X-RAY PHOTOEMISSION ENTERS THE LIFE SCIENCES

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ABSTRACT

Recent improvements in the lateral resolution of photoemission experiments open the way to their extensive application in the life sciences. We discuss some of the preliminary tests in this novel area, and a number of possible future developments.

I. FROM SPECTROSCOPY TO SPECTROMICROSCOPY

For decades, photoemission techniques have been the leading probes of electronic and chemical structures in materials science research.[1] For example, ESCA (Electron Spectroscopy for Chemical Analysis) finds very wide applications in virtually all branches of materials science. The applications of photoemission in the life sciences 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 very recently, the lateral resolution of photoemission experiments was very limited. One typical example is the double-pass cylindrical mirror electron energy analyzer (CMA), a very widely used instrument in photoemission research. The lateral resolution is determined by the "dense sphere" of the analyzer, and the order of magnitude, even under the best circumstances, does not exceed several tenths of a millimeter. Such a resolution is entirely inadequate for the heterogeneity of life-science systems.

The main limitation in improving the lateral resolution of photoemission experiments is the signal-to-noise level. In essence, with conventional photon sources, it is very difficult to achieve at the same time good energy resolution *and* good lateral resolution. Until recently, the most advanced commercial instruments did not exceed a resolution of the order of 100-200 micron, still inadequate for the life sciences.

In the past two years, commercial photoemission spectrometers such as the Scienta ESCA-300 and the Vacuum Generators ESCASCOPE have marked spectacular improvements, achieving lateral resolutions of the order of tens of

microns. This level of resolution is sufficient for some biological structures, *e.g.*, multicellular systems such as tissues. It is not sufficient, however, to study individual cells or subcellular structures.

In order to overcome the limitations of commercial instruments, it is necessary to use non-conventional photon sources of high intensity and brightness: the bending-magnet and insertion-device sources of synchrotron radiation. Very recently, major breakthroughs have, indeed, been produced in the lateral resolution of synchrotron-radiation photoemission. Submicron resolution levels have been reached by both the existing scanning photoemission microscopes based on synchrotron radiation: MAXIMUM at Wisconsin and PEM at Brookhaven.[1-4] This resolution level makes it possible, for the first time, to produce scanning photoelectron micrographs of individual cells, thereby opening the way to widespread applications of photoemission in the life sciences.

This short review presents the status of existing instrumentation, a brief outline of the first feasibility tests, a discussion of possible development avenues of this novel field, and a projection of the additional possibilities that will be opened by the advent of a new generation of ultrabright sources.

In essence, the achievement of high lateral resolution transforms conventional photoemission spectroscopy into a *spectromicroscopy*.[1] Conventional photoemission spectroscopy analyzes the energy and, in some cases, the direction of emission of the photoelectrons.[1] This delivers information on the energy and (for crystalline solids) crystal momentum of the ground electronic state. When the analysis is extended to core levels it becomes possible to obtain information on the chemical structure of the system; specifically, one can identify the component elements and the chemical status of such components. This last piece of information is delivered by the so-called *chemical shifts* of the core levels, that are determined by the local valence-charge distribution at the atom where the photoelectric process takes place.

Photoemission *spectromicroscopy* retains the capability of extracting chemical information as photoemission spectroscopy, and it adds the capability of doing so with high lateral resolution. For example, one can measure photoemission spectra from a small area of the specimen; or one can take micrographs reflecting the space distribution of a given element. One can push this technique beyond the mere identification of the element by taking micrographs of the chemical status of the element. These capabilities are complementary to those of pure microscopies, and make photoemission spectromicroscopy a very promising technique for both materials science and the life sciences.

Other microscopies possess spectroscopic capabilities. For example, scanning tunneling microscopy can perform the energy analysis of the states involved in the tunneling process. The analysis, however, is limited to a small range of energies. In principle, Auger spectromicroscopy could be a competitor for photoemission spectromicroscopy. However, Auger spectromicroscopy has never achieved energy resolutions comparable to those of photoemission,

thereby having a much more reduced capability of analyzing the chemical status of the elements. Furthermore, the primary probe puts on the sample an energy density several orders of magnitude higher than that of photoemission spectromicroscopy. In summary, photoemission spectromicroscopy emerges as the clear winner with respect to these competitors.

II. SCANNING AND ELECTRON IMAGING

The recent development of photoemission spectromicroscopes is taking place in two distinct directions: scanning instruments and electron imaging instruments.[1] In the first case, the lateral resolution is achieved by focusing the photon beam that extracts the photoelectrons from the sample. Micrographs are then created by scanning the beam over the sample (or the sample with respect to the beam), and measuring the emitted photoelectron intensity by means of an electron analyzer.

Substantial progress has been achieved in focusing soft-x-ray beams to a small spot. The photon energy range of interest is from a few electronvolts to hundreds of electronvolts — the energies required to effectively excite valence electrons and a number of core levels of diverse elements. Higher photon energies pose energy resolution problems, and make it more difficult to perform advanced chemical analysis. Focusing soft-x-rays is difficult because photons in this range are absorbed by all materials and are not easily reflected except at grazing angles of incidence.

The most significant advances were made with two kinds of focusing devices: the Wisconsin-Berkeley-Minnesota-Xerox-Frascati collaboration used Schwarzschild objectives, with reflectivity enhanced by multilayer coatings.[2,3] The Stony Brook-Brookhaven-IBM collaboration used micro reversed Fresnel zone plates.[4] Both approaches have been remarkably successful, and they operate in different spectral ranges, so they are largely complementary to each other. The corresponding instruments, MAXIMUM at the Wisconsin Synchrotron Radiation Center [2,3] and PEM at the Brookhaven National Light Source,[4] have recently achieved submicron lateral resolution. These instruments take advantage of the high brightness of the synchrotron radiation emitted by the insertion devices known as undulators. Other scanning instruments, based on the Kunz objective, are under development at HASYLAB in Hamburg and at Lund.[5,6]

In the case of electron-imaging devices, the lateral resolution is achieved by means of an electron optical system. Several devices of this kind have been implemented at synchrotron radiation laboratories; for example, a system based on a superconducting magnet has been implemented first at the Stanford Synchrotron Radiation Laboratory,[7] and then by Minnesota scientists at the Wisconsin Synchrotron Radiation Center. Also at Wisconsin, two other devices have been implemented by Argonne scientists [8] and by Tonner *et al.* of the University of Wisconsin-Milwaukee.[9]

Extensive descriptions of these scanning and electron imaging instruments

have appeared in recent reviews, and we refer the reader to these reviews for technical details. Here, we would like to address one important point: the complementarity of the different technical solutions.[1] We have already seen the complementarity between Schwarzschild objectives and reverse zone plates. There is also a fundamental complementarity between scanning (*i.e.*, photon focusing) devices, and electron imaging devices. In the first case, the focusing function depends on the photon energy, whereas in the second case it depends on the electron energy. Therefore, spectromicroscopy experiments that require changing the photon energy are more easily implemented in the electron-imaging instruments and *vice versa*. Modern photoemission includes all kinds of techniques, based on changing photon as well as electron energies. Therefore, no single kind of spectromicroscope can implement all photoemission spectromicroscopy experiments — hence, the complementarity of the two approaches.

III. FIRST TESTS IN THE LIFE SCIENCES

The generic possibility of applying photoemission spectromicroscopy to the life sciences does not mean that there are no obstacles in the practical implementation of these experiments. Several obstacles are peculiar to biological specimens. For example: photoemission spectromicroscopes work under ultrahigh vacuum, and when they use synchrotron radiation they are connected to the source, also under ultrahigh vacuum, without windows. Therefore, biological specimens must be prepared in such a way that they are compatible with an ultrahigh vacuum environment.

A similar problem exists for conventional electron microscopy, and it is solved by fixing and dehydrating the specimens. These specimens are also labelled or metal coated, thereby making them even farther from their natural state. From this point of view, photoemission spectromicroscopy offers distinct advantages: uncoated and unlabelled specimens can be used. This fundamental point was proved by De Stasio, Mercanti and Ciotti, in recent series of feasibility tests.[3]

The preparation procedure was applied to neuron networks on gold substrates. 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 2.5×10^5 cells/cm^2 in Basal Medium (Eagle's salt) containing 10% Fetal Calf Serum. 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), 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.[3] We proved that this preparation technique does not damage the external structure of cells, as shown by optical microscopy analysis and

by submicron-resolution photoelectron micrographs. The specimens were very stable: studies performed over several months did not detect any alteration.

The fixing and dehydrating technique was specifically tested for neuron cultures. However, the technique has very general applicability to every kind of cells. Therefore, the success of our feasibility tests demonstrates that the problem of ultrahigh vacuum compatibility can be solved in general without extensively damaging the external structure.

A second obstacle is created by the effects of the soft-x-ray beam. There are, in fact, two potential problems. First, the beam can damage the specimen. Second, it can cause charging, making it difficult to collect and analyze photoelectrons. Our feasibility tests are very encouraging for both of these potential problems.

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 experiments, and also in conventional electron microscopy, that is so widely used in the life sciences.

As far as charging is concerned, no such effects were detected in our tests. Such tests, however, were not extensive, and charging problems cannot be ruled out for future experiments. Effects of this kind could become a serious problem if and when photoemission spectromicroscopy will be performed with angular resolution.

Finally, we would like to mention the perceived problem of surface sensitivity. The escape depth of photoelectrons being very short at the relevant kinetic energies, the experiments probe only a region very near the surface, typically a few angstroms to a few tens angstroms. This could be regarded as a problem, since the technique does not explore the bulk of the structures. A similar problem existed in materials science, when surface-sensitive techniques made their first appearance. In the long run, however, the surface sensitivity was regarded more like an advantage than as a problem: it enabled the experimentalists to analyze with high sensitivity phenomena that are peculiar to surfaces and interfaces, proving their fundamental importance. We suggest that a similar evolution could occur in the life sciences, where surface-sensitive techniques like photoemission have not found very extensive applications.

We conclude this section by presenting some results from the first feasibility tests on neuron networks.[3] The results demonstrate that it is possible to perform life-science photoemission experiments with submicron resolution. Furthermore, they also emphasize the rapid progress of this field in recent months. Figure 1 shows the first photoemission micrograph of cells; the overall shape of neuron clusters is visible, but minute details are not imaged. The reason is the limited lateral resolution of the MAXIMUM instrument at the time when this

first test was performed.

The lateral resolution was dramatically improved after the test, reaching the 0.5 micron level early in 1990. The improvement is clearly visible when comparing Fig. 1 to Fig. 2, that shows a patchwork of six micrographs taken with the improved instruments. Note that minute details are now clearly visible, including the synapses that connect axons. Results like those of Fig. 2, repeatedly obtained over several days, definitely demonstrate that photoemission techniques can be used in the life sciences.

IV. POSSIBLE AVENUES OF DEVELOPMENT

Why is the submicron resolution so crucial to life-science experiments? The answer is obviously to be found in the scale of the typical microstructure of biological specimens. The most relevant is perhaps the size of cells. This ranges from 10-20 micron for some kinds of neurons to a fraction of a micron, *e.g.*, for cellular organelles. Other sizes are also quite relevant; for example, the most crucial function of a neuron network, the transmission of information, involves small structures such as dendrites and axons. The typical size of such structures ranges from 1-2 micron to 0.01 micron.

Photoemission studies with submicron resolution, therefore, can touch virtually every aspect of cell biology. We will discuss here only a few examples of possible experiments that exploit such a resolution. Consider, first of all, the crucial problem of the nerve pulse transmission. This is the central problem in neurobiology, and one of the most important problems in biology and physiology. We are, however, very far from its solution. Very little is known about the mode of pulse transmission; for example, it has not been clarified if this is a purely electronic process, or a process involving the transport of large masses such as ionized atoms or molecules.

The main impediment to the clarification of this fundamental issue is the scarcity of experimental information. Photoelectron spectromicroscopy could make a significant contribution, by revealing local microchemical features related to the pulse transmission. This, of course, requires performing spectromicroscopy with a resolution sufficient to study synapses. The recent tests on MAXIMUM demonstrate that synapses can, indeed, be observed in photoelectron micrographs. Feasibility tests are currently underway to move from a purely photoemission microscopy mode to a spectromicroscopy mode, and deliver the first pieces of information on the microchemical features of the synapse region. In particular, a search will be conducted for the chemical fingerprints of aminoacids, that have been hypothesized to participate in the pulse transmission mechanism.

A second important example of possible applications of photoemission spectromicroscopy in neurobiology is the role of metal atoms in pathological processes. It has been hypothesized that aluminum causes the neuropathology known as Alzheimer disease, a leading cause of death and incapacitation for

older people. A microchemical analysis of this problem would be extremely valuable, and photoemission spectromicroscopy could detect unusually large concentration of the aluminum atoms. This application, however, requires feasibility tests on the study of thin slices of cerebellar tissue as specimens.

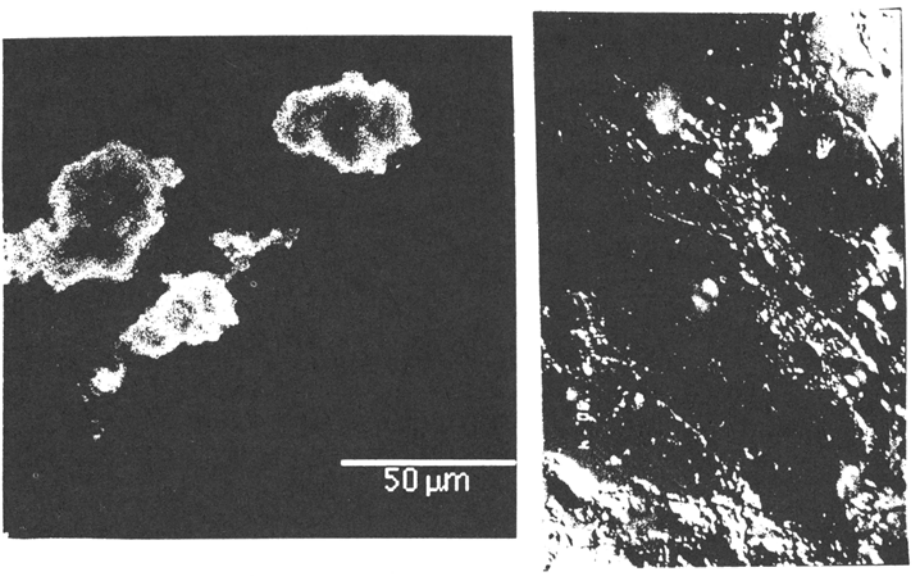

Fig. 1. The first photoemission micrograph of cells, showing clusters of neurons taken with a resolution of 2-3 micron using the MAXIMUM system at the Wisconsin Synchrotron Radiation Center. A visible micrograph of the same specimen is shown for comparison.[3]

Finally, we would like to mention another open problem in biology that can profit from the advent of photoemission spectromicroscopy: the analysis of calcium ions in calcium channels. These are channels through the cell membrane, that control the quantity and direction of the flux of calcium ions in and out of the cell. This mechanism determines the electrostatic potential drop across the membrane, and therefore a large number of mechanism involving passage through the membrane, *e.g.*, of sodium and potassium.

No other technique besides photoemission spectromicroscopy shows promise of delivering local quantitative information on the calcium channels. The alternate approach, based on the patch-clamp method, only delivers information averaging over an area larger than one micron. The use of photoe-

mission spectromicroscopy in this area, however, requires solving the problem of preparing the specimens without altering the channel structure and content. Preliminary tests in this direction are underway.

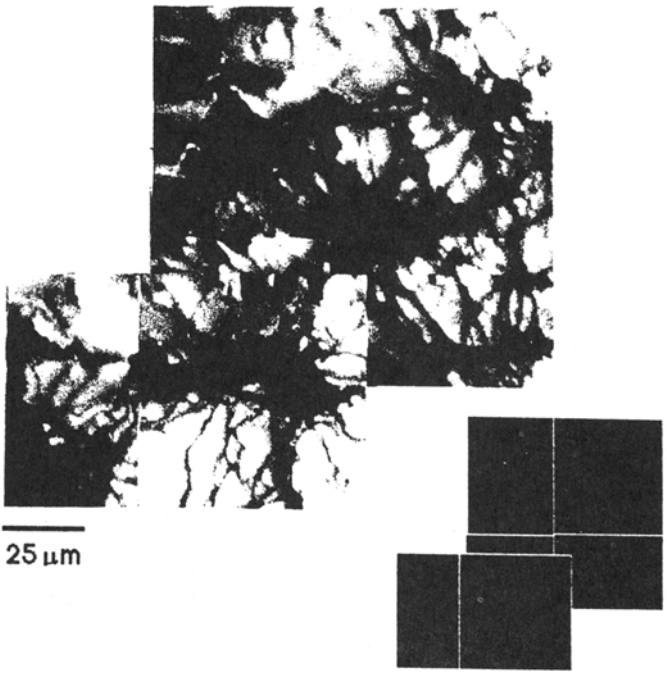

Fig. 2. Patchwork of six photoemission micrograph of a neuron network, produced by MAXIMUM with a resolution of 0.5 micron. Note the excellent imaging of individual cells and their connections, including dendrites and axons.[3]

These few examples give an idea of the substantial impact that photoemission will have on the life sciences when performed with high lateral resolution. It is fair to say that this novel research area is at a stage similar to that of materials science decades ago, immediately before the advent of surface-sensitive experimental techniques. As in that case, the extrapolation from previous experience gives a very limited picture of the future extent of this field. Most likely, we are not planning and not even imaging the most important experiments, that will originate from the evolution of this field and from the first results deliveder by this novel approach.

V. FUTURE INSTRUMENTATION

The recent breakthroughs such as the imaging of neuron networks on MAXIMUM demonstrate that photoemission microscopy is possible with the

present sources of synchrotron radiation.[2-9] The future of this field, however, is brightened (the pun *is* intended) by the advent of untrabright sources currently being commissioned.[1] Roughly speaking, with the existing sources the performances of photoemission spectromisroscopy are still limited by the signal level. For example, the lateral resolution will not be able to reach the diffraction limit of scanning photoemission spectromicroscopes.

With the increase in brightness provided by the new sources, the diffraction limit can be reached with signal to spare. For example, it will be possible to significantly decrease the data-taking time, that is now of the order of tens of minutes per micrograph. Again roughly speaking, the increase in brightness from the current to the new sources will be of at least three orders of magnitude. Of these, one is required to reach the diffraction limits in lateral resolution — and the others can be used for other performance improvements.

The improvement in brightness will originate from two factors.[1] First, a decrease in the size and angular divergence of the electron beam circulating in the storage ring used as source. This is expressed by saying that the new sources will be "low-emittance" machines, "emittance" being the parameter used in accelerator science to characterize, indeed, the size and angular divergence of the electron beam. The second factor in the brightess increase is the use of the insertion devices known as "undulators". These concentrate an exceptionally high level of brightness into a small spectral region. A few undulators are being used in the present synchrotron radiation sources; however, the new sources will be primarily based on these insertion devices.

Significant examples are the storage ring under construction at Berkely (Advanced Light Source or ALS) and Trieste (ELETTRA). With these new sources, photoemission spectromicroscopy will reach lateral resolution limits in the 100-angstrom range, breaking new ground in the life sciences. These life-sciences applications are, in fact, among the most exciting opportunities opened by the low-emittance undulator sources — and fully justify the considerable human, technical and financial resources invested in the development of these advanced facilities.

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