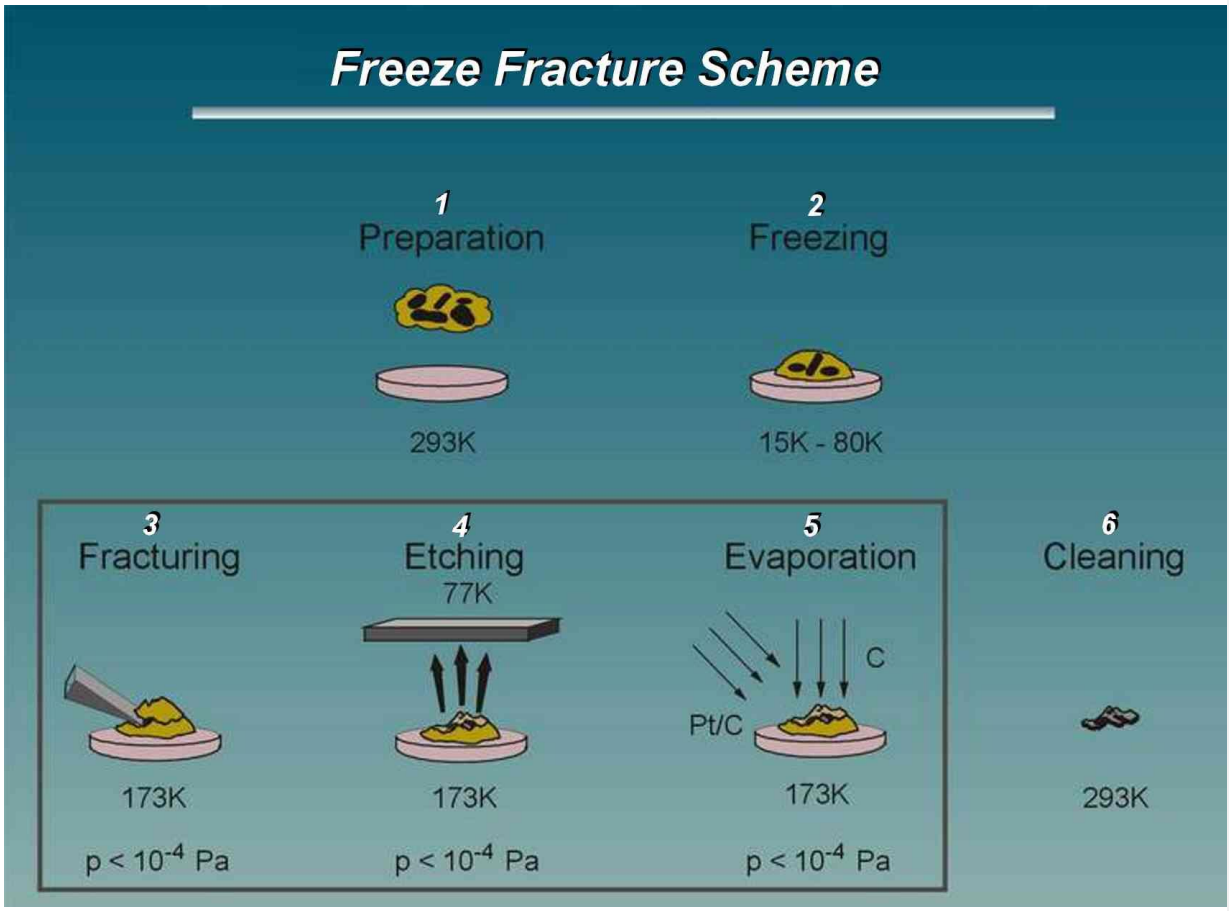


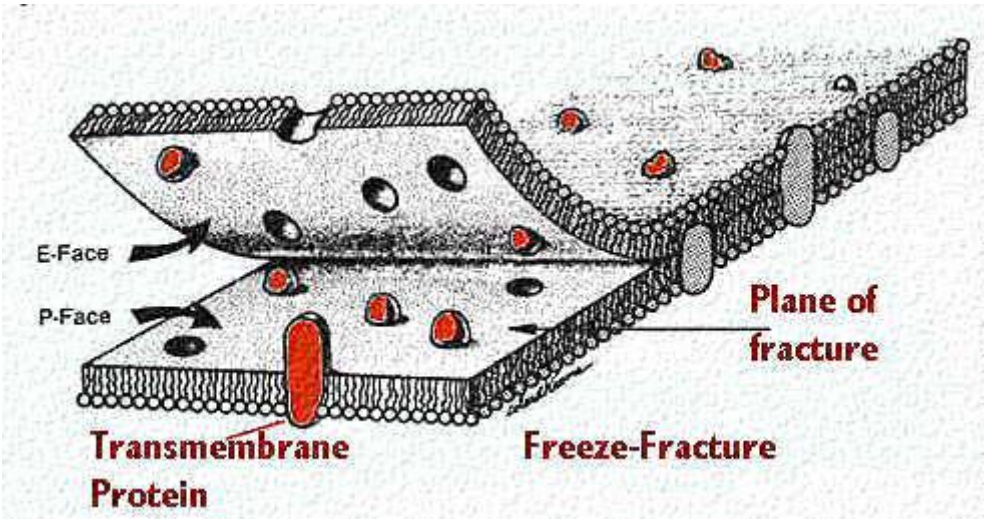
The specimen must be prepared under controlled conditions to a small size of approximately 1 mm^3 (1) and placed on a holder. Liquid samples can be placed between two thin metall sheets forming a sandwich. After freezing (Jet Freezing, Plunge Freezing, High Pressure Freezing), the frozen sample is transferred into a high vacuum freeze fracture device and kept at a temperature range between -100°C and -150°C. The sample is fractured with a microtome or alternatively in a double replica holder (3). Optionally, the fractured surface can be etched (4) by sublimation of surface water layers. Deeper laying features can thus be visualized. Subsequently, a replication of the surface is performed by deposition of a platin/carbon layer (approx. 2nm) under a definite angle combined with a stabilizing carbon layer (approx. 30nm) under 90° on to the fractured surface (5). After thawing the sample must be totally removed from the heavy metal replica. In the TEM only the replica is being analysed.

An Electron microscopic view of membranes

Surface specializations

An EM view of membranes via freeze fracture/freeze etching

The freeze-fracture/freeze etch technique starts with rapid freezing of a cell. Then the frozen cells are cleaved along a fracture plane. This fracture plane is inbetween the leaflets of the lipid bilayer , as shown by this cartoon. The two fractured sections are then coated with heavy metal (etched) and a replica is made of their surfaces. This replica is then viewed in an electron microscope. One sees homogeneous regions where there was only the exposed lipid leaflet (Is the exposed surface made of polar or nonpolar groups? *This figure was modified from Bloom and Fawcett, A Textbook of Histology, Chapman and Hall, N.Y., Twelfth Edition, 1994, Figure 1-3.* Consult the section on Membrane Architecture for the answer.)

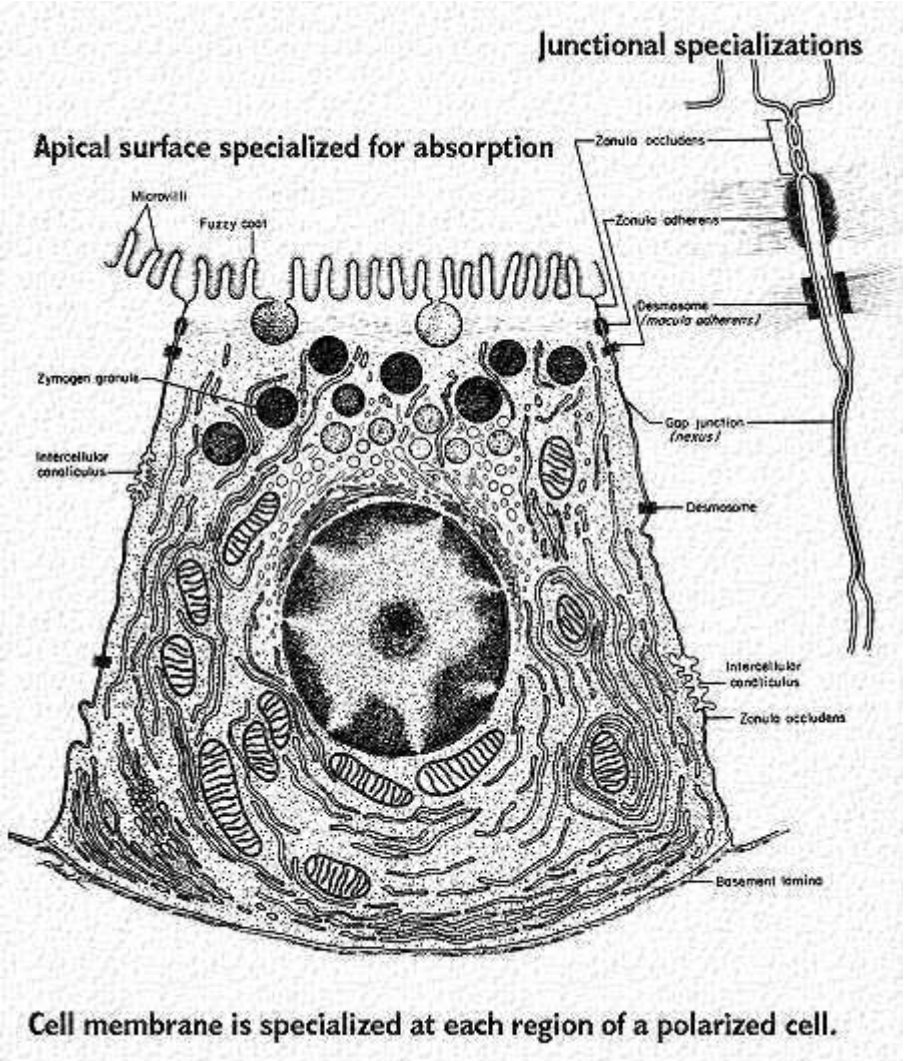


In certain areas of the cell, one also sees protrusions or bumps. These are coloured red in the cartoon. Sometimes one can see structure within the bumps themselves. These are the transmembrane proteins.

The following illustrations will show you some views of specialized regions in the membrane. The organization or structure of the transmembrane proteins can be visualized.

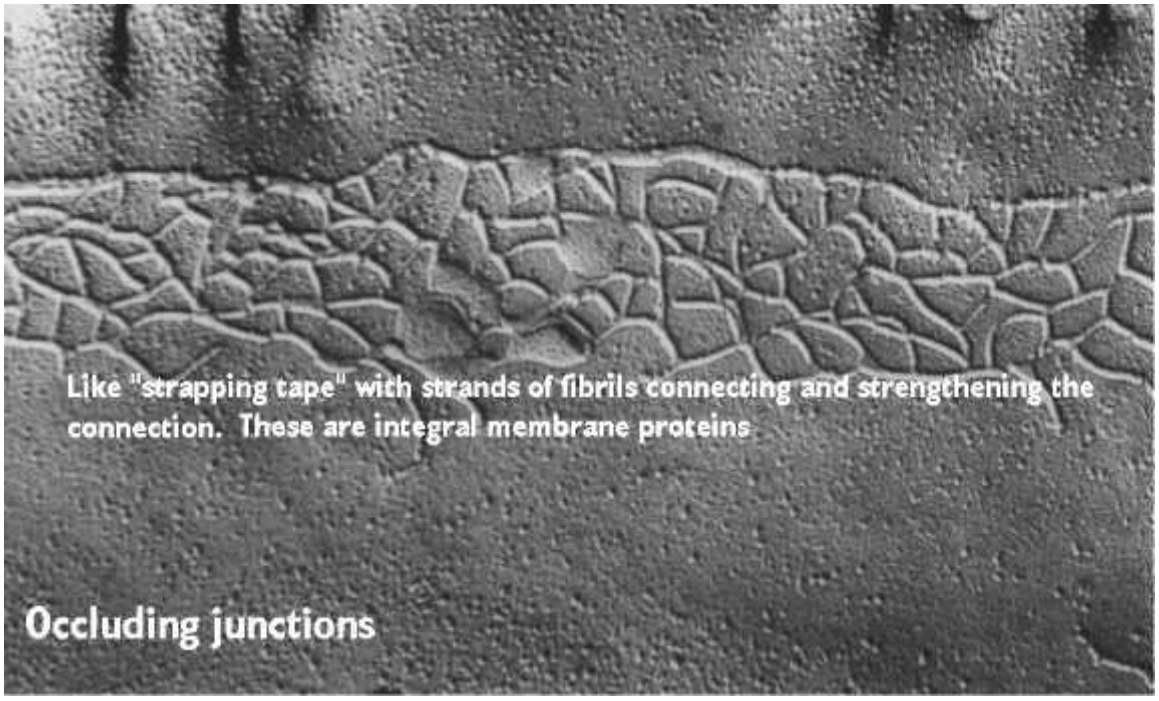
Membrane specializations: Junctions

The drawing is of a polarized cell. The top is specialized for absorption and the bottom for transfer of materials to the blood stream. The sides have specialized junctions that keep the nutrients from entering the space between the cells. For more information about the thick filaments associated with these junctions, please consult the intermediate filaments web page. *This figure was modified from Bloom and Fawcett, A Textbook of Histology, Chapman and Hall, N.Y., Twelfth Edition, 1994, Figure 2-10*



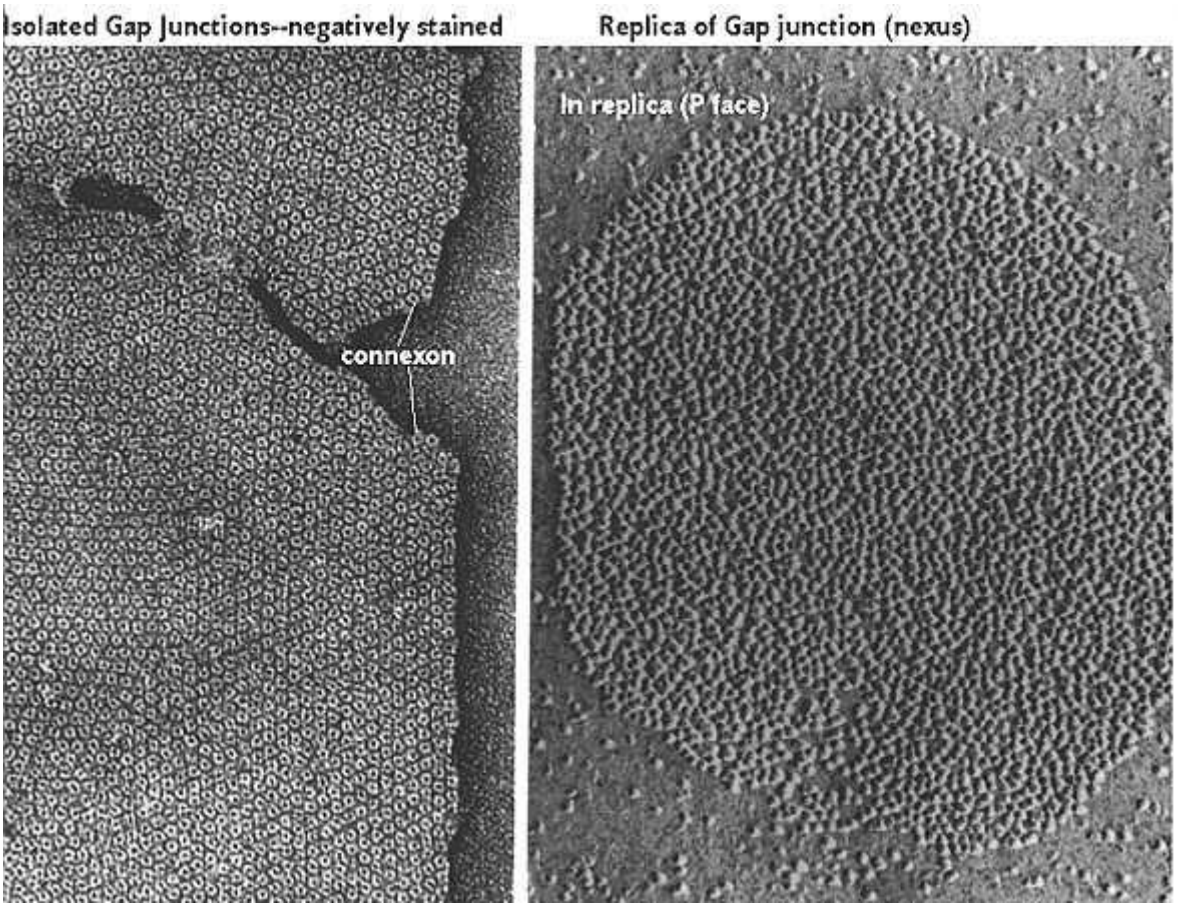
Tight Junctions

One of these is called a **tight junction or "occluding junction" (zonula occludens)**. This is shown as the top junction in the above drawing. At this site, membrane glycoproteins and associated "glue" bind the cells together like double-sided "strapping tape" The freeze-fracture/freeze etch view of this junction (shown below) illustrates the ridges in the plane of the exposed leaflet. These are the proteins that bind to the proteins from the adjacent cell. *This figure was modified from Bloom and Fawcett, A Textbook of Histology, Chapman and Hall, N.Y., Twelfth Edition, 1994, Figure 2-11.*



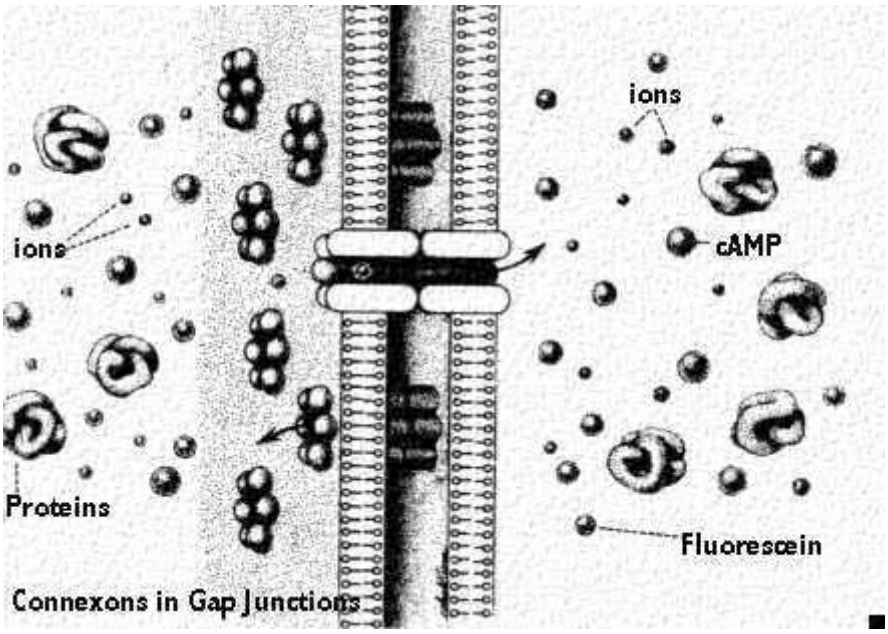
Gap Junctions

Another type of junction allows communication between cells. This type is called a **gap junction**. Small molecules or ions can pass through, as we will see by the following figures.



The above freeze-fracture /freeze etch image shows the internal view of the **gap junction** on the left. The proteins look like little donuts which reflects the fact that they are actually a channel. These proteins are "connexon" molecules. The side facing the cytoplasm (called the P face) is shown in the center panel. The region looks like aggregated lumps. Finally, the typical electron microscopic view is seen in the third panel. This shows a thin line between the two plasma membranes indicating a "gap junction". *This figure was modified from Bloom and Fawcett, A Textbook of Histology, Chapman and Hall, N.Y., Twelfth Edition, 1994, Figure 2-14.*

There are several ways to prove the cells are communicating by gap junctions. First, one can identify the connexon molecules by immunocytochemical labeling. Second, one can identify the actual junctional complex with freeze-fracture/freeze etch. To see if they are functional, however, one needs to inject one cell with a dye and watch to see if it is transferred to another cell.

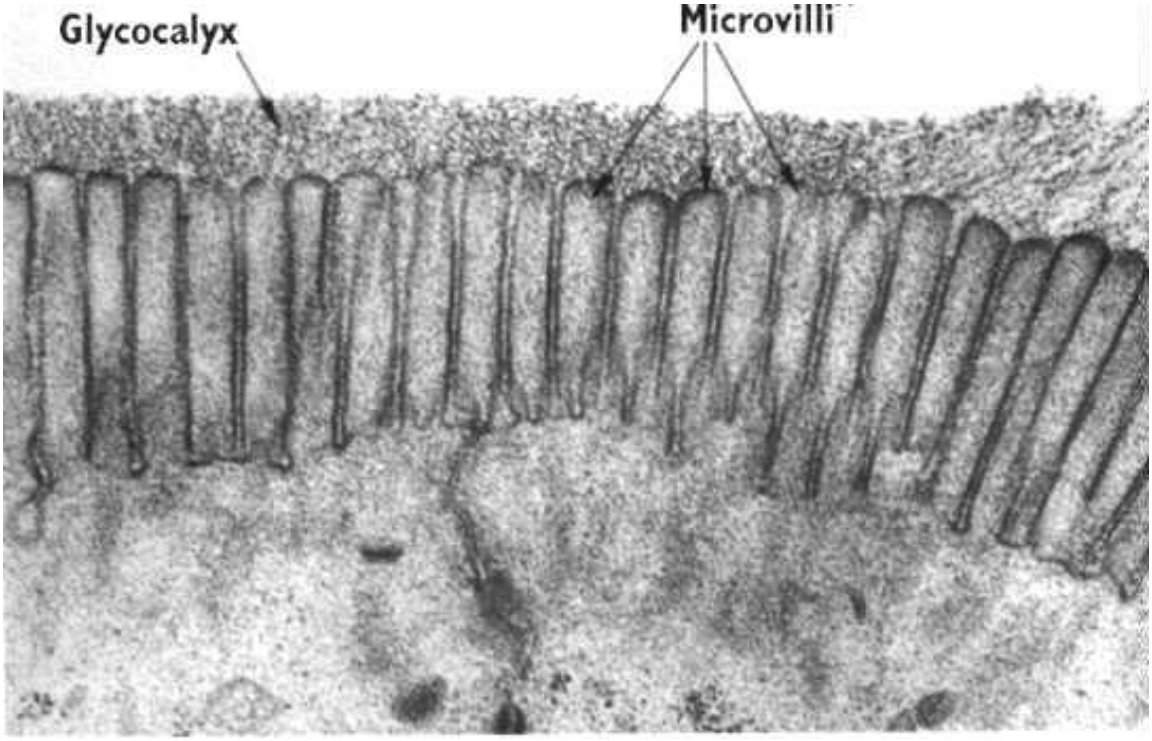


This cartoon diagrams a view of a gap junction showing molecules that can freely pass. Ions pass and in this way the cells can be electrically coupled together. Other small molecules that pass through include cyclic AMP (a second messenger) and the dye marker fluorescein. This last compound enables the scientist to study transport throught the gap junction.

This figure was modified from Bloom and Fawcett, A Textbook of Histology, Chapman and Hall, N.Y., Twelfth Edition, 1994, Figure 2-15.

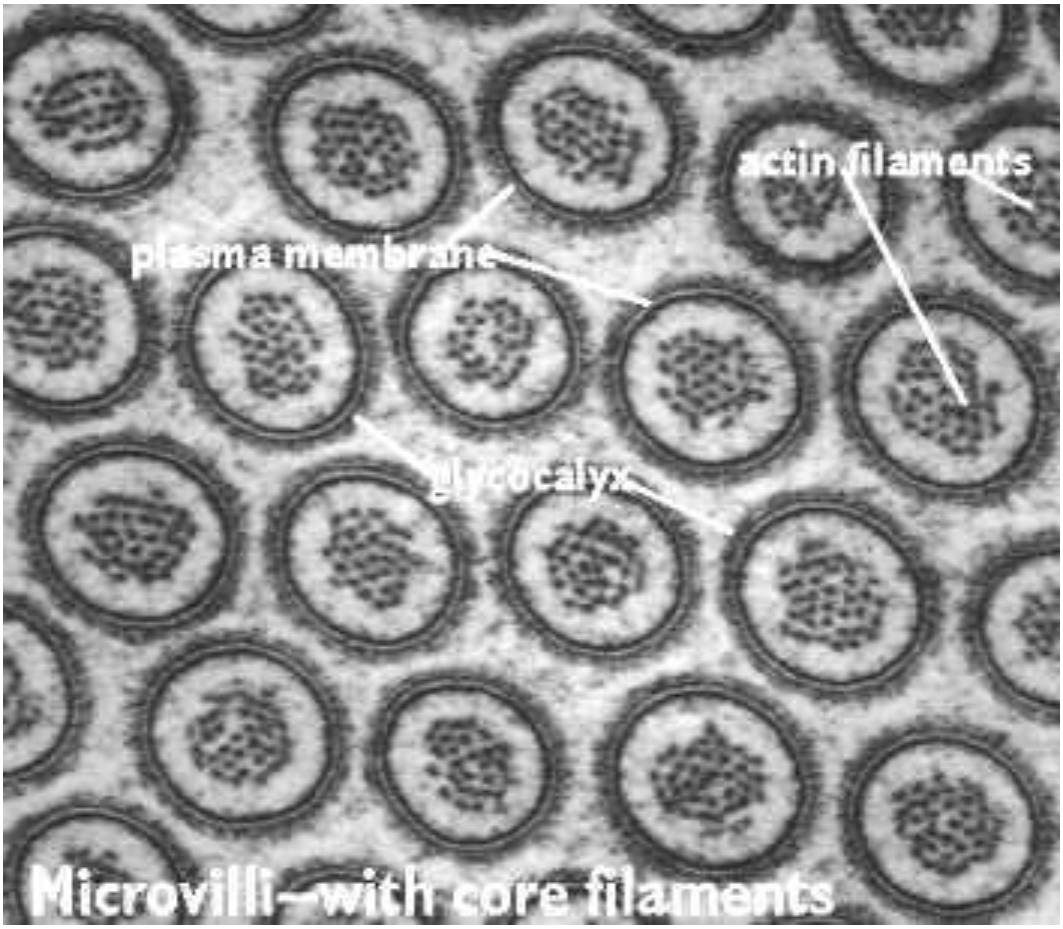
Membrane Specializations: Microvilli

The purpose of this final presentation is to introduce a surface specialization that projects from membranes called the microvillus. It is covered by a plasma membrane and encloses cytoplasm and microfilaments. Typically, microvilli are found in absorptive cells, whenever there is a need for an increase in surface area.



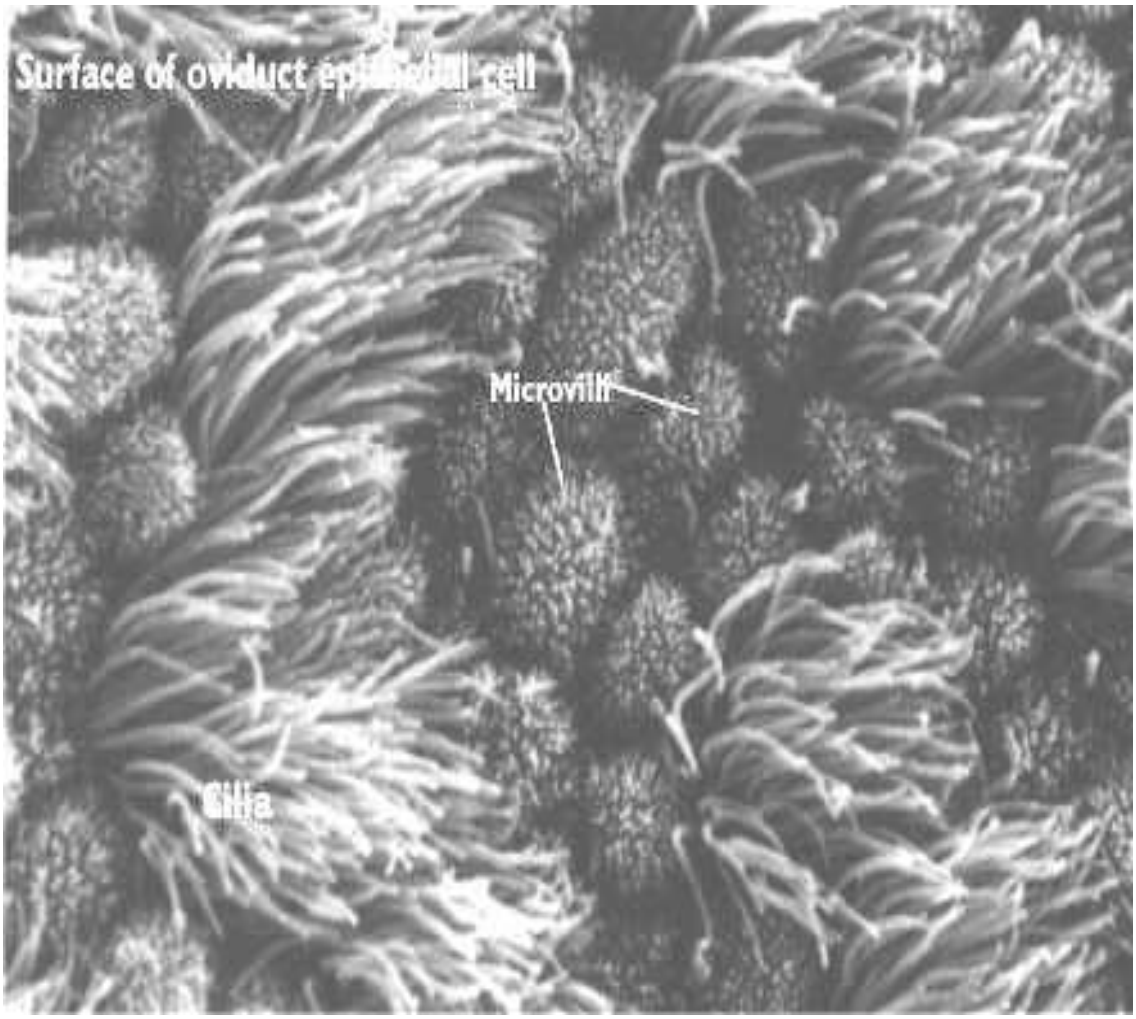
It is also covered by a glycocalyx which are peripheral glycoproteins that attach themselves to the membrane. It might be used to trap nutrients, protect against toxic subxtances, or adhere to substances needed for uptake. Enzymes used for the cell's function are stored in this region, depending on the cell type.*This figure was modified from Bloom and Fawcett, A Textbook of Histology, Chapman and Hall, N.Y., Twelfth Edition, 1994, Figure 2-17.*

The figures to the right show views of microvilli cut transversely.

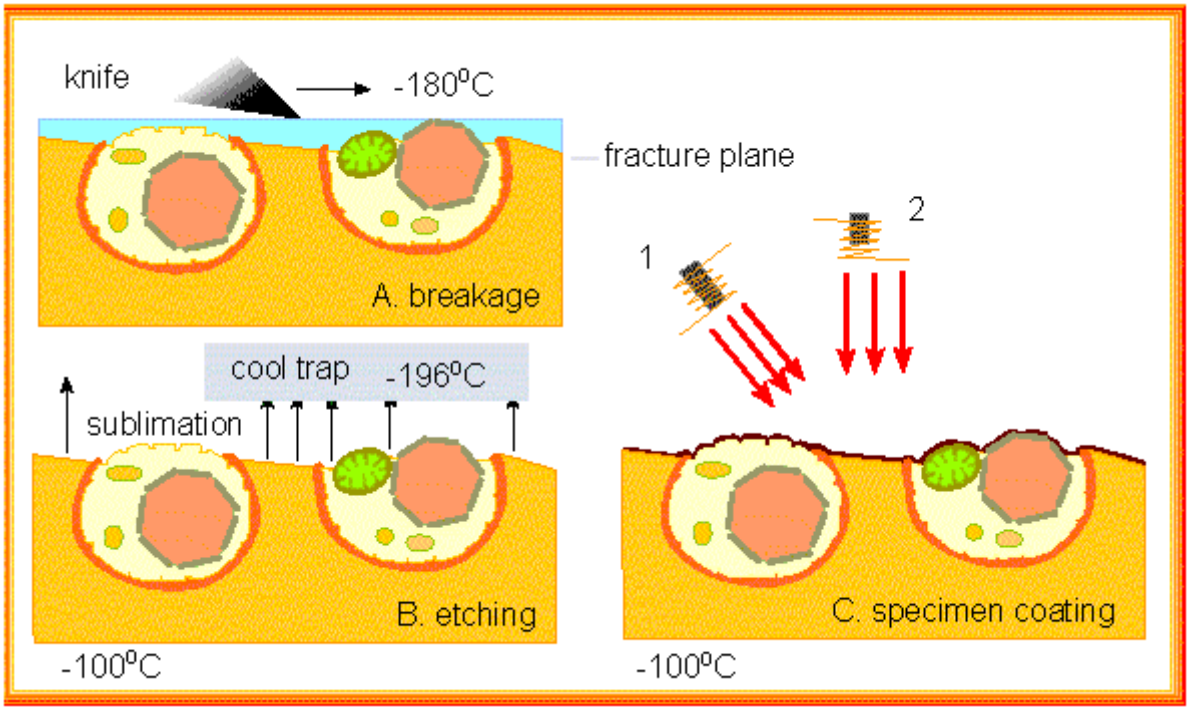


Note that the microvilli are lined with the Unit membrane . (top figure) The core of filaments may allow them to move, although such movement is not as great as that of cilia or flagella *This figure was modified from Bloom and Fawcett, A Textbook of Histology, Chapman and Hall, N.Y., Twelfth Edition, 1994, Figure 2-18*

The lower figure shows a scanning electron micrograph of the luminal surface of the oviduct. It illustrates one difference between cilia and microvilli. The longer projections are cilia, and the shorter projections are microvilli. For more information about the internal structure of cilia, consult the Cilia Web page

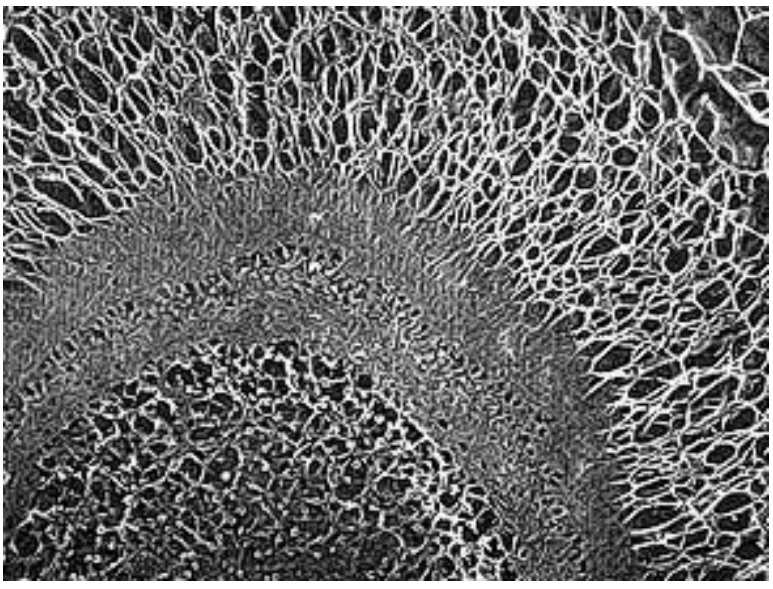


Freeze-Etching and Freeze-Fracture

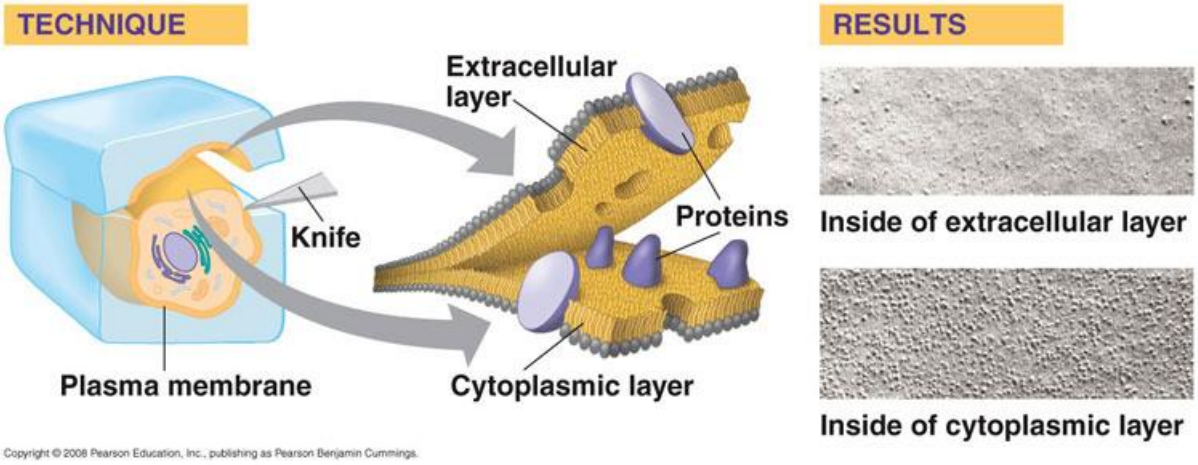


Freeze methods (like freeze-drying) offer an alternative to chemical fixation with often better preservation of the specimen's structures. Freeze-fracture and freeze-etching (H. MOOR and K. MÜHLENTHALER; University of California, Berkeley and Eidgenössische Hochschule Zürich, 1963) are well-suited for small specimen: cells and subcellular structures. The specimen is frozen and the frozen material is "broken" with the aid of a glass knife. The preparation splinters and the edges of breaking run along the membrane or between two half membranes.

Water is subsequently removed *via* freeze drying. It sublimates, i.e. it transforms out of its solid phase directly into the gas phase. This process etches the surface of the preparation. It is then coated and the metal coat is taken off and viewed in the microscope.

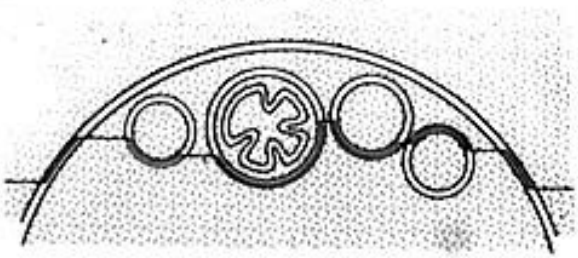


During the late 1970s, J. E. HEUSER (Washington University, St. Louis) developed the quick-freeze, deep-etch preparation of samples that avoids the crystallization of water. The (very small) specimens are frozen quickly in liquid nitrogen transferring the water into a glass-like state. This leads to a much better preservation of the sample. The method is suitable for the depiction of large molecules and molecular complexes (picture to the left). Here, too, an imprint is gained by coating. Contrary to negative staining a three-dimensional image of the specimen can be formed.

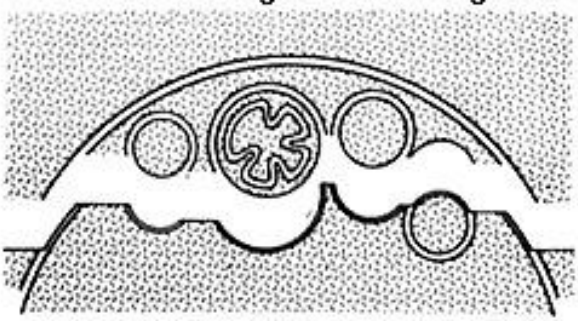


FREEZE-FRACTURE AND ETCHING

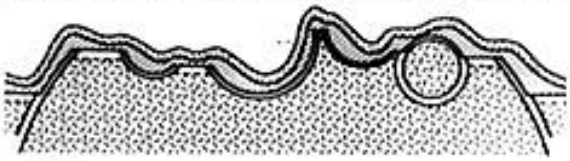
Frozen Cell



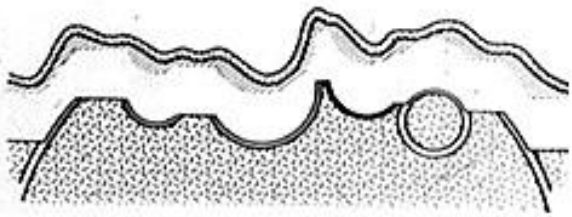
Fracturing and Etching



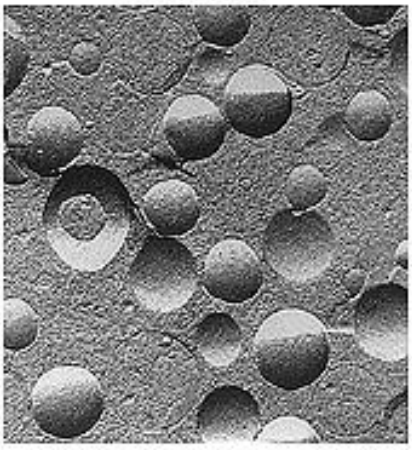
Replication of Fractured Surface



Clearing of Replica



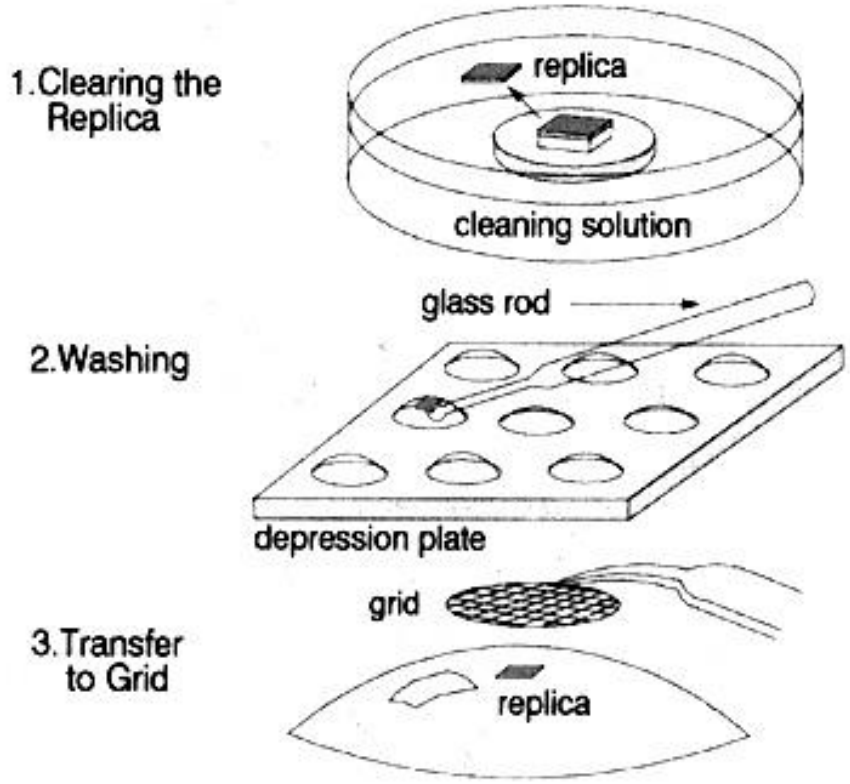
Replica of Fractured Membraneous Vacuoles



Replica of Fractured Eukaryotic Cell

Clearing the Replica

Carbon-platinum replicas are very fragile and brittle and cannot be directly manipulated without damage or breakage. After a small period of thwaing in the freeze-fracture apparatus, The fractured and coated specimen is removed and allowed to thaw at room temperature. As the underlying ice (tissue) melts, the replica is floated onto the surface of an aqueous solution of sodium hyperchlorite (bleach) which forms tiny bubbles which helps to lift the replica off the thawed tissue. The alkali also corrodes away any tissue left adhering to the replica.



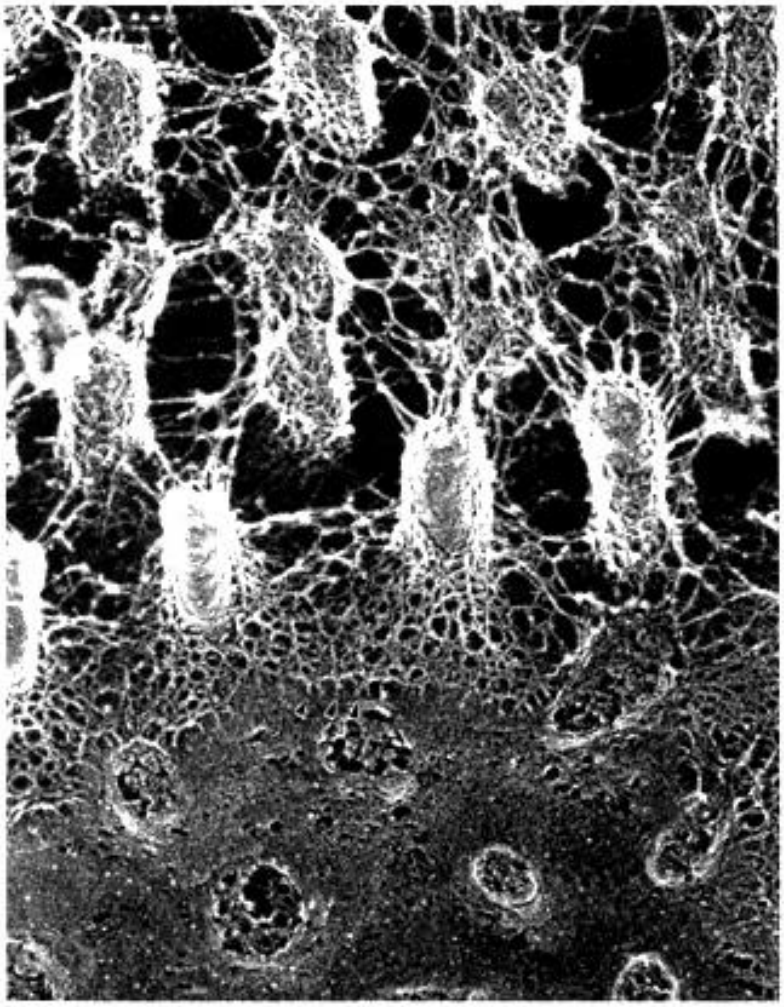
The replica is washed succesively in several changes of hypochlorite (tranferring with a smooth glass rod), the replica is picked up on a grid and examined with a transmission EM.

Cytoplasmic Formed Elements Revealed by Deep Etching

Non-membraneous organelles or formed elements such as microtubules or microfilaments must be differentiated from the general cytoplasmic background in which they lie.

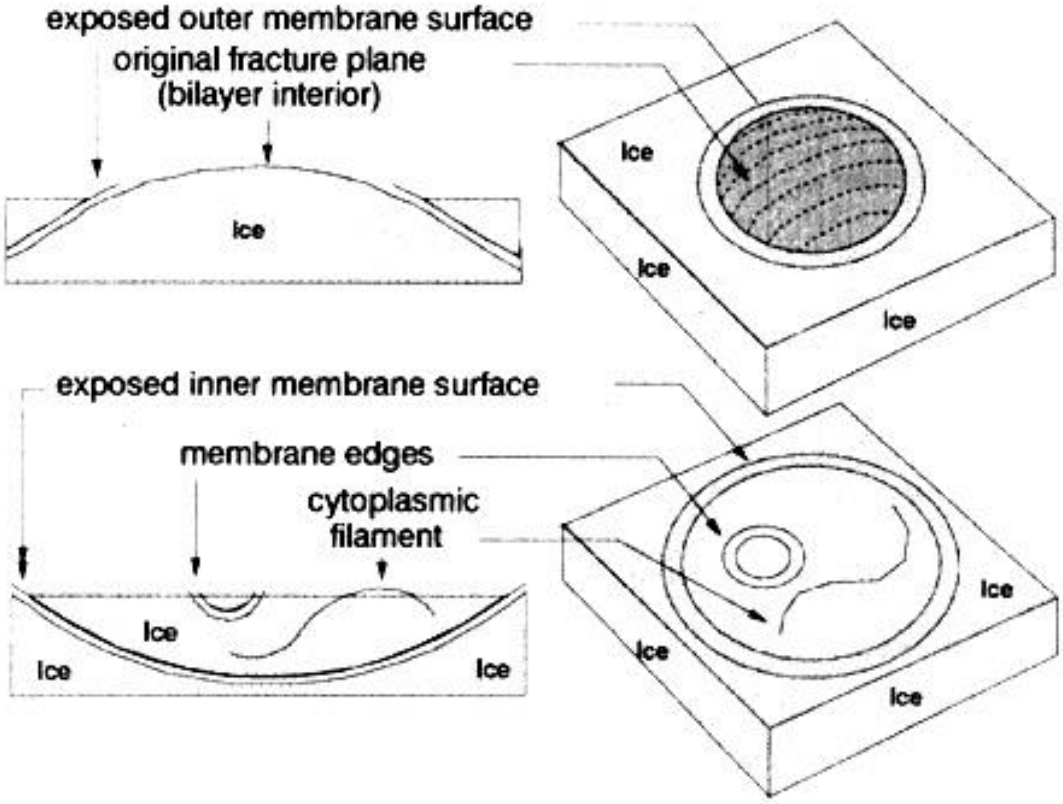
Deep-etching (sublimation of a greater amount of cell water) is an aid in differentiating some of these structures but the resulting granularity produced in the cytoplasmic background may obscure some classes of formed elements.

Deep-Etched Cell Surface with Microvilli and Microfilaments



Etching

The fractured surface is either immediately replicated or is first "etched" to remove some ice from the non-membraneous regions of the fracture face. Etching removes a layer of ice by sublimation thereby exposing true membrane surfaces embedded in ice and hidden from the cleavage surfaces. The true inner and outer surfaces of the membrane are thus exposed.



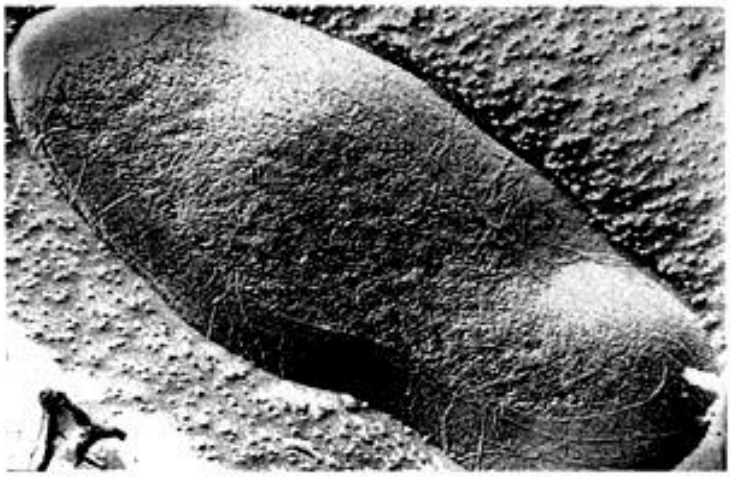
The cytoplasmic cross-fracture face will also be lowered slightly by etching so that membrane edges and other non-etchable features (cytoplasmic filaments and microtubules) will stand above it in relief.

Evidence For Split Membranes

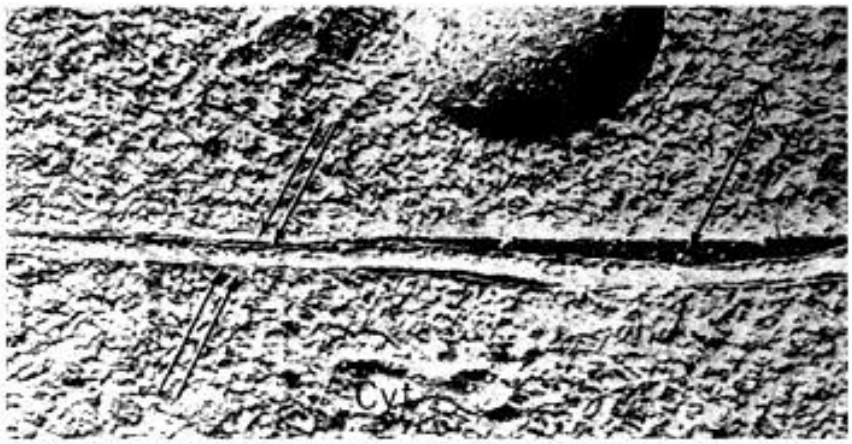
The assumtion that the two sheets (one in the replicated surface and the other in the disgarded piece are actually halves of the original membrane is supported by several lines of evidence:

The first is that neither surface is ecthable implying that they are comprised of membrane constituents and not ice.

If a recognizable marker such as fibrous actin is adhered to the cell surface prior to freezing and fracturing, it is never obsvervable on the original fractured surface. This indicates that the fracture plane does not reveal the membrane surface detail. Etching exposes the natural membrane surface and the actin that has adhered to it.



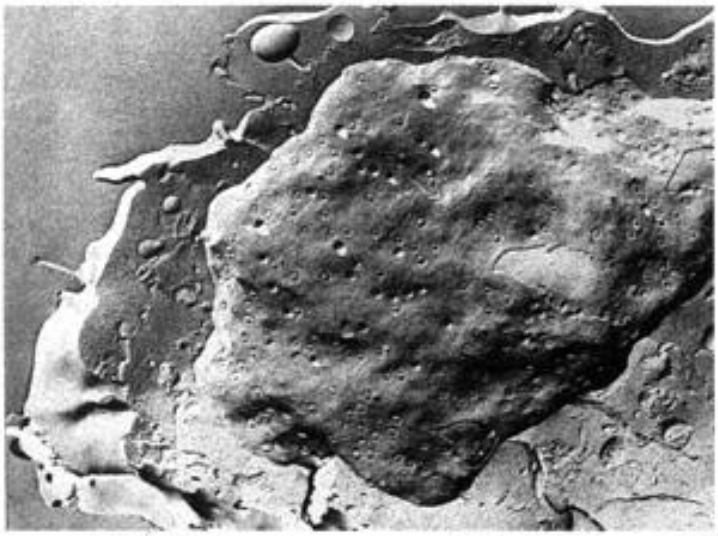
Direct evidence is also provided by instances where the fracture plane courses into an orientation normal to the plane of the membrane where the bilayer can be seen. In such cases, a continuity between the halves of the bilayer can be viewed directly.



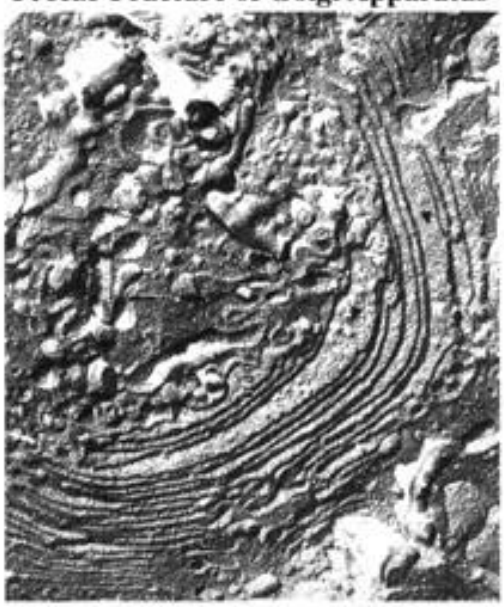
Freeze-Fracture Views of Organelles

Low temperature fracturing appears to preferentially expose extended en face views of membraneous organelles which are thus easily identified. Both face views and cross fractures of the nucleus golgi and mitochondria yield relatively unmistakable images.

Freeze-Fracture of Entire Cell Exhbiting Nucleus and Nuclear Pores



Freeze-Fracture of Golgi Apparatus



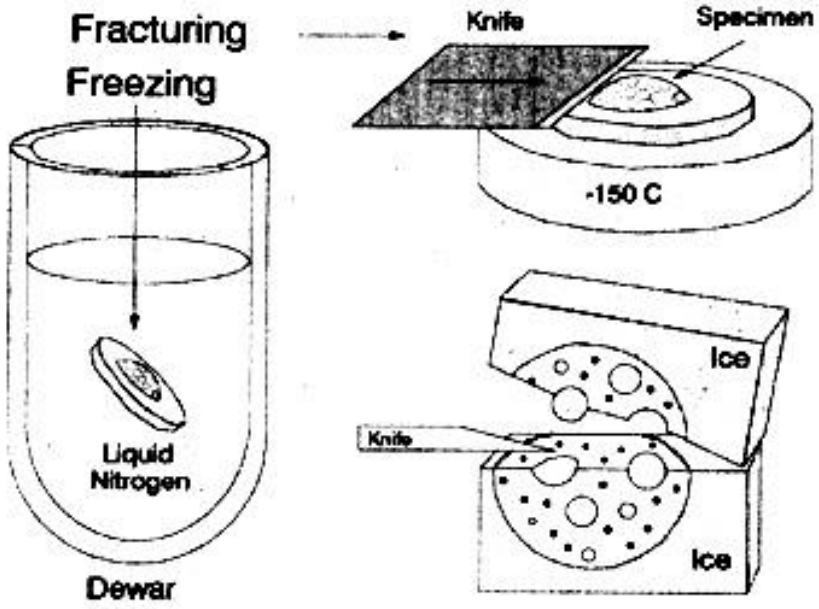
Cross-Fracture of Mitochondria

Fracturing

A freeze-fracture apparatus consists of a vacuum chamber which can be evacuated (10^{-6} Torr) and cooled to -100 C or lower. A fracturing tool (blade) is operated from outside the chamber and viewed with a binocular microscope.

A set of electrodes are positioned at 45 and 90 degree angles to the fractured surface for deposition of carbon and platinum in vacuo and the production of a metal "replica" of the surface.

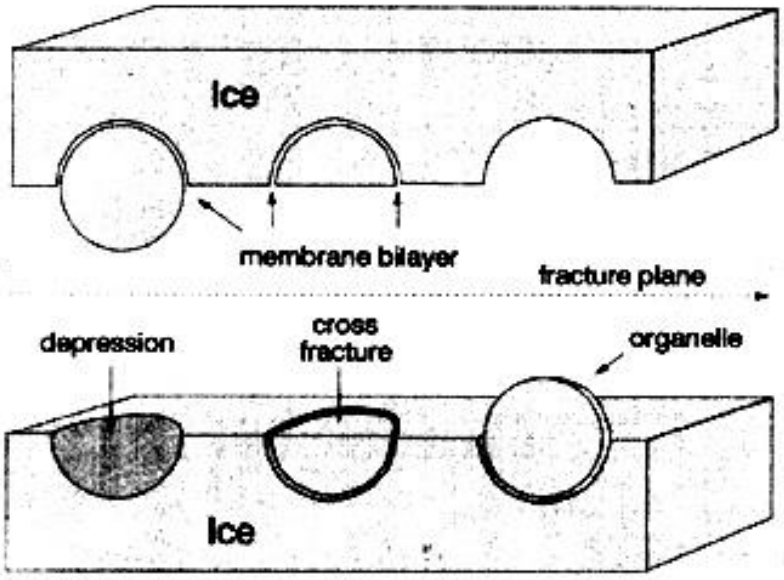
The fracturing produces a series of roughly hemispherical breaks in front of the knife edge and may course above or below or straight through whole cells and membrane systems.



The tendency of the fracture plane to course along the interior (hydrophobic) planes of membranes may be due to the different properties of the ice there which presents a weak domain.

Freeze-Fracture and Etching

Cryofixed specimens may be fractured exposing an internal surface of frozen tissue and cells. This surface is never smooth but represents an irregular relief which when replicated imparts a third dimension to ultrastructural analysis.



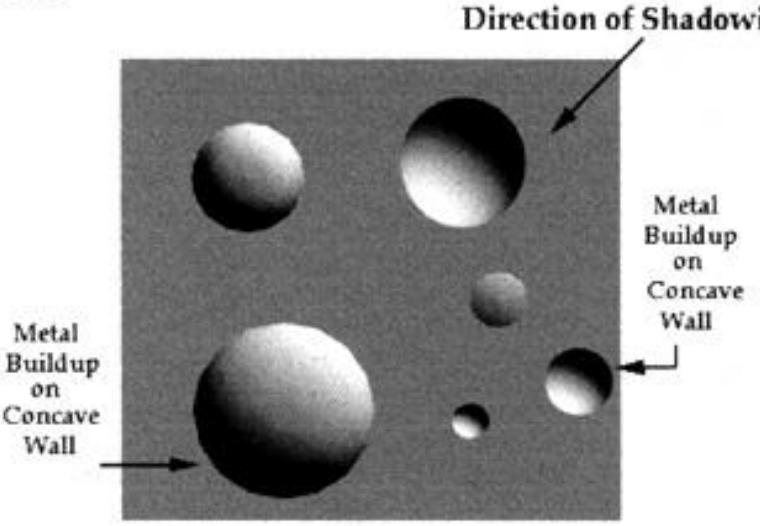
The fracture plane courses above or below organelles and other membraneous structures and sometimes directly through them at right angles to their plane (cross-fracture). The fracture plane exhibits a preference for the hydrophobic domain of membranes thereby separating their hydrophilic regions. This exposes the surface structure of the membrane interior by splitting it open.

The fracture plane is not observed directly rather a replica or cast is made of the fractured surface by shadowing it obliquely with heavy metal deposition. The resulting metal "replica" is retrieved, placed on a grid and viewed like a section with a transmission EM . Contrast results from different thicknesses of heavy metal producing a three-dimensional image corresponding to a template of the original fractured surface.

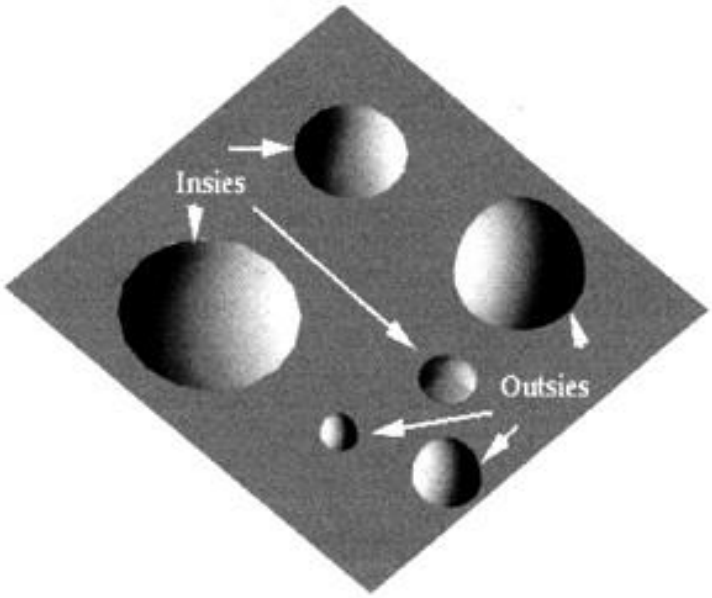
Interpretation of Freeze-Fracture Images

A knowledge of the direction of shadowing enables one to conclude whether a given structure in a replica is elevated (outsie) or depressed (insie) with respect to the general background. Thus if the buildup of metal shadowing material (dark on a positive print) on a given structure is similar to that of a known particle nearby, the structure must be similarily elevated.

En Face View

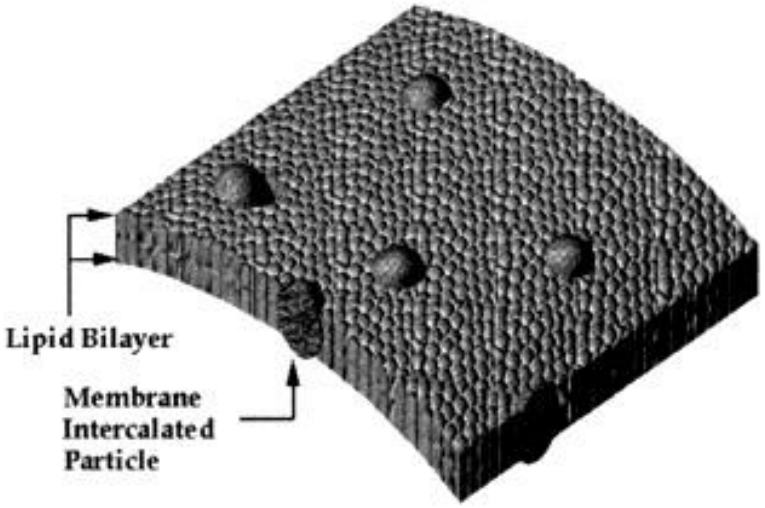


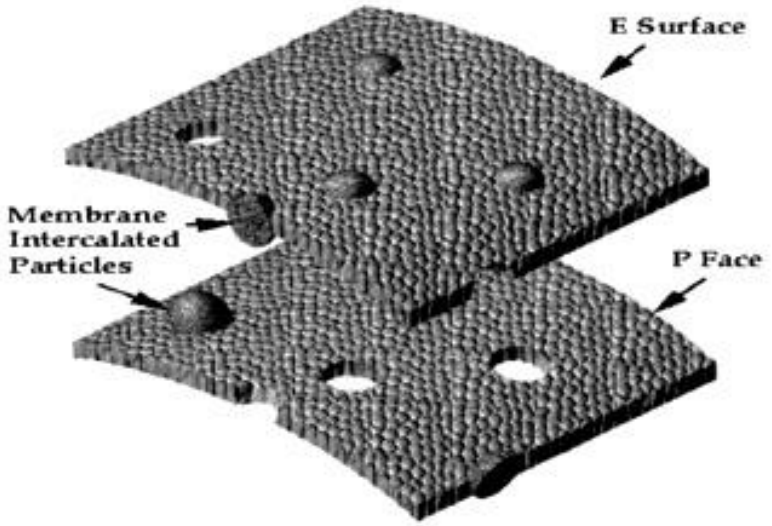
Isometric View



Intramembranous Particles in Freeze-Fracture Images

Intramembranous (intercalated) particles lack total compliment-arity and do not necessarily correspond to indentations or pits in the opposite fracture face.

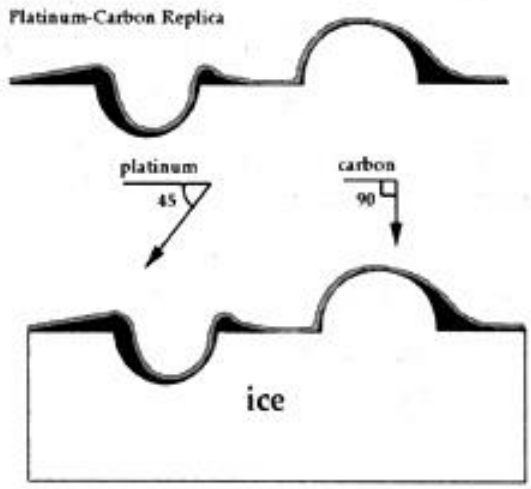




This may reflect a genuine membrane assymetry in which certain proteins are intercalated into only half of the lipid bilayer.

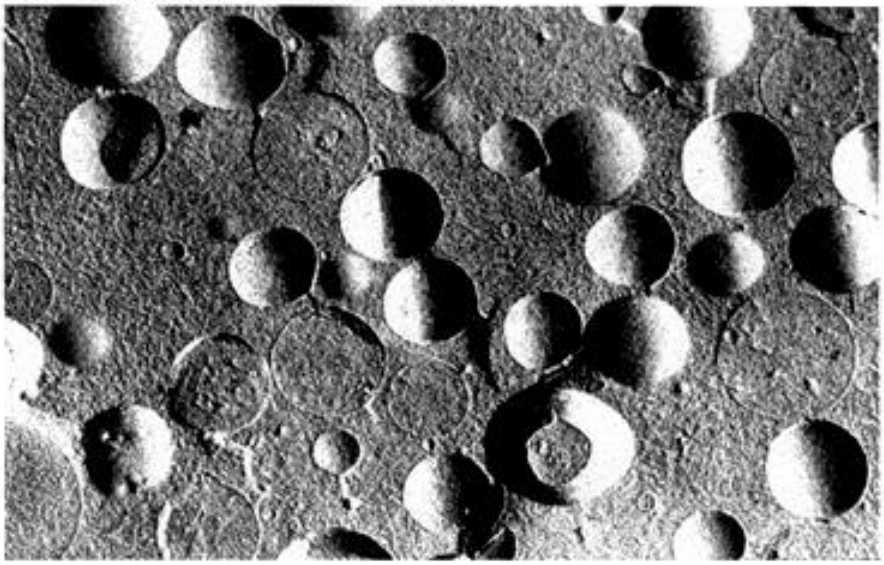
Replication

Replication of the fractured surface is accomplished by shadow casting the surface with platinum at an oblique angle (45 degrees). This produces a film 2-5 nm thick on the surface normal to the angle of the shdaow.



The thicknesses of the film at other regions is directly related to the slope of the shadowed surface in that region. The topology of the surface is converted into variations in the thickness of the shadow and finally into variations in electron density recorded on photographic film.

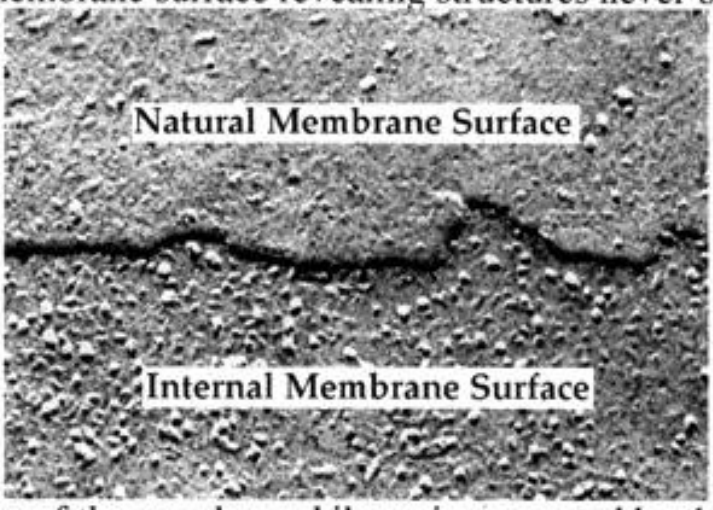
Following shadowing, a carbon backing is evaporated at a normal (90 degrees) angle to the surface. This inparts sufficient strength to the replica so that when it is removed from the surface it will not break.



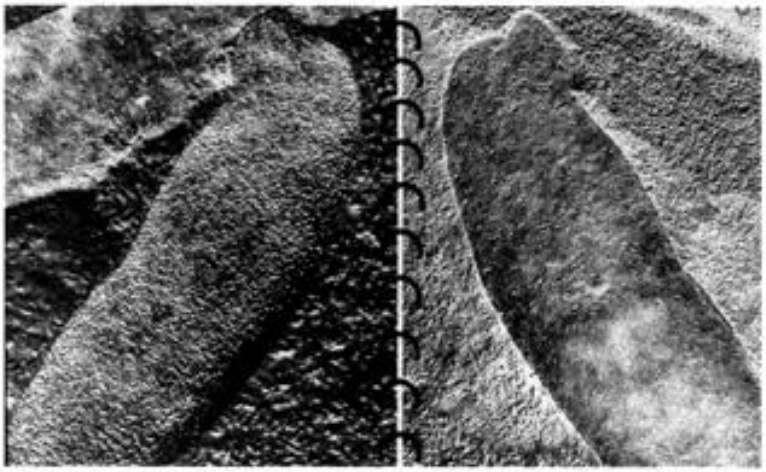
A replica of a fracture plane through a membraneous vesicle preparation reveals that the fracture plane sometimes courses over the top of a vesicle sometimes directly through the vesicle (cross fracture) or excavates the vesicle out of the fracture plane leaving only the bottom membrane half.

Fractured (split) Membranes

Literally a new dimension to membrane structure is revealed by freeze-fracture images since the original fracture plane splits the membrane bilayer (like a peanut butter sandwich) exposing its internal domain. Likewise etching exposes large areas of the natural membrane surface revealing structures never before seen.



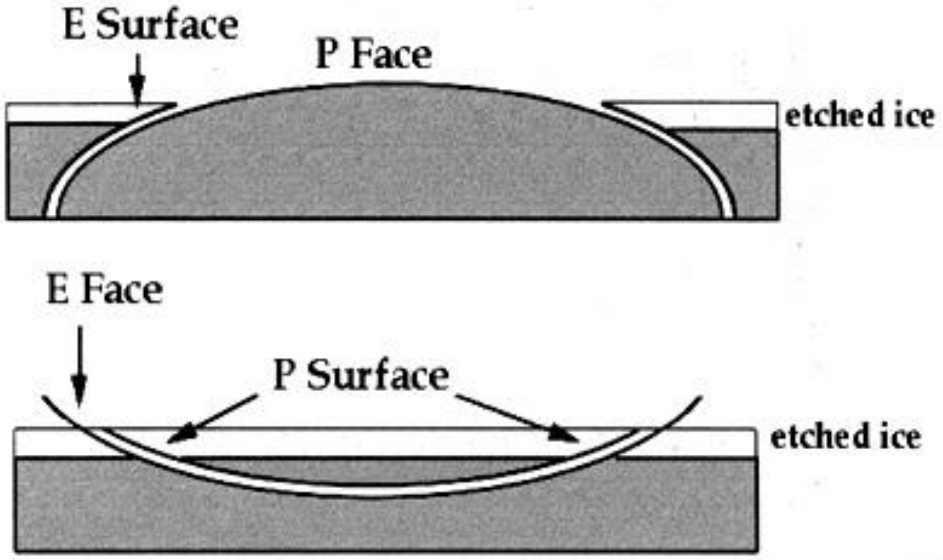
Splitting of the membrane bilayer is supported by the fact that if the fractured piece is recovered neither it nor the original fracture face it etchable (they are both membrane material and not ice).



Replicated halves of both fractured plane and recovered piece of a bacterial cell.

Notations for Membrane Surfaces

A special notation has been devised to indicate the various fractured and internal surfaces of membranes. The term "face" refers to a fracture face within a membrane exposed by the original fracture plane and "surface" refers to the natural surfaces of the membrane exposed by etching.

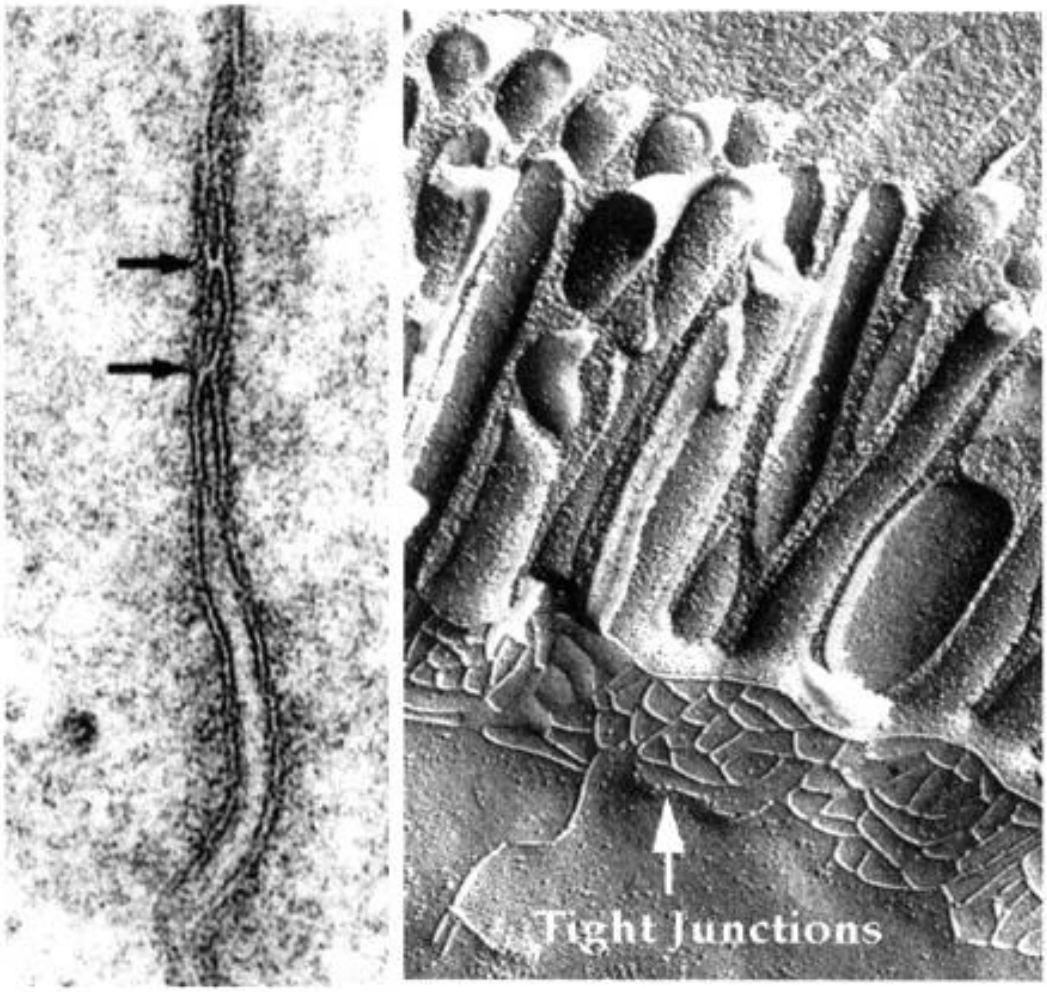


Fracturing of a membrane produces two faces: One is the face of the membrane portion left frozen to the cytoplasm (**P face**), The other is the face of the membrane left frozen to the extracellular space (**E face**). The **P surface** is the true outer surface of the membrane adjacent to the cytoplasm and the **E surface** is the true outer surface of the membrane adjacent to the extracellular space.

The P and E surfaces are only revealed by etching ice away from a portion of the original fractured specimen.

Freeze-Fracture Images of Tight Junctions (zonula occludens)

Sectioned membranes of adjacent epithelial cells which form tissue barriers exhibit such close association that their outer electron-dense leaflets appear fused. Freeze-fracture images of these regions reveal a complex of interconnecting ridges within the membrane bilayer. These effectively occlude movement of any material between the cells and thus perform an important tissue barrier function.



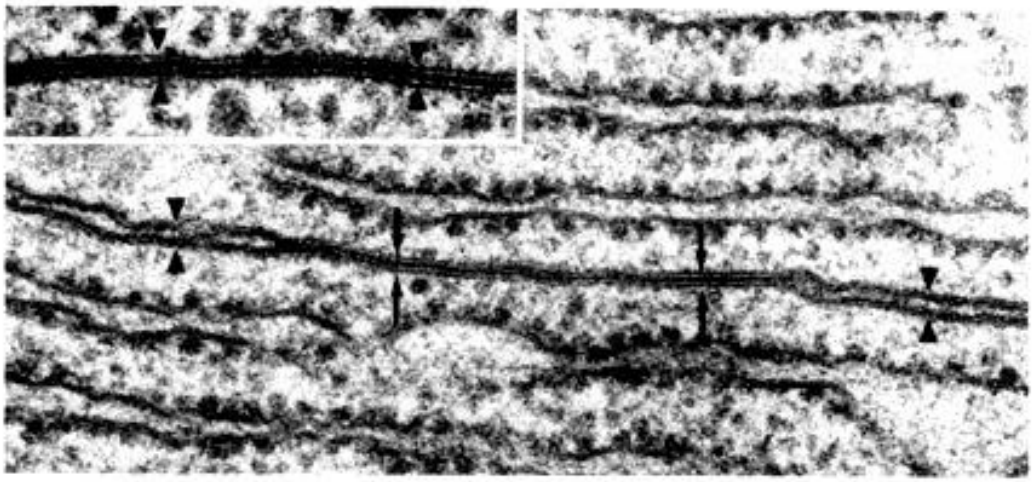
Membrane Intercalated Particles

A unique revelation provided by fractured membranes is the presence of discreet particles (@ 100 A dia.) which appear as bumps dispersed throughout the plane of E and P faces or aggregated into patterns. These particles are unique features of frozen and fractured membranes and have no readily observable counterpart in membranes in thin sections.

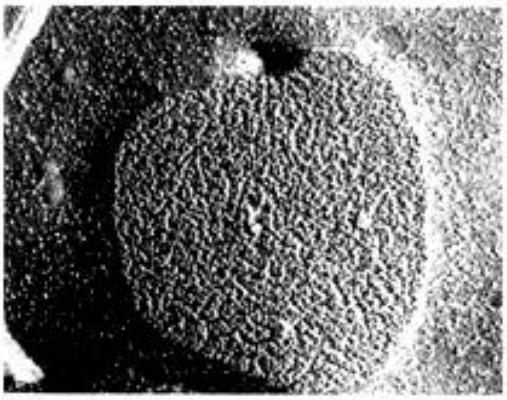
Evidence indicates that the particles are intergral membrane proteins, glycoproteins or aggregates thereof. The presence of observable globular proteins intercalated into the bilayer is consistant with and supports the "fluid mosaic model" of membrane structure

Freeze-Fracture Images of Gap (low resistence) Junctions

Thin sections through membranes of adjacent cells reveal close appositions (60 A gaps) which correlate with low-resistence coupling (ion transfer) between them.



Freeze-fracture images through these regions reveal islands or plaques of membrane intercalated particles indicating an aggregation of proteins. The plaques appear in both fracture faces suggesting that the proteins span the width of both adjacent membrane bilayers.



The proteins have been identified as "connexons" which form a pore connecting the cytoplasmic compartments of the cells and permit the passage of ions bwteen them