FREEZE-FRACTURE AUTORADIOGRAPHY: FEASIBILITY

KNUTE A. FISHER and DANIEL BRANTON. From the Department of Botany, University of California, Berkeley, California 94720. Dr. Fisher's present address is the Cardiovascular Research Institute, University of California, San Francisco, California 94143. Dr. Branton's present address is the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138.

Membrane splitting by freeze-fracture (2) could provide chemical information about the transmembrane distribution of constituents if the individual halves of the membrane could be analyzed separately. One means of analysis is the detection of membrane-incorporated radioisotopic labels by autoradiography after freeze-fracturing (6).

We have examined three model systems, two nonbiological and one biomembrane, that test the feasibility of combining freeze-fracture with electron microscope autoradiography (FARG) and that demonstrate FARG's potential in studies of biomembrane organization. We describe here a method for the formation and application of a dry, parlodion-stabilized monolayer of autoradiographic emulsion to frozen replicas of fractured surfaces (Fig. 1).

MATERIALS AND METHODS

Anion exchange resin beads (AG 1-X8, Minus 400 mesh; Bio-Rad Laboratories, Richmond, Calif.) sized by sequential slow-speed centrifugation (details given in reference 5) to submicron dimensions (95.6% were less than 1.5 μm in diam) were labeled with 125I-Na (New England Nuclear Corp., Boston, Mass.): 107 beads of OH- form, in 2 ml of distilled water, were equilibrated for 5 min at 22°C with 200 μCi 125I-Na pelleted, washed, and frozen. An oil-water emulsion was formed by dispersing oil droplets in water with sonication. Tritiated oleic acid, 0.5 mCi, partitioned into 5-μl linolenic acid, was added as a single 3.5 μl drop to 120 μl of glass distilled water and sonicated under the microtip of a Branson sonifier (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) for several seconds at 22°C to produce a milky white emulsion that was immediately micropipetted onto hats and frozen. Human erythrocyte ghosts prepared according to Dodge et al. (4) were labeled before rescaling with 125I-Na using coupled glucose oxidase-lactoperoxidase iodination (8).

Freeze-fracture was conventionally performed with a Balzers freeze-etch device (9), except that samples were placed on hat-shaped, matt-finish acetate supports (7).

Nuclear emulsion monolayers were strengthened and stabilized by applying partially gelled loops of emulsion to Parlodion films. A thin film of parlodion was cast by dropping 50 μl of 5% (wt/vol) Parlodion (Mallinckrodt Chemical Works, St. Louis, Mo.) in pentyl acetate onto a clean water surface and allowing the solvent to evaporate. The homogeneous transparent portion of the film was picked up by pressing a stopped, thick-walled Plexiglas or glass tube, 3.1 cm ID, against the floating film attaching it to the open end of the tube. Pressing a nichrome loop (2.9 cm OD) through the film attached it to the wire. The loop of Parlodion was then coated with a loop of 0.5% gelatin in distilled water (wt/vol) and applied to the side that contacted the replica. Films were placed on racks, air-dried at 22°C for at least 1 h and transferred to a darkroom kept at 15 ± 1°C and 70% relative humidity. Nuclear emulsion, Ilford L-4 (Ilford Ltd., Ilford, Essex, England), was weighed and diluted with distilled water, 1:1 (wt/vol), heated at 50°-52°C for 2-3 min, and transferred to a 35°-40°C water bath. The liquid emulsion was gently stirred for about 5 min and then a 4.2-cm ID nichrome wire loop was dipped into the emulsion, withdrawn, held vertically, and, after 15 s, applied to the Parlodion film (to the side untreated with gelatin).

After vertical drying, loops sacrificed to roomlight examination showed a central 0.6-cm wide band of copper-purple interference color containing a silver halide crystal distribution suitable for electron microscope autoradiography: a "monolayer" (10) as confirmed by transmission electron microscope examination of undeveloped controls. By interference color criteria, the thickness of the dry films was about 200 nm: 60 nm parlodion-gelatin plus 140 nm emulsion.

Dry emulsion monolayers were applied to frozen replicas either ex vacuo or in vacuo. For ex vacuo application, freeze-fractured and shadowed samples were kept frozen, removed from the Balzers freeze-etch apparatus,
transferred to a holder on a freezing post, and transported to the darkroom. Replicas were defrosted in flowing dry cold nitrogen gas, and coated by rapidly applying the dry, stabilized monolayer (in the dark), fitting it to replica contours with air pressure from a source of cooled dry N₂. Micrographs included here were produced using the ex vacuo method, which was developed to establish feasibility. For in vacuo application, prototype equipment including a transfer arm, transfer tubes, and magnetic stage were constructed to apply the monolayer to replicated samples retained in vacuo at -100°C after shadowing. Details of the method will be published later. Current studies by Zvi Ne'eman in D. Branton's laboratory have shown that the in vacuo method produces inexplicably high background levels. Further studies will therefore be required before the in vacuo method can be used.

After emulsion application, samples were stored in gas- and light-tight containers during exposure at -80°C. After exposure, samples were thawed, developed in Kodak Microdol-X, 1:3, for 4 min (Eastman Kodak Co., Rochester, N.Y.), given a 30-s stop bath, fixed in 20% sodium thiosulfate, pH 5.5, for 10 min, washed in tempered running water for 15 min, and given a final rinse in distilled water before applying an electron microscope grid and drying. All photographic chemicals were kept at 20 ± 1°C. grids were examined with Siemens 1 and 1A electron microscopes.

RESULTS
Dry monolayers of autoradiographic emulsion were stable when applied to thin Parlodion films. Parlodion-supported monolayers, 14.5 mm² in area, remained intact during pressure contouring to frozen replicas and immersion in photographic chemicals. Coating the film with gelatin assured adhesion of Parlodion to replica upon thawing. Because the Parlodion was positioned between replica and emulsion, chemographic grain deposition (observed when emulsion was in direct contact with replicated biological samples) was prevented, and direct exposure to photographic chemicals was assured. Despite the thickness of the gelatin-parlodion-emulsion sandwich, replica contrast was still high (Figs. 2 and 3) allowing easy examination of detail.

Examination of the freeze-fractured, iodinated exchange beads after FARG revealed silver grains clustered over cross-fractured areas (Fig. 2 a). Because the beads remained attached to the replica, they often obscured overlying silver grains. Nevertheless, examination at higher magnification of peripheral regions of bead fracture faces revealed numerous grains (Fig. 2 b). There was no evidence of lateral displacement of grains (emulsion) relative to replica in these samples, and controls showed only background numbers of randomly distributed grains. Background grain counts were high, however. This may have been due to pressure sensitization, to presence of label in the ice matrix, or to scatter from the thick underlying source of isotope.

Examination of freeze-fractured and autoradi-
FIGURE 2 Nonbiological test systems: (a-b) ¹²⁵I-labeled anion exchange resin beads; (c-d) ³H-labeled oil droplets in water. (a) Close inspection of labeled resin bead reveals numerous silver grains directly over cross-fractured areas. 15-day exposure at -80°C. Bar = 2.5 μm. x 10,200. (b) Detail of Fig. 2 a. The site of particle cross fracture (arrow) and clustered silver grains are obvious at higher magnification. Bar = 0.25 μm. x 101,600. (c) [³H]oleic acid in linolenic acid droplets in distilled water. Large cross-fractured lipid droplet (arrow) is obscured by silver grains. 9-day exposure at -80°C. Bar = 2.5 μm. x 11,600. (d) Same oil-water emulsion preparation as Fig. 2 c except 15-day exposure at -80°C. Smaller cross-fractured droplets showing lamellar faces. Bar = 0.5 μm. x 50,200.
**FIGURE 3** Biomembrane test system; lactoperoxidase-glucose oxidase $^{125}$I-labeled erythrocyte ghost membrane. (a) Silver grains directly over cross-fractured membrane. 8.5-day exposure at $-80^\circ$C. Bar = 1.0 $\mu$m. $\times$ 40,000. (b) Silver grains overlying extracellular fracture face indicating presence of radiisotope on exterior half of the membrane. 8.5-day exposure at $-80^\circ$C. Bar = 1.0 $\mu$m. $\times$ 41,400.
Fracture faces (Fig. 2 c) may have been due to the underlying droplet being of larger diameter than its fracture face and/or being misshapen during freezing. More grains were present over large droplets, and some smaller droplets possessed no grains. Occasionally, lateral displacement of silver grains could be seen in smaller cross-fractured droplets at higher magnification (Fig. 2 d). However, most autoradiographs showed no such displacement. Control replicas contained few background silver grains, randomly distributed.

Examination of freeze-fractured and autoradiographed 125I-labeled erythrocyte ghosts revealed silver grains associated with cross-fractured membrane (Fig. 3 a). A sample of 554.5 \( \mu m^2 \) of replica showed a total of 83 silver grains. Of that area, extracellular faces occupied 28.4 \( \mu m^2 \), protoplasmic faces 16.6 \( \mu m^2 \), and cross-fractured membrane about 3.8 \( \mu m^2 \). Silver grain centers (10) lying within 250 nm of membrane fracture faces were scored as membrane associated: extracellular faces (3), 6 grains (grain density = 0.36 grains/\( \mu m^2 \)); protoplasmic faces, 23 grains (0.81 grains/\( \mu m^2 \)); and cross-fractured faces, 12 grains (3.16 grains/\( \mu m^2 \)).

To provide some estimate of FARG efficiency (sensitivity), leaky ghosts were labeled with 125I to a sp act of about 385 disintegrations per day per ghost and exposed at \(-80^\circ C\) for 8.5 days (22.6 total disintegrations/\( \mu m^2 \) intact membrane). The grain yield was 0.103 grains/\( \mu m^2 \) split membrane (0.206 grains/\( \mu m^2 \) intact membrane). Efficiency was thus less than 1%.

Although none of the test systems presented here are especially suitable for evaluation of resolution, some comment can be made based on an analysis of data on 125I-labeled ghost. Cross-fractured membranes are sufficiently thin to be considered “line” sources of radioactivity. When 83 \( \mu m \) of line were evaluated, 34 grains fell within 2 \( \mu m \), 6 with centers directly above the line, 8 within 125 nm of the line, and 3 within 250 nm of the line. Thus, the half distance, defined as the distance within which 50% of the developed grains are found (10), was about 250 nm. 41% of the grains were found within 125 nm.

**DISCUSSION**

The requirement for highly labeled, identifiable point sources of radioactivity for testing FARG was satisfied by two test systems: iodinated anion exchange resin beads, and tritiated oil droplets, both as aqueous suspensions. Because the exchange beads remained attached to the replica, they provided unequivocal markers of isotope accumulation. The oil-water emulsion allowed better visualization of the grains and demonstrated that fluid systems (more sensitive to possible thawing or recrystallization artifacts) could be examined. Moreover, \(^3\)H as well as \(^125\)I was shown to be a useable isotope. Neither test system, however, was suitable for critical evaluation of efficiency or resolution.

The biologically significant part of this study was the demonstration that radioisotopically labeled, freeze-fractured membranes could be autoradiographed. Because the erythrocyte membrane half can be identified by its particle population, the association of silver grains with fracture faces easily can verify the presence of label as being with the extracellular or with the protoplasmic half of the membrane. But, in the present study, no effort was made to reseal the ghosts and, thus, \(^125\)I would label both sides of the membrane. Indeed, grains were found over both extracellular and protoplasmic fracture faces. However, even in experiments designed to determine which side of the membrane is labeled, grain distribution data must be interpreted carefully: little is known about the partitioning of membrane constituents during freezing and fracturing. For example, membrane polypeptides labeled only from the extracellular side may partition, upon fracturing, to the protoplasmic side and carry the label with them.

The above-described test systems demonstrate the feasibility of FARG. Dry monolayers of nuclear emulsion contain developable silver grains when exposed at \(-80^\circ C\) to radioisotopes of energies low enough to be compatible with electron microscope autoradiography, even when separated from the source by a platinum-carbon replica additionally coated with carbon, and by an interposed gelatin-coated Parlodion film. To use FARG, however, several conditions must be met. First, high specific activity, low energy labels are required. Second, the geometry of the labeled system must be appropriate, e.g., for only surface radioisotope to be detected, underlying isotopes must be far enough below the surface to avoid exposure of the emulsion. Third, for optimum resolution and quantitation, the emulsion must lie in close contact with the replica. Finally, it is desirable that the sample remain frozen, at low...
temperature, during application and exposure of emulsion. Given these conditions, present observations suggest that the resolution of FARG is similar to that of conventional electron microscope autoradiography (10). It should be emphasized, however, that since the number of grains counted was very small, the values for resolution and efficiency given here are only rough estimates.

A current limitation of the method is that low temperature reduces the number of developable silver grains (1), thus necessitating large doses of radioisotopes or long exposure times. For sidedness analyses, i.e., determination of isotope distribution across the plane of the membrane, the problem of geometry becomes paramount, and further experiments are required to demonstrate that the emulsion is equally well contoured to convex and concave faces. For examination of tissue, the problem of replica cleaning arises. In the present study, replica cleaning was sidestepped by using erythrocyte ghosts that were largely removed during photographic processing. The ex vacuo method was presented here because it provided the first evidence that FARG could be accomplished. The in vacuo approach, however, solves problems of frost removal from the replica surface (samples are kept in vacuo after shadowing), sample thawing (samples are retained at −100°C during emulsion application), or replica-emulsion contouring (emulsion is contoured by vacuum form fit). These advantages are appealing, but the development of in vacuo methodology still requires much work.

SUMMARY
We have shown that the combination of freeze-fracture with electron microscope autoradiography can be developed into a technique for correlating the molecular structure of the biological membrane with its chemical and functional characteristics. Within the limits of electron microscope autoradiographic resolution, FARG has the potential to detect the relative distribution of molecules in each half of the membrane and within the plane of the membrane. The use of radioisotopic labels in combination with freezing techniques requires minimal perturbation of the system being studied and may be suitable for the examination of substances which would be extracted or would diffuse during the normal fixation and embedding procedures used in standard electron microscope autoradiography.

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