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A HIGH VACUUM PHOTOELECTRON MICROSCOPE FOR THE STUDY OF BIOLOGICAL SPECIMENS.

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Abstract

A photoelectron microscope (photoemission electron microscope) has been designed and built for the study of organic and biological samples. The microscope is an oil-free stainless steel high vacuum instrument pumped by a titanium sublimation pump, an ion pump, and molecular sieve roughing pumps. The electron lenses are of the electrostatic unipotential type. The microscope is equipped with a dewar for sample cooling, an internal cryogenic camera, TV-image intensifier, and vibration isolation support. Applications include studies of biological cell surfaces, photosynthetic membranes and aromatic chemical carcinogens. A representative micrograph of mouse 3T3 cells is included. In some respects, photoelectron micrographs resemble scanning electron micrographs, but the basis for contrast is different in these two techniques.

KEY WORDS: Photoelectron microscopy, photoemission, biological cell surfaces

Introduction

Photoelectron microscopy has only recently emerged as a technique for studying biological samples[1-4] although its roots date back to the early days of electron microscopy.[5,6] A photoelectron microscope (PEM) is a type of emission microscope in which UV light is incident on the specimen and causes the emission of electrons. The emitted electrons are accelerated and imaged by an electron lens system. This instrument is also called a photoemission electron microscope. A recent overview and bibliography of photoelectron microscopy has been published in connection with the First International Conference on Emission Electron Microscopy held in Tübingen in 1979.[7] In addition, there are other useful reviews of the physical aspects and (nonbiological) applications of PEM.[8-10]

There are two principal sources of contrast, one arising from differences in photoelectron yields and the other from surface topography. In Fig. 1, PEM is compared to the more familiar SEM, TEM, and fluorescence microscopy techniques. PEM is used primarily for imaging surfaces, and the areas of application overlap those of SEM. However, PEM is not a scanning technique. It has in common with TEM an electron optical system for imaging the emerging electrons. PEM can also be thought of as an electron optical analog of the fluorescence microscope where images are formed by emitted electrons rather than by fluorescent light. PEM offers the possibility of extending certain fluorescence microscopy experiments to the much higher resolution

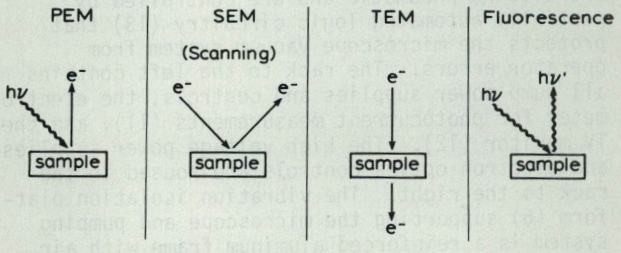


Fig. 1. Comparison of photoelectron microscopy with three other types of microscopies, showing the incident and emergent radiation. Only the secondary electron mode is shown for the SEM.

obtainable with electron optics. In this paper we describe a photoelectron microscope designed and built at the University of Oregon for the study of biological specimens.

Overall Design

The design goals include (1) minimization of sample contamination (2) control of specimen heating and (3) a sensitive detection system. To avoid sample contamination an oil free ultrahigh vacuum pumping system is used, and the microscope body is of stainless steel. Copper sealed ultrahigh vacuum flanges are used wherever practical in place of elastomer O-rings. Adjustable units, such as the specimen manipulator and aperture control, are metal bellows assemblies with x, y and z translators. Specimen heating is controlled by means of a small dewar connected to the specimen support and by the optional use of filters to block IR from the illuminating system. The third goal requires maximum signal detection efficiency while maintaining a clean vacuum system. This problem was solved using an internal cryogenic camera and a fiber optically coupled image intensifier-TV system.

A simplified diagram of the photoelectron microscope is shown in Fig. 2. UV light from short arc lamps (Cd doped Hg-Xenon, Advanced Radiation Corporation) is focused and reflected by the polished surface of the anode onto the specimen surface. This UV optics geometry optimizes the solid angle of the illumination.[11] Electrons released from the specimen by UV light are accelerated across a 3mm gap between the specimen and anode. The specimen, which serves as the cathode, is maintained at a negative potential (up to -40 kV) and the anode is at ground potential. Electrons passing through the anode aperture are focused by a conventional electrostatic lens system. The final image is received either on a phosphor-coated fiber optics output window connected to an image intensifier-TV system or on a photographic film.

A photograph of the photoelectron microscope is shown in Fig. 3. The microscope column (1) and camera system (2) are connected to the ultrahigh vacuum system through a port in the back side near the top of the microscope column. The pressure near the pumping port is 10^{-9} torr or lower without baking. The pumping system consists of a Varian Ti-Ball titanium sublimation pump backed by a Varian 220 liter/sec triode ion pump and three molecular sieve roughing pumps. All valves are electro-pneumatic and are controlled by partially automated logic circuitry (13) that protects the microscope vacuum system from operator errors. The rack to the left contains all pump power supplies and controls, the electrometer for photocurrent measurements (11), and the TV monitor (12). The high voltage power supplies and electron optics controls are housed in the rack to the right. The vibration isolation platform (6) supporting the microscope and pumping system is a reinforced aluminum frame with air cushions at the four corners.

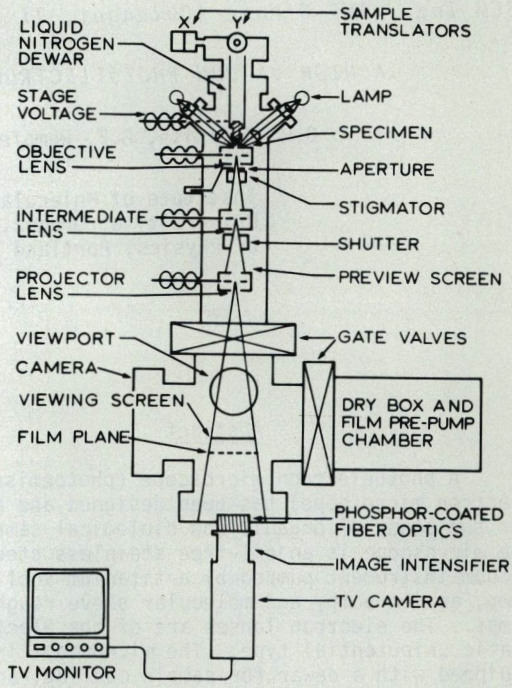


Fig. 2. Schematic diagram of the University of Oregon photoelectron microscope.

Electron Optical System

A three-stage electrostatic lens system is used to form the image of the specimen. The lenses are of the 3-electrode unipotential type. The outer electrodes are grounded and the center electrode is provided with an adjustable negative potential. The lens potentials are derived from a voltage divider supplied by the accelerating voltage source. The lens system has been tested to better than 20 Å resolution in the conventional TEM mode. However, resolution in PEM is limited by aberrations associated with the acceleration process in emission microscopy. Without aberration correction the theoretical resolution limit in PEM is about 50 Å. At present the resolution is conservatively 150 Å as demonstrated in Fig. 4. This performance is in the range of previous instruments designed for metallurgical and inorganic studies.[8,11] Further improvement in the resolution of this PEM is expected with refinements in the sample stage, increased shielding and UV wavelength control.

The object for the lens system is a virtual image of the specimen, formed in the acceleration process. In the normal imaging mode the objective lens is designed to focus on the object when the center electrode is at approximately cathode potential. An additional small adjustable d.c. voltage on the objective center electrode provides electrical focusing. The intermediate and projection lenses are identical. The magnification is selected by the voltage settings for these lenses and ranges up to x20,000 at the film plane.

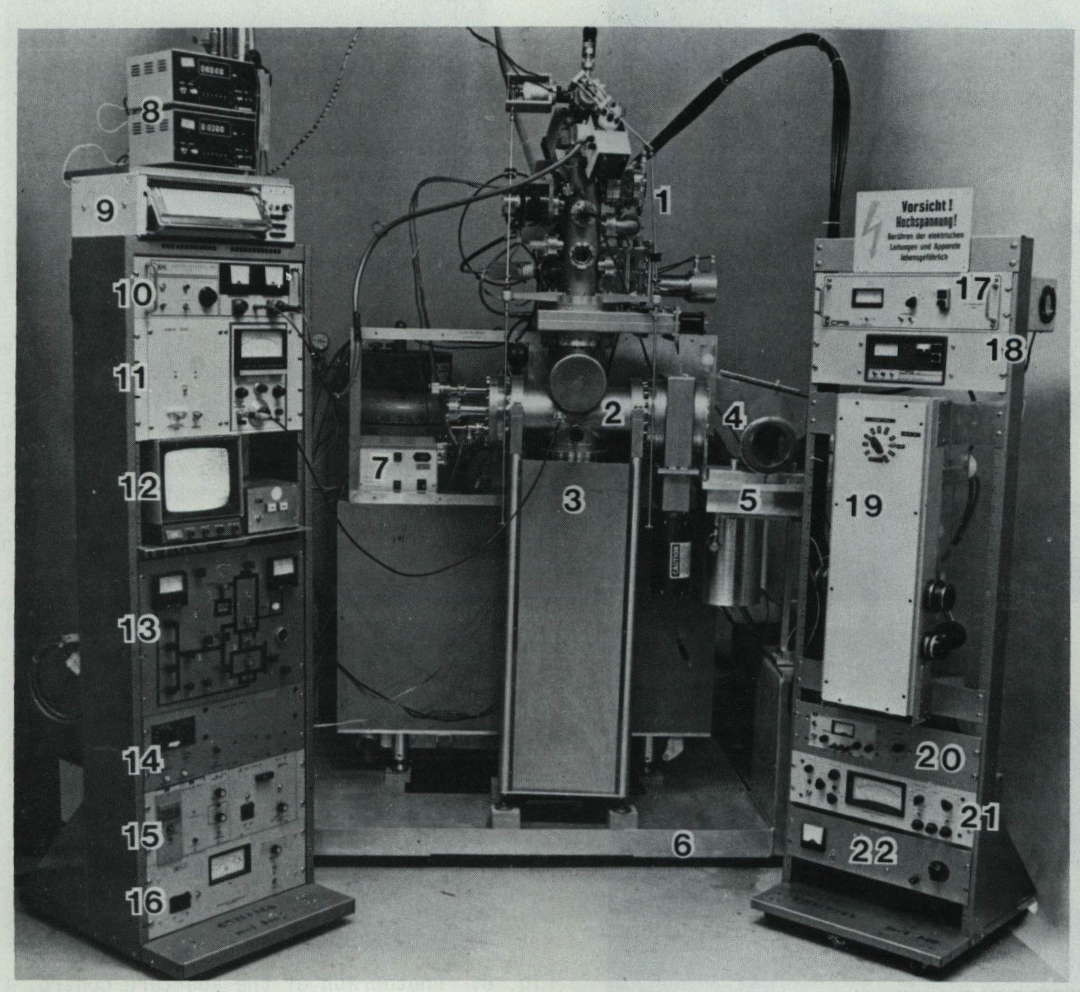
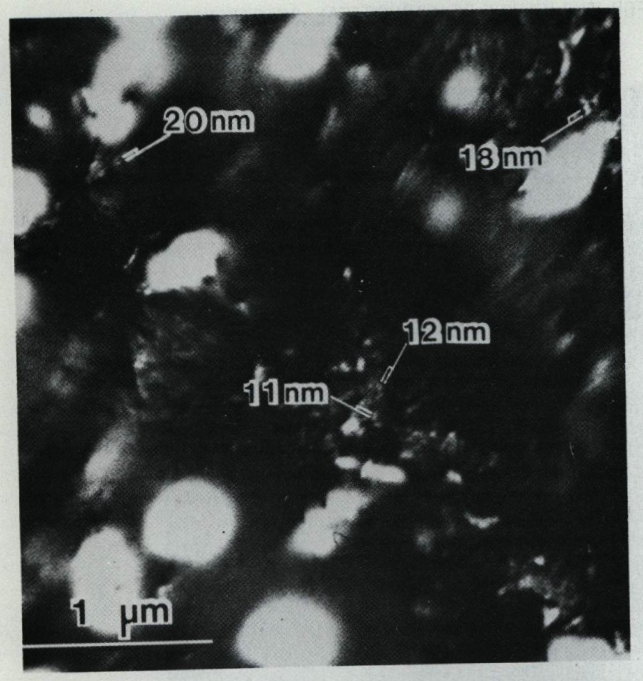


Fig. 3. Photograph of the University of Oregon photoelectron microscope. (1) microscope column, (2) internal camera system, (3) image intensifier and TV camera behind shield (Varo two-stage electrostatic image intensifier coupled by fiber optics to a Cohu 2810 series TV camera, 525 line-scan), (4) dry box, (5) film prepump chamber, (6) vibration isolation platform (Newport research), (7) UV lamp power supply (PEK), (8) specimen xy translator position indicators (Mitutoyo), (9) strip-chart recorder for electrometer, (10) UV lamp power supply (Zeiss), (11) electrometer (Keithley Instruments Model 602), (12) TV monitor, (13) vacuum system logic controls, (14) electronic shutter and timer controls, (15) titanium sublimation pump control (Varian Ti-Ball Control Unit), (16) ion pump power supply (Varian Vacion Control Unit), (17) 50 kV regulated DC power supply (CPS Model 101N), (18) 30 kV regulated DC power supply (CPS Model 100N), (19) Voltage divider for the electron optics system, (20) stigmator amplitude and azimuth controls, (21) photometer (for test purposes only), and (22) power supply for panel lights.

The projector lens was designed for distortionless projection at full (cathode) voltage, or when operated in appropriate combinations with the intermediate lens at lower voltage ratios. For very low magnification the objective lens is used with its power very much reduced as a field lens, and the image is formed by the projector lenses. A photograph of the unassembled lenses is shown in Fig. 5.

An aperture stop is located following the objective lens in the exit pupil plane (close to the rear focal plane) and within the re-entrant portion of the rear electrode. The stop can be moved in and out of the beam by means of the aperture control shown in Fig. 6. The micrometers provide a z-adjustment and one of the scanning motions. The large knurled knob controls the other scanning motion. The arm holding the aperture can be detented in either a horizontal or an upright orientation. During assembly to the microscope column, the arm shifts to the upright position shown in Fig. 6, placing the aperture at the desired elevation.



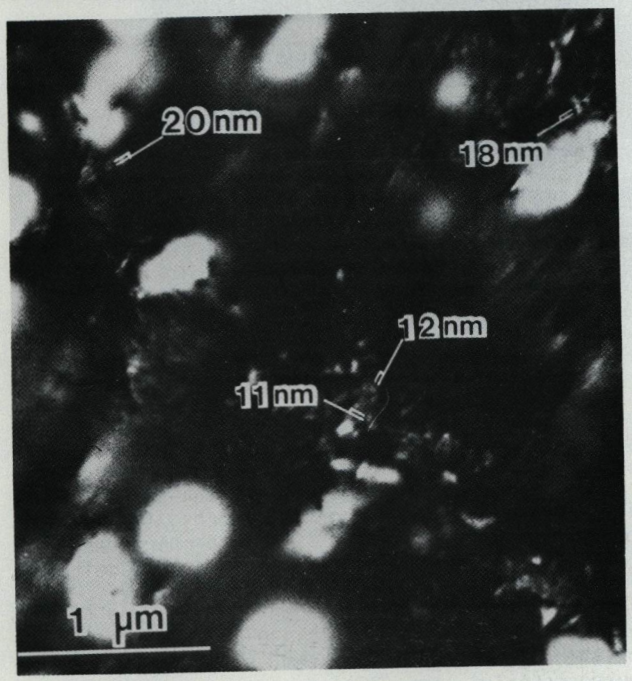


Fig. 4. Resolution test of the photoelectron microscope. Sample: beryllium-copper (2% Be) etched for 2-3 minutes at 24 °C with a freshly prepared 10% ammonium persulfate aqueous solution. The surface details used for the resolution measurement have not been characterized. The image did not change with time and there is no evidence for surface alteration by the UV irradiation. Accelerating voltage -30 kV, cathode-anode gap: 3.3 mm, aperture: 50 μm, electronic magnification: x9,000, illumination: two 100 W, Cd doped Hg-Xe short arc lamps, emulsion: Kodak electron image film 4463, exposure time: 15 sec.

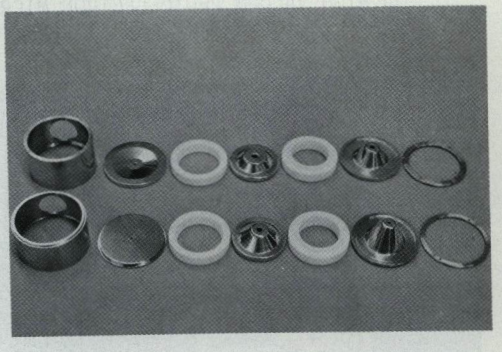


Fig. 5. Photograph of the disassembled projector lens (top) and objective lens (bottom). The intermediate lens is identical to the projector lens. From left to right: lens housing, front electrode, insulator, center electrode, insulator, rear electrode, and retaining ring. The center electrode of each lens is operated at a negative potential and the outer two electrodes are grounded. The outside diameter of the housing is 5.4 cm.

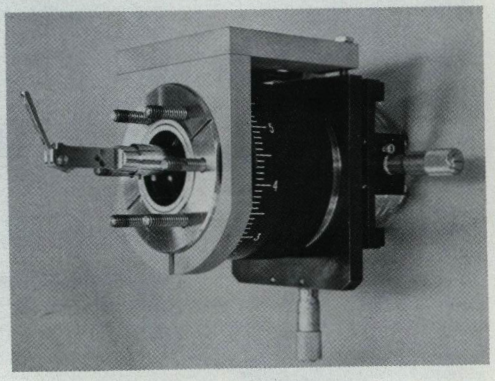


Fig. 6. Photograph of the aperture control (rear view). The flange has an outer diameter of 7 cm.

Astigmatism is compensated by a six-pole electrostatic stigmator located just below the aperture stop, as indicated in Fig. 2. An electrostatic shutter immediately below the intermediate lens is used during the process of recording the image on film. The shutter works on a deflection principle in which the shadow of a shutter stop covers the entrance to the projector lens when the shutter voltage is applied.

Camera and Viewing System

Recording of photoelectron images is done directly on photographic film in order to avoid any loss of information through the fiber optics window and video system. To prevent the photographic plates from becoming a source of contamination, a cryogenic camera was designed which can be cooled to 77 °K using liquid nitrogen. The camera may also be used at room temperature with plates dried over P_2O_5 under vacuum in the attached dry box and film pre-pump chamber. A diagram of the camera is shown in Fig. 7. More details are given elsewhere.[12]

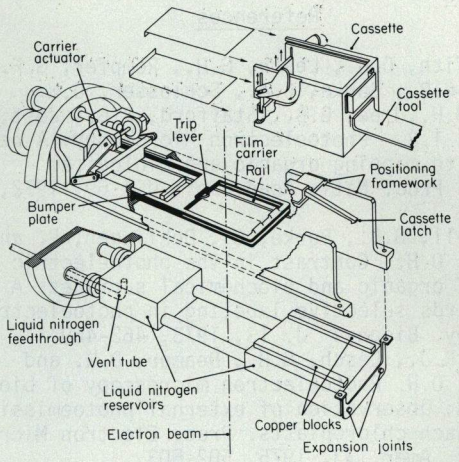


Fig. 7. Exploded view of the internal cryogenic camera showing the film cassette and film carrier mechanism (from [12]).

Except during exposure, the film in the camera is recessed in a cassette, and passage of the electron beam to the fiber optics output window is unobstructed. To make an exposure, the film is advanced from the upper chamber of the cassette to the film plane and after exposure it is returned to the lower chamber of the cassette. The cassette holds ten plates.

The image on the phosphor-coated fiber optics window is viewed on the TV monitor after enhancement by the two-stage image intensifier. The magnification on the monitor is about 20X that at the film plane. The image can also be viewed on a preview screen just above the projection lens or on the final tiltable screen above the film plane.

Applications

All substances emit electrons when exposed to sufficiently short wavelength light so, in principle, any specimen can be imaged using photoelectron microscopy. In practice, photoelectron microscopy is best utilized in applications where advantage can be taken of the very high sensitivity to topographical relief and differences in photoelectron quantum yield. Promising areas of application in biology include well-spread cells grown in tissue culture[13] and photosynthetic membranes.[14] An example of a photoelectron micrograph of tissue culture cells is shown in Fig.8. The cells are uncoated mouse 3T3 cells grown on the sample mount. Identifiable in the micrograph are the nucleus, nucleoli, fibrous elements of the cytoskeleton and some finer detail. The main source of contrast is topographical rather than photoelectron quantum yield contrast since much of the detail remains after metal coating.[15] Topographical contrast is caused by field disturbances produced by surface relief.[8,10,16] Electrons emitted from sloping surfaces are deflected by the disturbed field. The effect is more pronounced than in SEM because the sample is in a high electric field and the very low energy photoelectrons are readily deflected by this field. The sensitivity to topographical detail is so great that PEM is inappropriate for specimens with large relief such as rounded up cells. In this sense SEM and PEM are complimentary. SEM excels at imaging structures with large relief whereas PEM is most useful in examining fine surface detail on flatter structures.

To take advantage of photoelectron quantum yield contrast there must be significant differences in the photoelectron quantum yields of the surface components, or a photoelectron label must be introduced. The strategy is reminiscent of fluorescence microscopy, where either intrinsic fluorescence or fluorescence labels are used. In PEM, large conjugated molecules such as chlorophyll

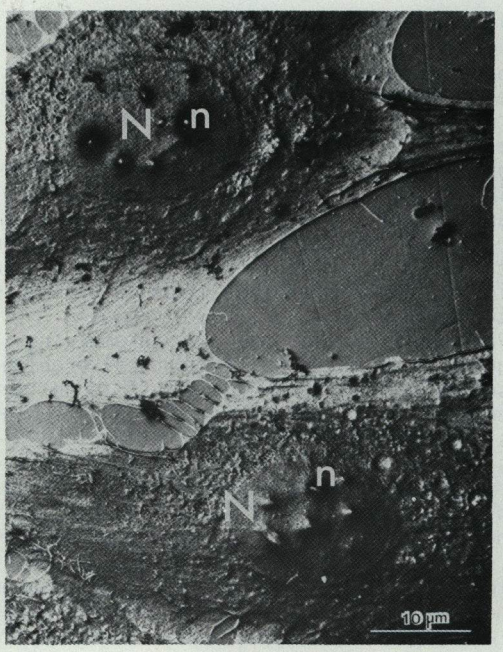


Fig. 8. Photoelectron micrograph of uncoated mouse 3T3 cells (a subculture of A31) grown in tissue culture. Indicated on the micrograph are cell nuclei (N) and nucleoli (n). Fibrous elements are evident throughout the micrograph. The region between the cells, in the center of the micrograph, shows a large number of fibrous elements and some retraction fibers. Cells were seeded on the stainless steel mount, grown overnight at 37 °C in Dulbecco's modification of Eagle's medium (Flow Laboratories) containing 10% fetal bovine serum. The culture was fixed in 2.5% glutaraldehyde, 0.05M cacodylate buffer, 6% sucrose, pH 7.4 for 15 minutes at 37 °C and then transferred to 4 °C and stored in fixative. Dehydration was accomplished through graded hexylene glycol before air drying. Some lipid is probably removed in this procedure due to the omission of a post fixation in OsO_4. PEM conditions are the same as in Fig. 4 except that the electronic magnification was x760 and the exposure time was 10 sec. Photoelectron microscopy of metal coated cells prepared by similar methods are presented elsewhere.[15]

have high relative photoelectron yields. Thus, PEM is a potential method for studying the distribution and organization of photosynthetic pigments in photosynthetic bacteria and chloroplasts of higher plants.[14] In cell surface studies it should prove possible to use photoelectron labels covalently attached to protein with high site specificity such as antibodies or the plant lectins. The advantage would be in the smaller size of the labels and higher resolution localization of membrane antigen molecules, since detection is based on photoemission as well as on label topography.

Photoelectron microscopy is an emerging technique and there are still technical problems to be solved. The origin of contrast is not yet completely understood and there are other factors such as substrate photoemission, reflection effects, and electric field effects that may contribute to the contrast and depth of information in certain cases.[17,18] As in SEM, sample charging can be a problem. A general study of sample supports and sample preparation methods to optimize the PEM images has yet to be undertaken. Nevertheless, the initial results here and in Europe are promising. For example, photoelectron images have been obtained of thin sectioned biological specimens,[1,4,19] patterns of fluorescent dyes,[1,2,20] photosynthetic bacteria,[14,21] chloroplasts,[14] chemical carcinogens such as the polycyclic aromatic hydrocarbons[22] and aromatic amines,[23] spermatozoa,[24] cells grown in tissue culture,[13,15] (e.g. Fig. 8) and TMV (this laboratory, unpublished data). The photoelectric properties of a number of biomolecule model systems in the solid state have also been examined,[13,25-30] contributing to a better understanding of the photoelectron images of biological specimens.

Conclusions

A photoelectron microscope has been designed specifically for the study of organic and biological specimens. In this instrument, sample contamination is minimized by the use of ultrahigh vacuum components, and specimen heating is controlled by cooling the specimen support with a liquid nitrogen dewar. A sensitive detection system is provided which is compatible with the clean vacuum design. Good photoelectron images are obtained from a variety of biological specimens. Since the contrast mechanisms are different from SEM and TEM, PEM has the potential of providing new information about the organization of biological surfaces.

Acknowledgements

We wish to thank D.L. Habliston for expert technical assistance and contributing to the design of the vacuum logic circuitry, W.A. Houle for help with selection of the vibration isolation table and Dr. K.K. Nadakavukaren for the mouse 3T3 specimens. We are also indebted to Drs. J.P. Revel, J.A. Weston, W. Engel and P.C. Jost for useful discussions. This work was funded by PHS Grant CA 11695 from the National Cancer Institute and NSF Grant PCM-7911501.

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Discussion with Reviewers

D.W. Deamer: One of the most exciting potential applications of the PEM method is to examine surfaces of freeze-fractured specimens, particularly chloroplasts and mitochondria which have membrane components that should be highly photoemittive. Are there any basic problems that would limit PEM in this regard?
Authors: As far as we know there are no fundamental reasons why freeze-fractured samples cannot be examined in the PEM. This experiment requires a high vacuum sample preparation chamber connected directly to the microscope so that the specimen can be transported and observed after fracturing (such a chamber is under construction). Freeze etching could be carried out in the PEM with simultaneous observation of the image. Some fractured surfaces might be too rough for observation in the PEM.

N.S. Allen: As this paper is being published in an SEM journal, could the authors explain a bit more carefully the advantages and differences of this method as compared to SEM, and possibly the x-ray scanning microscope?
Authors: We believe the advantages of PEM are (1) high sensitivity to fine topographical detail, (2) a new source of contrast (photoelectron quantum yield contrast) (3) higher depth resolution (short escape depth of the photoelectrons[25]) and (4) less damage to the biological specimen (the UV light causes less damage than ionizing radiation). As a consequence of the high sensitivity to topographical detail, PEM cannot match SEM in the visualization of objects with pronounced relief. PEM does not provide information regarding the elemental composition of the surface layers as does SEM with an x-ray detector. In short, SEM and PEM are complementary in their range of applications. (See also reference 9).

L. Wegmann: With regard to all the measures taken to obtain a very clean vacuum, would it not be permissable to speak of this as an ultrahigh vacuum PEM?
Authors: Yes, this PEM is an ultrahigh vacuum instrument. More important to us is that it is a clean vacuum system. Purists in surface physics would probably want to see the system bakable and all valves metal-sealed. This might be needed for some studies of freshly cleaned metal surfaces. However, even if trace quantities of oxygen and water vapor were present, the biological specimens are insensitive to them. Therefore, in order to increase reliability and decrease the cost, oil-free Viton O-ring sealed valves are used in place of metal sealed valves. Also, Rexolite insulators are frequently used in place of ceramic insulators in the electron lenses, so the microscope is not baked. However, if desired, ceramic insulators can be used and all Viton sealed valves could be replaced by metal-sealed valves.

R. Schwarzer: The question of contamination of the specimen surface in photoemission electron microscopy is important. What is the residual gas composition in the region of the specimen?
Authors: We do not use a residual gas analyzer but any nonmetal parts are analyzed first in a high resolution mass spectrometer. Rexolite, for example, showed no organic peaks in the mass spectrometer. There is no oil anywhere in the instrument. The microscope is backfilled with dry nitrogen gas, boiling off from a liquid nitrogen dewar connected to the system by a stainless steel bellows (i.e. no contact with plastics).

R. Schwarzer: It is not clear to me how you protect the specimen from heavy contamination when cooling the specimen support with a liquid nitrogen dewar. A cold trap which surrounds

the sample and includes the front (anode) electrode of the objective lens might shield the specimen sufficiently against warmer surfaces in the vicinity.

W. Engel: How is high voltage insulation and good heat conduction between specimen and Dewar obtained?
Authors: The cathode cup, which surrounds the specimen, is cooled by the dewar through a beryllium oxide rod which electrically insulates the cathode from ground potential. The specimen mount is cooled by contact with the cathode cup, so that the dewar above and cathode cup act as anti-contamination surfaces. Below there is the camera system which can be operated at liquid nitrogen temperatures, serving as another anti-contamination device. Even without the use of the liquid nitrogen dewar the base pressure is 10^{-9} torr or better. At 10^{-9} torr it takes about 17 minutes to form a monolayer and at 10^{-10} torr over two hours. These figures assume the worst case of a sticking coefficient of unity, which is unlikely for nitrogen gas especially with UV illuminating the specimen surface.

R. Schwarzer: The spectral reflectivity of the anode mirror affects the irradiance as well as the spectral composition of the UV radiation at the specimen. This is noticeable particularly with stainless steel mirrors at shorter UV wavelengths. What materials are your anode mirrors made of? Will you also attach a direct (non-reflecting) illumination system for studies of the dependence of image contrast on UV wavelength and polarization?
Authors: We currently use a polished stainless steel mirror anode because of its durability. We intend to use focal isolation for UV wavelength control in which case the mirror will affect only the irradiance. In this particular configuration of the system we are attempting to maximize the UV illumination rather than study the effect of polarization. No direct illumination system is planned in the near future.

R. Schwarzer: What are the characteristic data of your lens design: the focal length, the spherical and chromatic aberration coefficients of the emission (objective) lens, bores, thicknesses and distances of the electrodes?
Authors: The focal length of the objective lens is approximately 7 mm at full voltage. We do not have sufficient space here to provide a detailed description of the components of the electron optical system. Plots of the focal properties of the electron lenses and the aberration coefficients as a function of voltage ratio will be published later along with lens dimensions and a description of the cathode region, stigmator, and electronic shutter.

L.A. Staehlin: During the past few years you have made rather spectacular progress in improving the resolution of the photoelectron microscope, thereby significantly improving its potential usefulness for biological applications. What are, in your opinion, the major problems in specimen preparation techniques that need to be overcome to make the microscope a practical tool for biologists, particularly those that are presently utilizing fluorescent-labeled antibodies for their studies of cell surfaces and cytoskeletons?
Authors: Some of the sample preparation problems are the same as for SEM studies of uncoated biological specimens. One is sample conductivity. Further work is needed on sample preparation techniques that increase conductivity without altering the cell surface. Two other sample preparation problems that need more effort are the selection of sample substrates for PEM and the development of photoelectron labels.

W. Engel: Several techniques have been used in electron microscopy to detect immuno labels. Does the PEM offer advantages in detecting immuno labels?
Authors: Yes, we believe PEM has distinct advantages in detecting labeled antibodies and lectins on cell surfaces, but this is yet to be proven. Current SEM studies rely on recognizing markers by their size and shape. Often markers that are technically within the resolving power of the instrument cannot be distinguished from the natural surface detail of cells. PEM is more sensitive to the fine surface detail and has a higher depth resolution so distinguishing smaller markers should be possible. Moreover, once suitable photoelectron labeling methods are developed the high photoemission of the markers will make them much easier to see. The goal is to use a molecular marker; a marker that is small compared to the size of the antibody or lectin. In other words, the goal is a PEM analog of the fluorescent antibody method. Some progress has been made. Fluorescein, rhodamine and ANS dyes all have moderately good photoelectron yields and the yields of the chlorophylls are higher (see reference 14) and could be used as labels. All large π-conjugated molecules tested so far have relatively high yields. Furthermore, these aromatic molecules can emit electrons repeatedly over a period of time, greatly improving the statistics of image formation (in contrast to autoradiography where events only occur once so a high density of radioactive labels is required). We do not mean to leave the impression that photoelectron labeling will be accomplished quickly since there are many problems. However, fundamental advantages do exist and should be exploited.