High Pressure Freezing: Benefits and Limitations

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Rapid freezing of biological samples for subsequent processing and examination in the TEM has been around since the 1960's. Freezing, in general, is advantageous because it can provide an instantaneous cessation of all biological activity and halt ultrastructural rearrangement. The freezing "fixes" biological structures in water ice until such time that the ice can be substituted for a chemical fixative. Freezing is typically conducted at liquid nitrogen temperatures (-196° C), and freeze substitution (FS) is then carried out over days to weeks on dry ice (-80° C) (Severs *et. al.*, 1995). The advantages of freezing and freeze substitution can include improved preservation (particularly of tubular and vesicular structures) and increased antigenicity (Baskin *et. al.*, 1996). Also, background densities in many tissues are usually (but not always) higher. The main drawback is limited depth to which the various techniques can rapidly cause freezing (Slow freezing leads to the formation of large and damaging ice crystals), access to the necessary equipment, and expense.

High pressure freezing (HPF) came on the scene in the early 1980's with the development of an instrument out of the laboratory of Professor Hans Moore. HPF differs from other forms of freeze fixation (such as plunge, propane jet, or cold mirror freezing) in that pressures of approximately 20,000 atmospheres are applied at the instant of freezing. The high pressure serves to reduce the formation of large (>10 nm) ice crystals, which are the bane of good ultrastructural preservation and the major limitation of ambient pressure freezing techniques (Kiss and Staehelin 1995). Spectacular images of biological ultrastructure can be produced with the proper combination of HPF and FS (Kiss *et al.*, 1990).

A recent query to the Microscopy List Server (Microscopy@MSA.Microscopy.com) generated significant discussion on the potential benefits and limitations of HPF. Those responses make up the body of this article.

HPF shares many of the advantages and disadvantages of the more routine ambient pressure freezing protocols. The single-most important, and perhaps only, advantage of HPF over other freezing techniques is that it can allow for somewhat deeper penetration of adequate freezing. Standard ambient pressure freezing typically only reaches 10 to 40 μm into a tissue. Beyond that depth, large ice crystal formation causes serious artifacts. HPF can usually penetrate about twice as deep, although this is very tissue and specimen specific.

HPF has distinct limitations as compared to ambient pressure freezing or standard chemical fixation. Several of the respondents on the Microscopy listserver pointed out that one of the most serious drawbacks is related to the maximum tissue size that can be accommodated; a sample size which is set by freezing depth and the dimensions of the freezing chamber. Although studies of single cells or small organisms are quite feasible, overall sample size is limited to tissue no larger than about 12.5 mm in maximum dimension (*c.f.* Hohenberg *et. al.*, 1994). The depth to which adequate freezing can penetrate may actually be less than 20 μm in many samples (Severs *et al.*, 1995), for a maximum sample size of 40 μm in any dimension.

Heinz Fehrenbach (Technical University of Dresden) pointed out that for most investigations these limitations in sample size and freezing depth can have significant consequences. For instance, HPF is not applicable to the study of large or interior structures without sample excision. The surgical removal of tissue is well known to induce alterations in ultrastructure that will increase with time until fixation. Researchers who are interested in internal structures are therefore unable to use HPF and must rely instead on perfusion or infiltration of a conventional chemical fixative.

Because samples processed by HPF must be frozen one at a time, bulk processing of samples is not possible. Therefore, sampling bias is greatly enhanced and large studies comparing numerous specimens are typically not pos-

sible. Coupled with the fact that the freezing produced by HPF is usually quite uneven, the number of samples needed to find a site without freezing artifacts (and the consequent workload) is greatly increased. Note that in all HPF, the sample has to be placed in some kind of solution (usually a viscous protein or dextran solution) to exclude air and get maximum heat conduction. Therefore, tissue osmolarity might be a problem.

In addition to the above limitations, HPF is not without its own set of artifacts – some known and others suspected. Rosemary White (Monash University, Australia, pers. comm.) commented that the endoplasmic reticulum in many plant samples appears enlarged, and the endoplasmic reticulum membrane may become almost invisible after HPF treatment. Ding *et. al.*, (1992) report that microfilament bundles in HPF frozen cells have a "frayed or loosened appearance" when compared to those prepared with a propane jet freezer. They propose that this is an artifact of the pressure spike imposed by the HPF technique. Indeed, this pressure spike, which must undoubtedly be accompanied by a heating spike as well, remains an unexplored source of potential artifact. Deformation of whole cells and membrane tears (Craig and Staehelin, 1986) and the loss of peripheral vesicles in fungi (Hyde *et. al.*, 1991) have also been reported as artifacts specific to HPF tissue processing.

But probably the largest impediment to the widespread use of HPF in biological imaging is the availability and expense of the necessary instrumentation. Only two manufacturers have high pressure freezers on the market (Balzers and Leica) and they list at about $60,000 (US) for a complete setup. At this time, there are only six high pressure freezers in the United States, as compared to a (very rough) estimate of several thousand TEMs. At the present time, high pressure freezing is simply not an option for the vast majority of electron microscopists.

So what is a microscopist to do? Anecdotal stories on the internet and at scientific meetings claim that grants and publications are being rejected for not using HPF techniques. This author can certainly attest to that. General consensus among those responding to the original query to the microscopy net was: 1) the only advantage of HPF over other freezing techniques is the potential for deeper freezing, and even then the increase is minimal and tissue-specific; 2) several HPF specific artifacts have been noted but it is just too early to know what all of the limitations might be; 3) other forms of freezing and standard chemical fixation are and will continue to be very useful (and affordable) to the biological microscopist; and 4) when in doubt, use as many different fixation techniques as possible. ■

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