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X-Ray Secondary-Emission Microscopy (XSEM) of Neurons.

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Abstract. – We present the first X-ray secondary (photoelectron) emission microscopy (XSEM) pictures and video microimages of an uncoated and unstained neuron specimen. This novel kind of synchrotron radiation microscopy is suitable for local chemical analysis with a lateral resolution in the micron range. We explored the details of the neuron system, demonstrated chemical contrast by scanning the photon energy, studied in real time the photoelectron emitting properties of the specimen's components, and made preliminary tests of the radiation damage. These results significantly enhance the potential role of photoemission techniques in the life sciences and specifically in neurobiology.

Photoemission techniques such as electron spectroscopy for chemical analysis (ESCA) have been very widely used in materials science for decades [1], but not in the life sciences. This is primarily due to the lack of lateral resolution, since resolutions in the micron or submicron range are absolutely required for the local analysis of biological specimens. Recent instrumentation breakthroughs [2] have made it possible to change this scenario, and to obtain synchrotron radiation photoelectron microimages of biological specimens [3-7]. We present here the first results of this kind produced by an X-ray secondary (photoelectron) emission microscopy (XSEM) instrument [8-13].

The results were obtained on a fixed and dehydrated neuron specimen, prepared without any kind of metal-coating and without staining, and therefore fairly close to the natural architecture [7]. The results were in the form of high-resolution computer XSEM images, and of several hours of video images. The latter also included real-time studies of the photoelectron emission properties of the cells and of their interconnections, contrasted to those of the substrate.

We also performed some qualitative assessments of the radiation damage caused by the photon beam. An indirect comparison using images of photoresist microstructures [14] indicates that such damage is much more limited than in scanning electron microscopy. Finally, we demonstrated chemical contrast by scanning the photon energy and observing the corresponding changes in the relative intensity of different parts of the image.

The present experiments follow our successful tests [5-7] also on neuron specimens, with *scanning* photoemission microscopy, a technique that achieves high lateral resolution by focusing the X-ray beam with a photon energy bandpass device [15]. On the contrary, the XSEM achieves high lateral resolution by means of an electron optical system [8-13]. Both techniques can deliver extremely valuable chemical information, and they are largely complementary. For example, XSEM can produce chemical information by measuring the secondary (photoelectron) emission as a function of the photon energy [8-15], whereas changing the photon energy is difficult with a bandpass scanning photoemission microscope due to the nature of the X-ray optics.

The instrument used in the present work was described in ref. [8-15]; for these studies, the synchrotron radiation photons were extracted from the storage ring Aladdin of the Wisconsin Synchrotron Radiation Center. The XSEM consists of an objective lens to accelerate the electrons and form a first focussed image of the emissive surface, a contrast aperture in the back-focal plane (diffraction plane) of the objective lens to define the accepted solid angle of electron rays leaving the sample surface, a projective lens to provide additional, variable magnification, and a two-stage chevron micro-channelplate electron multiplier array (CEMA) and phosphor screen assembly.

The image formed on the phosphor screen is recorded using a TV-camera with an 18 mm diameter silicon-target-array low-light-level vidicon tube. The image is recorded at standard 30 Hz TV rates on magnetic tape, or digitized into frames of 512 horizontal by 480 vertical pixels using a Data Translation frame grabber and real-time image processing hardware (DT2861 and DT2858) in an 80386-based microcomputer. A schematic diagram of the system is shown in fig. 1.

The objective lens potential is used to focus the image for fixed sample position, and the projective lens bias is used to vary the magnification. The maximum magnification onto the CEMA detector is 600, with an additional 4 × magnification occurring in the optical stages using the TV-camera and display. A low magnification of around 50 can be achieved by turning off the projective lens.

The XSEM was extensively used for experiments in materials science [8-15]. In order to test the feasibility of similar experiments in the life sciences, we selected cerebellar granule cells (interneurons) because they are very well-known neural populations, for which extensive biochemical and structural information is available. Furthermore, they can grow *in vitro* with highly reproducible characteristics, automatically forming a monolayer, either as single cells or aggregates interconnected by a large number of neurites.

The neurons' property of growing as a monolayer over a flat substrate is almost ideal for photoelectron experiments. In fact, the insulating character of the cells could create a charging problem if the experiments were conducted on thick specimens. With monolayer thickness, there is a small but non-negligible conductance from the gold substrate, that prevents undesirable charging effects from taking place.

Also note that the neural systems have very characteristic shapes, and therefore are easily identifiable in microimages. This is certainly an advantage when testing a novel microscopy, since it makes it easier to distinguish cellular features from those due to possible contamination.

Cerebellar granule cells from 7-day-old rats were allowed to grow on a gold substrate. The cells were obtained [16] by enzymatical and mechanical dissociation of the cerebellar tissue

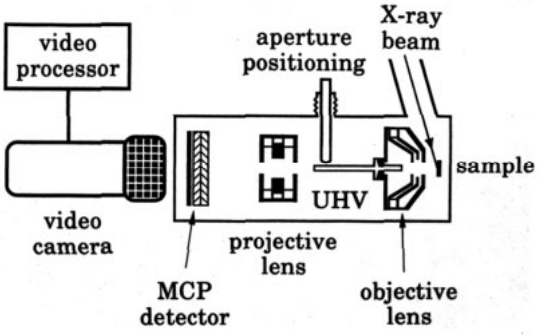

Fig. 1.

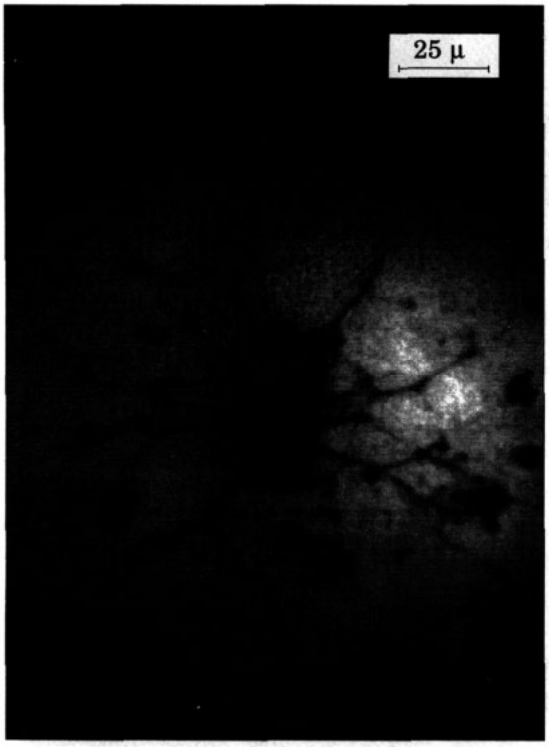

Fig. 2.

Fig. 1. – Schematic diagram of the XSEM instrument used in these experiments.

Fig. 2. – XSEM image of a small area of a neuron specimen on a gold substrate, showing a neuron aggregate (the large dark feature in the center), several neural fibers protruding from the aggregate, that connect it with individual granule cells (the small dark features). The photon beam was unmonochromatized.

and plated at a density of $2.5 \cdot 10^5$ cells/cm^2 in basal medium (Eagle's salt), containing 10% fetal calf serum. Dissociated cells were seeded in Petri dishes on a gold-coated stainless-steel substrate, treated with a 5 μg/ml of poly-L-lysine solution, and allowed to grow in an incubator at 37 °C in a 5% CO_2 humidified atmosphere.

After five days, the cultures were fixed for 10 min in a 4% solution of glutaraldehide in phosphate buffered saline solution (PBSS), carefully washed with DD water, and dehydrated at a pressure of 10^{-3} mbar, at room temperature for 24 hours [17]. The specimens so produced were found to be perfectly suitable for experiments under ultrahigh vacuum [5-7].

We also demonstrated that this preparation technique does not damage the external structure of the cells, as shown by optical-microscopy analysis and by submicron resolution photoelectron micrographs [5-7]. The specimens were found to be very stable over long periods of time: studies performed over approximately one year did not detect any alteration.

Our fixing and dehydrating technique was specifically tested for primary neuron cultures. However, the technique can be applied to almost every kind of cultured cells, as demonstrated on several tests on cells lines. The success of our tests demonstrates that the problem of ultrahigh vacuum compatibility can be solved without extensive damage of the cell structures and architectures.

Figure 2 shows an example of the many images taken in the course of these experiments. The image was obtained by accumulating the secondary-electron signal over several seconds

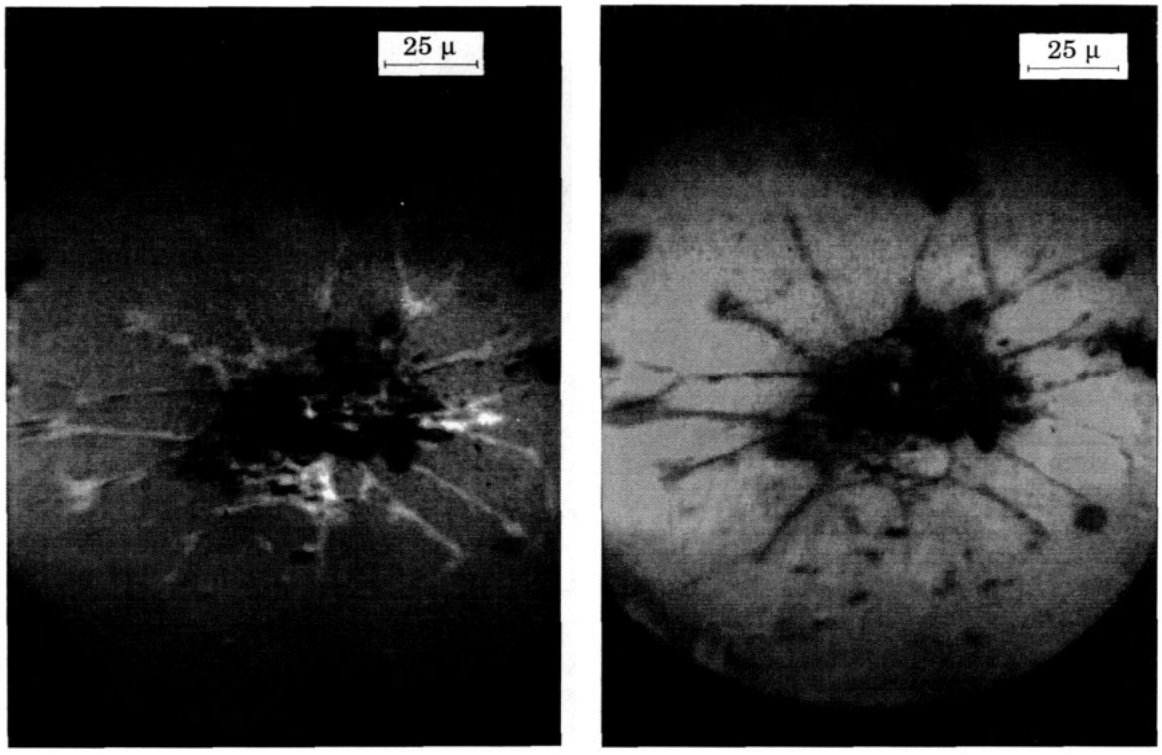

Fig. 3. – Two XSEM images of a portion of the neuron specimen, taken at photon energies of 135 (left) and 50 eV (right) *i.e.* above and below the gold absorption edge. The images show the reversed chemical contrast between substrate and components of the neural cells.

to reduce the random noise. Note the capability of XSEM to reveal fine details of the specimen, such as neural fibers that connect in this case the aggregate with several individual granule cells. Besides still images like this, with a large number of pixels, the experiments also produced several hours of video images.

We performed several tests relevant to the applicability of this microscopy to biological studies. First, by reducing the photon flux and taking video images in real time, we detected individual photoelectrons originating from different parts of the specimen. This enabled us to prove that photoelectrons are produced not only by the substrate, but also by the biological components of the specimen.

Second, we demonstrated the chemical contrast between different parts of the specimen by taking images at different photon energies. A nice example is shown in fig. 3, where we see two images of the same portion of the specimen, taken at two different photon energies, one above and one below the Au 4*f* absorption edge. Since the substrate is made of gold, it emits less secondary electrons when the photon energy is below threshold; hence, the reversed contrast that is clearly seen in fig. 3.

Third, we performed preliminary tests of radiation damage. These consisted in searching for evidence of photon-beam–induced damage in images taken over a period of several hours. No such evidence was found. In parallel experiments [14] we also observed the radiation damage for polymer films on silicon wafers. This damage was compared to the damage induced by an electron beam to obtain conventional electron micrographs of comparable quality: these tests clearly demonstrated that the photon-induced damage is orders of magnitude less than the electron-induced damage.

In summary, we demonstrated that the XSEM can be applied to the study of biological specimens with reduced damage with respect to other electron microscopies, and with the advantage of chemical-analysis capabilities. Experiments are currently underway that use this approach for microchemical analysis of trace elements in biological specimens. In general, these experiments demonstrate that photoemission techniques, thanks to their improved lateral resolution, are finally entering the domain of the life sciences.

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