

# HIGH RESOLUTION MICROCHEMICAL ANALYSIS USING SOFT X-RAY LITHOGRAPHIC TECHNIQUES

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## ABSTRACT

High resolution x-ray lithographic studies of cells from chick embryo hearts dried by the CO<sub>2</sub> critical point method have been made with soft x-ray radiation of different wavelengths. A marked difference in the relief replica in polymethyl methacrylate (PMMA) resulting from the differential absorption by the dried cells of carbon K<sub>α</sub> radiation at 4.48 nm and broad band synchrotron radiation (SR) with  $\lambda > 1.5$  nm demonstrates the potential usefulness of the technique in making high resolution ( $\approx 10$  nm) chemical identification of the constituents which make up the various parts of the cell.

**KEY WORDS** soft x-ray microscopy ·  
synchrotron radiation · microchemical analysis ·  
x-ray lithography · scanning electron microscopy

X-ray lithography, developed principally for the manufacture of miniature electronic devices, has now been used for high resolution studies of thin, stained, and unstained biological samples (7, 2) with a demonstrated resolution near 10 nm. The technique is a natural extension of contact micrography which was in vogue nearly two decades ago (1) but which suffered for want of higher film resolution and a lack of source intensity. By contrast, today the method has great promise because of the development of grainless films (x-ray resists such as polymethyl methacrylate [PMMA]) and in intense, potentially tunable x-ray source such as a monochromator coupled with a synchrotron radiation (SR) source (6). As a preliminary test of x-ray lithography as a microchemical analytical tool for biological samples, we have replicated two similar groups of cells with two different soft x-ray sources: one a carbon K<sub>α</sub>

(C-K<sub>α</sub>) line source available at the IBM Laboratories, and the other an intense, broad band synchrotron radiation source from the high-energy German electron synchrotron, (Deutsches Elektron-Synchrotron [DESY]) in Hamburg. We have found a substantial difference in the details of the resulting replicas. As a consequence, we suggest that the use of tunable soft x-rays can make it possible to obtain microchemical as well as microscopic information for biological samples. Recently, Polack et al. (4) demonstrated that chemical microanalysis of 30- $\mu$ m-thick geological samples could be carried out over large areas with a few micrometers' resolution. For these studies, tunable x-rays from an SR source at Orsay, France were used primarily at wavelengths near the iron and titanium K-absorption edges.

## MATERIALS AND METHODS

As the recording medium for our study, a 1–2- $\mu$ m-thick PMMA film was spun uniformly onto a 0.3-mm-thick, 25.4-mm-Diam, highly polished silicon disk. After the coating, the disk was washed, baked at 200°C in air,

cooled, and covered with specimens as follows.

7-d chick embryo heart fragments were grown for several days in Falcon plastic flasks (BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.) in McCoy's 5a medium supplemented with 20% fetal calf serum containing antibiotics. Cells were harvested by scraping them into fresh medium with a rubber spatula. Six prepared disks were overlaid with 5 ml of cell suspension and incubated at 37°C in a 5% CO<sub>2</sub>, 15% O<sub>2</sub>, and 80% N<sub>2</sub> mixture. After 24–48 h, the cells were placed in buffered (pH 7.3) fixative containing 2.5% glutaraldehyde and 2% formaldehyde for 1 h before they were dehydrated in ethanol and then critical-point dried with CO<sub>2</sub>. Before irradiation, the cells were examined thoroughly by light microscopy (incident light) and found to be well distributed on the PMMA substrate.

Three cell-coated resist disks were then irradiated for 25 h with 4.48 nm C-K<sub>α</sub> radiation which is just below the carbon K<sub>α</sub> absorption edge in the cells and the PMMA film. An exposure density of approx.  $5 \times 10^3$  J/cm<sup>2</sup> was incident upon the cell layer. The other three cell-coated resists were irradiated for ~8 min with broad band SR reflected at a glancing angle of 4° from a gold surface to eliminate hard radiation with wavelengths shorter than ~1.5 nm. In this case the soft x-ray exposure density density at the surface of the cells was estimated to be slightly in excess of 10<sup>3</sup> J/cm<sup>2</sup>.

Once irradiated, the disks were washed in ethyl alcohol and the cells were gently wiped from the x-ray-damaged PMMA surface with a Q-tip, in some cases leaving behind cell fragments which became part of our SEM control in the subsequent examination of the replica. Little or no meaningful structure was observed with the SEM in these cell fragments.

The amount of soft x-ray radiation reaching and damaging the resist depended upon the density  $\rho$  of the proteins, lipids, nucleic acid, and so on, making up the different parts of the cell. It also depended upon the thickness of the cell  $\tau$  and upon a product of the x-ray mass absorption coefficient  $\mu_i$  (associated with the  $i$ th component of the cell) and the fraction of the mass  $m_i$  making up that component. This is summarized by the usual formulation describing the intensity of transmitted radiation,

$$I = I_0 \exp[-|\rho\tau(\sum_i m_i \mu_i)|].$$

Image development was brought about by dissolving the radiation-damaged plastic from the surface of the disks in a solution of methyl isobutyl ketone (MIBK) and isopropanol (IPA) for 30 s at room temperature. A weak (MIBK:IPA, 1:3) solution was used for the C-K<sub>α</sub> irradiated samples and a stronger (1:1) solution was used for the disks which had received the lower density of radiation from the SR source.

The topological relief in PMMA which remained after development was then coated by evaporation in a vacuum of a 60:40 mixture of Au:Pd, and photographed with a JEOL-35 SEM having a nominal resolution of 10 nm, which matches very well the expected maximum resolution of the technique (2). In practice, however, we did not obtain this limit, partly because our resolution noticeably deteriorated as the replica was bombarded with 25–35 keV electrons in the SEM (note lines in Fig. 1).

## RESULTS AND DISCUSSION

Table I summarizes some of the results obtained from the analysis of micrographs used for Fig. 1. These two are typical and were chosen for comparison because their overall length (~80 μm), the length of their nucleus (~10 μm), and nucleolus (~2 μm), and the widths of the cell replicas were approximately the same. Note that there are gross differences in the thicknesses of the two replicas in the regions of the nucleus (*N*) and nucleolus (*NO*) and microtubules (*MT*) which run in the major cell processes and finally out into long microvilli, or microspikes (*MS*) of the type described by Taylor and Robbins (8) and by Vesley and Boyde (9). These differences no doubt reflect the differing chemical composition of the various parts of the cell and the associated difference in absorption for the narrow and broad band radiation.

Consider, for example, the nuclear regions of the two replicas. From computations such as those of Sayre et al. (5) and Henke (3), one has the mass fractions, component densities, and x-ray mass absorption coefficients which enable one to calculate the relative absorption for the different

TABLE I  
SEM Comparison of Some Replica Dimensions after Irradiation and Development

	IBM C-K <sub>α</sub> λ = 4.48 nm	DESY SR λ ≥ 1.5 nm	C-K <sub>α</sub> /SR
Maximum height of replica edge	~0.5 ± 0.1	0.5 ± 0.1	~1
Maximum height of nucleus replica above cell replica	~0.3	≤0.03	≥10
Maximum height of nucleolus replica above nucleus replica	~0.45	<0.03	>15
Maximum height of ridges replica	0.1 – 0.15	<0.03	>3
Maximum height of horizontal microspikes	≤0.07	≤0.28	~0.25

All dimensions in μm and are adjusted for 60° tilt angle of SEM stage. They are consistent with similar measurements taken for other cell replicas.

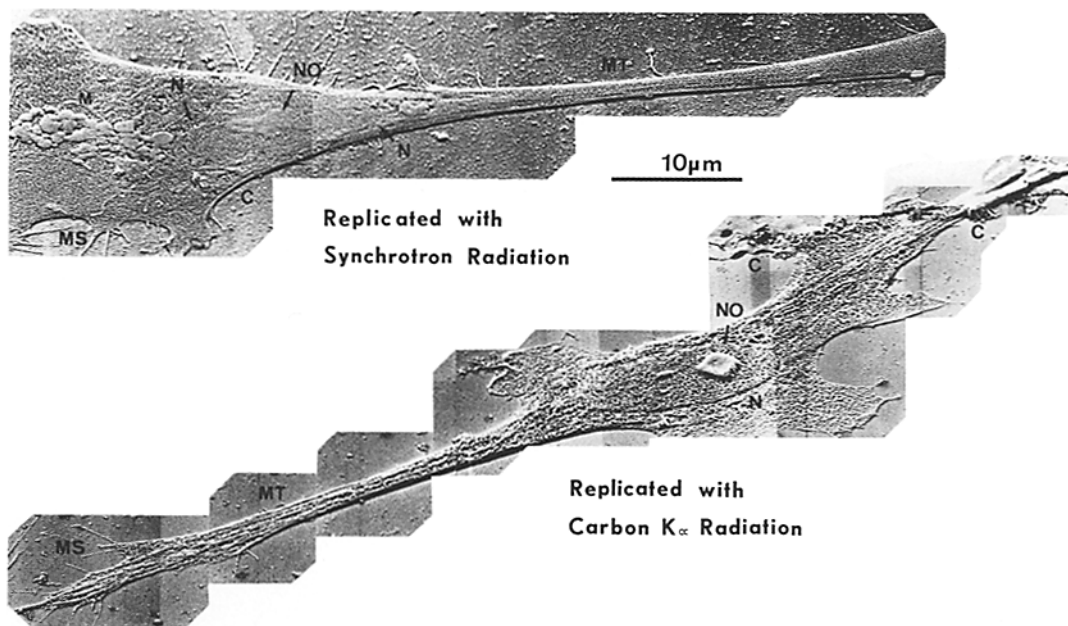


FIGURE 1 Two micrographs of chick embryo heart cells replicated with SR reflected at  $4^\circ$  from an Au mirror (upper left) and C- $K_\alpha$  radiation (lower right). The prominence of the nucleus ( $N$ ) and nucleolus ( $NO$ ) in the lower replica reflects the buildup of oxygen and phosphorus in the nucleic acid. In both replicas, the microspikes ( $MS$ ) and microtubules ( $MT$ ) are clearly defined. The grapelike structures to the left of the nucleus in the cell replicated with SR are mitochondria ( $M$ ) containing a quantity of magnesium which readily absorb x-rays at energies somewhat above the carbon  $K_\alpha$  absorption edge.

cellular constituents such as nucleic acid, lipids, and proteins. For C- $K_\alpha$  radiation incident on the cell samples, these calculations predict approx.  $e^5$  and  $e^{2.5}$  more absorption by nucleic acid (a dominant constituent of the nucleus and nucleolus) than by lipids and proteins, respectively. This difference is primarily due to the strong absorption above the L edges of the oxygen and phosphorus atoms abundantly present in the nuclear constituents. This prediction is consistent with the observed pronounced relief contours of the nucleus and nucleolus in the cell replicas produced with C- $K_\alpha$  radiation. It is the likely explanation of why the DNA component is so pronounced in the replica of the *Drosophila* salivary gland chromosome, reported by Feder et al. (2).

Estimates, furthermore, show that while the same difference in absorption between nucleic acids and proteins and lipids persists for lower energy x-rays, as the x-ray energy is raised above the carbon  $K_\alpha$  absorption edge the difference in absorption disappears. It is, therefore, not surprising that as the broad band SR source is used, the nucleus and nucleolus are much less pronounced. The grape-like structure to the left of the nucleus

in the SR replica is likely to be a group of mitochondria ( $M$ ) which are known to contain Mg and consequently absorb enough higher energy radiation to show greater thickness in the replica, even though the concentration of magnesium may be small.

An unsuccessful attempt was made to replicate live chick heart embryo cells by exposing eight different PMMA disks covered with live wet cells hermetically sealed with a  $2.2\text{-}\mu\text{m}$  film of mylar (E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.) to the SR source for periods ranging between 5 and 15 min. Although the method of cell preparation and encapsulation worked well, no acceptable replicas resulted. Time-lapse microscope studies, carried out by Professor R. C. Buck of The University of Western Ontario, subsequently showed that under our experimental conditions the cells remained very active, moving too actively during the exposure period to be replicated. These studies further showed that at temperatures near  $4^\circ\text{C}$ , the cells were effectively at rest and most likely could have been replicated. It should be further noted that, for the radiation levels used for these studies, few cells would be

likely to survive unharmed. It is recommended that, as part of any ongoing replication study of these or other living cells, detailed low-energy x-ray survival curves be taken as a function of the x-ray energy. No information now exists in the literature on this subject.

It is our pleasure to thank Dr. R. C. Buck and Dr. I. G. Walker, who not only assisted with the preparation of cell mounts and control studies but who, with Dr. M. Malachowski, contributed to our understanding of the problem. Special recognition must be given Facultés Universitaires, Notre Dame de la Paix, Namur, Belgique for use of their SEM facilities and where one of us (J. Wm. McGowan) was on sabbatical leave for the 1976-77 school year.

The project has been supported in part by the Canadian National Research Council and Medical Research Council, the U. S. Army, and the Veeco Fund.

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Received for publication 15 March 1978, and in revised form 2 October 1978.

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