Photoelectron microscopy in the life sciences: Imaging neuron networks

Delio Mercanti
Istituto di Neurobiologia del CNR, Viale Marx 15, 00100 Roma, Italy

Gelsomina De Stasio
ISM-CNR, Via E. Fermi 38, 00044 Frascati, Roma, Italy

M. Teresa Ciotti
Istituto di Neurobiologia del CNR, Viale Marx 15, 00100 Roma, Italy

C. Capasso, W. Ng, A. K. Ray-Chaudhuri, S. H. Liang, R. K. Cole, Z. Y. Guo, and J. Wallace
Departments of Physics and of Electrical and Computer Engineering, University of Wisconsin, Madison, Wisconsin 53706

G. Margaritondo
Institut de Physique Appliquée, Ecole Polytechnique Fédérale de Lausanne, Ecublens, Switzerland

F. Cerrina
Departments of Physics and of Electrical and Computer Engineering, University of Wisconsin, Madison, Wisconsin 53706

J. Underwood, R. Perera, and J. Kortright
Center for X-ray Optics, Lawrence Berkeley Laboratory, Berkeley, CA 94720

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Photoemission techniques like electron spectroscopy for chemical analysis are the leading electronic probes in materials science—but their impact in the life sciences has been minimal. 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, which deal with microstructures in the submicron range or smaller. This limit is being overcome with photoemission microscopes, such as our scanning instrument MAXIMUM. The first scanning photoelectron micrographs of a cellular system with submicron resolution are presented. Minute details of neuron networks are imaged on MAXIMUM, thereby opening the way to novel applications of photoemission in the life sciences. The details include individual neurons, axons, dendrites, and synapses, and composite large-area scanning micrographs were routinely produced with a lateral resolution of 0.5 μm.

We present the first successful scanning microimaging tests of neuron networks with photoelectrons, which reveal individual cells and their connections. In particular, we discuss the details of the specimen preparation, which are of general interest to spectromicroscopy experiments on biological specimens under ultrahigh vacuum conditions.

In recent years, the rapid increase of interest in the neurosciences has stimulated major improvements in the understanding of the biochemistry, physiology, pathology, and pharmacology of the nervous system. A number of studies have improved our knowledge on how neurons communicate and how they make complicated but well-defined networks.[1] Neurons in the central nervous systems and in peripheral nervous systems are organized in populations and subpopulations with different chemical and structural properties. Therefore it would be extremely useful to analyze the local chemical architecture of individual cells. Note that many unexplained aspects of the growth, development, differentiation, and aging of the nervous system must still be clarified. Photoemission spectromicroscopy, with its capability of providing advanced chemical information on a microscopic scale, is a promising new approach for the solution of these issues.[2]

Photoemission spectromicroscopy is the combination of photoemission spectroscopy with high lateral resolution. This technique,[2] which investigates for the first time both the morphology and the local chemistry of biological specimens, offers fundamental advantages over conventional techniques applied in neurobiology. In fact, multiple information can be obtained from a single specimen—whereas in conventional studies it is necessary to prepare different samples for morphological, architectural studies, and it is impossible to perform a local chemical analysis with lateral resolution on the scale of individual cells. It also appears possible to localize and follow the geometrical and steric displacement of a neuron circuit, at least *in vitro*.

For decades, photoemission techniques have been the leading probes of electronic and chemical structures in materials science research.[3] For example, electron spectroscopy for chemical analysis (ESCA) finds very wide application in virtually all branches of materials science. The applications of photoemission in life science are much more limited. We argue that the main cause of this disappointing situation is the heterogeneity of life science specimens. Space-averaged information is nearly useless for such systems and microscopy is, indeed, one of the leading experimental tools in the life sciences. Until recently, the most advanced commercial instruments did not exceed a resolution of the order of 100–200 μm, inadequate for life science experiments. Very recently, by the use of nonconventional photon sources of high intensity and brightness, such as bending magnets and insertion devices sources of synchrotron radiation, it was possible

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to increase the lateral resolution reaching submicron levels.[2,4]

Our project MAXIMUM is an example of such progress, and one of the two scanning photoelectron microscopes working with submicron resolution.[4,5] The present resolution of 0.5 μm was produced with the recent technical improvements, discussed in detail in Ref. 6. Further improvements appear feasible; with ultrahigh-brightness photon sources (ELETTRA at Trieste and ALS at Berkeley), it will be possible to reach the diffraction limit of a few 100 Å. The resolution of 0.5 μm was demonstrated for a number of biological and nonbiological specimens.

Achieving the necessary resolution is not the only obstacle in the application of photoemission microscopy to the life sciences. Several problems are peculiar to biological specimens. For example, the photoemission spectromicroscope works under ultrahigh vacuum, and it is connected to the synchrotron radiation source, also under ultrahigh vacuum, without windows. Therefore biological samples must be prepared in such a way that they are compatible with this vacuum environment. A similar problem exists for conventional electron microscopy and it is solved by fixing and dehydrating the specimens—but the samples are also labeled or metal coated, thereby making them even farther from their natural state.

We have demonstrated that photoemission spectromicroscopy offers the distinct advantage of being able to operate with uncoated and unlabeled specimens.[7] We selected cerebellar granule cells (interneurons) for our studies of this crucial point, for several reasons: they are a very well-known neural population, for which extensive biochemical and structural information is already available. They can be grown *in vitro* with highly reproducible conditions. They grow as a monolayer, either as single cells or aggregates interconnected by a large number of neurites (axons and dendrites).

Cerebellar granule cells from eight-day-old rats were allowed to grow on a gold substrate. The cells were obtained by enzymatic and mechanical dissociation of the cerebellar tissue and plated at a density of 2.5×10^5 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 ten minutes in a 4% solution of glutaraldehyde in phosphate buffered saline solution (PBSS), carefully washed with DD water, and dehydrated at a pressure of 10^{-3} mbar, at 25 °C for 24 h.

The specimens were stored in low vacuum desiccators, until used for the experiments. The specimens so produced were found to be perfectly suitable for experiments under ultrahigh vacuum. We also demonstrated that this preparation technique does not damage the external structure of cells, as shown by optical microscopy analysis and by submicron resolution of photoelectron micrographs. The specimens were very stable: studies performed over a period of six months did not detect any alteration.

The fixation dehydrating technique was specifically tested for primary neuron cultures. However, the technique can be applied to almost every kind of cultured cells, as demonstrated by several tests on cell lines. Therefore the success of our tests demonstrates that the problem of ultrahigh vacuum compatibility is solvable, in general, without extensive damage of the external cell structure. We are now pushing efforts to improve the specimen preparation technique by omitting the fixation step, in order to leave the structure and chemistry of the cells in a more natural state.

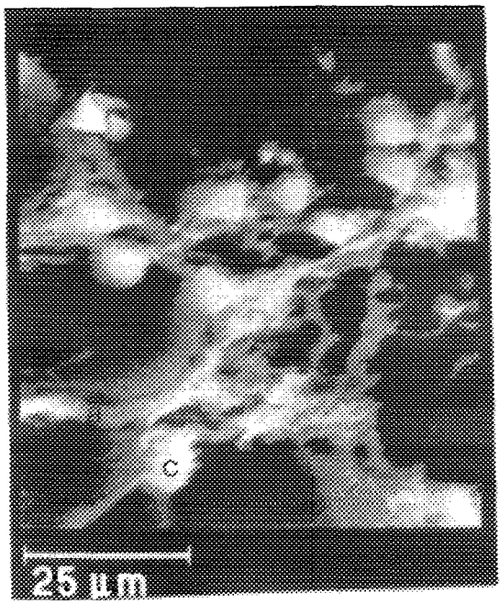

FIG. 1. A photoelectron micrograph of a neuron culture on a gold substrate obtained at MAXIMUM. The photon energy was 77 eV, and the image was produced by detecting the total photoelectric yield. The measured lateral resolution is 0.5 μm. Neuron granule cell bodies (such as *c*), axons (such as *a*), and synapses interconnecting axons are clearly distinguishable.

A second obstacle in applying photoemission microscopy to biological specimens is potentially created by the effects of the soft x-ray beam. There are, in fact, two possible problems *a priori*: the beam can damage the specimen, and charging effects can make it difficult to collect and analyze photoelectrons. Our experiments are very encouraging as far as both of these potential problems are concerned.[7] Photoelectron and visible micrographs taken on neuron networks before and after extensive exposure to the focused primary beam failed to detect beam-induced damage. It should be emphasized that the energy density of the primary beam compares very favorably with other techniques. Notably, it is orders of magnitude smaller in photoemission microscopy than in Auger microprobe experiments, and also in conventional electron microscopy, which is so widely used in the life sciences.

The quality of the results of our tests is demonstrated by the typical scanning photoelectron micrograph shown in Fig. 1. The picture of a neuron culture on a gold substrate, taken with a lateral resolution of 0.5 μm, shows the success of our preparation procedure. Minute details are clearly visible, such as neuron cell bodies, axons, and dendrites, and the interconnecting synapses. Further improvements are under-

way, as discussed in Ref. 6, and they should bring the resolution to levels even more suitable for life science experiments.[8]

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[8]We note that photoelectron microscopy can be implemented with electron optics rather than with photon focusing (i.e., in a scanning mode); see, for example, the excellent results described by G. H. Griffith in Appl. Surface Sci. **26**, 265 (1986), and in the references therein. However, the scanning mode is more suitable for the electron energy analysis that is required for spectromicroscopy.