

A SIMPLE METHOD FOR FREEZE-FRACTURE OF MONOLAYER CULTURES

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The customary method of preparing tissue culture cells for freeze-fracture has involved gently scraping fixed or unfixed cell layers off the culture dish, centrifuging the resulting suspension, and freezing portions of the pelleted cell layer. Although aldehyde-fixed, confluent cell layers will retain some of their integrity during this procedure, a great deal of information regarding morphology *in situ* and intercellular relationships is unavoidably lost. Delicate structures such as filopodia are usually broken during removal of the cell layer, and it is often difficult to orient regions of membrane specialization with respect either to the cell as a whole or to regions of interaction with neighbouring cells.

Recently, Pfenninger and Rinderer (9) have described a method for the *in situ* freeze-fracture of cells growing on 3-mm gold grids embedded in a thin collagen substrate. This apparatus has given excellent results, but has the disadvantages of complexity and high cost. Furthermore, in applications such as cell cycle studies where growth parameters may be dependent on the substrate upon which the culture is grown, it may be necessary to compare the biochemical properties and ultrastructure of cells grown on the same

substrate, and collagen may not always be the substrate of choice. The present report describes a simple technique for the freeze-fracture *in situ* of cultures grown on gold carriers coated with a thin layer of vacuum-deposited silicon monoxide. This coating can be deposited on growth chambers of any size and appears to be a suitable substrate for the growth of a number of cell lines. The method is inexpensive, yields large areas of cell membrane, and should be adaptable to use with other cell culture substrates.

MATERIALS AND METHODS

Cell Culture

Swiss 3T3 mouse embryo fibroblasts, obtained from Robert Holley of The Salk Institute (La Jolla, Calif.), were cloned before use in this study. TC-7 African Green Monkey kidney cells (obtained from Dr. Helene Smith, Naval Biomedical Laboratories, Oakland, Calif.), WI-38 human diploid lung fibroblasts (obtained from Dr. Leonard Hayflick of Stanford University at passage level 15; used at passage level 27), and NMuLi mouse liver epithelial cells (7; obtained from Dr. R. B. Owens, Naval Biomedical Laboratories) were used without cloning. For routine culture, 3T3, TC-7, and WI-38 cells were grown in Vogt and Dulbecco's modification of Eagle's medium

(13; DME, Grand Island Biological Co., Grand Island, N. Y.) containing 10% newborn calf serum (GIBCO). NMuLi cultures were grown in Eagle's medium (3; GIBCO) containing 10% fetal calf serum (GIBCO) and 10 $\mu\text{g}/\text{ml}$ insulin (Schwartz/Mann Div., Becton, Dickenson & Co., Orangeburg, N. Y.).

The cells were carried in 100-mm Falcon plastic dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and were incubated at 37°C in a 10% CO₂ atmosphere. Primary cultures of chick cells were prepared from 10-day old embryos as described by Rein and Rubin (10). Secondary cultures (1) were seeded on silicon monoxide-coated Falcon dishes in medium 199 (6; GIBCO) supplemented with 2% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.), 2% calf serum, and 1% chicken serum (Microbiological Associates, Inc., Bethesda, Md.). These cultures were incubated at 39°C in a 5% CO₂ atmosphere. Cultures were judged to be free of Mycoplasma by the lack of incorporation of [³H]thymidine into cytoplasmic areas of the cell after labeling with 0.05 $\mu\text{Ci}/\text{ml}$ [³H]thymidine for 24 h followed by autoradiography.

The growth curve of 3T3 cells on clean or silicon monoxide-coated tissue culture dishes was determined by seeding 5×10^4 cells per 35-mm dish. Each day, four coated and four uncoated dishes were removed and the number of cells per dish was determined by removing the cells with 2.0 ml 0.01% trypsin (DIFCO) in 25 mM Tris buffer, pH 7.4, containing 140 mM NaCl, 5 mM KCl, and 0.7 mM Na₂HPO₄; 0.5-ml samples were counted in a Model Fm Coulter counter. The life cycle parameters of cells at their saturation density on coated and uncoated dishes were analyzed by the flow microfluorimetry technique of Trujillo and Van Dilla (12).

Preparation of Cultures for Freeze-Fracture

Confluent cultures of 3T3 cells were prepared by a procedure similar to that of Pfenninger and Bunge (8). Cells were fixed for 15 min in 4% paraformaldehyde (Matheson, Coleman, and Bell Reagent grade) and 0.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) containing 0.1 M phosphate buffer and 1.4×10^{-4} M CaCl₂·2H₂O. Cultures were then fixed 45 min in 4% glutaraldehyde containing 0.15 M phosphate buffer and 1.4×10^{-4} M CaCl₂·2H₂O, washed three times in ice-cold 0.2 M phosphate buffer containing 1.4×10^{-4} M CaCl₂·2H₂O, and incubated for 45 min in cold 30% glycerol in 0.1 M phosphate buffer. All solutions had a pH of 7.0-7.4 at the temperature employed. Culture dishes were floated on the surface of a 37°C water bath during fixation and were transferred to an ice-water slush during washing and glycerination. Each solution was gradually replaced by the next one to minimize the osmotic shock to the cells. Osmotic concentrations of these solutions were approximately as follows: paraformaldehyde-glutaraldehyde, 1,500 mosmol/kg;

glutaraldehyde, 770 mosmol/kg, and 0.2 M buffer, 430 mosmol/kg.

Vacuum Coating

Vacuum coating with silicon monoxide was carried out in a Varian model VE-10 vacuum evaporator fitted with a 10 rpm rotating table and a liquid nitrogen trap. Small pieces of silicon monoxide (Ladd Research Industries, Burlington, Vt.) were placed in a tungsten wire basket situated 9 cm from the specimen. Evaporation was carried out for 20 s at a current of 18-20 amp and a vacuum less than 1.0×10^{-4} torr. Use of thinner coatings resulted in cracking and detachment of the coating after a few days in culture. 3-mm flat-topped specimen carriers (95% gold, 5% nickel; Balzers High Vacuum Corp., Santa Ana, Calif.) were polished with Wenol (Ladd) followed by sonications in absolute ethanol and petroleum ether before coating. For comparison of cell growth on Falcon plastic and silicon monoxide, a portion of each culture dish was masked during evaporation to provide an uncoated region, allowing comparison of cell growth on both substrates within the same dish.

Light Microscopy of Cells Grown on Silicon Monoxide-Coated Substrates

3T3 cells were grown on coated Balzers specimen carriers in 35- or 60-mm Falcon culture dishes. These cultures were prepared for microscopy either by fixation in cold 5% trichloroacetic acid followed by a standard Giemsa stain, or by simultaneous fixation and staining in 1% Crystal Violet in 25% ethanol. Cells were photographed on Kodachrome II professional Type A film (Eastman Kodak Co., Rochester, N. Y.) using a Leitz Orthomat camera on an Ortholux microscope fitted with a 22 \times objective and a Leitz Ultrapac vertical illuminator system. Cells growing on transparent substrates (Falcon culture dishes and fragments from a bacterial culture dish [Lab-Tek Products, Westmont, Ill.]) were photographed without fixation or staining on Tri-X film using either a Nikon or a Zeiss inverted tissue culture microscope equipped with phase-contrast optics.

Freeze-Fracture

3T3 cells were grown to their saturation density on silicon monoxide-coated 3-mm diameter, Balzers specimen carriers placed in the bottom of Falcon plastic dishes. After the cells had been fixed and glycerinated, the carriers were removed from the dish and the excess liquid carefully drained off with filter paper. The carrier was then sprayed with a 1% suspension of 9.7 μm polystyrene latex beads (Particle Information Service, Los Altos, Calif.), using a Pelco nebulizer (Ted Pella Company, Tustin, Calif.). A small copper hat (Fig. 1) was then placed over the cell layer on the raised central portion of the Balzers carrier, and the whole assembly

was frozen rapidly against the solid phase of partially solidified Freon 22 (E. I. duPont de Nemours & Co., Wilmington, Del.). The polystyrene latex spheres functioned as spacers to prevent the copper hat from resting directly on the cell layer. Good mechanical contact between the copper hat and the ice layer above the cells was ensured by drilling three 0.003-in holes in the base of each hat, and sonicating the hats in the 30% glycerol cryoprotectant to remove any air bubbles from the holes. The carriers with their attached hats were then placed in the depressions on the four-position specimen table of a Balzers BA 360M freeze-etching apparatus and held firmly in place by the specimen table clip and clamping ring. Specimens were fractured at stage temperatures between -125° and -100°C by knocking off the hats with the knife, and were immediately shadowed with platinum-carbon (7 s at 7.4 V using 10 cm of 0.004-in cleaned platinum wire on a standard Balzers carbon electrode) followed by carbon (10 s at 8.6 V). The specimens were then thawed and the cellular material digested away from the replica by chlorine bleach (5.25% sodium hypochlorite, 30 min) followed by two washes with distilled water. The replicas were quite firmly attached to the cell layer and tended to tear when the carriers were introduced into bleach. However, it was possible to alleviate this problem by soaking the specimen carriers overnight in 100% methanol and gradually replacing the methanol with distilled water before treatment with bleach. Replicas were picked up on clean 75×300 mesh copper grids and examined in a Siemens Elmiskop I electron microscope at an accelerating voltage of 80 or 100 kV. The hats were cleaned before each run by a brief immersion in dilute ammonium hydroxide followed by sonications in absolute ethanol and petroleum ether. Only the B face of the membrane at the lower cell surface and the A face of the membrane at the upper surface could be seen by this technique, as the

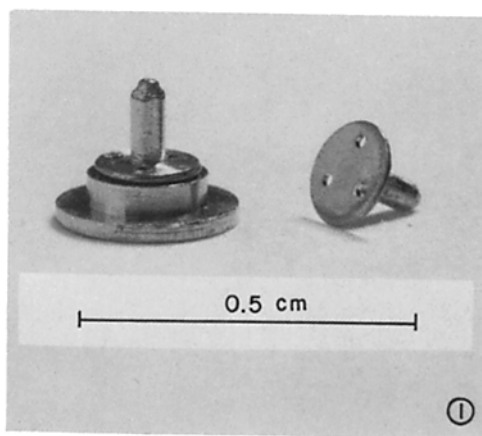


FIGURE 1 Copper hats with a Balzers flat-topped specimen carrier.

other two fracture faces are attached to the hat and cannot be retrieved at present.

In cases where very flat cells were fractured or when the opposing surfaces of the hat and carrier were slightly rounded, the fracture plane would frequently pass above the cell layer, either exposing only the tops of the cells or missing the monolayer altogether. This problem could be rectified by reducing the bead diameter to $4.7 \mu\text{m}$, and grinding the upper surface of the carrier and the bottom of the hat flat against a large piece of 0.25-in thick double ground and polished plate glass. The carriers were most conveniently ground by using no. 70C cement (Hugh Courtwright & Co., Chicago, Ill.) to attach a number of carriers to a small square of 0.125-in thick plate glass, then inverting the glass square over the grinding surface. Grinding was carried out in three stages, using a slurry of 800 mesh boron carbide (Nor-bite; Norton Co., Industrial Ceramics Div., Worcester, Mass.; particle size approximately $20 \mu\text{m}$) followed by $6 \mu\text{m}$ and $1 \mu\text{m}$ diamond paste (Buehler Ltd., Evanston, Ill.). A slurry of aluminum oxide in 20, 5, and $1 \mu\text{m}$ particle sizes (Universal Shellac and Supply Co. Ltd., Brooklyn, N.Y.) may also be used for this purpose.

RESULTS

Comparison of Cell Growth on Silicon Monoxide and Conventional Substrates

In order to evaluate the suitability of silicon monoxide as a tissue culture substrate, a comparison was made of the morphology of a number of different cell lines growing on coated and uncoated areas of Falcon plastic culture dishes. There was no visible difference in the density or morphology of 3T3 (Fig. 2 a, b), TC-7 (Fig. 2 c, d), or WI-38 cells (Fig. 2 e, f) growing on coated or uncoated areas of Falcon dishes. NMuLi cells (Fig. 2 g, h) exhibited the same growth pattern on Falcon plastic and silicon monoxide, although the morphology on either substrate tended to vary between different areas of the same dish. Chick embryo fibroblasts (Fig. 2 i, j) exhibited a more criss-crossed growth pattern, a higher frequency of cell vacuoles, and more bare patches in the monolayer on silicon monoxide; however, cell density seemed to be roughly comparable on the two substrates.

In order to determine that cells were actually growing on the oxide coating, rather than on areas where the culture dish might be accessible through microscopic cracks or bare patches, 3T3 cells were grown on fragments from a Lab-Tek bacterial culture dish, a substrate which cannot support the growth of this line of cells. While the cell layer on

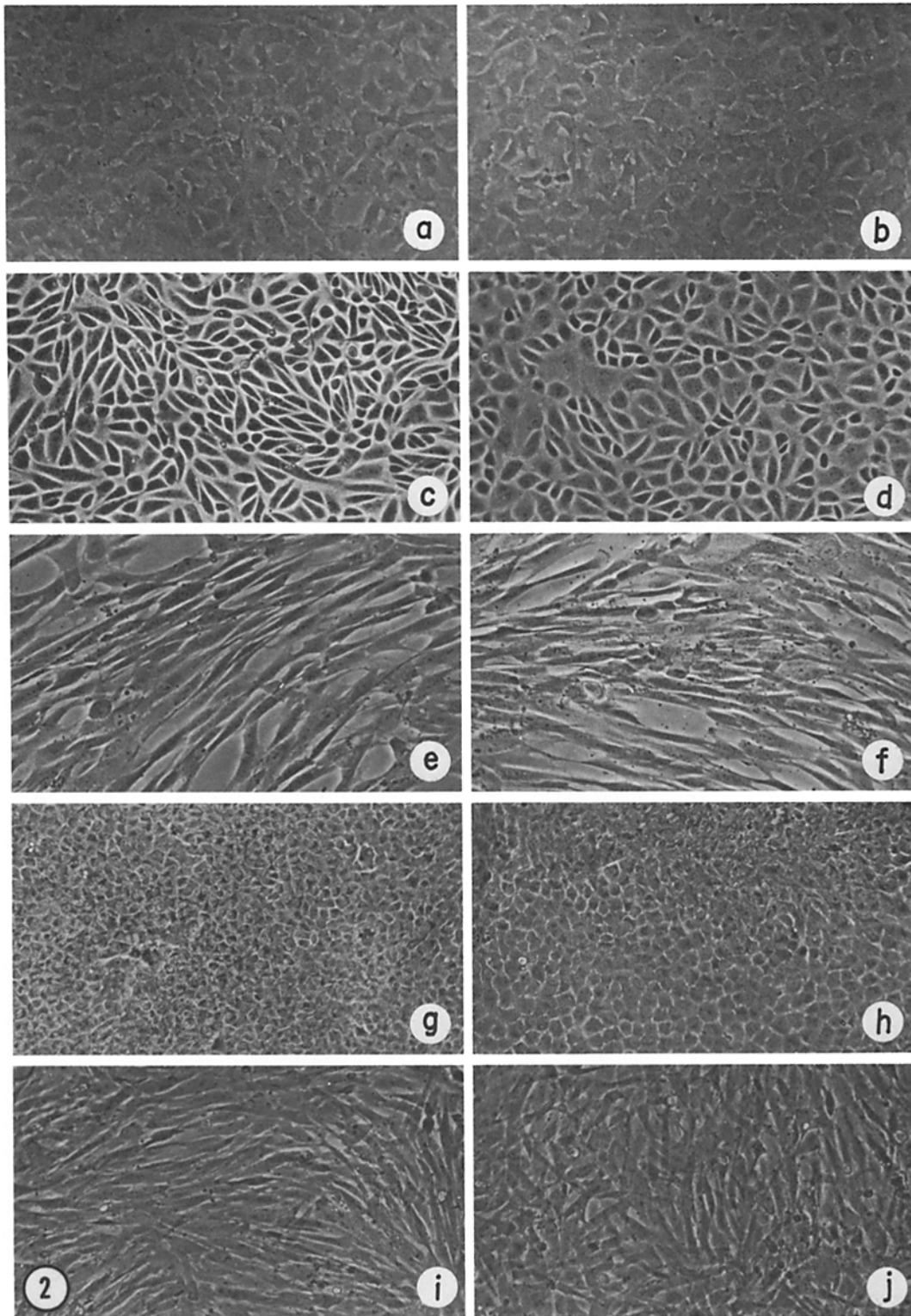


FIGURE 2 Cell growth on Falcon plastic (*a,c,e,g,i*) and silicon monoxide (*d,e,f,h,j*). (*a* and *b*) 3T3 cells; (*c* and *d*) TC-7 cells; (*e* and *f*) WI-38 cells; (*g* and *h*) NMuLi cells; (*i* and *j*) secondary chick embryo fibroblasts. $\times 100$.

the coated areas of these fragments appeared normal, cells in uncoated areas were sparse and tended to grow in clumps. A sharp boundary could usually be seen between the coated and uncoated areas. Gentle agitation of the plastic fragment caused the cell layer in the clear patches to detach and roll up to the edge of the coated area. This result suggested that 3T3 cells were weakly attached to this type of plastic, but had no difficulty in adhering to, and growing on, the silicon monoxide coating.

In similar experiments with coated and uncoated Balzers specimen carriers, 3T3 cells grew to confluency on both the silicon monoxide coat and the clean metal surface. Although it was not possible to observe fine details of cell morphology with the vertical illuminator system, cells growing on coated and uncoated specimen carriers appeared to have the same size and shape as those on Falcon plastic. Apparently, it would be possible to eliminate the silicon monoxide coating when using cell lines such as 3T3 which will grow on metal. However, in experiments with cell lines that do not grow well on metal or where it is desirable to employ the same substrate for ultrastructural and biochemical studies, use of the silicon monoxide coating is recommended.

To test whether the growth properties of 3T3 cells on silicon monoxide were any different from those grown on standard Falcon plastic dishes, the doubling time and saturation density were determined as described in Materials and Methods. The 3T3 cells used in these experiments had a doubling time of 15 ± 2 h and a saturation density of 6.2×10^4 cells/cm² growing on both Falcon plastic and the silicon monoxide coating. At their saturation density, 95% of the cells grown on both coated and uncoated dishes had a DNA content equivalent to cells in the G₁ part of their life cycle.

Freeze-Fracture of Monolayer Cultures

Replicas of confluent 3T3 cell monolayers fractured by this method showed large areas of cell membrane (Fig. 3). In preliminary observations, these cells appeared to be very flat, with a thickened portion over the nucleus sloping gently to thin peripheral sheets of cytoplasm which often extended for several micrometers. These peripheral sheets could be as thin as 0.06–0.07 μ m in places and showed extensive overlap with similar regions on neighbouring cells. Microvilli were small and infrequent on the upper and lower cell surfaces, but

large filopodia (*Fil*) were fairly common at the cell periphery. The stomata of numerous small vesicles (*v*) could be seen in localized areas of the membranes of both the upper and lower surfaces, and often appeared to be arranged in straight lines. In some cases, there was a very distinct boundary between areas of plasma membrane with many vesicular stomata and regions with no stomata. Both A and B fracture faces displayed numerous round intramembranous particles (IMP) 50–180 \AA in diameter, distributed at random across the matrix of the fracture face, with the density of IMP generally being greater on the A face. However, the density of IMP on the A face varied considerably between different regions of an individual cell membrane, with some areas being almost devoid of particles. A large protuberance filled with smooth-membraned vesicles (*Ves*) is visible on A₂; its nature is uncertain at present.

DISCUSSION

The technique described in this report has proven to be a simple and inexpensive method for fracturing a monolayer culture without removing it from the substrate. Our experience with freeze-fracture of cultures prepared by traditional methods¹ suggests that with these preparations, it is very difficult to interpret regional membrane specializations such as variations in the density of vesicular stomata or IMP. Preservation of the native cell shape by *in situ* fixation and fracturing makes it possible to determine how areas of membrane specialization are related to the cell as a whole and to regions of cellular interaction. Furthermore, since the cell layer remains attached to the substrate during preparation for fracturing, delicate structures such as filopodia (Fig. 3) are preserved intact.

This method of *in situ* fracturing presents several improvements over techniques I and II described by Pfenninger and Rinderer (9). Use of the silicon monoxide coating eliminates the necessity for the collagen-coated cellophane supporting film employed with cell cultures in technique I, while avoiding the complex apparatus required by technique II. The design of the hat minimizes the amount of metal separating the cell monolayer from the coolant during freezing and should provide faster freezing than the brass cylinders used in technique I. Since the carriers fit onto the standard Balzers specimen table, a special specimen stage is

¹ Collins, T. R. Unpublished observations.

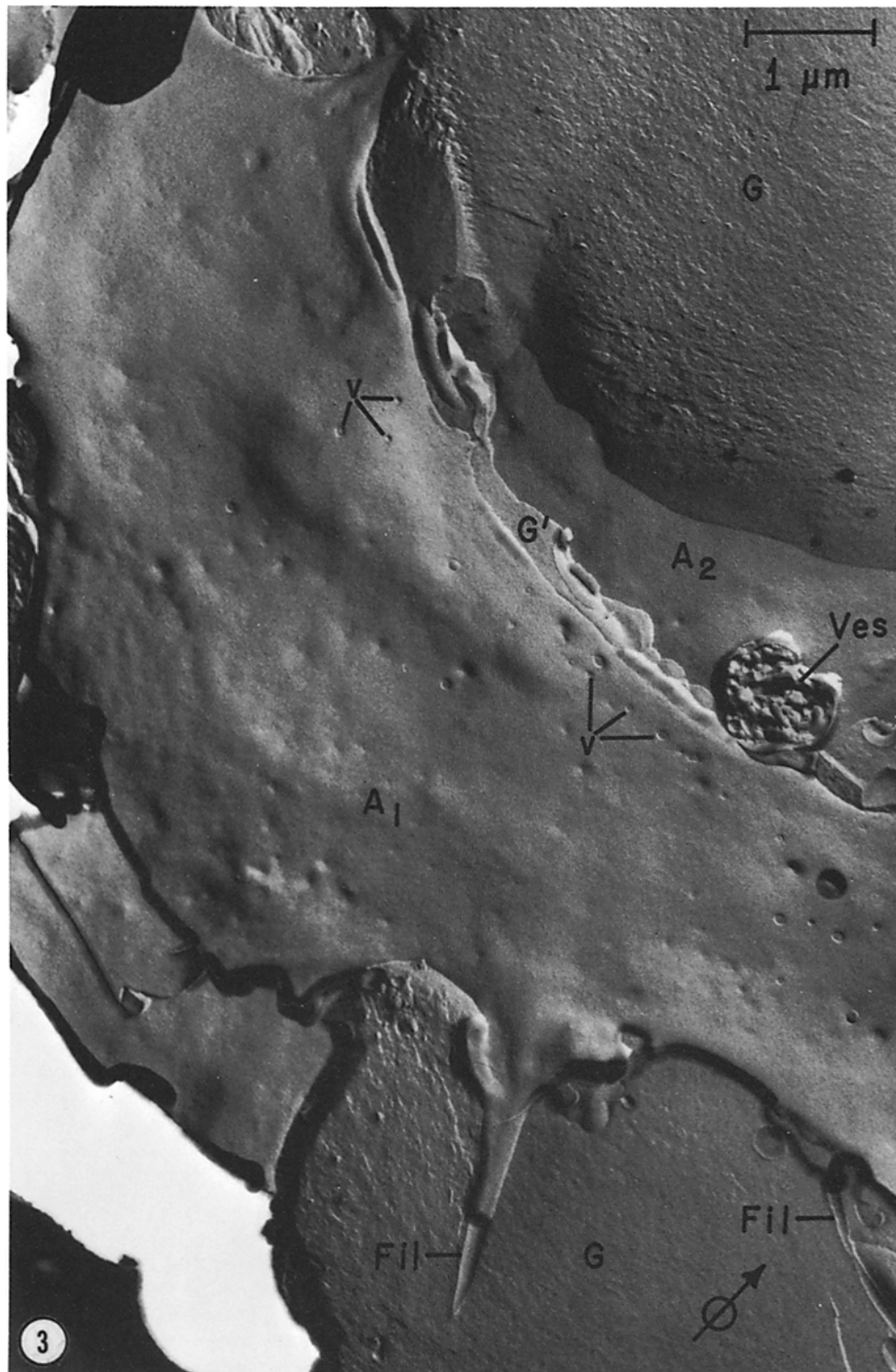


FIGURE 3 Freeze-fracture of a portion of a 3T3 cell growing on a silicon monoxide coated carrier. In the area marked A_1 , the fracture plane has followed the membrane of the upper surface near the cell periphery. A_2 is an adjoining A face which probably represents an underlying cell. G' is a narrow region of glycerol matrix separating A_1 and A_2 . The white area at the lower left represents a split in the replica. Shadow direction is indicated by the arrow, and shadows are white. G , glycerol matrix; v , pinocytotic or exocytotic vesicles; Fil , filopodia; Ves , bulge in A_2 filled with smooth-membraned vesicles. $\times 17,500$.

not required. The method should be adaptable to a number of different substrates, the only condition being that the layer of substrate applied to the gold-nickel carrier must be thin enough to permit good heat conduction during replication. For example, Hamamoto et al. (4) have recently used this technique to examine epithelial cell layers grown on Nucleopore filters. At present, the main limitation encountered with this method results from the necessity of using a vertical illumination system to observe the cells growing on the carrier. This problem does not arise with the method of Pfenninger and Rinderer, where the transparent collagen substrate extends across the open squares of the supporting gold grid. In addition, fracture faces produced by this technique seem particularly sensitive to "orange peel" contamination of the sort described by Moor (5; see especially Fig. 12), Dunlop and Robards (2), and Staehelin and Bertaud (11). However, contamination can be minimized by fracturing at -100°C and etching the specimen for 1 min with the cold knife positioned over the specimen table.² This problem is presently under study.

The silicon monoxide coating employed here appears to be an excellent substrate for freeze-fracture studies on cell cultures. Once coated, the carriers can be sterilized at 132°C without any apparent change in the oxide layer. 3T3 cells cultured on silicon monoxide show no modification of growth properties compared to cells growing on Falcon plastic, and other cell lines also appear to grow well on this substrate. The ability to coat culture vessels routinely with silicon monoxide allows comparison of the biochemical properties and ultrastructure of cells growing on the same substrate.

SUMMARY

A simple method is described for the freeze-fracture *in situ* of monolayer cultures grown on gold carriers coated with a thin layer of silicon monoxide. Preliminary observations on 3T3 mouse embryo fibroblasts indicate that this technique exposes large areas of cell membrane, making it possible to determine how areas of membrane specialization are related to the cell as a whole and to regions of cellular interaction. 3T3 cells cultured on silicon monoxide show no modification of growth properties compared to cells growing on

²Collins, T. R. Unpublished observations; and J. P. Revel, personal communication.

Falcon plastic, and other cell lines also appear to grow well on this substrate.

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