METHODS FOR THE FREEZE-FRACTURING OF NERVE TISSUE CULTURES AND CELL MONOLAYERS

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ABSTRACT

Two methods for freeze-cleaving of thin tissue layers are presented. Whereas a simple technique can be employed to fracture continuous, relatively firm tissue layers, a more sophisticated technique employing special carriers is needed to fracture very thin and incomplete layers, e.g., the fiber outgrowth of cultured nerve tissue or sparsely seeded isolated cells. Both methods basically consist of freezing the specimens sandwiched between two small metal carriers which are then fractured apart so that the cleavage plane runs through the tissue. In the resulting replicas of such thin specimens, large membrane areas are exposed, and new information is provided on the topography of membrane properties in entire cells or cell processes. The technique should also be useful for studies on the interactions of cells grown in culture.

By revealing the internal membrane structure, the freeze-fracturing method has opened new perspectives in biology. The conventional technique (Moor and Mühlethaler, 1963), however, has so far been limited to the preparation of replicas from tissue blocks or suspensions of small organisms, cells, organelles, or other particles. This has meant that cells and tissues grown in vitro could not be freeze-fractured without removal from their substrate. Thus investigations of cellular interactions and of regional differences in the plasmalemmal structure of polarized cells were not possible. The present paper introduces two methods which make it possible to cleave very thin layers of tissue and isolated cells grown in culture. The basic idea of these techniques is to sandwich specimens between a pair of carriers, to freeze this assembly, and to fracture the biological material by breaking the two carriers apart. This principle is similar to that of the methods used by other investigators to produce replicas of both fracture faces (Moor, 1971; Mühlethaler et al., 1973; Steere, 1973). A simple procedure (referred to in the following as technique I) can be applied to thin, flat tissue layers which form a relatively solid structure and can be handled microsurgically. For fracturing cell monolayers, nerve fiber outgrowth, and isolated cells grown in culture, a more sophisticated method utilizing special specimen carriers and a fracturing tool adapted to the Balzers freeze-etching apparatus (technique II) has been developed. Part of the technology has been presented earlier in abstract form (Pfenninger, 1972, 1974) and a paper describing the resulting biological data has been published separately (Pfenninger and Bunge, 1974).

All the parts required for technique II will soon be commercially available from Balzers High Vacuum Corp., Santa Ana, Calif.
METHODS AND RESULTS

Technique I

The areas of application of this method are: comparatively firm, but thin layers (not exceeding 300 μm thickness) of tissue dissected from animals such as omentum or from flattened (organotypic) cultures which consist of numerous cell layers and can be peeled off the culture dish.

Flat gold alloy specimen carriers (Balzers High Vacuum Corp.) and small brass cylinders, 1.9 mm in diameter and about 1.5 mm in length, are required for the following procedure. Three fine-bore holes (0.4 mm diameter) penetrate the cylinders longitudinally, and the surfaces of the two ends have been filed to be flat and smooth but not polished. A network of fine grooves has been scratched into the upper face of the flat carriers. Carriers and cylinders are carefully cleaned by sonication in several changes of acetone and alcohol and finally in the cryoprotectant solution. The tissue is first fixed with aldehydes (glutaraldehyde or a mixture of glutaraldehyde and formaldehyde; see, e.g., Bunge, 1973; Pfenninger and Bunge, 1974), briefly washed in buffer, and incubated at 4°C in 25% glycerol in buffer or in another cryoprotectant for 30 min. This layer is then cut into pieces similar in size to the upper face of a flat gold alloy specimen carrier. The pieces of the tissue film are placed in between a flat carrier and a well-centered brass cylinder (Fig. 1); excess fluid is removed with filter paper. The sandwiches are then transferred into liquid nitrogen-cooled Freon 22. Held in an upright position with a pair of curved forceps, the specimen sandwiches are rapidly immersed into the thin (previously thawed) superficial layer of liquid Freon and pressed against the solid, colder agent underneath. Subsequently, the specimens are transferred into liquid nitrogen for storage. For fracturing, four of the sandwiches are placed on a four-position specimen stage in the Balzers freeze-etching machine. Under high vacuum (better than 10⁻⁶ torr) at ~100°C, the brass rods are knocked off the flat carriers with the aid of the knife holder (without blade) so that the fracture runs through the tissue layer. It is important to lower the knife holder of the microtome only as much as necessary to hit the cylinders so that they will flip over during the fracturing process instead of sliding over the fractured face. Cylinders which have not fallen from the specimen stage can be pushed over the rim with the knife holder after it has been sufficiently

Figures 2-4 Freeze-fracture results obtained with technique I from the outgrowth of a 50-day old culture of rat spinal cord with dorsal root ganglia and meninges attached. Fig. 2 shows part of the plasmalemma (inner leaflet) of a nerve growth cone (gc) as identified on the basis of its shape and paucity of intramembranous particles (arrowheads). Note the contrasting abundance of such particles in the inner leaflet of the neighboring glial element (g). p, pinocytotic pits; v, vitrified glycerol-buffer surrounding the cellular elements. Figs. 3 and 4 illustrate intercellular junctions between meningeal cells (probably of fibroblastic nature) in the same spinal cord culture. A belt of tight junctions can be seen in Fig. 3. The outer plasmalemmal leaflet (e) is exposed, and the junctions appear as fine grooves which occasionally contain particles (arrowheads). The internal plasmalemmal leaflet (i) of one cell and parts of the external plasmalemmal leaflet (e) of its neighbor are seen in Fig. 4. These adjacent cells are interconnected by five large gap junctions (arrows) alone in the small area shown in the picture. Magnifications and calibrations: × 26,200, 1 μm (Fig. 2); × 58,400, 0.5 μm (Fig. 3); × 22,500, 1 μm (Fig. 4). Arrow on white disk, approximate shadowing direction for all three figures.
Two nerve growth cones in a 11-day old olfactory bulb culture. This freeze-cleave replica was obtained with the modified technique 1. The background is covered to a large extent by flattened supporting cells (g). Two of the several nerve fibers (n) present give rise to characteristically shaped growth cones (gc) whose external plasmalemmal leaflets are exposed. The asterisk marks a putative third growth cone lying underneath the other cellular processes, and the arrowheads point to the growth cone filopodia. co, collagen fibrils of the culture substrate embedded in vitrified glycerol-buffer. Magnification, × 7,600; calibration, 5 μm. Arrow on white disk, shadowing direction.
FIGURE 6 Cross section (A) and view from top (B) of the carriers used for technique II. The bottom platelet (b) is wider than the upper carrier (t), and its extending sides can be slid under the teeth of the fracture tool (Fig. 7). s, gold grid with specimen; cg, collagen-coated covering grid; h, opening in the upper carrier; arrow, plane of fracture (along Teflon-coated surface of upper carrier). For further description, see text. Calibration, 1 mm.

of platinum and carbon, respectively. Optimal film thicknesses, as judged from resolution, contrast, and stability of replicas, are 20–25 Å for the platinum and 250–300 Å for the carbon (as indicated by the film thickness monitor).

The thawed specimens are immersed in distilled water and then transferred into Clorox, a commercial bleach. After digestion of the adhering organic material (overnight), the replicas are washed in several changes of distilled water and picked up on Formvar-coated electron microscope grids. The transfers of the replicas from one solution into the other are always done in gradual steps. This gentle procedure avoids disruption of the replicas by changes in surface tension or convection currents.

The results of technique I are illustrated in Figs. 2–4. They show cultures of rat spinal cord with dorsal root ganglia and meninges attached (Pfenninger and Bunge, 1974) which were grown for 50 days in vitro. Fig. 2 is a survey picture showing some supporting (non-neural) cells (g) in the background and numerous cellular processes. The latter include one element which is most likely to be a nerve growth cone (gc) as suggested by its shape and low particle density (Pfenninger and Bunge, 1974). Figs. 3 and 4 illustrate some of the junctions between cells which are probably of meningeal origin. A belt of tight junctions as seen in the external leaflet (or B face) is illustrated in Fig. 3. Note the resolution of the fine grooves (arrows) containing some occasional particles. These particles are probably in relation with the ridges observed in the internal leaflet (Goodenough and Revel, 1970). Gap junctions are also observed very frequently and are revealed with great clarity as can be seen in Fig. 4.

Technique II

When the tissue layer to be fractured is so thin and incomplete that it cannot be handled as a consistent film another method has to be applied which involves the use of a supporting element for the cells or cell processes. Initially, collagen-coated cellophane was used as a supporting film and culture substrate. After fixation and glycerination of the cultures as outlined above, the desired areas including the cellophane film were cut out and placed between a flat specimen carrier and a brass rod as used for technique I. In order to guide the fracture plane into the tissue layer, a ring-shaped hydrophobic interface, a silicone-coated aluminum foil circle, was introduced just above the tissue (cf. Moor, 1971). The frozen samples were processed as for technique I. With removal of the brass rod, the fracture preferentially separated the hydrophobic silicone layer from the aqueous phase underneath and, in the opening of the ring, tended to continue within the cellular membranes (Pfenninger, 1972). Fig. 5 illustrates part of a replica which was prepared according to this procedure from the early fiber outgrowth of an organotypic rat olfactory bulb culture (Pfenninger and Bunge, 1974). Two nerve growth cones (gc) can be identified on the basis of their characteristic shape; they are surrounded by several supporting cells (g).

Good results could be obtained with the modified technique I (Pfenninger, 1972) but numerous complications during the preparation of the specimens required the development of an improved technique (referred to as technique II) which met the following requirements: (a) minimal loss of biological material, (b) large fracture area, (c) hydrophilic, transparent supporting film for the cul-
Figure 7 View from top (A) and from the side (B) of the fracture tool (dotted) with specimen sandwiches (hatched) inserted. A row of teeth (te; not shown in Fig. 7 B) holds the bottom carriers (b) down against the block of the tool, whereas the upper platelets (t) fit into the interspaces between them. However, the upper platelets extend over the hinged bar (hb) which can be lifted by advancement of the knifeholder (k). The hinged bar thus fractures the upper platelet away from the bottom carrier and tips it over (arrows and broken lines in Fig. 7 B) so that the fractured specimen on the bottom carrier is exposed for etching and/or replication, cf. also Figs. 11 A–C.

Supporting film and culturing of tissue: Honeycomb gold grids (3 mm diameter) are cleaned and autoclaved (or sterilized in 80% ethanol) as required for tissue culture purposes and submersed in a few drops of solubilized rat tail collagen which is thinly spread over the bottom of the culture dish (Bornstein, 1958). After polymerization of the collagen with NH$_4$OH vapors, thorough rinsing, and then “conditioning” (equilibration with two changes of the appropriate culture medium), the dish is ready to host the biological material. Isolated cells can be seeded into the dish containing the gold grids or, if the outgrowth of an explant is to be studied, small chunks of tissue are placed as near as possible to the edges of the grids. In the latter situation, it is advantageous to use gold grids whose broad rim has been cut off on two sides and to place the explants against these flat parts so that the early outgrowth...
FIGURES 8–10 Photographs of the parts and the assembly of the specimen sandwich for technique II. Fig. 8 illustrates (a) the two carriers, (b) bottom platelet with specimen grid and upper platelet, (c) assembled sandwich, and (d) the sandwich in the special specimen holder. The arrow points to the spring-loaded Teflon tip which gently presses the specimen against the two supporting wires of the holder. Scale in centimeters.

In Fig. 9, the tool for assembly and alignment of the sandwich can be seen. The two grooves at right angles provide for precise placement of the two carriers (large arrow). The notches (small arrows) allow one to easily remove the assembled sandwich with the specimen holder (Fig. 10) in which it is subsequently frozen.

FIGURE 11 Stages of the fracture process of technique II. In Fig. 11 A, one specimen sandwich (s) has just been inserted into the fracture tool (ft) which is mounted on the specimen stage (st) of a Balzers freeze-etching apparatus. Note that the bottom carrier is held down onto the block of the fracture tool by a row of teeth (te; see also in Fig. 11 C and in Fig. 7 A), whereas the upper platelet fits into the spaces between them and extends over the hinged bar (hb). The clamp to hold the blade in the knife arm (k) has previously been disassembled. Fig. 11 B, the knife arm has been moved forward so that it lifted the hinged bar (hb) and, thus, the upper platelet from the bottom carrier. In Fig. 11 C, the fracture process is completed and the knife arm has been retracted. The hinged bar tipped over the upper platelet (t) of the specimen sandwich so that the fractured specimen on the grid and the bottom platelet (arrow) is now exposed for replication. For further description, see Fig. 7 and text.

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will be visible soon in the grid openings and will be closer to the center of the grid which is the best fracture area (Fig. 6). After a period of growth, the cultures are fixed with aldehydes, washed, and glycerinated as described above. Then, the grids are removed from the dishes after careful cutting of the collagen layer around their edges (the thicker explants are cut away from the grids). Thus, even a very spread-out and loose network of cells or cellular processes can be preserved in its natural state on the collagen-coated grid and can be transferred onto a specimen carrier for freezing and fracturing.

Carriers and Assembly of Specimen Sandwich: As shown in Figs. 6 and 8, two different tempered stainless steel platelets are used to hold the specimens. The bottom consists of a rectangular platelet (3.9 × 5.7 × 0.2 mm) with four fine holes (0.2 mm diameter) and a square of scratch marks (3 × 3 mm) in the middle to indicate the position of the gold grid which will be placed on top. The thin upper platelet (3.5 × 6.5 mm, 0.025 mm thickness) is U shaped in a cross section and bears a hole of 2 mm diameter in such position that, after assembly of the sandwich, it will sit right above the center of the specimen grid (Fig. 6). After cleaning by sonication in acetone, the one side of the upper platelet which later faces the tissue is coated with a thin, but clearly visible, Teflon film applied with a spray (Fluoro-Glide, Chemplast Inc., Wayne, N. J.) and evened out by gentle buffing with a small cotton ball. Fig. 8 shows how the sandwich is put together. The gold grid with the biological material is transferred from the culture dish onto the lower carrier which has previously been cleaned by sonication in acetone and alcohol and rinsed in buffered glycerol. The upper carrier is then centered over the specimen grid and its hole is finally covered by a second collagen-coated grid. This is done with an alignment tool made of Plexiglass which ensures accurate and well-centered stacking of the various parts (Fig. 9). Excess fluid on the carriers is removed with filter paper; this has to be done with care since withdrawal of too much fluid will drain the fluid chamber above the specimen (Fig. 6 A) and thus result in problems with the fracturing process.

The cross section through the completed specimen sandwich (Fig. 6 A) is helpful for the understanding of the principle of the technique. After freezing, the specimens and supporting grid are anchored to the bottom platelet by the solidified glycerol-saline in the four holes and by adhesion to the metal surface. They are fixed to the top via the vitrified glycerol-saline and the grid covering the hole in the upper platelet. The relationship between the upper platelet and the biological material is of particular importance. As evident in Fig. 6 A, the Teflon-coated face of this carrier is just about level with the cellular material, and the margin of its hole overlaps the specimen grid and lies right adjacent to the cells and cell processes. Above is a small chamber filled with glycerol-buffer. Upon separation of the two carriers, the fracture starts along the Teflon-glycerol interface (Fig. 6 A, arrow). In the area of the opening of the upper platelet, the cleavage plane may enter the adjacent cellular membranes; the more cells or cell processes present in this area, the more likely is the fracture to continue intramembranously rather than splitting the vitrified fluid above. Thus the Teflon-glycerol interface guides the fracture (cf. the layer of Vaseline used by Moor, 1971) into

Figures 12-14 Electron micrographs of replicas obtained with fracture technique II from rat spinal cord cultures. In Fig. 12, a large portion of a supporting cell (g) in a 11-day old culture can be seen. The plasmalemma (inner leaflet) of most of the perikaryon (pe) which bears numerous small extensions (arrowheads) and of a large, branched process (pr) is clearly exposed. Asterisks, reconstituted collagen of the culture substrate. From the same culture, part of a nerve fiber bundle is shown in Fig. 13. The nerve fibers (n) are usually cylindrical, whereas another cell process (g) is of a very irregular shape typical of extensions of supporting cells. Fig. 14 shows a cross fracture through part of the cytoplasm of a glial cell in a spinal cord culture grown for 7 days. Note that technique II also provides satisfactory fracture results of the cytoplasmic contents of cells. Clearly identifiable in this micrograph are endoplasmic reticulum (er, cytoplasmic leaflet; er', inner leaflet), plasmalemmal vesicles (pv), and an outer mitochondrial envelope (m; cytoplasmic leaflet). pl, outer plasmalemmal leaflet. Magnifications, × 6,200 (Fig. 12), × 5,400 (Fig. 13), and × 37,100 (Fig. 14). Calibrations, 5 μm (Figs. 12 and 13), and 1 μm (Fig. 14). Arrow on white disk, approximate shadowing direction for all three figures.

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the right plane and brings it close to the specimen for its intramembranous course.

**FREEZING AND FRACTURING:** With the aid of a specially designed holder, the specimen sandwich is carefully removed from the alignment tool (Fig. 10) and then dipped quickly in the liquid nitrogen-cooled Freon 22 of which a thin superficial layer had been thawed previously. For fast freezing, it is important to bring the carrier immediately into contact with the solid Freon. About 20 s later, the sandwich is transferred into liquid nitrogen where it is released from the holder. The low mass of the specimen sandwich favors fast freezing, but it also warms up quite rapidly at room temperature so that quick handling of the samples throughout the procedure is crucial. For fracturing, a special tool as illustrated in Figs. 7 and 11 is required. It can hold up to three specimen sandwiches. This tool is mounted in place of the standard four-specimen holder of the Balzers freeze-etching apparatus. After precolling of the stage to -150°C and application of an excess amount of liquid Freon 22 to the tool, the samples are slid into the spaces separated by teeth which hold the extending sides of the bottom carriers down onto the block (Figs. 7 and 11). The longer sides of the upper platelets then extend over the bar (Fig. 7 B) which can be lifted with the knife holder of the Balzers unit (the clamp for the razor blade has been disassembled previously). After insertion of the carriers, the entire fracturing tool is covered by a thin metal sheet (not shown in Figs. 7 and 11) which is tipped over during the fracture process and falls off the specimen stage; this sheet prevents the upper platelets from flying away when they break off suddenly, and it avoids heavy frosting of the cold stage before closure and during evacuation of the vacuum chamber. The cleavage is performed at $T_0 = -110^\circ$ to $-120^\circ$C ($T_0$, temperature as indicated by the stage temperature control) and at a vacuum of better than $10^{-4}$ torr. Slow, smooth forward movement of the cold knife arm fractures the upper carrier platelet off the bottom and tips it over to the other side of the edge as shown in Figs. 7 B and 11 A–C and, this way, exposes the fractured specimen for etching and/or shadowing. Preparation of the replicas is then carried out as explained above.

Some of the results are illustrated in Figs. 12–16 (see also, Pfenninger and Bunge, 1974). Fig. 12 shows a glial cell in the outgrowth periphery of a 10-day old rat spinal cord culture. Note the extensive simultaneous exposure of various plasmalemmal regions of the same cell. The fibrillar material in the upper right-hand corner of the photograph is the reconstituted collagen culture substrate. In Fig. 13, a nerve fiber tract of the same culture as above can be seen. Some of the nerve fibers can be traced over lengths of many microns. The winding, large process which is probably of glial nature contrasts with the more cylindrical, straight neuronal fibers. Fig. 14 illustrates the cytoplasmic contents of a glial element which has been partly cross fractured (spinal cord culture grown for 5 days); note the elaborately branched endoplasmic reticulum both of whose membrane leaflets can be identified. This picture indicates that the contents of cells freeze-cleaved with technique II can also be studied although cross fractures through the cytoplasm of the flattened cultured cells are not so frequently found as fractures which run in the plasmalemma.

Isolated neurons from the rat superior cervical ganglion grown for 1–2 days in culture and rabbit blastocysts have also been fractured by this procedure. The blastocysts are too small to be placed...
FIGURE 18 Specimen temperature $T_s$ as a function of the temperature $T_o$ set on the temperature control unit of a Balzers BAF 300 freeze-fracture apparatus. All measurements were carried out under high vacuum of at least $10^{-4}$ torr. However, the $T_s$ values for $T_o = -150^\circ C$ at atmospheric conditions have been added. The upper curves CC and TLF represent the results for specimens on a standard cupped carrier (circled points) and on a technique II carrier platelet (asterisks), respectively, whereas the dashed line gives the theoretical curve for $T_s = T_o$. The lower two curves indicate the temperature difference $\Delta T = T_s - T_o$ as a function of $T_o$ for the two types of specimens (CC, cupped carrier; TLF, thin-layer fracture). For further details, see text. 

on regular freeze-etch carriers and then cut with a razor blade in the conventional way. However, after surgical removal from the uterus and fixation, they can very easily be placed on a supporting collagen-coated grid, sandwiched between the two special carriers, and frozen.

The elaborately branched terminal enlargement of a slender fiber in Fig. 15 is a nerve growth cone in a rat spinal cord culture grown for 7 days. Note the crisp appearance of intramembranous particles and fracture edges of membranes. This is in contrast to the image of the growth cone shown in Fig. 16 which was fractured at $T_o = -100^\circ C$ (instead of $-115^\circ C$). In this picture, a narrow rim along the outer membrane leaflets has apparently collapsed (see Figs. 17 A and B) and, at times, pits of varying size and shape can be found in both membrane faces.

The difference in the appearance of membrane structure at settings of $T_o = -115^\circ C$ vs. $-100^\circ C$ raises the question of the actual temperature $T_s$ of the specimen during the freeze-fracture-etch
process. The appropriate measurements have been carried out with Chromel-constantan thermocouples (0.13 mm diameter) in connection with an electronic ice point reference. The calibration of the equipment was tested before and after every measurement series with ice water and liquid nitrogen. The sensors were mounted in a small drop of 25% aqueous glycerol on a lower carrier platelet inserted into the fracture tool or, for comparison, on a regular cup-shaped gold alloy specimen carrier on the standard four-position stage. The measurements were carried out under high vacuum and, at every point, the temperature was allowed to equilibrate for at least 10 min, which turned out to be of great importance. The measurement series were repeated four times, and the mean values of these “specimen” temperatures $T_s$ were then plotted against the reading $T_o$ of the Balzers stage temperature control unit as shown in Fig. 18. The dashed line represents a theoretical curve where $T_o$ equals $T_s$. In reality, however, $T_o$ is considerably higher for the thin-layer fracture specimens (TLF) and, surprisingly, even more so for the regular cupped carriers (CC). The temperature difference $\Delta T$ as indicated by the two lower curves is slightly temperature dependent and, in the fracture temperature range, amounts to about 18°C and 21°C for the thin-layer fracture specimens and the regular specimens, respectively. The opening of the vacuum chamber at $T_o = -150°C$ increases the values for $T_o$ dramatically to $-101°C$ (TLF) and $-95°C$ (CC) within 2 min whereas shadowing with platinum for 15-40 s (to produce a replica of at least 25 Å) at $T_o = -95°C$ to $-100°C$ has no detectable effect on the specimen temperature. Unfortunately, technology did not allow temperature measurements during a simulated fracture process.

**DISCUSSION**

The material presented in this paper demonstrates two methods available for fracturing thin layers of tissue and shows that the resulting replicas are quite comparable in appearance to those obtained by the more conventional techniques. Both methods are based on the idea of guiding the cleavage plane through the tissue by sandwiching and freezing it between two carriers which are subsequently fractured apart. Thus we are taking advantage of the apparent phenomenon that the fracture preferentially runs through membranes. This new technique allows one to fracture (and, if frozen under proper conditions, to etch) cultured cells and to study their interactions without removing them from their substrate. The supporting film, the collagen, in the same time provides an adequate growth substrate for a large variety of cells. Even very sparsely seeded, individually growing cells can now be fractured and the topography of their membrane properties studied.

Although the results obtained with both techniques and especially with technique II are quite satisfactory, the following points should be considered. Freezing: Whereas the sandwich used for technique I contains a large amount of metal and freezing, therefore, tends to be slower than in regular specimens, the carriers developed for technique II are very light and can therefore be frozen very rapidly. Even without cryoprotectants, relatively good freezing can be achieved. However, the latter specimens also warm up much more rapidly so that all transfers have to be carried out quickly.

Fracturing: With techniques I and II, the process for fracturing is quite different from the cut-cleave procedure used for tissue blocks and for suspensions. In the latter case the advancement of the razor blade induces “micro-fractures,” whereas, in the former case the two ends of the assembled sandwich are pulled apart to induce a single fracture splitting the whole specimen into two. It is conceivable that the two cleavage procedures produce somewhat different artifacts, e.g., occasional deformation of structures (cf. Clark and Branton, 1968). But, so far, differences with respect to the conventionally prepared replicas and obvious deformation artifacts have not been encountered.

Temperature during fracturing: As described in Methods and Results, artifacts are found in material prepared according to technique II if it is fractured at $T_o = -100°C$, a temperature which normally gives satisfactory results. The temperature measurements show that the actual specimen temperature is considerably higher than one would expect from the setting of $T_o$. This difference $\Delta T$ is obviously due to convection of heat along the shaft of the specimen stage and infrared irradiation of the specimen and its support. In either case, the standard fracture procedure or technique II, the specimen temperature at $T_o = -100°C$ is near the critical range ($-80$ to $-70°C$ and above) in which recrystallization occurs and water is easily lost from specimens under high vacuum conditions. It is not clear why, at $T_o = -100°C$, there are usually no effects observed in regularly fractured specimens whose temperature is even higher than that.
of the thin-layer fracture preparations. A likely explanation seems to lie in the fact that the lower carrier platelet is slightly lifted off the fracture tool block during the cleavage process. This interruption of the thermal contact between specimen and stage may result in an increase of the specimen temperature during and after the fracture process. Such an increase, even if small, would lead to the loss of water from cells and organelles and to the collapsed appearance of membranes. This view is supported by the fact that these artifacts can be avoided if the cleavage is performed at $T_0 = -115^\circ C$. The results of the temperature measurement series clearly show that the conditions during the fracture-etch process can be accurately defined only if the temperature is measured right on the specimen carrier.

Further modifications of the techniques for thin-layer fracturing, in particular of technique II, will widen their range of application. First attempts indicate that future progress will permit the identification of the same cells in the living state under the phase-contrast microscope and in the freeze-fracture replica, deep etching of specimens frozen without cryoprotectants, and replication of both fracture faces.

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