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THE POTENTIAL ROLE OF PHOTOELECTRON MICROSCOPY IN THE ANALYSIS OF BIOLOGICAL SURFACES

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Abstract

The photoelectric effect provides the basis for an imaging technique useful for the study of biological surfaces. The photoelectron microscope (PEM) employs a UV lamp to photoeject electrons from the specimen surface. The electrons are then accelerated and imaged using electron optics. Photoelectron micrographs often resemble scanning electron micrographs, but the origin of contrast is different and these two techniques are complementary. Scanning Electron Microscopy (SEM) is unsurpassed in applications where specimens have pronounced relief or where elemental analysis is required. The advantages of PEM are a new origin of contrast, high sensitivity to fine topographical detail, short depth of information, and low specimen conductivity requirements. Photoelectron images of model systems, cell surfaces and cytoskeletal elements have been obtained.

KEY WORDS: Photoelectron microscopy (PEM)/ photoelectric effect/immunofluorescence/cell surface/cytoskeleton/carcinogen/colloidal gold/ vimentin/deoxyribonucleic acid/viruses.

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Introduction

A complete analysis of a biological surface requires a knowledge of the structures and locations of all of the components of that surface. In spite of the availability of several surface-directed analytical techniques, this remains a formidable task. One problem is that the same element can be combined in a myriad of ways to give compounds of strikingly different chemical properties. For example, a surface containing only carbon, hydrogen and oxygen could be a sugar or an aromatic carcinogen. An elemental analysis of this surface, although useful, would not yield sufficient information about its characteristics. A second problem is that the composition often changes rapidly with depth. The most common biological surface, for example, is the plasma membrane. The membrane is typically about 10 nm thick, with a composition that varies both laterally and vertically, and differs markedly in structure from the organization of the underlying cytoplasm. Any analytical approach to such a surface must contend not only with this complexity, but the fact that organic and biological specimens are poor conductors and are easily damaged by charged particle beams.

Photoelectron microscopy (photoemission electron microscopy or PEM) is now being developed to the point where it can begin to contribute to the analysis of biological surfaces. This imaging technique utilizes the photoelectric effect, wherein electrons are released from the specimen by the action of ultraviolet (UV) light. Applications of this technique in physics are reviewed elsewhere (Schwarzer, 1981; Pfefferkorn and Schur, 1979; Wegmann, 1972), and the ultrahigh vacuum photoelectron microscope developed at the University of Oregon for biological studies has been described in a previous volume in this series (Griffith et al., 1981a). Photoelectron microscopy is not yet an established method in cell biology but it qualifies as an emerging technique. The purpose of this paper is to review some of the recent preliminary applications of photoelectron microscopy to biological surfaces and to comment on the advantages and limitations of this technique.

Basic Principles

The photoelectron microscope is essentially a photoelectric cell with electron lenses built in to produce high spatial resolution. The key elements are (1) a source of UV light, (2) a vacuum chamber, (3) a specimen mounted on the flat cathode, (4) an electron field across the cathode-anode gap to accelerate the low energy photoelectrons, (5) a transmission electron microscope-like electron optical system consisting of an objective, intermediate, and projector lens, and (6) an image intensifier-TV monitor and camera. Neither the UV light nor the electron emission are scanned. A diagram of the electron optics in the region of electron emission is shown in Fig. 1. The electrons leave the specimen surface in many directions and are accelerated by the electric field along a set of approximately parabolic trajectories. Thus, the electrons appear to be coming from the virtual object at point f^* where the tangents to these parabolas intersect (Fig. 1b). The electrons then pass through the anode, which acts like a weak diverging lens to produce a slightly demagnified virtual object at a distance $-(4/3)f$ as shown in Fig. 1c (Rempfer et al., 1980a). After the fully accelerated electrons leave the anode region, the image is magnified by the conventional electron optics system (not shown) in essentially the same way as in transmission electron microscopy (TEM).

The practical lateral resolution of PEM, as in any microscope, is limited ultimately by the diffraction error and the type of sample under investigation. The resolution limit in PEM is determined by the wavelength of the emitted electrons and not, for example, by the much longer wavelength of the exciting light. For a typical 1.0 eV emitted electron, the diffraction error would be approximately 1 nm. However, this lower limit is increased by the presence of the aberrations of the accelerating field and of the objective lens. Currently, the practical resolution achieved in our laboratory (on a good day) is approaching 10 nm and the design goal of the present instrument when complete is 5 nm. Looking further into the future it has been suggested that upgrading of the present electron optics system to include aberration corrections, as is always done in light microscopy, could extend the resolution even closer to the theoretical diffraction limit (G. Rempfer, private communication).

There are other factors that can be just as important as resolution in determining the usefulness of imaging techniques in studies of biological surfaces. These include the depth of information (depth resolution), contrast mechanisms (both topographical and material contrast), depth of field, sample preparation and conductivity requirements, and sample damage. In general, most of these factors are favorable in photoelectron microscopy (Griffith et al., 1982). Specific advantages as well as limitations are discussed along with representative photoelectron micrographs in the following sections.

Specimen Preparation

Photoelectron microscopy shares many of the established specimen preparation techniques. All electron optical systems, including PEM, must be evacuated so that electrons are not scattered by gas molecules. For biological specimens this necessitates the use of either dehydrated or frozen specimens. For this purpose a number of stabilization, dehydration and freezing techniques have been developed for TEM or SEM and these are applicable for PEM. For example, the use of chemical fixatives such as glutaraldehyde or osmium tetroxide are compatible with photoelectron microscopic investigations. The newer cryofixation methods should also be applicable without modification. Specimen coating and replication are not used except in specialized applications. Uncoated biological specimens can be viewed directly in the photoelectron microscope.

A conductive substrate is required. The photoejected electrons forming the image are replaced by electrons from the cathode. The same molecules at the surface of the specimen can repeatedly emit electrons, thus enhancing the signal (Houle et al., 1979). Without a conducting substrate the specimen would rapidly charge. The first substrate used was stainless steel. More recently, round microscope coverslips, 5 mm in diameter, coated with a thin conducting layer of tin oxide have replaced stainless steel for many applications. The transparent discs can be dipped in a 1 mg/ml solution of alcian blue to increase adherence of cells in suspension or coated with serum albumin for cell growth on the substrate, sterilized and placed in tissue culture dishes. Cells will grow on the coated coverslips with no noticeable toxic effects from the tin oxide layer. The glass substrates also minimize light reflection, are more inert than metal supports (e.g., to osmium tetroxide), and make possible light optical experiments on the same cells examined by photoelectron microscopy.

Model Systems Studies

Photoemission studies involving uniform thin layers or patterns of organic compounds have provided much useful information about the depth of information and contrast mechanisms in photoelectron microscopy. All compounds will photoemit electrons when excited with light of sufficent energy, and the process occurs at all depths within the sample. However, only those electrons emitted within a thin region at the surface, defined by the electron escape depth, can escape from the specimen and contribute to the image. The electron escape depths, measured for a variety of organic model systems and electron kinetic energies, are very short, in the range of 1 nm to 10 nm (Burke et al., 1974; Houle et al., 1982). This provides an extremely short depth of information and effectively prevents structures below the surface from blurring the image detail at the surface.

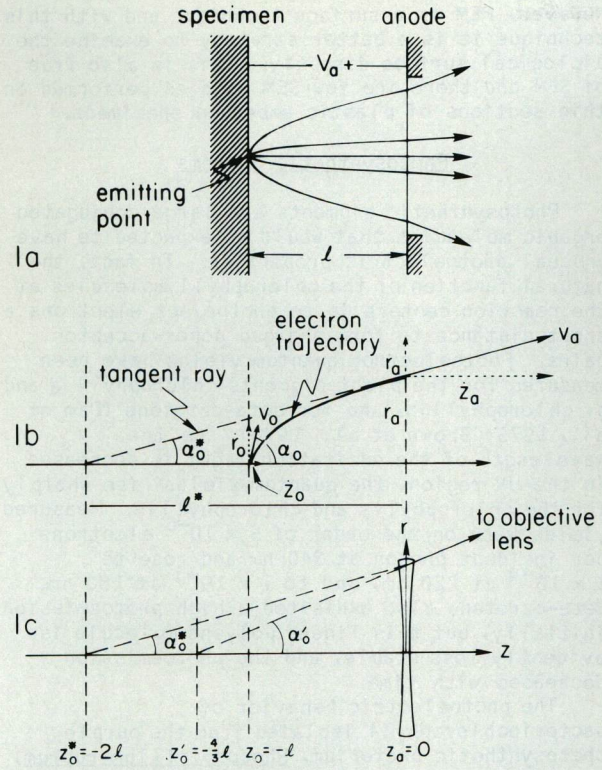

Fig. 1. (a) Electron trajectories for a point on the axis showing the curved paths in the accelerating region and the diverging action of the anode lens. Radial distances have been exaggerated. (b) Detail of the accelerating region showing the trajectory and the tangent ray defining the position of the virtual object at a distance ℓ^* from the anode. $\dot{r}_o$ and $\dot{z}_o$ are the components of the initial velocity $\underline{v}_o$, and $\dot{r}_a$ and $\dot{z}_a$ are the components of the final velocity $\underline{v}_a$. The angles made by the initial and final tangents are indicated by α_0 and α^*, respectively. (c) Electron optical equivalent for the special case of a uniform accelerating field combined with the diverging anode lens. α' is the angle of the ray after the diverging effect of the anode lens; $\underline{z}^*$ is the location of the virtual object for the anode lens; $\underline{z}'$ is the location of the virtual image formed by the anode lens; $\underline{z}_o$ is the location of the emitting point; $\underline{z}_a$ is the location of the anode lens. From Rempfer et al. (1980a) with permission.

Model systems studies have provided a good idea of the range of material contrast to be expected in biological studies. Material contrast results from differences in the ionization potentials (work functions) of different molecules. Some structures such as phthalocyanines and hemes are relatively bright in the photoelectron microscope (Schechtman, 1968; Dam et al., 1974a). In contrast, the amino acids, phospholipids, and saccharides are much less photoemissive (Dam et al., 1974b; Griffith and Dam, 1976; Dam et al., 1977). The large π-conjugated ring systems have strong optical

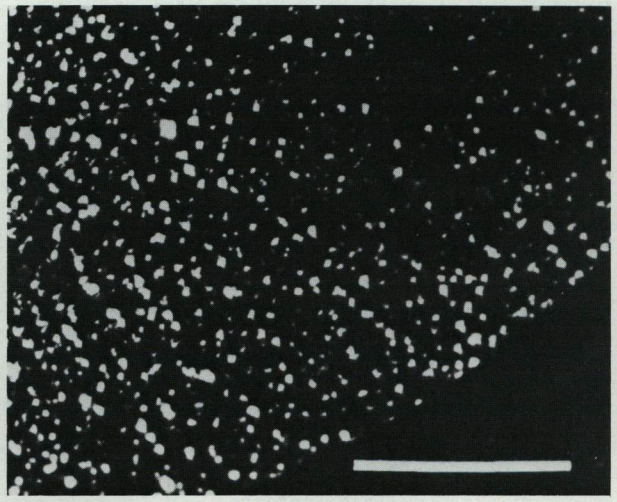

Fig. 2. Photoelectron micrograph of benzo[a]pyrene sublimed onto a thin layer of dimyristoylphosphatidylcholine. The bar represents 5 μm. From Houle et al. (1979) with permission.

absorption and evidently release electrons more readily, resulting in a higher electron quantum yield (the number of electrons released per incident photon). An example of material contrast is shown in Fig. 2. This photoelectron micrograph is of a model system prepared by subliming a small amount of the environmental carcinogen benzo[a]pyrene onto a continuous layer of phospholipids. The bright objects are crystallites of the carcinogen, which are photoemissive compared to phospholipids or protein. The high brightness is due to the conjugated structure of benzo[a]pyrene, not to its carcinogenic properties. Nevertheless material contrast provides one possible method of studying the interactions of carcinogens with membranes or nucleic acids, for example, without altering the structure of these molecules with heavy atom labeling (Houle et al., 1979).

Material contrast in PEM has also been observed to change after prolonged exposure to UV irradiation (Grund et al., 1979a; Nadakavukaren et al., 1979; Griffith et al., 1981b). After 10-60 min of illumination, surfaces with low photoelectron quantum yields tend to brighten proportionally more than the components that initially have high quantum yields. The net effect is a gradual overall brightening of the image accompanied by a decrease in material contrast. This situation has an analogue in fluorescence microscopy. Dyes with high fluorescence quantum yields are selected as labels because they will provide contrast against the low intrinsic fluorescence quantum yields of most biological specimens. However, this contrast is sensitive to the high intensity UV illumination in the microscope, and after a few minutes to an hour, the fluorescence of the dye gradually "bleaches" and the contrast diminishes or disappears entirely. In PEM, just the opposite, the "brightening effect" occurs.

Reducing agents such as stannane (SnH_4) can be used to control the brightening effect in PEM (Griffith et al., 1981b), just as n-propyl gallate can be used to slow fluorescence photobleaching (Giloh and Sedat, 1982). However, these effects can be used to advantage. In fluorescence microscopy, bleaching has provided a wealth of new information on lateral diffusion of lipids and proteins in wet membranes (Cherry, 1979). In PEM, the brightening effect permits higher practical magnifications and therefore a better opportunity to utilize the full resolution of the photoelectron microscope in cell surface studies. Both the bleaching and the brightening are evidently due to photochemical events, but these types of specimen alterations are not likely to produce gross structural rearrangements or any etching away of the surface such as can occur in charged particle beams.

Thin Sections

The first biological specimen imaged in the photoelectron microscope was a frozen and then dehydrated section of rat epididymis (Griffith et al., 1972). Grund and Engel and coworkers have subsequently examined unstained chicken liver specimens fixed in glutaraldehyde and embedded in methacrylate, Durcupan, Vestopal, Araldite, Epon or poly-N-vinylcarbazole and then sectioned by procedures commonly employed in transmission electron microscopy (Engel and Grund, 1974; Grund et al., 1978; Grund et al., 1979a,b; Grund et al., 1982). Images were obtained from specimens in all of these plastics and parameters such as section thickness, UV wavelength, and time of exposure were examined. The general conclusion from this work is that thin sections of biological materials prepared for transmission electron microscopy can also be observed by photoelectron microscopy. Cell types and cytological details are easily recognized in the photoelectron micrographs and correlate with known structures deduced previously from transmission electron micrographs. Beyond these general conclusions, these papers and especially the paper by Willig et al. (1979) contain some detailed conclusions about contrast formation that are now subject to substantial reinterpretation in light of new information about the specimens. It was thought initially that the specimens were truly flat. However, perhaps due to shrinkage of the embedding plastics or as a result of sectioning, the sections have been found not to be flat. Instead, there is a tendency for the surface to follow the contour of the embedded biological specimens and this contributes an additional contrast mechanism (Houle et. al., 1982). There is now general agreement on this point, although the origins of some of the contrast effects in the thin sections are not yet well understood. Thin sections will be useful in the future for some specialized work as in the polarized light studies of Schwarzer (1979), but probably will not be one of the main applications of photoelectron microscopy in cell biology. Thin sections are ideal for TEM in order to study the cross section of a preserved living organism. However, PEM is a surface technique and with this technique it is a better strategy to examine the biological surface directly. This is also true of SEM and there are few SEM studies performed on thin sections of plastic embedded specimens.

Photosynthetic Systems

Photosynthetic pigments are large conjugated organic molecules that would be expected to have unusual photoelectric properties. In fact, the natural function of the chlorophyll molecules at the reaction centers is to photoeject electrons a short distance to form charged donor-acceptor pairs. Photoelectron quantum yields have been measured for the plant pigments chlorophyll a and b, chlorophyllin, and for beta-carotene (Dam et al., 1975; Brown et al., 1978). As the wavelength of the excitation light is decreased in the UV region, the quantum yields rise sharply for the chlorophylls and chlorophyllin. Measured yields were on the order of 5×10^{-6} electrons per incident photon at 240 nm and rose to 1×10^{-4} at 220 nm, and to 1×10^{-3} at 180 nm. Beta-carotene also exhibited a high photoemission initially, but this linear polyene molecule is evidently less stable, and the photoemission decreased with time.

The photoelectric behavior of bacteriochlorophyll isolated from the purple photosynthetic bacterium, Rhodospirillum rubrum, has been examined as thin films and also as monolayers prepared in a Langmuir trough. The photoelectron quantum yield is very similar to that of the plant chlorophylls over the 180 nm to 230 nm range of excitation. The monolayer studies demonstrate the very high material contrast between the photoemissive chlorophyll and the much darker background of common lipids (Barnes et al., 1978). Based on this evidence, there is a good possibility that photoelectron microscopy can be useful in mapping the distribution of antenna chlorophyll structures and perhaps reaction centers, utilizing the intrinsic contrast provided by the photoemissive chlorophyll molecules. Very little work has been done in this area to date. Preliminary low magnification photoelectron micrographs of isolated spinach chloroplasts and of the photosynthetic bacterium Rhodospirillum rubrum have been obtained (Griffith et al., 1978). Photoelectron micrographs of the green bacterium Chloroflexus aurantiacus and of the purple membrane of Halobacterium halobium have also been reported (Birrell et al., 1979). Unlike the other photosynthetic systems, the principal chromophore of Halobacterium halobium is retinal, the same pigment that occurs in rhodopsin of the human eye. The amount of this pigment present is very small. The bulk of the functional unit on a molecular weight basis is protein and it is unlikely that the photoemission from the retinal would be strong enough to be detected, especially after the proteins have increased in photoemission as usually occurs upon exposure to the relatively high intensity illumination in the photoelectron microscope.

Surfaces of Cultured Cells

Conventional electron microscope methods for imaging the surface of cells include transmission electron microscopy (of thin sections and replicas) and scanning electron microscopy (of metal-coated specimens). Each of these affords a different view of the organization and structure of the cell surface, and each technique involves its own set of advantages and limitations. The technique of photoelectron microscopy adds another tool to the list of useful approaches to cell surface studies (Nadakavukaren et al., 1981; Griffith et al., 1982).

The typical PEM cell specimen is a culture of cells grown on a conductive substrate. The sample is washed in buffer, fixed in glutaraldehyde, and dehydrated, as is common procedure for all electron microscopy techniques. However, after the sample has been dehydrated, a major difference between PEM and the usual TEM or SEM sample preparation becomes apparent. Thin sections for TEM are prepared by embedding the specimen in resin, cutting exceedingly thin slices through it, and staining to give contrast. This gives a high resolution image but essentially a one-dimensional view of the cell surface. A three-dimensional TEM view can be also obtained by translating cell surface topography into a pattern of electron-dense regions through the preparation of a platinum shadowed, carbon coated replica. In this case the replica, and not the cell sample itself, forms the specimen. Sample preparation for SEM, which also gives a three-dimensional view, is simpler in that a thin metal coating is evenly applied over the entire sample surface and the image is recorded without further specimen treatment. In PEM the fixed, dehydrated cells are examined directly without staining, shadowing, or coating with metal. Because of the short escape depth for photoemitted electrons (10 nm or less) the information in a PEM micrograph of a cell reflects the surface of that cell, and not the photoemissive properties of structures beneath the cell surface. Even in the absence of variations in photoemission there is, fortunately, topographical contrast in the PEM image. The overall appearance of the PEM image of the cell surface is similar to that provided by a TEM replica or by SEM, in that the cells appear to be seen from a point above the sample and the general impression is one of three-dimensional structure.

Examples of photoelectron micrographs of uncoated cells are shown in Figs. 3-5. All three micrographs are of cell cultures grown on conductive glass discs and fixed with glutaraldehyde, but the cell lines and methods of dehydration differ. Fig. 3 is of human breast carcinoma cells dehydrated by a graded series of aqueous ethanol solutions up to 70% ethanol, followed by a final freeze-drying step to remove the remaining ethanol and water. Fig. 4 is a human fibroblast prepared in a similar fashion except that the cells were postfixed in osmium tetroxide and dehydrated through a graded series of aqueous ethanol solutions followed by amyl acetate after which the solvent was allowed to evaporate. Fig. 5 is a photoelectron micrograph of a cell sample that has been critical point dried. The critical point drying procedure is generally recognized as producing fewer artifactual effects than air drying, although there is shrinkage of the cell. The specimens shown here in Figs. 3-5 were neither coated nor stained.

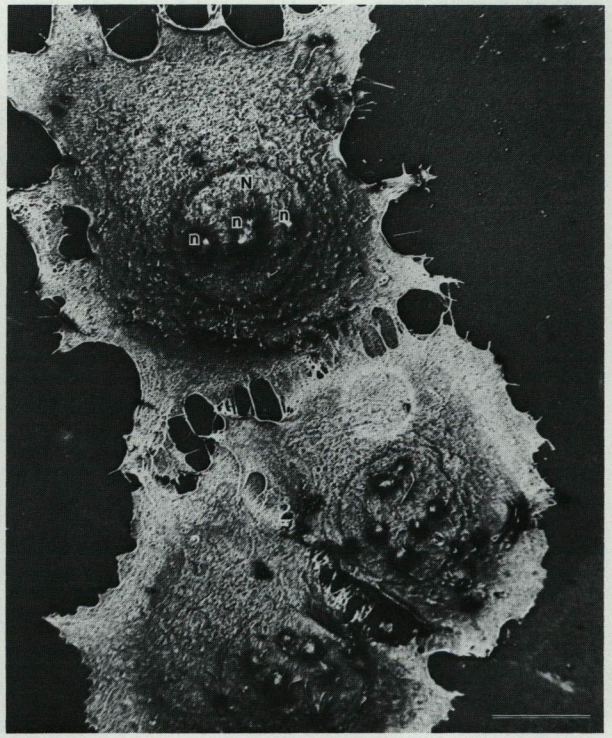

Fig. 3. Photoelectron micrograph of uncoated human breast carcinoma cells (cell line MCF-7) grown on a tin oxide coated glass overslip and fixed in 2.5% glutaraldehyde in 0.1 M Na cacodylate, 0.1 M sucrose, pH 7.4. After partial dehydration through a graded series of aqueous ethanol solutions the sample was freeze-dried in an ultraclean vacuum from 70% ethanol. N, nuclei; n, nucleoli. Bar = 10 μm.

Since the PEM image is formed by electron emission from the sample itself, sample characteristics that influence photoemission will affect the image. Two factors that can affect photoemission from the cell surface are charging and topography. Both of these perturb the electric field at the sample surface, which in turn can cause local deflections in the trajectories of the emitted electrons. If these perturbations are strong enough, the result can be distortions in the corresponding areas of the image. Charging is due to the relative nonconductivity of dehydrated cellular components, and is well known in SEM. Methods to increase sample conductivity for SEM have been and remain the object of considerable research (Hayat, 1978). However, to date the simplest

method for dealing with this problem is to coat the cell surface with a thin layer of conductive metal. The poor conductivity of the cell surface appears to be less of a problem in PEM, although there is undoubtedly some dependence on whether the sample is making good contact with the substrate. The design plans for the continued development of the photoelectron microscope at the University of Oregon include the technology required to examine frozen hydrated specimens, which may reduce conductivity problems even further.

Sample topography also affects the trajectories of photoemitted electrons, and this effect is a real advantage in that it is responsible for the unique sensitivity of PEM to cell surface detail. The origin of topographical contrast in the PEM image has been discussed previously (Rempfer et al., 1980a; Schwarzer 1981). Photoemitted electrons leave the sample surface with very low energies and then are accelerated by the electric field prior to entering the lens system. Because of their low initial energies, the emitted electrons are easily deflected by local perturbations in the accelerating field. The uniformity of this field in turn is affected by anything other than a plane surface. In practical terms, this means that a certain proportion of the electrons emitted from a sloping surface will not reach the image plane, and the result will be a corresponding dark region in the image. In samples with great depths and heights in the three-dimensional structure, the perturbations in the electrical field can become so large as to cause distortions in the image rather than useful topographical contrast. Fortunately, the topography presented by the average well-spread cultured cell is generally nicely imaged by PEM. This is illustrated in Figs. 3-5. The human breast carcinoma cells in Fig. 3 and the human foreskin fibroblasts of Figs. 4 and 5 are clearly visualized against the substrate. There has been some collapse or shrinkage of the cells in the dehydration processes, and many intracellular structures can be seen because the cell surface is draped over them. The clearest examples of this are the nucleoli, which appear as rounded bumps in the nuclear regions of the cells. However, the real power of PEM in visualizing fine structural details can be seen along the edges of the cells, for example in the right half of Fig. 4. Retraction fibers (arrows) are obvious, as is the linear pattern due to underlying stress fibers in the very thin cell periphery. Theory indicates that as the instrumentation improves the ability of PEM to image fine topographical detail will become even more evident (Rempfer et al., 1980a).

There are several other characteristics of PEM that are relevant to cell surface studies. One is the working depth of field, which is generally less in PEM than in TEM and SEM (Rempfer et al., 1980b), but is adequate for viewing most areas of these cells. In Fig. 5, however, the focal plane is at the substrate level and part of the nuclear regions of the fibroblasts are slightly out of focus. Another characteristic is material contrast which has been discussed above. The photoelectron quantum yields of the components of the cell surface do not differ significantly from one another, either initially, or after prolonged exposure to UV illumination induces the brightening effect. Consequently, the PEM image of the cell surface tends to be of the same overall intensity. Exceptions to this are occasionally seen as, for example, the bright regions towards the edge of the cell in Fig. 4. This is not completely understood, but may be due, in part, to UV light passing through the thinnest portions of the cells and reflecting back from the substrate. Since photoemission is a surface phenomenon, the effective addition (or cancellation) of the incoming and reflected light waves at the cell surface would increase (or decrease) the photoemission from that region (Dam et al., 1976; Houle et al., 1982).

Another consideration is sample damage. Although there is no beam of high energy electrons bombarding the specimen, the UV irradiation will undoubtedly cause some specimen deterioration with time. The only sample alteration that we have seen in cell surface studies at the magnifications we have used has been the time-dependent increase in photoemission from the sample. This brightening has been useful in that it permits observation of the cell surface at higher magnifications than are currently possible with the initial images.

Each of the techniques discussed has its own characteristic advantages and problems. TEM provides the highest resolution currently attainable, but sample preparation is an involved process and sample damage is a consideration. In SEM a variety of signals can be detected including X-rays, visible photons, backscattered electrons and low-energy secondary electrons. The secondary electron image is the most useful in topographical studies whereas the backscattered electrons and X-ray signals are useful in material contrast and analytical studies. The material contrast in SEM depends on the atomic number of the elements whereas material contrast in PEM (as in fluorescence microscopy) depends on the valence electrons. Both PEM and SEM can be used to examine cultured cells, but they differ in the types of samples that are optimal. PEM is especially sensitive to fine structural details on relatively flat surfaces, which can be difficult to image in SEM even with the use of very high tilt angles. Furthermore, the conductive metal coating that is applied to the SEM specimen can fill in and tend to obscure these details. On the other hand, the kinds of samples that exceed the useful range of topographical sensitivity in PEM are easily visualized by SEM. By directly visualizing the uncoated cell surface, PEM provides an alternative method for cell surface studies, which complements the established techniques of TEM and SEM.

Cytoskeletal Elements

Previous sections have already discussed the sensitivity of PEM to the details of surface topography and composition of biological

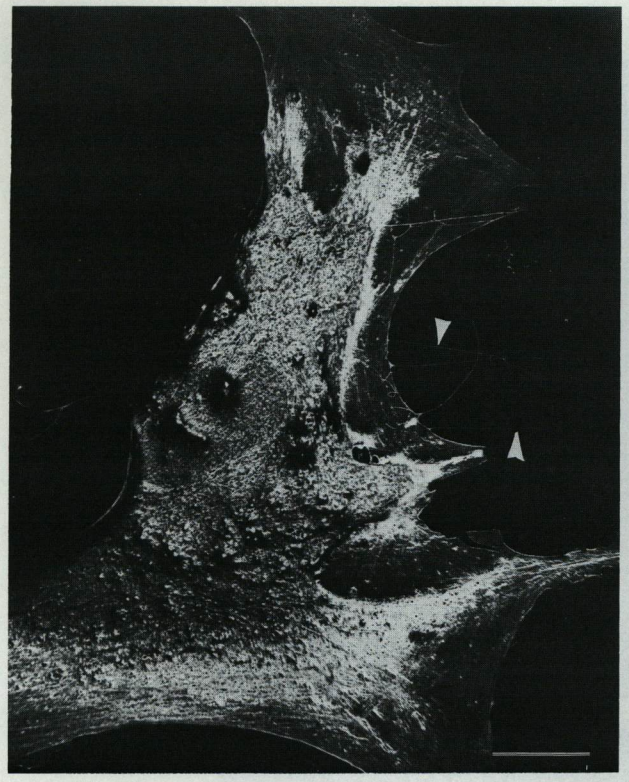

Fig. 4. Photoelectron micrograph of an uncoated human fibroblast (cell line FS-2) grown as in Fig. 3. The cells were fixed in 2.5% glutaraldehyde in 50 mM HEPES buffered saline, containing 0.8% NaCl, 0.05% KCl, 0.05% $CaCl_2$, pH 7.4; and additionally post-fixed in 1% OsO_4 in the same HEPES buffered saline, for 1.5 hours at room temperature. After washing in buffered saline the sample was dehydrated through a graded series of aqueous ethanol solutions to 50:50 ethanol:amyl acetate, then warm-air dried from 100% amyl acetate. Arrows identify some of the retraction fibers. Bar = 10 μm.

Fig. 5. Photoelectron micrograph of human fibroblasts (FS-2) that have been critical point dried. The cells growing on the conductive glass disc were fixed with 2.5% glutaraldehyde in 0.05 M HEPES saline buffer, post-fixed in 1% OsO_4 in 0.05 M HEPES buffered saline, then dehydrated through a graded series of aqueous ethanol solutions (70% to 100%), and critical point dried from CO_2 in a Polaron E3000 Series II critical point drying apparatus. The cell surface was not coated prior to microscopy.

specimens. Observations made while examining cell surfaces by PEM hinted at another promising application of this technique: as a tool for imaging the cytoskeleton. Photoelectron micrographs of whole cultured cells often show what appear to be ridges in the cell surface due to the presence of underlying cytoskeletal elements. This possibility has been investigated by preparing cells for immunofluorescence visualization of specific cytoskeletal elements, photographing the fluorescent images, then fixing and dehydrating the sample and locating the same cells in PEM (Nadakavukaren et al., 1983). By this approach it was possible to show that PEM can and does image both intermediate filaments and actin-containing microfilament bundles (stress fibers), provided that these structures either induce surface topography in the sample surface or are exposed in the preparative procedures.

An example is shown in Fig. 6. This rat fibroblast was prepared for indirect immunofluorescence of vimentin filaments and photographed by fluorescence microscopy to give the micrograph of Fig. 6A. The same cell, after subsequent glutaraldehyde fixation and dehydration, is also shown in the photoelectron micrograph of Fig. 6B and its enlargement in Fig. 6C. Many of the fibers of the vimentin-containing intermediate filament system in this cell can be traced in both types of micrographs (arrows). The smallest fibers that can be measured in the photoelectron micrographs and that can also be located in the fluorescence micrographs are 30 nm in diameter, consistent with a single vimentin filament decorated with two layers of antibodies on each side. Although the cell of Fig. 6 has been permeabilized to allow the penetration of antibodies, most of the cell surface and the components of the cytoplasm remain and obscure the PEM image of the cytoskeleton in the thicker areas of the cell. It should be noted that the contrast mechanism responsible for the imaging of these filaments in PEM is not material contrast from the presence of the fluorescent label, but is topographical contrast.

Nonionic detergents can also be used to solubilize nearly all but the detergent-resistant cytoskeleton, thus exposing this complex filament system to examination by photoelectron microscopy. Methods for preparing cytoskeletal samples and the conventional methods available for studying the architecture of the cytoskeleton have been reviewed by Bell in an earlier volume

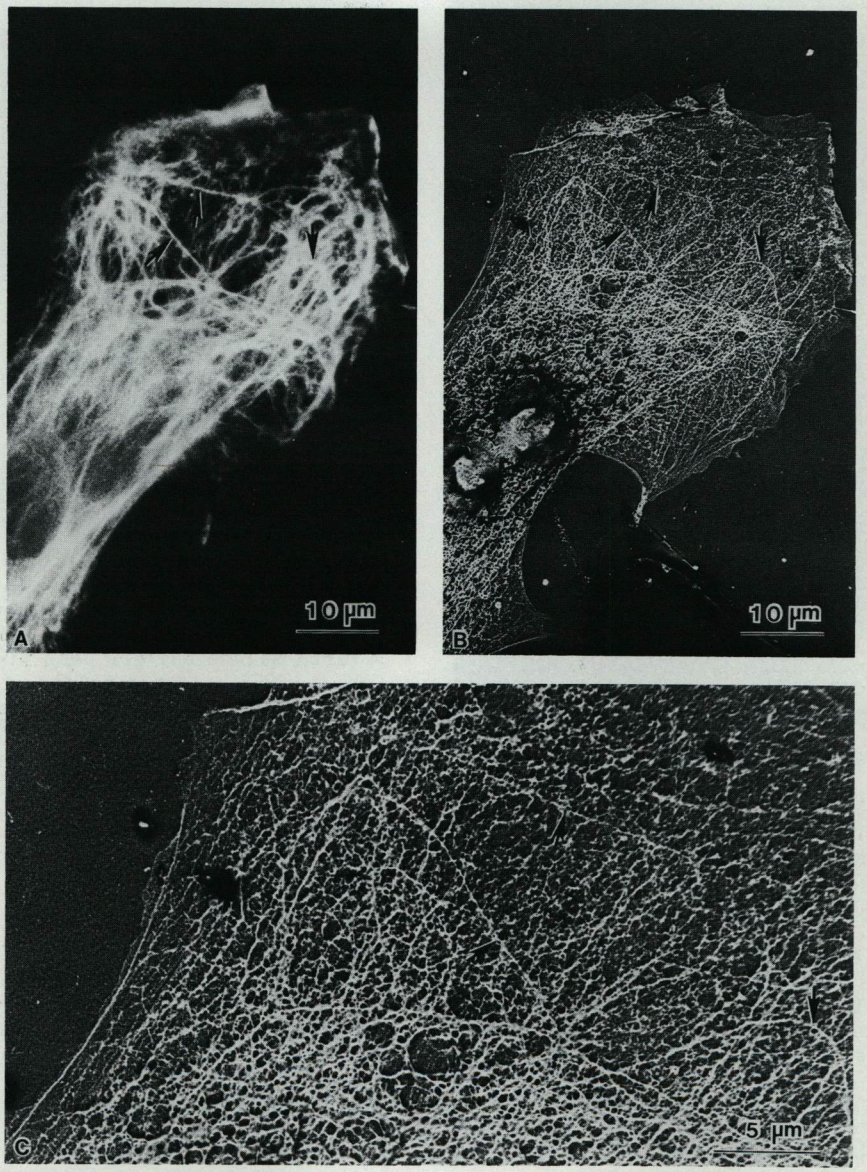

Fig. 6. RAT-1 fibroblast cells prepared for the immunofluorescent visualization of vimentin using mouse monoclonal anti-vimentin followed by rhodamine labeled goat anti-mouse Ig's. (A) fluorescence micrograph, (B) photoelectron micrograph, and (C) an enlargement of a selected area of (B). The cells were fixed essentially as in Fig. 3, but warm-air dried (for details of similar preparations see Nadakavukaren et al., 1983).

of this series (Bell, 1981). Detergent-extracted cytoskeletons can be imaged in TEM as stained whole mounts, platinum-carbon replicas, or thin sections; or in SEM after coating with metal (Bell, 1981). TEM offers the highest currently available resolution, while SEM excels at providing a view of the overall spatial organization of the specimen from different angles. However, PEM allows the isolated cytoskeleton to be imaged directly without staining or coating. Furthermore, as immunoelectron microscopy techniques for labeling specific cytoskeletal components are developed for PEM, this technique should provide valuable complementary information about the cytoskeletal elements.

Viruses and DNA

As the instrument resolution increases and sample preparation methods are better understood, progressively smaller objects can be seen. Tobacco mosaic virus (TMV) and the bacteriophages

T-4 and M-13 have been imaged by photoelectron microscopy (Houle and Griffith, 1983). These preliminary images do not, of course, compare with those of modern transmission electron micrographs in resolution, but the observed morphologies and dimensions agree with the known structures of these viruses. The photoelectron micrographs were taken without staining or metal coating and interesting variations in photoemission were observed that correlate with the amount of nucleic acids present. Recently, partially condensed bacteriophage lambda deoxyribonucleic acid (DNA) has been imaged by photoelectron microscopy, again without staining or coating (Houle and Griffith, 1984). Eventually, PEM may prove useful in studies of genetic regulation and chromosome structure, but such studies should be accompanied by further instrumental improvements. At present, the organization and function of cell surface components and the cytoskeletal elements of cells discussed above are more feasible with existing technology. In science it is difficult to look ahead, particularly where an emerging technique is involved, so we can expect surprises as photoelectron microscopy is applied to new problems in eucaryotic cell biology. The minimum gain will be a knowledge of the photoelectric behavior, one of the very few physical properties of the molecules of life that has not been explored in previous decades.

Summary

Over the past decade the quality of photoelectron micrographs has improved greatly. A variety of specimens have been examined. The images obtained correlate well with established optical and electron microscopy methods. Photoelectron microscopy can now be classified as an emerging technique in cell biology which will be used primarily for the study of relatively flat biological surfaces, particulary well-spread cells in culture and the surfaces of cellular organelles and components, such as the cytoskeleton and membranes. The imaging process is extremely sensitive to topography, which explains the requirement for relatively flat specimens and at the same time points out that this method is promising for the detection and study of very fine surface detail of normal and transformed cells. While estheticallly satisfying, we must recognize that good images are not sufficient. In order to be successful in cell biology an imaging technique must not only provide a faithful map of the surface, but must also provide a means of correlating structure and function. This will require the use of site-specific antibodies or plant lectins attached to markers that can be seen in the photoelectron microscope, just as fluorescent dyes mark the positions of proteins in fluorescence microscopy. Initially it was hoped that fluorescent dyes might be sufficiently photoemissive to act as markers in photoelectron microscopy (Griffith et al., 1972; Birrell et al., 1973). The organic dyes do photoemit and it is possible that some dyes can be used in specialized applications involving short exposures and high labeling ratios to improve contrast (Griffith et al., 1984). However, more promising at present is colloidal gold and other small particles that can be bonded to the site-specific proteins (Griffith and Birrell, 1983; Birrell et al., 1983). Colloidal gold is gaining in acceptance in TEM and SEM also, so this marker offers the opportunity to correlate the results of diverse imaging techniques on the same biological system.

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Discussion with Reviewers

Reviewer I: The purpose of your review is to cover recent promising applications of photo-electron microscopy to biological samples. Could you tell us where we can find references to work in both the development and applications of this technique in other areas?
Authors: The best single source is the Proceedings of the First International Conference on Emission Electron Microscopy (Pfefferkorn and Schur, 1979), although it is not available in many American libraries. This reference includes a comprehensive bibliography incorporating the bibliographies compiled by L. Wegmann and his associates at Balzers. Other applications in physics are brought more up to date in the review by Schwarzer (1981). Recently, Bethge and Klaua have described a horizontal PEM, or PEEM as it is usually called in Europe, attached to an ultrahigh vacuum chamber along with an Auger electron spectrometer and low energy electron diffraction apparatus. (Bethge, H., Klaua, M. 1983. Photo-electron emission microscopy of work function changes. Ultramicroscopy 11, 207-214.)

Reviewer V: This paper reviews the potential of an interesting and exciting technique for ultrastructural analysis of biological specimens. Particularly impressive are the correlative possibilities with immunofluorescent light microscopy. However, air drying is generally not an acceptable procedure for preparation of biological tissue for electron microscopy, especially for surface studies since it is just the surface structure that is most affected by the surface tension of evaporating liquids. What are the authors' plans for adapting other preparative techniques?
Authors: The most promising techniques for PEM sample preparation are the new cryofixation techniques being developed in several laboratories. We believe that it will be possible to study quick-frozen hydrated specimens directly in PEM, (water does not sublime at the temperature of the cold stage even at the high vacuum we use). This will require additional instrumentation for sample preparation and transfer since it is the actual frozen specimen and not a replica that is examined in the PEM. As to air drying, the simplest procedure is to air dry from water, but this is obviously unacceptable because of the high surface tension of water. In this preliminary exploratory work we have often used air drying from organic solvents (after step-wise dehydration), full well recognizing limitations in this approach. We are now also using critical point dried specimens. These samples in general give good images except in the nuclear region. Since this same question was also raised by other reviewers, we have since included a photoelectron micrograph of a cell prepared using critical point drying (Fig. 5).

G. Pfefferkorn: Cytoskeletal fibres are situated inside the cell. By drying of the cell in air a relief arises on the cell surface. By comparing with fluorescence light microscopy you have shown that there are cytoskeletal fibres. Are you sure that all filaments in your PEM-images are exclusively belonging to these cytoskeletal fibres? Many types of cultured cells - especially fibroblasts - are covered by a delicate fine-filamentous network containing glycoproteins, e.g., fibronectin.
Authors: The only cytoskeletal structures that have been positively identified are those that can also be correlated with the immunofluorescent micrographs. These include actin, vimentin, and keratin. We are currently working with antibodies to fibronectin. The preliminary results are quite encouraging in that they demonstrate that a direct labeling technique (using colloidal gold conjugated to the antibodies) can be employed which, in principle does not require correlation with immunofluorescence micrographs.

G. Pfefferkorn: In Fig. 3 the white spots (n) are called nucleoli. Have you proved by other methods (replica of SEM micrographs) that these spots are really peaks? These spots might be only deep holes, because PEM-imaging of deep holes often show in a blurred dark area white spots as an imaging phenomenon. Seen from the biological viewpoint it is the question if such cells might contain 6 or more nucleoli.
Authors: In PEM, both peaks and holes can give rise to white spots in the middle of a dark blurred area. The way in which peaks are distinguished from holes in PEM is to adjust the focus. If the lens must be made stronger (shorter focal length) to bring the central spot into focus, it is the top of a bump since the virtual object is closer to the objective lens. Conversely, if a longer focal length is needed to

focus the central area, the area is a hole. Sometimes holes and peaks are difficult to distinguish with any microscopic technique. However, in this case there is no ambiguity. The appearance of the nucleoli are well-known from a variety of studies. Malignant cells frequently have multiple nucleoli as seen in the carcinoma cells of Fig. 3, while normal cells characteristically have a single nucleolus (Fig. 4).

G. Pfefferkorn: It is possible to image cell cultures prepared on electrically conducting supports by SE in an SEM, too, without a conducting layer on the specimen. As supports are used silicon wafers or glass plates covered with a gold layer (e.g., Fomme, H.G., Pfautsch, M., Grote, M. 1980. Beitr. elektronenmikroskop. Direktabb. Oberfl. 13, 239).
Authors: The trend in SEM is to use thinner conductive coatings and better vacuum systems. However, conductive coatings are usually required especially at higher magnifications.

M. Beer: It is suggested that PEM is sensitive to some fine structural details which can be difficult to image by SEM with or without metal coating. Has a comparison been attempted of the images of particular specimens using PEM and SEM? For example, one could deposit onto carbon films DNA molecules, Actin filaments and Myosin thick filaments. These three samples would provide a range of thickness which would help in quantitative evaluations.
Authors: A comparison of PEM and SEM of biological specimens is planned as soon as we complete the current comparison of PEM with fluorescence microscopy. We expect PEM and SEM to be complementary, based on the theory of the origin of contrast. The specimens you mention would be good test objects for this comparative study, although carbon may be too photoemissive to use as a substrate, i.e. contrast reversal may occur.