

## FREEZE-FRACTURE OF MONOLAYER CULTURES

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Freeze-etching is an electron microscopy preparative technique which has been widely used in the study of the ultrastructure of procaryotic and eucaryotic cells. In general, use of the technique has been limited to studies of either tissue blocks or suspensions of cells, small organisms, organelles, or other particles. In order to freeze-etch cells grown *in vitro*, the cells must first be removed from their growth surface either by enzymatic digestion with trypsin (21), incubation in EDTA-solutions (19, 23), or by mechanical forces (6-11, 13, 17, 18, 21). These procedures introduce many artefacts and deprive us of the opportunity to study growth patterns of the cell lines. Direct observation of the freeze-fracturing of tissue cul-

ture cells grown on artificial surfaces has been hampered by technical problems which are encountered when one attempts to fracture split-thicknesses of monolayers of cells with a cryoultramicrotome.

Recently, several methods for the *in situ* freeze-fracturing of cell monolayers have been described (2, 3, 16). Each of these methods has produced useful replicas of freeze-fractured monolayers, but the methods are limited in the scope of their application by three factors: (a) cells are grown along the surface of the metal specimen holder which will be eventually mounted in the freeze-fracture device; (b) cells are grown on special supporting zones, either a collagen (16) or gelatin matrix (2)

or a layer of silicone monoxide (3); and (c) the techniques require the use of specialized equipment or accessories. Further, the method of Pfenninger and Rinderer (16) has the additional disadvantage of complexity and high cost.

We now report the development of a more versatile method for the *in situ* freeze-fracturing of cell monolayers. The method can be used on tissue cultures grown on standard plastic cover slips. The method yields large areas of fractured cell membranes and is easy to execute. It has the additional, important advantage that the same culture can be used for light microscopy and freeze-fracture electron microscopy, a feature which allows for the direct correlation of culture growth characteristics and freeze-fracture ultrastructure.

## MATERIALS AND METHODS

### Tissue Culture

Two categories of tissue cultures were examined: contact-inhibited cell cultures, derived from normal bovine endothelial cells which were cultured from thoracic aortas (4); and contact-uninhibited cultures grown from either TE-85 strain human osteosarcoma cells (12) or human breast carcinoma cells, strain A1Ab, cultured from a lung metastasis (20). Tumor lines were obtained from the Cell Culture Naval Biochemical Research Laboratory, Oakland, Calif. Both contact-inhibited and contact-uninhibited cultures were in monolayers at harvest. The tissue culture medium used for all three cell lines was RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 20% fetal calf serum (Reheis Chemical Co., Chicago, Ill.) for endothelial cells or 10% fetal calf serum for the human osteosarcoma and breast carcinoma cells.

Established cultures were subcultured on 25-mm round, plastic cover slips or 24 × 30-mm rectangular plastic cover slips (Polystyrene or Thermanox, Lux Scientific Corp., Thousand Oaks, Calif.) placed in 35-mm Falcon plastic dishes (Falcon Plastics, Div. of Bio Quest,

Oxnard, Calif.) or Multiplate-8 well containers (Lux Scientific Corp.) at an initial density of  $4 \times 10^4$  cells per dish or well. Cells were incubated at 37°C in a humidified 5% CO<sub>2</sub> air atmosphere. The cultures were re-fed with fresh tissue culture medium every 48 h.

Confluent cultures were fixed *in situ* for 30 min at 4°C by gradually replacing the tissue culture medium with 2% glutaraldehyde buffered with 0.1 M cacodylate, pH 7.3. Cultures were washed three times with cold 0.1 M cacodylate buffer containing 0.25 M sucrose. The plastic tissue-culture cover-slips were then routinely divided with a scissors into three parts: the first was processed for light microscopy, the second for thin-section electron microscopy, and the third for freeze-fracture electron microscopy. In some experiments, portions of cultures which were first examined by light microscopy were subsequently freeze-fractured.

### Freeze-Fracturing of Cultures

The part of the plastic cover slip for freeze-fracturing (or the part used for light microscopy) was soaked for 5 h in 25% (wt/vol) glycerol, buffered in 0.1 M cacodylate, pH 7.3. This cover slip was cut into 1.5 × 1.5-mm squares, and excess glycerol was carefully drained off with filter paper, leaving a thin layer of glycerol over the entire culture.

The central feature of our method is the use of a specimen-mounting medium, polyvinyl alcohol, which encourages fractures to propagate through frozen cell monolayers rather than through the mounting medium or along the specimen carrier. It was determined empirically that when confluent monolayers are immersed in Elvanol 51-05 solution (polyvinyl alcohol, E. E. duPont de Nemours & Co., Inc., Wilmington, Del.), quenched, and cleaved, the fracture face consists almost entirely of freeze-fractured cells. Large areas of both cell membranes and cytomembranes are exposed. Importantly, the ultrastructure of glutaraldehyde-fixed cells is not compromised by exposure to the mounting medium.

Elvanol 51-05 (E-51) solution was prepared by dissolving 20 g of Elvanol powder in 80 ml of 0.1 M phosphate-buffer, pH 7.4, containing 0.14 M NaCl (phosphate-buffered saline, PBS). To 80 ml of the EL-

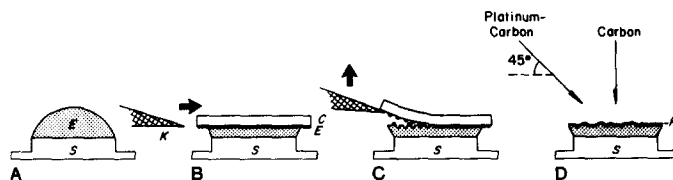
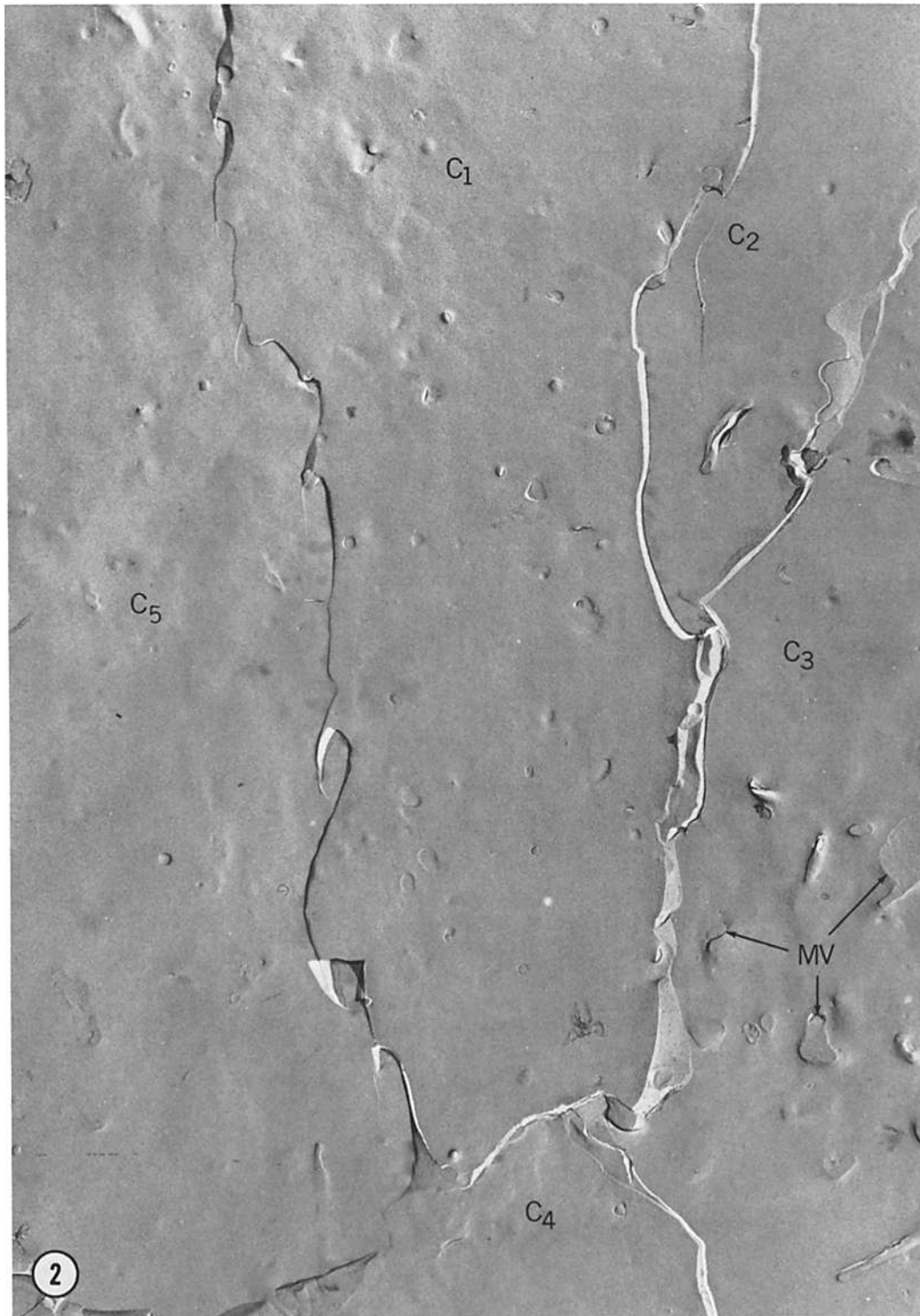


FIGURE 1 Method for freeze-fracturing of tissue culture monolayers. (A) A drop of Elvanol 51-05 (E) solution containing glycerol is placed on a specimen carrier (S). (B) A plastic cover slip (C) covered with a monolayer of fixed cultured cells is inverted onto the Elvanol 51-05. The specimen carrier and cover slip are quenched in Freon 22 at  $-150^{\circ}\text{C}$  and mounted on the Balzers specimen stage. The microtome knife (K) is advanced under the edge of the cover slip. (C) The cover slip is fractured away by raising the knife. (D) The fresh fracture face is shadowed with platinum-carbon and backed with evaporated carbon to form a coherent replica (R).



**FIGURE 2** Freeze-fractured monolayer of cultured TE-85 human osteosarcoma cells. Large areas of the fractured plasma membrane are demonstrated in five adjacent cells (C<sub>1</sub>-C<sub>5</sub>). MV, microvilli.  $\times 12,000$ .

vanol-PBS-mixture, 40 ml of 100% glycerol was added, and the mixture was stirred over-night at 4°C. Undissolved Elvanol was removed by centrifugation at 15,000 rpm for 30 min. DMSO (Fischer Scientific Co., Pittsburgh, Pa.) was added to the supernate to give a final concentration of 2% DMSO. The final solution was stored at -20°C. To prevent fungal growth in the working solution, either a crystal of thymol or 50 µg of methyl-*p*-hydroxybenzoate was added per milliliter of the E-51 solutions.

A large drop of the E-51 solution was pipetted onto the upper surface of a Balzers 3-mm gold flat-topped specimen carrier (Balzers High Vacuum Corp., Santa Ana, Calif.). A 1.5 × 1.5-mm square plastic cover slip bearing a monolayer of culture cells on its upper surface was inverted into the E-51 drop (Fig. 1). Because of the high viscosity of the E-51 solution, the droplet is only partially deformed, and an approx. 1-mm thick layer of E-51 solution separates the plastic cover slip from the specimen carrier. The specimen carrier and overlying inverted cover slip were rapidly quenched in Freon 22, cooled to -150°C in liquid nitrogen. The specimen carrier was mounted on an unmodified specimen stage of a Balzers BAF 301 freeze-etch machine, equipped with an electron beam evaporation device (EVM 052) and a QCS 201 quartz crystal-film-thickness monitor. The specimen was fractured at a stage temperature of -100°C. Fracturing was accomplished by lowering the cutting knife below the level of the plastic cover slip and lifting the knife until the cover slip separated from the specimen carrier (Fig. 1). The knife was then rotated to cover the fresh fracture face during heat etching. The fracture face was shadowed with platinum-carbon and backed with evaporated carbon as described by Moor and Mühlethaler (15). Replicas were retrieved by immersing the gold specimen carrier in a 0.9% NaCl solution. Replicas remained at the saline-air interface. Further cleaning was accomplished by bathing replicas in a 5% clorox solution.

## RESULTS AND DISCUSSION

Replicas produced by this method typically are large and often can be retrieved from the entire fracture face as a single coherent sheet. Detailed

within the replicas are large areas of PF and EF faces (1) of plasma membranes (Figs. 2-6) as well as cytomembranes. Images of the membrane fracture faces are indistinguishable from the images of freeze-fractured tissue blocks and particle suspensions which are obtained from specimens freeze-fractured with a conventional cryo-ultramicrotome. In general, replicas show no evidence of specimen contamination (5, 14, 22).

With the method described in this paper, freeze-fracturing is accomplished by mechanically separating an inverted cover slip from a mounting medium by raising the ultramicrotome knife. Success of the method depends upon the mounting medium's fulfilling the following essential conditions: (a) the medium must adhere sufficiently to both the plastic cover slip and the specimen holder so as to resist fracturing along the cover slip or the specimen holder; (b) the medium must penetrate the cell layer sufficiently to allow fracturing preferentially through the cells; and (c) the medium must not interfere with the harvesting or cleaning of the replica. The E-51 solution fulfills these conditions. Further, the E-51 solution has the advantage that it immediately liquifies after removal from the Balzers freeze-etch device, this facilitating the harvest of the replica.

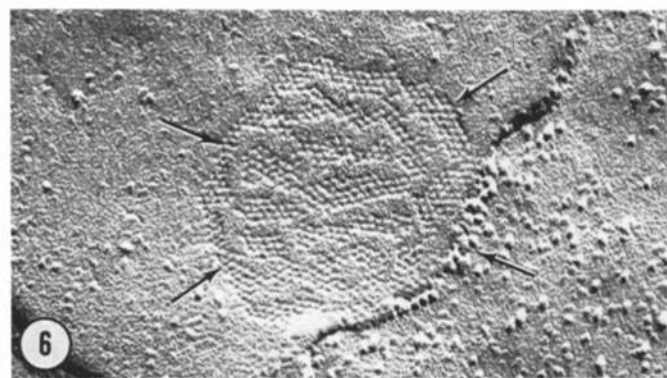
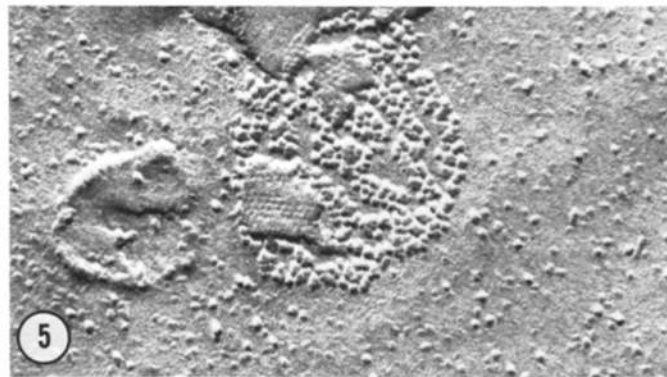
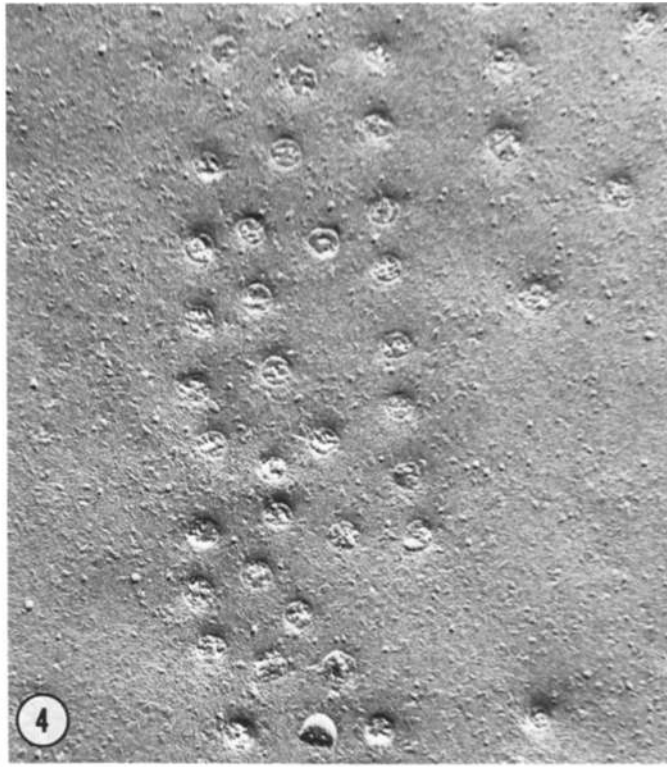
The method described herein offers several important advantages over methods previously described (2, 3, 16). With our method, the *in situ* freeze-fracturing of cell monolayers is performed without modification of standard and well-established tissue culture techniques. Plastic cover slips, being only slightly smaller than the bottom area of the culture dishes, offer essentially the same growth conditions as Falcon plastic dishes. In addition, other methods for monolayer freeze-fracturing use growth-supporting films, such as collagen-coated cellophane on 3-mm diam honeycomb gold grids (16), or silicon-monoxide- (3) or gelatin-(2) coated 3-mm Balzers specimen holders as

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FIGURE 3 Freeze-fractured intertwining filopodia at the surfaces of two TE-85 human osteosarcoma cells. Intramembrane particles (IMP) are present on both PF and EF fracture faces. × 60,000.

FIGURE 4 Freeze-fractured monolayer of cultured bovine aortic endothelial cells. Orifices of pinocytotic vesicles are sharply demarcated on the PF-face. × 66,000.

FIGURES 5 and 6 Gap junctions (nexuses) on fracture faces of A1Ab mammary carcinoma cells in culture. Figure 5: PF-face contains an array of 70-Å particles. Small fragments bearing the EF-face remain attached. × 120,000. Figure 6: The EF-face of a gap junction, demarcated by arrows, shows a hexagonal array of pits. × 120,000.



growth surfaces for cells. These organic and inorganic films may be inappropriate for certain tissue culture experiments. Further, with our method it is possible to preselect by light microscopy the areas to be freeze-fractured. This makes it possible to carry out correlative studies on the relationships between tissue culture growth characteristics and cell ultrastructure as visualized by freeze-etching.

#### SUMMARY

This paper describes a simple method for the freeze-fracturing of cells in monolayers or multi-layer tissue cultures. The method produces high quality replicas and is applicable to the study of virtually any tissue culture or organ culture system. It uses standard materials and equipment for both tissue culture and freeze-fracturing.

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