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Quantitative imaging and microanalysis with a scanning soft x-ray microscope

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Abstract. A scanning soft x-ray microscope has been developed that uses synchrotron radiation focused by a Fresnel zone plate to form a submicron beamspot on the specimen. Transmitted x-rays are detected and used to form a quantitative map of specimen absorptivity. Applications of the instrument to the imaging of whole wet cells and to the mapping of calcium in sections of bone are presented, with a resolution of 300 nm and an elemental sensitivity of 2 $\mu g\ cm^{-2}$.

1. Introduction

In scanning soft x-ray microscopy a quantitative map of specimen absorptivity is obtained. Sharp changes in the spectrum at absorption edges provide contrast and are the basis for elemental analysis by comparison of images taken at differing wavelengths. We have developed an instrument that uses monochromatised synchrotron radiation as its source. A collimated portion of this source is focused with a Fresnel zone plate to a submicron spot, across which the specimen is scanned under computer control. To illustrate the microscope's capabilities, we have imaged whole cultured neurons in a fixed but wet and unstained state and measured the distribution of calcium in thin sections of human skull tissue at a resolution of 300 nm.

The past decade has seen significant developments in x-ray microscopy. In its ability to image whole cells in their natural state it complements electron microscopy, though at more modest resolution. Notable results have been obtained using the technique of contact microscopy (Feder *et al* 1981, Mayne-Banton *et al* 1984, Panessa-Warren 1984, Cheng *et al* 1984, Bigler *et al* 1983), especially with a flash source (Feder *et al* 1985). Considerable success has been achieved in imaging with zone plate optics and synchrotron radiation (Rudolph *et al* 1984). Both of these approaches form the image on an x-ray sensitive recording medium, which must be viewed by electron or optical microscopy.

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In scanning microscopy the image is formed point by point in a serial fashion as the focused probe is moved across the specimen. This method is therefore well suited to simple electronic detection of the x-rays, and consequently the direct digital acquisition and processing of the image. By counting the transmitted photons, information is handled in an efficient manner with minimal noise. This results in minimising the radiation dose to the specimen (Sayre *et al* 1977a, b). In addition, a direct measurement of the absorptivity is obtained at each point in the specimen. The absorption coefficient can then be calculated if the thickness is known. Alternatively, the thickness can be determined if either the composition is known, or images are taken at more than one wavelength.

2. The microscope

The first scanning x-ray microscope was built by Horowitz and Howell (1972) (see also Horowitz 1978) almost 15 years ago. Our instrument is based on similar principles, but it is the first one to incorporate focusing optics to define a submicron probe, a tunable monochromatic soft x-ray source for absorptivity measurements and direct, computer-based image acquisition and processing. The microscope operates at beamline U15 of the ultraviolet ring at the National Synchrotron Light Source (NSLS). This beamline uses a toroidal grating monochromator with a resolving power of about 300 to select any desired wavelength in the 1.5–4.5 nm range (Williams and Howells 1983). A schematic diagram of the apparatus is shown in figure 1. A Fresnel zone plate, fabricated at IBM by electron beam lithography (Kern *et al* 1983, 1984), focuses the radiation to a spot of about 200 nm FWHM. Just before the zone plate the radiation passes through an ultrathin (120 nm of Si_3N_4) window (Feder and Sayre 1980) into an atmospheric environment. The specimen is placed at the focus, mounted on a stage that uses piezoelectric translators to scan it under computer control. X-rays transmitted by the specimen are detected by a flow proportional counter which counts single photons. The image is stored in computer memory, where it can be manipulated point by point, and displayed in real time on a colour television screen. A detailed description of the microscope can be found in Rarback *et al* (1984).

In the x-ray wavelength range above the oxygen K absorption edge (2.3 nm), water becomes relatively transparent, so organic matter stands out in the image of wet biological specimens. To study such material we used the wavelength of 3.2 nm, at

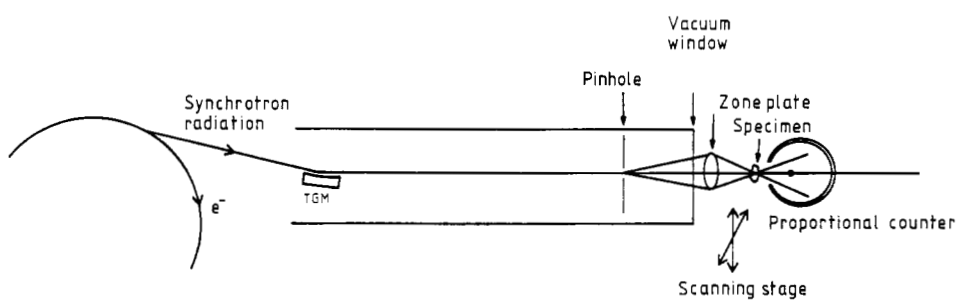


Figure 1. Schematic diagram of the apparatus (not to scale). The toroidal grating monochromator (TGM) selects the wavelength incident on the specimen, while the pinhole provides the required spatial coherence. The vacuum window separates the evacuated region of the beamline from the specimen area at atmospheric pressure. The zone plate focuses the radiation onto the specimen, which is scanned under computer control. Transmitted photons are detected by the flow proportional counter.

which nitrogen is also relatively transparent, so flux loss in the window and the residual air is minimised. (To further minimise the loss, we flushed the region around the specimen with helium.)

3. Results and discussion

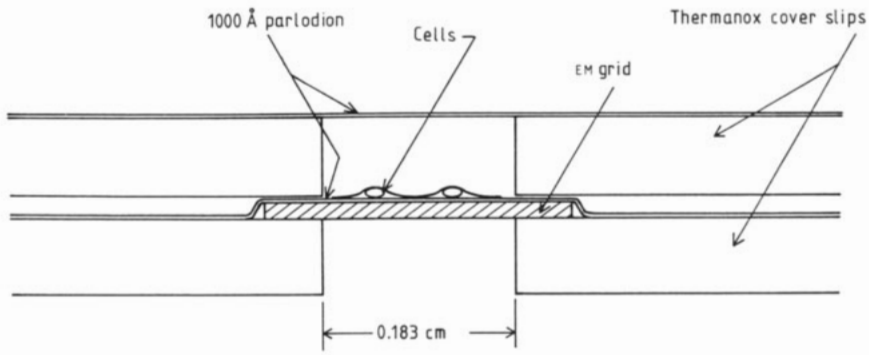
At a wavelength of 3.2 nm, the absorption coefficient in water is about 0.22 μm^{-1}, while in protein or lipid it is about 1.9 μm^{-1}. If, for example, a cultured neuron is assumed to have a thickness of 5 μm, consisting of 85% water and 15% protein, the net x-ray transmission is expected to be about 9%. Protein accounts for more of the attenuation than the water. The soft x-ray microscope thus appears well suited to imaging whole, wet, unstained, cultured cells. To investigate this capability, we have imaged cultured superior cervical ganglion neurons, grown on a layer of nitrocellulose about 100 nm thick, supported on an electron microscope grid. The cells were dissociated from newborn rats, and fixed in 2.5% gluteraldehyde after about 36 h in culture. The cells were kept wet in Earle's salts after fixation.

Figure 2(*a*) illustrates the way in which cells were grown on a grid supported by a plastic cover slip. To expose the cells in the microscope, the culture was drained and a second cover slip, the upper one in figure 2(*a*), was overlayed and sealed around the periphery with silicone vacuum grease. This cover slip, with its thin overlying window of nitrocellulose, formed a 'wet cell' for the microscopy. The culture was held vertical during exposure to the x-ray beam, and the thin layer of water remaining on the cells did not have an appreciable effect.

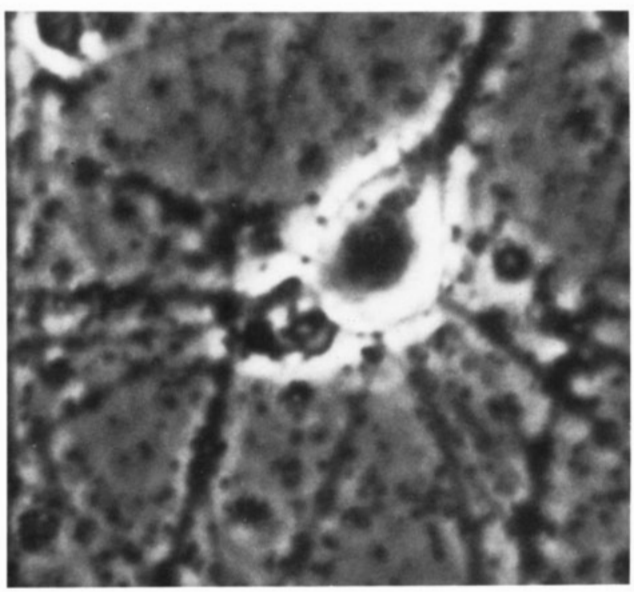
Figure 2(*b*) is a phase contrast micrograph of a cultured neuron and a number of neurites, made after several x-ray exposures. Figure 3(*a*) is an x-ray micrograph of the region shown in the phase contrast picture, made with 0.6 μm scanning steps. The false colour intervals are approximately 8% increments in transmitted x-ray intensity. Figure 3(*b*) is an enlarged view of the neuron, taken with 0.3 μm steps, which matches the resolution attainable with the optical micrograph.

The radiation dose given to the specimen during the exposures shown in figure 3 was about 10^4 rad. In order to achieve comparable contrast accuracy at five times the resolution, the dose would be about 10^6 rad (Sayre *et al* 1977a, b). This radiation dose would not be expected to produce gross disruption of tissue, and in fact may be compatible with survival of live cells. The future development of the microscope thus offers the promise of providing a powerful new tool for studies of cell biology. For neurons, the possibility that synaptic structures in culture can be resolved is particularly interesting.

To demonstrate absorption microanalysis, we examined sections of bone at 3.55 and 3.58 nm, where the absorption coefficient of calcium undergoes radical change (Kenney *et al* 1985). Chips of human skull tissue were fixed, dehydrated and embedded in epoxy. Sections, 0.2 μm thick, were cut with a diamond knife. They were floated on water, then heat-stretched and dried onto electron microscope grids. Figure 4(A) shows the image of part of such a section at 3.55 nm, where calcium is heavily absorbing, while figure 4(B) is the corresponding image at the other wavelength, where absorption in calcium is much smaller. Based on the data represented by these images, and the calcium absorption spectrum measured with the same apparatus, we calculate the amount of calcium present at each point in the image (Kenney 1985). The result is presented in figure 4(A–B). Here false colours represent equal linear increments in calcium content from 0 to 12 $\mu g\,cm^{-2}$. At the 5 standard deviation level in a single



(a)

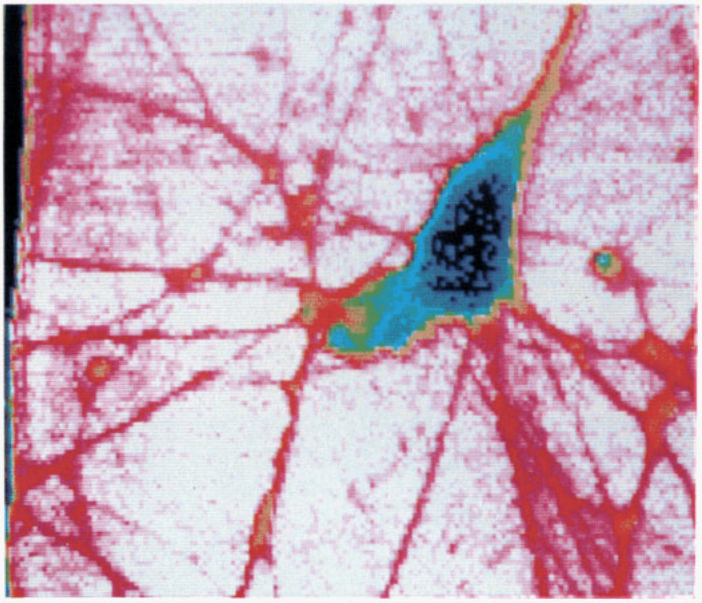


(b)

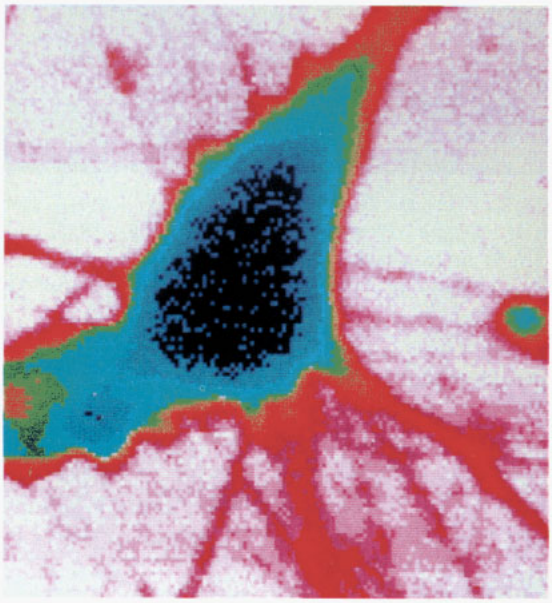
Figure 2. Scanning soft x-ray micrographs of a cultured neuron. (*a*) The wet cell, formed by placing a second cover slip and a thin window over the cover slip which supports the electron microscope grid on which the culture was grown. (*b*) Phase contrast micrograph of part of a culture of rat superior cervical ganglion neurons, showing one neuron and a number of neurites.

pixel our instrument can currently detect about 2 $\mu g\ cm^{-2}$, or roughly 5% calcium by mass. Heavily calcified regions in bone are known to be composed of various forms of calcium phosphate, mainly hydroxy apatite, while the organic matrix is made up of collagen, with cellular components and Haversian canals making up the balance. The detailed nature of the mineral component, and its variations under pathological conditions are under active investigation (Vaughan 1981). While the regions of calcification are clearly seen in the figure, individual crystals of hydroxy apatite are too small ($<0.1\ \mu m$) to be resolved.

The present instrument is limited in resolution and data rate by the zone plate and the radiation source, respectively. As a result, the measurement errors are in the range 10–30%, the resolution is 0.2 μm at best, and it takes close to an hour to form an



(*a*)



(*b*)

Figure 3. (*a*) Scanning x-ray micrograph of the cell and neurites shown in figure 2(*b*), taken with 0.6 μm steps, imaged in 'false colour' from digitised x-ray transmission data. The colour steps correspond to approximately 8% changes in transmission. (*b*) Enlarged view of the cell shown in (*a*), taken with 0.3 μm steps.

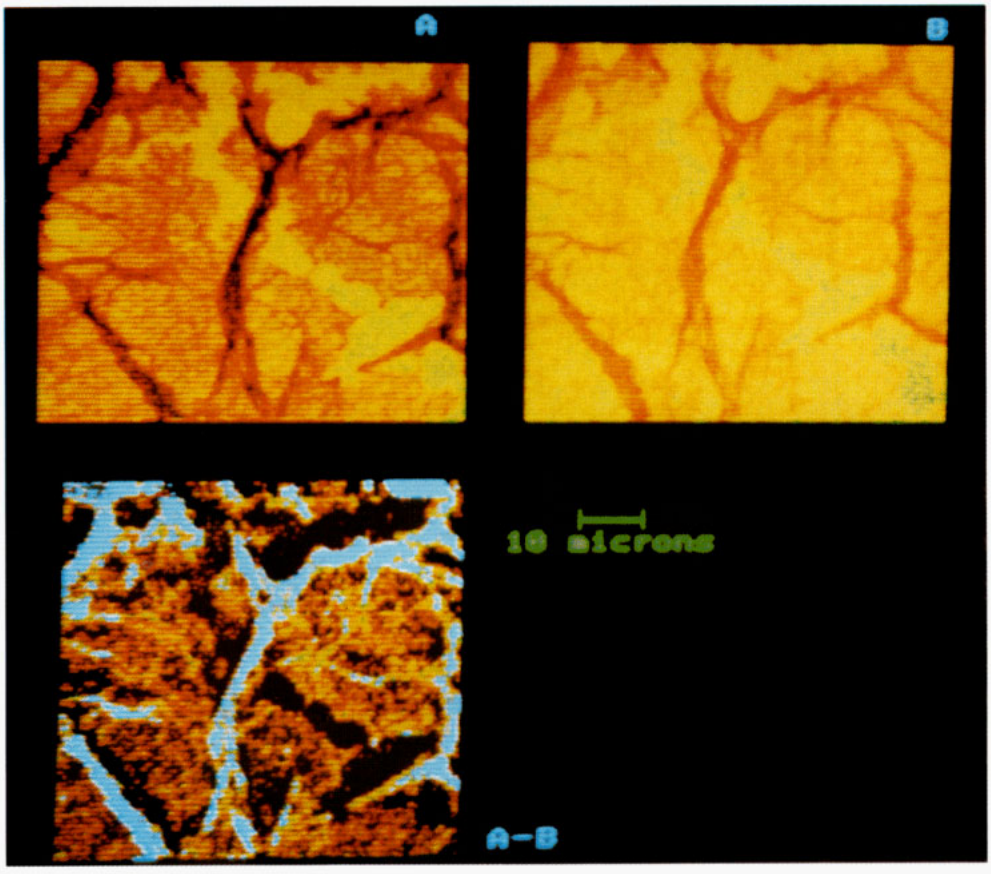


Figure 4. X-ray micrograph of 0.2 μm thick section of human skull tissue. $60 \times 60\ \mu m^2$ area scanned in 0.5 μm steps. (A) X-ray wavelength 3.55 nm (Ca highly absorbing). (B) X-ray wavelength 3.58 nm (Ca relatively transparent). (A–B) Ca map calculated from the data of (A) and (B). Black regions contain less than 1.5 $\mu g\ cm^{-2}$ of Ca, blue regions contain more than 10 $\mu g\ cm^{-2}$, while other shades indicate intermediate amounts.

image of the type presented here. In collaboration with the NSLS, we are engaged in the construction of a new undulator-based beamline that will remove the intensity limitations. We anticipate that higher resolution zone plates will also become available in the near future. The next generation microscope is expected to generate images in less than one minute with a resolution of less than 0.1 μm and with elemental concentration measurements of one per cent accuracy or better.

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Résumé

Imagerie quantitative et microanalyse à l'aide d'un microscope à balayage utilisant des rayons X de faible énergie.

Les auteurs ont développé un microscope à balayage utilisant des rayons X de faible énergie; ceux-ci proviennent du rayonnement d'un synchrotron focalisé par un dispositif de Fresnel, de manière à obtenir un impact de taille inférieure au micromètre au niveau de l'échantillon. Les rayons X transmis sont détectés et utilisés pour déterminer la répartition spatiale de l'absorption dans l'échantillon. Les auteurs présentent l'application de cet appareil à l'imagerie de cellules entières en phase humide et à la détermination de la répartition du calcium dans des couples d'os, la résolution étant de 300 nm et la sensibilité de détection d'un élément atteignant 2 $\mu g\ cm^{-2}$.

Zusammenfassung

Quantitative Darstellung und Mikroanalyse mit einem Abtast-Röntgenmikroskop.

Entwickelt wurde ein Abtast-Röntgenmikroskop, bei dem mit Hilfe einer Fresnel-Zonenplatte Synchrotronstrahlung auf einen submikroskopischen Bereich des zu untersuchenden Objektes fokussiert wird. Die durch das Objekt gehenden Röntgenstrahlen werden nachgewiesen und für eine quantitative Darstellung des Absorptionsverhaltens des Objektes verwendet. Die Verwendung des Gerätes bei der Darstellung von Zellen und von Kalzium in Bereichen des Knochens wird vorgestellt. Dabei beträgt die Auflösung 300 nm und die Empfindlichkeit 2 $\mu g\ cm^{-2}$.

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