CHAPTER 28

"Tips and Tricks" for High-Pressure Freezing of Model Systems

Kent McDonald[*], Heinz Schwarz[†], Thomas Müller-Reichert[‡], Rick Webb[§], Christopher Buser[¶], *and* Mary Morphew[#]

[*]Electron Microscope Laboratory, University of California, Berkeley, California 94720

[†]Electron Microscopy Unit, Max Planck Institute of Developmental Biology, 72076 Tübingen, Germany

[‡]Medical Theoretical Center, TU Dresden, 01307 Dresden, Germany

[§]Centre for Microscopy and Microanalysis, University of Queensland, 4072 Queensland, Australia

[¶]Department of Molecular & Cell Biology, University of California, Berkeley, California 94720

[#]Boulder Laboratory for 3D Electron Microscopy of Cells, University of Colorado, Boulder, Colorado 80309

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Abstract

High-pressure freezing (HPF) has been around since the mid-1980s as a cryopreparation technique for biological electron microscopy. It has taken quite some time to "catch on" but with the recent interest in cellular tomography and electron microscopy of vitreous cryosections it has been used more frequently. While HPF is relatively easy to do, there are a number of steps, such as loading the sample into the specimen carrier correctly, that are critical to the success of this method. In this chapter we discuss some of the "little" things that can make the difference between successful or unsuccessful freezing. We cover all aspects of HPF, from specimen loading to removing your sample from the carriers in polymerized resin. Our goal is to make it easier and more reliable for HPF users to get well-frozen samples for their research.

I. Introduction

In the past decade and more, there have been numerous articles about how to do high-pressure freezing—from a detailed account of using the BAL-TEC HPM 010 (McDonald, 1999) or Leica EM PACT (Studer *et al*., 2001) to more recent works that cover much the same territory (Kaech, 2009; Vanhecke and Studer, 2009). This is in addition to many other "instructional" papers that have addressed the application of high-pressure freezing techniques for specific model system organisms such as flies (McDonald, 1994; McDonald *et al*., 2000), yeasts (McDonald and Müller-Reichert, 2002), and worms (Müller-Reichert *et al*., 2003; McDonald, 2007). We distinguish these papers from regular research papers by the fact that they primarily cover the "how to" aspects of HPF rather than focus on the scientific results. They are like the Methods and Materials section of regular articles, expanded to give details that most editors will not allow. We usually refer to these types of articles as "methods papers" as opposed to research articles. They are the kind of account found in books like the present publication. In this chapter, we would like to highlight some of the "little" things that one can do to make a difference between success and failure for HPF, or just make it a lot easier to do; what are often called "tips and tricks". Some of these are from our own cumulative experience of more than 70 years of high-pressure freezing, but others come from colleagues' work, both published and unpublished. This is not to say that others have not come up with the same ideas but if they are unpublished we have no way to acknowledge their contributions. There will also be items that we miss and we apologize to the authors concerned in advance.

II. Rationale

The items that follow cover all aspects of high-pressure freezing, from preparing the samples and sample carriers to removing the samples from the carriers embedded in

resin. They have in common the idea that the method being discussed will make it easier or more reliable to get well-frozen cells and tissue samples.

III. Methods

A. Working with Cellulose Microdialysis Capillary Tubing

1. History

The use of cellulose capillary tubing for high-pressure freezing was the idea of Hohenberg *et al*. (1994). Since then it has found applications with samples such as plant protoplasts (Tiedemann *et al*., 1998), single *Caenorhabditis elegans* embryos (Muller-Reichert *et al*., 2007), 3D mouse breast cancer organoids and strips of cochlear cells (Triffo *et al*., 2008), and correlative microscopy and tomography of worms (Müller-Reichert *et al*., 2007) to name some.

2. Where to Buy

There are a number of sources for regenerated cellulose hollow fibers as they are called in the industry: Packs of 100 capillaries are available from Leica Microsystems (contact your local Leica Microsystems sales representative for prices and availability). In the United States, they are available from Spectrum, 23022 La Cadena Dr., Suite 100, Laguna Hills, CA 92653, USA. In the past, we ordered the Spectra/Por Hollow Fiber Bundles, 200 μm inner diameter, Cat. no. 132 290. However, this part number no longer exists at Spectrum Labs, but if you go to http://www.spectrapor.com/cell/MaxCarts.html, you will find the so-called "hollow fiber bioreactors" and some contain regenerated cellulose hollow fibers with inner diameters of 200 μm (Prod. No. 430-011) and 192 μm (Prod. No. 400-004) and molecular weight cut-off values of 30 and 20 kD, respectively. It costs around $US 500 for these "bioreactors" but it appears that they might contain thousands of fibers about 20 cm in length. The alternative at Spectrum seems to be Parts Nos. 132294 and 132295 that come 20 fibers to a package for $US 112.

In Europe, capillaries may also be purchased much cheaper from the original MICRODYN-NADIR Diaperm Dialysis Module LD OC 02, which contains 1280 cellulose hollow fibers (each with an active length of 245 mm) per cartridge. This lab module is produced and distributed under the article number 00020401 by MICRODYN-NADIR GmbH, Rheingaustrasse 190–196, D-65203 Wiesbaden; E-mail: info@microdyn-nadir.de. The price is 155 € per module. The same module is also available from Reichelt Chemietechnik GmbH, D-69126 Heidelberg (www.rct-online.de) as Thomapor MICRODYN-LD OC02, Cat. No.72571 for 390 €.

Problem 1: Cutting and sealing samples into capillary dialysis tubing requires more information than given in the original reference (Hohenberg *et al.,* 1994). In this article it was recommended that a scalpel or opthamological punch could be used to cut the

capillary tubing. This is true as far as it goes, but for some samples it is also necessary to cut and crimp the tubing so that the cells inside do not leak out during subsequent processing. A new scalpel will almost always cut the tubing cleanly and it will be totally open at the ends. If you want to cut and crimp at the same time, try the following method as suggested by Andreas Kaech, University of Zurich (pers. comm.)

Solution:

1. Take a number 11 scalpel blade and cut or break off the tip so the new tip is about 1 mm across and put the blade in a scalpel handle (Fig. 1A).
2. Use a whetstone to make the tip into a chisel shape (Fig. 1B–C).
3. Test the blade by cutting dialysis tubing filled with dye under 1-hexadecene in a shallow plastic dish. Use a rocking motion perpendicular to the tubing long axis to make the cuts. You should not have to press down hard to make the cut.

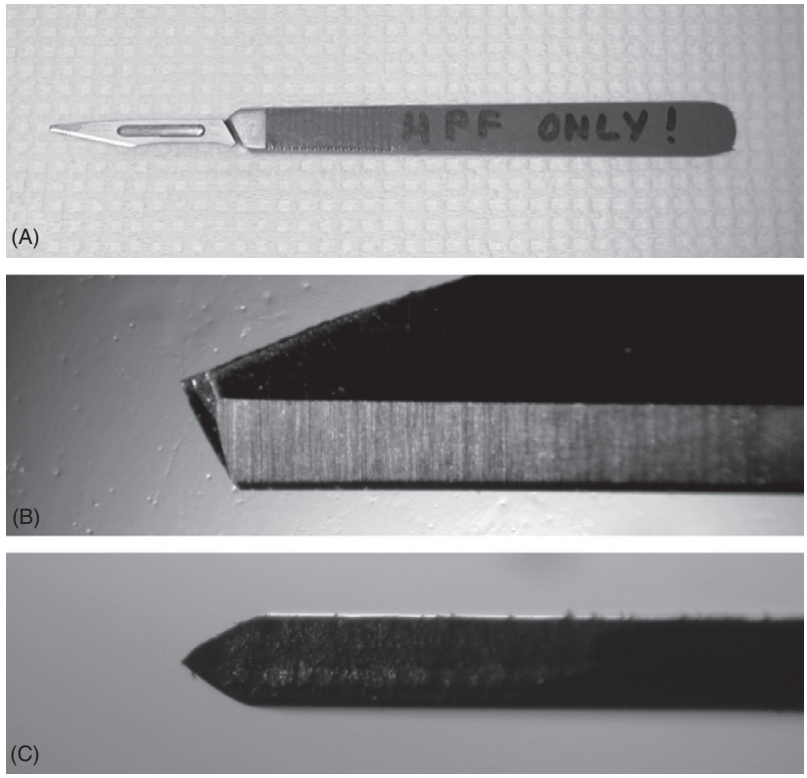

Fig. 1 Details of the crimping tool for sealing microdialysis capillary tubing. (A) The tool should be mounted in a scalpel handle and labeled such that no one will throw away the blade. Depending on the frequency of use, these tools can be used for months before reshaping. (B) Side view of the tip of the crimping tool. (C) View from the top of the tip.

4. Continue shaping and testing until a single motion both cuts and seals the tubing. This has to be done for both ends. The result should look like Fig. 4A in Müller-Reichert *et al*., 2008.
5. When the procedure is comfortable and reproducible, use on your tissues and cells of choice.

Problem 2: Capillary tubing can be used to isolate single cells, which can then be frozen and processed in the tubing. Getting cells into the tubing and sealing it can be tricky. Thomas Müller-Reichert solved this problem by using nail polish to seal some capillary tubing into a micropipette (Müller-Reichert *et al*., 2007).

Solution:

1. Cut pieces of microdialysis tubing into 3–4 cm lengths (Fig. 2A).
2. Seal the tubing into the pipette tip with nail polish and let dry (Fig. 2B).
3. Attach the tip to a pipette and place the tubing into the liquid from which the cells will be withdrawn. The tubing will curl, and then straighten out as the walls fill with liquid.
4. Carefully draw the single cell into the tubing, transfer to a shallow plastic Petri dish with enough 1-hexadecene to cover the tubing, and cut the tubing near the pipette tip with the crimping tool (see Section above).

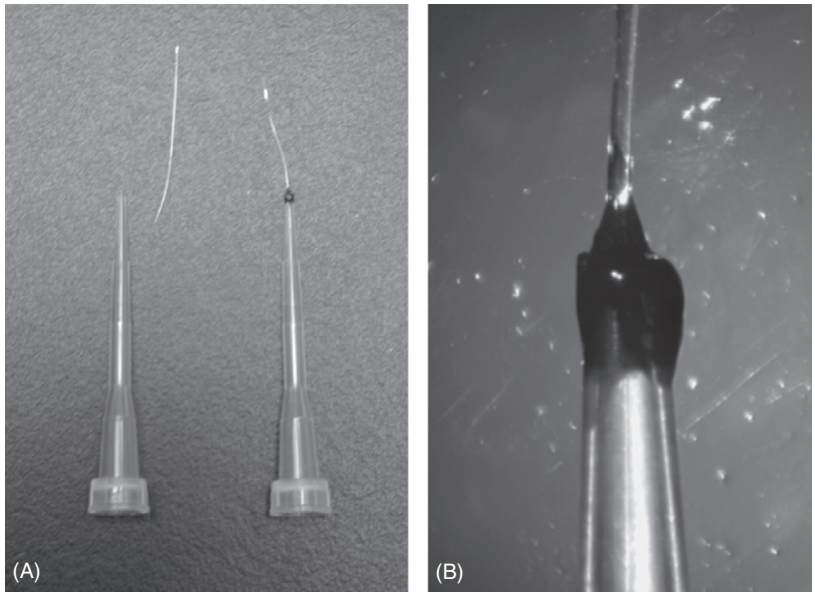

Fig. 2 Capillary tubing mounted into micropipette tips for use in controlled movement of small objects into the tubing. The tips are the small size used in 0.5–10 µl pipetmen. (A) A pipette tip and piece of capillary tubing on the left prior to assembly. On the right, a tip after sealing the tubing into the tip with nail polish. (B) Detail of the nail polish seal at the tip. It is important to make sure the polish completely seals the tubing.

5. Cut the tubing with cell into the appropriate length for the HPF specimen cup you are using. For the Wohlwend-style cups, that can be about 1.8 mm, and for Leica cups about 1.4 mm.
6. Using forceps to pick up the tubing by one of the crimped ends, place it in the cup, cover with cryoprotectant to fill the cup, and freeze.

B. Making Up 20% Bovine Serum Albumin

Problem: Making up 20% bovine serum albumin (BSA) in small quantities (less than 5 ml) can be difficult because the volume of the powder is about the same as the volume of the liquid and it does not go into solution readily. It tends to clump together and requires vortexing and heating for 5–10 min before it dissolves. If you are only using 20% BSA as a filler solution for the HPF planchettes, you may only require less than 1 ml total. If you want to use 20% BSA as a filler, try this idea from Rick Fetter of Howard Hughes Medical Institute, Janelia Farms Research Campus (pers. comm.) for making up small quantities very easily.

Solution:

1. Put 850 µl of solution (whatever you want to use for your cells/tissues) in a 2 ml Eppendorf tube. Make sure there is no solution on the side of the tube above the meniscus or the BSA will stick to it and not go into solution.
2. Weigh out 200 mg of BSA and layer it on top of the solution in the tube.
3. Spin for 15–20 s in a microfuge at 15,000 × G.

Some say that 20% BSA will extract membranes from cells, but Paul Verkade (University of Bristol, UK) reminds us that if you make up the BSA with medium containing at least 5% or more fetal calf serum, then extraction will not be a problem.

C. Working with a Very Small Volume of Cells

Problem: Sometimes you may only have a few microliters of cells and concentrating them and loading them into an HPF specimen carrier is a challenge. While the specimen carriers typically hold less than a microliter, there is often a lot of transfer loss going from a tube or filter to the carrier. Here is an idea from Alisdair McDowall of Cal Tech, Pasadena (pers. comm.) that optimizes concentrating and loading with mimimal loss.

Solution:

1. Using a flame source, such as a cigarette lighter, seal the ends of some 200 µl pipette tips (Fig. 3).
2. Concentrate the cells of interest into about 100 µl of solution in a regular small tube such as an 0.5 ml or 2 ml Eppendorf.
3. Resuspend the cells in the Eppendorf, then pipette them into the sealed pipette tip.

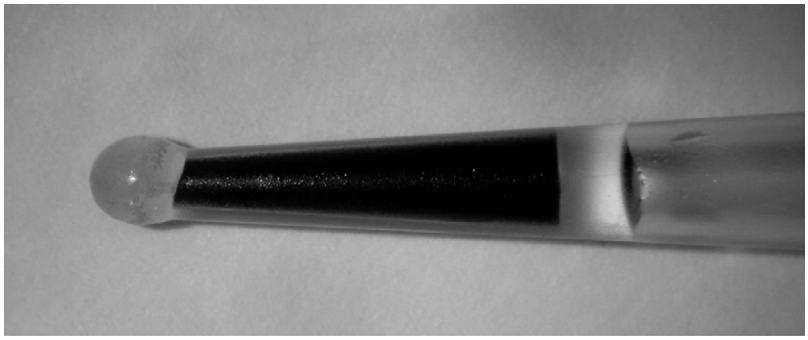

Fig. 3 A 200 µl pipette tip showing how the end looks after sealing with a flame. After centrifugation, the biological material (dark) is concentrated at the tip with a small amount of supernatant at the top to keep it from drying out.

4. Place the pipette tip plus cells into a 0.5 ml Eppendorf tube without cap, then this assemblage into a 2.0 ml Eppendorf tube without cap.
5. Create a balance tube with the same components.
6. Spin down until the cells are concentrated in the bottom of the sealed pipette tip.
7. Carefully remove most, but not all, of the supernatant above the cells with wicking paper (Fig. 3).
8. Using a very sharp blade, carefully cut off the tip of the pipette so that there is an opening for the concentrated cells to be extruded.
9. With a mouth pipette, or micropipetter, carefully extrude cells from the pipette tip directly into an HPF specimen carrier until full, then freeze immediately.
10. If there is enough material to fill more than one specimen carrier, take care not to let the very tip of the pipette with cells to dry out. Putting it up against a small piece of paper saturated with medium in the bottom of a 2 ml tube can work. Better yet, have some 1% (or less) agarose in a dish or tube and plunge the tip/cells into that immediately after extrusion for HPF.

D. Using Yeast Paste as a Filler for Difficult-to-Freeze Cell Suspensions for Vitreous Cryosectioning

Problem: Most cell suspensions (bacteria, tissue culture (TC) cells) can be concentrated and used to fill the entire volume of a specimen cup. However, some cells do not freeze well when processed this way, especially in cups 100 µm or more deep. Yeast (*Saccharomyces cerevisiae*) cells, on the other hand, freeze very well in even very deep (400 µm or more) specimen cups. Difficult cell types can be mixed with yeast to give a hybrid mix, and this will sometimes improve their quality of freezing. Mark Ladinsky of Cal Tech, Pasadena (pers. comm.) has used this strategy effectively for cutting vitreous sections of mammalian tissue culture cells.

Solution:

1. Set up cultures of yeast and the other cell type to grow in solution to early log phase.
2. Concentrate the cells by centrifugation, or another means if spinning them down could be harmful.
3. Mix the concentrated cell types in at least a 1:1 ratio. Having more yeast than the other cell type will improve the chances of good freezing.
4. Load the mixture into HPF specimen cups and freeze.

E. Using Slot Grids with the Wohlwend-Style Specimen Cups as a Variable Depth Spacer for Difficult-to-Freeze Cell Types

Problem: Some cell types do not yield 100% well-preserved tissue when frozen in the standard 100 μm deep specimen cups. With Wohlwend-style cups it is possible to buy from Wohlwend Engineering (Sennwald, Switzerland; Technotrade International, Manchester, NH, USA) specimen cups with depths of 25 and 50 μm. An alternative method to create shallower depths is to use TEM slot grids as spacers. Rick Fetter of HHMI Janelia Farms Research Campus developed this strategy to improve the yield of well-preserved *C. elegans* worms. More information on known slot grid thicknesses can be found in McDonald *et al*., (2007).

Solution:

1. Place a piece of filter paper in a small Petri dish and saturate with 1-hexadecene.
2. Place Type B (one flat side, one 300 μm deep well on the other side) specimen cups, flat side down on the saturated filter paper. Also place the slot grid of choice on the filter paper.
3. After blotting to remove excess hexadecane, put a specimen cup (flat side up) in the tip of the HPF specimen loader (Fig. 4A).
4. Put a slot grid on top of the specimen cup in the loader (Fig. 4B).
5. Fill the cavity of the slot grid with sample.
6. Blot and place a specimen cup (flat side down) on top of the slot grid/sample (Fig. 4C). This step needs to follow the previous step quickly so the sample does not dry out.
7. Close the tip of the specimen loader and freeze the sample (Fig. 4D).

In Fig. 4, the slot grid used was very thin, about 27 μm, and this is a good size for cell suspensions such as bacteria. For thicker samples, a thicker slot grid can be used. In Fig. 5, a slot grid about 65 μm thick was used to freeze *C. elegans* worms. The worms were slightly compressed at this thickness, but by having both sides of the worm touching metal, the chances of good freezing are improved.

F. Freezing Monolayers of Tissue Culture Cells on Sapphire or Aclar® Disks

Problem: TC cells grown on a substrate are among the most widely used model systems in cell biology today. The challenge is how to grow them for high-pressure

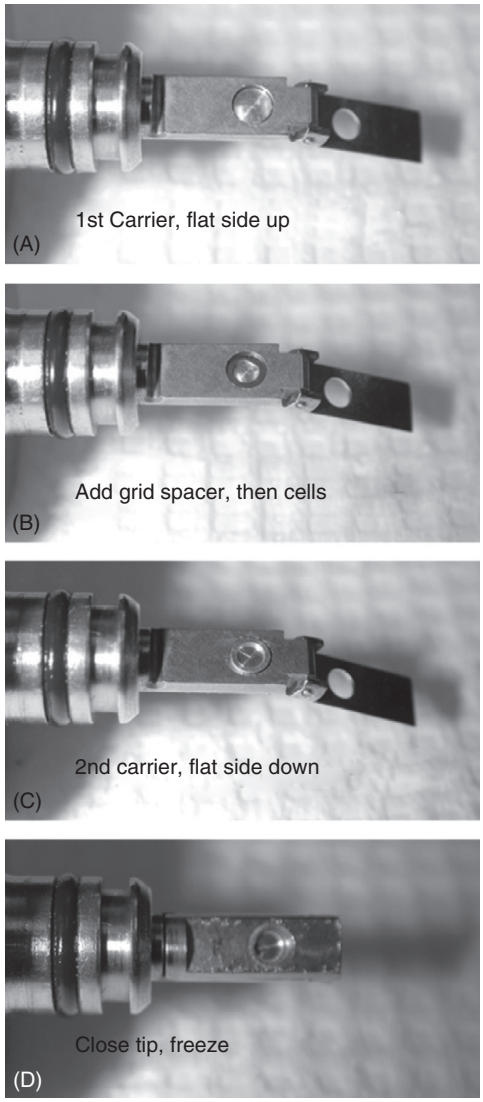

Fig. 4 A sequence of steps for using EM slot grids as spacers for creating shallow wells in ABRA HPM 010, BAL-TEC HPM 010, and Wohlwend HPF Compact 01 high-pressure freezers. (A) Place a Type B (flat on one side) specimen carrier flat side up in the specimen holder. (B) Add a slot grid as a spacer, and then fill with cells, or place a sapphire disk cell side down over the spacer. (C) Place a second Type B carrier on top of the slot grid or sapphire disk, flat side down. (D) Close the tip, secure it into the holder, and freeze.

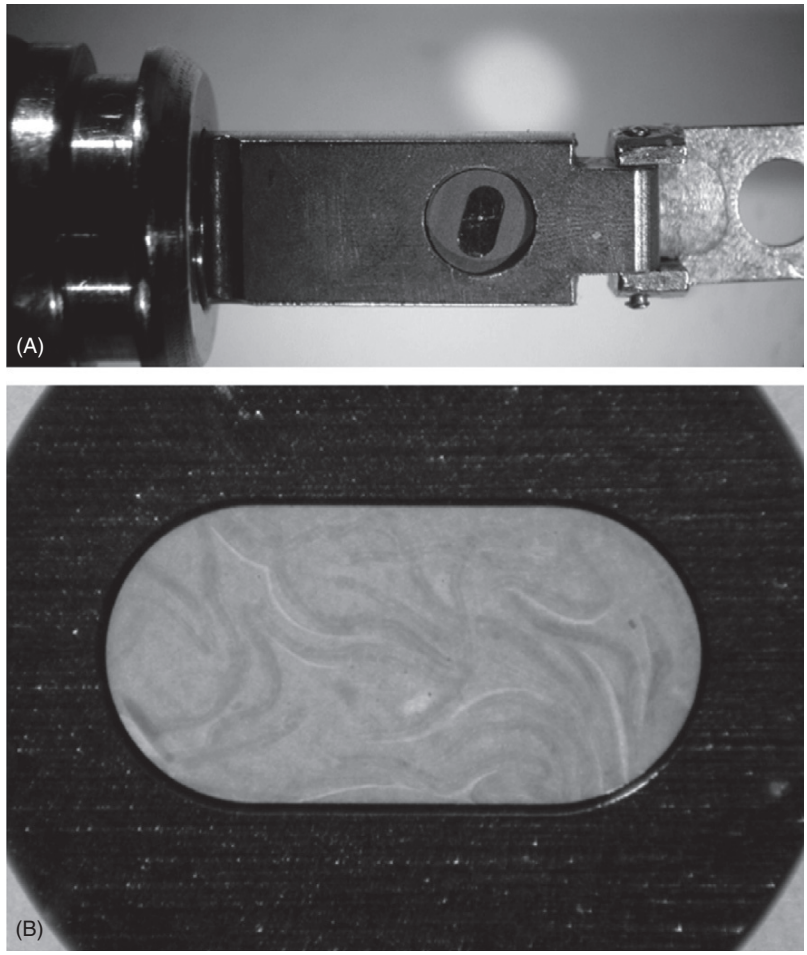

Fig. 5 Use of a slot grid thicker than the one shown in Fig. 4. (A) A slot grid in the tip prior to adding *C. elegans* worms in a paste of thick *Escherichia coli* in the slot. (B) After freezing, freeze substitution, and infiltration with resin, the worms are still trapped in the slot because the *E. coli* paste forms a solid mass after freeze substitution.

freezing and recover them for sectioning, in some cases sectioning the same cell that was observed in a light microscope. In the sections that follow we will describe the various ways in which TC cells can be prepared for high-pressure freezing and how to process them after that. We will discuss only TC cells grown on or adhered to substrates as opposed to those grown in solution and frozen in bulk. The substrates that can be used for high-pressure freezing of adherent TC cells include sapphire disks, Aclar® disks, filter membranes, HPF specimen cups, Formvar-coated gold grids, chips of Thermanox plastic Mylar® film, and microcarrier beads.

Solutions:

1. Sapphire Disks

a. History of Use Sapphire disks were first used to grow cells for high-pressure freezing by Schwarb (1990) in the Martin Mueller lab in Zurich, and later by Tse *et al*. (1997), Hess *et al*. (2000), Erlandsen *et al*. (2001), and Riepert *et al*. (2004) among others. They are preferred over glass coverslips because the coefficient of heat transfer is much greater at liquid nitrogen temperatures (Echlin, 1992). Before plating cells on them, it is best to coat with carbon or gold because cells often prefer these substrates, but more importantly, it is easy to scratch an asymmetric figure in the carbon/gold to indicate which side of the disk the cells are on.

b. Where to Buy Sapphire disks are available through Leica Microsystems at a cost of $510 per 100 for the 3 mm disks that fit the Wohlwend, Abra, and Leica HPM 100 high-pressure freezers, and $639 per 100 for the 1.4 mm disks that fit the Leica EM PACT instruments. Engineering Office M.Wohlwend GmbH (Bifig 14, CH-9466 Sennwald, Switzerland) sells the 3 mm size for either $344 per 100 (50 μm thick) or $373 per 100 (120 μm thick), and the 1.4 mm size for $442 per 100. One can also order directly from Rudolf Bruegger, S.A. in Minusio, Switzerland where ordering in bulk (500 or more) may save a little over the Wohlwend price. For example, for 500 pieces, the cost is $324 per 100.

c. Carbon or Gold Coating We use two different approaches to coating depending on whether we are planning to do correlative microscopy or not.

1. No correlative microscopy. Place the sapphire disks in a dish or other container that will fit into the coating device. Some evaporators have a metal plate with holes and slots for holding grids (Fig. 6A). We like to use the adhesive side of a Post-it® note to keep the disks separate yet put many in a small area. Evaporate enough carbon or gold onto the disk such that it will be easy to see in a stereomicroscope an asymmetric figure such as the letter "F" scratched into the coating.
2. Correlative microscopy. When using disks for this purpose it is useful to evaporate the pattern of an EM finder grid onto the surface. For the 3 mm disks used in ABRA HPM 010, Leica HPM 100, and Wohlwend Compact HPF 01 machines, this is straightforward because you just have to put an EM finder grid on top of the disk before evaporation. To keep the grid from sliding off the disk it is necessary to put them into a recess of some sort. This is where the EM grid holder plates that come with some evaporators come in handy. However, there are other easy solutions to this problem. If you have reasonable access to a machine shop, then they could make a grid holder by copying the type shown in Fig. 6A–B. Alternatively, one can use Teflon® immunostaining pads, such as Cat. No. 10526-1 from Ted Pella, Inc. (Fig. 6C). These have shallow conical depressions that will accommodate a sapphire disk and the covering finder grid nicely. There are many finder grid patterns available but we prefer those that have a relatively large "F" on the rim because it is easy to see in a stereomicroscope (e.g., EMS Cat. No. LF-135Cu; Fig. 6B and D).

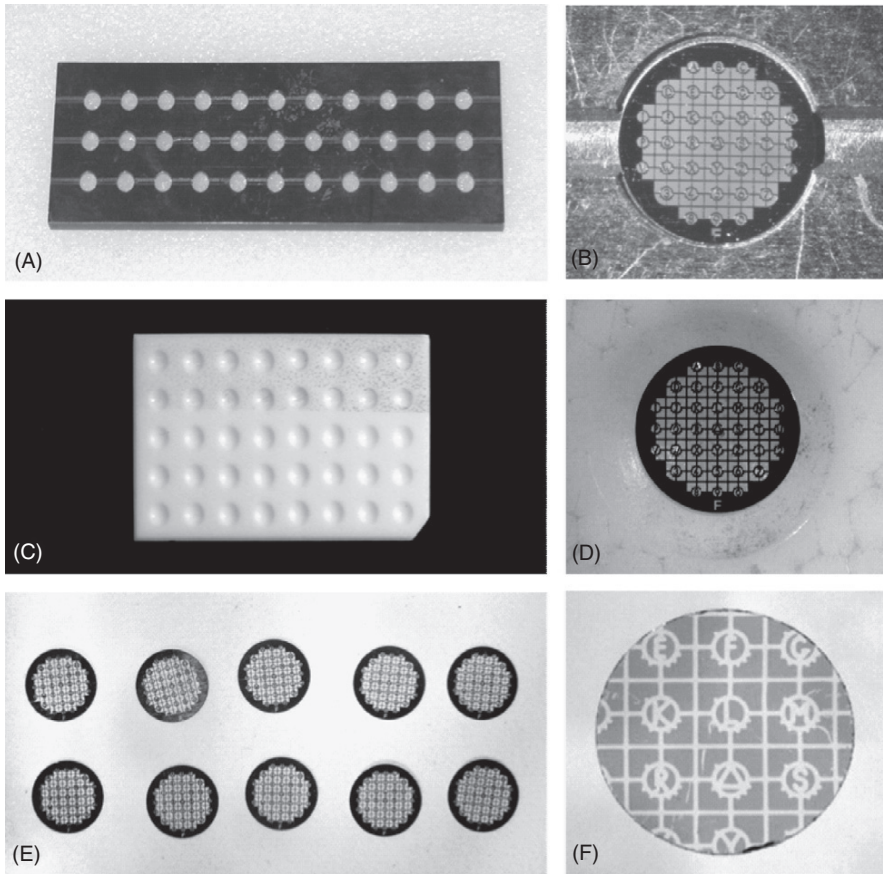

Fig. 6 Different ways to carbon coat sapphire disks with a finder grid pattern. (A) A brass grid holder with recessed holes for holding sapphire disks and finder grids. (B) Close-up of a recess shown in (A) showing the grooves on either side for picking up the sapphire disks/grids. (C) A Teflon® well plate with hemispherical depressions that can be used to hold sapphire disks and finder grids. Place a disk in the well and then put a finder grid on top. (D) Detail of (C). (E) A row of finder grids stuck down on the sticky part of a Post-it® note paper. Underneath each grid but not visible here are 1.4 mm sapphire disks used in the Leica EM PACT HPF machines. (F) The pattern on one of the 1.4 mm disks following carbon coating. Note that a heavy carbon coat is laid down, making it easier to see the pattern when handling the cells and in the resin after polymerization.

For sapphire disks in the Leica EM PACT system the approach is slightly different. Because the disks are only 1.4 mm in size they will not fit easily into one of the holders mentioned above. But here we can use the adhesive side of a Post-it® note again to hold not only the disk, but the finder grid also. First, put the sapphire disks on the adhesive about 5 mm apart. Then, place the finder grid over the disk (Fig. 6E) in a way that some asymmetric letters and/or numbers will be visible after coating. Fig. 6F shows one such pattern that is possible. With a gloved finger, press down the grid over the disk so that it sticks to the adhesive of the Post-it®.

Even if you are not going to do correlative microscopy on the same cell, using finder grids can have some advantages for processing TC cells. It can be a guide for remounting and identifying the best regions of the grid to section. This is illustrated in the Supplementary Materials file labeled SD Remount from Capsule.

d. Stabilizing the Carbon Prior to Plating Cells Before immersing the disks in medium it is recommended that the coating be stabilized so that it will not float off or curl in the liquid. For the gold, this step is not necessary. To stabilize, place the coated disks in a glass dish and bake overnight at 120°C. If the disks are to be used right away, there is no need to glow discharge them. If they are stored in a refrigerator at 4°C until use, they will remain hydrophilic for up to a month. Otherwise, glow discharge the disks before using them as cell substrates. The 3 mm size can be stored in a grid box, or on Post-it® note adhesive. For the smaller 1.4 mm size, the Post-it® note approach works, or you can store either type of disk in a Gel-Pak® box (Ted Pella, Inc., Cat. No. AD-22T-00-X8). These boxes are expensive, but they are reusable and very handy for not only sapphire disks, but also for specimen cups and other small items. The gels can be cleaned easily by stripping clean the surface with adhesive tape.

e. Culturing Cells Disks can be sterilized by ultraviolet radiation or heating in a microwave for 5 min at 800 W (Jimenez *et al*., 2006). Make sure that after the medium is added and inoculated with cells the disks have the coated side up. Also check to ensure that there are no air bubbles collecting around the edges of the disk that might cause the disk to rise and float at the surface of the medium. This is especially critical for the small Leica EM PACT disks. If this problem recurs then consider tacking the disk down with a small amount of agarose or petroleum jelly.

f. Attaching Cell Suspensions Another strategy for getting cells onto sapphire disks is to let them settle onto the disk and attach before freezing. Some cells will do this naturally, while others may need a favorable substrate, such as fibronectin, collagen, and Matrigel®. One can also use poly-L-lysine to attach cells (Sawaguchi *et al*., 2003).

g. High-Pressure Freezing How one goes about freezing sapphire disks depends on whether or not correlative microscopy is being done. Within the category of correlative microscopy it also depends on the time resolution required. By correlative microscopy we mean looking at a cell in the light microscope, then looking at the same cell by electron microscopy. If the cell needs to be frozen within 4–5 s of making observations, then the Leica EM PACT2 with Rapid Transfer System is the method of choice. This technique is covered in detail by Paul Verkade in his article using HPF for correlative LM–EM with about 5 s time resolution (Verkade, 2008). We would only add that there is a new rapid transfer system (RTS) loading device made by Marine Reef International that facilitates long observation times prior to freezing (Fig. 7). For those who may be interested in acquiring such a loader, they can be obtained from Marine Reef International by contacting Paul DeGeorge by email at pdegeorge@marinereef.com. The price is the same as the regular RTS loader from Leica.

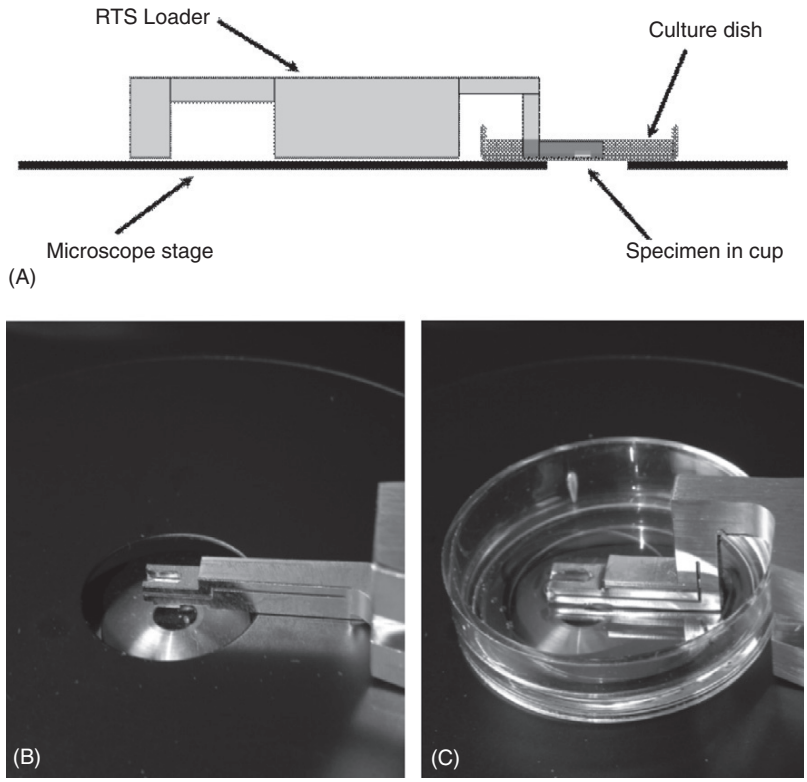

Fig. 7 A new loader for the Leica EM PACT2-RTS high-pressure freezer that allows longer incubation and observation times for cells prior to freezing. (A) Schematic representation of a loader in a culture dish on an inverted microscope stage. (B) The current RTS loader on a microscope stage. Observation times are rather short (5 min or so) because the tip is sitting on a coverslip (not shown) and the only fluid around the cells is what is trapped between the specimen carrier and the coverslip. (C) The new Marine Reef loader shown inside a culture dish on the microscope stage.

If there is no great need for speed of transfer from the light microscope to the high-pressure freezer, then it is possible to use the freezing methods described in the next sections. Except for the first method of putting disks inside the specimen cup, all the techniques described below pertain to the BAL-TEC, ABRA, or Wohlwend machines. For every method, the specimen cups, washers, and spacer grids should be coated with a release agent such as 1-hexadecene or 1% lecithin in chloroform before freezing. This will facilitate releasing the disks from the carriers under liquid nitrogen or at later stages in processing.

1. *Putting disks inside the specimen cup*. This is the method described by Reipert *et al*. (2004) for freezing 1.4 mm sapphire disks in the Leica EM PACT system. You remove the disk from the culture dish, dip it into 20% BSA solution made up with culture medium, load it into the cup, and freeze in a 100 μm deep membrane carrier.

If you like using the small sapphire disk you could also use any of the other HPF machines by loading into a 50 μm deep carrier and covering with a 25 μm deep carrier, or a flat carrier and a slot grid spacer.

2. *Sandwiching the disk between two flat carriers*. Place a carrier flat side up in the tip of the specimen loader. Remove the disk from the culture dish by picking it up from the sides with fine tweezers, and put it on top of the flat specimen carrier in the loader. Make sure the cells are facing up. You can either dip the disk in 20% BSA or use the culture medium alone. BSA will help the cells stay in place during subsequent processing because it forms a solid layer after freeze substitution. Place a Chien-style, 27 μm thick slot grid (see Fig. 4, and also McDonald *et al*., 2007) on top of the disk, then another specimen carrier flat side down. Close the tip of the loader and freeze immediately.
3. *Sandwiching two disks separated by a slot grid between spacer rings* (Hawes *et al*., 2007). BAL-TEC AG used to sell open washers that could be used to hold sapphire disks in the HPF specimen holder. Whether RMC or Leica Microsystems still sell these remains to be seen. To load, place a spacer ring in the tip of the HPF specimen loader, then a sapphire disk with cells up, a Chien-style slot grid, and then another sapphire disk with cell side down. Finally, place another spacer ring on top, close the tip of the loader, and freeze.

h. Freeze Substitution The thing to note about freeze substitution of TC cells on a substrate is that the substitution time can be drastically reduced compared to solid tissues. Hawes *et al*. (2007) describe a method that takes 6.5 h to reach −50°C at which point they infiltrate with Lowicryl HM20 for about 24 h, then another 4 days for complete polymerization of the resin. They believe the initial quick processing to the resin step results in improved ultrastructural preservation. Furthermore, only culture medium is used in freezing and the freeze substitution (FS) medium is acetone plus 2% or 0.2% uranyl acetate depending on whether immunolabeling is going to be performed. For more details consult the original paper by Hawes *et al*. (2007). For those preferring to use more traditional organic solvent-fixative combinations it is likely that shorter substitution times would also yield excellent results.

i. Embedding For any of the substrates used to grow TC cells it is necessary to embed them in resin and remove them after polymerization. There are different ways to do this, so in the following text we describe methods for using flat-bottomed capsules and embedding the disks in a thin layer of resin between Teflon®-coated microscope slides.

1. *Flat-bottomed capsule embedding*. Flat-bottomed capsules are available from Ted Pella, Inc. (Cat. No. 133-P) and perhaps other vendors. We prefer to use the stiff polypropylene capsules because they retain their shape when heated, especially in a microwave oven. To embed, place the capsules in a rack and put a piece of Aclar® plastic cut out with a paper punch in the bottom of each one. After labeling each capsule, fill partially with resin, then place the sapphire disk, cell side up, in the bottom of the capsule. Fill completely with resin and place in the oven for polymerization.

2. *Thin-film embedding of cells on disk substrates*. This method allows one to locate a particular cell as in the case of correlative LM–EM or to select a specific cell for its stage in mitosis. Once located, a given cell or group of cells can be cut from the thin film of resin and easily remounted in any orientation onto a blank resin block for sectioning. This technique is an adaptation of a method previously described (Reymond and Pickett-Heaps, 1983). The details of the procedure are as follows:

   a. Following freeze substitution and solvent rinses, gently wash disks (sapphire, Aclar®, or filter membranes) and remaining freezer hats out of the substitution vessel (cryovial) and into a small glass Petri dish or other glass dish. This can be done by holding the cryovial at a right angle to the glass dish and flushing the vial with acetone from a Pasteur pipette, taking care not to let the substrate with cells dry out at any time.
   b. After the disks are all transferred to a glass dish, it is best to work under a stereomicroscope. Remove the aluminum hats from the dish, leaving behind the substrates.
   c. Orient the disks so that the cells are "up" or are facing the operator. When the disks have been shadowed with a finder grid pattern prior to cell plating (see Section "Carbon or Gold Coating") this is easily done by observing the correct orientation of the letters. Otherwise, the cell side can be determined by making a small scratch on either surface to see if cells come off. When all of the disks are in the proper orientation, complete the infiltration of the samples with epoxy resin.
   d. Prepare microscope slides by coating with PTFE Release Agent Dry Lubricant (MS-143E liquid, or MS-122 spray, Miller-Stephenson, Danbury, Connecticut). Let the coating dry thoroughly (about 10 min) and then wipe the slide clean with a lab wipe.
   e. Using a forceps, remove the adhesive liner of a Secure-Seal Imaging Chamber (Electron Microscopy Sciences, Cat. No. 70327-20S, Hatfield, PA, USA) and apply the spacer, adhesive side down, onto the prepared slide and press gently. Remove the other adhesive liner.
   f. Use a forceps to transfer the disks from the resin bath to the chamber, carrying over some resin from the forceps. You may put 1–5 disks per chamber.
   g. Cover the chamber with a Thermanox® plastic coverslip, 22 mm diameter (Electron Microscopy Sciences, Cat. No. 72282, Hatfield, PA, USA), or a piece of 7.8 mil Aclar® film (Electron Microscopy Sciences, Cat. No. 50425-10, Hatfield, PA, USA) and transfer to the oven to polymerize for 48 h.

j. Remounting for Oriented Sectioning Depending on how the disks were embedded in the previous section, the remounting procedure will be different.

Disks in flat-bottomed capsules: The block is first removed from the capsule, and then resin is trimmed away from the edges of the sapphire disk. After suspending the trimmed capsule/disk in liquid nitrogen vapor for 30 s or so it is possible to separate the disk from the resin with a pair of forceps. If correlative microscopy was done and only

one cell is of interest, the block can be trimmed around that cell and sectioning can begin right away.

Disks in a thin layer of resin between slides:

a. Remove the slide chambers from the oven and cool completely to room temperature. Remove the Thermanox® or Aclar® cover and use a #11 scalpel blade to release the epoxy wafer (containing the sapphire disks) from the microscope slide.
b. Invert the wafer from its original orientation so that the cells are now facing "down". Use the scalpel blade to scrape off any resin from the back of the sapphire disk and work the scalpel around the edges of the sapphire disk to expose the edges of the disk. Gently pry the sapphire off the resin wafer. Often the disk will release in one piece but it may fracture into a few pieces. If using an Aclar® substrate (see Section III.F.2; Jimenez *et al*., 2006, 2009), simply cut away resin from the edges of the Aclar® and peel it off with forceps.
c. Once the sapphire disk/ Aclar® is removed, the cells remain in the resin at the very surface of the now-inverted wafer. If using the finder grid shadowed disks, the grid pattern will transfer to the resin wafer, as well. You may tape this wafer to a microscope slide and scan its surface to locate a cell of interest.
d. Using a #11 scalpel blade cut a small square of resin containing the relevant area and mount it onto a blank resin block using remount glue, Epoxy 907 adhesive (Miller-Stephenson, Danbury, Connecticut). Resin squares can be oriented for either cross sections or for en face sections.
e. If using filter membranes (Morphew and McIntosh, 2003) there is no need to remove them since they can be sectioned.

k. Sectioning Because TC cells are generally only a few microns thick, sectioning them en face can be challenging. However, there are ways of shaping the block face and orienting it to the knife edge that permits rather easy sampling of cells from their very bottom nearest the substrate on up to their topmost regions. Trim the block as a rectangle with an aspect ratio of about 3 to 1, with the small side about 350–400 μm in size. When aligning the block surface to the knife edge prior to cutting, tilt the top of the block slightly forward of vertical so that the knife encounters this area first when sections are beginning to cut. The tilt angle can be only a few degrees so as you section, the cells nearest the bottom of the section represent the bottom of the cell, and as you move toward the top of the section, you will be seeing areas that represent more upper regions of the cell.

2. Aclar® Disks

a. History In the mid-1960s, investigators were searching for an alternative to glass coverslips as a substrate for TC cells that would be embedded in resin for EM. While there were some earlier reports with different plastics, it was the discovery of Aclar® 33C (Honeywell, Morristown, NJ) by Masurovsky and Bunge (1968, 1989) that revealed the desirable properties of this plastic that is widely used today. They noted

that it was inert to chemicals routinely used for EM specimen preparation, that it was optically clear for light microscopy, including fluorescence, and that it stripped easily from polymerized resin without losing any cells.

Although Cook (2004) used Aclar® as a substrate for high-pressure freezing algal cells, it was Jimenez *et al*. (2006) who have fully explored the potential of this plastic for high-pressure freezing of cultured animal cells. We will summarize some aspects of their work in the sections that follow, but anyone interested in using Aclar® for HPF should consult this original paper.

b. Where to Buy Aclar® 33C is sold by most EM supplies vendors and it should be noted that it comes in two thicknesses: 2 and 7.8 mil. For many EM applications, researchers prefer the 7.8 mil thickness because it is less likely to curl and is easy to handle. However, for making high-pressure freezing disks it is important to use the 2 mil (51 μm) thickness.

c. Making Disks and Coating Jimenez *et al*. (2006) used an early model of the Wohlwend HPF machine (sold by Leica as the EM HPF) for freezing so the disks were 3 mm in diameter. They were punched out with a revolving punch plier available at hardware stores. To make 1.4 mm disks for the Leica EM PACT system may require a custom-made punch of that size. Most cells will attach and grow on Aclar® without any coating but some researchers prefer to put down fibronectin, collagen, or some other material that favors cell adherence and growth. As for carbon coating it is not necessary usually and if one wants to make an asymmetric figure in the plastic to show which side the cells are on, it is easy to scratch or cut the plastic for this purpose. In a more recent study (Jimenez *et al*., 2009), the authors have shown that, for correlative microscopy using the Leica EM PACT system, one can also prepare disks with the carbon-coated pattern of a finder grid.

d. Culturing Cells on Disks A big problem with small Aclar® disks is their tendency to float in culture medium. To prevent this, Jimenez *et al*. (2006) used a hot wire to tack the disks to the bottom of plastic culture dishes. One may also be able to use a small dab of vacuum grease, agarose, or some other inert sticky substance to affix the cells to the bottom of the dish. This group also used microwave irradiation for 5 min at 800 W for sterilization.

e. High-Pressure Freezing and Freeze Substitution HPF can be carried out as described above in Sections 1.g.1 and 1.g.2 for sapphire disks. Jimenez *et al*. (2006) use 20% dextran as a cryoprotectant, but we prefer 20% BSA because, in our experience, dextran becomes brittle after freeze substitution and is more difficult to section. Freeze substitution could also be carried out as above for sapphire disks.

f. Embedding, Remounting, and Sectioning As above, except that removing the Aclar® disk is done simply by peeling it off. Just trim around the edge with a razor blade to expose the Aclar®–resin interface, insert a forceps under the Aclar®, and lift it off.

3. Other Methods

Most of the other methods for freezing TC cells can be found in published accounts so there is little need to reprint the details here. See the following techniques and references: filter membranes (Morphew and McIntosh, 2003); HPF specimen cups (Sawaguchi *et al*., 2003); Formvar-coated gold grids (Ladinsky *et al*., 1999); chips of Thermanox® plastic (Marsh *et al*., 2001); Mylar® film (McEwen *et al*., 1998); and microcarrier beads (Hagen, C., and Grünewald, 2008).

G. Separating Frozen Tissues from HPF Carriers under Liquid Nitrogen or during Infiltration and Embedding

Problem: Some cell types and/or fillers make it difficult to separate the biological sample from the specimen cup following HPF. Attempts to "dig out" the sample with forceps or other tools while in LN_2 can break the sample into small pieces. Failing to remove the sample may mean it has to be embedded in the metal carrier and separated after resin polymerization.

Solution: Early HPF work was often done with freeze fracture in mind and some special carriers were designed to create a "dome" of frozen material that could then be fractured. Coating the top piece of the HPF sandwich with lecithin dissolved in chloroform made it possible to remove it cleanly under liquid nitrogen, leaving the biological material in the bottom piece (Craig *et al*., 1987). For today's carriers that are like simple cups, one can coat them with either lecithin or 1-hexadecene, and that aids in the removing the material from the cup in one piece under liquid nitrogen.

1. For two-piece Wohlwend-style carriers, coat both the cup and the top with a thin layer of 1-hexadecene prior to filling and freezing. Flat-sided carriers used as a "top" piece can be put flat-side down on a piece of filter paper saturated with 1-hexadecene and blotted just before putting onto the bottom piece and freezing. Use a small paint brush or similar tool to coat the insides of cups, then blot dry with paper.
2. For Leica membrane carriers, cups, or biopsy carriers, coat with 1-hexadecene and blot dry prior to filling and freezing.
3. After HPF and while under liquid nitrogen you can try to tease out the sample by gently inserting the tip of a needle, or the tip of a number 11 scalpel blade at the edge of the cup to dislodge the sample. This works best for whole pieces of tissue such as plant leaves or disks of cartilage. For many samples it may be best to proceed with the freeze substitution and try removing the sample during the resin infiltration steps. It is not uncommon for some types of samples, such as pellets of cells, or samples in 20% BSA to come out of the cups during freeze substitution, especially if there is some agitation of the tubes.
4. If you prefer to try lecithin, you can usually find lecithin powder at health food stores. Make a 100 mg/ml solution in chloroform, then paint it onto the surfaces that you want to release from the sample, and let it dry. Fill with material and freeze. Remove sample from the carriers as above for 1-hexadecene.

H. Freezing Brain and Other Soft Materials, or Pathogenic Samples

Problem: Some tissues, brain being one of the best examples, are so flaccid that taking out a small piece for freezing is extremely difficult without creating mechanical damage. A biopsy needle may be the best approach, but not everyone has them in the small size necessary, and even with this tool there may be damage.

We are also confronted at times with users who want to high-pressure freeze pathogens of one kind or another. This poses all kinds of risks, not least the fact that any material on the outside of the HPF carrier may be aerosoled in the LN_2 exhaust.

Solution: Fix the tissue before freezing. In particular, brain and other animal organ tissues should be perfusion fixed, not by dissection and immersion methods. After fixation, the tissue will harden significantly and it will be easier to take the smaller pieces necessary to fit into HPF specimen carriers. Use of a biopsy system after fixation should also be a valid approach. A relatively recent paper by Sosinsky *et al*. (2007) provides good examples of what can be achieved with this hybrid approach.

For pathogens, especially certain bacteria, do not assume that overnight fixation in glutaraldehyde will kill the cells. Have the researcher plate out cells after fixation to see if they are still viable. One of us (KM) has had the experience that TB cells (*Mycobacterium tuberculosis*) were fixed overnight in 2% glutaraldehyde but some remained viable the next day. For additional containment of cells, it may also be a good idea to concentrate them in sealed microdialysis tubing for freezing and subsequent handling. Finally, instead of fixing them in just glutaraldehyde prior to freezing, use a cocktail containing glutaraldehyde and a small percentage of osmium as the primary fix. After 20–30 min in this cocktail, transfer to glutaraldehyde fix only for the remaining fixation time.

I. Using Agarose as a Filler

Problem: Many cell types are very difficult to freeze. One of the reasons for this can be finding a filler to surround the cells during the freezing process that does not upset the cells at all. Agarose can be used as a very effective filler in low concentrations and most cell types do not seem to be affected by being suspended in it. Low-gelling-temperature agarose (e.g., Sigma A9045) will remain perfectly liquid at 37°C and exhibit great clarity allowing easy visualization of the sample within the gel even after freeze substitution. It will also hold the cells together during subsequent processing such as freeze substitution. The agarose has the ability to bind a high percentage of the water mainly through hydrogen bonding and so changes the freezing properties of this water. It is easy to test if cells are at all affected by the agarose by growing them in media containing agarose and observing if they grow and behave as normal. Percentages between 0.7 and 1% are sufficient to be used as a filler. It can be used as a filler in the place of hexadecane or yeast paste in all situations, e.g., surrounding cellulose microdialysis capillary tubing.

Solution:

1. It is important to keep all equipment coming in contact with the agarose warm to prevent it gelling prior to freezing. A heating block and a warming platform (or a microscope heating stage) can be used for this. Micropipette tips are stored in the heating block. The end of the rapid loader for the Leica EMPACT or the loading arm for the BalTec HPM010 or Wohlwend is rested on to the platform to keep it warm and the freezing hats or carriers are stored on the platform.
2. Make up agarose in the culture media in which the cells are growing. If using cells growing on sapphire or Aclar disks, use 1%, while if using free growing cells, use 2% as there will still be some liquid remaining with the cell pellet, which will dilute this down.
3. Melt agarose and keep at 37°C in heating block.
4. If cells are growing on disks, place a drop of molten agarose in a Petri dish on the warming platform. Remove the disk from the growth media and place it into the warmed agarose dipping it a couple of times to ensure it is surrounded by agarose. Place the disk into the freezing hat and adjust the level of liquid with paper points.
5. If the cells are free then they should be spun gently into a pellet. They should then be put into the heating block to keep warm and the supernatant removed. A volume of agarose equal to the size of the pellet should be added and this mixed thoroughly. A volume of this mixture is then added to the freezing hats or carriers.

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The authors want to thank their colleagues in the high-pressure freezing community for the many fruitful discussions about techniques over the years. The origins of some of these "tips and tricks" are somewhat obscure by now and we are sure that many have been developed independently in different labs. We have tried to identify the origins as best we can, but no doubt some citations have been missed and we apologize to those who may have been unintentionally left out.

References

Cook, M. E. (2004). Cytokinesis in *Coleochaete orbicularis* (Charophyceae): An ancestral mechanism inherited by plants. *Am. J. Bot*. **91**, 313–320.

Craig, S., Gilkey, F. C., and Stähelin, L. A. (1987). Improved specimen support cups and auxiliary devices for the balzers high pressure freezing apparatus. *J. Microsc. (Oxford)* **148**(1), 103–106.

Echlin, P. (1992). "Low Temperature Microscopy and Analysis." Plenum, NY. (pp. 568).

Erlandsen, S. L., Bittermann, A. G., White, J., Leith, A., and Marko, M. (2001). High-resolution cryoFESEM of individual cell adhesion molecules (CAMs) in the glycocalyx of human platelets: Detection of P-selectin (CD62P), GPI-IX complex (CD42a/CD42bα,bβ), and integren GPIIbIIIa (CD41/CD61) by immunogold labeling and stereo imaging. *J. Histochem. Cytochem*. **49**, 809–819.

Hagen, C., and Grünewald. (2008). Microcarriers for high-pressure freezing and cryosectioning of adherent cells. *J. Microsc*. **230**, 288–296.

Hawes, P., Netherton, C. L., Mueller, M., Wileman, T., and Monaghan, P. (2007). Rapid freeze-substitution preserves membranes in high-pressure frozen tissue culture cells. *J. Microsc*. **226**, 182–189.

Hess, M. W., Mueller, M., Debbage, P. L., Vetterlein, M., and Pavelka, M. (2000). Cryopreparation provides new insight into the effects of brefeldin A on the structure of the HepG2 Golgi apparatus. *J. Struct. Biol*. **130**, 63–72.

Hohenberg, H., Mannweiler, K., and Mueller, M. (1994). High-pressure freezing of cell suspensions in cellulose capillary tubes. *J. Microsc*. **175**, 34–43.

Jimenez, N., Humbel, B. M., van Donselaar, E., Verkleij, A. J., and Burger, K. N. (2006). Aclar discs: A versatile substrate for routine high-pressure freezing of mammalian cell monolayers. *J. Microsc*. **221**, 216–223.

Jimenez, N., van Donselaar, E. G., de Winter, D.A.M., Vocking, K., Verkleij, A. J., and Post, J. A. (2009). Gridded aclar: Preparation methods and use for correlative light and electron microscopy of cell monolayers, by TEM and FIB-SEM. *J. Microsc*. **237**, 208–220.

Kaech, A. (2009). BAL-TEC HPM 010 high-pressure freezing machine. *In* "Handbook of Cryo-Preparation Methods for Electron Microscopy" (Cavalier, A., Spehner, D., and Humbel, B. M., eds.) pp. 101–128. CRC Press, Boca Raton, FL.

Ladinsky, M. S., Mastronarde, D. N., McIntosh, J. R., Howell, K. E., and Staehelin, L. A. (1999). Golgi structure in three dimensions: Functional insights from the normal rat kidney cell. *J. Cell Biol*. **144**, 1135–1149.

Marsh, B. J., Mastronarde, D. N., Buttle, K. F., Howell, K. E., and McIntosh, J. R. (2001). Organellar relationships in the Golgi region of the pancreatic beta cell line, HIT-T15, visualized by high resolution electron tomography. *Proc. Natl. Acad. Sci. U.S.A*. **98**, 2399–2406.

Masurovsky, E. B., and Bunge, R. P. (1968). Fluoroplastic coverslips for long-term nerve tissue culture. *Stain Technol*. **43**, 161–165.

Masurovsky, E. B., and Bunge, R. P. (1989). Aclar film in biological electron microscopy. *J. Electron Microsc. Tech*. **12**, 172–173.

McDonald, K. (1994). Electron microscopy and EM immunocytochemistry. *In* "*Drosophila melanogaster*: Practical Uses in Cell and Molecular Biology" (L.S.B. Goldstein and E. Fyrberg, eds.). *Methods Cell Biol*. **44**, 411–444.

McDonald, K. L. (1999). High pressure freezing for preservation of high resolution fine structure and antigenicity for immunolabeling. *Methods Mol. Biol*. **117**, 77–97.

McDonald, K. L. (2007). Cryopreparation methods for electron microscopy of selected model systems. *Methods Cell Biol*. **79**, 23–55.

McDonald, K. L., Morphew, M., Verkade, P., and Müller-Reichert, T. (2007). Recent advances in high pressure freezing: Equipment and specimen loading methods. *Methods Mol. Biol*. **369**, 143–173.

McDonald, K., and Müller-Reichert, T. (2002). Cryomethods for thin section electron microscopy. *In* "Guide to Yeast Genetics and Molecular and Cell Biology, Parts B and C." (C. Guthrie, and G. Fink, eds.). *Methods Enzymol*. **351**, 96–123.

McDonald, K. L., Sharp, D. J., and Rickoll, W. (2000). Preparing thin sections of *Drosophila* for examination in the transmission electron microscope. *In* "*Drosophila*: A Laboratory Manual" (W. Sullivan, M. Ashburner, and S. Hawley (eds.) 2nd edn., pp. 245–271, CSHL Press, Cold Spring Harbor, NY.

McEwen, B. F., Hsieh, C. E., Mattheyes, A. L., and Rieder, C. L. (1998). A new look at kinetochore structure in vertebrate somatic cells using high-pressure freezing and freeze substitution. *Chromosoma* **107**, 366–375.

Morphew, M. K., and McIntosh, J. R. (2003). The use of filter membranes for high-pressure freezing of cell monolayers. *J. Microsc*. **212**, 21–25.

Müller-Reichert, T., Mantler, J., Srayko, M., and O'Toole, E. (2008). Electron microscopy of the early *Caenorhabditis elegans* embryo. *J. Microsc*. **230**, 297–307.

Müller-Reichert, T., O'Toole, E. T., Hohenberg, H., and McDonald, K. L. (2003). Cryoimmobilization and three-dimensional visualization of. *C. elegans* ultrastructure. *J. Microsc*. **212**, 71–80.

Müller-Reichert, T., Srayko, M., Hyman, A. A., O'Toole, E., and McDonald, K. (2007). Correlative light and electron microscopy of early *Caenorhabditis elegans* embryos in mitosis. *Methods Cell Biol*. **79**, 101–119.

Reipert, S., Fischer, I., and Wiche, G. (2004). High-pressure freezing of epithelial cells on sapphire coverslips. *J. Microsc*. **213**, 81–85.

Reymond, O. L., and Pickett-Heaps, J. D. (1983). A routine flat embedding method for electron microscopy of microorganisms allowing selection and precisely orientated sectioning of single cells by light microscopy. *J. Microsc*. **130**, 79–84.

Sawaguchi, A., Yao, X., Forte, J. G., and McDonald, K. L. (2003). Direct attachment of cell suspensions to high-pressure freezing planchettes. *J. Microsc*. **212**, 13–20.

Schwarb, P. (1990). Morphologische grundlagen zur zell-zell interaction bei adulten herzmuskelzellen in kultur. Ph.D. thesis, Swiss Federal Institute of Technology, Zurich.

Sosinsky, G. E., Crum, J., Jones, Y. Z., Lanman, J., Smarr, B., Terada, M., Martone, M. E., Deerinck, T. J., Johnson, J. E., and Ellisman, M. H. (2007). The combination of chemical fixation procedures with high pressure freezing and freeze substitution preserves highly labile tissue ultrastructure for electron tomography applications. *J. Struct. Biol*. **161**, 359–371.

Studer, D., Graber, W., Al-Amoudi, A., and Eggli, P. (2001). A new approach for cryofixation by high-presure freezing. *J. Microsc*. **203**, 285–294.

Tiedemann, J., Hohenberg, H., and Kollmann, R. (1998). High-pressure freezing of plant cells cultured in cellulose microcapillaries. *J. Microsc*. **189**, 163–171.

Triffo, W. J., Palsdottir, H., McDonald, K. L., Lee, J. K., Inman, J. L., Bissell, M. J., Raphael, R. M., and Auer, M. (2008). Microaspiration for high pressure freezing: A method for ultrastructural preservation of fragile and sparse tissues for TEM and electron tomography. *J. Microsc*. **230**, 278–287.

Tse, F. W., Tse, A., Hille, B., Horstmannn, H., and Almers, W. (1997). Local Ca^{++} release from internal stores controls exocytosis in pituitary gonadotrophs. *Neuron* **18**, 121–132.

Vanhecke, D., and Studer, D. (2009). High pressure freezing Leica EM PACT. *In* "Handbook of Cryo-Preparation Methods for Electron Microscopy" (Cavalier, A., Spehner, D., and Humbel, B. M., eds.) pp. 129–156. CRC Press, Boca Raton, FL.

Verkade, P. (2008). Moving EM: The rapid transfer system as a new tool for correlative light and electron microscopy and high throughput for high pressure freezing. *J. Microsc*. **230**, 317–328.