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**ATTACHMENT OF PLASMA MEMBRANES OF CULTURED CELLS TO SILICON CHIPS
FOR HIGH MAGNIFICATION IMAGING IN SCANNING ELECTRON MICROSCOPY**

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Abstract

In the membrane preparation method described in this paper, a polylysine-coated silicon chip is adsorbed to the exposed apical surface of a cell monolayer. Upon removal, the adsorbed chip separates the plasmalemma from the residual bodies of the cultured cells. This sandwich-membrane separation approach simplifies access to the cytoplasmic aspects of both the apical and the basal plasmalemma which remains on the culture substrate and is covered to a varied extent by cytoplasmic infrastructures. To stabilize the attached membranes, small crosslinking agents are used in a controlled osmium impregnation. Large crosslinkers are avoided since they induce thickening of fine structures. Optimal conditions for attachment of plasmalemma of cultured adrenal endothelial cells to the cationic chip are defined. Effective cleaning procedures of the chips and useful molecular weights of polylysine are determined by quantitating colloidal gold adherence to the surface of the chips. The specimens are examined by high resolution scanning electron microscopy.

KEY WORDS: Cell splitting, sandwich-membrane separation method, membrane adsorption, polylysine coated Si chips, silicon chip, high resolution scanning electron microscopy, SE-I signal contrast, colloidal gold, preparation techniques, internal structures of cells.

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Introduction

For conventional scanning electron microscopy (SEM) imaging, cellular specimens are coated with a 10-20 nm thick metal film to improve electrical conductivity and signal generation (Echlin, 1972). Such thick coatings are not useful for high magnification work because they obscure the fine surface structures of the specimens. In high magnification imaging, the metal is primarily used for contrast enhancement of surface features and for stabilization of surface fine structures against volume loss caused by beam damage (Peters, 1986a). Only very thin and continuous metal films of 1-2 nm thickness are suitable for high resolution SEM, but do not scatter the electrons of the probe sufficiently to prevent specimen charging. The electrical conductivity of tissues can be adequately increased by a controlled osmification (Peters and Green, 1983; Peters, 1985) using thiocarbonylhydrazide (TCH) as a small covalent crosslinker (Hanker et al., 1964) and avoiding osmium black coating (Kelley et al., 1973). Osmium black precipitation at the surface of the specimen can be prevented if the surface of interest is exposed before the osmification procedure is performed. In that way, diffusible non-bound reagents and reaction products can be effectively removed by extensive washing. In contrast, osmium black accumulations beneath the surface are not completely dissolved and removed during washing; therefore, an exposure of surfaces after osmium impregnation is not appropriate for high magnification work.

Application of this high resolution preparation procedure is not limited to *in situ* existing surfaces. Other surfaces can be accessed preceding dehydration and drying, either before or after conventional fixation (aldehydes and buffered osmium tetroxide). From bulk tissue samples, thin slices can be made after fixation and used to visualize the extracellular aspect of the plasmalemma on the exposed cells (Peters, 1985; Milici et al., 1986). Membranes of single cells and cells in culture are accessed with a variety of other methods in which, in a first step, cell membranes of interest are attached to a solid support and, in a second step, are exposed by removing the parts of the cell which cover the surfaces of interest.

Several different ligands are applicable to

bind the plasmalemma either to flat coverslips or small round beads, utilizing ionic, covalent or membrane specific ligand-cell membrane interactions. Cationic ligands were found to interact with anionic surface charges at the plasmalemma. Polylysine binding to cell surfaces as well as to glass has been intensively studied (Nevo et al., 1955). Polylysine was adsorbed to clean glass, plastic or metal supports and used for cell adsorption (Kennedy and Axelrad, 1971; Tachibana and Ishikawa, 1973), or attachment of cell organelles (Mazia et al., 1974), or as support for cell cultures (Yavin and Yavin, 1974). Other cationic ligands were also applied, i.e., protoamine sulfate (Steinhardt et al., 1971; Vacquier, 1975) or Alcian blue (Sommer, 1977). Nitrocellulose coating of DEAE Sepharose beads was also found suitable to adsorb plasma membranes (Gotlib and Searls, 1980). To improve the stability of polylysine coating, a covalent binding through various organic ligands to the substrate was applied on glass beads (Kalish et al., 1978; Jacobson et al., 1978) or on polyacrylamide beads (Cohen et al., 1977 and 1980). Other ways to bind membranes to the substrate involved covalent, direct binding (Blechi and Bächli, 1979; Eshdat and Prujansky-Jakobovits, 1979) or covalently bound membrane-specific ligands, i.e., IgG bound to polyacrylamide beads (Ito and Palade, 1978) or lectins bound to glass beads (Aplin and Hughes, 1981).

Poly-L-lysine hydrobromide (polylysine) is most commonly used to coat support surfaces and adsorb on them plasmalemmal specimens which are then processed for SEM or transmission electron microscopy (TEM) imaging. Three different methods have been applied to recover parts or all of the plasmalemma: a "freeze fracture-membrane splitting method", a "cell lysis-squirt cleaning method", and recently a "sandwich-membrane separation method". The freeze fracture splitting of attached membranes allowed the separation of extracellular half-membranes which can be chemically analyzed (Park and Pfeifhofer, 1974; Fisher, 1974; Jacobson et al., 1974) and whose surface (E face) can be imaged by TEM methods (Fisher, 1975; Nermut and Williams, 1976 and 1977). To allow access to the cytoplasmic aspect of intact cell membranes, two different approaches were followed. Either the plasmalemmal area by which the cells attach is used or the area which faces the medium is recovered. The first approach removes the bulk of the cell bodies by lysis and mechanical force produced by a jet of liquid and is characterized as a lysis-squirting method (Nermut, 1982 and 1983). Initially applied for chemical analysis of the remaining membranes (Jacobson et al., 1974; Cohen et al., 1977; Jacobson and Branton, 1977), the approach was extended to SEM and to TEM imaging by negative and positive staining (Clarke et al., 1975; Mazia et al., 1975). Then, Pt-C replicas were used for fine structure analysis by TEM (Blechi and Bächli, 1979; Aggeler and Heuser, 1980; Lang et al., 1981). The "apical" membranes of attached cells are separated from the cell bodies by a sandwich separation method. In this case, a second ligand coated surface is placed over the cell layer and the apical plasmalemma is adsorbed to it. Then,

CONTROLLED Os IMPREGNATION on Silicon Chips

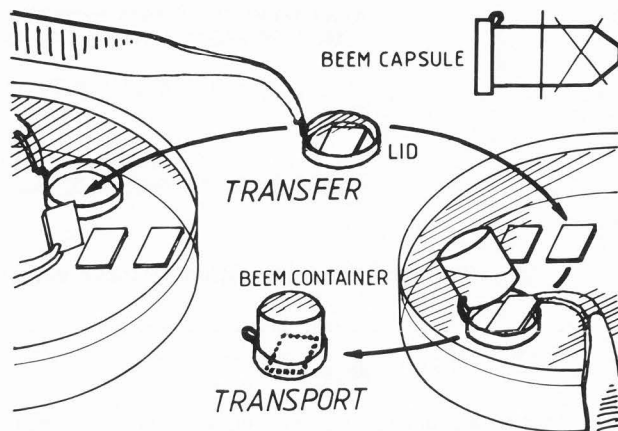


Fig. 1. Handling of silicon chips during controlled osmium impregnation. The chips are transferred from one Petri dish to another in liquid filled lids of BEEM capsules. Finally, the chips are mounted in BEEM containers made from capsules cut in half.

after separating the sandwich, the membranes attached to the second surface are processed for TEM replica imaging either by freeze-drying (Aggeler and Werb, 1982) or critical point (CP)-drying (Aggeler et al., 1983) or for SEM imaging (Peters et al., 1984 and 1985). Since preservation of the integrity of membrane structures was of great concern in CP-dried specimens, it was recommended to stabilize the adsorbed membranes by a coat of large-sized crosslinkers, i.e., lysine-glutaraldehyde complexes (Boyles, 1984) or tannic acid (Aggeler et al., 1983; Rutter et al., 1985). However, these procedures lead to a recognizable increase in the dimensions of small features and this makes this approach of little use for fine structure imaging.

For high magnification SEM, the adsorbed membranes were found to be sufficiently well preserved by the stabilization effects of the controlled osmification. This approach had the advantage of imaging particles as small as ~ 5 - 10 nm whose size - we assume - is not grossly affected by the preparation procedure. However, effective adsorption of the membranes to the support became essential. In this paper, artifacts of membrane detachment and their prevention are described.

Materials and Methods

Materials.

Silicon chips were cut from ~ 0.25 - 0.27 mm (10-12 mil) thick wafers of a 1:0:0 orientation and a diameter of ~ 7.5 cm (3 inch) (Cincinnati Milacron Co., Lebanon, OH). Wafers of this type cut easily into rectangular chips 5 x 7 mm in size. Wafers of any dopant-type can be used, i.e., nitrogen/antimony. Si chips are now

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available from Ted Pella, Inc., Redding, CA (Cat. No. 16007, 16008). Poly-L-lysine hydrobromide and all other chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO). Colloidal gold was prepared by Frens' procedure (1973).

Preparation Procedure.

Bovine adrenal cortex endothelial (BACE) cells (Peters et al., 1985) were grown on gelatin-coated silicon chips (5 x 7 mm in size) and transferred into a deep Petri dish containing a hypotonic (~200 mOsmol) cytosolic (H-C) buffer composed of 70 mM KCl, 5 mM MgCl₂, 3 mM EGTA, and 30 mM Hepes, pH 7.0 (Hirokawa and Heuser, 1981). After washing the cells by agitating the chip in the buffer for 5 - 10 sec, the cells were sandwiched between another, polylysine coated chip which adsorbed to their previously exposed "apical" plasmalemma. After 30 - 60 sec the chips were separated and adsorbed plasmalemmal fragments and complementary cell remnants were immediately fixed for 30 min in 1.6% formaldehyde (freshly prepared from paraformaldehyde), 1% glutaraldehyde in 70 mM KCl, 5 mM MgCl₂, 3 mM EGTA buffered to pH 7.0 with 0.1 M Na cacodylate-HCL (buffer F, ~300 mOsmol). The chips with specimens were transferred from one reagent to another under liquid in simple small containers (Fig. 1), made from the lid of BEEM (Better Equipment for Electron Microscopy, Inc., Bronx, NY) capsules (regular type, size 00: E.F. Fullam Inc., Schenectady, NY). After fixation, the chips were transferred into the same buffer and washed for 15 min, treated for 30 min in 1% OsO₄ in buffer F, and washed 6 times for 15 min each using the same buffer at each step. The specimens were subsequently stabilized by a 10 min treatment with saturated thiocarbonylhydrazide in 150 mM KCl then washed extensively 8 times for 15 min each with 150 mM KCl, and reacted for 10 min with 1% OsO₄ in 150 mM KCl to obtain a controlled osmium impregnation (Peters and Green, 1983). After repeated

washes in H₂O (4 times, 10 min each), the chips were mounted into BEEM containers (made from capsules cut in half) and processed with an exchange technique (Peters, 1980a) through dehydration in ethanol (using a linear concentration gradient between H₂O and ethanol) followed by substitution with Freon 113 (via a linear concentration gradient between dried ethanol and Freon 113). The specimens were finally CP-dried in CO₂ and coated with a 1-2 nm thick continuous chromium film by high-energy sputtering (Peters, 1980b, 1986b and c).

Microscopy.

Specimens were examined by SEM at 30 kV in the SE-I image mode (Peters 1982 and 1984a) in a JEOL JFSM 30 provided with a cold field emission gun. Specimen-specific SE collection was achieved by shielding the pole piece with a converter plate and biasing the plate positively (Peters, 1984a and 1985). The specimen was either biased positively or negatively in order to increase signal collection efficiency.

Stereo Micrographs.

Stereo micrograph pairs were taken with a 4° stereo angle and mounted vertically for viewing with stereo prisms. A newly introduced "KMQ System" (Prismatic Stereo Viewer: MA-302-0; Marivak Ltd., Halifax, NS B3K1X5, Canada) is recommended because it uses stronger prisms than the "System Nesh" (Viewer B8010 040 45; Balzers High Vac. Corp., Nashua, NH). Moreover, the KMQ System uses modified prisms which enables easier stereo adaptation. The KMQ prisms are held with their flat surface facing the eyes. When viewing the same stereo pairs in the Nesh system, the prisms have to be held with their flat surface facing the micrographs because the prisms are mounted on reverse.

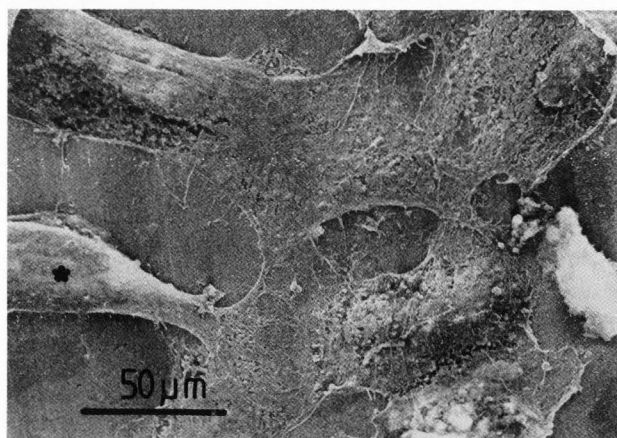


Fig. 2. BACE monolayer after retrieval of plasmalemma by the sandwich-membrane separation method. Most cells exhibit an exposed cytoplasm. Some cells remain intact (asterisk).

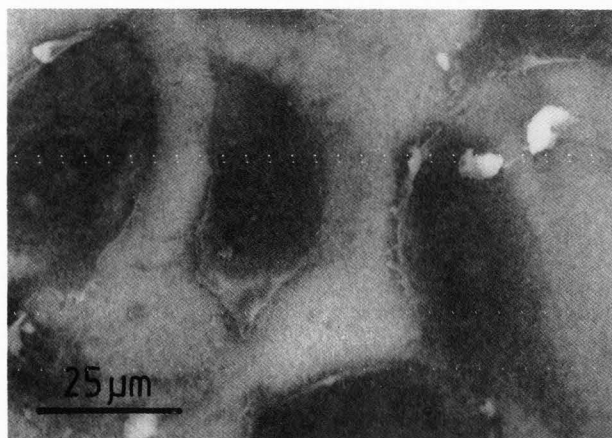


Fig. 3a. SE image of apical plasmalemma (PL) attached to the polylysine coated silicon chip used for membrane separation. Thin membrane areas are imaged in a material contrast.

Results

The sandwich-membrane separation method is especially suited to prepare the plasmalemma of cells grown in monolayers. However, a strong binding of the membranes was found to be necessary to stabilize the membranes during CP-drying. Visualization of the Internal Structures of Cultured Cells.

Both chips of the sandwich were processed for imaging. On the chip used for culturing the cells, most of the cell remnants revealed exposed internal structures (Fig. 2). However, some cells were left intact but were flattened (Fig. 2: asterisk).

Large patches of plasmalemma were found on the polylysine coated silicon chips (Fig. 3a). At low magnifications the membranes appeared dark due to a material contrast produced on the silicon

background. The backscattered electron (BSE) image was useful to assess the thickness of the membrane-attached infrastructure (Fig. 3b). If no other filamentous material was present, the BSE signal given by plasmalemmal patches was similar (Fig. 3b: asterisks) to that generated on the bare silicon substrate. However, thick clumps of remaining cytoplasmatic materials produced strong topographic particle contrast. In the thin regions, the plasma membrane appeared as a smooth continuous sheet imaged at higher magnifications mainly with topographic contrasts (Fig. 4). The cytoplasmic aspect revealed submembranous filamentous material surrounding vesicles and coated pits (Peters et al., 1985).

Most of the infrastructure and cell organelles of the cytoplasm remained on the chip used for cell culture. At higher magnification, in areas where little of the cell bodies were left,

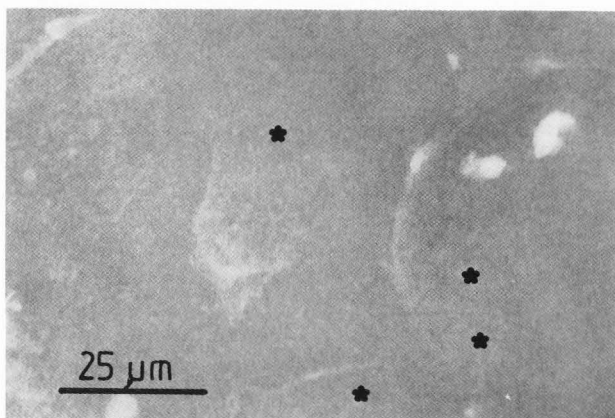


Fig. 3b. BSE image of the same specimen as used in figure 3a. Thin areas of PL (asterisks) are not recognized by BSE. Thicker structures are imaged in topographic contrasts.

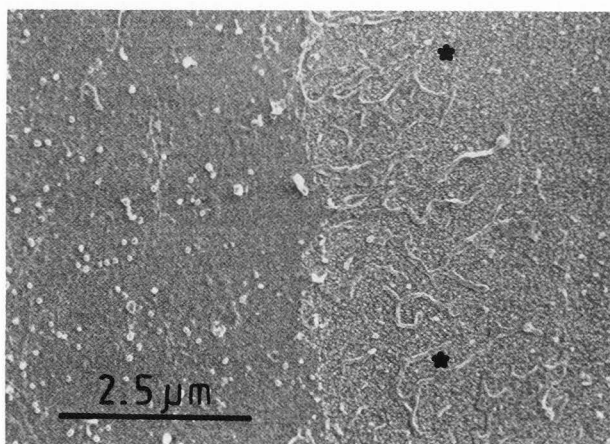


Fig. 4. Edge of an attached membrane sheet bordered by a zone of disintegrated material (asterisks). Topographic contrasts are produced entirely in the thin metal coating.

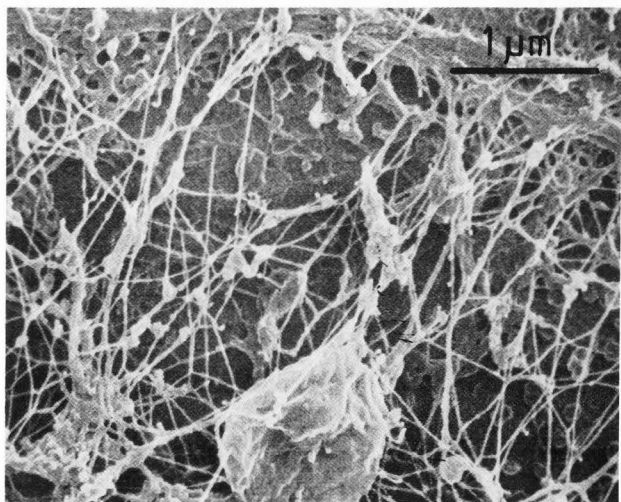
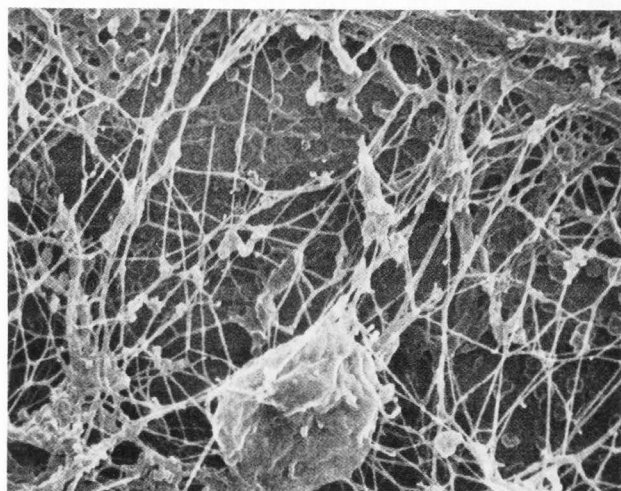


Fig. 5. Stereo pair of an area from figure 2. The basal plasmalemma is obscured by dense cellular infrastructure and stress fibers (top). Vesicles can be seen attached to the membrane.

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cytoplasmatic infrastructure could be observed (Fig. 5). A network of filaments and enclosed organelles lay on the basal plasmalemma. Submembranous stress fibers were recognizable as well as individual (striped) vesicles attached to the cytoplasmic face of the membrane by thin extensions of their surface coat (Peters et al., 1985).

Detachment of Cell Membranes.

The large pieces of plasmalemma, adsorbed to the polylysine coated chip, were often surrounded by a zone of disintegrated membrane (Fig. 4: asterisks) which formed irregular disrupted patches and elongated thin tubules, ~ 25 nm in diameter. In this zone the membrane apparently had a reduced mechanical contact with the chip's surface when the cell body flattened off and extended briefly onto the adjacent cationic surface during the sandwich procedure.

In routine preparations, sometimes intact membranes were not found at all. On entire chips, the remaining membranous materials appeared disintegrated, although the mechanical pressure during the adhesion period was unchanged. In areas of cell contact, membranes either disintegrated in punctured sheets (Fig. 6) or formed tubules (Fig. 7). However, the membrane stayed partially intact when covered by filamentous material (Fig. 8). Apparently, the mechanical support provided by the filaments (asterisk) helped prevent total disintegration. A similar effect was found on membrane patches covered by a polygonal network of clathrin (Fig. 9). Some chips were completely free of any organic material. Several preparation parameters expected to influence the binding strength of the membranes to the cationic surface of the chips were examined, i.e., buffer composition, pH, temperature (4 versus 20°C), pressure and contact time during sandwiching, agitation during washing and length of fixation. However, no significant influence on membrane stability was observed.

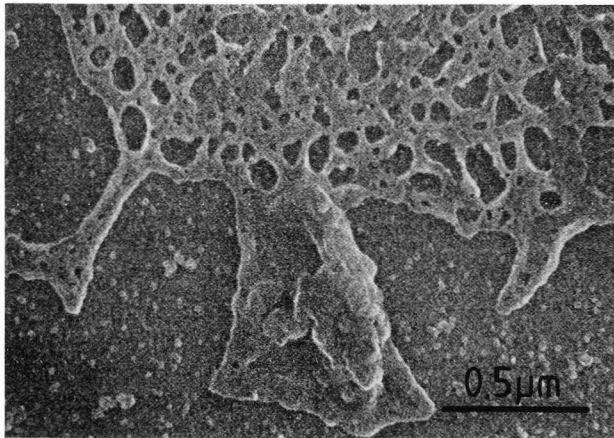


Fig. 6. Membrane partially detached due to insufficient adsorption. First holes appear, then the membrane rolls up.

Possibly, in cases of membrane detachment, the polylysine coating was discontinuous or inefficient.

Charge density of polylysine coated chips.

The relative charge density on polylysine coated silicon chips was tested with colloidal gold, mixed 1:1 (vol/vol) from gold preparations with particles of 20 nm and 40 nm average diameters. The polylysine coated chips were agitated for 30 seconds in the colloidal gold mixture. Following a washing step of 30 seconds in H_2O , the chips were dehydrated in 3 steps of 30%, 60% and absolute ethanol (10 seconds each) and air dried. For cationic coating of the chips a 0.2% (w/vol) solution of 330 kD MW poly-L-lysine hydrobromide in H_2O was applied for 15 min, followed by 10 sec agitated rinsing in water and air drying. As a control for the cationic coating, uncoated chips cleaned in chloroform were labeled with colloidal gold. Such chips were free of gold granules except for a few defined areas where obviously some dirt still adhered.

First, different cleaning procedures were tested to prepare the surface of the chips for polylysine coating and successive gold labeling. Boiling for 1 hr in concentrated sulfuric acid (Fig. 10) or rinsing for 30 seconds in organic solvents, i.e., chloroform (Fig. 11), acetone (Fig. 12) or Freon 113 (Fig. 13) were applied. Conventional acid cleaning gave the least binding assessed by counting the number of particles per μm^2 . Chloroform increased binding by $\sim 70\%$, acetone by 100% and Freon by 150%. Although the larger gold particles were less frequent ($\sim 10\%$ of the 20 nm particle density), they followed the same trend of increased binding.

Secondly, other different molecular weight polylysine preparations were applied to Freon cleaned chips. A 400 kD MW polylysine coating bound gold particles of similar frequency as obtained with the 330 kD MW polylysine. However, a 17 kD MW preparation gave only $\sim 10\%$ of that

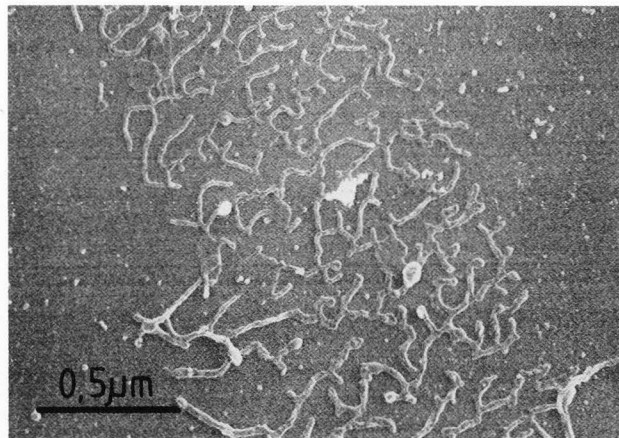


Fig. 7. Disintegrated membrane forming tubular structures which are still in part adhering to the chip.

Stereo-micrographs are mounted vertically for viewing with stereo prisms only.

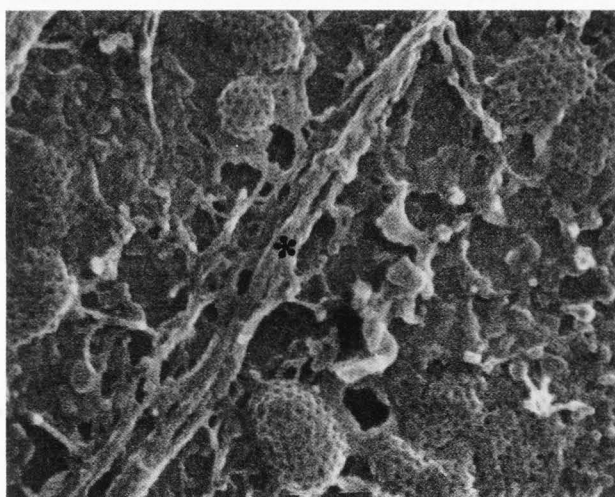
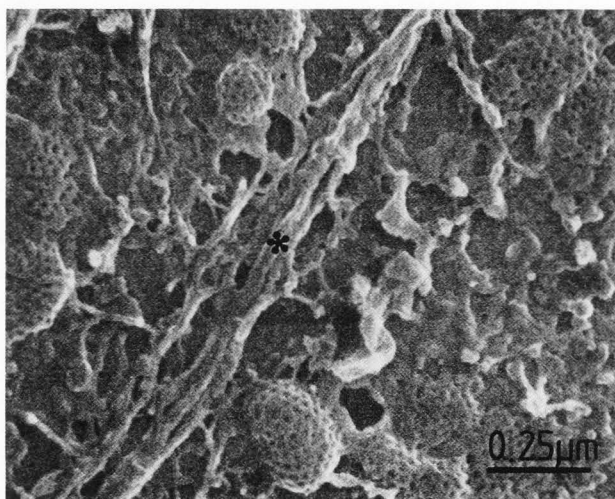


Fig. 8. Stereo pair of partially detached membrane stabilized to a certain extent by fibrillar infrastructure (asterisk).

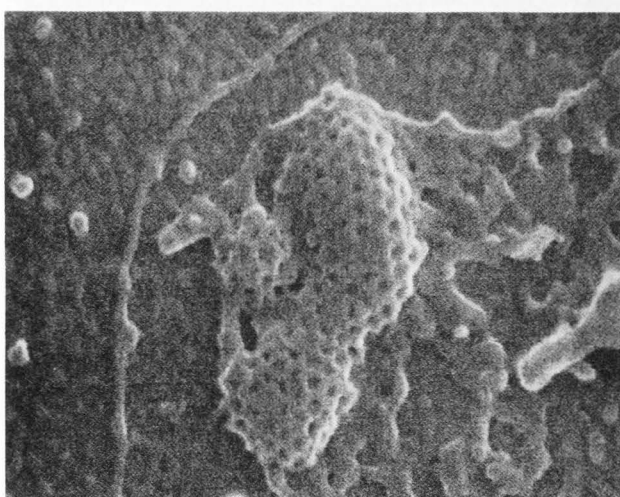
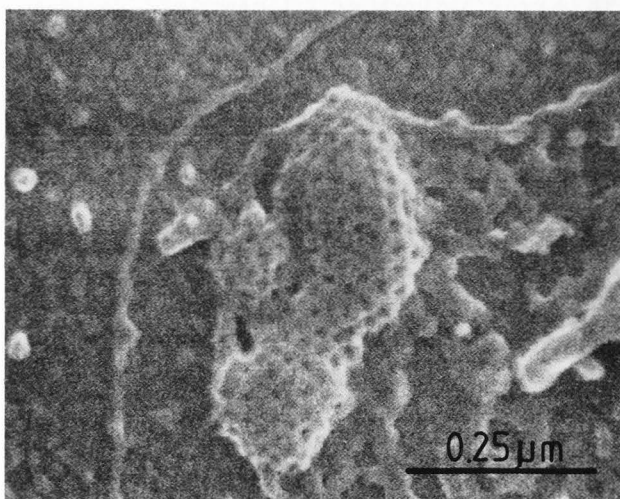


Fig. 9. Membrane patch stabilized by a clathrin coat. Other membrane areas are disintegrated (right) or detached then lost during preparation (left).

binding.

Comparison at the same magnification of the patterns of disintegrated membranes (Figs. 6 and 7) and adhering gold (Figs. 10-13) may illustrate the effects an insufficient charge density might have on the stabilization of membranes.

Discussion

The sandwich-membrane separation method described here is, in principle, identical to the established method (Aggeler and Werb, 1982) but is modified in the following ways to suit high magnification SEM imaging (Peters et al., 1985): i) silicon chips are used as supports for cells and membranes; ii) thin continuous metal coatings are used for contrast enhancement; iii) an

additional step of controlled osmification is added to stabilize molecular structures and to increase electrical conductivity; and iv) stronger adhesion of membranes to the support is provided so as to eliminate large size crosslinkers and to preserve specimen structural details on a nm-scale. These modifications are essential for high resolution SEM because the coated specimens are imaged without further processing required for TEM replica imaging.

Specimen Supports.

The use of silicon chips as supports for cells and plasmalemma is very advantageous in high magnification microscopy since they have a high electrical conductivity and are chemically inert when compared to glass (Broers et al., 1975). Additionally, cells in culture grow as well on silicon chips as on glass (Biemersderfer et al.,

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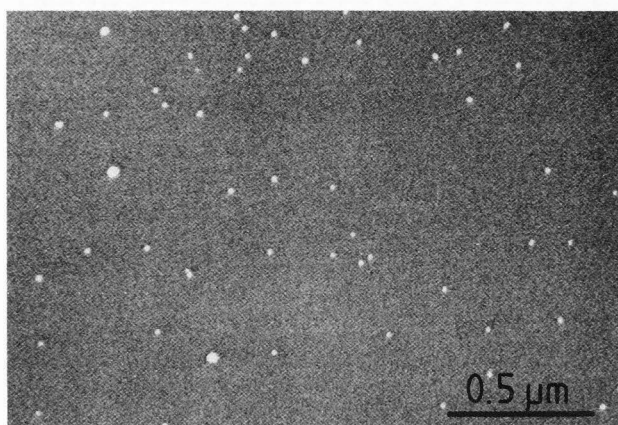


Fig. 10. Acid cleaned chip coated with 330 kD MW poly-L-lysine hydrobromide and gold labelled. Label density taken as standard.

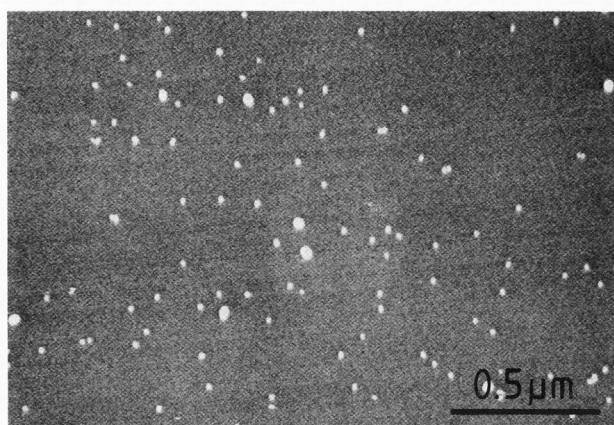


Fig. 12. Acetone cleaned chip, coated and labelled as in figure 10. 100% increase of label density.

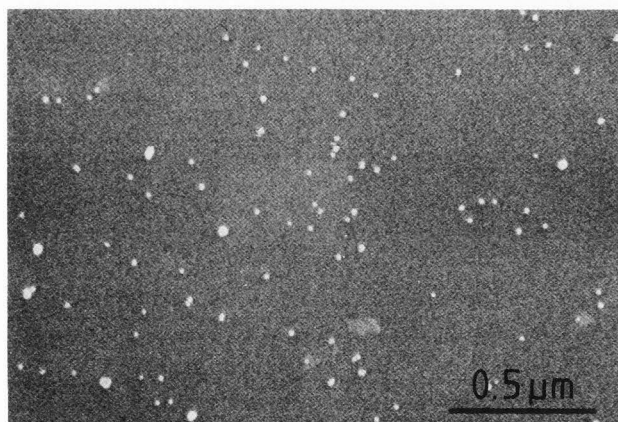


Fig. 11. Chloroform cleaned chip, coated and labelled as in figure 10. 70% increase of label density.

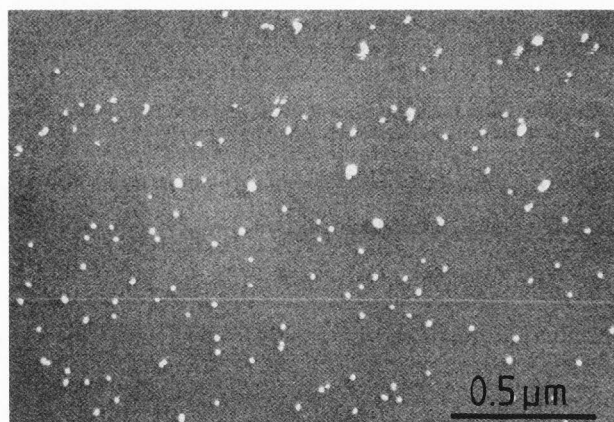


Fig. 13. Freon 113 cleaned chip, coated and labelled as in figure 10. 150% increase of label density.

1978). The low electrical resistance of the silicon supports eliminates the need to coat the support with metal. Chips allow use of much thinner films to coat the specimens than in conventional procedures since the conductive path from the probe site to the electrical ground of the specimen holder is shortened to the direct line through the support. On high resistance supports, i.e., glass, mica or plastic, heavy metal coating is needed to cover the whole surface of the supports and to access the electrical ground. In that case, sufficient metal must also accumulate at the perimeter of the specimens to provide contact between the specimen surface and the metal film on the support.

The silicon chips are cut into pieces, 5 x 7 mm in size, to fit into the lid of BEEM capsules. In the lid they may be held by the attached part of the capsule proper which remains connected to the lid after the capsule is cut in half. The assembly makes a small container in which a chip is held under liquid during all successive

preparation steps (Peters, 1980a and c). However, during controlled osmification, chips have to be handled individually (Fig. 1) to avoid osmium black accumulation in the capsules.

Microscopy.

Silicon chips are suited for the imaging of small or thin specimens (Male and Biemersderfer, 1978) since they are composed of a homogeneous low atomic number material. This property facilitates imaging of small details in high resolution SE contrast since the background signal is reduced in level and contrast (Peters, 1984a). The high resolution SE-I signal is imaged only at high magnifications and is recognizable in thin bright rims of a few nm in width, outlining edges and particles (Peters, 1984b and 1985). At low magnifications (<25,000 x), this signal does not contribute to the contrast which is entirely produced by the conventional type II SE signal generated by scattered electrons of the electron probe. At low magnifications (Figs. 2 and 3), the SE-II signal images thin membranes on the silicon

support in material contrast. Although the BSE signal strength is not affected by the membranes, more SE are generated by emerging BSE on the surface of the silicon than on the carbonous, chromium-coated membranes. At higher magnifications (Fig. 4), when the exit area of extensively scattered BSE ($\sim 3-5 \mu\text{m}$ in diameter) accounts for a substantial part of the imaged specimen surface, the SE signal component, produced by these BSE, is reduced to a background signal and the fraction of SE produced in the metal coating, contribute topographic contrasts to the image. These contrasts are similar on the membranes and the support since they both are covered evenly by the same metal.

Membrane Adhesion.

The physio-chemical binding of the plasmalemma to a polylysine-coated surface is affected by the strength of ionic interactions between the cationic surface layer of the chips and the anionic glycocalyx of the membranes. It was already shown that the binding technique has the following constraints (Nermut, 1982): i) the cell layer must be a uniform monolayer and the cell surfaces must be smooth; ii) the medium in which the sandwich is produced must be free of small proteins or divalent ions; and cell lysis before attachment must be avoided; iii) hydrophobic surfaces prevent attachment, thus dishes and tweezers must be clean; and iv) high salt and high pH reduce adhesion. However, even under these conditions not all membranes show sufficient attachment (Nermut, 1983) due to a premature rupture of cells during the sandwiching procedure or insufficient ionic interaction of the plasmalemma with the cationic surface. Cell rupture could be reduced by preincubating the intact cells with tannic acid (Rutter et al., 1985), however, such treatment of living cells may alter composition and fine structure of the plasmalemma. Little attention was given to the ionic binding of the membranes to the cationic surface under these conditions.

Satisfactory attachment of red blood cell (RBC) membranes was obtained with a surface coat from 2 - 4 kD MW poly-L-lysine hydrobromide (Fisher, 1975; Nermut and Williams, 1977), but this coating was not sufficient for the BACE cells used here. The reason for their lack of adhesion may be found not only in an insufficient charge density on the surface of chips and cells, but also in the steric inaccessibility of charged groups. Endothelial cells have a glycocalyx which extends further into the extracellular space and is expected to be more heterogeneous than that of the RBC membranes. Two layers of cationic ferritin bound to the BACE plasmalemma accessing a 12-24 nm wide zone of negatively charged moieties (Milici et al., 1986). On other endothelial cells, ectodomains of membrane proteins were imaged as ~ 15 nm high rod-like particles (Peters, 1985). However, also much longer filamentous proteoglycans are expected to extend above the membranes. Therefore, 20 and 40 nm negatively charged colloidal gold particles were chosen as a probe to demonstrate accessibility of cationic charges on the silicon surface.

The surface charges accessible to the gold on 17 kD MW polylysine-coated chips were very few and indicated that steric inaccessibility might have

been a major problem for membrane attachment. Nevo et al. (1955) demonstrated that polylysine of small MW forms an interlaced flat layer on glass surfaces. Only large MW forms can be expected to extend into the fluid phase sufficiently high to be accessed by negatively charged cell surface moieties of various sizes. Conventionally, 80 - 250 kD MW polylysines are used to attach cells other than RBC (Jacobson and Branton, 1977; Cohen et al., 1977). Here, for membrane attachment, 330 - 400 kD MW were successfully applied. However, a surprisingly low labelling density of charged sites were found by applying the gold probe. Although it is not conclusive as to what extent the gold density is representative of the total charge density, it can be used as a relative measure for charges accessible to the marker.

Conventional acid cleaning of glass (Nermut, 1982), sufficient for RBC membrane attachment, did not give the highest labelling density. Organic solvent rinsing improved adhesion of the polylysine. Silicon chips are produced with ultraclean surfaces and shipped in an inert gas atmosphere. However, during the cutting procedure and during conventional storage they might be contaminated. Freon cleaning gave the highest labeling density and is now used routinely in the preparation of membrane substrates.

A small marker of 5 - 10 nm in diameter might label positively charged surfaces with higher density than the gold particles used here to assay charge accessibility. Native ferritin or small colloidal gold may be suitable to determine the total charge densities on the substrate. A further improvement in cationizing chips is expected from covalent binding of polylysine to silanized surfaces using glutaraldehyde.

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Discussion with Reviewers

E. deHarven: For the benefit of the reader who is not equipped to do high-energy chromium sputtering could you please indicate what are the differences between the physical characteristics of a 1-2 nm continuous chromium film obtained by high-energy sputtering and those of the thinnest continuous

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chromium film obtained (at similar substrate temperature) by evaporation?

Authors: The films differ in two properties which concern thickness and evenness of mass distribution on the surface. Both film properties affect extensively surface imaging at high magnification by altering the strength of SE-I contrasts and the size of smallest detectable features. For details, please refer to some publications which specifically address these effects, i.e. Peters, 1985 and 1986a, b, c.

E. deHarven: This technique permits to "visualize internal structures of cultured cells". Among these structures, coated pits seem to be the only ones which can be positively identified. All the others are described as "submembranous filamentous material", without any precise identification of the type of cytoskeletal element involved. What can we expect from a method which apparently cannot identify all the types of cytoskeletal microfilaments, so clearly identified in transmission EM?

Authors: You may like to consider that the cytoplasm is a well ordered, dense complex of soluble and structural and regulatory elements. Several "techniques" are established (and referred to in the text) which may be applied to uncover the structures of interest by "extracting" undesirable elements. Such preparative techniques may or may not be applied independently of the imaging modes (TEM or SEM) and of the preparative "methods" described here and used to access the cytoplasmic "aspect" of the plasmalemma. However, our approach will allow better imaging of fine structural details and better accessibility to the cytoplasmic face of the plasmalemma with probes such as antibody-gold conjugates. In this study, no attempts were made to describe and identify cytoskeletal microfilaments.

P.B. Bell, Jr.: What was the rationale behind using this particular buffer formulation for fixation?

Authors: The fixative, buffer salt formulations, concentrations and pH were varied to determine optimal membrane binding to the silicon chips while still maintaining the ultrastructure as seen by other techniques. We opened the cells without prior fixation to maintain membrane flexibility and to prevent formation of fixative-induced vesicles at the membrane. The paraformaldehyde-glutaraldehyde fixation mixture is a mild fixation used in many electron microscopic techniques and is known to preserve antigenicities as well as structures. The fixative formulation used yielded optimal results in both respects.

P.B. Bell, Jr.: What was the reason for pretreating with hypotonic buffer? What effect does this treatment have on the structure/ultrastructure of the cytoplasm and membranes? How much of the resulting appearance of the sample is an artifact of this hypotonic shock to the cells?

Authors: The cell surfaces have to be cleaned before attachment but lysis of the cells has to be prevented. We tried for this purpose different buffer compositions, i.e., 70, 115 and 230 mM KCl

and pH 6.0 and 7.0. Buffer containing 70 mM KCl at pH 7.0 gave best results in our system concerning integrity of attached plasmalemma and membrane associated structures (clathrin coated pits and striped vesicles). Since the membrane associated components are physically held in position after attachment to the second chip, we do not think that the buffer treatment altered those structures significantly. High salt concentrations left more filamentous material on the membrane, undesirable in our case. We think that the sandwich-membrane separation method is less suited for analysis of the fine structure of the cytoplasm, since most of the cytoplasmic components are extracted during the procedure.

P.B. Bell, Jr.: The one picture (Figure 5) of the cytoplasmic "infrastructure" shows a network of very "clean" filaments. Are these representative of the cytoskeleton? If so, would you please comment on the apparent absence of the so-called microtrabecular lattice? Is the absence of the later related possibly to the hypotonic treatment?

Authors: The filamentous infrastructure seen here is certainly only a remnant of the cytoskeleton since extensive extraction was applied to the plasmalemma. Microtrabecular lattice is observed in intact cells without extensive extraction and after critical point drying. The different preparation procedures produce a very different appearance of filaments.

P.B. Bell, Jr.: Did you try protamine sulfate or Alcian Blue as an alternative to poly-L-lysine?

Authors: Protamine sulfate was not tried yet but Alcian Blue was used as recommended by Sommer (1977) and Nermut (1982 and 1983). However, adhesion of our cells was not satisfactory. Therefore, we first tried to analyze the mechanism involved in adhesion by the established polylysine procedures.

J.R. Sommer: The methodology would indeed be well suited to study cationization comparatively. In the absence of other explanations, of course, there always remains steric interference to be blamed for things to work or not work.

Authors: Unfortunately, on a level of macromolecular interaction, steric accessibility of active groups is the most basic phenomenon to deal with. We designed a system which can easily be adapted to any other specific problem. We do not think that the topographic distribution of charged sites is similar on all cell membranes. Therefore, no standard test is expected to work using only one probe. Also, the preparation (extraction) technique, buffer composition and fixation may be varied to expose and preserve different features of interest. No procedure is expected to be universally applicable.

J.R. Sommer: What caused the author to print the stereo-pairs on top of each other rather than side-by-side? Those of us who cross their eyes to view these things have difficulties to turn the page. There is no telling the distortions those must suffer that need a stereo viewer.

P.B. Bell, Jr.: I have problems...to buy special viewers just to view the stereo micrographs when

most of us either already have horizontal viewers or can fuse the two images without viewers.

Authors: If you do not have prisms, use only one of the stereo micrographs. The prints are much larger than conventional horizontal stereo prints and thus reveal sufficient details.

However, to appreciate stereo information don't cross your eyes or suffer eye strain during SEM sessions just to save a few dollars necessary to buy a pair of these inexpensive (US \$5), light, pocket size prisms. Those of "us" who apply this technique are delighted to use the system for instant stereo viewing. No longer do we have to carry fragile, complex lenses, no longer are we restricted to certain stamp-like formats or dependent on complex mounting equipment to view a simple pair of stereo micrographs. Only a minority of microscopists routinely use stereo information. This is regrettable especially in SEM where the instrumental conditions are ideally suited for stereo imaging. We believe that the unpopularity of stereo imaging is caused by the expense and complexity of the viewing equipment and by the additional time consuming step required prior to viewing, i.e., printing and mounting stereo micrographs at a specific size with a specific center-to-center distance. The prisms are a simple flat piece of plastic held close to the eyes at no specific optical distance. No strain is put on the eyes when looking through the prisms. More importantly, any stereo pair can be viewed independent of size, format or center-to-center distance. Even the complex alignment used for conventional stereo pairs is not necessary. For example, wouldn't "you" also like to take freshly developed TEM negatives to a light box and view them -- still wet -- in stereo without overlapping the negatives to establish the required center-to-center distance? How about taking two SEM Polaroids and evaluate their stereo information an instant after finishing the last frame and even before coating them? No cropping or overlapping of the 4x5 positives is required. This ease of stereo viewing makes the prisms very valuable in daily routine.

Stereo information was always encumbered by its complex, time consuming processing for viewing and presentation. The prisms open a way to use it in a practical imaging routine, especially at high magnifications. One has to realize, however, that prisms are useful for stereo viewing only, i.e., accessing stereo information. More precise height measurement requires, in any case, sophisticated methods and expensive equipment.