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Photoelectron spectromicroscopy with synchrotron radiation: applications to neurobiology

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Abstract

We show that photoelectron spectromicroscopy with synchrotron radiation has been successfully exported from materials science to the life sciences. The transfer is well beyond mere feasibility tests. Photoelectron spectromicroscopy is in fact used for real, long-term studies in neurobiology. Specific examples are discussed, first to see in practice some applications to microchemical analysis of physiologic elements, and then to discuss present and future instrumentation development issues. This last point is illustrated by briefly presenting the MEPHISTO project and the CERVIN project for the Swiss Light Source.

Keywords: Photoemission; Spectromicroscopy; Neurobiology; Synchrotron radiation

1. Introduction

At its development, photoelectron spectromicroscopy was proposed as a technique of interest for life-science applications [1]. The achievement of high lateral resolution in photoelectron experiments is certainly important for materials science applications [2]. But it is even more fundamental for the extension of these techniques to the life sciences, whose spatial scale is set by the dimensions of cells and cell structures: significant contributions are difficult without submicron resolution [1].

Different types of photoelectron spectromicroscopy techniques have indeed been used in life-science tests in the past several years [1]. Most of the initial effort was devoted to feasibility experiments and the refinement of the basic techniques. We are no longer in this initial stage: long-term research programs have been in operation since the end of the 1980's, using a variety of photoelectron spectromicroscopy approaches to investigate the local chemistry of neurobiological systems [1,3]. The present review presents the status of this effort, and discusses some of its future directions, opportunities and problems.

The future issues are strongly related to the ongoing progress in the instrumentation. The feasibility of high lateral resolution in photoelectron spectroscopy was indeed the result of improvement in the photon sources, most notably, the advent of high brightness [2,4]. In turn, the photon source improvements stimulated progress in other parts of the instrumentation. This process is still underway and in fact accelerating.

Because of their importance, therefore, instrumentation issues deserve special attention in our review. The second part of the article is devoted to them, and their discussion is based on specific examples. First, we discuss the applications of photoelectron spectromicroscopy instrumentation in the complementary

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field of transmission spectromicroscopy [5]. Second, we present the newly constructed MEPHISTO system (Microscope A Emission de PHotoélectrons par Illumination Synchrotronique de Type Onduleur—Photoelectron Emission Microscope by Synchrotron Undulator Illumination) [6]. Third, we briefly disuss the possible impact of the planned commissioning in 2000 of SLS (Swiss Light Source)—the first member of the fourth generation of synchrotron facilities [7].

2. A practical example: physiologic elements in brain cell cultures.

Most experiments in our neurobiological photoelectron spectromicroscopy program investigated the uptake of toxic and physiologic elements by brain cells in culture [1,3]. We discuss here the specific example of physiologic elements before and after ashing the cells in cold oxygen plasma [8].

Ashing is a procedure used to reduce the thickness of tissue sections or to investigate the chemical properties of biological specimens [9–11]. This procedure, in particular, is supposed to eliminate certain elements (carbon, nitrogen and hydrogen) and to enhance the relative concentration of others, thus making it easier to investigate these latter. The effects of ashing on the structure of specimens have been extensively analysed [9]. However, the chemical effects are much less well-known.

We analysed these effects, by comparing ashed and non-ashed neurobiological specimens with photoelectron-imaging spectromicroscopy [12–14].

2.1. Experimental procedure

Synchrotron photoelectron-imaging spectromicroscopy consists of sending onto the system monochromatized X-rays emitted by a synchrotron light source (the Wisconsin Synchrotron Radiation Center storage ring Aladdin, in our case) and detecting the photoelectrons thus produced. The photoelectron intensity vs. photon energy curves reproduce the optical absorption coefficient of the system, and from characteristic spectroscopic features one can extract information on the presence and status of each element [15].

We studied ashed and non-ashed neurobiological specimens with the MEPHISTO spectromicroscope [6] (described later in Section 4.3), with a spatial resolution of 0.2 μm. The specimens were primary cultures of rat cerebellar granule cells. A detailed description of our specimen preparation process can be found in Refs. [13] and [14]. In short, cells extracted from rat cerebellum were allowed to grow for 8 days on gold-coated silicon substrates, pretreated with 10 μg ml^{-1} of poly-L-lysine solution. Selective techniques were used to obtain a prevailing population of granule cells [16–18].

At the end of the growth period, the cultures were washed, fixed with para-formaldehyde and dehydrated. Some parts of the cultures were analysed in this form, and others were first ashed with a cold plasma (150 C, Plasma-Processor 300E, Techn. Plasma GmbH, München) in the presence of oxygen for $\geq$ 24 hours.

Previous tests were systematically performed to assess the possible presence of problems such as radiation damage or charging, with negative results. We are therefore confident that our present results are immune from such problems. Specifically, samples exposed for hours to the monochromatized X-ray beam (whose size was of the order of 0.3×0.3 mm^2) did not exhibit any detectable evidence of damage, such as spectral changes. Instead, substantial damage was observed after several minutes of exposure to a much more intense unmonochromatized beam (which was never used in our present experiments).

It should be noted that this kind of spectromicroscopy can investigate biological specimens without previous coating and labeling. Therefore, it studies systems in a state more similar to the natural one than most electron microscopies.

2.2. Typical results

Fig. 1 shows an ashed granule cell culture imaged by MEPHISTO. In these micrographs the contrast is originated by the different photoelectron emission yield of different elements. The images were acquired at 140 eV photon energy, for which the phosphorus photoelectron yield is higher than that of gold; therefore, the phosphorus-containing cells appear brighter than the surrounding gold substrate.

We then selected microscopic areas on micrographs like those of Fig. 1 and acquired X-ray absorption spectra by scanning the photon energy, thus revealing

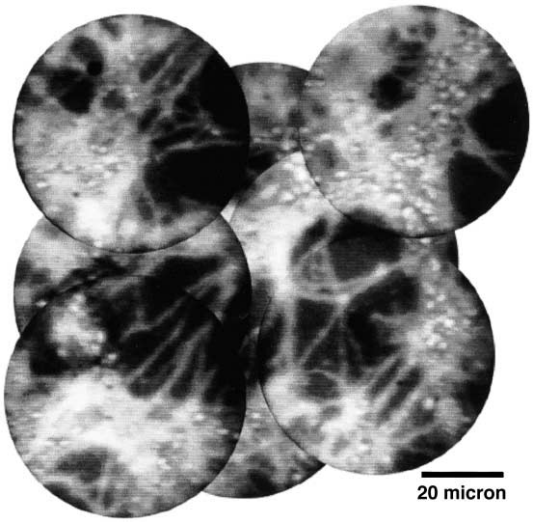

Fig. 1. Patchwork of micrographs of an ashed granule cell culture on a gold-coated silicon substrate. The images were obtained using the recently commissioned MEPHISTO microscope, illuminating the specimen with monochromatic photons of 140 eV.

the chemical composition of specific features such as cell structures. Fig. 2 shows results obtained in the spectral regions of the carbon 1s, calcium 2p, phosphorus 2p, potassium 2p and sulphur 2p edges. All spectra were normalized to the monochromator yield curve.

We see that carbon is almost completely removed by ashing. We also note that the traces of carbon left after ashing appear in a different chemical status with respect to the non-ashed sample. The first spectral feature, in fact, occurs at 285 eV (aromatic carbon) before ashing and at 286 eV after ashing. Such a difference may be related to a more oxidized C state created by oxygen-plasma ashing.

The most important point clarified by our results is the enhancement of the calcium, phosphorus, potassium and sulphur concentration by ashing. Quite interestingly, thus, ashing makes it possible to investigate the properties of calcium, potassium and sulphur in neurobiological specimens, which are of crucial importance for a large variety of fundamental physiological functions.

Such elements are in fact not visible prior to ashing. Phosphorus, on the other hand, was detectable even before ashing. Why is phosphorous not removed by ashing like, for example, carbon? We believe that this is because it is mostly present as a phosphate, and therefore already completely oxidized. To test this hypothesis, we took spectra on a dried droplet of water solution of $FePO_4$; a comparison with cell spectra (Fig. 2(c)) confirms that the P oxidation state is similar. The 142 and 145 eV peaks of the non-ashed sample spectrum are the Cls second-order peaks (at one half the energy of the corresponding features of Fig. 2(a)).

3. Special sample preparation techniques

One should note that high surface sensitivity is a general feature of this class of experimental technique, even though its degree changes substantially from one approach to another. Surface sensitivity is not always a positive factor; in fact, in some cases it is quite undesirable because it makes it difficult to investigate the inner parts of the cell.

These difficulties can be overcome, at least to some extent, by specialized sample preparation techniques. For example, we recently optimized [19,20] a cell decapping technique that makes it possible to selectively remove the outermost portions of cells, exposing their inner parts.

The decapping procedure consisted of letting a membrane filter adhere to the cell membranes and peeling it off. The peeling off step removes an increasingly large part of the cell as the adhesion times increase. The specific procedure was as follows. The samples were soaked with CS buffer (100 mM PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid) from Sigma), 5 mM EGTA (ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid from Sigma), 2 mM $MgCl_2$ from Carlo Erba, pH 6.8) [21], and covered with membrane filter (Millipore HAWP 0.45 mm pore size).

The CS buffer causes the adhesion of the cell membrane to the membrane filter. After a certain adhesion time varying from 30 s to 5 min, during which we soaked continuously to offset evaporation, the membrane filter was quickly peeled off, removing part of the cells. At this point, the decapped cell culture was rapidly fixed with 4% glutaraldehyde in PBS (phosphate buffer saline solution). After 20 min in the fixing solution, the cell cultures were rinsed twice in doubly distilled H_2O.

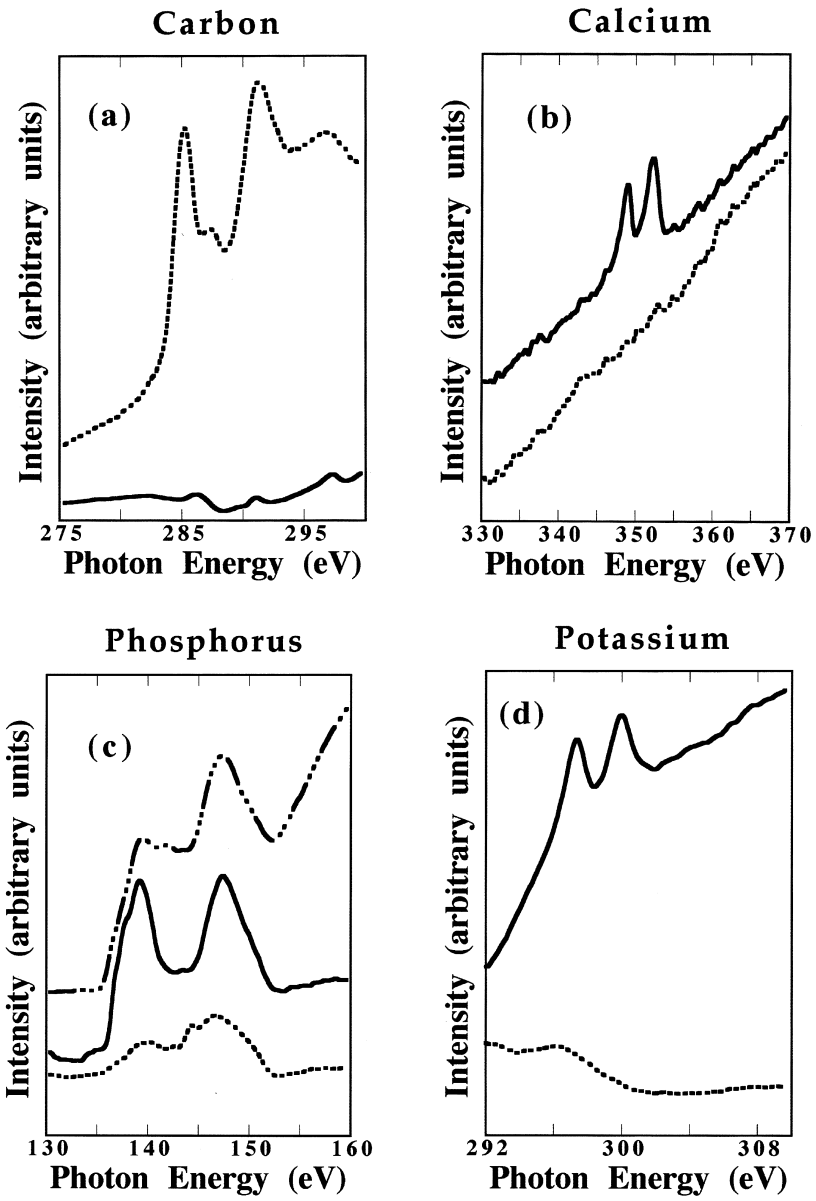

Fig. 2. (a) X-ray absorption curves in the Cls edge spectral region taken on ashed and non-ashed neuron cell culture. The solid line refers to ashed cells, the dashed line to non-ashed cells. It is evident that carbon is almost completely removed by ashing. The spot sizes from which the two spectra were taken was $20 \times 20\ \mu m^2$. (b) Similar data for the Ca2p edge spectral region. (c) Spectra of the P2p absorption edge. Also shown is a reference spectrum taken on a dried droplet of $FePO_4$ in water (dashed-dotted line). (d) Spectra of the K2p edge. (e) Data for the S2p edge. It is clear that the relative Ca, P, K and S contents are strongly enhanced by ashing.

The results of the procedure were characterized with atomic force microscopy (AFM) [20] and with spatially-averaged photoemission spectroscopy, observing the correct trends of the different elements for increasing amounts of cell membrane removal. Fig. 3 shows [22] an example of a specimen prepared with our decapping procedure: the XSEM microimage of a portion of a decapped glial cell culture. Extensive

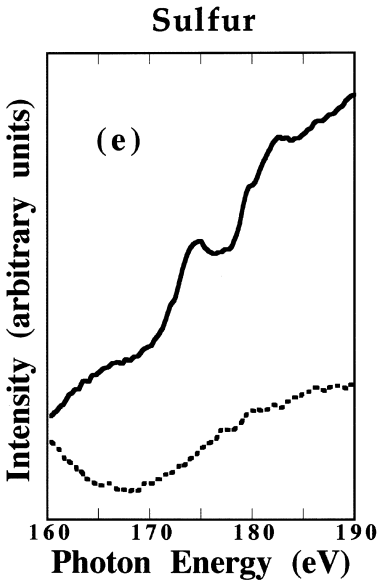

Fig. 2. Continued.

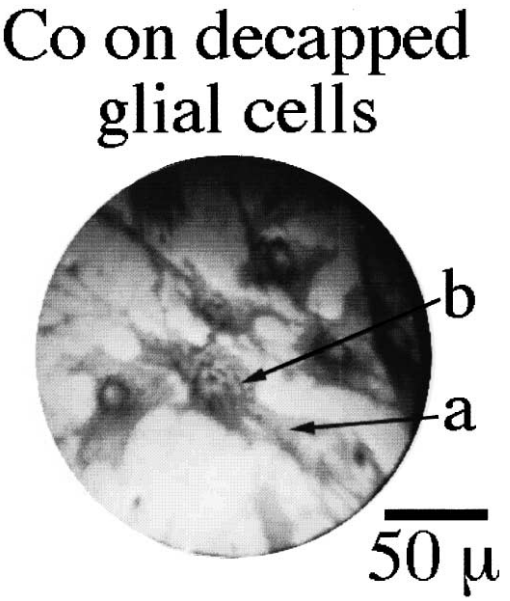

Fig. 3. XSEM microimage of a portion of a decapped glial cell culture. Data from Ref. [22].

characterization suggests [20] that an increase in the adhesion time to the membrane filter corresponds to an increase in the removed cell portion.

4. Instrumentation issues

4.1. General considerations

The development of real life-sciences experimental programs based on photoelectron spectromicroscopy [1,3,22] has revealed a series of problems. For example, most of the experiments in this domain have been performed [3] with XSEM-like [12] techniques (which, as we have seen, are based on detecting the yield of secondary photoelectrons as a function of the photon energy). The main reason is the rapidity of the data-gathering procedure: with this approach, images can be obtained in real time (a 512×512 pixel video frame can be typically obtained in 1/30 s), and the time per spectrum can be reduced to a few minutes. The rapid data-gathering is essential in life-science experiments which require the analysis of a large number of specimens.

However, the spectroscopic capabilities of XSEM-like approaches are not as advanced as those of scanning photoelectron spectromicroscopy [2,4] (based on a focused photon beam and on the energy analysis of the photoelectrons). The comparison is essentially the same as for photoemission spectroscopy compared with absorption spectroscopy [4]: with the former, one can select the final states and therefore easily deconvolve their effects from initial-state effects, whereas this deconvolution is quite complicated in absorption spectroscopy. Why, then, is scanning spectromicroscopy not used for life-science studies as widely as XSEM-like approaches?

One reason, as we have seen, is the rapidity of the XSEM data and image taking. This rapidity, in particular, makes it very easy to optimize the specimens position as far as both focusing and selecting the most interesting areas are concerned. Furthermore, XSEM-like approaches are less surface-sensitive and probably less likely to produce radiation damage, since the photon beam does not have to be strongly focused.

However, an enhanced role of scanning spectromicroscopy in the life sciences would be highly desirable. This requires a serious effort for the development of the required instrumentation. The effort concerns not only the spectromicroscopy instrumentation itself, but also the quality of the photon source [4].

This last aspect is affected by a real revolution at

the present time, with the recent commissioning [4] of the first third-generation synchrotron sources. These facilities are characterized by ultrahigh brightness and have in general superior characteristics with respect to the previous generations.

The positive impact of high brightness on microscopy and spectromicroscopy is based on some fundamental points [2,4]. One of the consequences of the Liouville theorem is the conservation of brightness along an ideal (loss-less) beamline [2,4]. This implies that applications requiring concentration of X-rays are much helped by a high source brightness level.

However, high brightness does not automatically lead to better spectromicroscopy experiments. Its full exploitation requires progress in all aspects of the instrumentation. We will briefly discuss this point in the remaining parts of our review.

4.2. Flexibility. the transmission mode

Instrumentation development efforts require very substantial resources from the financial, technical and human points of view. Luckily, the return of this effort is enhanced by the flexibility of its products: new spectromicroscopy systems can be used advantageously in a variety of modes, most of which find applications in the life sciences.

We present here one example of this flexibility. This is a recently implemented [5] soft X-ray transmission spectromicroscopy technique derived from the XSEM [12].

Transmitted X-rays were converted with a photocathode into photoelectrons, which were subsequently electron-optically processed by an XSEM producing submicron-resolution images. Test images demonstrated the excellent contrast due to the chemical differences between silicon features and a silicon nitride transmitting window. We also obtained X-ray transmission vs. photon energy curves for microscopic specimen areas.

This work is closely related to contact X-ray microscopy using image-converter detectors. Early attempts can be traced to the work of Huang, who used a tungsten target as the X-ray source [23]. Polack and Lowenthal considered the effects of chromatic aberration as a limiting factor in the spatial resolution of transmission X-ray microscopy with an emission electron microscope as a detector [24]. They constructed an electromagnetic emission microscope with a Castaing–Henry energy filter, which was briefly used on the ACO synchrotron storage ring. Our approach reaches better performances than any previous X-ray transmission spectromicroscopy technique based on electron optics [25].

Fig. 4 shows the scheme [5] of our transmission spectromicroscopy apparatus. The X-ray beam for the tests was produced by the Aladdin storage ring at the Wisconsin Synchrotron Radiation Center, and monochromatized by a 6-meter toroidal grating monochromator. Fig. 5 and Fig. 6 refer to the first successful tests [5] which demonstrated the following performances: lateral resolution better than 0.5 μm and energy resolution (of the incident X-rays) of 0.1 eV.

In Fig. 5(a), we can see silicon microstructures on an Si_3N_4 membrane. The photocathode consisted of a 50-Å evaporated Au coating on the Si nitride membrane. The photoelectrons were electron-optically

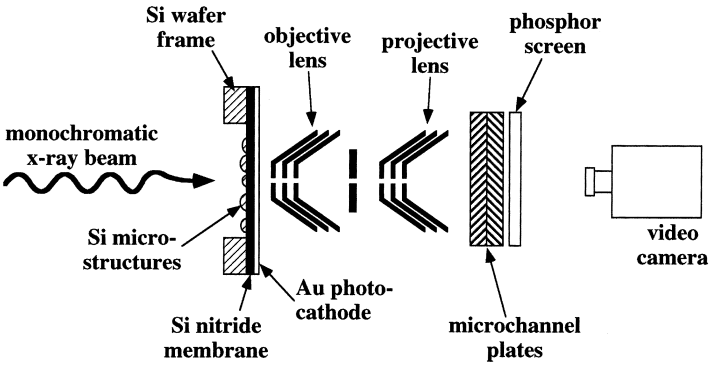

Fig. 4. Schematic diagram of our approach [5] to X-ray transmission spectromicroscopy. The specimen shown here is the one of the first test.

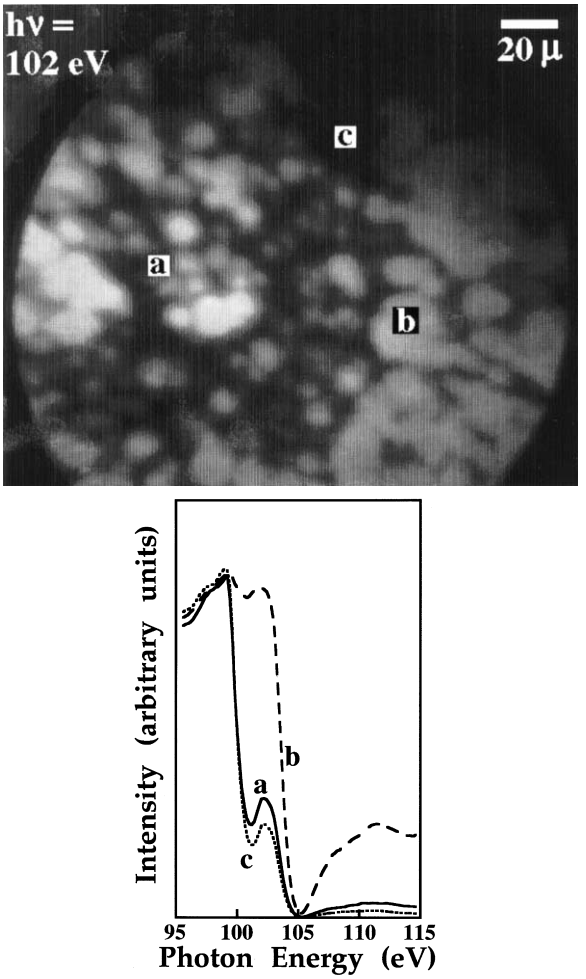

Fig. 5. (A) X-ray transmission micrograph showing silicon microstructures (dark areas) over a silicon nitride membrane. The photon energy was 102 eV, intermediate between the Si2p edges of silicon and silicon nitride. Intensity vs. photon energy curves were taken in the areas labeled a, b and c (see Fig. 5B). No image processing was used to enhance the contrast or otherwise modify the data. (B) Transmitted X-ray intensity as a function of photon energy simultaneously taken in the three areas a, b and c of Fig. 5A. The curves were normalized and corrected for the background to obtain two isosbestic points at 99.2 and 105 eV. Data from Ref. [5].

processed by the XSEM which, as it is known, consists of an objective lens followed by an aperture stop and by a projective lens [9]. The photocathode coating was kept at a negative voltage bias of 4–8 kV and the first element of the objective lens was at ground. After the XSEM, the beam was intensified by a double microchannel plate and produced a visible image on

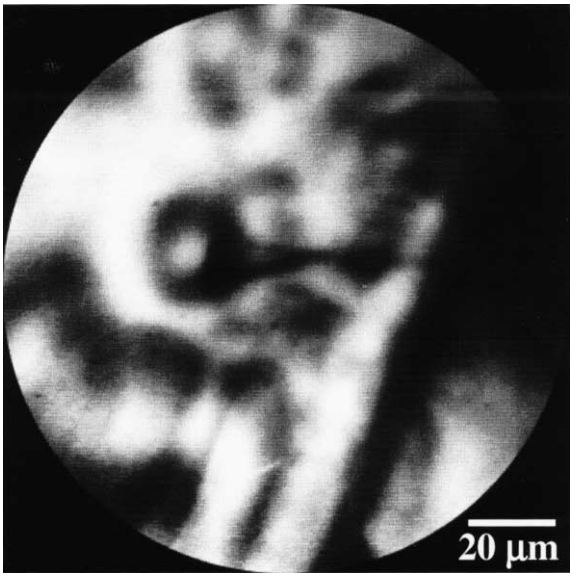

Fig. 6. Microimage of a self-standing aluminum sample, without photocathode or supporting membrane. Data from Ref. [5].

a phosphor screen. The real time visible image was viewed and captured by a video system. With the same apparatus, we took X-ray transmission curves from many local image areas by scanning the photon energy and measuring the local intensity. This is directly related to the photon intensity transmitted through the specimen.

The transmission micrograph of Fig. 5(a), obtained at a photon energy of 102 eV, is a typical result of the first test. At 102 eV, silicon nitride is transparent whereas the silicon microfeatures absorb photons appearing as dark areas. The lateral resolution is better than 0.5 μm.

Fig. 5(b) demonstrates the spectromicroscopic capabilities of our approach. Transmission curves as a function of photon energy were simultaneously taken in the areas labeled as a, b and c in Fig. 5(a). All three curves exhibit features corresponding to the silicon and silicon nitride Si2p absorption edges (100 and 104 eV). In curves a and c, which correspond to silicon microstructures in Fig. 5(a), we see a strong silicon edge and a relatively weak silicon nitride edge. In contrast, the silicon nitride edge is most prominent in curve b, which indeed corresponds to a bright area of Fig. 5(a)

Note that no image processing was done on Fig. 5(a). Even so, we can appreciate a very high

contrast in spite of the small sample thickness, which demonstrates the high sensitivity of the apparatus to chemical differences. This is of fundamental importance in life-science applications such as the microchemical analysis of thin tissue sections.

We devoted a specific effort [5] to finding the optimum thickness of the Au photocathode. In principle, this should be a thickness comparable to the photoelectron escape depth. In practice, however, the thickness cannot be decreased below a certain level without compromising the good conductivity of the photocathode, required in the high-field region between the sample and the objective lens.

We sought the optimum by evaporating different thicknesses of Au on one side of many different silicon nitride membranes. In order to create a reference for imaging, we put plastic microspheres (from Merck Fractogel) on the other side. Tests were performed for 50, 100, 200 and 500 Å thick photocathodes, which all produced good images. However, the signal level was lowest for the 50 and 500 Å specimens. This indicates that the escape depth falls between 100 and 200 Å as expected.

Fig. 6 shows typical results of another set of tests: a microimage of a selfstanding aluminium sample, without photocathode or supporting membrane. Note that the microimage of Fig. 6 is not a transmission micrograph because the photons, in this and only this case, reached the sample from the same side as the electron optics. Spectra similar to those of Fig. 5(b) were produced by photons from the opposite side.

4.3. The MEPHISTO project

The full exploitation of ultrabright synchrotron sources (such as ELETTRA, the ALS in Berkeley, Max-Lab, SRRC-Taiwan and PAL-Korea) requires novel ideas for spectromicroscopy instrumentation. One significant example in that regard is the already mentioned Swiss-Italian-American MEPHISTO project [6]. The most important objective of this program is to push to new levels the spatial resolution of XSEM-like instruments while reducing as much as possible the aberrations in the image produced by the electron optical system.

Fig. 7 shows an artist's view of the system. The basic philosophy is still quite similar to that of the XSEM or PEEM (photo-electron emission microscope). However, significant improvements are designed to achieve the previously stated objectives.

Specifically [6], the number of optical elements has been increased and the maximum accelerating voltage has also been augmented. The design lateral

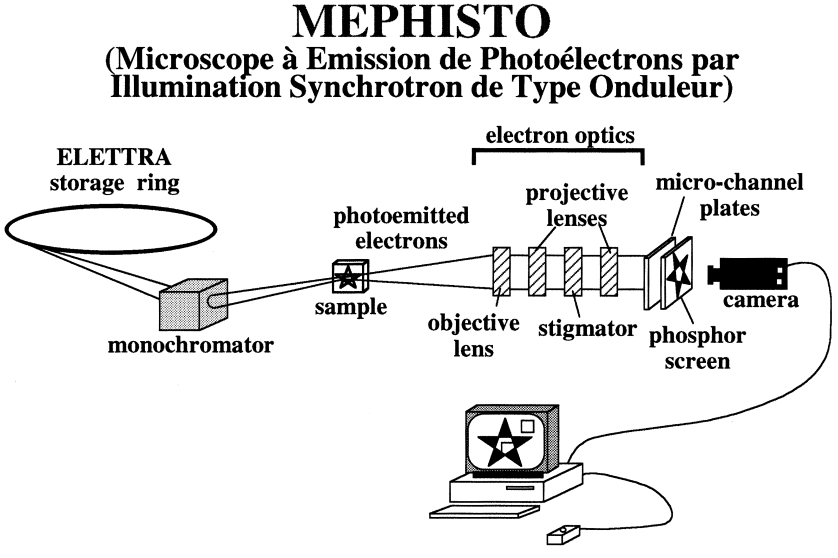

Fig. 7. Artist's view of the MEPHISTO spectromicroscope.

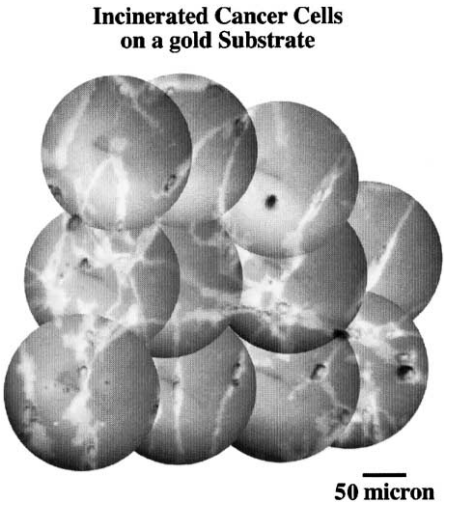

Fig. 8. An example of an photoelectron microimage produced [26] by the first version of our new MEPHISTO instrument: a patchwork of a portion of a culture of C6 cells (a cell line originally from a human glioma). Prior to data-gathering, the cells were incinerated.

resolution is better than any other instrument of the same class operating at the present time. The higher acceleration voltage also improves the instrument performances as far as chromatic aberrations are concerned.

The development of MEPHISTO began in 1995. The construction of the first version of the instrument (with a preliminary electron optics system) was initiated on October 18 of the same year and the first images were delivered on November 20. Although not yet reaching the final design performances, this first version already produced real data with a satisfactory resolution. The second version of the instrument has brought the resolution to $\approx$ 0.2 micron, and further improvements are underway.

Fig. 8 shows another example of an image produced by MEPHISTO. The micrograph patchwork shows a portion of a culture of C6 cells (a cell line originally from a human glioma). Prior to data taking, the cells were incinerated. The main objective of this study is to investigate the uptake of boron by cancer cells for the optimization of BNCT (boron neutron capture therapy) [26].

5. Towards the future: the SLS project and the CERVIN program

The struggle for brighter synchrotron sources has not ended with the third generation of facilities. Recently, the Paul Scherrer Institute of Villigen, Switzerland, developed the blueprints of a new soft X-ray source whose top brightness will exceed by a factor of five the performances of the third-generation sources [7]. This increase is expected to significantly impact the domain of spectromicroscopy and its applications in the life sciences [7].

The new source, code-named SLS, is still in the planning stage. The project has received seed financial support by the Swiss government, and a final decision is expected early in 1997. The commissioning is foreseen for the year 2000.

How will SLS impact spectromicroscopy? Basically, it will enable us to add a new dimension to the current experiments. Consider the evolution of photoemission-based techniques [2,4], which is a direct result of the progress of the synchrotron sources. First, angular resolution was added, but spatial resolution, time resolution and ultra-high energy resolution were not possible. With the third-generation sources, each one of these performances is possible, but one cannot achieve two of them simultaneously. Perhaps the only exception to this is the XSEM which combines spatial resolution and rapid data-gathering. We have seen, however, that the spectroscopic capabilities of the XSEM technique do not match those of scanning spectromicroscopy, for which time resolution is currently impossible.

With the SLS, a combination of two of the above performances becomes possible. The most exciting possibility is to combine spatial resolution, spectromicroscopy, with either time resolution or with ultra-high energy resolution. Tentatively, submicron spatial resolution should be compatible with 1-second time resolution or better in the scanning mode, and with 10-meV energy resolution at low photoelectron energies (a few 10 eV).

A specific plan is being proposed [27] by two Swiss universities for the development of the corresponding facilities. The plan involves the Applied Physics, Experimental Physics and Micro-Optoelectronics Institutes of the Ecole Polytechnique Fédérale of Lausanne, together with the Experimental Physics

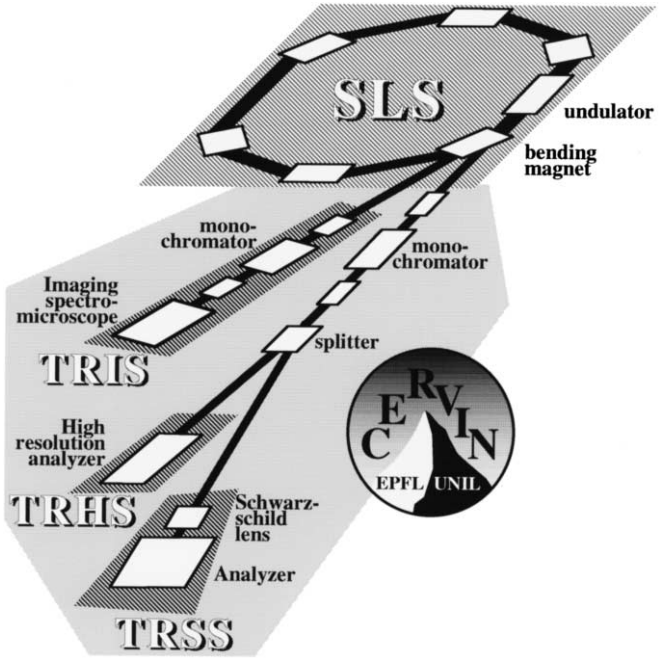

Fig. 9. Artist's view of the CERVIN facility [27] for the Swiss Light Source [7] with its three branches: ''Time-Resolved Scanning Spectromicroscopy'' (TRSS), ''Time-Resolved High-resolution Spectroscopy'' (TRHS), and ''Time-Resolved Imaging Spectromicroscopy'' (TRIS).

Institute of the University of Lausanne. The code-name of the project is CERVIN (Cooperation for an Experimental Radiation Venture in Nanoscience), the Swiss-French name of the Matterhorn.

Fig. 9 shows an artist's view of the CERVIN facility [27]. This consists of the combination of three different branches, two of which are dedicated to spectromicroscopy. Monochromatized undulator radiation is exploited in two branches: the ''Time-Resolved Scanning Spectromicroscopy'' (TRSS) branch and the ''Time-Resolved High-Resolution Spectroscopy'' (TRHS) branch. In addition, a bending magnet will feed photons to a third branch: ''Time-Resolved Imaging Spectromicroscopy'' (TRIS), an XSEM-like instrument capable of extremely fast data-gathering.

As the detailed plans for SLS and the CERVIN facility are being completed, the future for spectromicroscopy and its life science applications appears quite exciting. The very name of the new project makes this point: with SLS, one can climb to the top of synchrotron radiation performances—the Matterhorn-Cervin. The actual climbing is made possible by specialized equipment such as the CERVIN system. And after reaching the top, the sky is the limit!

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