SECTION 1

Basic Transmission and Scanning Electron Microscopy

CHAPTER 1

High Pressure Freezing and Freeze Substitution of Schizosaccharomyces pombe and Saccharomyces cerevisiae for TEM

Stephen Murray
TEM Service Facility
Paterson Institute for Cancer Research
University of Manchester
Manchester, United Kingdom

Abstract

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Abstract

The use of standard room temperature chemical fixation protocols for the ultrastructural preservation of yeast and subsequent observation under the electron microscope is fraught with difficulties. Many protocols require the use of enzymatic digestion of the cell wall in order to facilitate the entry of fixatives into the cell interior. Others rely on the use of permanganate-based fixative solutions, which whilst enabling overall preservation of the cell, does require multiple centrifugation, washing, and resuspension steps. This often results in the significant loss of sample volume whilst the use of permanganate can cause extraction of cytoplasmic components. The use of low temperature techniques and in particular high pressure freezing (HPF) and freeze substitution (FS) overcomes many of these problems.

With the recent advances in cryotechnologies and in particular the development of commercially available equipment such as the high pressure freezer, the level of ultrastructural preservation attainable in electron microscopy has increased markedly. It is now possible to capture dynamic time sensitive events and to place them in their ultrastructural context with a level of resolution which at the present time can only be achieved with electron microscopy.

I. Introduction

The use of high pressure freezing (HPF) and freeze substitution (FS) as a method of preserving yeast can now be considered a mainstream technique. As with all procedures in electron microscopy, there are numerous HPF and FS protocols published in the scientific literature, with each individual worker having a preference for a particular processing regime. Some are designed with one specific aim e.g. preservation of epitopes (Monaghan and Robertson, 1990; Monaghan et al., 1998; Neuhaus et al., 1998) while others would seem to be counterintuitive e.g. addition of water to the FS cocktail (van Donselaar, et al., 2007; Walther and Ziegler, 2002). All of the protocols have one single aim, the preservation of the tissue or cell being investigated as close to the native state as possible. The ultimate is of course to observe the sample in a frozen fully hydrated state with out addition of chemical fixatives (Al-Amoudi et al., 2004; McDowall et al., 1983, 1984; Michel, 1991). However, for the purposes of this chapter, the author deals purely with a HPF and FS protocol he has found to work consistently well with both wild type and mutant strains of *Schizosaccharomyces pombe* and *Schizosaccharomyces cerevisae*.

II. Materials and Instrumentation

HPF was performed using a Bal-Tec HPM010 high pressure freezer (Bal-Tec AG Principality of Liechtenstein) and using interlocking brass hats as the specimen carrier (Swiss Precision, Inc., Palo Alto, CA, USA). Subsequent FS was performed
using an automatic FS unit (Leica AFS; Leica Microsystems, Vienna) with the FS solution being contained in 1.5-ml conical Eppendorf centrifuge tubes (catalogue 0030 120.086; Eppendorf UK, Ltd., Cambridge, UK).

Yeast cultures were vacuum concentrated onto 0.45 μm membrane filters (catalogue # VLP02500; Millipore UK Ltd., Watford, UK) using a KNF Laboport N86KT.18 dry vacuum pump (KNF Neuberger UK Ltd.) and Sartorius suction flask and 25 mm glass filter holder assembly (catalogue 16672 & 16306; Sartorius Ltd., Epsom, UK). To facilitate quick vacuum release, an in line Edwards AV10K manual air admit valve and T-piece assembly were placed between the vacuum pump and the suction flask. An image of the filtration system can be seen in Fig. 2. To aid sample loading into the specimen carriers, any standard stereo zoom dissecting microscope and cold light source may be used. Removal of the yeast sample from the filter and its subsequent loading into the brass hat was achieved using a pointed cocktail stick. Fig. 1 shows additional useful tools for the handling, manipulation, removal, and separation of the sample carriers following HPF include two pairs of Dumont Dumoxel #3 medical grade tweezers catalogue T5272 (Agar Scientific, Essex, UK) used to manipulate the brass hats and load them into the specimen rod. Two flat bladed screwdrivers with fine tips, CK Xonic 4880X, one with 1.5 mm tip, the other with 2.5 mm tip (catalogue 2508619214 & 2508618794; RS Components, Corby, UK). The screwdrivers are used to pry the brass hat assembly apart once frozen.

![Fig. 1](image-url)  
**Fig. 1** Tools used during loading and manipulation: (1) Dumont No. 3 forceps, (2) Pointed applicator sticks for paste scraping/loading, (3) Interlocking brass sample carriers, (4) Fine blade screwdrivers for separating hats following freezing, (5) Insulated cryoforceps for handling frozen sample, (6) Dumont No. 6 forceps for pushing sample carrier from specimen rod, (7) Millipore membrane filter 0.45 μm, 22 mm.
A pair of Dumont Dumostar #6 medical grade tweezers (catalogue T5277; Agar Scientific, Essex, UK) is useful to push the frozen brass hat assembly from the HPF specimen rod should it be required. The frozen samples are placed into prelabeled 1 ml CryoPlus tubes held in the work chamber of the HPF (catalogue 72.377;
Sarstedt, Leicester, UK). In order to hold the vials and allow easy attachment of the lids, the author has found it invaluable to use a Sarstedt Cryorack 40 cut to size and fixed firmly to the bottom of the HPF cryo work chamber (see Fig. 3) using Araldite or epoxy resin (catalogue 93.856.040; Sarstedt, Leicester, UK).
Other chemicals and consumables mentioned in this article are commonly available from electron microscopy supply companies.

III. Procedures

A. Preparation of Sample Holders
1. Clean the Brass Hats as Follows
   - Ultrasonicate for 5 min in acetone
   - Rinse in double distilled water
   - Ultrasonicate in 70% ethanol
   - Dry using a hair dryer
   - Place in a plastic Petri dish lined with hardened filter paper ready for use

B. Filtration of Yeast Sample
The cells are grown to mid log phase equivalent to 0.4–1.0 \times 10^7 \text{ cells/ml}. At this density approximately 50–100 ml of culture will be required to produce a paste which should be slightly glossy and have an apple sauce consistency. It should be noted that the degree of filtration required to produce the paste varies from strain to strain. If upon removal of the membrane filter the paste is found to be too wet, excess moisture can be removed by carefully dabbing the underside of the membrane filter onto hardened filter paper. If the paste is too dry, nothing can be done. Too much fluid in the paste will result in poor freezing, too little will result in poor FS and embedding.

1. Place a fresh membrane filter into the filter assembly. Turn on the vacuum pump and ensure that the vacuum release valve is fully closed.
2. Pour the yeast culture into the filter assembly and top up as required.
3. At the moment the last visible trace of solution disappears, turn off the pump and open the vacuum release valve.
4. Unclamp the filter funnel and remove the membrane filter.
5. Using a cocktail stick carefully scrape some of the paste from the filter and load it into the bottom of the brass hat pair. The hat should be overfilled and no air bubbles should be seen. Air bubbles will result in poor freezing.
6. Place the top part of the brass hat onto the bottom and press down using the forceps.
7. Quickly place the complete assembly into the specimen rod of the HPF and clamp into position.
C. High-Pressure Freezing of the Sample

Following filtration, the filling of the specimen carrier and the loading and freezing of the sample should take between 20 and 30 s. While this procedure can be carried out by a single operator, it is much better to have an additional person to assist in the filtration and sample loading of the brass hats. Additionally, the HPF sample rod will need to be defrosted between each freezing cycle. The author has found the purchase of an additional specimen rod invaluable in speeding up the process. While one rod defrosts and dries out, the other can be put into use. All tools used subsequent to the freezing process must be precooled to LN2 temperature before handling the sample.

1. Insert the specimen rod into the freezing chamber of the HPF and secure with the locking bolt.
2. Initiate the freezing cycle by pressing the “Jet” button.
3. As soon as the Jet button has been pressed, allow a couple of seconds to pass before removing the locking bolt and rapidly transferring the specimen rod into the cryo work chamber of the HPF.
4. Allow the end of the rod to rest for a few seconds in the liquid nitrogen before unclamping the specimen holder.
5. Under nitrogen and on the metal working platform of the cryowork chamber, push the brass hat from the specimen holder using the precooled No. 6 forceps if necessary.
6. Using the two precooled screwdrivers, separate the two halves of the brass hat by prising them apart. Take care at this stage, it is very easy to apply too much pressure and catapult the brass hat out of the cryowork station.
7. Place the separated halves of the specimen carrier into the relevant prelabeled vial using precooled No. 6 forceps.
8. Once all samples have been frozen place the lids on the cryovials and transfer them either to a storage Dewar or the FS unit.

D. Freeze Substitution of Yeast

After much experimentation, the author has found that the addition of water to the FS cocktail has resulted in the optimal preservation of his particular samples. It was of particular importance that the nuclear membranes, the spindle pole bodies and microtubules be well delineated and preserved. The protocol which follows fulfills these requirements and has the added bonus that subsequent staining of ultrathin sections requires the use of Reynolds lead citrate only (Reynolds, 1963).

1. Programming of the AFS
   1. Set the first temperature step to $-90 \degree C$ and the time to 72 h
   2. Set the first slope to $+5 \degree C/h$
1. High Pressure Freezing and Freeze Substitution

3. Set the second temperature step to $-20 \, ^\circ C$ and the time to 2 h
4. Set the second slope to $+5 \, ^\circ C/h$
5. Set the final temperature step to $+4 \, ^\circ C$ and the time to 4 h
6. Place the AFS into pause mode and allow the temperature to reach $-90 \, ^\circ C$

2. Prepare the Freeze Substitution Cocktail

1. Prelabel the 1.5 ml Eppendorf tubes as required. Up to ten 1.5 ml Ependorfs can be accommodated at one time.
2. Dissolve 0.2 g of crystalline osmium tetroxide in 9.5 ml of acetone.
3. Add 500 ml of 2% aqueous uranyl acetate to the acetone/osmium mix.
4. Dispense 1 ml into each of the Eppendorfs.
5. Place the Eppendorfs into the AFS and allow the temperature to equilibrate.
6. Due to the toxicity of osmium tetroxide and uranyl acetate Steps 2–4 of the above must be carried out in a fume hood.

3. Transfer of Specimens to AFS

1. Place the cryovials containing the samples into a small transfer container filled with liquid N$_2$.
2. Using precooled No. 3 forceps, quickly transfer the specimen carrier from the cryotube to the relevant pre labeled Eppendorf tube in the AFS.
3. Once all samples have been placed into the AFS, cancel the pause function to begin FS cycle.

4. Resin Infiltration and Embedding of Samples

During FS, the samples will readily separate from the specimen carrier. After 2 h at $+4 \, ^\circ C$, the FS cocktail is rinsed out with fresh acetone. This can be done with the Eppendorf carrier in the AFS or, it can be transferred to a fume hood and placed over ice.

1. Wash the specimens three times with fresh acetone over a 1 h period at $+4 \, ^\circ C$.
2. During the third wash, remove the empty specimen carriers from the Eppendorfs.
3. Carefully transfer each sample from the Eppendorf into a prelabeled 7-ml glass vial containing 2 ml of acetone. The transfer can be effected either by using fine point forceps (No. 3) or a wide bore disposable plastic Pasteur pipette.
4. Infiltrate the sample with Spurr’s (Spurr, 1969) resin/acetone mixtures. If possible, degas the resin under vacuum prior to dilution with acetone
   - 1:7 resin/acetone 3 h
   - 1:3 resin/acetone overnight
• 1:1 resin/acetone 4 h
• 3:1 resin/acetone 4 h or overnight
• 100% resin 4 h (with no lid on sample vials)
• 100% resin 4 h (under vacuum if available)
• 100% resin overnight (under vacuum if available)
• Place a small volume of fresh resin into prelabeled Beem capsules
• Carefully transfer each sample from the glass vial to the Beem capsule using a pointed wooden stick or wide bore plastic Pasteur pipette and centralize in the capsule
• Fill the capsules with resin
• Polymerize the resin for 48 h at 60 °C or 24 h at 70 °C under vacuum if possible.

5. Once polymerized trim a standard trapezoid block face and proceed to cut 50–70-nm ultrathin sections and collect on formvar/carbon coated grids.
6. Post stain the sections for 5 min using Reynold’s lead citrate in the presence of sodium hydroxide pellets.
7. Wash sections to remove stain.
8. Carefully remove excess water and allow to dry.
9. Image under the TEM at 80 kV.

IV. Comments and Problems

It has been reported that the presence of water in the FS medium in excess of 1% severely reduces the ability of acetone to replace the sample ice (Humbel and Mueller, 1986). Certainly, the addition of water to the FS medium would seem counterintuitive, but it has been demonstrated in numerous publications that addition of water to the FS medium dramatically enhances the visibility of membranes and in particular the nuclear envelope in yeast (Fig. 4). It is possible to achieve improved membrane visibility using other protocols (Giddings, 2003), but this often requires the use of two different FS mediums and has the considerable challenge of washing and replacement steps being carried out at −90 °C.

It has been reported that the use of epoxy resin as a fixative during FS of Caenorhabditis elegans resulted in a more complete preservation of cellular proteins and membranes (Matsko and Mueller, 2005). Results with yeast show some improvement in membrane visibility (Fig. 5) when compared to a standard osmium/acetone protocol (Fig. 6), but are not as remarkable as those in which water is added to the FS medium (Fig. 4). It should also be noted that the concentration of water in the FS medium is important. There appears to be virtually no difference in terms of membrane visibility in yeast when only 1% water is added, compared to FS medium containing no water (Fig. 6). Another intriguing observation is that in the presence of high water concentration (10%) S. cerevisiae appears to be well
preserved while *S. pombe* is very poorly preserved and shows distinct ice damage (Fig. 7), which would seem to imply that effects of water addition are not only concentration dependant but also sample dependant. It is recommended that a water concentration of 5% be used initially and if necessary adjustments made during subsequent FS runs.

One common artifact induced by HPF particularly when freezing yeast is the rupture of the cell wall and/or the nuclear envelope (Fig. 8). In the author’s experience, this occurs in 5–10% of yeast cells both wild type and mutant (an observation also made by M. Morphew, personal communication).
1. Safety

It is extremely important when using cryogenic equipment that the manufacturer’s recommendations and safety instructions are followed. It is essential that the safe handling and use of liquid nitrogen and a thorough understanding of the very specific hazards associated with it are clearly understood before any work commences. Many of the chemicals used for FS and embedding are at the very least irritants and many are extremely toxic and even carcinogenic. They should therefore always be handled in a fume hood with the operator wearing suitable protective clothing and gloves. When using resins, standard latex or nitrile gloves do not afford sufficient protection. Instead, the use of vinyl gloves is recommended. Once contaminated, gloves should be changed immediately. The exhaust from the AFS should be placed into the fume hood. Care should be exercised when handling the polymerized resin blocks. If it is necessary to file or hacksaw the blocks, the operator should wear gloves and a mask and all debris and dust removed immediately.

2. Vacuum Concentration of the Yeast Culture and Loading of Specimen Carriers

It is very important that the yeast paste produced by vacuum filtration is neither too wet nor too dry. The ideal paste should have an apple sauce consistency. When scraping the paste from the Millipore filter, care should be taken not to rip it with the tip of the cocktail stick. When loading the sample carriers, it is essential for good freezing that no air pockets are present in the sample or the carrier. The paste will dry very quickly so speed is important when loading the sample carrier. The paste is also quite sticky and during handling with forceps, the specimen carriers will quite happily attach themselves to the tips. This can provide the operator with minutes
of endless amusement as they try to place the sample into the specimen freezing rod. It is best to have two pairs of forceps in order to overcome such difficulties.

3. High Pressure Freezing with the Bal-Tec HPM010

- Ensure that the alcohol reservoir contains sufficient isopropanol for the run.
- Ensure that the instrument has been thoroughly dried by running the air function for at least an hour prior to cool down.
Ensure that the compressor outlet pressure and alcohol pressure settings are correct.

Ensure that there is sufficient LN2 in the supply dewar for the instrument cool down and freezing run.

Ensure that the output pressure from the supply dewar is correct.

Fill the cryowork box with liquid nitrogen to the correct level and allow to equilibrate.

Replace and grease the O-rings on the sample freezing rods and the recording rod.

Fig. 7  Tolerance of (A) *S. pombe* and (B) *S. cerevisiae* to FS medium containing 10% added water. Samples freeze substituted in medium containing 2% osmium tetroxide/0.1% uranyl acetate/10% water in acetone. Bars 500 nm.
Carry out three test shots using the recording rod and ensure that the recorded values are within range.

Grease the O-ring on the sample freezing rods every five shots and replace every 50 or at the start of each run.

Precool all instruments to be used in handling the frozen specimen in the cryowork box.

Try and insulate the end of the forceps with tape to prevent cold burns when handling.

Allow 90 s to pass between each shot.

Always defrost and dry the rod between each shot.

Take care when manipulating the frozen sample and handling the precooled forceps/tools; wear two pairs of nitrile gloves, this affords only minimal protection from the extremely cold tools but it is better than nothing. Cryogloves cannot be worn because of the severe restriction to dexterity.

Take care when screwing the specimen carrier clamp into position on the freezing rod, it is very easy to trap the nitrile gloves in the assembly.

As soon as the “Jet” button has been pressed, count quickly to four and then remove the locking pin and specimen rod.

When removing the sample from the rod always keep it within the work box and at LN2 temperature.

When separating the sample carrier halves with the screwdrivers, take care not to apply too much force—the whole sample can be catapulted out of the box and lost for good.

Fig. 8  Ruptured nuclear envelope (Arrow) of high pressure frozen *S. pombe*. Note the presence of chromatin (Cr) in the cytoplasm of the cell. Bar 500 nm.
4. Freeze Substitution, AFS and Embedding Issues

On the whole FS and the use of the AFS is fairly trouble free. Major issues include poor preservation and/or ice damage. This can result from a number of causes all of which are operator related:

- Failure to pre cool instruments used for handling of the frozen specimen.
- Poor transfer or handling of specimen after freezing or during FS.
- Yeast paste too wet or too dry prior to freezing.
- TF setting on the AFS too high or AFS lid left open for long periods causing freezing of the FS medium.

In its grossest form ice damage can manifest itself as lattice type pattern or large holes within the cell. Subtle damage is more difficult to interpret. In general, segregation or granularity of the chromatin or within the cytoplasm is an indicator that ice damage has occurred (Fig. 9).

Resin embedding of yeast can be problematic and poor infiltration is not uncommon. If the initial paste was too dry prior to freezing, subsequent embedding is adversely affected. A slow incremental infiltration protocol together with vacuum infiltration of the samples once in pure resin is a major help in reducing these problems. It is, however, important to stress that continuous pumping of the sample during vacuum infiltration can be detrimental to good polymerization. This is because the individual resin components have different vapor pressures and continuous pumping reduces the effective concentration of the more volatile components. As a result the original formulation of the resin is changed and with it, the polymerization and hardening characteristics. It is better to attain a specific level (100 mb) of vacuum in the chamber and then isolate it from the pump.

Fig. 9  Ice damage in the nucleus (Nu) of *S. pombe*. Bar 500 nm.
The use of a slow speed angled rotator is also beneficial during the infiltration steps.

The original formulation of Spurr’s resin is no longer available due to the toxicity of one of its major components, ERL4206. However, a modified formulation which replaces the original ERL4206 with ERL4221D is available from TAAB laboratory supplies, UK. This particular formulation is only slightly more viscous than the original and is to be recommended.

Acknowledgment

I thank Victor Alvarez and Iain Hagan of the Cell Division group, for the provision of S. pombe and S. cerevisiae cultures.

I also thank Sandra Rutherford for expert technical assistance. This work was supported by CRUK and carried out at The Paterson Institute for Cancer Research, University of Manchester, UK.

References


