Freeze-Fracture Cytochemistry in Cell Biology

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Abstract

The term freeze-fracture cytochemistry embraces a series of techniques which share the goal of chemical identification of the structural components viewed in freeze-fracture replicas. As one of the major features of freeze fracture is its ability to provide planar views of membranes, a major emphasis in freeze-fracture cytochemistry is to identify integral membrane proteins, study their spatial organization in the
membrane plane, and examine their role in dynamic cellular processes. Effective techniques in freeze-fracture cytochemistry, of wide application in cell biology, are now available. These include fracture-label, label fracture, and the freeze-fracture replica immunolabeling technique (FRIL). In fracture-label, samples are frozen and fractured, thawed for labeling, and finally processed for viewing either by critical-point drying and platinum–carbon replication or by thin-section electron microscopy. Label-fracture involves immunogold labeling a cell suspension, processing as for standard freeze-fracture replication, and then examining the replica without removal of the cellular components. Of greatest versatility, however, is the FRIL technique, in which samples are frozen, fractured, and replicated with platinum–carbon as in standard freeze fracture, and then carefully treated with sodium dodecylsulphate (SDS) to remove all the biological material except a fine layer of molecules attached to the replica itself. Immunogold labeling of these molecules permits the distribution of identified components to be viewed superimposed upon high resolution planar views of replicated membrane structure, for both the plasma membrane and intra-cellular membranes in cells and tissues. Examples of how these techniques have contributed to our understanding of cardiovascular cell function in health and disease are discussed.

I. Introduction

Freeze-fracture electron microscopy was established as a major technique in ultrastructure research over 30 years ago. The success and credibility of the technique arose from its ability to provide compelling images of membranes that were entirely novel in their scientific content, set in the context of cellular features that were instantly recognizable from existing knowledge. The landmark paper by Moor and Mühlethaler (1963) presenting the first successful freeze-fracture micrographs of cells attracted wide interest, but interpretative uncertainties initially hindered full exploitation of the technique. From the early 1970s, however, with interpretative controversies resolved, the scene was set for the technique to flourish; and this it did over the following two decades, profoundly shaping our understanding of the structural organization of the plasma membrane, membrane-bound organelles and other components of the cell. With the supply of new discoveries diminishing, and molecular techniques in the ascendant, interest in freeze fracture declined in the 1990s, but a revival is currently taking place, stimulated by the development of effective approaches in freeze-fracture cytochemistry. This chapter aims to give a brief overview of the state-of-the-art in this field, focusing on those techniques that are currently contributing to new advances in cell biology.

An understanding of the principles and methodology of freeze-fracture cytochemistry requires a basic knowledge of how standard freeze fracture works. The utility of freeze fracture depends critically on the tendency of the fracture plane to follow a plane of weakness in the hydrophobic interior of frozen membranes, splitting them into half-membrane leaflets. This creates, at low magnification,
spectacular three-dimensional perspectives of cellular organization in which *en face*-viewed membranes are brought to the fore. At high magnification, details of membrane structure are seen at macromolecular resolution. In particular, the distribution and organization of integral membrane proteins (seen as intramembrane particles) are viewed in the membrane plane.

There are four essential steps in making a standard freeze-fracture replica: (1) rapid freezing of the specimen, (2) fracturing it at low temperature (−100 °C or lower), (3) making a replica of the newly exposed frozen surface by vacuum-deposition of platinum and carbon, and (4) cleaning the replica to remove the biological material. The replica is then examined in the transmission electron microscope. For a detailed protocol on how to carry out freeze fracture, see Severs (2007).

In routine application, the freezing step is often preceded by pretreatment with glutaraldehyde fixation and glycerol cryoprotection. Ultrarapid freezing techniques, such as copper block impact freezing and propane jet freezing, overcome the need for cryoprotection, and provide opportunities for examining specimens frozen directly from the living state and capturing rapid, dynamic cellular events (Escaig, 1982; Heuser, 1981; Müller et al., 1980). An optional step of etching, involving vacuum sublimation of ice, may be interposed between fracturing and replication to reveal the surface structure of cells and their components. One particularly effective technique for imaging surface structure is the combination of ultrarapid freezing, extended etching and low-angle rotary shadowing, a technique popularized as the “quick-freeze/deep-etch technique” (Heuser, 1981, 1989; Heuser and Salpeter, 1979).

Freeze fracture and associated techniques revolutionized the way we look at membranes. Nevertheless, without a means to identify the chemical nature of the structural components visualized, the functional role of these components all too often remained a matter of intelligent guesswork rather than firmly established fact. For this reason, the combination of cytochemistry with freeze fracture was an eagerly sought goal. The technical challenges involved in developing effective techniques in freeze-fracture cytochemistry were considerable, however, and took several decades to overcome. Ironically, with the major ultrastructural discoveries of standard freeze fracture in place, interest in freeze-fracture electron microscopy ebbed at the very time that some of the solutions to effective freeze-fracture cytochemistry were emerging. This has left something of a dearth in expertise to exploit the opportunities afforded today.

II. Basic Rationale: Solving the Problems of Combining Freeze Fracture with Cytochemistry

Originally, the combination of cytochemistry with freeze fracture was thought to be impossible because of the need to clean the replica scrupulously (e.g., with sodium hypochlorite or chromic acid) in order to remove all biological material
that would otherwise obstruct the electron beam and interfere with visualization of
detail in the replica. Such cleaning inevitably leads to loss of any label that might be
attached to the biological material before or after freeze fracture. Thus, the only
labeling procedure applied in the early days of freeze fracture involved attaching
relatively nonspecific markers to the surfaces of cells in suspension, and then
etching to expose these markers for visualization as shadowed particles on a
margin of the true surface of the membrane, adjacent to the membrane fracture
face (Pinto da Silva and Branton, 1970; Tillack and Marchesi, 1970).

During the 1980s, however, more sustained and increasingly imaginative
attempts at further development of freeze-fracture cytochemistry gathered pace
(see Severs, 1995a). An early attempt to solve the problem of losing preattached
label along with the biological material at the cleaning stage came with “the
sectioned replica technique,” in which the replica was viewed en face, together
with labeled biological material still in place, from within a thin section (Rash
et al., 1989). Perturbation of membrane structure before freezing using specific
lipid binding agents (e.g., filipin and tomatin) offered another avenue; here the
structural alterations, rather than an attached label, acted as the marker (Severs
and Robenek, 1983). This approach enjoyed a spell of popularity in the 1980s but,
with the adoption of a more critical approach to interpretation, proved to have
more restricted applications than many researchers initially anticipated (Severs,
1995b, 1997; Severs and Simons, 1983).

Significant breakthroughs came with the introduction of colloidal gold cyto-
chemistry to electron microscopy in the early 1980s. Gold particles, because of
their high electron density and small size, were quickly recognized to be ideal
markers to use in conjunction with replicas, and this stimulated experimentation
with new ideas for retaining label in such a way that it could be viewed super-
imposed on replicated structural detail. Experimental strategies for integrating
gold labeling into the freeze-fracture procedure were tried at each of the key steps
in the freeze-fracture procedure: (1) before the freezing step, (2) after fracturing,
and (3) after replication. Notable among the techniques developed were “label-
fracture” (Pinto da Silva and Kan, 1984) in category (i) and “fracture-label”
(Pinto da Silva et al., 1981a,b) in category (ii). Among the techniques in category
(iii) were “replica-staining label-fracture” (Andersson Forsman and Pinto da Silva,
1988), “replica-label-whole mount” (Rash et al., 1989) and the “SDS freeze-
fracture replica labeling technique” otherwise known as freeze-fracture replica
immunolabeling (FRIL) (Fujimoto, 1995 ). These various techniques differ in precisely how freeze fracture and cytochemistry are combined, what type of information is obtained, and which types of specimen are amenable to study. For
a detailed discussion of the full range of techniques, the reader is referred to an
earlier comprehensive review (Severs, 1995a). In this article, we will concentrate
on three techniques that have stood the test of time, “fracture-label” (category
(i)), “label-fracture” (category (ii)), and “FRIL” (category (iii)).
III. Methods

A. Freeze-Fracture Nomenclature

An understanding of these three freeze-fracture cytochemistry techniques requires a grasp of the nomenclature used for describing the aspects of the membrane viewed and accessible to label with freeze fracture and etching (Branton et al., 1975). The nomenclature is best explained by envisaging the membrane as consisting of two halves—a P half which lies adjacent to the protoplasm, and an E half which lies adjacent to the extracellular, exoplasmic, or endoplasmic space (Fig. 1). The term fracture face is reserved for the interior views of membranes exposed by freeze fracturing, while the term surface is used for the true, natural surfaces of the membrane that may potentially be exposed by etching. The fracture face of the P half is thus termed the P face, while that of the E half is termed the E face. The true surfaces of the membrane are correspondingly designated the P surface and the E surface, respectively. When applying the nomenclature to intracellular membranes, the term “P” encompasses cytoplasm, nucleoplasm, the matrix of mitochondria, and the stroma of chloroplasts, while the term “E” is used to designate the spaces between inner and outer membranes of all double membrane-bound organelles (nucleus, mitochondria, and chloroplasts), and the lumina of all single-membrane organelles (Fig. 2).

Fig. 1  Nomenclature for describing the aspects of the plasma membrane revealed by freeze fracture and etching. The membrane comprises a lipid bilayer with intercalated proteins. The half-membrane leaflet adjacent to the extracellular space is termed the E half; that adjacent to the protoplasm is termed the P half. The term fracture face is reserved for the interior views of membranes exposed by freeze fracturing, while the term surface is used for the true, natural surfaces of the membrane that may potentially be exposed by etching. The fracture face of the P half is thus termed the P face (or PF), while that of the E half is termed the E face (or EF). The true surfaces of the membrane are correspondingly designated the P surface and the E surface (PS and ES), respectively.
B. Fracture-Label

In “fracture-label,” cytochemical labeling is done immediately after samples have been manually freeze fractured under liquid nitrogen and thawed. This exposes for labeling the membrane halves created by freeze fracture. Fracturing the sample allows access of the label to these membrane halves, and other components, within tissue samples and cells. The labeled specimen may be examined by thin sectioning (Pinto da Silva et al., 1981b) or as a replica (Pinto da Silva et al., 1981a).

The basic procedure for fracture-label is straightforward (Pinto da Silva et al., 1986). Tissue pieces or cell pellets (cross-linked in a gel of bovine serum albumin)
are aldehyde-fixed and rapidly frozen. Fracturing under liquid nitrogen is routinely done by repeatedly crushing samples to give multiple fractures or by using a blade for directed fracturing of individual specimens. The fractured pieces are then thawed in the presence of aldehyde fixative, rinsed and blocked, and the cytochemical procedure of choice carried out.

For the replica approach, the labeled samples are critical point dried or freeze dried, and a platinum-carbon replica of the fractured surface prepared at room temperature. This step can be done using a standard vacuum evaporator if a freeze-fracture machine is not available. The platinum–carbon shadowing results in the gold marker particles being partially embedded in the replica. Because the shadowed platinum and carbon of the replica come into direct contact with the gold, the replicas are carefully cleaned using sodium hypochlorite rather than chromic acid so that the biological material is removed without dislodging the gold that is directly attached to the replica. For the thin-section approach, the samples are simply embedded by standard procedures, and sections cut at right angles to the plane of fracture.

A drawback of fracture-label is that exposure to aqueous media at the thawing stage leads to reorganization of the fractured half-membrane leaflets into a discontinuous bilayer. Underlying cytoplasmic or extracellular components are exposed for labeling in addition to epitopes of membrane proteins partitioning with a given membrane half. Aldehyde prefixation is essential to minimize these structural changes but frequently has an adverse effect on epitope preservation and hence ability to label the sample. Glutaraldehyde was extensively used in the original protocols in which lectins and other cytochemical methods not involving antibodies were applied. Owing to the deleterious effects of this fixative on epitope preservation, however, formaldehyde fixatives (freshly prepared from paraformaldehyde) are normally employed when immunogold labeling is to be undertaken. This means that ultrastructural preservation is likely to be less than ideal. A useful compromise can be to include a trace of glutaraldehyde (0.05–0.3%) with the formaldehyde fixative, but the efficacy of this approach will depend on the specific antigens to be detected and the antibodies available.

Another limitation of fracture-label is that because samples are thawed and dried before replication, the planar views of membranes have a ruffled appearance; the crisp, smoothly contoured and highly detailed faces familiar from standard freeze fracture are lost. Despite these drawbacks, fracture-label has proven to be a useful technique for analyzing the spatial organization of peripheral proteins at the cytoplasmic interface of the membrane, and has the merit that it can be done in laboratories that do not have a freeze-fracture machine.

C. Label-Fracture

The label-fracture technique provided the first really effective solution to the problem of how to retain gold markers for viewing in conjunction with high-quality freeze-fracture replica details of membranes (Kan and Pinto da Silva, 1989; Pinto da Silva, 1989; Pinto da Silva and Kan, 1984). An explanatory
Diagram and summary protocol is given in Fig. 3. In brief, a suspension of cells is immunogold labeled and then freeze-fractured and replicated in the standard manner. Instead of cleaning the replica, it is simply rinsed in distilled water and examined with the cells that have been fractured still attached. Where a cell is convexly fractured, the bulk of the cellular remnants completely obstructs the electron beam, preventing visualization of any detail. However, where a cell is concavely fractured, all that remains attached to the replica is the E half of the membrane together with the label attached to the E surface. A half-membrane leaflet is so thin when viewed en face that there is little interference with visibility of the replicated detail. Thus, label attached to the E surface of the half-membrane leaflet is seen superimposed upon a standard E face view of the membrane’s fracture face.

The images produced by label fracture are stunning, but the limitations of the technique are that it is restricted for use with cells in suspension or the luminal portions of the membranes of tissues, and only the E face of the plasma membrane can be viewed structurally in combination only with labeling of the E surface; components of the P half of the membrane remain inaccessible to study.

Nevertheless, the realization that stabilizing fractured membrane leaflets attached to the replica could be used as a vehicle to view label in conjunction with the usual detail visible in a standard freeze-fracture replica stimulated and underpinned subsequent development in techniques. Apart from the extension of this concept in “fracture flip” (giving extended replica views of labeled membrane surfaces (Pinto da Silva et al., 1989) and dual replica techniques (“simulcast” (Ru-Long and Pinto da Silva, 1990) and “composite replica technique” (Coleman and Wade, 1989) where surface label is combined with replicas of both the surface and the membrane fracture face), it provided the foundation for FRIL (Fujimoto, 1995), which has become the preeminent technique in freeze-fracture cytochemistry today.

D. Freeze-Fracture Replica Immunolabeling

In the FRIL technique, invented by the late Kazushi Fujimoto, conventional freeze-fracture replicas are first prepared; the biological material is then dissociated using sodium dodecylsulphate (SDS) (Fujimoto, 1995, 1997). The SDS removes the bulk of the biological material, leaving a single lipid monolayer and associated integral and surface proteins adherent to the replica. As in label-fracture, this remaining layer is so thin that it does not obstruct the electron beam. The proteins (or less commonly, the lipids) are then localized by immunogold labeling. This reveals their spatial distribution superimposed upon a standard planar freeze-fracture view of the membrane interior (Fig. 4). Unlike “label-fracture” which is restricted to E-surface labeling of cells in suspension, both membrane halves are accessible to labeling. Moreover, because samples are freeze fractured prior to the cytochemistry step, target epitopes deep within tissues are rendered accessible for labeling.
1. Cells in suspension are immuno-gold labeled

2. Labeled cells are frozen and fractured

3. Platinum-carbon replica is made

4. Replica examined with fractured cell remnants still attached

**Fig. 3** The key steps in label-fracture. A suspension of cells is immunogold labeled, rapidly frozen and processed for standard freeze fracture (1). The fracture may split the plasma membrane by traveling upwards and over the cell (revealing the P face), or downwards and under the cell (revealing the E face); alternatively, it may cross-fracture the cell (2). A platinum–carbon replica of the frozen, fractured surface is made, as in conventional freeze fracture (3). The replicated specimen is rinsed in distilled water and the replica, with attached cell fragments, examined in the electron microscope (4). Where cells are convexly fractured or cross fractured, the mass of cellular material attached to the replica completely obstructs the electron beam, so no structure is visible (X). However, where cells are concavely fractured, the electron beam penetrates the very thin membrane monolayer, so that the gold label on the E surface is viewed superimposed upon an E-face replica view of the membrane.
1. Frozen cell

2. Freeze-fracture splits membrane

3. Replication preserves fracture face structure

4. SDS removes cellular material leaving surface membrane components attached to the replica

* Epitopes available for labeling

**Fig. 4** The key steps in FRIL. Tissue or cell samples are frozen and fractured (1 and 2), and then replicated with platinum–carbon (3), as in the standard freeze-fracture technique. The replicated specimen is treated judiciously with SDS to remove the cellular components apart from those attached to the replica. The cellular component of choice is then immunogold labeled. On examination in the electron microscope, the fine layer of cellular components is essentially transparent to the electron beam; the electron dense gold label is clearly visible against the replica, marking the target molecule in the membrane plane.
For the SDS to work, glutaraldehyde prefixation has to be avoided. Thus, in the original technique, samples are prepared by ultrarapid freezing. An alternative is to give a brief treatment in glycerol (with no glutaraldehyde fixation beforehand) and then use standard rapid freezing techniques. Typical preparation conditions involve treatment of the replicated specimens in Tris-buffered 5% SDS (with sucrose), pH 8.3, overnight at room temperature followed by thorough washing in PBS and blocking with 1% BSA before immunogold labeling.

More than any other technique, FRIL has meant that freeze-fracture cytochemistry has come of age. Whether applied to tissues or cell suspensions, FRIL gives superb high-resolution images in which structural and compositional information are combined. The scope of scientific information is unique to the technique, and hence its application is currently having a substantial impact in solving questions in cell biology that have hitherto been impossible to address with other ultrastructural, cell biological or molecular approaches.

### IV. Discussion: Impact on Topical Questions in Cell Biology

As an illustration of how freeze-fracture cytochemistry has recently contributed to advances in cell biology, we will look at two areas of research, the spatial organization of plasma membrane proteins in cardiovascular cells, and the role of lipid droplets and their associated proteins.

#### A. Spatial Organization of Proteins in the Plasma Membrane

The cardiac muscle cell is uniquely specialized to contract constantly, without tiring, 3 billion times or more in an average human life-span. A detailed picture of the membrane organization of the cardiac muscle cell—to which freeze-fracture cytochemistry has made major contributions—underpins our understanding of the exquisite machinery through which individual cellular contractions are harnessed to create the heart beat.

The plasma membrane of the cardiac muscle cell is seen in conventional freeze-fracture replicas to be studded with abundant, scattered intramembrane particles, typically 3–10 nm in diameter (Fig. 5A and B). These particles represent the complement of specific channels, transporters, and receptors that endow the plasma membrane of the cardiac muscle cell with its unique electrical, transport, and signal detection/transduction properties. Upon depolarization, influx of calcium through L-type calcium channels in the plasma membrane triggers the opening of calcium release channels in the junctional sarcoplasmic reticulum (SR) membrane, resulting in a major release of calcium into the cytoplasm that triggers myofibril contraction (calcium-induced calcium release). Label-fracture demonstrates that L-type calcium channels are organized in discrete clusters in the plasma membrane, facing underlying junctional SR cisternae (Fig. 5C and D) (Gathercole et al., 2000; Takagishi et al., 1997). Such close spatial apposition of L-type calcium
Fig. 5  Cardiac myocyte plasma membrane; structure and localization of L-type Ca channels by label fracture. (A) Planar freeze-fracture view of the plasma membrane (E-face), showing regular arrays of transverse tubule openings (T), and smaller vesicular structures, the caveolae (c). At the site of the transverse tubule openings, the plasma membrane curves upwards; before freeze-fracture, these were finger-like extensions of the plasma membrane projecting upwards, at right angles to the plane of the page. (B) High magnification freeze-fracture views disclose a heterogeneous collection of particles (3–10 nm in diameter) which represent the integral proteins of the membrane. (C) L-type calcium channels are shown by label-fracture to be aggregated in the plasma membrane (encircled). These clusters of channels lie adjacent to sacs of junctional sarcoplasmic reticulum in the cytoplasm (D), enabling the calcium-induced calcium release process that triggers contraction. The example in (D) shows a junctional sarcoplasmic reticulum sac (asterisk) apposed to transverse tubule membrane (T). The junctional SR is continuous with the free SR network (sr) that surrounds the myofibril along the length of the sarcomere. Scale bars: (A) 1 μm; (B) and (C) 100 nm; (D) 200 nm. From Severs BioEssays 22: 188–199 (2000).
channels (in both the peripheral plasma membrane and transverse tubules of the myocyte) to calcium release channels in the junctional SR facilitates optimal coupling of plasma membrane Ca\textsuperscript{2+} influx to SR Ca\textsuperscript{2+} release into the cytoplasm.

The plasma membrane of the myocyte not only serves as a vehicle to carry the electrical impulse but has to sustain the force of contraction without damage, and transmit this force both from cell to cell and laterally across the tissue. The role of fasciae adherentes junctions in cell-to-cell force transmission, and of the desmosome/intermediate filament cytoskeleton, is well established. In addition, the membrane skeleton, comprising networks of peripheral membrane proteins closely applied to the entire cytoplasmic aspect of the lateral plasma membrane, plays a key mechanical role. Freeze-fracture cytochemistry has helped unravel how these proteins are organized and interact. For example, fracture-label combined with double immunogold marking demonstrates, at the plasma membrane interface, a direct molecular interaction between the carboxyl-terminal domains of dystrophin (a peripheral membrane protein of the membrane skeleton) and \(\beta\)-dystroglycan (an integral membrane protein which in turn binds to laminin via \(\alpha\)-dystroglycan on the extracellular side of the membrane) (Fig. 6A) (Stevenson et al., 1998). Dystrophin, however, is organized independently from spectrin, despite overlapping mechanical roles (Fig. 6B) (Stevenson et al., 2005).

Coordinated contraction of the cardiac chambers requires a precisely orchestrated spread of electrical excitation from cell to cell throughout the heart. The sites of electrical coupling between individual cardiac muscle cells that mediate this process are formed by gap junctions, clusters of transmembrane channels which span the closely apposed plasma membranes of neighboring cells. Gap-junctional channels are composed of connexins, a multigene family of conserved proteins.

**Fig. 6** (A) Fracture-label demonstration of direct molecular interaction between the carboxyl-terminal domains of dystrophin and \(\beta\)-dystroglycan (pairs of arrows: large gold markers, \(\beta\)-dystroglycan; small gold, dystrophin). (B) FRIL demonstration that spectrin (labeled with 15 nm gold) is distributed independently from dystrophin (10 nm gold). Scale bars: 100 nm.
The specific connexin type or mix of connexin types is a major determinant of the functional properties of gap junctions. Three connexin types — connexin43, connexin40, and connexin45 — are differentially expressed, in various combinations and relative quantities, in different, functionally specialized subsets of cardiac myocyte (Severs et al., 2004). For example, while myocytes of the ventricle predominantly express connexin43, those of the atrioventricular node express connexin45, a connexin that forms low conductance channels, contributing to the slowing of conduction that ensures sequential contraction of atria and ventricles. A notable feature of distal Purkinje fibre myocytes, which distribute the impulse to the contractile cells of the ventricle, is the presence of high levels of connexin40, a connexin that gives high conductance channels, facilitating rapid distribution of the impulse at this stage of the cycle.

FRIL is ideally suited to exploration of the diversity of connexin expression in cardiac myocytes and other cardiovascular cells in relation to their functional properties (Severs et al., 2001; Yeh et al., 1998) (Fig. 7). The technique has similarly contributed to the diversity of connexin expression in neurones (Kamasawa et al., 2005, 2006; Nagy et al., 2004; Rash et al., 2005). A protocol for FRIL, as applied to the study of gap junctions, has been published by Dunia et al. (2001).

Fig. 7  FRIL demonstration of the co-assembly of three connexin types, Cx37 (5 nm gold, small arrows), Cx40 (10 nm gold, large arrow) and Cx43 (15 nm gold, large arrowhead) within the same gap-junctional plaque. This example comes from aortic endothelial cells. Scale bar: 100 nm.
B. Lipid Droplets

Until recently, the lipid droplet was arguably the most neglected organelle in cell biology, widely envisaged as little more than a relatively inactive intracellular storage depot for excess lipids. Recent research, to which FRIL has made a crucial contribution, has completely transformed this view by overturning long-held concepts on the biogenesis, structure, dynamic nature, and functions of the lipid droplet and its associated proteins.

Structurally, the lipid droplet consists of a hydrophobic neutral lipid core (containing cholesterol esters and triacyl glycerol) enveloped by a single monolayer of phospholipids. The assembly, fusion and degradation of lipid droplets, resulting in storage and release of their lipid components, is controlled by a series of proteins, in particular lipid transport proteins, acyl-CoA synthetases, caveolins and PAT family proteins (the collective term for perilipin, adipophilin and TIP47). As a storage depot, lipid droplets not only serve as a source of cellular fuel and constituents for membrane construction, but also provide precursors for lipid signaling molecules and hormones. They are thus intimately involved in the cellular influx and efflux of lipids and in the signaling and transcriptional networks central to lipid homeostasis in health and disease. Lipoatrophy (lack of mature lipid-droplet containing adipocytes) leads to diabetes and fatty liver pathology, while lipodystrophy (abnormal fat distribution), resulting from sedentary lifestyle and excess food intake, is associated with obesity and diabetes. Moreover, lipid accumulation is a critical step in the pathogenesis of atherosclerosis which, by causing coronary heart disease, is a principal cause of death and disability throughout the world.

Understanding how lipid droplets form in the cell is thus fundamental to our knowledge of these disease conditions. The mechanism of lipid droplet formation that has gained general acceptance holds that neutral lipids accumulate within the lipid bilayer of the endoplasmic reticulum (ER) membrane from where they are budded-off, enclosed by a protein-bearing phospholipid monolayer originating from the cytoplasmic monolayer of the ER membrane, to give a cytoplasmic lipid droplet. This idea has the superficial attraction of explaining how the lipid droplet could acquire both its outer phospholipid monolayer and the proteins necessary for its function, but unfortunately required something of a leap of imagination in the capabilities of the principal imaging methodologies applied to support the idea (i.e., fluorescence confocal microscopy and immunogold label thin-section electron microscopy).

Results from FRIL refute the prevailing view on several counts (Robenek et al., 2006b). First, freeze fracture, by permitting unique three-dimensional views of the spatial relationships of membranes and organelles, demonstrates unequivocally that at sites of close association, the lipid droplet is not situated within the ER membrane, but adjacent to it (Fig. 8). Both ER membranes clearly lie external to and follow the contour of the lipid droplet, enclosing it in a manner akin to an egg-cup (the ER) holding an egg (the lipid droplet) (Fig. 8A). Freeze-fracture cytochemistry further demonstrates that the PAT family protein adipophilin is
Fig. 8  Freeze-fracture views of lipid droplets and their association with endoplasmic reticulum (ER) membrane from lipid laden macrophages (i.e., macrophages fed acetylated low density lipoprotein to induce lipid droplet formation). (A) Lipid droplet situated in a cup formed from ER membranes. Both ER membranes are visible (seen in P-face and E-face view), following upwards and over the contour of the lipid droplet from below. The lipid droplet has been convexly fractured, and lies beneath (i.e., adjacent to and not within) both ER membranes exposed. (B) Similar view to (A), but with labeling for adipophilin using the FRIL technique. Abundant gold label is visible on the ER membrane (P-face) immediately adjacent to the lipid droplet. (C) Lipid droplet seen in concave fracture. FRIL demonstrates abundant labeling for adipophilin in the outer phospholipid monolayer surrounding the lipid droplet (P-face) exposed in this view. Scale bars: 200 nm.
concentrated in prominent clusters in the P-half of the ER membrane at the site of the closely apposed lipid droplet (Fig. 8B), as well as in the lipid droplet surface apposed to the ER (Fig. 8C). Adipophilin is thus strategically placed to play a role in lipid droplet growth by facilitating lipid transfer from the ER to the droplet. The evidence from these studies indicates that lipid droplets originate and develop adjacent and external to specialized domains of the ER membrane enriched in adipophilin, not within the bilayer of the ER as previously supposed.

The prevailing view is further discredited by the spatial distribution of caveolin 1, a putative mediator of intracellular lipid transport (Robenek et al., 2003, 2004). As with other lipid droplet associated proteins, caveolin 1 is proposed to traffic to the lipid droplet from the ER membrane by the budding process. As this process involves the budding off and enclosure of the lipid accumulation in the P half of the ER membrane, the model requires that lipid droplet proteins such as caveolin originate from this membrane leaflet. Contrary to this prediction, FRIL demonstrates the caveolin 1 is situated in the E-half of the ER membrane. As this membrane half does not participate in the proposed mechanism of lipid droplet production, caveolin is actually in a location in the ER membrane that would make it impossible to gain access to the forming lipid droplet.

Fig. 9 FRIL images demonstrating that PAT family and other proteins are distributed not only at the lipid droplet surface but also in the cross-fractured lipid droplet core. These examples come from adipocytes and show labeling for perilipin and caveolin. (A) Example in which perilipin label is seen predominantly in the outer phospholipid monolayer (P face) of the lipid droplet. (B) Example of a lipid droplet in which perilipin label is seen throughout the core. (C) Example in which abundant caveolin label (18 nm gold) is seen in the core, and perilipin label (12 nm gold) is predominantly in the phospholipid monolayer (seen in P-face view). Scale bars: 200 nm.
The prevailing wisdom holds that caveolin and the PAT family proteins are confined exclusively to the droplet surface and that the latter are specific to lipid droplets and not present in any other organelle or membrane system of the cell. FRIL, however, demonstrates that in macrophages and adipocytes (1) PAT family proteins and caveolin are distributed not only in the surface but also throughout the lipid droplet core (Fig. 9); and (2) PAT family proteins are integral components of the plasma membrane (Fig. 10) (Robenek et al., 2005a,b). Under normal culture conditions, these proteins are dispersed in the P half of the plasma membrane (Fig. 10A). Stimulation of lipid droplet formation by incubation of the cells with acetylated low-density lipoprotein leads to clustering of the PAT family proteins in raised plasma membrane domains (Fig. 10B). Fractures penetrating beneath the

![Fig. 10](image_url)

**Fig. 10** FRIL images demonstrating that PAT family proteins are present in the plasma membrane, and undergo profound changes in distribution under conditions of lipid loading. (A) View of the plasma membrane (P face) of a normal cultured adipocyte after labeling for perilipin. The perilipin is widely distributed throughout the membrane. (B) Upon lipid loading, the perilipin becomes clustered in elevated domains in the plasma membrane. (C) Fractures that penetrate beneath the plasma membrane demonstrate that lipid droplets lie beneath the elevated protein-rich domains, as seen in this example from an adipophilin-labeled lipid laden macrophage. Scale bars: 200 nm.
plasma membrane demonstrate that lipid droplets are closely apposed to these domains (Fig. 10C). A similar distribution pattern of labeling in the form of linear aggregates within the clusters is apparent in the P half of the plasma membrane and the immediately adjacent outer monolayer of the lipid droplet (Robenek et al., 2005b). The aggregation of the PAT family proteins into such assemblies may facilitate carrier-mediated lipid influx from the extracellular environment into the lipid droplet. The findings point to a common cellular mechanism of intracellular lipid loading in the macrophage as part of the pathogenesis of atherosclerosis and in the adipocyte during development of obesity.

A related area to which FRIL has shed new light is the mechanism of milk fat globule secretion (Robenek et al., 2006a). Milk fat globule formation involves the trafficking of what is essentially a lipid droplet with an outer phospholipid monolayer (termed the “secretory granule”) to the cell surface; the granule is then enveloped by a portion of plasma membrane and released from the cell as a milk fat globule. The milk fat globule thus has a lipid bilayer derived from the plasma membrane exterior to the phospholipid monolayer enclosing the neutral lipid core (Fig. 11). The molecular mechanism of the secretory process is proposed to involve

![Fig. 11](image)

**Fig. 11** Freeze-fracture view illustrating the structure of a milk fat globule secreted from a human mammary epithelial cell. The globule consists of a lipid droplet core surrounded by a phospholipid monolayer, which in turn is surrounded by a membrane bilayer derived from the plasma membrane which enwraps the droplet during secretion. These different structures are revealed as the fracture plane skips between them (bilayer seen in P-face view; phospholipid monolayer in E-face view; core cross-fractured). Scale bar: 200 nm.
formation of complexes between butyrophilin in the plasma membrane with
cytosolic xanthine oxidoreductase; the resulting complexes are then believed to
interact with adipophilin on the outer surface of the lipid droplet to enwrap the
secretory granule in plasma membrane.

The reality demonstrated from FRIL, however, is that the topological distribu-
tion of the relevant proteins makes the proposed mechanism impossible (Figs. 12
and 13). Adipophilin is actually more abundant in the plasma membrane domains
to which secretory granules are apposed in the mammary epithelial cell, and in the
bilayer surrounding the secreted milk fat globule, than in the monolayer enclosing
the lipid droplet (Fig. 12). Xanthine oxidoreductase is diffusely distributed in the
lipid droplet monolayer. Importantly, butyrophilin in the plasma membrane is
concentrated in a network of ridges that tightly appose and match the protein’s

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**Fig. 12** FRIL images demonstrating the distribution of adipophilin and butyrophilin in the secreted
milk fat globule. (A) Abundant adipophilin label is seen in the bilayer P face. (B) Double labeling reveals
that adipophilin (18 nm gold) is also abundant in the phospholipid monolayer P face, while butyrophilin
(12 nm gold) is present both in the bilayer E face and phospholipid monolayer P face. Scale bar: 200 nm.
distribution in the monolayer of the lipid droplet (Fig. 13). While adipophilin-rich domains in plasma membrane may well be linked to secretory granule positioning at the cell surface, butyrophilin–butyrophilin interactions between monolayer and bilayer mediate envelopment of the granule by the plasma membrane and its release from the cell (Robenek et al., 2006a).

**Fig. 13** FRIL image demonstrating pattern of butyrophilin labeling in concavely fractured milk fat globule. Note the network pattern of butyrophilin distribution in both the bilayer E face and phospholipid monolayer P face. This mirror distribution suggests a role for butyrophilin-butyrophilin interactions in the secretory process. Scale bar: 200 nm.

V. Concluding Comment

The examples discussed illustrate the recent impact of freeze-fracture cytochemistry in advancing our understanding of selected aspects of cardiovascular cell biology. The information that this approach provides is unique; without its wider application, substantial gaps in our knowledge of how membranes function will remain. Further exploitation of freeze-fracture cytochemistry may be expected as the scope and power of this technique become more widely appreciated.
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References


11. Freeze-Fracture Cytochemistry


