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# "Half" Membrane Enrichment: Verification by Electron Microscopy


Abstract. *Membranes of intact erythrocytes bound to polylysine-treated glass fracture nonrandomly when covered with thin copper and frozen. Electron microscopic examination of the glass side reveals extensive areas of outer "half" membrane (B face) and of the copper side, inner "half" membrane (A face). This technique allows the ultrastructural examination of square-centimeter areas of fractured membrane and the chemical analysis of these membrane "halves."*


During freeze-fracture the erythrocyte membrane is split along an interior plane (*1*) producing two fracture faces that are structurally asymmetric. The A face, cytoplasmic side, contains many particles, whereas the B face, extracellular side, contains fewer particles (*2*). At the chemical level, it is currently thought that the erythrocyte membrane is highly asymmetric in the transmembrane distribution of its phospholipids (*3, 4*) and major polypeptides (*4, 5*). This concept is derived from numerous chemical and physical experiments designed to examine membrane structure, often by utilizing permeant or nonpenetrating reagents or enzymatic degradation of intact or lysed cells (*3–5*). Another conceptually and experimentally different approach to answer questions of membrane structure would be to exploit the property of membrane splitting during freeze-fracture and isolate portions of the membrane "halves" in quantities suitable for bulk analysis (*6, 7*). In such an approach one of the most important aspects is the verification and quantitation of half membrane enrichment. The purpose of this report is first to describe a method for the preparation of square-centimeter areas substantially enriched in outer half membrane (complementary to areas substantially depleted in outer half membrane) and second to provide electron microscopic evidence for this enrichment and depletion.

When cells are frozen in bulk, the fracture meanders randomly. Nonrandom splitting of the membrane on one side of the cell is relatively rare. However, if the cell is bound, flattened, to a smooth substrate and the other side is free and thus of irregular shape, the fracture preferentially follows the flattened side, especially if this geometry is reinforced by large numbers of contiguous cells similarly bound over a large area. I have tested a variety of materials and techniques following this principle. The most reproducible procedure is described here.

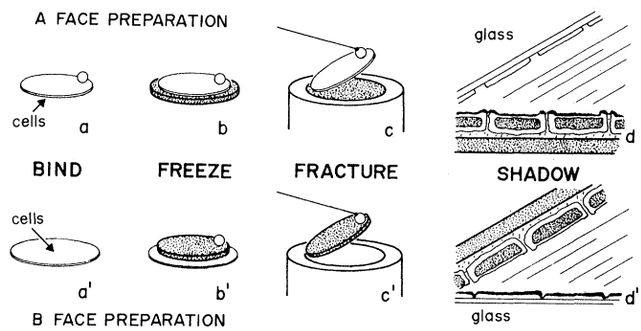


Fig. 1. Four steps—binding, freezing, fracturing, and shadowing—are common to the preparations of inner half membrane (a to d) and outer half membrane (a′ to d′). Replicas of A faces are prepared by binding erythrocytes to the lower side of a positively charged glass disk with a pull ring (a), applying the disk to a larger copper disk and freezing (b), transferring the frozen sandwich to a stage that holds the copper disk while the glass is pulled free in vacuo (c), and shadowing the copper side (d). Replicas of B faces are prepared by binding erythrocytes to the upper side of the glass disk (a′), applying a smaller copper disk (b′), transferring them to a cold stage that retains the glass disk while the copper is pulled free (c′), and shadowing the glass side (d′).

Because the outer surface of the erythrocyte possesses a net negative charge at physiological *p*H (*8*), it will bind strongly to a surface that possesses a net positive charge. Adsorption of polycationic polylysine (*9, 10*) to clean, optically smooth cover glass provides such a surface (*11*). Disks of Corning No. 1 cover glass (9 mm in diameter for A face preparations, 12 mm for B face) are cleaned with acid, rinsed in distilled water, and dried with filtered compressed air or dry $N_2$. Ten microliters of 5 m*M* poly-L-lysine, molecular weight 2000 (Sigma Chemical Company), is applied for 30 seconds at 22°C to one side of the cleaned cover glass, which is then washed with distilled water and dried with compressed air. After the cleaning and polylysine application the surface of the glass must remain totally hydrophilic.

Human erythrocytes from freshly drawn blood are washed three times with 0.155*M* NaCl, followed by three washes with phosphate-buffered saline (PBS). Twenty microliters containing $1.4 \times 10^8$ erythrocytes in PBS are applied to the positively charged glass disk and unbound cells are washed off with PBS. The use of fresh blood and its thorough washing and proper dilution are essential for the reproducible generation of a homogeneous closely packed monolayer of cells.

For A face preparations, erythrocytes are applied to the polylysine-treated side of a glass disk 9 mm in diameter (Fig. 1a). Unbound cells are removed by washing, and the cell side, wet with about 10 μl of residual buffer, is placed against a dry copper disk (Fig. 1b). For B face preparations erythrocytes are applied to a glass disk 12 mm in diameter and unbound cells are washed free by immersing the disk in buffer at 4°C and agitating for 60 seconds. The untreated side is then wiped dry quickly. Drying ensures close contact to the stage and quickness prevents lysis due to buffer evaporation from the cell side. A copper disk with pull ring is gently placed against the cell side (Fig. 1b′). For both A face and B face preparations the copper had been flattened, ground smooth, and made hydrophilic by dipping in nitric acid, rinsing in distilled water, and drying with compressed air. A platinum pull ring had been attached with low-temperature epoxy to the 9-mm glass disks.

The copper-erythrocyte-glass sandwich is immediately frozen in liquid Freon 22 at −150°C. Interference colors are occasionally seen, suggesting that substantial fracturing occurs during freezing, a probable result of thermal expansion differences be-

tween copper and glass. The sandwich is transferred to a Balzers freeze-etch machine and placed on a flat cold-stage that has a threaded, flanged cap designed to retain the bottom disk (copper, Fig. 1c, or glass, Fig. 1c'). The upper disk is pulled free by using a braided nylon line attached to a rotary feed-through and to the pull ring. After disk detachment at −95°C and $2 \times 10^{-6}$ torr the sample is etched for 10 minutes, to remove ice crystals deposited during fracturing, and shadowed (Fig. 1, d and d') (*12*). Replicas of A faces are cleaned by floating on distilled water containing 2.5 percent sodium dodecyl sulfate (weight to volume), followed by three distilled water washes. Replicas of B faces are examined without cleaning since residual biological material is minimal.

Before fracturing, the monolayer of blood cells appears pink. After fracturing, the glass surface appears transparent and colorless, with occasional patches of pink. To determine the fraction of transparent area, glass disks from freeze-fractured (unshadowed) and dried preparations are placed in a photographic enlarger and "printed" at × 15; the total area is measured and the area of pink patches subtracted. The transparent portion ranged from 71.6 to 99.8 percent of the total with a mean ± standard deviation of 89.5 ± 10.5 percent (total area = 4.08 $cm^2$; $N = 6$).

Electron microscopic examination of replicas from the copper side revealed extensive areas of A fracture faces surrounded by strips of etched ice (Fig. 2a). At higher magnification the exterior "surface" of the membrane exposed by etching could be seen between the fracture faces and the intercellular ice (Fig. 2c). Examination of replicas from the transparent portion of the glass surface revealed extensive areas of B fracture face (Fig. 2b). As found for A face preparations, B faces at higher magnification (Fig. 2d) were remarkably similar in particle distribution to faces of conventionally freeze-fractured erythrocyte suspensions.

Quantitative electron microscopy with calibration standards and random photography of 4465 $\mu m^2$ of replica taken from the transparent portion of glass (pooled data of three experiments, area measured by planimetry) revealed that 3518 $\mu m^2$ or 78.8 percent of the glass was covered by half membrane. Of that membrane less than 1.0 percent represented A face. Among the random micrographs several entire half membranes from single cells were observed and measured by planimetry. The profiles were continuous, uninterrupted by a central torus, indicating that the concave portion of the erythrocyte flattens against the glass. Profile area equaled 46 ± 6.3 $\mu m^2$ ($N = 19$). Thus, after freezing and fracturing, 33 percent of the outer surface or 16.5 percent of the outer plus inner surfaces remained attached to glass (*13*).

This approach has several advantages over conventional techniques for the structural examination of the biological membrane. Because the plane of fracture is fixed (it follows the plane of the glass) the shadow angle is known and thus structural dimensions can be accurately determined. Because square-centimeter areas of half membrane can be produced, regions of structural specialization that occur at low frequency can be studied readily. Moreover, large, well-defined areas are readily compatible with many techniques for surface analysis, such as x-ray photoelectron spectroscopy.

As a conceptually different means to examine the chemical composition of half membranes, bulk freeze-fracture is expected to make its greatest contribution. One approach is simply to treat the glass and the copper with appropriate solvents and compare the concentrations of components extracted from each portion, which gives a measure of their distribution across the plane of the membrane. For such analyses the method has been scaled to larger areas. The distribution of cholesterol across the plane of the membrane has been examined and will be reported elsewhere (*14*).

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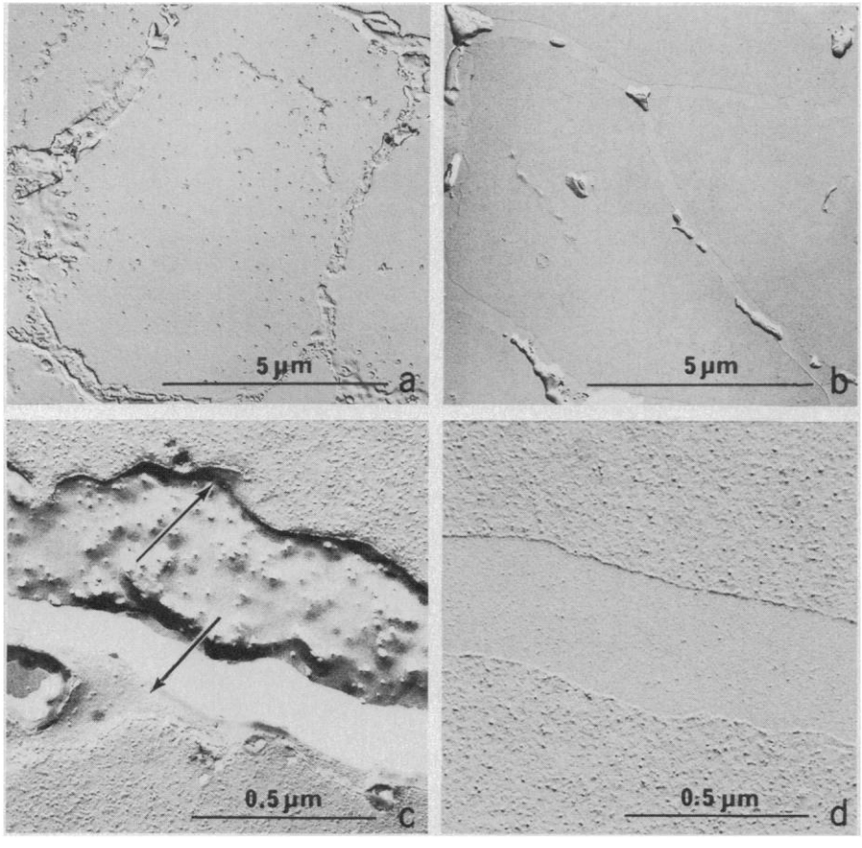


Fig. 2. Human erythrocytes bound to glass and freeze-etched: (a) low magnification showing portions of six A faces (× 6,300); (b) portions of five B faces (× 6,300); (c) detail of A face, etched ice revealing surface of membranes (arrows) contiguous to fracture faces that contain characteristic inner half membrane particle populations (× 51,000); (d) detail of B face, glass surface separates two outer half membranes (× 51,000). Shadow direction: bottom to top.

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## Retinal Capillaries: Proliferation of Mural Cells in vitro

Abstract. *Capillaries from bovine, monkey, and human retinas maintained in tissue culture produced a monolayer of cells. Autoradiographic and electron microscopic evidence indicated that the mural cells (intramural pericytes) were the cells that proliferated. Since intramural pericytes are damaged selectively in diabetes mellitus, their availability in culture will be useful in seeking means to control diabetic retinopathy.*

Vertebrate retinal capillaries contain two cell types: endothelial cells and mural cells, or intramural pericytes (*1, 2*). Endothelial cells line the vessel lumen while mural cells are embedded in the vascular basement membrane and surround the vessel with extensive cytoplasmic processes. Details of the physiological roles of these cells are largely unknown (*3, 4*); however, abnormalities in their biochemistry and function may be critical factors in the pathogenesis of several retinal vascular diseases which are common causes of blindness. For example, in diabetic retinopathy mural cells are affected early and degenerate, leaving capillaries with a preponderance of endothelial cells (*3, 5*). The biochemical events that precede these anatomical changes are unknown. Their elucidation would be an important step toward the understanding and eventual treatment of many retinal vascular diseases.

The capability of maintaining cells in tissue culture greatly facilitates studies of their biochemistry, growth, and function. While long-term culture of endothelial cells from human umbilical vein has been reported by several investigators (*6, 7*), to the best of our knowledge such a system of sustained growth has not been described for retinal vascular cells (*8*). We now report methods whereby cellular proliferation from bovine, rhesus monkey, and human retinal capillaries can be obtained regularly. Our observations indicate that the cells which grow under the imposed culture conditions originate from the mural cells of the retinal capillaries.

Eyes from rhesus monkeys, cattle, and humans were usually processed for culture 4 to 8 hours after enucleation (*9*). Eyes were cleansed in a physiologic saline solution and rinsed in tincture of benzalkonium chloride (0.13 percent) before dissection. Retinas were removed from the eyes, minced in modified Earle's salt solution, homogenized, and filtered as described by Meezan and co-workers (*10*), using sterile technique. Vessels remaining on the 85-$\mu$m sieve were washed into sterile plastic petri plates with Ham's F-12 medium containing 5 percent fetal calf serum; 2 m*M* glutamine; penicillin, 200 unit/ml; and streptomycin, 200 $\mu$g/ml. The plates were incubated at 37°C in an atmosphere of 95 percent air and 5 percent $CO_2$.

Isolated retinal capillaries could be identified by their size and structure (Figs. 1A and 2A). Endothelial cell nuclei were distinguished by their location within the capillary wall and their ellipsoidal shape, with the long axis parallel to the lumen. Mural cell nuclei had round profiles which protruded from the capillary wall like "bumps on a log." Occasionally blood cells were seen trapped within the vessels. Perivascular glia or neural cells were seen only rarely adherent to the isolated vessels. We never observed cellular proliferation from these infrequent contaminants.

Approximately 1 to 2 percent of the isolated vessels displayed growth. When placed in culture media, the vessels behaved as explants, producing an advancing sheet of polygonal cells (each about 35 by 70 $\mu$m) by day 8 to 12. The configuration of some vessels varied as loosely adherent branches shifted positions (Fig. 1B). During the first 2 to 4 days after isolation, the vessels often appeared to entwine upon themselves, making it difficult to distinguish cell types by their morphological appearance (Fig. 1, C to E). By day 9 large cells with ovoid nuclei and multiple nucleoli could be seen projecting from the closely packed cluster of vessel strands (Fig. 1F). An advancing sheet of cells was usually apparent by day 17 (Fig. 1G) and the establishment of a dense monolayer by day 30 (Fig. 1H). Rate of growth was slow, with several proliferating vessels requiring 2 to 4 months to fill a 60-mm culture plate. Older cultures generally displayed cells in monolayers of uniform density. However, some of these cultures contained cells of varying size and packing. Sometimes cells were found irregularly heaped on each other. Cells were never found to abut one another in either regular epithelioid or typical fibroblast patterns (*6, 7, 11*). Several cultures have been subcultured many times over an 8-month period without any change in morphological appearance or growth characteristics.

Electron microscopy of cells grown from rhesus monkey retinal capillaries for 30 days in culture revealed ultrastructural characteristics suggestive of metabolically active, somewhat specialized cells. Smooth endoplasmic reticulum, Golgi, and coated vesicles were prominent near the cell cen-

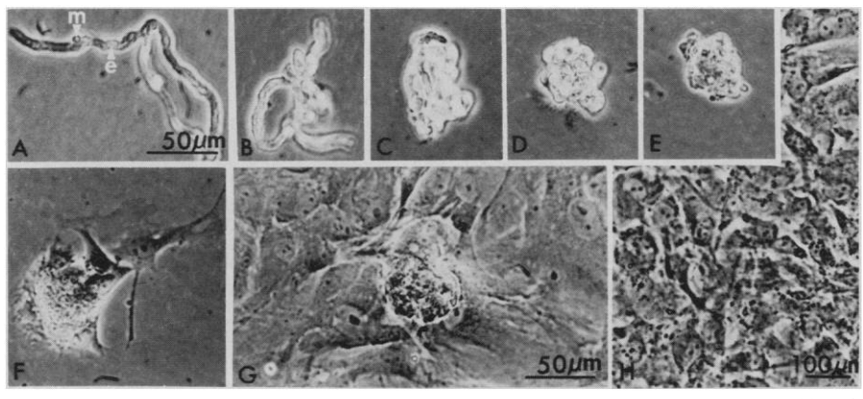


Fig. 1. Phase contrast photomicrographs of a single rhesus monkey retinal capillary observed in culture for more than 30 days: (A) day 0, (B) day 1, (C) day 2, (D) day 3, (E) day 6, (F) day 9, (G) day 17, and (H) day 30. Abbreviations: *e*, endothelial cell; *m*, mural cell (A to G, × 265; H, × 81).