHOENBORN: Can you use a scanning procedure to increase resolution?

BEESE: The resolution is actually decreased in a scanning mode. The resolution in the scanning x-ray microscope has been limited to 2,000 A

by the zone plate used to focus the x-rays. However, within the last couple of weeks a zone plate has been made that should reduce the spot size down to 800 Å. At present our technique permits us to see features that are at 100 Å resolution.

STUBBS: Could you clarify the limitations of scanning x-ray microscopy?

BEESE: There is another way to use soft x-rays to produce images. The one I have described is a very simple contact technique. A more complicated method uses synchrotron radiation and focusses the beam to a very small spot. This focussing is done using a zone plate and then the spot is moved with a high precision stage across the sample, measuring the absorption. The resolution depends on the quality of the zone plate. This plate is constructed using the same techniques that we use to produce our images, namely x-ray lithography.

STEWART: I admire the fact that you can produce such beautiful images, or indeed, any images at all. You have shown images of objects such as fibroblasts. Have you been able to use test resolution objects to get a more quantitative idea of the resolution obtainable in different sorts of specimens?

BEESE: We are working on that in two ways. First, we are using synthetic structures such as lines ~ 80 Å across via the STEM and these structures have been replicated. Second, we have begun a collaboration with Mike Lamvick at Duke to investigate tropomyosin paracrystals, which will allow us to study resolution and also to investigate radiation damage.

MAKOWSKI: What is the effect of near-field diffraction when one examines thick samples?

BEESE: Diffraction is a problem with all of these images. In addition to absorption by the sample there is diffraction that is particularly noticeable from the edges. This limits the resolution to the square root of the wavelength times the sample thickness, $\sqrt{\lambda s}$. For a one-micron-thick object in a typical experiment, the diffraction-limited resolution is ~550 Å.

MAKOWSKI: As the system is used right now, how thick an object can you actually look at and get images from the surface?

BEESE: Specimens over 5μm thick have been imaged. The specimen shown in Fig. 4 is a 1 μm sample of muscle; strong fresnel diffraction rings appear.

SALEMME: What is the smallest linewidth that has been replicated by x-ray lithography?

BEESE: An image has been made with a 175-Å line (Flanders, D. C. 1980. *App. Phys. Letts.* 36:93–96.)

POLLARD: I am interested in the potential of this technique for solving biological problems, but I am concerned because your Fig. 4 shows a piece of muscle, but it has no features that I recognize.

BEESE: This is a bad figure for showing features normally apparent in our x-ray images of muscle. It was included mostly to show how confusing diffraction can be.

POLLARD: I think we need help with determining what can be learned with this technique. Perhaps your Fig. 6 is difficult to interpret because it was a scanning electron microscope image of the three-dimensional resist. The picture of a fibroblast shown in your summary was easier to interpret and resembled a transmission micrograph of a dried fibroblast. Could you expand on what you consider to be the potential of this for studying biological specimens?

BEESE: X-ray imaging should complement electron microscopy as we look at thick, unstained, wet, possibly living specimins. In the cases of the bone or the fibroblast, the images are similar to TEM images. By comparing the electron microscopic images and the x-ray images we are building up a library of information to relate the two techniques. In the bone pictures the lamellae and the canaliculi are clearly evident. In the difference image the calcium lies roughly where you expect it to, along the collagen fibers. This is an example of where you can interpret things fairly easily.

LEWIS: You used three types of x-ray sources: a synchrotron, a stationary target, and a pulsed plasma source. In the stationary target and the synchrotron sources the bone sample was in vacuum and does not represent a living system. You studied a living platelet but a single pulse of the plasma source essentially destroyed the specimen. What are the different dosage levels that cause no damage, moderate damage, or sufficient damage to destroy the sample completely? This is important for making three-dimensional reconstructions from multiple images and studying dynamic processes.

BEESE: At present, the flash source literally vaporizes the sample. This is not due to the dose that is required to make the image, but rather to a shock wave that follows the soft x-rays. We are using filters to try to eliminate the unwanted radiation so that the sample is only receiving the dose necessary to do the imaging. When considering radiation damage from electrons, a rule of thumb is that 10^9 rads is a destructive dose; at 10^6 rads the sample is no longer living, but there may be no morphological changes. The corresponding measurements have not been made for soft x-rays, and these experiments should be done. Our exposures are at a dose of roughly 10^7 rads.

KAPLAN: I assume that the projecting material shown in Fig. 5*A* represents the nuclear pores. In our experience with monolayer cultures, the nuclear pores can be seen extending beyond the cell surface.

BEESE: The SEM image gives you a false sense of three-dimensionality. The "high" portions of the resist correspond to regions that absorb very strongly and the lower regions correspond to areas where the specimen did not absorb strongly. The result is a projection of all of the density in the sample. In this example we believe that the density fluctuations are due mainly to the chromosomes.

KAPLAN: Thank you for clarifying this figure, for now it seems to me that the x-ray image has the absorption pattern to be expected from a nucleus with dispersed chromatin. We are doing scanning electron microscopy with cells grown on microspheres. When the focus is changed slightly one gets a stereoscopic view. Have you tried that, or has the sample been destroyed after a single image?

BEESE: Stereo images have been made of samples by tilting the sample with respect to the reference beam. Viewed in the electron microscope, the resist is tilted in the same way. In this manner it is possible to obtain an impression of the three-dimensional structure in a thick sample.

KAPLAN: Is it possible to use microspheres as test patterns to aid in interpretation of your images?

BEESE: Yes. To scale our images we put polystyrene latex spheres on the surface of the sample. These spheres help in the alignment needed when subtracting images and they also provide us with a gray scale for the images.

RUBEN: It seems to me that you have two problems. The first is interpreting the image in relation to biological objects. The second is getting high enough resolution in the resist to understand it in detail. One possible experiment would be to expose blood platelets or a similar sample to the x-rays and then prepare by demembraning the cell by deep etching the cell itself, making a replica of it, floating it off, developing the resist, doing the same with it, and then comparing the two images.

BEESE: There are many good experiments to do and that is certainly one of them.

LANGMORE: Wouldn't the TEM rather than the SEM provide you with more quantitative readout of the photoresist images?

BEESE: Yes. The early work had to be done using SEM because the silicon substrate on which the resist was spun was opaque. The resists we use now are usually spun on a very thin silicon nitride membrane which is relatively transparent to electrons. The readout of the resist is an area with room for improvement. We are considering scanning tunneling microscopy, and also looking at replicas of the surface. There have been several discussions with George Ruben concerning replicas and stereo pairs of these replicas so that we can obtain quantitative height information.

BLUM: Can high-Z materials such as uranium or tungsten be used for shadowing the surface and getting a better distribution of secondary x-rays through the sample?

BEESE: Perhaps that's something we should consider.

MAKOWSKI: How linear is the etching of the resist with x-ray exposure?

BEESE: The dissolution rate as a function of dosage is not a linear function. Using standard curves, it should be possible to translate the height information and find the absorption of the sample.

MAKOWSKI: What is the resolution in this technique?

BEESE: The resolution is limited by current readout methods and by diffraction effects, but the potential resolution is $\sim$ 100 Å. Diffraction is probably the most serious limitation for thick samples.

HENDRICKSON: My concern is not so much with resolution as it is with contrast. Is it realistic to think of looking at carbon, nitrogen, oxygen, sulfur, and phosphorus or is it only useful with something like calcium where you can really distinguish the distribution?

BEESE: The contrast depends upon the difference in the absorption coefficients and the concentration. The difference in concentration should be at least several percent. An interesting example involves the carbon edge where there is a 1.5-fold difference in absorption between protein and lipid.

BETZIG: I'm concerned with the resolution. On the one hand there is a decrease in the resolution with the square root of the wavelength due to diffraction. On the other hand if you go to eshorter wavelengths there is a decrease in resolution ecause of secondary electrons which break bonds in the resist. It would seem that if you plot these trends you would find a wavelength giving the optimal resolution. Do you know what that would be?

BEESE: That depends on how thick your specimen is. For thick samples where diffraction is significant the wavelength should be $\sim$ 10 Å. However, if you have a thin sample, the optimal wavelength is $\sim$35 Å.