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# Using Rapid Freeze and Freeze-Substitution for the Preparation of Yeast Cells for Electron Microscopy and Three-Dimensional Analysis

**Thomas H. Giddings Jr.**[*], **Eileen T. O'Toole**[†], **Mary Morphew**[†], **David N. Mastronarde**[†], **J. Richard McIntosh**[*,†], and **Mark Winey**[*]

[*]Department of Molecular, Cellular and Developmental Biology, Porter Bioscicnces, University of Colorado–Boulder, Boulder, Colorado 80309

[†]Boulder Laboratory for 3D Fine Structure, Porter Bioscicnces, University of Colorado–Boulder, Boulder, Colorado 80309

## I. Introduction

The analysis of yeast organelles, such as their spindle pole bodies (SPBs), often benefits from the ability to image the relevant structures in an electron microscope. For example, electron microscopy (EM) of yeast SPBs has been used to describe the organelle itself, its duplication cycle in mitotic and meiotic cells, the curious fusion of SPBs that occurs during karyogamy, and SPB morphology in cells mutant in genes encoding SPB components or regulatory factors. Furthermore, components of the SPB have been localized to the organelle using electron microscopic analysis of immunolabeled preparations. The requisite morphological and immunological data have been obtained by several methods: electron microscopy of whole cells, of isolated nuclei, or of cellular fractions enriched for SPBs. Most of these applications have used relatively standard chemical fixation and embedding techniques that have been described elsewhere (e.g., Byers and Goetsch, 1991; Wigge *et al.*, 1998). Advantages of standard chemical fixation are its convenience and quickness (for a detailed protocol visit: http://mcdb.colorado.edu/labs/winey/protocols.html). The principal limitations of standard chemical fixation derive from the fact that this method relies on the diffusion of chemical fixatives into the cell, a process that is slow relative to the dynamics of cytoplasmic constituents such as microtubules (MT) and membranes (Gilkey and Staehelin, 1986). Moreover, because the cell wall surrounding yeast cells limits diffusion of both fixatives and resins, wall components must be removed by degradative enzymes prior to postfixation and embedding. Such problems have motivated alternative approaches to preparing yeast samples for EM, including the application of cryofixation and freeze-substitution described here and the groundbreaking study of isolated SPBs embedded in vitreous ice using cryo-electron microscopy and tomography to reveal exquisite structural detail within this organelle (Bullitt *et al.*, 1997).

Rapid freezing and freeze-substitution of yeast cells (Tanaka and Kanbe, 1986; Ding *et al.*, 1993; Winey *et al.*, 1995) and other fungi (Heath and Rethoret, 1982; Heath *et al.*, 1984) for electron microscopy have been found to result in morphological preservation that is generally superior to that seen after chemical fixation. Figure 1 shows images of cross sections through microtubules prepared by rapid freezing and freeze-substitution (Fig. 1a) and by standard chemical fixation (Fig. 1b). Following freeze-substitution, the microtubules are round and of the expected diameter and their protofilaments can sometimes be seen. Perhaps more significantly, the observations that the shape of the nucleus and other organelles is not distorted and that microtubules can exhibit preferred packing arrangements suggest that the architecture of entire MT arrays such as the mitotic spindle are faithful representations of the living state (Winey *et al.*, 1995). SPBs are also well preserved; the multiple layers or plaques of the SPB are well contrasted, and the microtubules organized by the SPB appear straight (Fig. 1c). Indeed, rapid freezing and freeze-substitution work very well for both mitotic and meiotic cells (Fig. 2) without the special modifications that are necessary for good quality chemical fixation of meiotic cells. The cells remain in the normal culture environment until freezing, without exposure to osmotic stresses and without removal of the cell wall. These may be the key factors responsible for the minimal extraction or rearrangement of components that we and others have observed. Baba *et al.*, (1989) have used freeze-substitution to reconstruct entire mating factor arrested cells. Our own experience confirms that rapidly frozen and freeze-substituted cells exhibit excellent preservation of morphology. The techniques we use are described in the following sections.

## II. How Rapid Freezing and Freeze-Substitution Work

Accurate preservation of cellular structures depends in part on the speed with which cellular processes are stopped. Rapid freezing results in the almost instantaneous fixation of the cell (ca. 10 ms), which is orders of magnitude faster than the time needed for chemical fixatives to diffuse into a cell and cross-link its components (Horowitz *et al.*, 1990). A potential problem with cryofixation is, however, the formation of ice crystals that distort the structure of the sample. This has been solved largely by employing extremely rapid rates of cooling such that crystalline ice is not formed but rather intracellular water is vitrified (Gilkey and Staehelin, 1986). This has been achieved by plunging thin (<20 $\mu$m) layers of cells into liquid ethane or propane, or by propane jet freezing. However, the rate of heat transfer through aqueous media is slow enough to limit the depth of vitrification to about 20 $\mu$m from the surface of the sample. Even with such thin samples it is sometimes useful to add to the growth medium a molecule that will not enter the cells, not cause a significant increase in osmotic pressure, and yet reduce the freezing point of the medium. Such a nonpenetrating “cryoprotectant” permits supercooling of the extracellular medium, resulting in delayed and minimized ice crystal formation. That in turn increases the rate of intracellular freezing and minimizes ultrastructural damage from ice crystal formation. A more effective solution to this problem relies upon instruments known as high-pressure freezers. These devices apply pressure (upward of ~2050 bar) to the samples prior to freezing, which greatly limits the formation and growth of ice crystals, favoring the formation of vitreous ice (Gilkey and Staehelin, 1986; Moor, 1987; Dahl and Staehelin, 1989). The principal benefit of high-pressure freezing is that larger samples can be well frozen, including pellets of yeast cells,

and even whole embryos of *Drosophila* or sea urchin embryos (McDonald and Morphew, 1993). Pieces of plant (Craig and Staehelin, 1988; Staehelin *et al.*, 1990) or animal (Royer *et al.*, 1996; Kirschning *et al.*, 1998) tissue can also be preserved in this way.

After freezing, the cells must be further fixed by freeze-substitution and then embedded in resin in preparation for viewing in the electron microscope. Freeze-substitution involves replacing the frozen water of the cell with an organic solvent at low temperature, thus avoiding the damaging effects of dehydration that occur at ambient temperature (Steinbrecht and Muller, 1987). The solvent usually contains a chemical fixative for cross-linking cellular components as well as a stain to provide increased contrast of particular organelles in the electron microscope. At the temperatures commonly used for freeze substitution (ca. −90° to −80°C), chemical reactions are very slow. Indeed, some workers have found that covalent cross-linking of proteins by aldehydes does not occur until significantly higher temperatures are reached as the sample is warmed up after freeze-substitution (Horowitz *et al.*, 1990). Our results suggest, however, that some stabilization of structure is obtained by chemical fixatives such as glutaraldehyde introduced at low temperatures, based on the observation of improved preservation of cellular ultrastructure even when such specimens are never warmed above −50° C throughout the entire procedure. Following freeze-substitution, the samples are ready for embedding, sectioning, and microscopy. For yeast cells, the slow freeze-substitution and embedding procedure described in detail later allows the fixation and staining of cells with the cell walls intact, leading to excellent preservation. Our finding is that these cells are suitable for the analysis of most organelle systems. The preservation and visualization of particular organelles of interest can usually be optimized to yield excellent results.

## III. Rapid Freezing Techniques

As listed earlier, there are a variety of tactics to freeze cells fast enough to minimize ice crystal formation. We have made extensive use of two of those methods: plunge freezing and high-pressure freezing. For plunge freezing, cells are grown on a membrane and then immersed rapidly into a liquid ethane bath. The advantage of this technology is that it is inexpensive and easy to set up anywhere. The disadvantage is that only a small number of cells are frozen and the samples must be handled carefully to prevent loss. Moreover, the yield of well-frozen cells is lower than that achieved with high-pressure freezing (Ding *et al.*, 1993). Nonetheless, well-frozen cells prepared this way are indistinguishable from those prepared by high-pressure freezing.

### A. Plunge Freezing

We have used a method for plunge freezing modified from Tanaka and Kanbe (1986) that has worked well for preserving the fine structure of the SPB and mitotic spindles of the fission yeast *S. pombe* (Ding *et al.*, 1993). In this protocol, cells are transferred to small (3×3 mm) squares of PCTE (Polycarbonate Track-Etch) membrane that have been presoaked in growth medium and placed on agar plates. After an additional period of growth, the cells will stick to the membrane. The membrane containing the attached cells is picked up with forceps, which are then attached to a guillotine-type plunge freezer and plunged into liquid ethane or propane cooled to near its freezing point by a bath of liquid nitrogen. The

membrane is then transferred to liquid nitrogen for storage. The cells remain attached to the PCTE membrane throughout freezing, freeze-substitution, dehydration, and resin infiltration. After infiltration, the membranes are flat-embedded between two glass slides and polymerized in a 60°C oven, resulting in a thin wafer of polymerized resin containing the membrane. Cells of interest can then be viewed under the light microscope and excised for thin sectioning.

### B. High-Pressure Freezing

Three commercial models of high-pressure freezers are available. Our work has been done with the BAL-TEC HPM-010 (Technotrade International, Manchester, NH). This particular instrument, as well as the Leica EM HPF (Leica, Inc., Deerfield, IL), is able to freeze about 2 μl of cell paste per freezing run, and each run takes only minutes to achieve. These instruments are able not only to rapidly freeze relatively large samples with a quick turn around time, they produce samples with a uniform and reproducible high quality of freezing. The latest model of the BAL-TEC HPM-010 can produce a data trace of pressure and temperature for each freezing run so that the operator is assured of consistent performance. Leica recently introduced a lower cost, but smaller capacity high-pressure freezer, the Leica EM PACT.

Yeast cultures undergo minimal manipulation prior to high-pressure freezing. Cultures are generally grown in liquid to an OD of between 0.2 and 0.6 so that the culture will be in log phase growth at the time of freezing or at the start of the experiment (e.g., shift to nonpermissive temperature). The culture conditions, temperature and stirring, are maintained throughout the sampling period. An aliquot of cells, usually 5–10 ml, is removed and concentrated by vacuum filtration on a Millipore filter (Type HA, 0.45 μm pore size). The filtering is controlled carefully to avoid drying the layer of yeast on the filter. The yeast are scraped off the filter with a spatula and applied to a brass specimen carrier (Dahl and Staehelin, 1989; McDonald 1999; Swiss Precision, Inc. Palo Alto, CA; or Ted Pella Inc., Redding, CA). The complete specimen carrier consists of a mated pair of brass pieces that completely enclose the cell concentrate. The harvesting and loading processes take less than 2 min. In a matter of a few more seconds, the specimen carrier is loaded into the high-pressure freezer and the freezing process is immediately initiated by simply pressing a button. After freezing, the sample is transferred quickly to liquid nitrogen. Prior to the start of freeze-substitution, the interlocking specimen carriers must be separated under liquid nitrogen.

## IV. Freeze-Substitution Protocols

Once the cells are frozen, a variety of a freeze-substitution protocols may be used. In the first phase, the samples are warmed to −90° or − 80° C and maintained at that temperature for 2–4 days. During this time the sample is immersed in an organic solvent such that the frozen water in the sample dissolves in the solvent and diffuses out of the sample. We routinely use acetone as the solvent because it yields the best overall preservation of ultrastructure. Methanol may also be used. We normally carry out freeze-substitution in 2-ml cryovials (such as Nunc or Corning) containing about 1 ml of freeze-substitution media. These can be prepared in advance and stored under liquid nitrogen. The samples, still

attached to the brass specimen carriers, are transferred to the cryovial. Care should be taken to avoid any warming of the sample at this stage. The cryovial can be kept in a shallow layer of liquid nitrogen so it is kept cold without liquid nitrogen filling the vial. Forceps used to transfer the specimen carriers should be chilled in liquid nitrogen before they touch the specimen. Once loaded and capped, the cryovial can be warmed to the freeze-substitution temperature. A minimal system consists of dry ice in a Styrofoam box, which maintains a temperature of −80°C. An aluminum block with holes to accommodate the cryovials serves to keep them upright and to slow the rate of warming once the block is transferred to a −20°C freezer. A −80°C freezer can be used instead of the dry ice, but consideration must be given to the potential for fixative to leak and contaminate other valuable samples and reagents in the freezer.

A more versatile device for freeze-substitution can be constructed from a metal block suspended in the gas phase above liquid nitrogen in a large storage dewar. A thermocouple to measure the temperature of the block, a heater, and an automatic temperature controller can maintain the desired temperature for freeze substitution. A very simple prototype of such a device is shown in Kiss and McDonald (1993). Commercially available freeze-substitution units include features for controlled, automatic warming, easier fluid exchanges, and UV polymerization at low temperature. Examples include the Leica EM AFS and the BAL-TEC FSU-010.

Freeze-substitution protocols can be customized to some degree to enhance either membrane or cytoskeletal staining or for the preservation of antigenicity for immunolabeling. To obtain the best contrast for microtubules and SPBs, one can freeze-substitute in 2% osmium tetroxide plus 0.1% uranyl acetate in acetone. After approximately 3 days at −80°C, the samples can be warmed to −20°C for 2 h, 4°C for 2 h, and finally room temperature for 1 h. At this point the freeze-substitution medium is replaced with plain solvent and the samples are pushed out of the brass HPF specimen carriers. The carriers can be cleaned and reused.

Membrane staining can be increased by incorporating tannic acid into the freeze-substitution protocol (Ding *et al.*, 1993). Initial freeze-substitution can be done either in 0.1 % tannic acid in acetone or in this solution with the addition of 0.5% glutaraldehyde. After freeze substitution at −80°C, this initial medium is rinsed out with several changes of acetone at −80°C over 2–4 h. Acetone with 2% osmium tetroxide is added at −80°C and allowed to infiltrate for several hours. The samples are then warmed to −20°C for about 16 h followed by 4–6 h at 4°C. The fixative is rinsed out with acetone and the samples can then be warmed to room temperature and prepared for embedding.

We have obtained excellent morphological preservation of yeast while retaining antigenicity with a combination of aldehyde-based freeze-substitution and embedding in Lowicryl HM20. Freeze-substitution is done in 0.25% glutaraldehyde plus 0.05–0.1% uranyl acetate in acetone. After the initial substitution at −90°C to −80°C, the samples are kept at −20°C for 4–6 h. The freeze-substitution medium is rinsed out with acetone at −20°C prior to embedding (see later).

## V. Embedding Protocols

Embedding of freeze-substituted material can be very similar to the methods used for samples prepared by other methods, except that the process of infiltration is started with more diluted embedding resin and is drawn out for a longer time period. After freeze-substitution, most of the cells remain in clusters that are large enough that no centrifugation is required during embedding. Either Spurr's or Epon-Araldite is suitable for morphological studies. Samples can be infiltrated for about 8–16 h at each resin concentration, starting with 1:7 (resin:acetone), followed by 1:3, 1:1, and 3:1. Finally, three changes of 100% resin over about 24 h should be sufficient to complete the infiltration.

Samples embedded in Lowicryl HM20 have been labeled successfully with antibodies to a variety of different antigens. Fusion proteins that include the green fluorescent protein (GFP) epitope have fared particularly well with this protocol (e.g., Chial *et al.*, 1998; West *et al.*, 1998). After freeze-substitution with 0.25% glutaraldehyde and 0.1% uranyl acetate in acetone, the samples are infiltrated in Lowicryl HM 20, diluted in acetone at a ratio of 1:3 for 2 h, then 1:1 for 2 h, followed by 3:1 overnight, all at −20°C. Four changes in 100% Lowicryl HM20 are carried out the next day, and two more changes the following day. Finally, the samples are loaded into BEEM capsules, cooled to −50°C, and polymerized under UV at that temperature.

## VI. Sectioning and Imaging

Once cells are embedded in resin, they are handled like any other sample for electron microscopy. We routinely cut serial thin sections of about 50 nm thickness. These relatively thin sections are used for yeast because of the dense staining of the nuclei and cytoplasm, but the small size of the cells (5 to 10 $\mu$m) makes it feasible to collect serial sections of entire cells. This compares to the thicker sections (up to 80 to 90 nm) that have been used for chemically fixed samples (Byers and Goetsch, 1991). Sections up to 400 nm thick may be used for high-voltage EM tomography (see later). We routinely stain sections on the grids with 2% uranyl acetate in either water or 70% methanol, followed by staining in Reynold's lead citrate. Adjustments are made as needed, such as omitting or shortening the staining steps to permit easier visualization of immunogold label in samples prepared for immuno-EM.

Those new to yeast morphology and microscopy techniques may find the following hints useful. One is to use very small block faces in preparation for sectioning. A trapezoid face that is longer than it is tall helps in finding the same cell on successive serial sections. The small size of yeast cells makes it possible to view several dozen cells in sections cut from even a very small block face. Furthermore, the small block face allows more sections to be applied to each grid. We have been able to place ribbons containing serial sections of entire cells on a single grid. Another point to keep in mind is that yeast cells have dense nucleoplasm and cytoplasm that is packed with ribosomes. The density of yeast cells is very apparent in cells prepared by the high-pressure freezing/freeze-substitution (HPF/FS) protocols because there is very little extraction of material. The normal density of these cells is sometimes mistaken for poor sample preparation. However, excessively dense-staining

cytoplasm can indicate poor fixation and embedding. In those cases, the cells may be partially collapsed and the cell wall shape may be irregular. Many published images are available for comparison to distinguish between these possibilities (Figs. 1 and 2; McDonald *et al.*, 1996; O'Toole *et al.*, 1997, 1999; Winey *et al.*, 1995, 1997).

Finally, the identification of SPBs in sections of whole cells can be difficult, but a search strategy can help. We generally scan sections of cells that exhibit part of the nucleus, looking for microtubules at any orientation. The microtubules are then tracked section to section with the expectation that the microtubules will lead to the SPB. Also, the cellular morphology of the budding yeast aids in the identification of key structures and cell cycle stages. Large budded cells are expected to have the nucleus at the bud neck or extended through the bud neck with one SPB facing the bud neck or at the leading edges of the mitotic nucleus that span the bud neck. Finally, the identification of SPBs is usually based on both the layered morphology of the organelle and the association of microtubules. In poorly prepared samples of haploid strains, SPBs can be confused with nuclear pore complexes, which are approximately the same size (around 90 nm in diameter; Byers, 1981). To address this issue, we often analyze SPBs in diploid cells where the organelle is larger (Byers, 1981).

## VII. Immunolabeling for Electron Microscopy

An important consideration in the study of yeast cell structure in general and of SPBs and spindles in particular is the localization of protein components. As with structural studies, it is often important to carry out such work on whole cells. The Boulder Laboratory for 3D Fine Structure has found that GFP can be used effectively as a general tag for immuno-EM (Fig. 3; e.g., Chial *et al*., 1998). The reiterated epitope tags *myc* (13×) and *pk* (3×) have also worked for immuno-EM, but the signal-to-noise ratio appears to be somewhat lower than with GFP (M. Morphew, unpublished observation, Chial *et al*., 1999, respectively). Effective, high-resolution immunolocalization requires that cellular morphology be well preserved while antigenicity is also retained so that cellular structures can be recognized for the interpretation of the localization data. Our protocol therefore begins with the same rapidly frozen cells as described earlier. Modifications of the freeze-substitution and embedding protocols have been listed previously.

In order to facilitate the localization of SPBs and tracking of MTs, we generally retrieve serial sections for immuno-EM, usually about 40–60 nm thick. These are picked up on formvar-coated nickel slot grids. For immunostaining, the blocking solution consists of 0.8% bovine serum albumin, 0.1% gelatin [IGSS quality, Amersham; nonfat dry milk has also worked in PBST buffer (10 m*M* sodium phosphate, pH 7.3, 150 m*M* sodium chloride, 0.1% Tween)]. The samples are exposed to the blocking solution for 0.5–1 h and then floated on the primary antibody diluted in blocking solution. Labeling is often done for either 2 h at room temperature or overnight at 4°C. After a thorough rinse with a stream of PBST, the grids are floated on the appropriate secondary antibody–gold conjugate. Colloidal gold ranging from 5 to 20 nm in diameter, conjugated to a variety of secondary antibodies, is available commercially. Secondary labeling is usually done at room temperature for 1 h using a 1:20 dilution in blocking solution. The grids are then rinsed in PBST followed by

distilled water and allowed to dry. The samples are then stained as described earlier to provide adequate contrast on the structures of interest without obscuring the visualization of the colloidal gold particles.

## VIII. Three-Dimensional Modeling of Spindle Pole Bodies and Other Organelles

Fast-frozen, freeze-substituted cells have been used for routine morphological analysis and for immuno-EM analysis of SPBs and spindles. We have also used cells prepared with these methods for three-dimensional (3D) modeling of yeast organelles, such as mitotic spindles, SPBs, and nuclear envelopes. Two strategies have been used to construct three-dimensional models. One is reconstruction from serial thin sections (Ding *et al*., 1993; O'Toole *et al*., 1997; Winey *et al*., 1995, 1997) and the other is electron tomography (O'Toole *et al*., 1999). These two approaches to creating and visualizing three-dimensional models of structures in cells are complementary. Serial sections permit the reconstruction of comparatively large volumes (e.g., 1 × 1 × 10 $\mu$m), but the resolution of the reconstructions is anisotropic. EM tomography is more suitable for smaller volumes (e.g., 2 × 1 × 0.5 $\mu$m) and the resolution is almost isotropic at ~6 nm. All our work with these methods has used programs developed by the Boulder Laboratory for 3D Fine Structure (Kremer *et al*., 1996; Mastronarde, 1997), which are freely available from our website, http://bio3d.colorado.edu. Similar 3D reconstruction approaches have also been developed in other EM facilities, and a number of review articles on these topics are available (e.g., McEwen and Marko, 1999).

Relevant to this volume, reconstruction from serial thin sections has been used to track microtubule arrays in the mitotic spindles of *S. pombe* and *S. cerevisiae* (for review, see McDonald *et al*., 1996). Microtubule numbers and arrangements in 3D are informative about SPB function during the cell cycle. For this type of modeling, spindles that are in approximate cross section are sought, and an EM stage capable of tilt about any axis is used to get the spindle microtubules in sharp cross section (as shown in Fig. 1). Digitized images of the serial cross sections of spindle microtubules are then aligned to each other. Next the microtubule cross sections are marked by placing a model point in the center of each microtubule that is present in the aligned, serial sections. These aligned points are used to define curves in space, which represent the trajectories of individual microtubules. The resulting model can then be projected in 3D (Fig. 4). Microtubule polarity is inferred by tracking each microtubule to the SPB from which it emerges; that end of the microtubule is defined as the minus end. These models are informative about mitotic spindle organization and its rearrangement during mitosis. A number of analytical tools have been developed to provide quantitative descriptions of microtubule number, length distribution, and mutual proximity to aid in the identification of distinct functional classes of microtubules (McDonald *et al*., 1992; Mastronarde *et al*., 1993; Ding *et al*., 1993; Winey *et al*., 1995).

Reconstruction from serial thin sections yields detailed and accurate maps of relatively large structures that are easily recognized on thin sections and aligned as successive slices of a 3D object. For example, we have constructed models of mitotic spindles over 9 $\mu$m long that would be very difficult, if not impossible, to analyze by any other technique. Furthermore, the use of immuno-EM techniques coupled with reconstruction allows for the mapping of

spindle or SPB components in three-dimensional reconstructions (e.g., Bridge *et al*., 1998). Finally, the accurate description of structures in wild-type cells provides the benchmark for comparison for strains mutant in genes that affect spindle structure and organization (O'Toole *et al*., 1997).

Electron tomography is a method that creates three-dimensional reconstructions from material in a thick section of a cell or of isolated organelles or protein complexes. The technology is well suited to irregularly shaped organelles and to organelles that are small enough that serial thin sectioning (40–80 nm) does not divide the structure finely enough to be informative. Tomography works by collecting serially tilted images of a thick section (250–500 nm) and then computing a three-dimensional reconstruction of the structure by back projection algorithms. Cryoelectron tomography of frozen-hydrated specimens has been used for the analysis of isolated centrosomes (Kenney *et al*., 1997) and of isolated yeast SPBs (Bullitt *et al*., 1997). We have used tomography to examine the structure of SPBs *in situ*, using thick sections of cells prepared as described earlier. The use of dual axis tomography to view SPBs *in situ* has revealed details of SPB structure and duplication not observed previously (O'Toole *et al*., 1999). As shown in Fig. 5 (see color insert), data from tomograms are generally displayed as slices that may be cut from the 3D reconstruction at any orientation or as data from a model of the organelle under study that combines data from many tomographic slices. With sufficient data, one can build models based on volume data in 2- to 3-nm units, resulting in isotropic resolution of 5–6 nm. It is clear that tomography is a powerful tool in the analysis of a wide range of yeast organelles. Such data will be valuable for the comparison of wild-type and mutant strains and in the study of organelle assembly processes. Furthermore, the development of techniques to label proteins in cells destined for tomographic examination would contribute substantially to the utility of tomography in the analysis of SPBs, centrosomes, or other organelles.

## IX. Conclusion

Fast-frozen, freeze-substituted cells have proven to be outstanding samples for both morphological and immuno-EM applications. We have used these samples to examine SPBs and spindle microtubules and to localize components of SPBs or spindles in 3D. Morphological analysis has included three-dimensional reconstruction of microtubule arrays and tomography of SPBs. All of this work has been accomplished using whole cells where the SPB or spindles are *in situ*, yielding significant structural detail, including information about structures that are not seen in isolated organelles (e.g., O'Toole *et al*., 1999). Such images can be used for the comparison of a variety of organelles in wild-type and mutant strains, and as such these morphological tools should be useful to many as they probe the role of individual gene products in the structure or function of yeasts.

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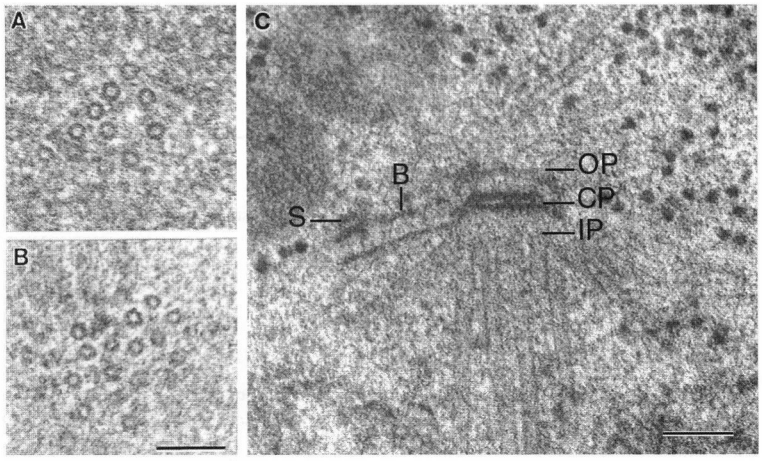


**Fig. 1.**

Microtubule and SPB detail in rapidly frozen, freeze-substituted cells. Microtubules in cross section (A) from rapidly frozen, freeze-substituted cells are shown for comparison with those seen in chemically fixed cells (B). From Winey *et al.* (1995), with copyright permission of The Rockefeller University Press. Note the round appearance of the microtubules in cells prepared by rapid freezing and freeze-substitution. The SPB (C) in rapidly frozen, freeze-substituted cells shows valuable detail in the layers of the organelle (IP, inner plaque; CP, central plaque; OP, outer plaque), as well as in the bridge (B) and in the satellite (S). Bar: 0.1 $\mu$m.

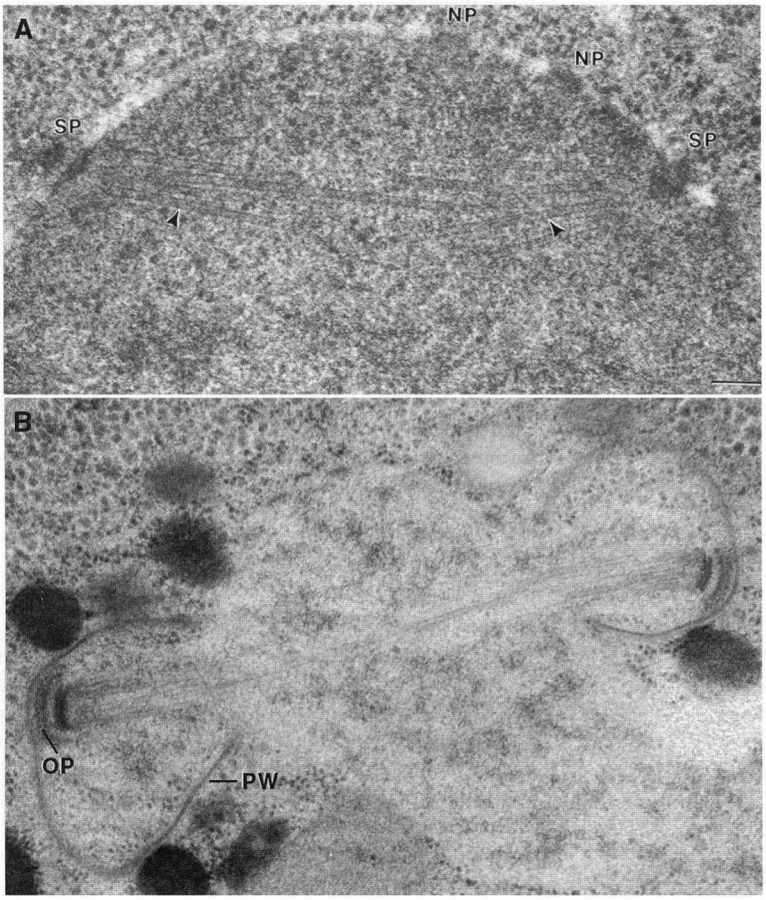


**Fig. 2.**

Mitotic and meiotic spindles in rapidly frozen, freeze-substituted cells. In a longitudinal view of a mitotic spindle (A), SPBs (SP) and nuclear pore complexes (NP) are indicated. Nuclear, spindle microtubules are indicated by arrowheads. From Winey *et al.* (1995), with copyright permission of The Rockefeller University Press. A similar view of a meiosis II spindle (B) reveals not only the spindle microtubules and the SPBs, but also the heavily modified outer plaque (OP) on the SPBs, as well as the prospore wall (PW). Bar: 0.1 $\mu$m.

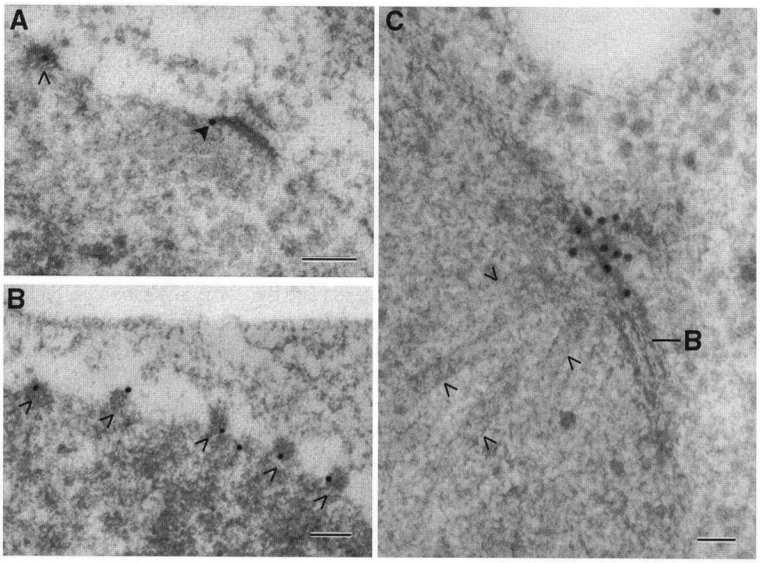


**Fig. 3.**

Immuno-EM of Ndc1-GFP (A and B). [from Chial *et al*. (1998), with copyright permission of The Rockefeller University Press] and of Spc42-GFP [C; see Adams and Kilmartin (1999)]. Cells were prepared as described in the text and stained with antibodies against GFP (see Chial *et al*., 1998). Ndc1p is a membrane protein found at the periphery of the SPB (A, arrowhead) and at nuclear pore complexes (A and B, carets). (C) Spc42p spans the central and outer plaques of the SPB (Donaldson and Kilmartin, 1996; Chial *et al*., 1998). The bridge (B) and the nuclear microtubules (carets) are apparent in this image. Bar: 50 nm.

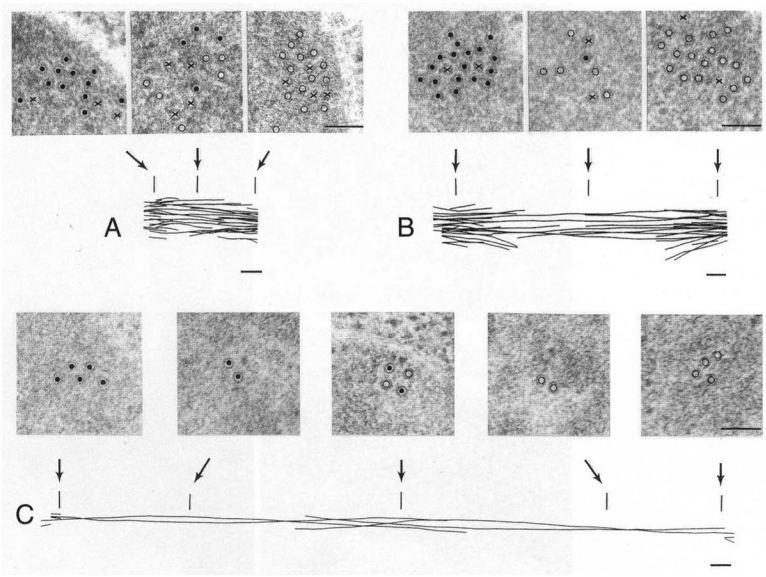


**Fig. 4.**

Representative spindle reconstructions from serial thin sections reprinted from Winey *et al*. (1995), with copyright permission of The Rockefeller University Press. The microtubule arrays in short (A), medial (B), and long (C) spindles are shown such that the SPBs will be found at the ends. Representative cross sections through the spindle microtubules are shown and their position in each spindle model is indicated by an arrow and tick mark. The polarity of individual microtubules was inferred by tracking each microtubule to the SPB from which it originates (see Winey *et al*., 1995). Microtubules in the cross-section images marked by a closed circle originated from the left SPB; open circles mark those originating from the right SPB. The few microtubules marked by an X had both ends so close to the SPBs that a polarity could not be assigned (see Winey *et al*., 1995, for discussion). Bars: 0.1 $\mu$m.

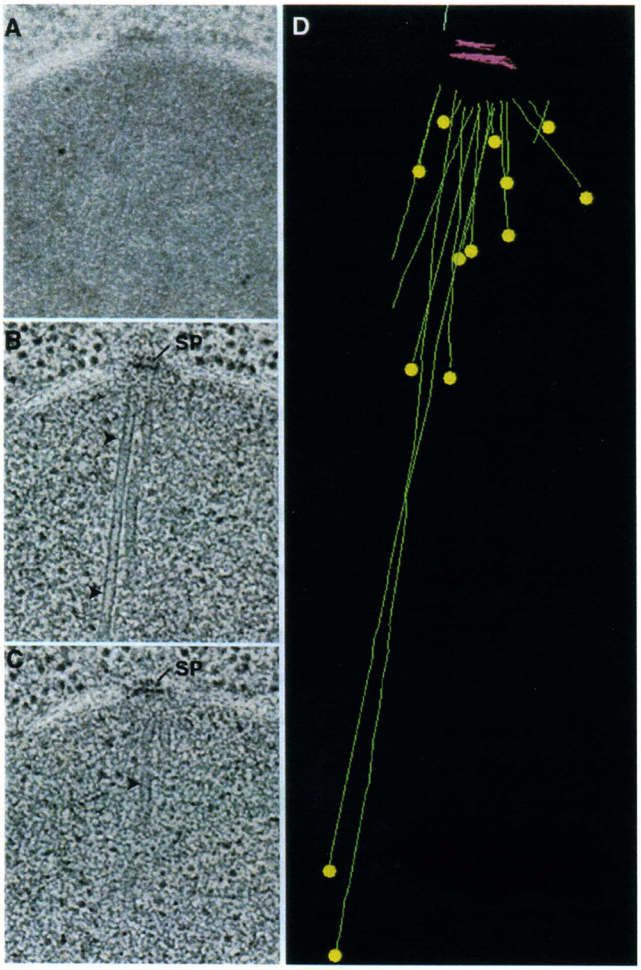


**Fig. 5.**

An overview of electron tomography of yeast SPBs. Adapted from O'Toole *et al*. (1999), with permission from the American Society for Cell Biology. Tomograms are computed from a series of views of a thick section, tilted about two orthogonal axes of a thick section (Mastronarde, 1997). (A) An image of a thick section (300 nm) containing a single SPB in G1. Data from the tomogram can be viewed by computing a number of "slices" extracted from the 3D reconstruction at any chosen level or orientation (B and C). For these manipulations, the IMOD program was used (Kremer *et al*., 1996). Here the SPB (SP) is shown, as are nuclear microtubules (arrowheads). Model points can be placed on structures of interest in serial tomographic slices, and the resulting 3D model of the structure can be displayed (D). The SPB in this thick section is shown in pink, a cytoplasmic microtubule in light blue, and nuclear microtubules in green. Microtubules for which plus ends (SPB distal) were located in the thick section are marked by yellow dots. This SPB in a G1 haploid cell has enough nuclear microtubules to have attachments to all of the kinetochores [see O'Toole *et al*. (1999) for discussion].