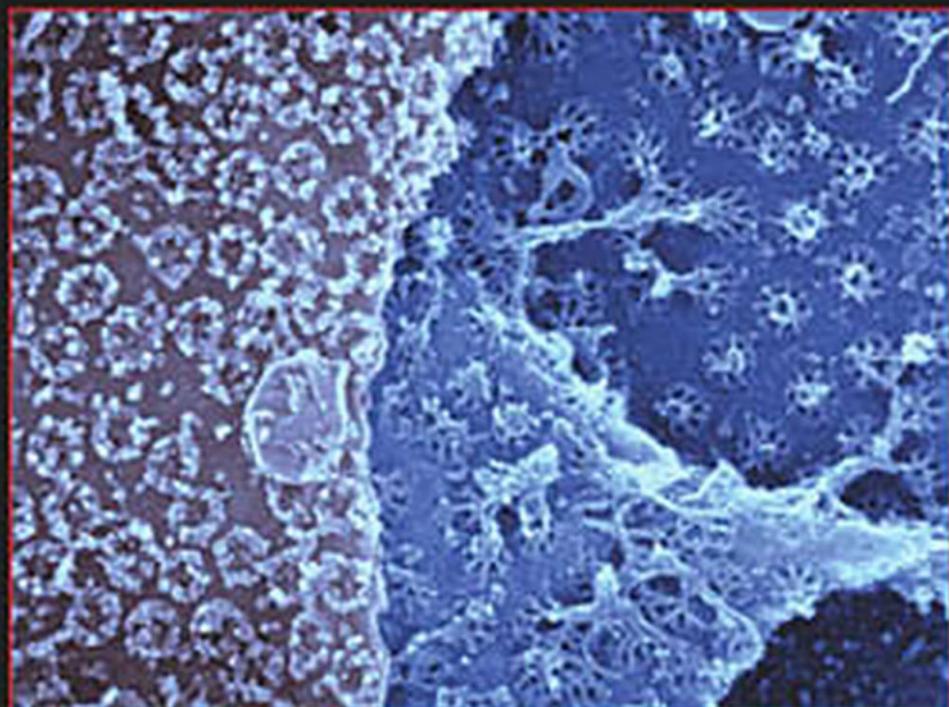


Methods in Cell Biology • Volume 88

INTRODUCTION TO ELECTRON MICROSCOPY FOR BIOLOGISTS



Edited by
Terence D. Allen



Methods in Cell Biology

VOLUME 88

Introduction to Electron Microscopy for Biologists

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Methods in Cell Biology

VOLUME 88

Introduction to Electron Microscopy for Biologists

Edited by

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Structural Cell Biology
Paterson Institute for Cancer Research
Manchester



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CONTENTS

Contributors

xiii

PART I Exploring the Organisation of the Cell by Electron Microscopy

SECTION 1 Basic Transmission and Scanning Electron Microscopy

1. High Pressure Freezing and Freeze Substitution of *Schizosaccharomyces pombe*
and *Saccharomyces cerevisiae* for TEM
Stephen Murray
 - I. Introduction 4
 - II. Materials and Instrumentation 4
 - III. Procedures 7
 - IV. Comments and Problems 10
 - References 17

2. Electron Probe X-ray Microanalysis for the Study of Cell Physiology
E. Fernandez-Segura and Alice Warley
 - I. Introduction 20
 - II. Rationale 21
 - III. Methods 22
 - IV. Equipment 37
 - V. Discussion 38
 - References 40

3. Preparation of Cells and Tissues for Immuno EM
Paul Webster, Heinz Schwarz, and Gareth Griffiths
 - I. Preparing Biological Specimens for Examination by Electron Microscopy 46
 - II. Vitrification and Chemical Fixation for Immunolocalization 48

III. Vitrification Followed by Freeze Substitution	48
IV. Chemical Cross-linking (or Fixation)	49
V. Embedding in Resin for Sectioning	50
VI. Cryosectioning for Immunocytochemistry: The Tokuyasu Method	50
VII. The Starting Material	51
VIII. Protocols	51
IX. Alternative Approaches to Freeze Substitution	53
X. Correlative Microscopy	54
References	55
4. Quantification of Structures and Gold Labeling in Transmission Electron Microscopy	
<i>John Lucocq</i>	
I. Introduction	59
II. Sampling and Stereology	60
III. Quantities Displayed on Sections—Gold Labeling and Profile Data	62
IV. Quantities in Three Dimension	69
V. Spatial Analysis	79
References	80
5. Combined Video Fluorescence and 3D Electron Microscopy	
<i>Alexander A. Mironov, Roman S. Polishchuk, and Galina V. Beznoussenko</i>	
I. Introduction	84
II. Rationale	84
III. Method Steps	85
IV. Immunolabeling for EM with NANOGOLD	87
V. Immunolabeling for EM with HRP	88
References	95
6. From Live-Cell Imaging to Scanning Electron Microscopy (SEM): The Use of Green Fluorescent Protein (GFP) as a Common Label	
<i>Sheona P. Drummond and Terence D. Allen</i>	
I. Introduction	98
II. Rationale	99
III. Methods	99
IV. Summary	103
V. Concluding Remarks	104
References	107

7. Immunolabeling for Scanning Electron Microscopy (SEM) and Field Emission SEM	
<i>Martin W. Goldberg</i>	
I. Introduction	110
II. Methods	120
III. Procedure	122
Conclusions	129
References	129
8. Immunogold Labeling of Thawed Cryosections	
<i>Peter J. Peters and Jason Pierson</i>	
I. Introduction	132
II. Preparation of Carbon- and Formvar-Coated Copper Grids	132
III. Aldehyde Fixation of Cells for Cryoimmuno Labeling	134
IV. Embedding Samples for Cryoimmuno Labeling	136
V. Cryosectioning for Cryoimmuno Labeling	138
VI. Immunogold Labeling	140
VII. Reagents and Solutions	143
VIII. Future Outlooks	146
References	148
9. Close-to-Native Ultrastructural Preservation by High Pressure Freezing	
<i>Dimitri Vanhecke, Werner Graber, and Daniel Studer</i>	
I. Introduction	152
II. Rational	155
III. Material	156
IV. Methods	158
V. High Pressure Frozen Samples	159
VI. Discussion	160
References	162
10. High-Pressure Freezing and Low-Temperature Fixation of Cell Monolayers Grown on Sapphire Coverslips	
<i>Siegfried Reipert and Gerhard Wiche</i>	
I. Introduction	166
II. Materials and Instrumentation	167
III. Procedures	167
IV. Comments and Pitfalls	174
References	180

11. Freeze-Fracture Cytochemistry in Cell Biology	
<i>Nicholas J. Severs and Horst Robenek</i>	
I. Introduction	182
II. Basic Rationale: Solving the Problems of Combining Freeze Fracture with Cytochemistry	183
III. Methods	185
IV. Discussion: Impact on Topical Questions in Cell Biology	191
V. Concluding Comment	201
References	202

PART II Electron Microscopy of Specific Cellular Structure

SECTION 1 The Cell Membrane

12. Three-Dimensional Molecular Architecture of the Plasma-Membrane-Associated Cytoskeleton as Reconstructed by Freeze-Etch Electron Tomography	
<i>Nobuhiro Morone, Chieko Nakada, Yasuhiro Umemura, Jiro Usukura, and Akihiro Kusumi</i>	
I. Introduction	208
II. Protocol for Visualization of the Three-Dimensional Structure of the MSK of the Cytoplasmic Surface of the Plasma Membrane	212
III. 3D Structure of the Cytoskeleton-Plasma Membrane Interface	219
IV. Electron Tomography Clarified that Some of the Actin Filaments are Laterally Bound to the Cytoplasmic Surface of the Plasma Membrane	231
References	232
13. Visualization of Dynamins	
<i>Jason A. Mears and Jenny E. Hinshaw</i>	
I. Introduction	237
II. Methods and Materials	240
III. Discussion	251
IV. Summary	253
References	254

SECTION 2 The Cytoskeleton

14. Correlated Light and Electron Microscopy of the Cytoskeleton

Sonja Auinger and J. Victor Small

I. Introduction	258
II. Materials and Methods	259
III. Methods	260
IV. Results and Discussion	266
V. Summary	271
References	271

15. Electron Microscopy of Intermediate Filaments: Teaming up with Atomic Force and Confocal Laser Scanning Microscopy

Laurent Kreplak, Karsten Richter, Ueli Aebi, and Harald Herrmann

I. Introduction	274
II. Rationale	277
III. Visualization of Intermediate Filaments <i>in vitro</i> and in Cultured Cells	278
IV. Materials	293
V. Conclusions and Outlook	294
References	295

16. Studying Microtubules by Electron Microscopy

Carolyn Moores

I. Introduction	300
II. Molecular Electron Microscopy	302
III. Cellular Electron Microscopy	311
IV. Outlook	314
References	315

SECTION 3 Extracellular Matrix and Cell Junctions

17. Electron Microscopy of Collagen Fibril Structure *In Vitro* and *In Vivo* Including Three-Dimensional Reconstruction*Tobias Starborg, Yinhui Lu, Karl E. Kadler, and David F. Holmes*

I. Introduction	320
II. Electron Microscopy of Isolated Collagen Fibrils	327
III. Fibroblast/Fibril Interface in Developing Tendon	334
IV. Discussion	340
V. Conclusions	341
References	341

18. Visualization of Desmosomes in the Electron Microscope	
<i>Anthea Sothorn and David Garrod</i>	
I. Introduction	348
II. Rationale	351
III. Methods	351
IV. Materials	360
V. Discussion	364
VI. Summary	364
References	365
SECTION 4 The Nucleus	
19. A Protocol for Isolation and Visualization of Yeast Nuclei by Scanning Electron Microscopy	
<i>Stephen Murray and Elena Kiseleva</i>	
I. Introduction	368
II. Materials and Instrumentation	370
III. Procedures	372
IV. Comments and Problems	385
References	386
20. Scanning Electron Microscopy of Nuclear Structure	
<i>Terence D. Allen, Sandra A. Rutherford, Stephen Murray, Sheona P. Drummond, Martin W. Goldberg, and Elena Kiseleva</i>	
I. Introduction	390
II. Rationale	393
III. Methods	393
IV. Colloidal Gold in the SEM	400
V. Immunolabeling Protocol	403
VI. CPD for High Resolution SEM	404
VII. Discussion	407
References	408
21. Electron Microscopy of Lamin and the Nuclear Lamina in <i>Caenorhabditis elegans</i>	
<i>Merav Cohen, Rachel Santarella, Naama Wiesel, Iain Mattaj, and Yosef Gruenbaum</i>	
I. General Introduction	412
II. <i>In Vitro</i> Assembly of Ce-Lamin Filaments	414
III. Preparation of Embryos and Adults for Transmission Electron Microscopy Using Microwave Fixation	416

IV. Preparation of <i>C. elegans</i> Embryos and Adults for Conventional Transmission Electron Microscopy by High Pressure Freezing Combined with Freeze Substitution	419
V. Preembedding Immunogold EM Staining of Lamina Proteins in <i>C. elegans</i> Embryos	423
VI. Postembedding Immunogold EM Staining of Lamina Proteins in <i>C. elegans</i> Embryos	425
VII. Summary	427
References	427
22. Visualization of Nuclear Organization by Ultrastructural Cytochemistry	
<i>Marco Biggioera and Stanislav Fakan</i>	
I. Introduction	432
II. Cytochemical Contrasting Approaches	432
III. High Resolution Autoradiography	441
IV. Immunocytochemistry	441
V. Molecular <i>in situ</i> Hybridization	443
VI. Identification of Nucleic Acids by Means of Enzymatic Reactions	444
VII. Targeting of Intranuclear Substrates using Enzyme-Colloidal Gold Complexes	446
VIII. Concluding Remarks	446
References	447
23. Scanning Electron Microscopy of Chromosomes	
<i>Gerhard Wanner and Elizabeth Schroeder-Reiter</i>	
I. Introduction	452
II. Materials and Methods	454
III. Chromosome Preparation	456
IV. Chromosome Structure in SEM	458
V. Chromosome Analysis in SEM	460
VI. Outlook	470
References	473
PART III Cells and Infectious Agents	
24. Infection at the Cellular Level	
<i>Christian Goosmann, Ulrike Abu Abed, and Volker Brinkmann</i>	
I. Introduction	477
II. Methods and Materials	478
III. Overview and Conclusion	494
References	495

25. Electron Microscopy of Viruses and Virus-Cell Interactions

Peter Wild

I. Introduction	498
II. Methods	500
III. Material	515
IV. Discussion	516
V. Summary	520
References	521
Index	525
Volumes in Series	537

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PART I

Exploring the Organisation of the
Cell by Electron Microscopy

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SECTION 1

Basic Transmission and Scanning Electron Microscopy

CHAPTER 1

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

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-
- Abstract
 - I. Introduction
 - II. Materials and Instrumentation
 - III. Procedures
 - A. Preparation of Sample Holders
 - B. Filtration of Yeast Sample
 - C. High-Pressure Freezing of the Sample
 - D. Freeze Substitution of Yeast
 - IV. Comments and Problems
 - References

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 to 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 in 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 cerevisiae*.

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. The screwdrivers are used to pry the brass hat assembly apart once frozen.

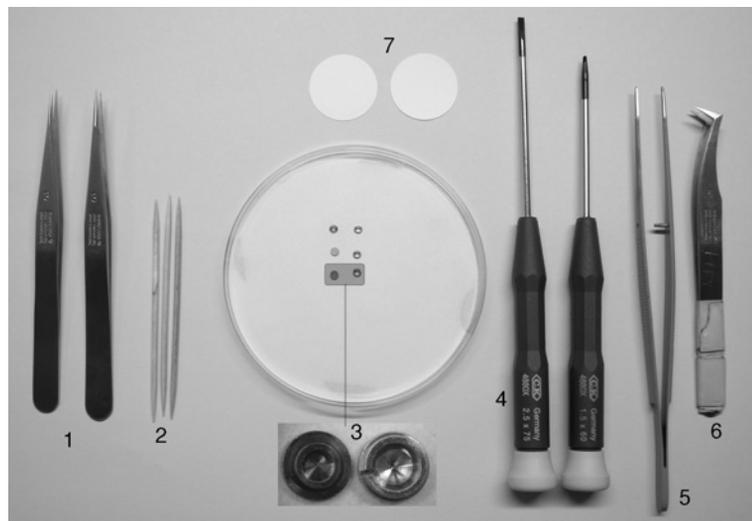


Fig. 1 Tools used during loading and manipulation: (1) Dumont No. 3 forceps, (2) Pointed applicator sticks for paste scraping/loading, (3) Interlocking brass sample carriers, (4) Fine blade screwdrivers for separating hats following freezing, (5) Insulated cryoforceps for handling frozen sample, (6) Dumont No. 6 forceps for pushing sample carrier from specimen rod, (7) Millipore membrane filter 0.45 μm , 22 mm.



Fig. 2 Filtration system for sample harvesting (manual air inlet valve on the right).

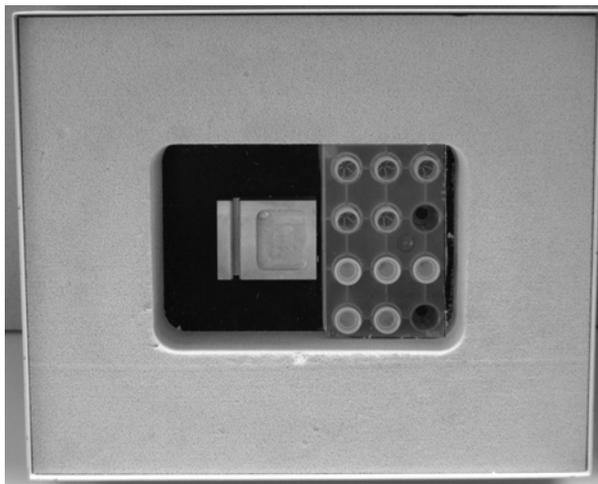


Fig. 3 Cryowork box with cryotubes and holder.

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 pre-labeled 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 Sarsted 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 $0.4-1.0 \times 10^7$ 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 $-90\text{ }^{\circ}\text{C}$ and the time to 72 h
2. Set the first slope to $+5\text{ }^{\circ}\text{C/h}$

3. Set the second temperature step to -20°C and the time to 2 h
4. Set the second slope to $+5^{\circ}\text{C/h}$
5. Set the final temperature step to $+4^{\circ}\text{C}$ and the time to 4 h
6. Place the AFS into pause mode and allow the temperature to reach -90°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 μl 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 $+4^{\circ}\text{C}$, 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 $+4^{\circ}\text{C}$.
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 pre-labeled 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 counter intuitive 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

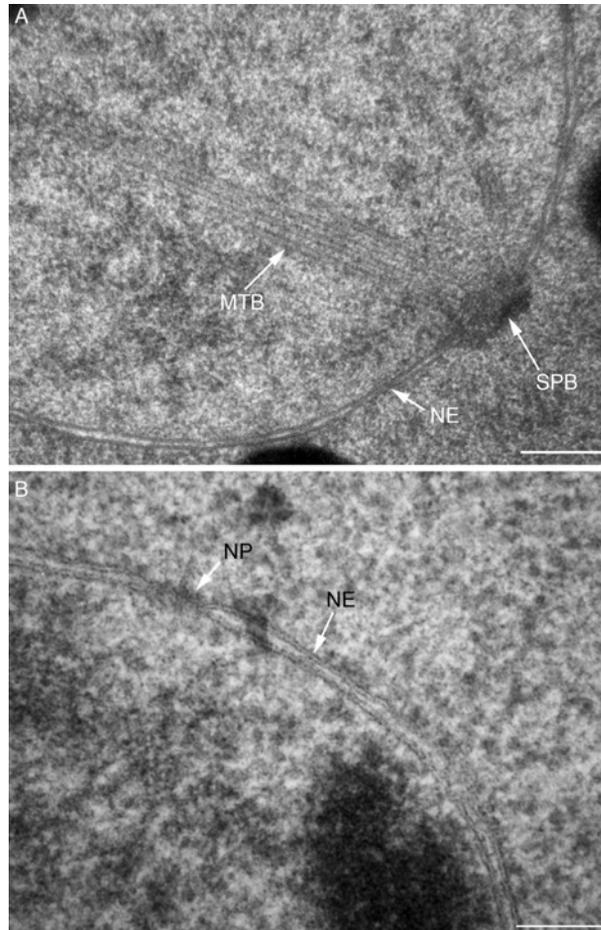


Fig. 4 Nuclear Envelope (NE) of *S. pombe* (A) with Spindle Pole Body (SPB) and Microtubules (MTB) and (B) Nuclear Pore (NP). Sample freeze substituted in medium containing 2% osmium tetroxide/0.1% uranyl acetate/5% water in acetone. Each element of the NE is clearly visible, demonstrating typical membrane structure. Bars (A) 200 nm and (B) 100 nm.

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).

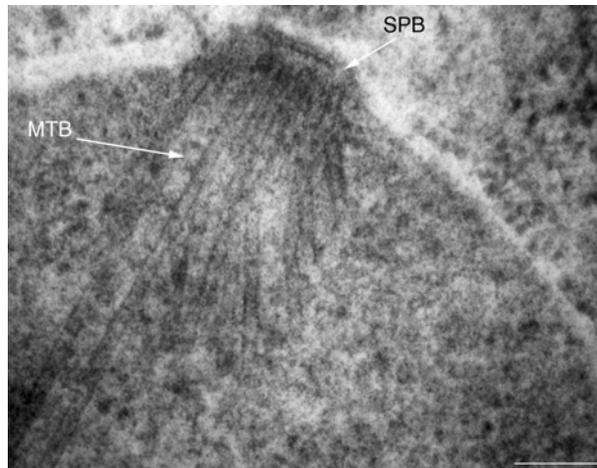


Fig. 5 SPB and MTB of *S. cerevisiae* freeze substituted in epoxy/araldite embedding mix. Bar 100 nm.

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