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Cryo-Fracture or Freeze-Fracture, a Method to Expose Internal Tissue Surfaces and Cell Surfaces for Viewing in the Scanning Electron Microscope

Jeannette Taylor
Emory University, Atlanta, Georgia
jvtaylo@emory.edu

Cryo-fracture, in conjunction with critical point drying is a method used to prepare biological samples in order to expose, for viewing via scanning electron microscopy, those naturally occurring surfaces which might otherwise remain obscure. For example, the Bowman's capsule and tubules of a kidney, tiny blood vessels on any organ, inter-cellular spaces in liver or alveoli in the lungs. Also, some surfaces, not normally exposed at all such as the membrane surface of a nuclear envelope, mitochondria or chloroplasts or the cytoplasm of a cell, can be brought to light with this method. Herein is a review of the development of cryo-fracture and how it is currently used at our facility.

Cryo-fracture of biological material was introduced by G. H. Haggis in 1970 (1). Haggis presented two methods of freeze fracture. In one method, the tissue samples, about 0.5 mm in one dimension, were fixed for 24 hours in 2% glutaraldehyde in a buffered sucrose* solution, pH 7.4, and rinsed in distilled water for 4 hours to wash off any buffer salts that might crystallize on the tissue surface. The fixed tissue was plunge frozen in Freon 22 at -150°C, fractured at -170°C by a chilled blade and freeze dried at -80°C for 15 hours. A 20 to 30 nm coat of gold was evaporated onto the mounted samples for examination in the scanning electron microscope. An alternative method proposed was to plunge freeze unfixed (washed or unwashed) tissue and fracture it as above then freeze dry, mount and coat for viewing. As the sample prepared using both these methods were frozen while wet with water, there was ice crystal damage.

Combining critical point drying with freeze fracture was introduced by W. J. Humphreys, B. O. Spurlock, and J. S. Johnson in 1973 (2) as an alternative to freeze drying the freeze fractured tissue. The authors pointed out that critical point drying takes less time than the 15 hours for sublimation in freeze-drying. One major change in the freeze fracture method of Haggis was that the tissues were fixed and then dehydrated to 100% ethanol prior to freezing and fracturing in liquid nitrogen (-196°C). This technique was elaborated and refined in 1974 (3) by the same authors who described utilizing the Parafilm packets used to contain the dehydrated samples in the 100% ethanol during the freezing and fracturing steps. Substituting ethanol for the water in the specimens prior to freezing appeared to have eliminated ice crystal damage. The ethanol, held in place by the Parafilm packet, also filled all the voids and supported the tissue through the trauma of fracture. The authors described using a 2 cm strip of Parafilm wrapped around a 2 mm wooden dowel to form the cylindrical packets. The sealed Parafilm packets, filled with 100% ethanol and the ethanol-dehydrated tissue were submerged in liquid nitrogen until frozen. The frozen cylinder was next placed on a metal block chilled by liquid nitrogen and with a chilled razor blade, gently fractured while submerged in liquid nitrogen. The fractured pieces were immediately placed into a container of fresh 100% ethanol and allowed to thaw. Critical point drying followed.

The fracture surfaces, described as distinctly smoother and shinier, were mounted face up. A layer of 20 to 30 nm of gold was evaporated onto the samples prior to viewing in the scanning electron microscope. The authors cryo-fractured onion root tips, leaves, and the perused kidney and liver of a mouse.

The authors, Humphreys, Spurlock, and Johnson, ran a parallel study of tissues prepared through critical point drying after which they were placed into propylene oxide, embedded, and thin sectioned for transmission electron microscopy (TEM). Though these samples appeared to be well preserved, at the request of a reviewer, the authors followed with another parallel study in 1975, this time embedding and sectioning samples that had been not only prepared by freeze fracture and critical point drying but also coated and viewed in the scanning electron microscope. The gold-coated fractured surfaces were cut in cross-section exposing the plane of the fracture, the organelles at that edge and the metal coating (4). This work demonstrated that there was no cytoplasmic loss or shift at the freeze-fracture surface. The authors did note, however, that the evaporated coat of 37 nm of gold had an effect of obscuring any detail their scanning electron microscope might have been capable of resolving and suggested a thinner metal coating.

In 1977, G. H. Haggis, with B. Phipps-Todd, (5) presented a modification of the freeze fracture method, wherein the samples are fixed, dehydrated to 100% ethanol, frozen in liquid nitrogen and critical point dried. Their modification was to first infiltrate small (<0.5 mm) pieces of tissue samples with dimethylsulphoxide (DMSO), freeze in Freon 22 chilled by liquid nitrogen, fracture the tissue and then let it thaw in fixative containing 25% DMSO, followed by dehydration and critical point drying in that order. Their aim as they described it was to "wash out" the soluble proteins at the fracture face, thus revealing the 3D structure of the internal membranes, cytoskeleton, and cytoplasmic and structures of the plant and animal tissues which they used. They did have to contend with ice crystal damage but the DMSO helped to reduce it. The samples started in an appropriate buffer to which was added DMSO in 5% steps until 25% DMSO was reached. Haggis and Phipps-Todd described the 25% DMSO as a compromise between a higher concentration to reduce ice crystal damage and a lower concentration to reduce possible tissue damage by the DMSO itself.

As was noted previously, the fracture face of a fixed, dehydrated, freeze-fractured sample revealed little detail. This new freeze-fractured, thawed, fixed protocol offered whole nuclei, fibers, globules, lobes, fractured nuclei, chloroplasts and vacuoles. Haggis and Phipps-Todd prepared samples using both fracture methods but critical point dried their tissue, using CO_2 as the transition medium, rather than freeze- drying as Haggis had previously done. This fracture protocol seems to open up the cell to easy viewing but a lot can be lost. Using both methods in tandem would provide a fuller picture of cell organization. Alternatively, perhaps a very light fixation could be employed to just firm up the organelles.

A further refinement on the pretty much standard freeze-fracture method of first fixing the tissue, dehydrating to 100%

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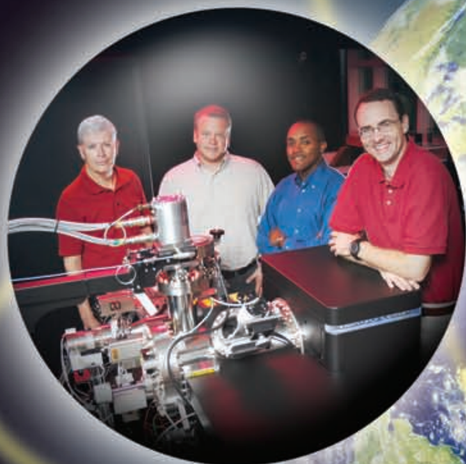

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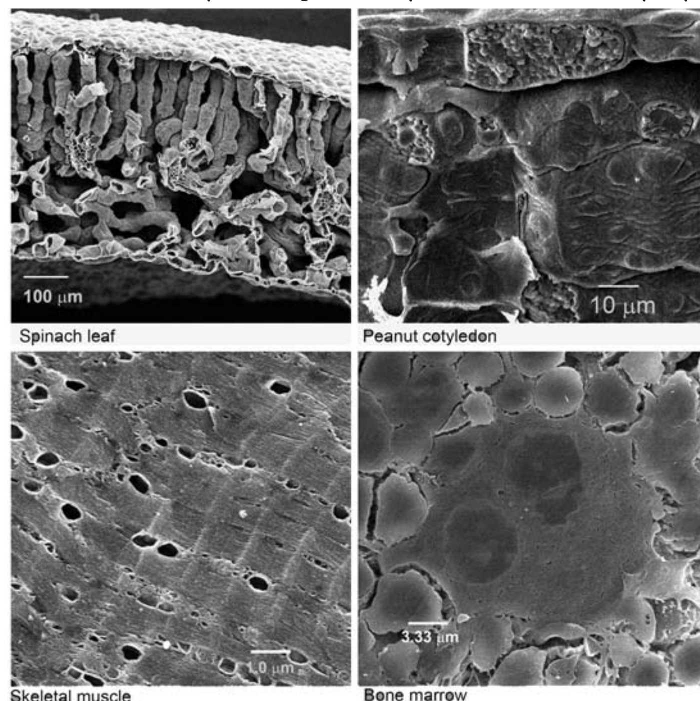
ethanol and then freeze-fracturing the tissue followed by critical point drying was made in 1981 by R. Apkarian and J. C. Curtis (6). Their study of ovarian follicles of immature rats employed cryofracture (same as freeze-fracture) to prepare samples for viewing in the scanning electron microscope. The authors made three refinements in the protocol. The fixative was highly oxygenated. The fixed samples were washed in distilled water for a long time to eliminate salt deposits on the sample surface. Sample dehydration was performed using a linear gradient apparatus to provide a very gentle dehydration. And last, the specimens were fractured using a modified Smith-Farquhar tissue chopper. In this modification to the freeze-fracture method, the 100% ethanol infiltrated samples were sealed in little Parafilm packets pre-filled with 100% ethanol, plunge frozen in a chilled Freon 22 slush, and transferred to liquid nitrogen, in which the submerged samples were fractured. The Smith-Farquhar chopper was equipped to hold liquid nitrogen in a trough and the height of the razor was adjusted so that when it was released to fall on the sample, it would only just cleave the top, not cut through the sample. The shock of the impact propelled the fracture and the fractures were well controlled. The fracture pieces were then placed into fresh 100% ethanol to thaw after which they were critical point dried, mounted, and coated.

A. E. Hotchkiss, V. J. Martin, and R. P. Apkarian employed this same method in 1984 in their study of the planulae of a hydrozoan (7). The samples were fixed, rinsed with buffer, rinsed briefly with distilled water, dehydrated to 100% ethanol, sealed in little Parafilm packets filled with 100% ethanol, plunge frozen in chilled Freon 22 at -160°C and fractured in liquid nitrogen at -196°C using the modified Smith-Farquhar tissue chopper, after which the samples were placed into fresh 100% ethanol and critical point dried, mounted and coated.

The cryo-fracture protocol as currently practiced at our facility is based on a long history, which began with simply fracturing frozen fixed and unfixed wet tissue. The next development produced what is essentially the protocol as practiced here today, that is, ethanol dehydration prior to cryo-fracture followed by drying using the critical point method. However, many refinements have been made over the years to tissue handling and to the process of critical point drying. Freons are no longer used as they are now recognized as an environmental hazard.



Spinach leaf; Peanut cotyledon; Skeletal muscle; Bone marrow

200 nm

Skeletal muscle

The cryo-fracture protocol followed at our facility is to fix the small pieces of tissue, rinse in buffer and distilled water and dehydrate through 100% ethanol, seal the tissue inside little Parafilm packets filled with 100% ethanol, freeze the samples in liquid nitrogen and gently fracture the samples using a liquid nitrogen-chilled razor blade while submerged in liquid nitrogen. The complete infiltration with 100% ethanol would seem to be adequate protection against ice crystal formation in the tissue. One point to make about using the Parafilm packets is that at no time are any samples exposed to air. The samples are placed into the packets and sealed while everything is submerged in 100% ethanol. The freshly cryo-fractured tissue is removed to a container of fresh 100% ethanol to thaw and the Parafilm discarded. Critical point drying, mounting and coating proceed from there.

The 12 nm of sputtered gold applied to samples for conventional scanning electron microscopy (SEM) in our facility is just a third of the thickness of that used by Humphreys, Spurlock, and Johnson in their work and the 1 to 2 nm of sputtered chromium applied to samples requiring high resolution SEM imaging might seem almost invisible in a comparative TEM cross section.

The images are SEM micrographs of a Cryo-fractured spinach leaf, peanut cotyledon, mouse bone marrow and mouse skeletal muscle. Please note the nicely preserved organelles and membranes such as fat storage droplets in the peanut. Some membranes look like sheathes broken away revealing the cell contents. But particularly note the nearly flat plane of the fracture. In the marrow one can see nuclei in cross section and in the muscle one can see the banding; higher magnification shows detail.

These samples were sputter coated with 12 nm gold then viewed using a Topcon DS130 SEM at 10 kV. ■

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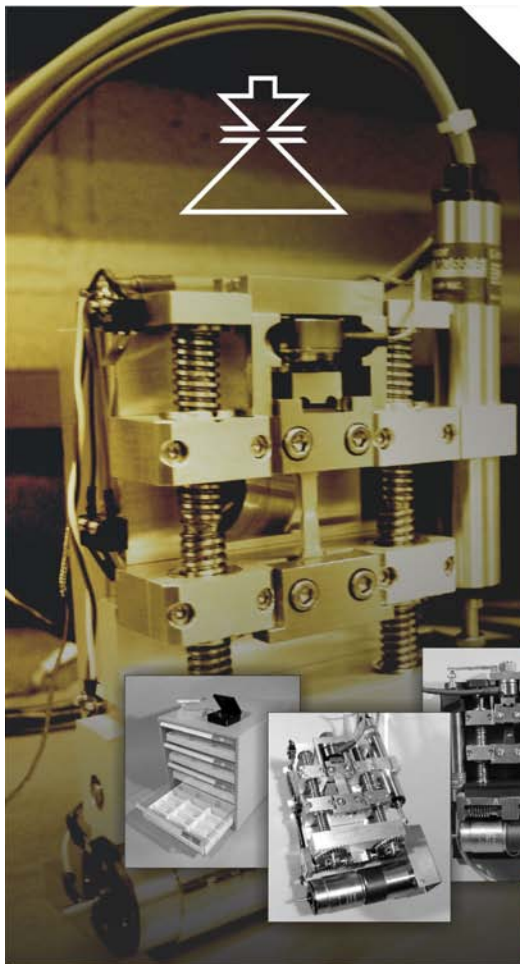

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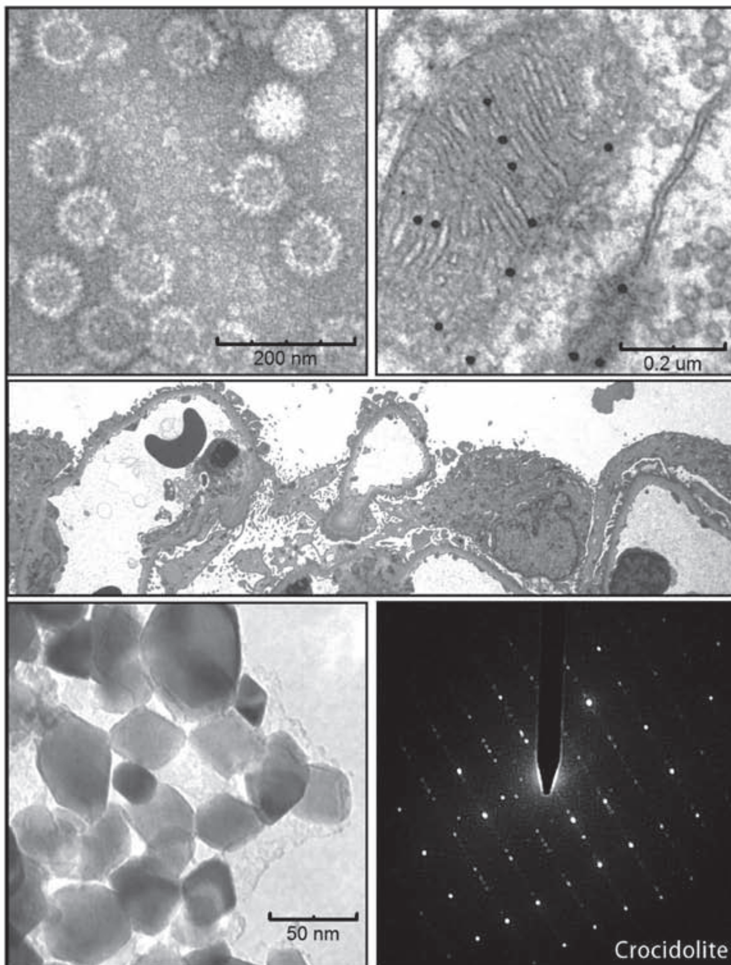

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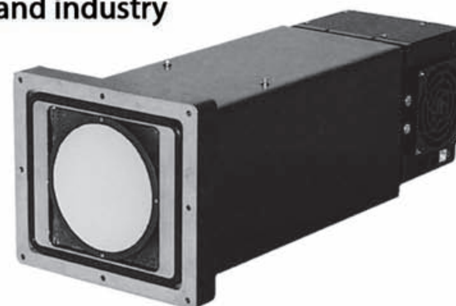

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