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HIGH VOLTAGE ELECTRON MICROSCOPY AND LOW VOLTAGE SCANNING ELECTRON MICROSCOPY OF HUMAN NEOPLASTIC CELL CULTURE

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Abstract

Improved procedures were developed to correlate cell culture data with the images provided by advanced ultrastructural technologies. These procedures were compatible with the two main types of cellular behavior: adherent, spreading (melanomas, rhabdomyosarcomas) and non-adherent in suspension (leukemias).

The ultrastructure and function of spreading neoplastic cells primarily depend on surface properties of the attaching substrates. Therefore, the films used for cultured cell whole-mount ultrastructural analysis must have adherence features identical to those of standard cell culture vessels. Improved procedures were developed to produce the polystyrene films of required qualities. These films allowed processing of cells for electron microscopy including chemical fixation, cryoimmobilization, and immunolabelling. Furthermore, these polystyrene films permitted observations of the same cell in the high voltage electron microscope to reveal the internal organization and in the low voltage scanning electron microscope to reveal the surface topography.

Neoplastic cells in suspension may dramatically change their ultrastructure as a result of interactions with substrates or other cells. Therefore, immobilization of cellular processes must occur rapidly while cells remain in suspension. These processes were cryoimmobilized by high pressure freezing through the use of the newly designed specimen carrier. Procedures allowing high yield attachment of cryo-fixed neoplastic cells to aminopropyl-derived glass carriers enabled observations of cell surface topography. Furthermore, freeze-substitution and drying of freeze-fractured cells revealed their three-dimensional internal organization in the low voltage scanning electron microscope.

Key Words: human neoplastic cell culture, polystyrene films, high pressure freezing, high voltage electron microscopy, low voltage scanning electron microscopy.

Introduction

Cell culture has proved to be a valuable technology in diagnostic and research oncology, including experimental chemo-, radio- and immuno-therapy (Furth and Greaves, 1989). Furthermore, cell culture has recently become an accepted and integral part of gene therapy (Nichols, 1988). Usually, cell cultures are grown in disposable, sterilized polystyrene vessels (dishes, flasks) filled with cell culture media and maintained in incubators providing controlled temperature, gas composition and humidity (Priest, 1971). In this environment cells either adhere to and spread on the surfaces of cell culture vessels, or remain suspended in medium. Based upon standardized cell culture techniques, numerous analytical procedures have been developed, including fluorescently activated cell sorting, radioimmunoassays, cell counting, and polyacrylamide gel electrophoresis. These procedures, involving vast quantities of cells, make feasible the physiological and statistical characterization of cell populations. Simultaneous morphological studies with the aid of electron microscopy provide ultrastructural background.

The knowledge of intracellular organization of adherent and spreading cultured cells is derived primarily from transmission electron microscopy of ultrathin sections (Richters and Valentin, 1973; Barber and Burkholder, 1974) or freeze-fracture replicas (Pfenninger and Rinderer, 1975; Collins *et al.*, 1975; Heuser and Kirschner, 1980) and also from scanning electron microscopy of cell fractures (Haggis, 1982; Ris, 1988; Kelley *et al.*, 1973; Ip and Fischman, 1979) or extracted cytoskeletons (Trotter *et al.*, 1978; Trotter and Kelley, 1979; Lindroth *et al.*, 1987; Bell *et al.*, 1989). Surface architecture of dried and metal coated (Boyde and Vesely, 1972; Porter *et al.*, 1972; Boyde *et al.*, 1973; DeBault, 1973; Porter and Fonte, 1973; Albrecht and MacKenzie, 1976; Brunk *et al.*, 1980; Peters, 1986) or frozen hydrated (Pawley *et al.*, 1991) cells is recognized primarily with scanning electron microscopy. Growth of adherent cells on formvar films supported by gold grids provides a foundation for the study of whole-

mounts of fixed-dried (Buckley and Porter, 1973; Wolosewick and Porter, 1976; Bell, 1984; Ris, 1985; Bell *et al.*, 1989), freeze-dried (Bridgman and Reese, 1984), negatively stained (Small and Celis, 1978), and frozen-hydrated (O'Toole *et al.*, 1990; Frederik *et al.*, 1991) cells by means of high voltage electron microscopy, intermediate voltage electron microscopy, scanning transmission electron microscopy, and conventional transmission electron microscopy.

The ideal, but the most technically demanding, approach to the ultrastructural analysis of spreading cells utilizes both high voltage electron microscopy to reveal cellular interiors and scanning electron microscopy to study surface topography of the same cell (Barber, 1974; Fonte and Porter, 1974; Barber, 1976; Wetzel and Albrecht, 1989; Malecki *et al.*, 1990). However, the advantages of this technology for cell culture studies are offset by differences in the surface properties between polystyrene vessels and formvar films which compromise the data correlation. Recent advances in production of polystyrene films have allowed cultures of human endothelial cells, or fibroblasts, followed by their processing for integrated high voltage electron microscopy and low voltage scanning electron microscopy (Malecki *et al.*, 1989; Malecki *et al.*, 1990). The first objective of the current project was to develop improved technology utilizing polystyrene films for integrated high voltage electron microscopy and low voltage scanning electron microscopy of human neoplastic cultured cells.

Ultrastructural study of cells in suspension requires that these cells must be rapidly immobilized while they remain suspended in culture medium. This can be accomplished by either fast freezing or chemical fixation techniques. Both techniques are undergoing continual improvements. Advances in high pressure freezing represent an important development in this field (Meryman, 1966; Moor and Riehle, 1968; Moor and Hoechli, 1970; Riehle and Hoechli, 1973; Müller and Moor, 1984; Gilkey and Staehlin, 1986; Studer *et al.*, 1989). Through the use of this technique, a sample up to 230 μm thick was frozen without forming ice segregation patterns (Riehle and Hoechli, 1973). A volume of culture medium corresponding to that sample would allow human neoplastic cells to remain freely suspended in this medium. In procedures published thus far, specimens within carriers for high pressure freezing had to be sealed using: hexadecene (Studer *et al.*, 1989), yeast paste with methanol (McDonald and Morphew, 1989), or polyvinyl alcohol/polyvinylpyrrolidone mixture (Müller and Moor, 1984). However, these procedures were not satisfactory for the current project primarily because of unknown side effects of substances used as sealants on very sensitive human cells (Rapatz and Luyet, 1965; Mazur, 1966; McIntyre *et al.*, 1974). The second goal of the current project was to design a specimen carrier for high pressure freezing of neoplastic cell suspension that would require no sealants, cryoprotectants, or fixatives.

Ultrastructural analysis of cells immobilized in suspension requires also attachment of whole cells to carriers for subsequent processing and direct observation with scanning electron microscopy. However, these cells being too thick to be transparent for electrons even at 1 MeV require ultramicrotomy for transmission electron microscopy followed by computer reconstruction of entire cells. Two methods of fixed cell attachment were utilized in the earlier studies: **a.** aspiration of chemically fixed cells through Nucleopore filters (Wetzel *et al.*, 1974; Oliver and Albrecht, 1987), or of high pressure frozen, freeze-substituted cells through Durapore filters (Malecki *et al.*, 1991); **b.** binding by electrostatic forces to cover slips coated with polycations (Mazia *et al.*, 1975; Sanders *et al.*, 1975; De Harven *et al.*, 1975; De Harven *et al.*, 1984). However, application of these methods resulted in a variable or selective cell yield (Nermut and Eason, 1989; De Harven *et al.*, 1984) as well as dramatic alteration of cell physiology and structure (Wetzel *et al.*, 1974). In response to this problem, the new reliable procedure was recently developed allowing attachment of cells fixed in suspension to aminopropyl-derived glass cover slips (Malecki and Ris, 1991, 1992). The modification of this procedure for ultrastructural studies of high pressure frozen non-adherent human neoplastic cells was the third objective of the current project.

The ultimate goal of the entire project was to develop technologies permitting direct and adequate correlation between data on cultured neoplastic cell functions and their structural background. Ideally, developed technologies should be compatible with standard cell culture environments and should allow subsequent ultrastructural studies by means of advanced integrated microscopy.

Methods

Polystyrene film / gold grid carriers

In order to study the ultrastructure of whole-mounts of adherent and spreading cultured neoplastic cells, I designed a carrier facilitating polystyrene film manufacturing, cell growth and processing for electron microscopy. Procedures for manufacture of these carriers are illustrated in Figure 1. Polystyrene cell culture dishes (Corning) were dissolved in amyl acetate (0.5% solution w/v) (Malecki *et al.*, 1989). Cleaned glass slides were dipped into this solution and left to dry. Subsequently, 6 x 6 mm squares were scratched on the thin layer of polystyrene film coating the slide. These film squares

Figure 1. Assembling of the polystyrene film/gold finder grid carrier. **(a)** polystyrene film floating on distilled water, **(b)** gold, finder grid, **(c)** frame made of gold foil, **(d)** the whole polystyrene film/ gold grid carrier assembled.

Figure 2. Assembling of the specimen carrier for high pressure freezing. **(a)** gold or glass base, **(b)** teflon sleeve, **(c)** gold cap, **(d)** the carrier assembled.

Figure 3. Assembling of the glass cover slip/gold grid carrier or the mica disc/gold grid carrier. **(a)** 3 mm diameter round glass cover slip or disc punched from freshly cleaved mica sheet, **(b)** tabbed, gold, finder grid, **(c)** silicone rubber, **(d)** the whole mica disc/gold grid carrier assembled.

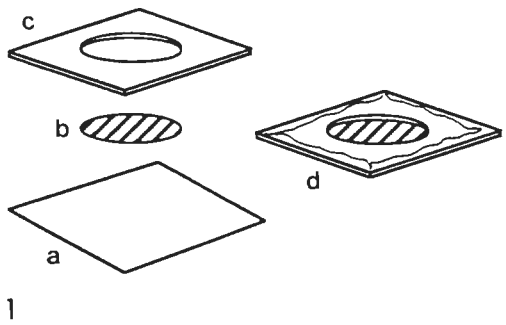
Figure 4. Modification of a standard cell culture dish (Corning). **(a)** bottom with the drilled opening, **(b,d)** glass cover slips, **(c)** cover, **(e)** the whole modified vessel.

Figure 5. Assembling the flat-bottom light-transparent centrifuge tube. **(a)** upper portion of 15 ml centrifuge tube (Corning), **(b,d)** glass coverslip, **(c)** tube cap with the drilled hole, **(e)** the whole flat bottom tube assembled.

(a) were floated onto a distilled water surface. Cleaned gold finder grids (Pella) were placed onto the tops of the floating film squares (b). Finally, the filmed grids were picked up with the gold frames (5 mm x 5 mm squares with 2.8 mm central hole, or 3 mm diameter rings) (Aldrich). These frames provided strong support and prevented films from braking (c). The frames had bent corners to facilitate handling during processing for electron microscopy and the film edges overlapped the frame rim. This created the polystyrene film/gold grid carrier (d). Any remaining water droplets were blotted with a filter paper. After drying, the films in the carriers were exposed to oxygen-plasma glow discharge (Amstein and Hartman, 1975; Ramsey *et al.*, 1984). Additionally, polystyrene dishes obtained in their untreated by manufacturer form (Corning) were exposed to the same treatment as the polystyrene films manufactured in this project. Neoplastic cells adhered firmly to and spread promptly on the polystyrene films of the carriers located in cell culture vessels (dishes or flasks).

Carriers for high pressure freezing

For high pressure freezing of non-adherent cell suspension, I designed a new specimen carrier allowing cell



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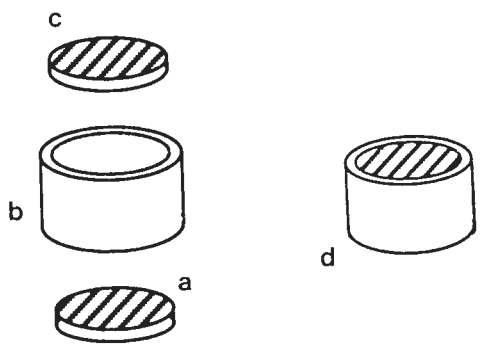

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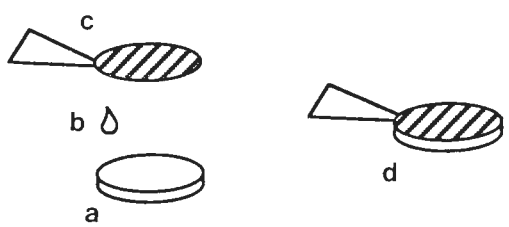

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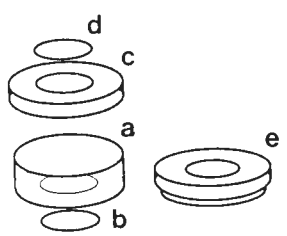

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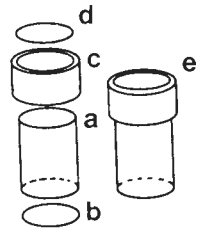

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growth within undisturbed cell culture medium followed by rapid freezing. The carrier manufacture is illustrated in Figure 2. A teflon (DuPont) cylinder (b) was attached onto the gold base (a) to create the carrier well. The carrier cap (c) fitting into the inner diameter of teflon tubing was prepared. Cell suspension was injected into the carrier well, which was then tightly closed with the gold cap (c).

Glass cover slip/gold grid carriers

Neoplastic cells in suspensions either chemically fixed or cryoimmobilized and freeze-substituted were attached to the carriers, I designed, to facilitate processing and electron microscopical observations. Figure 3 illustrates the carrier manufacture for this purpose. Three mm diameter round glass coverslips (Münnahütte Glassarbeitung, FRG) were cleaned with 40% hydrochloric acid in ethanol. Also as an alternative, 3 mm diameter mica discs (a) were cut from freshly cleaved mica sheets using a puncher (Balzers). Glass cover slips or mica discs were attached to tabbed, gold, finder grids (Pella) (b) with silicone rubber (Corning) (c). This created the glass cover slip/gold grid carrier. These carriers were cleaned in acetone, dried and then soaked for 2-24 hours in 2%-5% 3-aminopropyl-triethoxy-silane (Aldrich) in electron microscopical grade acetone at 45 °C (Robinson *et al.*, 1971; Buechi and Bachi, 1979; Malecki and Ris, 1991). Finally the carriers were washed in acetone and phosphate buffer saline.

Cultures of human, adherent, spreading, neoplastic cells

Human Malignant Melanoma cells (HTB63 HT 144), Human Rhabdomyosarcoma cells (HTB153 MS 729) and Human Adenocarcinoma cells (HTB 151 MS 696) from American Type Culture Collection were studied. Cells were grown either on polystyrene film/gold grid carriers, or polystyrene coated glass cover slip (mica disc)/gold grid carriers placed into polystyrene cell culture dishes filled with Dulbecco's medium (Irvine Scientific) containing 10% Fetal Bovine Serum and no antibiotics. Cultures were maintained in the environment of 5% carbon dioxide at 37 °C within an incubator (Queue). For high resolution light microscopical observations, living cells adhering to the carriers were located within the modified cell culture dishes (Figure 4) and maintained within an incubator attached to the inverted light microscope (Nikon).

Cultures of human neoplastic cells in suspensions

Leukemia cells (HTB 176) from American Type Culture Collection were grown suspended in RPM1 1640 supplemented with 20% Fetal Bovine Serum and no antibiotics. Adherent cells (melanomas, rhabdomyosarcomas) were detached from substrates with non-enzymatic dissociating solution (Sigma) and resuspended in fresh culture medium. Cell suspension remained in cell culture flasks or in the carriers for high pressure freezing, which were placed on a rotating-tilting (Pella) table inside an incubator (Queue). For the high resolution light microscopical observations, living cells were injected into flat bottom tubes (Figure 5) maintained within an incubator attached to the inverted light microscope (Nikon).

Chemical fixation and processing for the EM

Neoplastic cells spreading on polystyrene films were fixed by immersion in the fixative 4% formaldehyde, 0.2% glutaraldehyde (prepared from stock solutions available from Polysciences) (modified from Karnovsky, 1965) in isotonic phosphate buffer saline (Bell, 1984) containing 3.0 mM calcium chloride, 0.3 mM magnesium chloride and warmed up to 37 °C. The fixation was continued in 1% glutaraldehyde in the same buffer for 1 hour at room temperature. Then samples were transferred into the postfixative: 0.05% osmium tetroxide, 0.05% ferricyanide (modified from Elbers *et al.*, 1965; McDonald, 1984) and kept for 15 minutes at 4 °C in the darkness to eliminate photo-activation (Bullock, 1984). After dehydration with ethylene glycol and ethanol at low temperatures (Malecki and Small, 1987), cells were critical point dried from carbon dioxide (Anderson, 1951; DeBault, 1973; Ris, 1985). Dried cells were argon-ion beam sputter-coated with platinum (Franks *et al.*, 1980).

Cells suspended in culture medium were fixed by rapid injection into the fixatives described above. After being fixed with glutaraldehyde and washed with phosphate buffer saline, cells were transferred into flat bottom tubes (Figure 5). 3-aminopropyl-triethoxy-silane treated glass cover slip (mica disc)/gold grid carriers were placed onto bottoms of these tubes. Fixed cells remaining in suspensions were spun down onto the carriers (Malecki and Ris, 1991, 1992). The maximal g-force, having no effects on the cell geometry (diameter, spherical shape) was determined for the each line. Further treatment of cells attached to the carriers was the same as that of spreading cells.

Fast freezing and processing for the EM

Neoplastic cells spreading on polystyrene films were cryoimmobilized either by plunging into melting ethane or by high pressure freezing. Subsequently these cells were viewed either directly in the microscope using cryostages (Gatan) (O'Toole *et al.*, 1990; Pawley *et al.*, 1991), or after freeze-substitution, rehydration, immunogold labelling, electroconductive staining and drying (Malecki, 1992), or after freeze substitution (methanol containing osmium), drying, and planar magnetron sputter coating (Van Harreveld and Crowell, 1964;

Humphreys *et al.*, 1974; Barlow and Sleigh, 1979; Bridgman and Reese, 1984; Malecki *et al.*, 1990).

Non-adherent cells remained suspended in culture medium filling the carrier for high pressure freezing (Figure 2). These carriers were maintained in an incubator. Transfer from an incubator into the high pressure freezer specimen chamber (Balzers High Pressure Machine 010) took less than 5 seconds. Temperature of the specimen chamber was maintained at 37 °C. Pressure increased within the specimen chamber first to 200 bar (2×10^7 Pa) and subsequently above 2300 bar (2.3×10^8 Pa). A cap of the carrier worked, at this time, as a piston transferring pressure onto cell suspension. Cooling from 0 °C, to -50 °C lasted 10 ms, while temperature below -100 °C was reached within 20 ms. After pressure has been released, temperature below -100 °C was maintained for 7 seconds, allowing transfer of specimens into liquid nitrogen. For surface topography studies, cells frozen in medium containing the low serum concentration (1%) were freeze-substituted, washed and spun down onto aminopropyl-derived glass carriers (Figure 3). For internal organization studies, frozen cells were fractured and freeze-substituted (Humphreys *et al.*, 1974; Malecki, 1992). Further treatment of the freeze-substituted cells or cell fractures was the same as that of spreading cells.

High Voltage Electron Microscopy

The AEI Mark II Type 7 high voltage electron microscope was operated at 1 MeV. The microscope was equipped with axis-centered rotation and low-temperature (Gatan) stages. Performance of the microscope was improved by digital controls over the objective lens current and linked digital memory (Pawley, 1980, 1981). Additionally, the microscope was equipped with a stereo-imaging system, which allowed three-dimensional observations in real time directly by the microscope and determination of optimal stereo-tilt angles during recording of stereo-pairs (Malecki, 1990). The images were either recorded on SO 163 Kodak films, or stored in memory of an image processor (Quantex).

Low Voltage Scanning Electron Microscopy

The Hitachi S-900 low voltage scanning electron microscope was operated either at 1.5 - 15 kV in the secondary electron (SE) mode or at 2.5 - 15 kV in the backscattered electron (BSE) mode. The microscope was equipped with a cryostage (Gatan). The performance of the microscope had been greatly improved by an oil-free vacuum that reduced contamination and by modification of the stigmator controls (Pawley, 1987). The microscope was also equipped with a double-tilt real-time stereo imaging system (Pawley, 1988). The images were recorded on Polaroid 52 films. Images of uncoated samples were recorded by collection of 2-10 frames with fast scanning rates.

Image Processing

The image processor (Quantex) directly linked to the high voltage electron microscope permitted preliminary processing of images (averaging, subtraction, alignment) and observations of three-dimensional structures directly by the microscope in real time using the stereo-imaging system (Malecki, 1990). The images on prints were scanned on a MacScanner (MacIntosh) and further processed (by: thresholding, counting, histograms, etc.) using the MacintoshII computer.

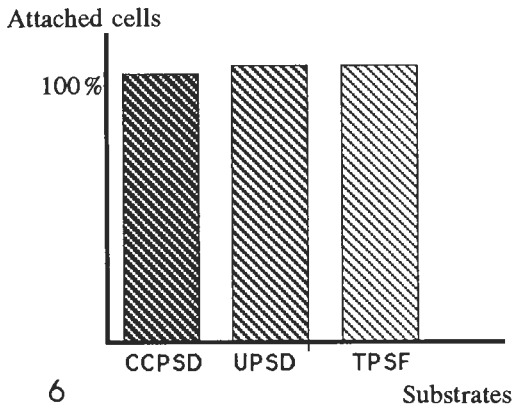
Results

Adherent, spreading neoplastic cells.

Polystyrene films were suitable substrates for growth of neoplastic cells. The composition of the films was identical to that of cell culture dishes from the same batch (Corning). Surfaces of both films and dishes were identically treated by a glow discharge. Due in large part to these characteristics, the films were identical as substrates to commercially-manufactured, standard, disposable cell culture polystyrene vessels. Indeed, in adhesion assays performed on melanoma, rhabdomyosarcoma and adenocarcinoma lines, cells adhered to polystyrene films with plating efficiency identical to that with polystyrene dishes (Figure 6). This test was especially important for evaluation of the metastatic potential of neoplastic lines by adhesion assays. Furthermore, spreading rate and cell geometry did not differ between the prepared polystyrene films and the commercially available polystyrene dishes (Figure 7). Unlike formvar films, polystyrene films produced values for plating efficiency and spreading rate equal to those of oxygen plasma treated polystyrene cell culture ware.

Malignant melanoma cells spreading on the polystyrene film are shown in Figures 8-17. Replacing portions of culture dish bottoms and covers with glass coverslips (Figure 4) created a strain-free optical pathway and permitted the use of polarized, or Nomarski optics together with phase contrast light microscopy in high resolution work (Figures 8, 9). Light optical properties of the polystyrene films were identical to those of polystyrene vessels. The films were sufficiently thin that cells on the carriers located at the bottoms of standard cell culture flasks were still in the correction range of the long working distance phase con-trast objective lens of the light microscope (Figure 8). Thus, the optical features of the polystyrene films allowed high resolution light microscopy during cell adhesion and spreading, as well as during processing for electron microscopy.

Mechanical properties of the films were important during their manufacture and during cell growth and



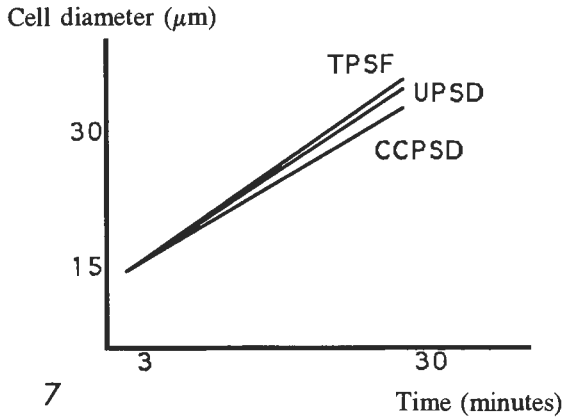


Figure 6 (at left). Adhesion assay (number of attached cells per substrate) of malignant melanoma cells. Cells were seeded onto: **a)** polystyrene dishes glow discharge treated by the manufacturer to enhance cell culture growth (Corning) - CCPSD, **b)** polystyrene dishes untreated by the manufacturer (Corning), but exposed to oxygen plasma together with thin polystyrene films in this project - UPSD, and **c)** thin polystyrene films manufactured and oxygen plasma glow discharge treated in this project. These cells were allowed to attach for 30 minutes. Then unattached cells were washed off with buffer. Histograms of attached cells were prepared on MacintoshII after scanning and thresholding of recorded images. The results were compared to CCPSD which was considered 100%.

Figure 7 (at right). Spreading rate (cell mean diameter over time) of malignant melanoma cells on various substrates. Cell diameters were measured after 3 and 30 minutes of spreading. Abbreviations of substrates identical to those of Figure 6.

Figures 8-12 (on the facing page). Malignant melanoma cell spreading on the polystyrene film assembled into the carrier. The carrier was placed into the modified cell culture dish to ensure a strain-free light optical pathway. Arrowheads indicate structures selected at consecutive steps of observations. The living cell in the light microscope (Figures 8-9). The same cell identified with the coordinates of the gold finder grid and observed in the high voltage electron microscope (Figure 10) and the low voltage scanning electron microscope (Figures 11 and 12).

Figures 8-9. The phase-contrast (**Figure 8**) and the Nomarski (**Figure 9**) light microscopical images of the same living cell. The cell developed numerous filopodia and ruffles (Figure 8). Cellular fibrils could be revealed due to a strain-free optical path (Figure 9).

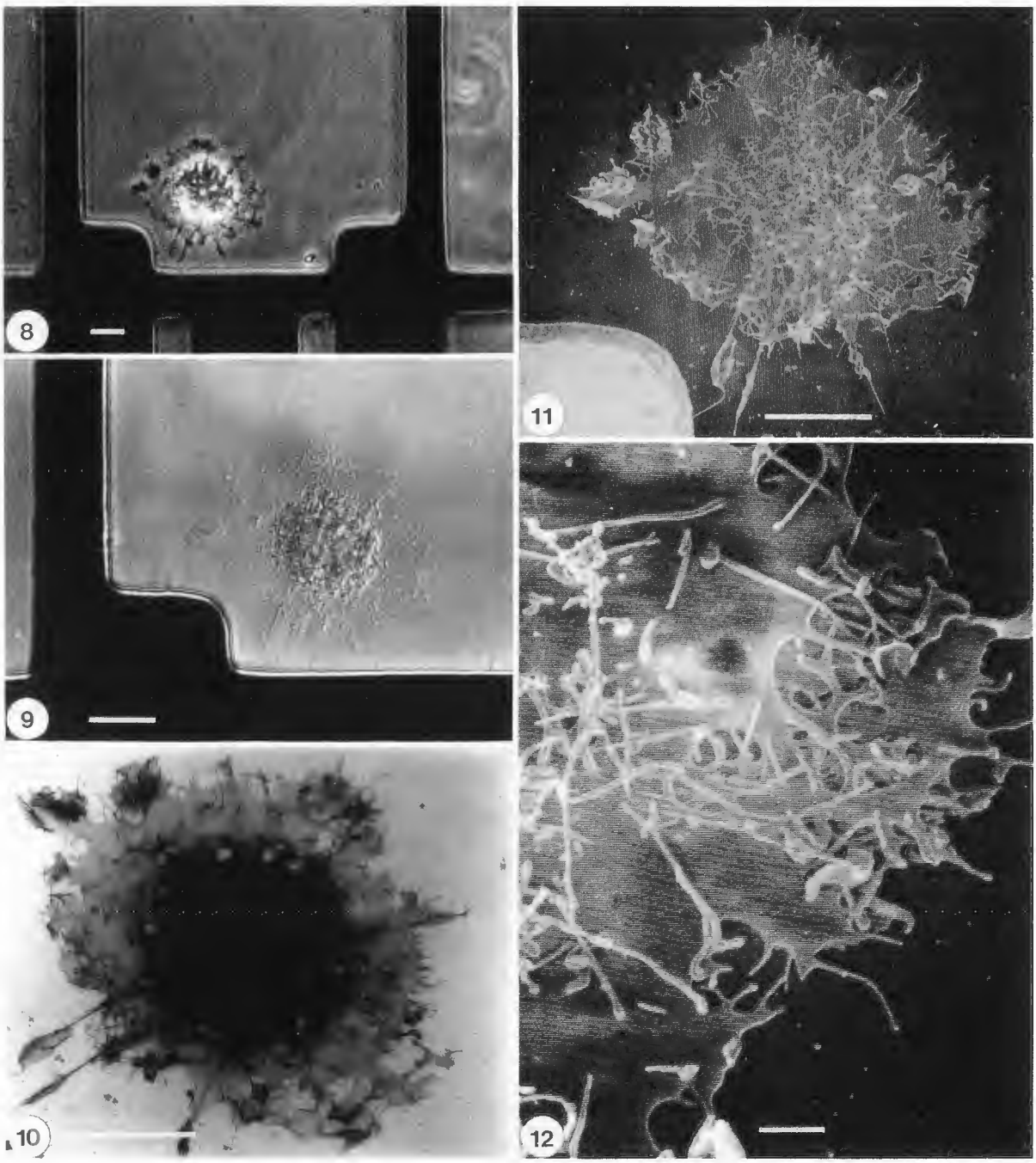
After recording with the light microscope, the cell was fixed with aldehydes only, dehydrated and critical point dried. No staining or coating was applied. Integrated high voltage electron microscopy and low voltage scanning electron microscopy demonstrated complete integrity of polystyrene films after processing for electron microscopy and exposure to an electron beam.

Figure 10. The same cell as in Figures 8 and 9 viewed in the high voltage electron microscope. This image demonstrates transparency of the whole cell on the polystyrene film for the electrons at 1 MeV.

Figure 11. The surface topography of the same cell as in Figures 8-10 observed in the secondary electron mode of the low voltage scanning electron microscope, recorded with fast scan averaging (8 x 1.5 sec/frame - 500 lines).

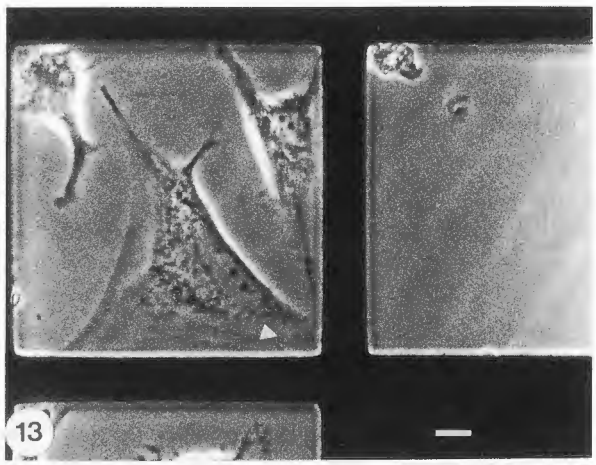
Figure 12. The area indicated by the arrows in Figures 8-11 observed in the low voltage scanning electron microscope at 1.5 kV. Even with the fast scanning, charging of the cell surface occurred. However, the good preservation of the membrane integrity can be appreciated.

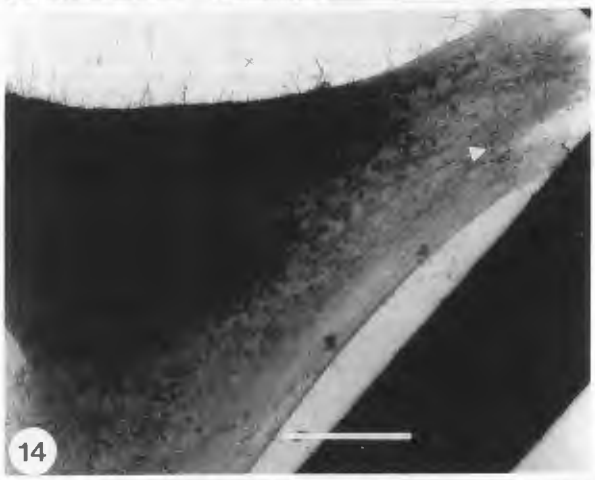
Bars = 5 μm (Figs. 8-11); 1 μm (Fig. 12).

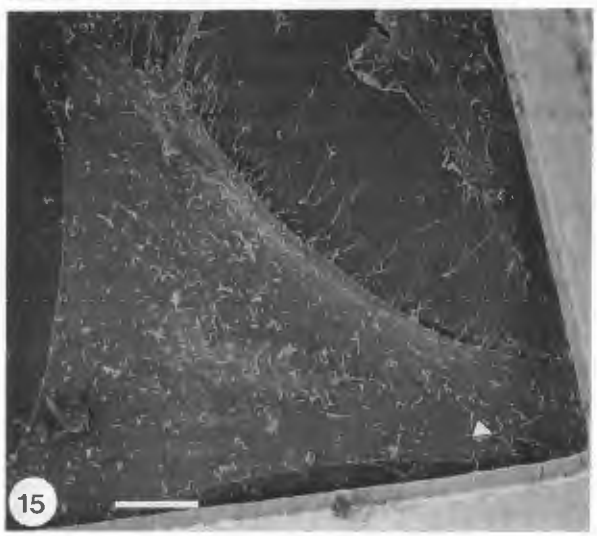


processing for electron microscopy. During manufacturing, the films had to be floated onto a water surface with the sharpest possible angle, between a slide and the water surface. During processing bending had to be avoided, otherwise thin fractures could already be seen already with a light microscope. Also excessive heating (cooling) leading to the expansion (contraction) of gold grids might lead to fractures (wrinkles); which could enlarge during further steps of processing. In this respect, the carrier frame was crucial as it provided films with strong support. Polystyrene films prepared as described above did not require additional supportive coating.

Light microscopical observations indicated that neoplastic cells behaved on polystyrene films as on regular polystyrene cell culture vessels. Furthermore, they could be processed for electron microscopy. To determine suitability of these films for electron microscopical imaging, their ability to withstand a high vacuum and







Figures 13-17. Malignant melanoma cell after 24 hours spreading on the polystyrene film. The carrier was located within a standard polystyrene cell culture vessel prior to seeding cells. After light microscopical observations of the living cell, this cell was processed for electron microscopy. The identical cell areas of the gulf within the cell edge are indicated by arrowheads. The exact correlation between the living cell structure revealed with the light microscope and the ultrastructure of this cell after processing for the electron microscope can be achieved. **Bars = 5 μm (Figs. 13-15).**

Figure 13. The phase-contrast light microscopical image of the living cell. The arrows indicated the gulf of the cell edge (its ultrastructure was subsequently revealed in integrated high voltage electron microscopy and low voltage scanning electron microscopy (Figures 14-17). Note the surface pattern of polystyrene flask. The polystyrene film retained its integrity.

Figure 14. The intracellular organization of the same cell (Figures 13 and 15) observed in the high voltage electron microscope at 1 MeV. Staining with osmium-ferricyanide enhanced significantly contrast.

Figure 15. The surface topography of the same cell (as in Figures 13 and 14) observed in the low voltage scanning electron microscope-secondary electron mode at 1.5 kV. The sample was ion-beam sputter coated with platinum to enhance secondary emission; thus, to facilitate surface topography imaging.

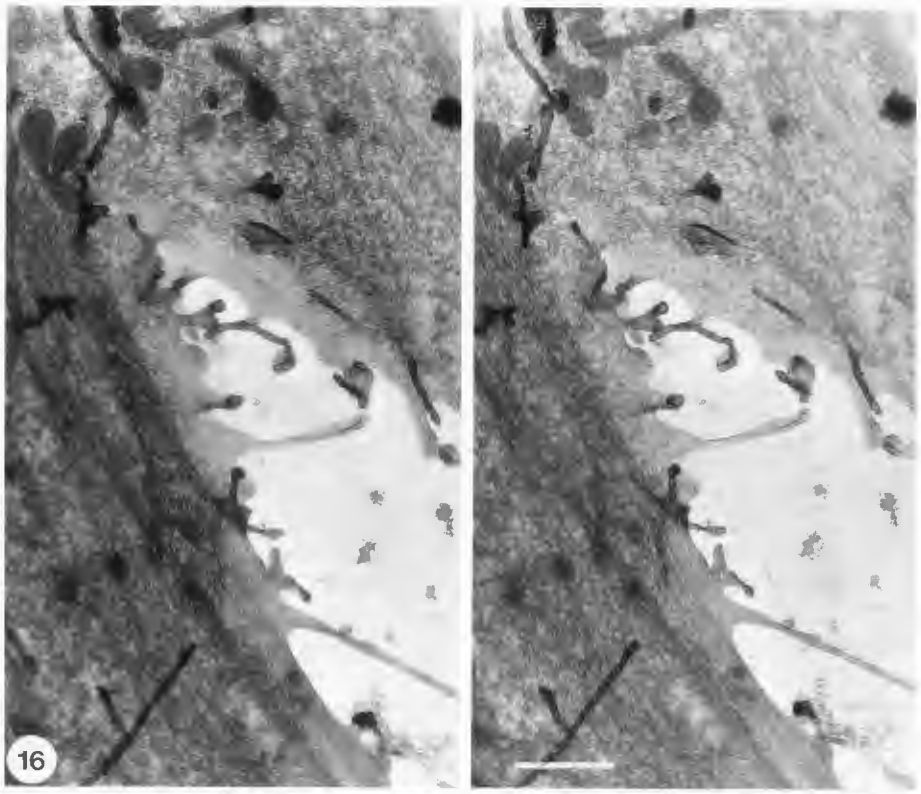
Figures 16-17 *(facing page).* Integrated high voltage electron microscopy and low voltage scanning electron microscopy of the malignant melanoma cell three-dimensional ultrastructure (the gulf in the cell edge indicated with arrows in Figures 13-15). **Bars = 500 nm.**

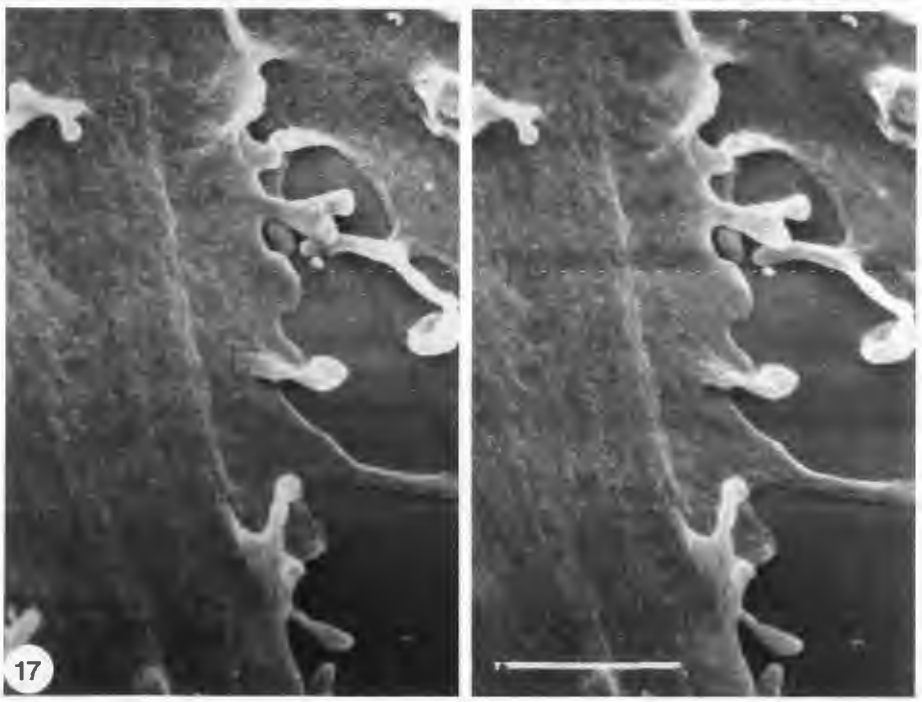
Figure 16 (stereo-pair). The stereo-pair recorded with the tilt ±4° in the high voltage electron microscope revealed the three-dimensional intracellular organization.

Figure 17 (stereo-pair). The stereo-pair recorded in the low voltage scanning electron microscope with a tilt angle of ±4°, of the same area observed previously in the high voltage electron microscope (Figure 16). The cell surface topography (Figure 17) can be exactly correlated with the intracellular organization (Figure 16).

their stability under an electron beam had to be determined.

Cells grown on polystyrene films, photographed with the light microscope (Figs. 8, 9, 13) and then fixed and critical point dried were subsequently observed in





the high voltage electron microscope (Figs. 10, 14, 16). The film integrity was perfectly preserved with no fractures present even though these films were not supported by any evaporated layers (Fig. 10). Films were very stable under an electron beam within a wide range of beam spreading. This stability allowed recording of micrographs at 30,000 x (Fig. 16) and up to the highest magnifications. Cellular three-dimensional organization was observed either directly by the high voltage electron microscope with the attached stereo-imaging system, or

after recording pairs of tilted specimen images. Both methods required exceptional substrate stability. The stereo-pair recorded in the high voltage electron microscope (Fig. 16) demonstrates that polystyrene films possess the required stability.

The same melanoma cell previously observed with the light microscope and afterwards in the high voltage electron microscope was subsequently observed in the low voltage scanning electron microscope (Figs. 11, 12, 15 and 17). The image of the cell spreading on polystyrene film, then fixed with aldehydes and dried (Figs. 11, 12), demonstrates the preserved film integrity without masking of fractures (wrinkles) by any evaporated coating layer. Reduced accelerating voltage (below 1.5 kV) resulted in the decreased specimen charging and beam damage. Presence of a grid, on the film top, greatly facilitated work in the low voltage scanning electron microscope operating at 1.5 kV and below. A finder grid pattern was easy to recognize and thus worked as a reference system (Fig. 11, 15) allowing fast and accurate localization of light microscopically selected cells. When cells were grown on formvar films supported from below increased voltage (up to 5 kV) was required for recognition of a grid pattern. Recording of images, of critical point dried but uncoated specimens, in the low voltage scanning electron microscope had to be performed at fast scanning rates to prevent charge build-up (Figs. 11, 12). To improve electro-conductivity and secondary emission, samples were argon-ion beam sputter-coated with 1-2 nm of platinum (Figs. 15, 17). Integrity of the film did not suffer during ion bombardment. Platinum layer helped to reveal the cell surface detailed topography (Fig. 17). Figures 8-17 clearly demonstrate durability of these films during processing and observations with integrated high voltage electron microscopy and low voltage scanning electron microscopy.

Polystyrene films provided appropriate supports for neoplastic cells during preparation protocols involving harsh extraction and immunogold labelling. Consequently, the same immunogold labelled microtubules were observed with the high voltage electron microscope (Fig. 18) and the low voltage scanning electron microscope (Figs. 19, 20). Furthermore, high electron transparency of polystyrene significantly enhanced the signal to noise ratio in the low voltage scanning electron microscope-backscattered electron mode, this permitted clear verification of fibronectin receptor immuno-gold labelling (Fig. 22), detected on the cell surface previously observed in the low voltage scanning electron microscope-secondary electron mode (Fig. 21 at 200,000x).

As an alternative to chemical fixation, cells spreading on polystyrene were cryo-immobilized either by plunging them into melting ethane, or by high pressure freezing. Polystyrene provided sufficiently strong support so that cells either frozen hydrated or freeze-substituted rehydrated immunogold labelled might be studied by means of electron microscopy (Malecki, 1992).

In conclusion, polystyrene films proved their reliability for ultrastructural studies on adherent and spreading cultured neoplastic cells.

Figures 18-22. Imaging techniques of immunogold labelling of human fibroblasts grown on the polystyrene film. The bundle of labelled microtubules is indicated with arrows in Figures 11-13. The group of labelled fibronectin cell surface receptors is indicated with arrows in Figures 14 -15. **Bars = 500 nm (Figs. 18-20), and = 50 nm (Figs. 21, 22).**

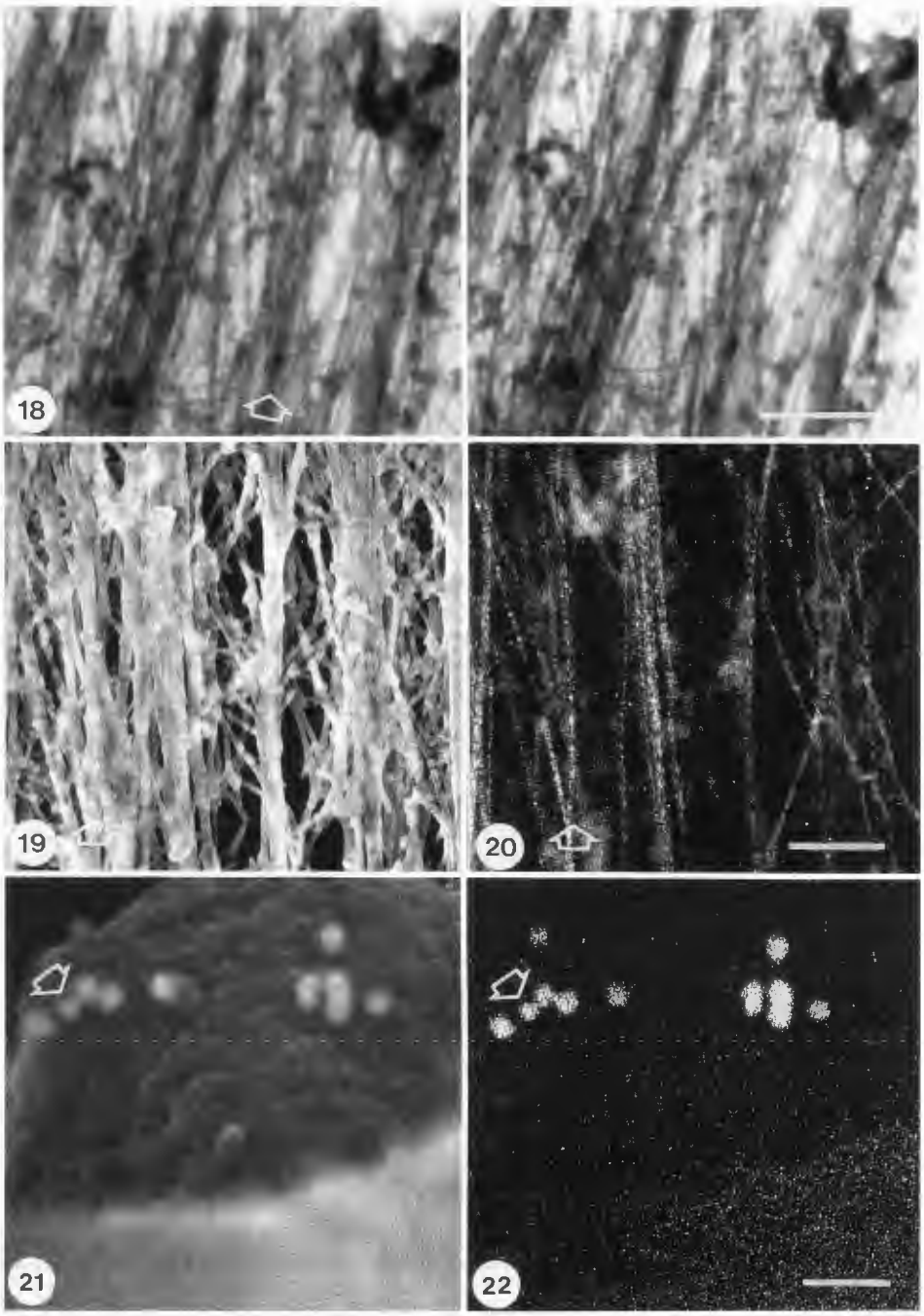
Figure 18 (stereo-pair). The three-dimensional organization of microtubules labelled with antibodies conjugated to 3 nm gold particles and observed in the high voltage electron microscope. The gold particles (black dots) on the microtubules are clearly seen. The 3 nm gold particles were the smallest detectable correlatively in the high voltage electron microscope and the low voltage scanning electron microscope without silver enhancement and image processing.

Figure 19. The area observed previously in the high voltage electron microscope (Figure 18) was subsequently observed in the low voltage scanning electron microscope-secondary electron mode at 1.5 kV. Note the cell extraction caused by buffered 0.5% Triton X100 used to enhance immunolabelling.

Figure 20. The atomic number contrast image recorded at 15 kV in the low voltage scanning electron microscope-backscattered electron mode of the same area as Figures 19-20. Distribution of individual gold particles can be verified.

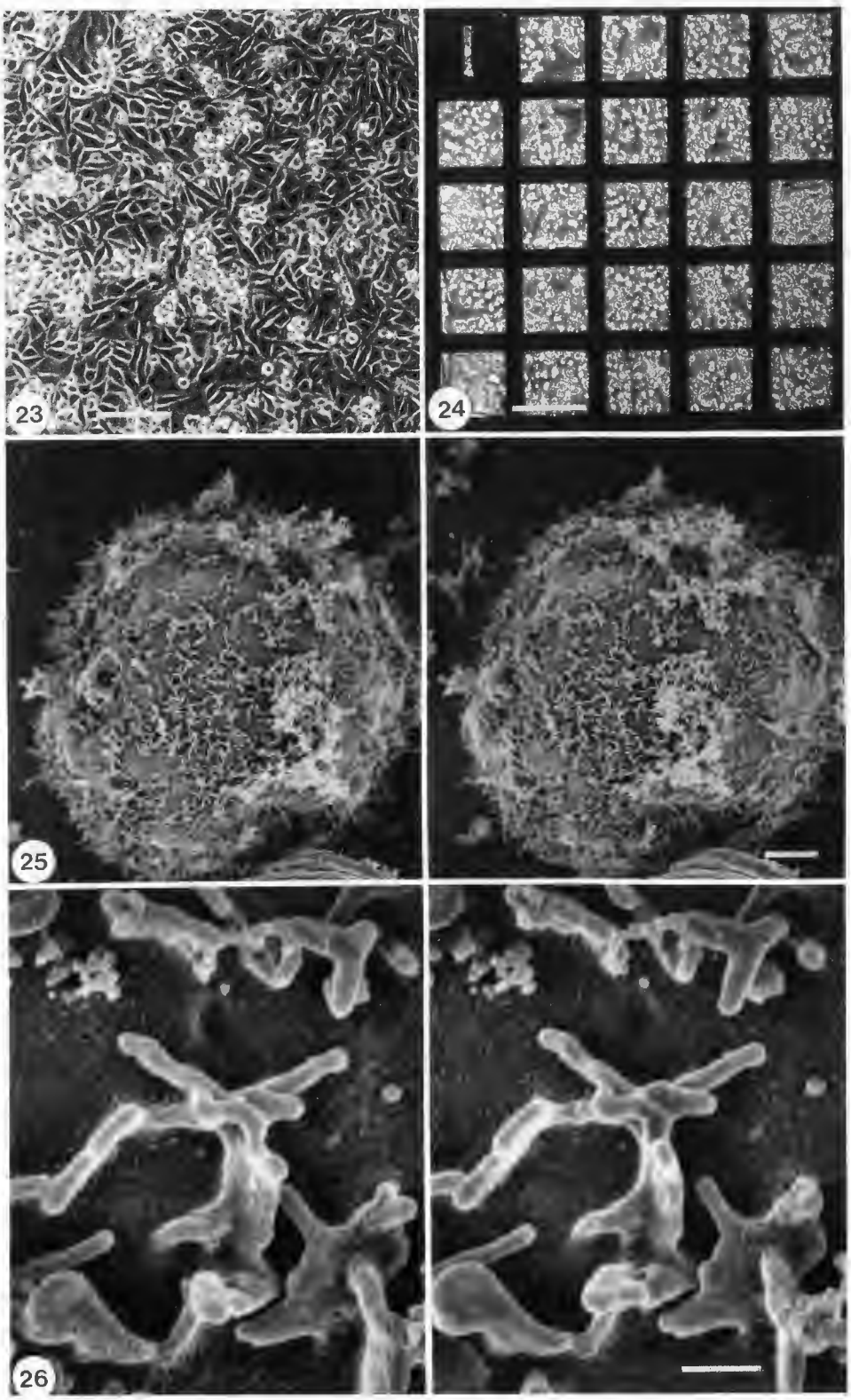
Figure 21. The immunogold labelling of the cell surface fibronectin receptor with the antibodies conjugated to 10 nm gold particles observed in the low voltage scanning electron microscope-secondary electron mode at 1.5 kV. The gold core is covered with a shell of antibodies creating a complex much bigger than the receptor itself (about 3 x 12 nm).

Figure 22. The gold distribution verification in the same cell area as in Figure 21, in the low voltage scanning electron microscope-backscattered electron mode at 15 kV.



Neoplastic cells in suspensions.

Rhabdomyosarcoma cells detached from substrates (Figs. 23-27) and non-adherent leukemia cells (Figs. 28-32), both remained suspended in culture medium. For chemical fixation these cells were injected directly into fixative. Injecting allowed immediate transition from the cell culture environment into fixative; thus, immediate contact of the cell surface with the full strength fixing agent was achieved. Fixed and washed cells were spun down onto aminopropyl derived glass carriers. Approximately 98% of cells spun down onto these carriers remained attached after washing with buffer (Figures 24, 27). More than 96% of cells remained attached to these carriers after further processing involving critical point drying (Figures 24, 27). For cryo-immobilization, cell suspension was injected into the carrier and high pressure frozen. Subsequently freeze-substituted cells also attached to the aminopropyl derived glass carriers with high yield (Figure 29). Cells also remained attached to the carriers during embedding into resins for subsequent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Figures 23-26. Rhabdomyosarcoma cells (Figure 23) were detached from polystyrene flasks, fixed in suspension and subsequently attached to the mica disc/gold grid carrier treated with 3-aminopropyl-triethoxy-silane. Selection of the cell with the light microscope (Figure 24), followed by the same cell identification with the gold finder grid coordinates in the low voltage scanning electron microscope (Figure 25) and the selected area surface topography ultrastructure at high magnification (Figure 26).

Figure 23. Rhabdomyosarcoma cells growing in polystyrene dish. Cells have spindle-like geometry. Mitotic cells rounded up. Some cells grew on top of others. Bar = 200 μm.

Figure 24. The phase-contrast light microscopical image of cells fixed in suspension and attached to the carrier. Observations were performed within the flat-bottom centrifuge tube. The cell spherical geometry is preserved. Bar = 200 μm.

Figure 25 (stereo-pair). The three-dimensional image of the cell from the same sample as in Figure 24 after critical point drying from carbon dioxide and ion-beam sputter coating with platinum observed in the low voltage scanning electron microscope operating at 1.5 kV. Bar = 5 μm.

Figure 26 (stereo-pair). The high magnification image of the cell surface topography in the central area of the cell recorded in Figures 24, 25. Filopodia protrude from the cell surface. Note the preserved integrity of the cell membrane. Bar = 250 nm.

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ultramicrotomy (Malecki and Ris, 1991). Transparent carriers (mica discs, glass cover slips) allowed evaluation of samples at all procedural steps. Attached finder grids worked as durable reference systems allowing identification of selected cells in the electron microscope using coordinates of gold finder grids (in cases of cells missing after drying, portions of remaining membranes were found attached to the carriers in the former locations of cells, indicating that forces of attachment were stronger than those of cell integrity). Therefore, electron microscopical images were considered representative of the entire cell population. Additionally, living cell spherical geometry was well preserved after both chemical fixation (Figure 25) and high pressure freezing (Figure 29). Furthermore, cells had well preserved membrane integrity (Figures 26, 30). Thus, the procedures were optimal for evaluation of large sample areas

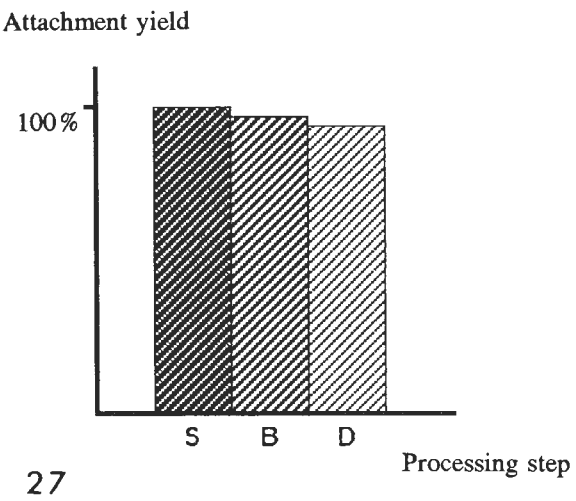

Figure 27. Attachment yield of rhabdomyosarcoma cells fixed in suspension. S-cells fixed in suspension and spun down onto the aminopropyl-derived glass carrier; B-cells remaining attached after washing with phosphate buffer saline; D-cells remaining attached to the carrier after critical point drying.

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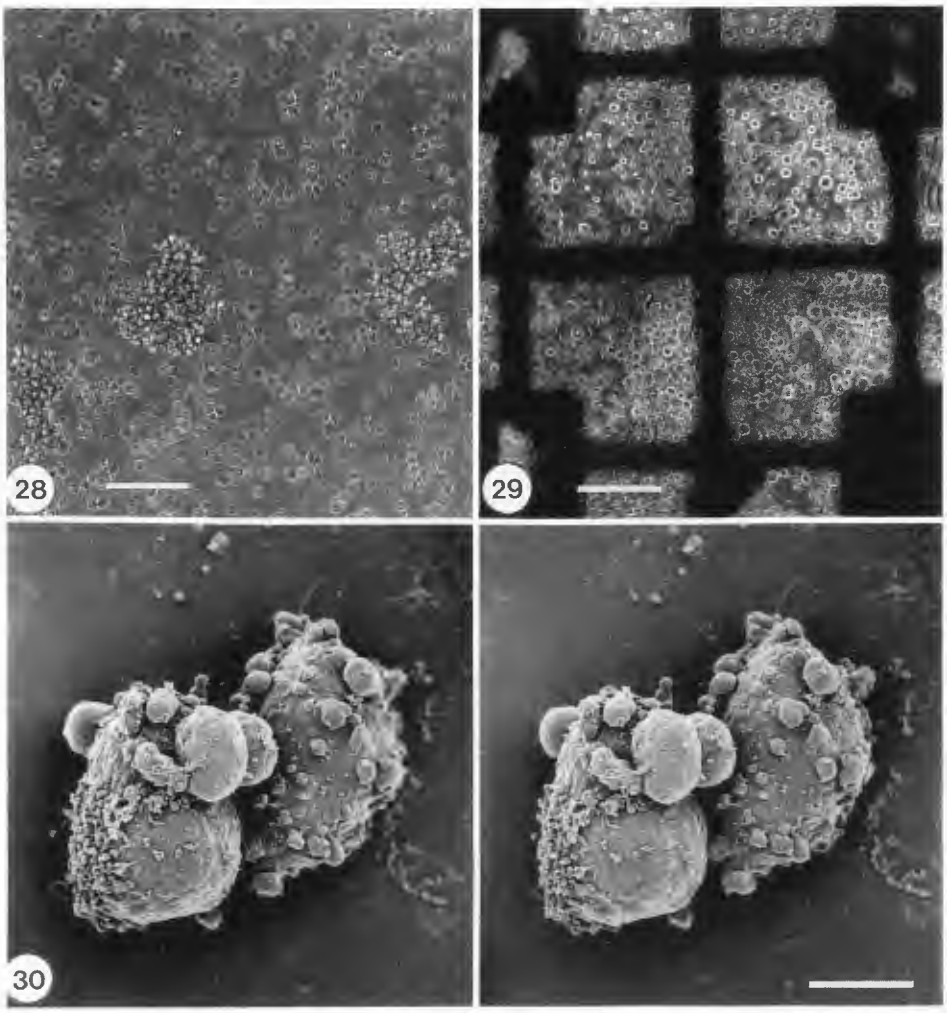
at low magnification (correlative to other analytical procedures such as cell sorting etc.), followed by selection of individual cells and finally high resolution ultrastructural analysis of neoplastic cell surface topography at very high magnifications.

In conclusion, the described procedure of attachment of fixed cells to the aminopropyl derived glass cover slip/gold grid carriers proved to be suitable for ultrastructural analysis of non-adherent human neoplastic cell surface topography.

Neoplastic cells, high pressure frozen, fractured, freeze-substituted and dried allowed observations of the cellular interior three-dimensional organization with the low voltage scanning electron microscope (Figures 31, 32). In this approach, in contrast to studies performed on freeze-fracture platinum replicas for transmission electron microscopy, large areas of freeze-fracture faces of many, entire cells and cell aggregates were available for observation. The sample was representative of the entire cell population; thus permitted correlation with other analytical methods. For this study, it was also particularly important that fast freezing made immobilization of intracellular processes possible at almost the same time as those processes on the cell surface. Success in freezing and further processing of cell suspension was clearly demonstrated by the chromatin preservation.



Figures 28-30. Leukemia cells were grown in polystyrene flasks (Figure 28), they were injected into the carrier and high pressure frozen. Subsequently these cells were freeze-substituted and attached to the aminopropyl-derived glass cover slip/gold grid carrier (Figure 29). Finally cryo-fixed cells attached to the carrier were critical point dried and coated (Figure 30).

Figure 28. Living leukemia cells in polystyrene flask observed with the phase-contrast light microscopy. Cells are spherical, but many cells have irregular protrusions from their surfaces. Some cells form aggregates. Bar = 200 μm.

Figure 29. Attached to the carrier cryofixed cells observed with phase contrast light microscopy. Cells maintained their spherical geometry observed in the living state. Bar = 200 μm.

Figure 30 (stereo-pair). The image of leukemia cells selected from the sample presented in Figure 29, after drying and coating, observed with the low voltage scanning electron microscope. Well preserved cell spherical geometry can be appreciated spatially. Membranes preserved their integrity. Cellular protrusions observed in the living state can be analyzed ultrastructurally. Bar = 5 μm.

Figures 31-32 (facing page). Leukemia cells suspended in Dulbecco medium were high pressure frozen using the carrier described in this report with no sealants, no cryoprotectants, or no fixatives. Subsequently frozen cells were fractured, freeze-substituted, critical point dried from carbon dioxide and coated with platinum. Images were recorded using the secondary electron mode in low voltage scanning electron microscope at 1.5 kV.

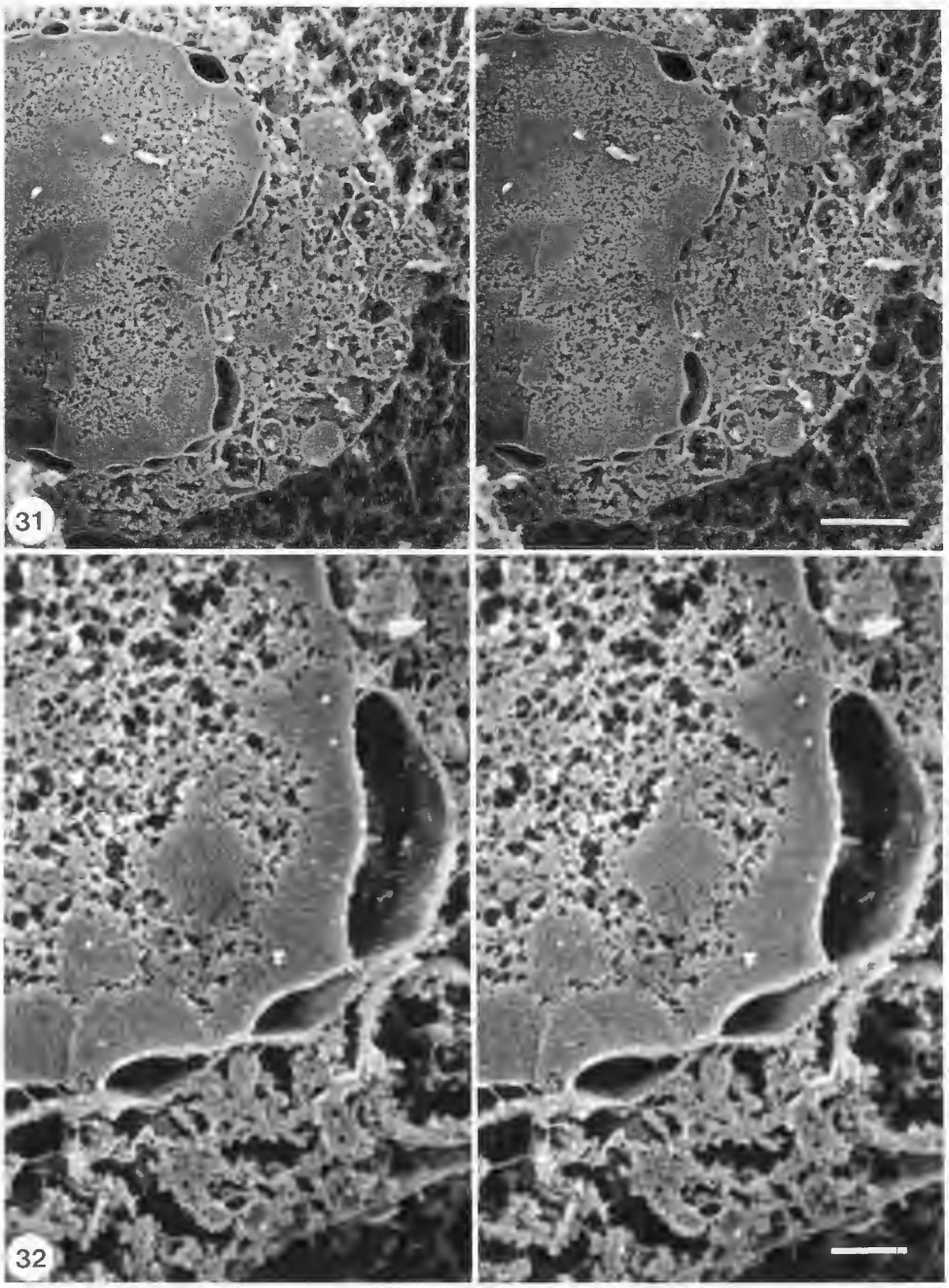


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Figure 31 (stereo-pair). The image of a human leukemia cell. This leukemia cell has preserved its spherical geometry. The three-dimensional internal organization is revealed with clear distinction of the cell membrane, the nuclear envelope and cell organelles. Bar = 1μm.

Figure 32 (stereo-pair). The image at high magnification of internal organization of the nucleus selected area of the same cell as observed in Figure 31 (area indicated with arrows on Figure 31). Membranes of the nuclear envelope have well preserved integrity. The clear distinction between eu- and hetero-chromatin is present. Bar = 250 nm.

The nuclear organization was very well preserved allowing clear distinction between eu- and heterochromatin (Figure 31). Extreme sensitivity of chromatin to damage by ice crystal formation implied, that the chromatin organization was the most sensitive indicator of appropriate freezing (Sitte *et al.*, 1987; Richter and Dubochet, 1990; Sorber *et al.*, 1990). The nuclear envelope also was well preserved (Figure 32). Although the very compact intracellular matrix obscured the image of structures located far below the fracture plane, it revealed the absence of ice segregation patterns indicating optimal freezing and further processing. The intracellular organization was further revealed by extraction procedures before freezing and during freeze-substitution (Malecki, 1992). These results were obtained with no sealants, no cryoprotectants, no fixatives, which could affect cell structure and function. The crucial element for the success of this quality specimen preparation was the new design of the specimen carrier for high pressure freezing. The carrier cap was tight enough to seal the carrier to prevent any leaks of culture medium. It also prevented any loss of cells by the first 200 bar ethanol burst and subsequent liquid nitrogen driven rapid pressure increase above 2300 bar. The cap also worked as a piston efficiently transferring pressure. Specimen fractures perpendicular to the cooling surface allowed determination of the sample thickness, so that its central portions were free from ice segregation patterns. This thickness was approximately 200 μm. Additional advantage of the designed carrier was, that when fracture planes were selected parallel to the freezing surfaces, they were immediately close to this freezing surface. This feature made it feasible to reveal interiors of cells frozen with the optimal cooling rates.

In conclusion, the newly designed specimen carrier for high pressure freezing allowed rapid cryo-immobilization of human neoplastic cells, while these cells remained suspended in undisturbed cell culture medium.

Discussion

Adherent, spreading neoplastic cells.

Polystyrene films provided adherent and spreading cultured neoplastic cells with the identical substrates as standard polystyrene cell culture vessels (dishes, flasks). They were like portions of these vessels, but portable. Final surface properties of the films suitable for growth of cells could be attributed to implantation of oxygen atoms within carboxyl groups into polystyrene chains during glow discharge (Amstein and Hartman, 1975; Ramsey *et al.*, 1984; Pratt *et al.*, 1989). Furthermore, these films supported by gold grids assembled into carriers provided cells with a strong support during processing and imaging of cellular ultrastructure in the high voltage electron microscope and in the low voltage scanning electron microscope. The stability of polystyrene films in the high voltage electron microscope could be explained by the fact, that the mass loss at 1 MeV was significantly reduced, as compared to intermediate and conventional transmission electron microscopy at 80 - 400 keV (Glaeser, 1974). Nevertheless, polystyrene films were more stable than formvar films under the same beam current. This was attributable to the fact that the mass loss of polystyrene was significantly reduced as compared to formvar or collodion exposed to the same beam current (Baumeister and Hahn, 1978).

For cultures of neoplastic cells, there are three major implications of polystyrene film technology. First, identical surface properties for cell adhesion and spreading of polystyrene dishes and films allow the use of standard cell culture routines with the exception that some polystyrene films assembled into carriers are placed into standard, commercially available cell culture vessels. Cells on films and those cells within flasks or dishes have identical conditions for growth. At the desired time, cells grown on films are removed from cell culture vessels and processed for ultrastructural studies with integrated high voltage electron microscopy and low voltage scanning electron microscopy on cell whole-mounts. Cells remaining within vessels are studied with standard analytical procedures including cell sorting, radioimmunoassays, cell counting, etc. Finally, straight correlation between all applied methods is possible.

Second, various conditions (concentrations, temperatures, times) established for adsorption of different proteins onto surfaces of polystyrene cell culture vessels are identical for the polystyrene films. This is particularly useful for studies of ultrastructural aspects of the neoplastic cell adhesion to the isolated components of extracellular matrix, basement membranes, or cellular membranes (Ruoslahti, 1991).

Third, polystyrene films coated with isolated components of basement membrane appear to be very suitable for growing monolayers of human endothelial cells serving as the blood vessel wall model (Malecki *et al.*, 1989), or fibroblasts as the connective tissue model (Malecki *et al.*, 1990). Both these models are essential for functional and structural studies on mechanisms of tumor cell invasion and metastasis (Kramer and Nicolson, 1979; Niedbala *et al.*, 1985).

Neoplastic cells in suspension.

The newly designed specimen carrier allowed fast cryoimmobilization of the neoplastic cells suspended in culture medium. The cooling rate was the critically important factor for avoiding ice segregation patterns. It must not fall below the optimal values, even when freezing at high pressure (Riehle and Hoechli, 1973). The

thermal coefficient was assumed to remain constant across homogeneous cell suspension and to be equal to that of pure water. Therefore, the freezing rate at a given point of a specimen was a function of the distance from the freezing surface. This criterion was used to determine empirically the thickness of specimens and thus the appropriate height of the carrier, so that at no point of the specimen was the cooling rate below optimal. An equally essential factor was pressure. Pressure exerted on specimens was essential for good preservation of the ultrastructure. The carrier cap operating as a piston, during the hyperbaric phase of freezing process, ensured efficient transfer of pressure within the specimen chamber onto cell suspension. The third factor strongly influencing the observed cell images was the fracture plane localization with regard to the freezing surface. This factor was primarily determined by the specimen carrier construction. Carriers used in earlier studies were composed of two symmetrical planchets with specimens sandwiched between them (Mühlethaler *et al.*, 1970; Moor *et al.*, 1980; Müller and Moor, 1984; Craig *et al.*, 1987; Studer *et al.*, 1989). In practice, fracture planes passed roughly through the specimen central portions. Thus, cells observed in the electron microscope were mostly those cells far from the freezing surface, hence frozen under the worst conditions. The asymmetrical carrier designed for this project ensured fracture planes close and parallel to the cooling surface; thus, cells frozen in the optimal conditions were revealed for observations. Additionally, fractures perpendicular to the freezing surface permitted evaluation of the spatial distribution of freezing phenomena. Consideration of these three factors resulted in the carrier design, which ensured very good ultrastructure preservation of neoplastic cells frozen while they remained suspended in culture medium uncontaminated by sealants, cryoprotectants, or fixatives.

Procedures developed in this project to study the ultrastructure of human neoplastic cells in suspension have three major implications for further projects. First, the electron microscopical sample is representative of the entire cell population. For cells remaining in suspension after fixation or freeze-substitution, this is due to very strong bonds (resistant to electron microscopical processing steps) between derived glass carrier aminopropyl-groups and cryo-fixed cell free aldehyde groups (Malecki and Ris, 1991, 1992). For cryo-fractured cells, this is due to obtaining large fracture areas through the entire cell suspension. These features allow exact correlation between ultrastructural images and quantitative data available from cell sorting, radioimmunoassays, etc. Ultrastructural analysis is performed in the direct extension of cell sorting or radioimmunoassays with the same samples for further electron microscopical processing.

Second, cryo-immobilization of cellular phenomena followed by freeze-fracture, substitution and drying opens up opportunities to study three-dimensional internal organization within individual cells as well as within cell aggregates. Furthermore, observation of sample freeze-fracture large areas (containing many cells) can be followed by high resolution work (selected cell structures) at very high magnifications in the low voltage scanning electron microscope.

Third, fast freezing of individual cells suspended in volumes of culture media that significantly exceed cell volumes offers the opportunity for freezing cell aggregates suspended in culture medium. This technique decreases dangers of intercellular interaction reorganization either by penetration of chemical fixatives or by attachment of living cells to carriers. It allows analysis of these phenomena with high temporal resolution. This is particularly important for studies on mechanisms involved in formation of tumor cell aggregates or interaction between neoplastic cells with natural killer cells (Haji Karim and Carlsson, 1978; Collins *et al.*, 1981; Trinchieri, 1989; Malecki and Walther, 1991).

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References

Albrecht RM, MacKenzie AP (1976) Cultured and free-living cells. In: *Principles and Techniques of Scanning Electron Microscopy*, vol 3, Hayat MA (ed), Van Nostrand Reinhold, New York, pp 109-153.

Amstein CF, Hartman PA (1975) Adaptation of plastic surfaces for tissue culture by glow discharge. J Clin Microbiol **2**:46-54.

Anderson TF (1951) Techniques for the preservation of 3D structures in preparing specimens for EM. Trans NY Acad Sci **13**(II):130-134.

Barber TA (1974) Preparation of single cells for correlative high voltage and scanning electron microscopy. In: *Proc 32nd Meeting EMSA*, Claytor's Publ, Baton Rouge, LA, pp 96-97.

Barber TA (1976) Correlative scanning and high voltage transmission electron microscopy of single cells. In: *Principles and Techniques of Scanning Electron Microscopy*, vol 5, Hayat MA (ed) Van Nostrand Reinhold, New York, vol 5, pp 122-143.

Barber TA, Burkholder PM (1974) Correlative SEM and TEM study of the in vitro macrophage migration response to antigen challenge. Scanning Electron Microsc **1974**:683-690.

Barlow DI, Sleigh MA (1979) Freeze substitution for preservation of ciliated surfaces for SEM. J Microsc **115**:81-95.

Baumeister W, Hahn M (1978) Specimen supports. In: *Principles and techniques of Electron Microscopy*, vol 8, Hayat MA (ed), Van Nostrand Reinhold, New York, vol 8, pp 1-112.

Bell PB (1984) The preparation of whole cells for electron microscopy. In: *The Science of Biological Specimen Preparation for Microscopy and Microanalysis*, Revel JP, Barnard T, Haggis GH (eds), Scanning Microsc Intl, AMF O'Hare, IL, pp 45-59.

Bell PB, Lindroth M, Fredriksson BA, Liu XD (1989) Problems associated with the preparation of whole mounts of cytoskeletons for high resolution electron microscopy. In: *The Science of Biological Specimen Preparation for Microscopy and Microanalysis 1988*, Albrecht RM, Ornberg RL (eds.), Scanning Microsc Suppl **3**: 117-136.

Boyde A, Vesely P (1972) Comparison of fixation and drying procedures for preparation of some cultured cell lines for examination in the SEM. Scanning Electron Microsc **1972**:265-272.

Boyde A, Weiss RA, Vesely P (1973) SEM of cells in culture. Exp Cell Res **71**:313-324.

Bridgman PC, Reese TC (1984) The structure of cytoplasm in directly frozen cultured cells. I. Filamentous networks and cytoplasmic ground substance. J Cell Biol **99**:1655-1668.

Brunk UT, Collins VP, Arro E (1980) The fixation, dehydration, drying and coating of cultured cells for SEM. J Microsc **123**:121-131.

Buckley LK, Porter KR (1973) Advances in EM of whole cells grown in vitro. J Cell Biol 59: **2**:73a.

Buechi M, Bachi T (1979) Immunofluorescence and electron microscopy of the cytoplasmic surface of the human erythrocyte membrane and its interaction with Sendai virus. J Cell Biol **83**:338-347.

Bullock GR (1984) The current status of fixation for electron microscopy. J Microsc **133**:1-15.

Collins TR, Bartholomew JC, Calvin M (1975) A simple method for freeze fracture of monolayer cultures. J Cell Biol **67**:904-911.

Collins VP, Fredriksson BA, Brunk UT (1981) Changes associated with the growth stimulation of in vitro cultivated spheroids of human glioma cells. Scanning Electron Microsc **1981**;II:187-196, 186.

Craig S, Gilkey JC, Staehlin LA (1987) Improved specimen support cups and ancillary devices for the Balzers high pressure freezing apparatus. J Microsc **148**: 103-106.

DeBault LE (1973) A critical point drying technique for SEM of tissue culture cells grown on plastic substratum. Scanning Electron Microsc. **1973**:317-324.

De Harven E, Lampen N, Polliack A, Warfel J, Fogh J (1975) New observations on methods for preparing cell suspensions for scanning electron microscopy. Scanning Electron Microsc **1975**:261-268.

De Harven E, Soligo D, Lampen N (1984) Scanning electron microscopy of human leukocytes. Scanning Electron Microsc **1984**;II:773-782.

Elbers PF, Ververgaert PM, Demel R (1965) Tricomplex fixation of phospholipids. J Cell Biol **24**:23-30.

Fonte VG, Porter KR (1974) Visualization in whole cells of herpes simplex virus using SEM & TEM. Scanning Electron Microsc **1974**:827-834.

Franks J, Clay CS, Peace GW (1980) Ion beam thin film deposition. Scanning Electron Microsc **1980**;I:155-162.

Frederik PM, Stuart MCA, Bomans PHH, Busing WM, Burger KNJ, Verkleij AJ (1991) Perspectives and limitations of cryo-electron microscopy. J Microsc **161**:253-262.

Furth M, Greaves M (1989) Molecular Diagnostics of Human Cancer. Cold Spring Harbor Lab Press. Cold Spring Harbor, New York.

Gilkey JC, Staehlin LA (1986). Advances in ultrarapid freezing for the preservation of cellular ultrastructure. J Electr Microsc Techn **3**:177-210.

Glaeser RM (1974) Radiation damage and resolution limitations in biological specimens. In: *High Voltage Electron Microscopy*, Swann PR, Humphreys CJ, Goringe MJ (eds), Academic Press, New York, pp 370-378.

Haggis GH (1982) Contribution of scanning electron microscopy to viewing internal cell structures. Scanning Electron Microsc **1982**;II:751-763.

Haji Karim M, Carlsson J (1978) Proliferation and viability in cellular spheroids of human origin. Cancer Res **38**:1457-1467.

Heuser JE, Kirschner MW (980) Filament organization revealed in platinum replicas of freeze dried cytoskeletons. J Cell Biol **86**:212-234.

Humphreys WJ, Spurlock BO, Johnson JS (1974) Critical point drying of ethanol infiltrated, cryofractured biological specimens for scanning electron microscopy. Scanning Electron Microsc **1974**:275-282.

Ip WS, Fischman DA (1979) High resolution scanning electron microscopy of isolated and in situ cytoskeletal elements. J Cell Biol **83**:249-254.

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol **27**:137a.

Kelley RO, Dekker RA, Bluemink JG (1973) Ligand mediated osmium binding: its application in coating biological specimens. J Ultr Res **45**:254-258.

Kramer RH, Nicolson GL (1979) Interactions of tumor cells with vascular endothelial cell monolayers. Proc Natl Acad Sci USA **76**:5704-5708.

Lindroth M, Bell PB, Fredriksson BA (1987) TEM, SEM, STEM studies of sputter-coated cytoskeletons. Scanning **9**:47-56.

Malecki M (1990) Stereo imaging system for HVEM. In: *Proc 12th Intern Congress Electr Microsc*, vol 1, Peachey LD, Williams DB (eds), San Francisco Press, San Francisco vol 1, pp 178-179.

Malecki M (1992) High and ambient pressure freezing of human neoplastic cell cultures. J Electr Microsc Techn (in press).

Malecki M, Ris H (1991) Preparation of cell suspensions for ultrastructural studies. Scanning **13**:82-83.

Malecki M, Ris H (1992) Surface architecture and intracellular organization of non-adherent neoplastic cells as revealed by scanning electron microscopy. Scanning (in press).

Malecki M, Small JV (1987) Immunocytochemistry of contractile and cytoskeletal proteins. Protoplasma **139**:160-169.

Malecki M, Walther P (1991) High pressure freezing of cell aggregates for ultrastructural studies with LVSEM and HVEM. Scanning **13**:68-69.

Malecki M, Goodman SL, Albrecht RM (1989) Enhanced endothelial cell adherence and spreading on basement membrane coated polystyrene thin films suitable for LM, LV-HRSEM and HVEM imaging. In: *Proc 47th EMSA Meeting*, San Francisco Press, San Francisco, pp 1002-1003.

Malecki M, Paddock S, Schatten G (1990) Integrated Microscopy Resource for biomedical research at the University of Wisconsin-Madison. EMSA Bulletin **20**:123-127.

Malecki M, Pawley J, Ris H (1991) High pressure freezing of cells in suspension for LVSEM. In: *Proc 49th EMSA Meeting*, San Francisco Press, San Francisco, pp 64-65.

Mazia D, Schatten G, Sale W (1975) Adhesions of cells to surfaces coated with polylysine. J Cell Biol **66**:198-200.

Mazur P (1966) Physical and chemical basis of injury of single-celled micro-organisms subjected to freezing and thawing. In: *Cryobiology*, Meryman HT (ed), Academic Press, New York, pp 214-316.

McDonald K (1984) Osmium-ferricyanide fixation improves microfilament preservation and membrane visualization in a variety of animal cell types. J Ultrastruct Res **86**:107-118.

McDonald K, Morphew MK (1989) Preservation of embryo ultrastructure by high pressure freezing and freeze-substitution. In: *Proc 47th EMSA Meeting*, San Francisco Press, San Francisco, pp 994-995.

McIntyre JA, Gilula NB, Karnovsky MJ (1974) Cryoprotectant-induced redistribution of intramembraneous particles in mouse lymphocytes. J Cell Biol **60**:192-203.

Meryman HT (1966) Review of biological freezing. In: *Cryobiology*, Meryman HT (ed), Academic Press, New York, pp 1-114.

Moor H, Hoechli M (1970) The influence of high pressure freezing on living cells. In: *Proc 7th Intern Conf Electr Microsc, Grenoble*, vol 1, Favard P (ed) Soc Franc de Microsc Electr, Paris, pp 445-446.

Moor H, Riehle U (1968) Snap freezing under high pressure. In: *Proc 4th Eur Reg Conf Electr Microsc*, vol. 2, pp 33-34.

Moor H, Bellin G, Sandri C, Akert K (1980) The influence of high pressure freezing on mammalian nerve tissue. Cell Tissue Res **209**:201-216.

Mühlethaler K, Wehzli E, Moor H (1970) Double fracturing methods for freeze etching. In: *Proc 7th Intern Conf Electr Microsc, Grenoble*, vol 1, Favard P (ed) Soc Franc de Microsc Electr, Paris, pp 449-450.

Müller M, Moor H (1984) Cryofixation of thick specimens by high pressure freezing. In: *The Science of Biological Specimen Preparation for Microscopy and Microanalysis*, Revel JP, Barnard T, Haggis GH (eds), Scanning Microsc Intl, AMF O'Hare, IL, pp 131-138.

Nermut MV, Eason P (1989) Cryotechniques in Macromolecular Research. In: *The Science of Biological Specimen Preparation for Microscopy and Microanalysis 1988*, Albrecht RM, Ornberg RL (eds), Scanning Microsc Suppl 3: 213-226.

Niedbala MJ, Crickard K, Bernacki RJ (1985) Interactions of human ovarian cells with human mesothelial cells grown on ECM. Exp Cell Res **160**:499-513.

Nichols EK (1988) *Human Gene Therapy*. Harvard University Press. Cambridge, Mass.

Oliver JA, Albrecht RM (1987) Colloidal gold labelling of fibrinogen receptors in epinephrine- and

ADP-activated platelet suspensions. Scanning Microsc **1**: 745-756.

O'Toole E, Wray GP, Kremer JP, McIntosh JR (1990) HVEM Cryomicroscopy of Frozen Hydrated PTK Cells. In: *Proc 12th Intern Conf Electr Microsc*, vol 1, San Francisco Press, San Francisco, 500-501.

Pawley JB (1980) Recent improvements to the EM7 at the Madison Integrated Biotechnology Resource. *Proc 38th Meeting EMSA*, Claytor's Publ, Baton Rouge, LA, pp 8-11.

Pawley JB (1981) Digital fine control for the objective lens current of the EM-7. *Proc 39th Meeting EMSA*, Claytor's Publ, Baton Rouge, LA, 304-305.

Pawley JB (1987) LVSEM: A new way of seeing biology. *Proc 45th Meeting EMSA*, Claytor's Publ, Baton Rouge, LA, 550-551.

Pawley JB (1988) Beam deflection for stereo imaging and for compensation of misalignment caused by SE collector field on the Hitachi S-900. Inst Phys Conf Ser **1**:233-234.

Pawley JB, Walther P, Shih SJ, Malecki M (1991) Early results using high resolution, low voltage, low temperature SEM. J Microsc **161**:327-335.

Peters KR (1986) Metal deposition by high energy sputtering for high magnification electron microscopy. In: Advanced techniques in biological electron microscopy, Koehler JK (ed), Springer Verlag, pp 101-166.

Pfenninger KH, Rinderer ER (1975) Methods for the freeze fracturing of nerve tissue cultures and cell monolayers. J Cell Biol **65**:15-28.

Porter KR, Fonte VG (1973) Observations on the topography of normal and cancer cells. Scanning Electron Microsc. **1973**:683-688.

Porter KR, Kelley E, Andrews PM (1972) The preparation of cultured cells and soft tissues for SEM. In: *Proc Vth Ann Stereoscan Coll*, Cambridge Sci Instr, Cambridge UK, pp 1-15.

Pratt KJ, Williams SK, Jarrell BE (1989) Enhanced adherence of human endothelial cells to plasma discharge modified polyethylene terephthalate. J Biom Mater Res **23**: 1131-1147.

Priest JH (1971) *Human Cell Culture*. Publisher Press, Thomas, Springfield, IL, pp 1-29.

Ramsey WS, Hertl W, Nowlan ED, Binkowski NJ (1984) Surface treatment and cell attachment. In Vitro **20**:802-808.

Rapatz G, Luyet B (1965) Effects of cooling rates on the preservation of erythrocytes in frozen blood containing various protective agents. Biodynamica **9**:333-350.

Richter K, Dubochet J (1990). High-resolution study of DNA in vitrified sections. In: *Proc 12th Intern Conf Electr Microsc*, vol 1, San Francisco Press, San Francisco, pp 488-489.

Richters A, Valentin PL (1973) TEM of tissue culture preparations on polystyrene coverslips. Stain Technol **48**:181-188.

Riehle U, Hoechli M (1973). The theory and technique of high pressure freezing. In: *Freeze Etching: Techniques and Applications*, Benedetti EL, Favard P (eds) Soc Franc de Microsc Electr, Paris, pp 31-61.

Ris H (1981) Stereoscopic EM of Chromosomes.In: *Methods in Cell Biology*, vol 22, Academic Press, pp 77-96

Ris H (1985) The cytoplasmic filament system in critical point dried whole-mounts and plastic-embedded sections. J Cell Biol **100**:1474-1487.

Ris H (1988) Application of LVSEM in the analysis of complex intracellular structures. In: *Proc 46th EMSA Meeting*, San Francisco Press, San Francisco, pp 212-213.

Robinson PJ, Dunnill P, Lilly MD (1971) Porous glass as a solid support for immobilization or affinity chromatography of enzymes. Biochim Biophys Acta **242**: 659-661.

Ruoslahti E (1991) Integrins. J Clin Invest **87**:1-5.

Sanders SK, Alexander EL, Brayden RC (1975) A high-yield technique for preparing cells in suspension. J Cell Biol **67**:476-480.

Sitte H, Edelmann L, Neumann K (1987) Cryofixation without pretreatment at ambient pressure. In: *Cryotechniques in Biological Electron Microscopy*, Steinbrecht RA, Zierold K (eds) Springer Verlag, pp 87-113.

Small JV, Celis JE (1978) Direct visualization of the 10 nm (100 Å) filament network in whole and enucleated cultured cells. J Cell Sci **31**:393-409.

Sorber CWJ, De Jong AAW, Den Breejen NJ, De Bruijn WC (1990) Quantitative energy-filtered image analysis in cytochemistry. Ultramicrosc **32**:55-68.

Studer D, Michel M, Müller M (1989) High pressure freezing comes of age. In: *The Science of Biological Specimen Preparation for Microscopy and Microanalysis 1988*, Albrecht RM, Ornberg RL (eds), Scanning Microsc Suppl **3**:253-269.

Trinchieri G (1989) Biology of natural killer cells. In: *Advances in Immunology*, vol 47, Dixon FJ (ed), Academic Press, New York, pp 187-376.

Trotter JA, Kelley RO (1979) A novel technique for high resolution analysis of the cytoskeleton. Anat Rec **195**:7-14.

Trotter JA, Foerder BA, Keller JM (1978) Intracellular fibres in cultured cells: Analysis by scanning and transmission electron microscopy and by SDS-polyacrylamide gel electrophoresis. J Cell Sci **31**:369-392.

Van Harreveld A, Crowell J (1964) Electron microscopy after rapid freezing on a metal surface and substitution fixation. Anat Rec **149**:381-386.

Wetzel B, Albrecht RM (1989) The evolution of correlative techniques for electron microscopy. In: *The Science of Biological Specimen Preparation for Microscopy and Microanalysis 1988*, Albrecht RM, Ornberg RL (eds), Scanning Microsc Suppl **3**:1-6.

Wetzel B, Cannon GB, Alexander EL, Erikson BW, Westbrook ES (1974) A critical approach to the scanning electron microscopy of cells in suspension. Scanning Electron Microsc **1974**:581-588.

Wolosewick TJ, Porter KR (1976) Stereo HVEM of whole cells of human diploid line WI 38. Am J Anat **147**:303-324.

Discussion with Reviewer

T.D. Allen: You have been successful in dissolving tissue culture plastics to generate thin film for cell growth suitable for electron microscopy, and the rationale for this is to provide a comparable substratum to tissue culture flask growing surfaces. In producing these films, however, you have dissolved tissue culture plastic in amylacetate to make the films. In doing this you must have altered the special surface treatment during manufacture which allows the tissue culture plastic to be either hydrophobic (as in the case of bacteriological tissue culture plastic) or hydrophilic (mammalian tissue culture plastic). In our own experience the manufacturers always refuse to specify exactly how they make growing surfaces of tissue culture plastic ware hydrophilic, and how sure can you be that you have exactly replicated the manufacturer's procedure of surface treatment.

Author: The biocompatibility of polystyrene cell culture ware can be changed through three different procedures: a) glow discharge (Amstein and Hartman, 1976, text reference), b) oxidation (Curtis *et al.*, 1986), and c) protein adsorption (McKeehan and Ham, 1976; Gospodarowicz and Tauber, 1980). Glow discharge has been used by Corning to improve surface properties of cell culture ware (Ryan, 1989). The exact technological parameters (pressure, plasma current, voltage, duration, frequency) are proprietary; however, the implications of changes in these parameters for cultured cell behavior have been described (Ramsey *et al.*, 1976). Physical surface properties can be evaluated by measuring contact angles, counting hydroxyl groups, or measuring surface charges. However, the polystyrene biocompatibility can best be evaluated by measurements of cell plating efficiency, spreading rate and doubling time. The measurements performed in this project demonstrate the same biocompatibility qualities of: a) polystyrene dishes plasma discharge treated to enhance suitability for culture commercially available (Corning), b) polystyrene dishes untreated by manufacturer (Corning), but exposed to oxygen plasma discharge in this project, and c) thin polystyrene films (suitable for the integrated microscopy) manufactured in this project.

Additional References

Curtis ASG, Forrester JV, McInnes C, Lawrie F (1986) Adhesion of cells to polystyrene surfaces. J Cell Biol **97**:1500-1506.

Gospodarowicz D, Tauber JP (1980) Growth factors and extracellular matrix. Endocr Rev **1**:201-207.

McKeehan WL, Ham RG (1976) Stimulation of clonal growth of normal fibroblasts with substrata coated with basic polymers. J Cell Biol **71**:727-734.

Ryan JA (1989) *General Guide for Identifying and Correcting Common Cell Culture Growth and Attachment Problems*. Corning Glass Works BVP3/89: 1-9.