Fracture faces of frozen membranes: 50th anniversary

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ABSTRACT In 1961, the development of an improved freeze-etching (FE) procedure to prepare rapidly frozen biological cells or tissues for electron microscopy raised two important questions. How does a frozen cell membrane fracture? What do the extensive face views of the cell's membranes exposed by the fracture process of FE tell us about the overall structure of biological membranes? I discovered that all frozen membranes tend to split along weakly bonded lipid bilayers. Consequently, the fracture process exposes internal membrane faces rather than either of the membrane's two external surfaces. During etching, when ice is allowed to sublime after fracturing, limited regions of the actual membrane surfaces are revealed. Examination of the fractured faces and etched surfaces provided strong evidence that biological membranes are organized as lipid bilayers with some proteins on the surface and other proteins extending through the bilayer. Membrane splitting made it possible for electron microscopy to show the relative proportion of a membrane's area that exists in either of these two organizational modes.

Odd as it may seem, tedium paved the way to the astonishing discovery that the membranes of a frozen living cell can easily be cleft into two sheets. In 1961, shortly after starting a two-year National Science Foundation Postdoctoral Fellowship with Fritz Ruch in Frey-Wyssling's department at the ETH in Switzerland, I realized that the motivating hypothesis for my project was misguided. Ruch convinced me to endure a few months carefully proving and explaining why prior investigators' qualitative data were misleading, but it was dull research (Branton and Ruch, 1964). The tedium motivated me to find a project that would open new insights rather than simply falsify a hypothesis.

I was intrigued by the freeze-etching (FE) methods being developed in the department by Hans Moor. Initiated by Russel Steere, FE was a method of preparing biological material for the electron microscope without chemical fixation, dehydration, and embedding (Steere, 1957). Steere's idea was to rapidly freeze a living specimen in a tiny block of ice, plane away overlying ice with a scalpel to expose the material of interest, etch away (sublime) a small amount of water from the exposed frozen material in a vacuum, and then make a replica of the exposed, etched surface material that could be viewed in an electron microscope. Studies of baker's yeast (*Saccharomyces cerevisiae*) by Moor and his colleagues showed FE to be the first really productive alternative to the usual fixation–dehydration–embedding routines and associated artifacts (Moor and Muhlethaler, 1963).

The "plane away overlying ice" step envisaged by Steere often fractured the frozen material in a plane that followed any membrane's contours for many microns. This resulted in surprisingly expansive three-dimensional views of many cellular membranes. Such in-plane views had never been seen in thin sections of biological material. Only later, after the correct locus and physical basis of the fracture process were established, would the signal importance of these views become fully apparent.

My initial work focused on easily grown onion root-tip cells whose freeze-etched cellular features could be compared with similar cells that had been visualized following classical methods. At first, freeze-etched root tips seemed to present only three-dimensional versions of the usual structures seen in fixed, sectioned root tips. But unfamiliar 7–10 nm diameter particles were observed on most of the membrane faces. Moor had observed similar particles on yeast endoplasmic membranes and assumed they must be ribosomes, even though they were much smaller than any known ribosomes (Moor and Muhlethaler, 1963). Although Moor and his colleagues insisted that the similar particles in my root-tip cells must also be ribosomes, I was reluctant to use this designation. Thus, in my first paper reporting freeze-etch results, written jointly with Moor
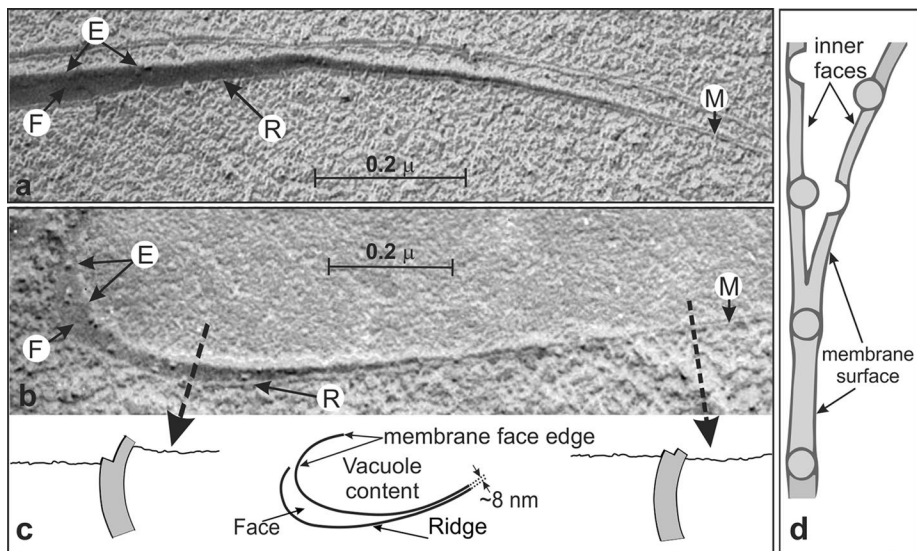
FIGURE 1: Membrane splitting. (a) FE replica of endoplasmic reticulum membranes in an onion root-tip cell showing the membranes in face views (F) on the left and in cross-fractured views (M) on the right; (b) FE replica of a vacuole membrane showing the vacuole membrane in face view (F) on the left and in cross-fractured view (M) on the right. The dashed lines point to diagrams in c, left and right, corresponding to hypothetical cross-sectioned views of the material under each dashed line. (c) At center, a labeled diagram interpreting the vacuole image in b to emphasize how the ridge at the base of the membrane face (R) merges with the membrane face edge (E) to produce the image of a single cross-fractured membrane (M) when the plane of the replica surface is perpendicular (or very close to perpendicular) to the membrane's surfaces. (d) Diagram of a membrane in the process of splitting. Reproduced or redrawn from Branton (1966).

(Branton and Moor, 1964), we compromised and concluded that "further studies … will establish a definitive correlation between surfaces seen in freeze-etched preparations and structures visualized by other techniques."

At issue was not only the nature of particles seen on the freeze-etched membrane faces but also the identity of the membrane faces on which the particles were found. Newly appointed as an assistant professor at University of California, Berkeley, I undertook a thorough reexamination of the replicas made during my post-doctoral research. Doing so revealed a previously overlooked feature: a small ridge at the base of every exposed membrane face. When observed in views that extended from a face-on view into a cross-section of the same membrane, the edge of the face-on view and the small ridge at the base of the face-on view merged and became continuous with, and indistinguishable from, the two ridges that *together* represented the FE appearance of a cross-sectioned membrane (Figure 1, a–c). These observations led me to postulate that the fracture process that exposes the membrane faces cleaves through a central region of the membrane, splitting it in two (Branton, 1966). If true, none of the membrane faces seen in freeze-etch replicas represent the surface of membranes. Instead, the membrane faces seen in replicas are either of the two

inner faces created when a membrane is split through its center (Figure 1d).

What is the molecular organization of biological membranes that can explain this stunning tendency to cleave in two? Unfortunately, there was no universally accepted explanation for the molecular organization of biological membranes in the 1960s. Many membrane functions were recognized, and it was known that membranes contain a heterogeneous group of proteins and amphiphilic lipids. It was also generally agreed that membrane surfaces are hydrophilic, their interiors hydrophobic. But the common molecular organization of the membrane matrix that accounted for this limited scope of agreement was much disputed. Investigators assumed one of three possible models: proteins on the outside surfaces and lipids in the center (the PLP model; see Figure 2a); or lipids on the outside surfaces and proteins in the center (the LPL model; see Figure 2b). Both of these models for membranes assumed that the membrane matrix was interrupted by specialized protein-containing assemblies that account for the differentiated functions performed by the cell's various membrane types. A third model for membranes explicitly questioned the lamellar continuity implicit in PLP and LPL models and proposed instead a membrane composed of subunits self-associated into a sheet-like structure by hydrophobic bonds (Figure 2c). A review of the available evidence in 1963 (eventually published and updated in 1969; Branton, 1969) led me to conclude that the topological assignment of protein on the outside surfaces, lipid in the center (the PLP model, Figure 2a) was fully convincing in at least one membrane system—myelin—but at best only persuasive in a few others. Definitive evidence for most membrane types was simply not available, and electron microscopy of classically prepared specimens failed to establish the location of the membrane's lipids and proteins within its ~8-nm thickness.

The striking tendency of all cell membranes, including myelin (Branton, 1967), retinal outer-segment rod disks (Clark and Branton, 1968), and chloroplast lamellae (Branton and Park, 1967), to split along some inherent midmembrane plane of weakness ruled out subunit models and made LPL models unlikely. Both these models postulate amino acid chains extending through the membrane interior. Such covalently linked chains would not create an easily cleaved plane of weakness. In contrast, the PLP model that postulates a continuous lipid bilayer does predict a plane of weakness through the middle of a frozen membrane. The hydrophobic (entropic) bonding that stabilizes a lipid bilayer is attributable to the bilayer sequestering
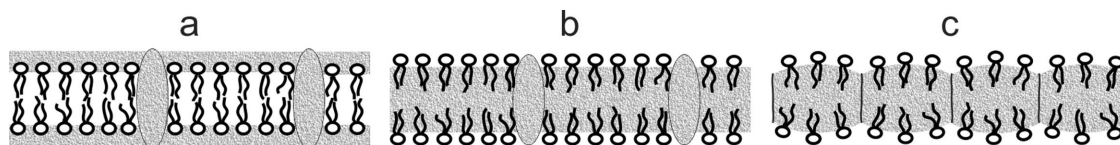

FIGURE 2: Membrane models c. 1965. Diagrams of (a) protein–lipid–protein (PLP), (b) lipid–protein–lipid (LPL), and (c) subunit models for biological membranes. Redrawn from Branton (1969).

hydrocarbon chains away from liquid water molecules (Frank and Evans, 1945; Kauzmann, 1959). Absent *liquid* water in a frozen specimen ready to undergo FE, entropic bonding would no longer be important, and only relatively weak van der Waals forces would hold the two lipid layers together. Thus the PLP model with a common lipid bilayer in all biological membranes explains why frozen membranes are easily split in two, while cleavage along a central plane in all membranes observed in FE provided strong new evidence for the interior location of lipids postulated by the PLP model.

Because FE produces expansive three-dimensional interior views that follow membrane contours, investigators were for the first time able to see differentiated assemblies and transmembrane proteins that could account for active transport and other functions. The appearance of very smooth, particle-free fracture faces in myelin membranes is consistent with myelin's role as a metabolically inert insulator around nerve axons (Branton, 1967), whereas the fracture faces of most of the other membrane systems with known metabolic functions—such as the cell membrane (Branton and Moor, 1964), the nuclear membranes (Branton, 1966), chloroplast lamellae (Branton and Park, 1967), sarcoplasmic reticulum (Baskin and Deamer, 1969), or retinal rod outer segments (Clark and Branton, 1968)—all exhibit fracture faces studded with different sizes and densities of particles or ridge-like protuberances that were assumed to be active, possibly transmembrane, proteinaceous assemblies. By showing the existence of conformational differences between various membrane systems, FE not only substantiated the PLP model but also provided the first morphological evidence for the range of structural complexity within this model.

I decided to summarize my FE investigations and evidence for membrane splitting in a poster and brief oral presentation at the 1966 meeting of the American Society for Cell Biology (ASCB) in Houston. Both the poster and oral presentation showing views of many different membrane systems were among the first FE specimens ever seen by most ASCB members. The images' sharp detail and extensive three-dimensional views of membranes elicited great interest, but my interpretation of the images as showing membrane splitting met substantial skepticism.

Richard McIntosh, at that time a student at Harvard University, was one of the skeptics who approached me after my talk. After politely explaining his doubts about my FE interpretation, he hurried off to another talk. His parting words were "Dan, why don't you do an experiment?" "Hadn't I been doing experiments?," I asked myself, but quickly realized McIntosh, and probably many others, would be convinced only by a quantifiable experiment that did not depend on subjective interpretations of electron micrographs.

By the time my return flight landed in California, I had conceived such an experiment using a model membrane system. Blodgett and Langmuir's descriptions of transferring lipid monolayers on water to solid surfaces (Blodgett, 1935) were well known and led me to conclude that successive experiments with one or both monolayers of a lipid bilayer radioactively labeled should yield quantitative evidence about where cleavage occurred when fractured in the frozen state. I sketched out how a ^{14}C-labeled lipid bilayer on a glass slide could be frozen and fractured while in contact with a buffer solution.

Because I had never seen anyone actually form a lipid monolayer on water or transfer the monolayer to a solid substrate, I asked colleagues and postdocs in other research groups whether anyone had done such experiments. David Deamer, a new postdoc in a neighboring lab, soon identified himself as the person I was seeking. Deamer was interested in the bilayer experiment I proposed and agreed to demonstrate layer formation and help me get started. As it happened, our experiments proceeded at such a rapid pace that we worked together until their completion.

Using ^{14}C-labeled stearic acid, a toy jack, and other improvised equipment, we quickly but conclusively demonstrated that frozen stearate bilayers were cleaved apart in the plane between their hydrocarbon tails (Deamer and Branton, 1967). At Dave's suggestion, we went on to measure the half-times of stearate molecule exchange between the two layers before freezing—the first demonstration of lipid "flip-flop." A stearate bilayer is, of course, not a biological membrane, but it is similar in that it has an extensive central hydrophobic region and surface hydrophilic regions.

The publication of our stearate model membrane results demonstrating the ease with which the hydrophobic bonds that stabilize a bilayer are split apart convinced most researchers that FE does indeed reveal the internal faces of split biological membranes. Later evidence that the particles seen on the freeze-fractured membrane faces can be attributed to transmembrane proteins (Yu and Branton, 1976) and the growing realization of the important role hydrophobic interactions play in determining membrane protein folding (Singer, 1971) established that FE can be a morphologically important extension of biochemical approaches to fractionation and separation of membrane components.

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