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High-Resolution Photoelectron Microimaging of Neuron Networks.

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Abstract. – Neuron networks have been imaged with the scanning photoelectron microscope MAXIMUM, showing details in the submicron range such as axons, dendrites and their synapses. These results, obtained with the scanning photoemission microscope MAXIMUM, demonstrate that photoemission can reach the lateral resolution required for the life sciences, and prepare the way to the future use in the life sciences of techniques such as ESCA.

Photoemission techniques like ESCA are the leading electronic probes in materials science—but their impact in the life sciences has been minimal [1]. A critical problem is that the lateral resolution in ordinary photoemission does not exceed a few tenths of a millimeter. This space-averaged probe is nearly useless for most of the fundamental problems in biophysics and biochemistry, that deal with microstructures in the submicron range or smaller. This limit is being overcome with photoemission microscopes such as our scanning instrument MAXIMUM [2, 3]. We present the first photoelectron micrographs of a cellular system with submicron resolution. Minute details of neuron networks were imaged on MAXIMUM, thereby opening the way to novel applications of photoemission in the life sciences.

It should be noted that this technique is not important for the production of images, since its lateral resolution will be ultimately set by the diffraction limits of soft X-rays. Its interest is related to the capability of performing chemical analysis on a microscopic scale, and specifically to provide information on the elements of the specimen and on their chemical

status. This is a well-known capability of ESCA, but it is precisely the lack of sufficient lateral resolution that has made it impossible to apply such a capability in the life sciences. Our present result is an important feasibility test, since it shows that nothing prevents photoemission, given a sufficient signal level, from reaching the minimum lateral resolution required for the life sciences.

The tests were performed on uncoated and unlabelled neurons, and revealed axons, dendrites and synapses. The lateral resolution, characterized with several different techniques, was 0.5 μm. These resolution levels were achieved by focussing the ultrabright synchrotron radiation emitted by a 30-period undulator on the Aladdin storage ring of the Wisconsin Synchrotron Radiation Center; microimages were produced by scanning the focussed beam on the specimen's surface and collecting the photoelectrons so produced [2]. Focussing in the soft–X-ray range was achieved by means of a multilayer-coated Schwarzschild objective [4].

In 1989, we obtained from MAXIMUM the first photoemission micrographs of neurons. A neuron network is an almost ideal system to test the technique, since it includes a variety of microstructures (cell bodies, dendrites, synapses etc.), of sizes ranging from several microns to the submicron domains and with different shapes. In the 1989 tests, the image quality was severely limited by the lateral resolution, approximately 3 μm at that time. Recently, we implemented a new focussing system, that enabled us to improve the resolution to the submicron level required for experiments in the life sciences. This made it possible to dramatically improve the quality of the micrographs of neuron networks–in particular, to image dendrites whose width is in the submicron range.

The lateral resolution was repeatedly measured using two different methods: first, by gradually inserting a knife edge, taking the derivative of the signal and estimating its full width at half maximum. Second, by imaging several objects of known dimensions such as micropatterned metal overlayers. Both methods consistently gave a full width at half maximum of 0.5 to 0.55 μm after optimization of the focussing conditions.

The neuron network specimens were prepared from eight-day–old rat brains. The cells were obtained by enzymatic and mechanical dissociation of the nervous tissue 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 [5]. Cells were seeded in Petri dishes on a gold-coated stainless-steel substrate treated with a 5 mg/ml poly-L-lysine solution, and allowed to grow in an incubator at 37 °C in a 5% CO_2 atmosphere. After five days, they were fixed by a 4% glutaraldheyde solution in PBSS (phosphate buffered saline solution) [6], carefully washed with DD water, and dehydrated at a pressure of 10^{-3} mbar, at room temperature for 24 hours.

The resulting specimens are perfectly suitable for experiments under ultrahigh vacuum. We proved that this preparation technique does not damage the external structure of cells, as shown by optical microscopy analysis and by the photoelectron micrographs themselves. The specimens were very stable: studies performed over several months did not detect any alteration. Note that the neuron networks are left in a near-natural state, without coating or labelling as it would be required for other electron microscopy techniques.

The quality of the photoelectron microimages is evident in fig. 1 and 2. Figure 1 shows individual neurons and their interconnections. The reproducibility of the microimaging system was tested, for example, by taking a series of partially overlapping photoelectron micrographs, and combining them as shown in fig. 2. Many components of the neuron network are visible in this patchwork, including neuron clusters (C), isolated neurons (B), axons (A), synapses (S), dendrites (D), and glial cell, most likely an astrocyte (G). Two axons and their connecting synapse (S) are particularly clear on the lower part of the patchwork. Figure 3 shows an optical micrograph taken on the same specimen, for comparison with the photoelectron micrographs and also to assess the specimen's quality.

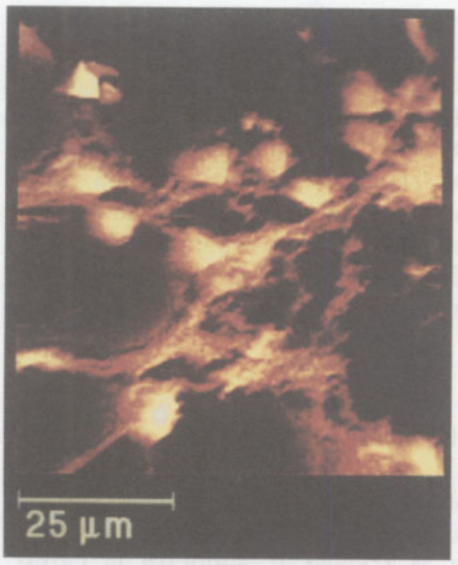

Fig. 1.

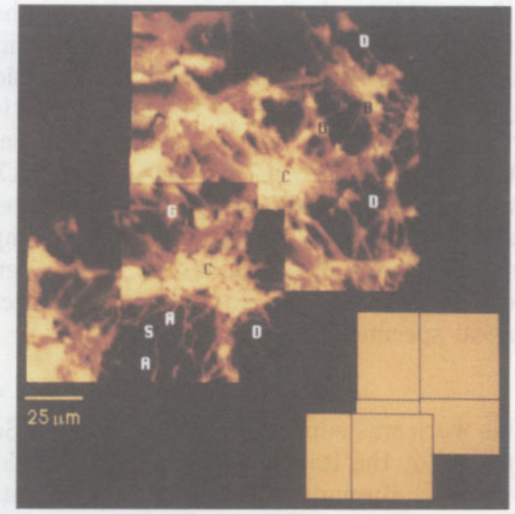

Fig. 2.

Fig. 1. – Scanning photoelectron micrograph of a $(75 \times 75)\,\mu m^2$ area of a neuron network on a gold substrate, showing a cluster of neurons. The measured resolution was 0.5 µm. Note the individual neurons and their interconnecting dendrites and axons.

Fig. 2. – Patchwork combination of six partially overlapping micrographs. A = axon; S = axon-axon synapse; D = dendrite; B = cell body; G = glial cell; C = cluster of cell bodies. The combination scheme of the patchwork is shown on the lower right-hand side of the figure.

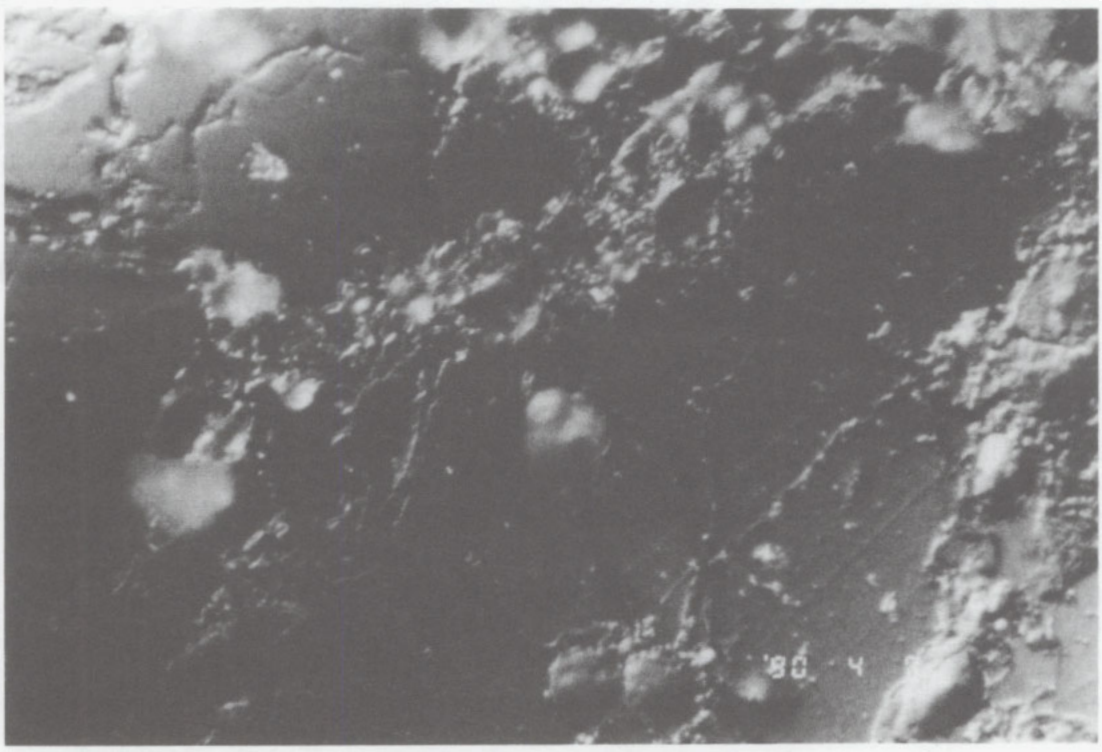

Fig. 3. – Optical micrograph of a portion of the neuron network specimen.

Fifteen additional photoelectron micrographs besides those of fig. 1 and 2 demonstrated the reliability of MAXIMUM in producing high-quality images. These results prove the feasibility of studying submicron details of biological systems with photoemission microscopy and spectromicroscopy. The high-resolution photoelectron micrographs were obtained over a period of five days of synchrotron radiation beamtime, immediately before the beginning of the final construction stage of MAXIMUM. This stage will further improve the characteristics of the instrument including lateral resolution. The instrument will be used for microchemical analysis of the different components of neuron networks; note that since the signal-to-noise level will be improved by energy filtering of the electrons, the present successful tests demonstrate that such a spectromicroscopy technique is feasible for our biological specimens.

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REFERENCES

[1] MARGARITONDO G. and CERRINA F., *Nucl. Instrum. Methods A*, **291** (1990) 26; MARGARITONDO G., *Introduction to Synchrotron Radiation* (Oxford, New York, N.Y.) 1988.

[2] CERRINA F., MARGARITONDO G., UNDERWOOD J. H., HETTRICK M., GREEN M. A., BRILLSON L. J., FRANCIOSI A., HÖCHST H., DELUCA jr. P. M. and GOULD M. N., *Nucl. Instrum. Methods A*, **266** (1988) 303; CERRINA F., CROSSLEY S., CROSSLEY D., GONG C., GUO J., HANSEN R., NG W., RAY-CHAUDHURI A., MARGARITONDO G., UNDERWOOD J. H., PERERA R. and KORTRIGHT J., *J. Vac. Sci. Technol. A*, 8 (1990) 2563; HALBACH K., CHIN J., HOYER E., WINICK H., CRONIN R., YANG J. and ZAMBRE Y., *IEEE Trans. Nucl. Sci.*, 28 (1981) 3136; WINICK H., BOYCE R., BROWN G., HOWER N., HUSSAIN Z., PATE T. and UMBACH E., *Nucl. Instrum. Methods*, **208** (1983) 127.

[3] ADE H., KIRZ J., HULBERT S., JOHNSON E., ANDERSON E. and KERN D., *Nucl. Instrum Methods*, **291** (1990) 126; KING P. L., BORG A., KIM C., PIANETTA P., LINDAU I., KNAPP G. S., KEENLYSIDE M. and BROWNING R., *Nucl. Instrum. Methods*, **291** (1990) 19; TONNER B. P., *Nucl. Instrum. Methods*, **291** (1990) 60.

[4] LOVAS I., SANTY W., SPILLER E., TIBBETS R. and WILCZYNSKY J., *Proc. SPIE*, **316** (1980) 90; CERRINA F., *Imaging Science*, **30** (1986) 80; LAI B., CERRINA F. and UNDERWOOD J. H., *Proc. SPIE*, **563** (1985) 174.

[5] LEVI G., ALOISI F., CIOTTI M. T. and GALLO V., *Brain Res.*, **290** (1984) 77.

[6] CIOTTI M. T., MERCANTI D. and LEVI G., in *A Dissection and Tissue Culture Manual of the Nervous System*, edited by A. SHAHAR, J. DE VELLIS, A. VERNADAKIS and B. HABER (Alan R. Liss) 1989, p. 291.