[4] Immunoelectron Microscopy of Aldehyde-Fixed Yeast Cells

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Introduction

Immunolocalization of antigens in cells requires simultaneous preservation of structure and antigenicity. The difficulties of immunolocalization methods are centered around the methods used to preserve structure, which as a rule tend to degrade the ability of antigens (usually proteins) to be recognized by their cognate antibodies. The value of immunolocalization for cell biology was early recognized in the case of yeast at the level of the light microscope: immunolocalizations and, even more important, colocalizations using reagents labeled differentially with different fluorophores were developed and came into general use in the early 1980s.[1-4] However, application of immunolocalization in electron microscopy of yeast lagged behind, largely because of the difficulty of adequately preserving both structure and antigenicity with the sample preparation methods then in general use.

The advent of useful immunoelectron microscopy (immuno-EM) for yeast was the realization by van Tuinen and Riezman[5] that sodium metaperiodate ($NaIO_4$) can be used to facilitate the infiltration of resin into intact yeast cells (i.e., cells with their cell walls in place). Robin Wright (working in Jasper Rine's laboratory) used this method with a variety of new acrylic resins just coming into use and found that one of them, L.R. White, gives particularly good results with metaperiodate-treated yeast cells.[6] We adopted Wright's technique and, over the last decade, have optimized and extended it so that we can now routinely preserve structures and still see most antigens, with a frequency of successful localization comparable to that reported for other cell types, including mammalian cells.[7-11] The critical element

[1] M. N. Hall, L. Hereford, and I. Herskowitz, *Cell* **36,** 1057 (1984).
[2] A. E. Adams and J. Pringle, *J. Cell Biol.* **98,** 934 (1984).
[3] J. Kilmartin and A. E. Adams, *J. Cell Biol.* **98,** 922 (1984).
[4] P. Novick and D. Botstein, *Cell* **40,** 405 (1985).
[5] E. van Tuinen and H. Riezman, *J. Histochem. Cytochem.* **35,** 327 (1987).
[6] R. Wright and J. Rine, *in* "Methods in Cell Biology" (A. Tartakoff, ed.), Vol. 31, p. 473. Academic Press, New York, 1989.
[7] D. Preuss, J. Mulholland, C. A. Kaiser, P. Orlean, C. Albright, M. D. Rose, P. W. Robbins, and D. Botstein, *Yeast* **7,** 891 (1991).
[8] D. Preuss, J. Mulholland, A. Franzusoff, N. Segev, and D. Botstein, *Mol. Biol. Cell* **3,** 789 (1992).
[9] J. Mulholland, D. Preuss, A. Moon, A. Wong, D. Drubin, and D. Botstein, *J. Cell Biol.* **125,** 381 (1994).
[10] J. Mulholland, A. Wesp, H. Riezman, and D. Botstein, *Mol. Biol. Cell* **8,** 1481 (1997).
[11] J. Mulholland, J. Konopka, B. Singer-Kruger, M. Zerial, and D. Botstein, *Mol. Biol. Cell* **10,** 799 (1999).

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that we found necessary to preserve the larger structures and organelles was the maintenance of a suitable osmotic environment during fixation, which we accomplish with the addition of slightly submolar concentrations of sorbitol.

As suggested earlier, the ability to colocalize different antigens on the same specimens is a very important technology for modern cell biology. For the case of immuno-EM, we have reduced to practice two general approaches that can accomplish such colocalizations. One of these exploits the possibility of using antibodies derived from different animal species (e.g., rabbit and guinea pig) as primary recognition reagents; these can then be visualized using secondary antibodies, differentially labeled with different sizes of gold particles, that specifically recognize the antibodies of the two animal species. The other method exploits the ability, in serial sections, of capturing the opposing faces of adjacent sections from the microtome and labeling them differentially, followed by analysis of separate and merged images. This allows the use of primary antibodies raised in a single organism for colocalization at the level of ultrastructure.

In what follows, we specify the current methods we use in detail; we discuss only those issues that relate directly to yeast. It should be mentioned that we do not cover cryopreservation methods, which are given in the chapter by McDonald and Müller-Reichert[12] elsewhere in this volume. We see these methods as complementary and distinguished in considerable part by the reality that the chemical methods use equipment and materials generally found in electron microscopy laboratories, whereas the cryopreservation methods require special, often expensive, equipment as well as expertise. Thus for many routine studies, chemical methods are both adequate and accessible. The literature on chemical methods for immuno-EM in general is extensive and has been reviewed excellently and thoroughly by Griffiths.[13]

Overview and Rationale for Chemical Fixation and Processing of Yeast Cells for Immuno-EM

Culture Density and Media

For most immuno-EM studies of yeast, the culture density (OD_{600}) should be equal to or less than 0.5 at the time of fixation. There are several reasons for this recommendation. First, at this density the morphology and molecular content of organelles and structures will be those of actively growing cells; in rich media (e.g., YEPD) at the relatively low OD_{600} of 0.8, yeast cells are slowing their exponential growth rate and already have significantly altered their gene expression patterns.[14]

[12] K. McDonald and T. Müller-Reichert, *Methods Enzymol.* **351,** [6], 2002 (this volume).
[13] G. Griffiths, "Fine Structure Immunocytochemistry." Springer-Verlag, Berlin, 1993.
[14] J. DeRisi, *Science* **278,** 680 (1997).

Second, the yeast cell wall becomes less porous as the culture density increases,[15] making cells more refractory to processing for EM. This is particularly important because our method, when applied to exponentially growing cells, does not require removal of the cell wall. To ensure that cells are really growing exponentially, it is recommended that cultures have been maintained at a low density (<0.5) for three or more generations.

Aldehyde Fixation

To provide adequate fixation with minimal loss of antigenicity, immuno-EM methods generally employ a mixture of formaldehyde and glutaraldehyde. Both aldehydes are uncharged and can diffuse rapidly into cells and both react with the amino groups of proteins. However, the monoaldehyde formaldehyde produces many fewer cross-links than the dialdehyde glutaraldehyde. The reaction of aldehydes with amines also produces protons and thus a drop in intracellular pH is expected during fixation. Neither aldehyde reacts significantly with carbohydrates or lipids, and any retention of these molecules in the fixed cell is through cross-linking of the associated or surrounding protein components. The rationale behind using both aldehydes is based on the reality that the extensive cross-linking that would occur if adequate concentrations of glutaraldehyde were used would severely limit the immunoreactivity of many antigens. For this reason, glutaraldehyde is used in relatively low concentrations in the presence of ca. 10-fold higher concentrations of formaldehyde that serve to complete fixation with a minimal loss of antigenicity. We recommend starting with a glutaraldehyde concentration of 0.4% together with 4% formaldehyde. If this regime fails to provide an expected level of signal, decreasing the glutaraldehyde concentration to 0.2 or 0.1% should be tried. This tactic is limited by the consequence that lower concentrations of glutaraldehyde may result in displacement or complete loss of antigen as well as insufficient preservation of morphology.

The chemistry of aldehyde fixation is extremely complex and therefore the use of aldehydes in EM protocols has, for the most part, been determined empirically. However, there are a few facts about the chemistry of formaldehyde relative to fixation that are worth pointing out. At low concentrations (less than 2%), formaldehyde exists predominately as monomeric methylene glycol. At higher concentrations (10% or more), formaldehyde exists predominately as methylene glycol polymers. Large methylene glycol polymers form polymethylene cross bridges more quickly and more stablely than monomeric methylene glycol,[16] thus making it the preferred reagent for fixation. When diluted from high concentrations (10–37%), formaldehyde initially retains a high proportion of these large

[15] J. De Nobel and J. Barnett, *Yeast* **7,** 313 (1991).
[16] T. Johnson, *J. Electron Microsc. Tech.* **2,** 129 (1985).

polymers. This argues for preparing formaldehyde for fixation from concentrated stocks. Concentrated formaldehyde can be purchased as a 37% methanol-stabilized stock (100% formalin) or at lower, methanol-free, concentrations of 10 or 16%. Good results can be obtained with commercial stocks of 10 or 16%, but methanol containing formaldehyde is not recommended for histochemical studies. We make fresh formaldehyde stocks (30 to 35%) from paraformaldehyde (the solid, polymerized form of formaldehyde). We have found that freshly prepared formaldehyde gives better and more consistent results than those obtained from commercially prepared formaldehyde.

Osmolarity

The yeast plasma membrane, like those of other eukaryotic cells, appears to remain semipermeable during fixation with the low concentrations of aldehydes typically used in immuno-EM methods.[13,17,18] Therefore, changes in the osmolarity of the external medium during fixation can cause movement of water in either direction across the membrane, resulting in loss of both morphology and antigens. These changes can be observed not only in the electron microscope, but also in changes in the appearance of the sensitive structures (e.g., the vacuole and the actin cytoskeleton[19]) in immunofluorescence light microscopy. We investigated the effects of fixative osmolarity on the preservation of yeast morphology and antigenicity in immuno-EM by fixing wild-type yeast cells under different osmotic conditions starting at 0 *M* sorbitol and increasing in 0.25 *M* increments to 1.5 *M* sorbitol; fixation was accomplished with 4% formaldehyde and 0.4% glutaraldehyde in 0.04 *M* potassium phosphate buffer, pH 6.7. Using affinity-purified antibodies directed against carboxypeptidase Y (CPY) and antibodies directed against actin, we found that aldehyde fixation with osmotic support greatly improves antigenic and morphological preservation in *Saccharomyces cerevisiae* (Fig. 1). We can summarize the importance of Fig. 1 and many subsequent applications of this technique as follows.

1. Aldehyde concentrations (ca. 4% formaldehyde and 0.4% glutaraldehyde in phosphate buffer) and fixation times (i.e., 30–60 min) used for immuno-EM leave the yeast plasma membrane semipermeable and osmotically active.

2. Fixation in hypotonic conditions (i.e., no added sorbitol) causes rupture of the vacuole in a high percentage of cells (ca. 60%). Hypotonic fixation also disrupts the cortical actin patch and its attachment to the plasma membrane. We

[17] Q. Bone, *J. Cell Biol.* **49,** 571 (1971).

[18] A. M. Glauert, "Practical Methods in Electron Microscopy," Vol. 3. North-Holland, New York, 1975.

[19] J. Pringle, R. Preston, A. Adams, T. Stearns, D. Drubin, B. Haarer, and E. Jones, *in* "Methods in Cell Biology" (A. Tartakoff, ed.), Vol. 31, p. 357. Academic Press, New York, 1989.

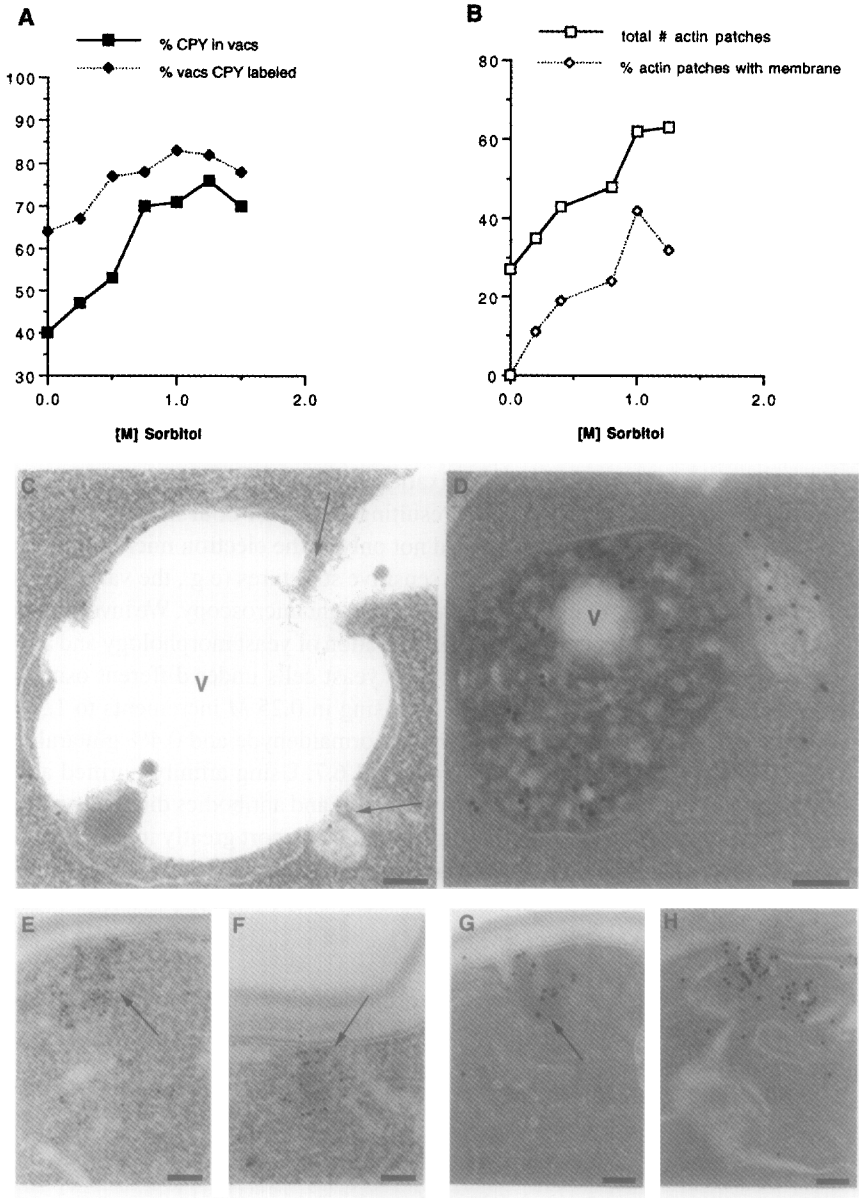

FIG. 1. Fixation in the presence of sorbitol improves the preservation of yeast ultrastructure. (A) Quantitative analysis of anti-CPY (carboxypeptidase Y) localization demonstrates an increase in the localization of CPY (as a percentage of the total number of CPY localizations) to vacuolar structures that correlates with fixation in high osmolarity. A total of 39 cells per each of the seven

attribute these artifacts to osmotically induced swelling of the cell and its organelles (especially the vacuole).

3. Cells fixed in buffered fixative containing 0.2 *M* sorbitol show a degree of ultrastructural preservation equivalent to cells fixed directly in growth medium, using buffered fixative containing no sorbitol. We find that still higher concentrations of osmotic support produce an even better preservation of the organelle structure.

4. Buffered fixative (see later) containing 1 *M* sorbitol appears to approximate the internal osmolarity (isotonic) of yeast cells growing in YPD (2% glucose).[20–22]

[20] J. Conway and W. Armstrong, *Biochem. J.* **81,** 631 (1961).

[21] W. N. Arnold and J. S. Lacy, *J. Bacteriol.* **131,** 564 (1977).

[22] W. N. Arnold, *in* "Yeast Cell Envelope: Biochemistry, Biophysics, and Ultrastructure" (W. N. Arnold, ed.), Vol. 1, Chapter 3. CRC Press, Boca Raton, FL, 1981.

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different fixative osmolarities were observed and quantified for CPY localization. Of the cells fixed in buffered fixative (0 *M* sorbitol), only 40% of the CPY localizations were to vacuole structures. Of the cells fixed in high osmolarity (0.8 to 1.25 *M* sorbitol), 70% of the CPY localizations were to vacuolar structures. The percentage of vacuole structures that had CPY labeling also increased with increasing amounts of sorbitol. The level of CPY localization to the vacuole, as well as the number of vacuole structures that label, appears to plateau at approximately 1 *M* sorbitol, perhaps representing saturation of the available CPY antigen. The total number of anti-CPY localizations per cell section, on average, did not change relative to the fixative osmolarity. (B) Cells fixed in the presence of added sorbitol have an increased number of antiactin localizations and an increased number of cortical actin patches compared to cells fixed without or little added sorbitol. While actin-localizing patches were observed in cells fixed in buffered fixative (0 *M* sorbitol), no plasma membrane-associated patches (% actin patches with membrane) were observed. Plasma membrane-associated patches were evident only after osmotic support was added to the fixative. Quantification of antiactin localization was done on 15 different cells, at each of the seven different fixative osmolarities. All cells counted were at the same approximate point in the cell cycle as judged by relative bud and neck size. (C and D) Good morphological preservation of vacuolar structures is indicated by vacuoles that are surrounded by an intact membrane and are filled with electron-dense polyphosphate. (C) The cell shown was fixed in buffered fixative without sorbitol; note that the vacuole membrane is ruptured and autolysis of the surrounding cytoplasm is evident (arrows). Also, note the low level of CPY localization and that some of the CPY is located in the cytoplasm. (D) In contrast, the cell shown was fixed with the same protocol as (C) except that 0.75 *M* sorbitol was added to the fixative. Note that the vacuole membrane of (D) is intact and that the vacuole contains dense polyphosphate and is well labeled with anti-CPY antibodies; also note the well-labeled and preserved late endosome to the right of the vacuole. (E–H) Good preservation of the actin cytoskeleton is indicated by actin patches that are electron dense and surround a finger-like invagination of the plasma membrane [J. Mulholland, J. Konopka, B. Singer-Kruger, M. Zerial, and D. Botstein, *Mol. Biol. Cell* **10,** 799 (1999)]. (E and F) The cortex of cells that were fixed in buffered fixative without sorbitol. Note that antiactin localizations (arrows) are in dispersed patches at the cell cortex; these actin patches do not contain any plasma membrane. (G and H) In contrast, the cells shown were fixed in the presence of 0.75 *M* sorbitol. Note that the cortical actin patches of these cells are electron dense and contain invaginations of the plasma membrane; note also that actin localization is not as dispersed as it is in (E) and (F). V, vacuole. Bars: 0.1 μm.

5. The best overall ultrastructural preservation of yeast organelles is achieved when using a slightly hypotonic fixative. The exact concentration of sorbitol used will vary with growth conditions, strain background, and mutants. We currently start at 0.75 *M* sorbitol in 0.08 *M* potassium phosphate buffer, 4% (w/v) formaldehyde, and 0.4% (w/v) glutaraldehyde for cells grown in YPD (2% sugar). Cells grown in minimal media tend to require less osmotic support than those grown in rich media, and we recommend starting with 0.5 *M* sorbitol and the same concentration of phosphate buffer. Cells grown in high concentrations of sugar will require more osmotic support during fixation (e.g., cells grown in 5% sugar generally require 1 *M* sorbitol). Osmotically hypersensitive mutants may require less osmotic support (e.g., actin mutants have better ultrastructure using 0.25 to 0.5 *M* sorbitol).

Buffers

As pointed out earlier, fixation with aldehydes causes a drop in intercellular pH and therefore the buffering capacity of the fixative vehicle should be important. We have used mainly phosphate buffer (0.08 *M*, pH 6.8) for immuno-EM, although comparable concentrations of cacodylate (0.1 *M*, pH 6.8) and PIPES (0.1 *M*, pH 6.8) have also given good results. To help minimize the extraction of cellular components and to stabilize membranes and polypeptides, many EM methods recommend the addition of millimolar amounts of Mg^{2+} and Ca^{2+} to buffers.[23] However, these cations tend to precipitate out of phosphate buffer and therefore we avoid adding them. In fact, the absence of these cations and a concentration of phosphate buffer of 0.08 *M* appear to give better overall ultrastructural preservation than the more commonly recommended 0.04 *M* phosphate buffer with diverse cations. For an extensive discussion about buffers and their use in EM methods, see Johnson[24] and Griffiths.[13]

Fixation Time and Temperature

At the aldehyde concentrations used for immuno-EM of yeast, fixation should be adequate within 30 min at 25°.[25] We generally fix for 60 min. However, low molecular weight antigens may not be retained with short fixation times, especially at lower concentrations of glutaraldehyde, and the retention of some antigens may require longer fixation times (several hours). On the other hand, extended (i.e., overnight) incubation of fixed cells in phosphate buffer could result in an extraction of cellular proteins (particularly low molecular weight antigens) and

[23] M. A. Hayat, "Principles and Techniques of Electron Microscopy: Biological Applications," 3rd Ed., p. 17. CRC Press, Boca Raton, FL, 1989.
[24] T. Johnson, *J. Electron Microsc. Tech.* **2,** 129 (1985).
[25] F. Flitney, *J. R. Microsc. Soc.* **85,** 353 (1966).

phospholipids.[26] It is therefore recommended that fixation not exceed 2 hr, with 1 hr being the standard, and that cells be processed into resin as soon as possible. Overnight incubation in phosphate buffer and fixative is not recommended.

There has been no systematic analysis of the effect of fixation temperature on yeast ultrastructure. Theoretical considerations can be used to argue that fixation should be done on ice to slow or block any deleterious physiological reactions that might not be immediately inhibited by fixation. While this may be important in samples where penetration of the fixative is slow, such as in tissue, it should not be a consideration when fixing yeast because infiltration of the fixative is rapid. We generally fix cells at room temperature. Cultures grown or shifted to elevated temperatures can also be harvested and fixed, as described later, at room temperature without any noticeable effects on ultrastructure.

However, in the special case of rapidly reversible, temperature-sensitive mutants, it may be important to fix at the same temperature as the culture or, even better, employ cryofixation methods.[12] For example, when studying a reversible, temperature-sensitive mutant blocked in membrane fusion, it could be imagined that shifting the cells to a permissive temperature during fixation might release the block, allowing membrane fusion to occur prior to sufficiently fixing the cells. Similarly, the mutant protein could be associated with a particular complex and organelle at nonpermissive temperature and the shift to room temperature may reverse this association. This might allow the protein to be relocated during fixation and subsequent processing steps.

Metaperiodate Treatment, Dehydration, Resin Infiltration, and Polymerization

As described earlier, one of the early innovations in the immuno-EM of yeast was the use of sodium metaperiodate to facilitate the infiltration of resin.[5] Sodium metaperiodate attacks the glycol groups of the mannan fibrils of the cell wall, cleaving 1,2-diols, thereby making the wall more permeable. This "loosening" of the cell wall is essential for the adequate infiltration of resin.[6] However, the action of sodium metaperiodate generates free aldehyde groups that can be a source of nonspecific antibody background. Treatment with 50 m*M* ammonium chloride is used to block these free aldehydes.[5]

There is some concern that treatment with periodate might result in the oxidation and denaturation of some proteins and eliminate sensitive epitopes. These concerns are based on early studies that demonstrated oxidation of amino acids by sodium metaperiodate.[27] Those studies, however, were done at concentrations of metaperiodate sixfold higher than those used for immunohistochemistry with incubation times on the order of hours, not minutes. We suggest using metaperiodate for the minimum time required to provide good infiltration of resin, typically

[26] R. Salema and I. Brandao, *J. Submicrosc. Cytol.* **5,** 79 (1973).
[27] J. Clamp and L. Hough, *Biochem. J.* **94,** 17 (1965).

10 min. If there is reason to suspect sodium metaperiode for the loss of antigen, other methods of making the wall permeable should be considered[28] and of course traditional enzymatic removal of the wall remains possible.[29]

After cells are fixed they must be dehydrated; all cellular water is removed by first replacing it with ethanol and then resin. Once polymerized, the resin provides support for the ultrastructure of the cell so that it can withstand sectioning, introduction into the high vacuum of the electron microscope, and interaction with the electron beam. How water is removed from the cell is very important to the ultimate results obtained, and the regime of dehydration should be tailored to the experimental aims of the researcher. For example, EM methods originally designed for structural studies of the yeast microtubule cytoskeleton initiate dehydration with 95% ethanol, and then proceed to dehydration at 100% ethanol. This rapid dehydration moves water quickly out of the cell, thereby maximizing the extraction of ribosome-dense cytoplasm and thus improving the resolution of microtubules. For immuno-EM, retention of antigens is a main objective and slower, more gradual removal of cellular water is required to preserve them in place. Therefore, dehydration is done on ice and is started at 50% ethanol, moving stepwise through higher concentrations until 100% ethanol is reached.

Once completely dehydrated, the cells are infiltrated with resin. LR White is a low viscosity acrylic resin that can be polymerized with heat or at low temperatures with the use of a chemical accelerant or ultraviolet light. Because polymerization of LR White is inhibited by oxygen, LR White polymerization is done in dry gelatin capsules. Standard plastic embedding capsules (Beem) are not used because they tend to adsorb gases. It should be noted that LR White resin shrinks both during polymerization and in the EM. Infiltration of the resin is done in steps of increasing concentrations, and proper infiltration is a slow process. As described later, infiltration starts with two parts of 100% ethanol and once again proceeds stepwise to 100% resin. Cell wall mutants or strains found to be difficult to infiltrate require longer infiltration times and perhaps more gradual steps before 100% resin is achieved. Unlike the original protocol of Wright and Rine,[6] we do not recommend the application of vacuum during infiltration because it appears somehow to disrupt the ultrastructure of the vacuole.

LR White is polymerized at 47° for 48 to 72 hr. A stable polymerization temperature (±2°) is required for proper polymerization.[6] A standard heat block heater that can maintain this temperature, equipped with a custom-drilled aluminum block that fits the gelatin capsules, is recommended for this step. The capsules in the block are covered with foil during polymerization. We have made several aluminum heat blocks and have learned that the diameter of the holes should just allow the nondried

[28] O. Rossanese, J. Soderholm, B. Bevis, I. Sears, J. O'Connor, E. Williamson, and B. Glick, *J. Cell Biol.* **145,** 69 (1999).

[29] B. Byers and L. Goetsch, *Methods Enzymol.* **194,** 602 (1991).

gelatin capsule to fit. Drilling the holes to this specification will assure that the dried gelatin capsule will fit properly and can be removed easily. It is important to fully dry the capsules before use (see protocol later) lest they become permanently stuck in the heat block during polymerization.

Common Artifacts and Problems

There are at least five common artifacts that are observed when using chemically fixed yeast cells and LR White resin for immuno-EM studies. The first is plasmolysis, which is the separation of the plasma membrane from the cell wall (Fig. 2A). Plasmolysis generally occurs when cells are transferred to hypertonic media. In immuno-EM, plasmolysis will occur in fixative that contains too much sorbitol. If a large percentage of cells exhibit plasmolysis, the first step is to redo the EM procedure, reducing the amount of sorbitol added to the fixative.

However, it is generally the case that some cells (usually less than 10%) fixed and embedded in LR White resin will have areas where the plasma membrane has separated from the cell wall. This separation is caused by shrinkage of the LR White resin during polymerization as well as when the section is first exposed to the electron beam. If the section is shrinking severely and cells are developing holes between the wall and plasma membrane, it may be useful to first condition the sections. To condition the section, the beam is defocused so that the amount of energy hitting the section is decreased and spread over the whole section. After a few seconds, the beam is focused more and the area of view is moved around so that the section is allowed to shrink as evenly as possible. Once stabilized, higher magnifications and a more focused beam can be used.

However, if there are many holes or it is observed that many of the cells are separating from their cell walls and falling out of the section, then the resin has not been infiltrated properly. To remedy this problem, sections can be picked up on Formvar-coated grids and, after immunolocalization, they can be further stabilized by coating with carbon.[23] Alternatively (and better overall), the experiment can be repeated with fresh resin and longer infiltration times. Also note that extended incubation (>30 sec) in lead citrate stain (pH 12, see later) may cause cells to fall out of the section. Therefore, if cells are falling out of the sections, it may be helpful to check the sections for stability in the EM prior to lead staining.

A second artifact is blebbing and vesiculation of membranes. Vesiculation and blebbing of membranes, particularly the plasma membrane, can occur when little or no glutaraldehyde is used with the standard 4% formaldehyde. Weakly fixed cells are especially sensitive to membrane rearrangements if the fixative is hypertonic, and plasma membrane blebs are often observed at areas of the cell surface that have suffered plasmolysis (Fig. 2A).

The third common artifact is lumenal distention of the nuclear membrane and endoplasmic reticulum (ER) (Fig. 2B). We believe that this artifact is caused by a

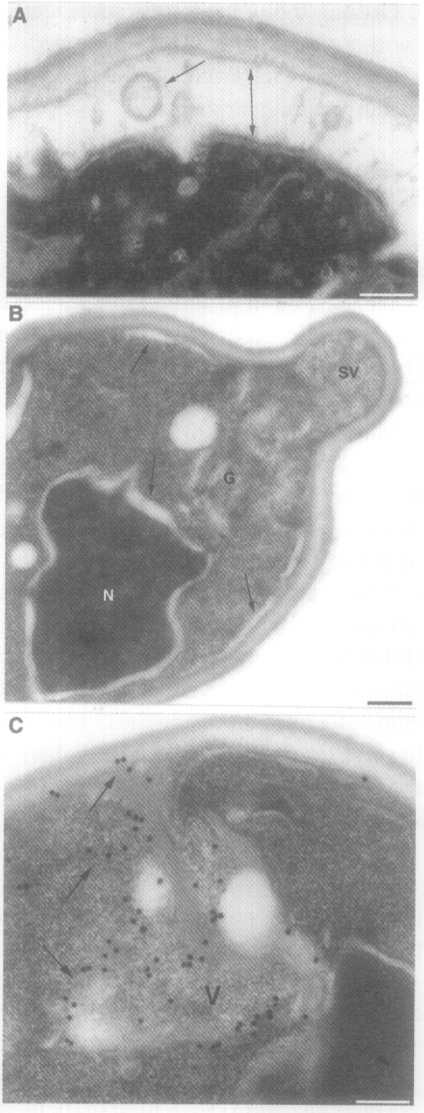


FIG. 2. Artifacts of aldehyde fixation and LR White resin embedding (see text for details). (A) Cell shown exhibits an extreme example of plasmolysis; double-ended arrow indicates the separation between the plasma membrane and the cell wall. Also note the blebbing and vesiculation of plasma membrane (single-ended arrow). (B) Cell shown exhibits artifactual distention of ER and nuclear membranes (arrows). Also note that the Golgi membranes (G) are poorly preserved, whereas the secretory vesicles (SV) of the bud are well preserved. (C) Cell shown illustrates the ultrastructural effects of vacuole rupture. Note that the fusion of the vacuole with the cell surface and autolysis of the cytoplasm results in the localization of CPY (anti-CPY, 15 nm gold) to the cell surface and cytoplasm (arrows). N, nucleus; V, vacuole. Bars: 0.1 μm.

combination of weak fixation and LR White shrinkage. When there is little protein content within the ER lumen, the extent of cross-linking to and across the ER lumen is minimal and is perhaps the most weakly fixed area of the cell. During the initial exposure to the electron beam, the resin shrinks and pulls the weakly fixed ER and nuclear membranes apart. This artifact is frequently eliminated by postfixing the sections in 8% glutaraldehyde after immunolocalization reactions but before electron-dense staining (see later).

The fourth artifact is lysis of the vacuole (Fig. 1C). Fixation in either hypotonic or hypertonic fixative can result in lysis of the vacuole. In an extremely hypertonic fixative, shrinkage of the cell may cause the plasma membrane to contact and fuse with the vacuole membrane, causing rupture and mixing of vacuolar and cell surface proteins (Fig. 2C).

A fifth artifact, or limitation, of our method is the loss of microtubule fine structure while preserving the immunoreactivity of tubulin (Figs. 4C and 4D). Fixation appears to be sufficient to keep the tubulin subunits in place but no individual tubules can be resolved; they appear to have disassembled *in situ*. We have yet to find a fixation condition that preserves both microtubule fine structure and immunoreactivity. This limitation may be overcome by using freeze substitution techniques in combination with light (0.01%) OSO_4 staining (see Ref. 12).

Standard Protocol for Aldehyde Fixation and LR White Resin Embedding

Fixation with Osmotic Support

1. Grow culture to early exponential phase ($OD_{600} < 0.5$) in YEPD or SD medium (2% sugar). We typically process 50–100 ml per sample; this will produce two to three blocks of material for sectioning. However, as little as 5 ml of sample can be processed with slight modification of this protocol (see later).

2. Quickly harvest cells using a disposable 0.45-μm filter unit (Corning) with a vacuum of 20 to 15 in Hg ("house vacuum"). Swirl while filtering to a final volume of 5 ml; important—do not filter dry. Disconnect vacuum and quickly add 25 ml of fixative (see later) directly to the cells. Swirl to cover cells, and use a 10-ml disposable pipette to resuspend the cells by pipetting up and down. The less time between harvesting the sample and adding the fixative the better; it should be possible to take a sample through filtration and addition of fixative in about 1 min.

3. Transfer the cell suspension from the filter unit to 50-ml conical tubes (Corning) and incubate for 60 min at room temperature with occasional mixing.

Sodium Metaperiodate Treatment

4. Collect cells in a low-speed centrifuge (e.g., clinical IEC on setting 6 for 2 min or in a Sorval at 3000 rpm, SS34 rotor for 5 min) at room temperature. Use minimal speed to avoid packing the cells too densely. Pour off supernatant.

5. Resuspend and wash cells by centrifugation (same speed and time) using different buffers as follows:

1× (5 ml) 0.50 *M* sorbitol in 0.08 *M* potassium phosphate buffer (pH 6.7)
1× (5 ml) 0.25 *M* sorbitol in 0.08 *M* potassium phosphate buffer (pH 6.7)
1× (5 ml) 0.08 *M* potassium phosphate buffer (no sorbitol) (pH 6.7)

The cell pellet can be resuspended using very gentle vortexing.

6. Transfer cells to disposable glass test tubes (13× 100 mm) with a Pasteur pipette. Centrifuge and resuspend cells in 5 ml of 1% (w/v) $NaIO_4$ (in H_2O, Fluka, Ronkonkoma, NY). Make solution just prior to incubation. Incubate for 10 min at room temperature.

Ammonium Chloride (NH_4Cl) Treatment

7. Centrifuge and wash cells at room temperature using double-distilled water (or water of equivalent or higher purity obtained by other means). Cells will now clump together due to modification of their cell walls. These clumps can be resuspended easily by mixing with a wooden applicator (VWR, West Chester, PA). Cell clumps should be <1 mm.

8. Centrifuge and resuspend cells in 5 ml of 50 m*M* NH_4Cl (in H_2O, Sigma, St. Louis, MO). Incubate for 15 min at room temperature.

Dehydration

9. Centrifuge and resuspend cells serially in an ice-cold ethanol/H_2O series (2–5 ml each) as follows: 50% ethanol, 70% ethanol, 80% ethanol, 85% ethanol, 90% ethanol, 95% ethanol, 100% ethanol, 100% ethanol (this is the second such resuspension), and 100% ethanol (for this final resuspension, use a fresh bottle of ethanol at room temperature).

Dehydration should be done for 5 min/step on ice with ice-cold ethanol (except the final 100% step as noted earlier). Centrifugation should be very brief (ca. 2 min) and can be done at room temperature; we use an old-fashioned table-top IEC clinical centrifuge. Do not compact cells too densely. Pellet should be a little loose. Use wooden applicators to resuspend cells in ethanol. In the higher concentrations of ethanol, cells will become less clumped so care must be taken not to pour off cells when recovering the pellets. To avoid damage to cells due to the rapid evaporation of ethanol, cells at the 90% ethanol step and beyond should have the ethanol removed and immediately covered with the next ethanol concentration (or resin).

Infiltration

10. Centrifuge cells and pour off ethanol. Immediately cover and resuspend in 2 ml of a 2 : 1 (v/v) mixture of ethanol : LR White resin (medium hard, Polysciences

Inc., Warrington, PA). Place, covered with Parafilm, on a rotator for 1 hr at room temperature. We use a multipurpose rotator (VWR) with a drum diameter of 6 inches rotating at approximately 20 rpm. The cell/resin mixture can be stored at −20° overnight if needed, although it is preferable to bring the cells through the next step.

11. Pellet cells and pour off ethanol/resin and resuspend in 2 ml of 1 : 1 ethanol : LR White resin. Place, covered with Parafilm, on a roller overnight at room temperature.

12. Pellet cells and pour off ethanol/resin and resuspend in 1 ml of 1 : 2 ethanol : LR White resin. Cover with Parafilm and place on a roller for 1–2 hr at room temperature.

13. Pellet cells and remove resin with a Pasteur pipette; resuspend in 1 ml 100% LR White resin. Place on a roller for 1 hr at room temperature. After 1 hr, the cell/resin mixture can be stored at −20° for at least 1 month with a little noticeable change in morphology. However, storage may result in increased extraction of the cytoplasm and may result in movement or extraction of the antigen.

14. Pellet cells and aspirate resin with a Pasteur pipette and resuspend in approximately 0.5 ml 100% LR White resin. If the cells are stored at −20°, bring them to room temperature prior to uncovering and proceeding with step 14, otherwise condensation will wet the sample.

Embedding and Polymerization

15. Transfer cells and resin using a Pasteur pipette to gelatin capsules (size 00, Ted Pella, Redding, CA); capsules should be about half full. A 100-ml culture at an OD_{600} of 0.5 should produce enough material for at least three capsules. It is very important to dry gelatin capsules in an oven (60–70°) for 24 hr prior to using; dry capsules will shatter when crushed between fingers. Labels should be written with pencil (No. 2 works best) because some inks appear to be soluble in LR White resin.

16. Top off the capsules with fresh 100% LR White resin and add labels and gelatin caps. Allow cells to settle to the bottom of the capsules for approximately 15 to 30 min at room temperature. Place capsules in a heat block set at 47°. Cover the heat block (several layers of aluminum foil will work) to keep the temperature stable. Polymerize for 2–3 days at 47°.

Fixation of Low Numbers of Cells

As little as 5 ml of a cell culture ($OD_{600} < 0.5$) can be fixed and processed for immuno-EM. However, the main problem with processing small numbers of cells is that when they are transferred into gelatin capsules, the cells spread out over the whole surface of the capsule tip. This forms a very thin layer of cells that can be trimmed away easily or sectioned through. To avoid this problem, we employ two methods that concentrate the cells at the tip of the capsule.

In both methods, cells are first fixed by taking an aliquot of the culture and adding it, with quick mixing, to a equal volume of 2× fixative and buffer. Because medium is being carried over, the amount of osmotic support needed during fixation can be reduced to a final concentration of about 0.5 *M* sorbitol for rich media and 0.25 *M* for minimal media. Fixation is then as described earlier; there is no need to resuspend the cells in fresh fixative.

For the first method, after fixation and the final wash, the cell pellet is resuspended in approximately 0.1 ml of 2% low melt agarose (in 0.08 *M* potassium phosphate buffer, pH 6.7) that has been held at 37°. The pellet is resuspended quickly and then placed on ice to solidify the agarose. The agarose cell pellet is then broken up into pieces no larger than 1 × 1 mm and processed as described earlier. Several of these agarose pieces can be place in a gelatin capsule and polymerized; the rest can be stored in 100% resin at −20°.

For the second method, after being fixed in medium as described earlier, cells can be processed normally until the final resin step. At the final resin step, cells are resuspended in approximately 0.2 ml of 100% resin and transferred to dry, size 4 gelatin capsules (Polysciences, Inc.). This capsule is then placed in the larger standard size 00 capsule and is filled with resin, labeled, and polymerized as usual. This technique produces a thin column of cells at the tip and center of the larger capsule (we call this a "corn dog"). The polymerized block is then trimmed down to the cells at the center. This method can also be used in combination with the agarose method and will make locating agarose-embedded cells a little easier.

Reagents

We use double-distilled water or water purified through ion-exchange systems (e.g., Milli-Q). Although it may not matter for many of the steps, we believe that the immuno-staining protocols, at least, require very pure water.

Buffered fixative (prepare in a hood):
0.08 *M* potassium phosphate buffer (pH 6.7),
0.75 *M* sorbitol (Sigma),
4% Formaldehyde (make fresh, see later), and
0.4% Glutaraldehyde (8% EM grade, snap vial; Polysciences). The final pH should be between pH 6.5 and 7. Check it.

These are the final concentrations for the fixative (i.e., 5 ml of sample plus 25 ml of fixative). Therefore, calculate amounts based on the 30-ml/sample but bring the volume of fixative to 25 ml/sample; the 5 ml of sample will bring the final volume to 30 ml. Fixative is made within 2 hr of processing cultures and is divided into 25-ml aliquots in 50-ml polypropylene conical centrifuge tube (Corning).

1 *M* potassium phosphate buffer (pH 6.7):
75.77 g dibasic potassium phosphate (K_2HPO_4) [0.435 *M*],
76.89 g monobasic potassium phosphate (KH_2PO_4) [0.565 *M*], and
800 ml doubly distilled H_2O or Milli-Q water;
Mix. Check pH (should be pH 6.7). Bring volume to 1 liter.
Filter sterilize.

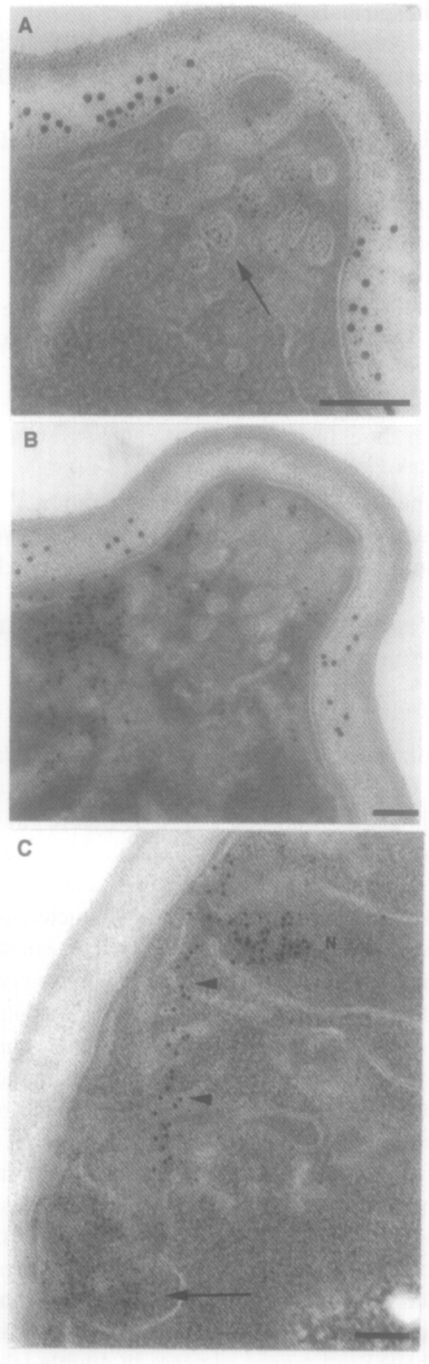
Formaldehyde stock (weigh and prepare in a hood):
Weigh 6–8 g paraformaldehyde (Polysciences, Inc.) in a 50-ml conical tube (Corning, Inc.).
Add 20 ml double-distilled (or equivalent purity) water; shake vigorously.
Add 0.5–1 ml 5*N* NaOH (freshly made, less than 1 week old) and shake vigorously.
Heat to 65–70° in water bath (approximately 15 min).
Shake. Solution should be clear; if not, return to water bath or add a little more NaOH.
Measure volume and calculate (w/v) concentration.
Add required amount of formaldehyde stock to fixative.
Repeat in separate tubes if more formaldehyde is needed.

Overview and Rationale for Postembedding, Immunogold Localization on Yeast Cell Sections

Antibodies and Gold Conjugates

Immuno-EM localization experiments are done using either polyclonal [usually immunoglobulin G (IgGs)] or monoclonal antibodies (usually IgMs). It is essential that the antibody be well characterized before immuno-EM is undertaken. At a minimum, Western blot analysis should be done to confirm that the antibody reacts with a single (preferably) protein band of the expected molecular weight. Immunofluorescence light microscope characterization of the antibody should be the next step. Light microscopy provides the easiest and fastest method for characterizing antigen–antibody interactions in the fixed cell. Optimal fixation conditions and antibody dilution can be determined efficiently at the light level and, once optimized, immunofluorescent light microscopy can be used to provide an overview of the concentration and distribution of the antigen. This overview is virtually essential if one is to interpret successfully immunolocalization results at the level of ultrastructure. If an antigen cannot be localized at the light level, there is usually no point in attempting localization in the electron microscope.

It is strongly recommended that affinity-purified antibodies be used in immuno-EM experiments. In our experience, unfractionated rabbit immunosera produce so much nonspecific background that they are essentially useless for immuno-EM. However, we have had some success using guinea pig immunosera; nevertheless, we would not depend on such a reagent for primary characterization of an antigen. Instead, we have used guinea pig sera in double-label experiments (the


A
B
C
N

other antibody is typically an affinity-purified rabbit antibody) where the antigen has already been characterized biochemically and immunohistochemically using an affinity-purified antibody (typically also rabbit).

For immuno-EM, antibodies are usually used at concentrations 10 times greater than those used for immunofluorescent microscopy; we titrate every preparation for both purposes. IgG antibodies in a typical purification can be used at a dilution between 1 : 1 and 1 : 50, and monclonal IgM antibodies are used at a dilution between 1 : 1 and 1 : 10 (supernatant) or between 1 : 1 and 1 : 1000 (ascites fluid). These are general rules; we have had success with monoclonal culture supernatant at 1 : 100 (see Figs. 3C and 5C for example) and affinity-purified IgG antibody at 1 : 250. It is essential to find the optimum concentration for each preparation. Antibodies directed against the carbohydrate moieties of glycoproteins (e.g., anti-1,6-mannose) are often so effective that they can be used at high dilution (1 : 2000). This may reflect a relative lack of degradation of the antigenic structures by fixation or, more simply, the relative accessibility of the epitopes themselves.

It should be noted that we have had limited success employing monoclonal antibodies for immuno-EM. It may well be that the reason for this is the uniqueness of the epitopes recognized; in contrast, polyclonal antibodies generally recognize several epitopes on target proteins. If monclonal antibodies are to be used, it is helpful to prepare samples fixed at different concentrations of glutaraldehyde (e.g., 0.4% and 0.1%) to determine whether the single epitope is sensitive to glutaraldehyde fixation.

Colloidal gold has become the most popular particulate marker for immuno-EM, mainly because gold particles of defined size are produced easily and can be adsorbed to a number of affinity molecules such as immunoglobulins and protein A. The dense gold particles are recognized easily and resemble no natural cellular structures. The production of colloidal gold and its adsorption to proteins for immuno-EM has been well described by Roth[30] and reviewed in Jan *et al.*[31]

[30] J. Roth, *Histochem. J.* **14,** 791 (1982).

[31] L. Jan, M. Leunissen, and J. De May, *in* "Immunogold Labeling in Cell Biology" (A. Verkleij and J. Leunissen, eds.), Chapter 1, p. 3. CRC Press, Boca Raton, FL, 1981.

FIG. 3. Examples of double localizations using lectins and antibodies (see text for details). (A) A high magnification image of a small bud demonstrating double localization of two lectins. Con A (Con A–biotin detected with antibiotin–5-nm gold secondary) labels the secretory vesicles (arrow) and cell surface; WGA (WGA–15-nm gold conjugate) labels the chitin ring at the neck of the small bud. Note the lack of WGA labeling of the cell wall of the small bud; also note the difficulty in visualizing 5 nm gold due to the granularity of the lead citrate staining. (B) Image of a small bud demonstrating the antibody localization of actin (antiactin, detected with a 10-nm gold secondary) together with the localization of chitin (WGA–15 nm gold). (C) Double localization of two antibodies shows the localization of nuclear and cytoplasmic microtubule spindles (arrow heads) and the actin cytoskeleton (cortical actin patch, arrow). Tubulin was localized using a mouse monoclonal antitubulin supernatant applied at a 1 : 100 dilution and detected with an antimouse IgG and IgM–10-nm gold secondary applied at a 1 : 50 dilution. Actin was localized using affinity-purified (rabbit) antiactin applied at a 1 : 30 dilution and detected with an antirabbit IgG–5-nm gold secondary applied at a 1 : 50 dilution. Bar: 0.2 μm.

We use gold particles of different sizes (from 5 to 15 nm in diameter) adsorbed to a secondary antibody [most commonly goat IgG or IgM directed against the immunoglobulin(s) of the organism (rabbit, guinea pig, mouse, or rat) the primary antibody was raised in]. Alternatively, gold particles can be adsorbed to protein A, which binds specifically and tightly enough to IgG molecules of most mammalian species. We always perform such indirect immunolocalization reactions sequentially, first incubating the section with the primary antibody, followed by extensive washing to remove antibodies bound nonspecifically, and only then incubating with the secondary affinity molecule to which the gold particles are bound. High-quality secondary gold conjugates of various sizes are available commercially (see later) and are generally used at a dilution of 1 : 50–1 : 100.

Lectins

Lectins are proteins that bind a diverse group of carbohydrate molecules. They are especially reactive with the sugar groups of glycoproteins and lipids. Very high levels of labeling can be achieved with lectins (as well as antibodies) because the reactive carbohydrate molecules are abundant and are not cross-linked by aldehyde fixation. However, unlike labeling with affinity-purified antibodies, lectins can exhibit a range of avidity for different sugar linkages under diverse conditions. This potential lack of specificity must be kept in mind when interpreting the localization of lectins.

Concanavalin A (Con A) and wheat germ agglutinin (WGA) are the most commonly used lectins for affinity localization in yeast.[19] Con A has a high-avidity interaction with α-mannose linkages (less for α-glucose and α-*N*-acetyl-D-glucosamine) of glycosylated yeast proteins and can be used to label the secretory pathway, from the Golgi to the plasma membrane and cell wall. WGA has an affinity for β1,4 linkages between *N*-acetylglucosamine residues and therefore is useful for labeling chitin-rich components of the yeast cell wall (e.g., bud neck and bud scars). WGA labeling is particularly useful for discriminating between mother and daughter cells; newly formed daughter buds and cells have little to no detectable chitin (see Figs. 3A and 3B).

Both Con A and WGA can be purchased directly conjugated to gold of various sizes. We have tried Con A and WGA gold conjugates manufactured by BBInternational Inc. (Ted Pella, Inc.) and have had good labeling with the WGA–gold conjugates (Figs. 3A and 3B). In our hands, Con A–gold conjugates from BBInternational and from Sigma failed to produce an adequate signal. We have been successful in using a Con A–biotin conjugate detected with antibiotin–gold antibody, both from BBInternational (Fig. 3A). It is also possible to purchase purified lectin and colloidal gold sols with which lectin–gold conjugates can be made easily.[30,31]

Blocking

As with any immunohistochemical procedure, nonspecific antibody interactions must be blocked to reduce the background and optimize the signal-to-noise ratio of the antibody. Nonspecific interactions are generally caused by molecules in the section that are "sticky" and tend to adsorb the antibody. This stickiness can be caused by a variety of weak interactions, such as charge and hydrophobicity, or by reactive groups, such as unblocked free aldehydes generated by periodate treatment. Reduction of nonspecific background is achieved in two ways: (1) by beginning the process with reagents that block the sticky molecules so that nonspecific interactions with the immunoglobulins cannot occur and (2) by washing sections extensively after antibody localizations to remove any weak interactions that manage to occur despite the blocking.

Blocking is best done with a mixture of molecules, typically proteins, that do not react with the antibodies used in the immunolocalization. Ovalbumin, gelatin, and bovine serum albumin (BSA) are often used, as is "normal" serum; our experience favors mixtures of the pure proteins. Salt and weak detergents are used to inhibit further nonspecific interactions. Caution is warranted, as blocking agents, particularly salts and detergents, can efficiently block the desired, specific antigen–antibody interactions if used at a high enough concentration. It should also be remembered that some "background" localization can be specific. For example, unwanted "spurious" localization of the antibody could be caused by the presence of an authentic epitope in a protein other than the one of interest. Fortunately, these cases can generally be detected by the preliminary Western blot and light microscopy screening recommended earlier. Harsh or careless processing for immuno-EM may also cause displacement of the antigen from the normal location in the cell, thus producing a signal at locations not predicted by immunofluorescence or biochemical characterization. Again, most of these artifacts can be detected by proper control experiments.

We routinely use a mixture of ovalbumin, BSA, and Tween 20 in phosphate-buffered saline (PBS, "standard IEM block," see later). When this mixture is not effective, we employ instead glycine, fish gelatin, or both. We also sometimes add purified mannan to the blocking mixture (recommended by Ben Glick, University of Chicago) to avoid a particularly common problem in yeast: the nonspecific localization of both affinity-purified and, especially, unfractionated serum antibodies to the cell wall or other heavily glycosylated materials in the cell. This background is sometimes not observed at the light level because the cell wall is removed for immunofluorescence light microscopy.

Controls

Microscopy, by its nature, tends to be used as a qualitative method, despite the fact that robust quantitative techniques are available for immuno-EM and should

be applied whenever possible.[13,32] The qualitative nature of most EM experiments makes it even more imperative that microscopists do basic controls necessary to show that the immunolocalization results are meaningful. As indicated earlier, antibodies should have been well characterized with Western blots and immunofluorescent light microscopy prior to using them in immuno-EM experiments. Assuming that this has been done, with controls appropriate to those methods, a few essential controls remain to be conducted at the EM level. Many of these are particularly easy with yeast, and make this organism, despite its small size and other difficulties, an attractive subject for morphological study with affinity reagents.

Tests for Specificity of Primary Antibody. If the protein antigen is encoded by a nonessential gene, then the best and most logical control is immuno-EM examination of a deletion mutant. If the gene encoding the protein target is an essential gene, then a mutant can nevertheless sometimes be found that encodes a protein that exhibits a greatly reduced affinity for the antibody (e.g., *sec4-8*[33]). One can screen for this phenotype in Western blots and by immunofluorescence light microscopy. A complementary approach to finding evidence for antibody specificity is to examine, by immuno-EM, strains that have been genetically altered to overproduce the antigen and observing a suitable increase in the antibody localization signal(s). It should be noted that often these methods indicate that a particular antibody has a specific signal over a background of relatively nonspecific localization (or even some specific localization to alternative structures). In such cases the deletion and/or overproduction controls can allow one to interpret immunolocalization nevertheless. Finally, a purified antigen, if available, can also be incubated with the antibody (or other affinity molecule) and then immuno-EM reactions done. The degree of depletion of the signal is a measure of specificity of the antibody.

Tests for Specificity of Secondary Antibody. With indirect detection methods it is necessary to also control for the specificity of the secondary antibody (or protein A) to which the gold particles are attached. This is done easily by eliminating the primary antibody from the immunolocalization protocol. If the secondary goldconjugate is specific for the primary antibody, then its application in the absence of primary antibody should give little or no staining. This control should be done every time, without fail, because secondary gold conjugates degrade with age. This degradation can produce free gold particles as well as aggregates of immunogold conjugates that can stick to sections.

Occasionally, the affinity of the detection molecule for its target needs to be confirmed. This can be done using dot-blot assays. If testing the affinity of immunoreagents, then purified immunoglobulins (IgG or IgM) or normal serum obtained

[32] M. Clark, *Methods Enzymol.* **194,** 608 (1991).

[33] B. Goud, A. Salminen, N. Walworth, and P. Novick, *Cell* **53,** 753 (1988).

from the organism that the immunogold conjugate is directed against is applied to nitrocellulose and probed with the secondary immunogold conjugate. If employing nonimmunodetection methods, then purified reactive material is blotted to nitrocellulose and probed with the affinity molecule–gold conjugate. The gold signal is then detected by amplification using silver enhancement.[34]

Positive Controls. Finding no signal is, unfortunately, not a rare event. Any primary antibody–gold conjugate combination that has previously given good, reproducible immunolocalization results should routinely be used as a positive control for the entire process.

Double-Label Immunoelectron Microscopy

Double Labeling on Same Surface of a Section. In order to carry out successful double labeling, it is necessary to have two specific antibody reagents and also two distinct secondary reagents labeled with alternative sizes of gold particles. It is particularly important that the secondary reagents used to localize one primary antibody not react with the other primary antibody, its secondary antibody–gold conjugate, or with complete complexes containing both. This problem is usually surmounted by the use of affinity-purified, primary antibodies obtained from different organisms. For example, a rabbit primary antibody is used in combination with a primary antibody from a guinea pig or mouse. These two primary antibodies are localized and detected using different secondary gold conjugates directed against immunoglobulins of the organisms in which the primary antibodies were raised. To discriminate between the localization of the different antibodies, the secondary antibodies are conjugated to different sized gold particles. We generally use 10- and 15-nm gold conjugates in the double-label experiments. We avoid using 5-nm gold conjugates because it is difficult to visualize them against the dense cytoplasm (stained ribosomes are appropriately 5 nm in size) (see Figs. 3A and 3B). We also avoid 20-nm gold conjugates because their large size appears to produce a decrease in signal, which is most likely due to steric hindrance.

Thus, in the example just given, we might use goat antirabbit 15 nm gold (GaRab-15) and a goat anti-mouse 10 nm gold (GaGP-10) (see Fig. 3C). To guard further against false colocalization due to the antibodies adsorbing to each other, all the antibodies are usually first cross-adsorbed against normal serum of the opposite species. If control experiments demonstrate that the antibodies used really do not cross-react, then primary antibody incubations can be done together, followed (after washing) by detection using a mixture of the secondary gold conjugates.

However, it will generally be the case that the primary antibodies of interest will have been raised in rabbits. In such cases, two different rabbit antibodies will

[34] M. Moeremans, G. Daneels, A. Van Dijck, G. Langanger, and J. De Mey, *J. Immunol. Methods* **74,** 353 (1984).

need to be localized, and a different approach to prevention of cross-localization is needed. One way to prevent cross-adsorption of the immunoreagents is to carry out the two labeling reactions serially. After the first primary and secondary gold antibodies have been applied, the complexes formed are modified (blocked). Two common blocking methods have been published: one involves application of protein A or Fab fragments that "coat" the first primary antibody, immunogold complex.[35] In the other, aldehydes are applied to cross-link and block (presumably by denaturation) the first primary antibody–immunogold complex.[36] We have not attempted the protein A or Fab blocking method but have successfully used the aldehyde blocking method using the protocol given later.[11]

With all multiple labeling techniques that label the same surface of the section, the possibility must be taken seriously that the localization of the first antibody–immunogold complex will sterically hinder access to the other antigen. Therefore, it is important to reverse the sequential order of the labeling experiments, as well as to localize the antigens singularly.

Double Labeling Using Two Different Surfaces. Two different methods can be used to avoid the problems inherent in conducting multiple localizations on the same surface of the section; in one of these we label opposite faces of the same section and in the other we label the adjacent faces of serial sections.

The first method is accomplished by floating the grid and sections on immunolocalization reagents rather than submerging the grid and sections in the reagents.[37] If this method is not done carefully, antibodies applied to one side will come in contact with the opposite side of the section, thus giving erroneous localization results. An additional problem with using the opposite sides of the same section is that localized antigens can be separated by as much as 80 nm of embedded cellular material. This separation introduces a significant loss of resolution that can greatly complicate interpretation.

The second method uses serial sections for the localization of two or more antigens and it avoids all the problems inherent in methods that apply antibodies to the same or opposite sides of the same section.[13] This method, which we call "adjacent-face double localization," uses two immediately sequential sections (80 to 100 nm thick, silver to gold interference color) that are picked up on two separate, 200-mesh nickel grids. These sections are cut to be almost large enough to cover the entire 3.05-mm grid. Each section is then subjected to a standard (single), immunogold localization but each with a different primary antibody. Only one side of each of the sections is exposed to the antibody; the side that is exposed to the antibody on each of the two sections is the side or "face" that was contiguous with

[35] H. J. Geuze, J. W. Slot, P. A. van der Ley, and R. C. Scheffer, *J. Cell Biol.* **89,** 653 (1981).

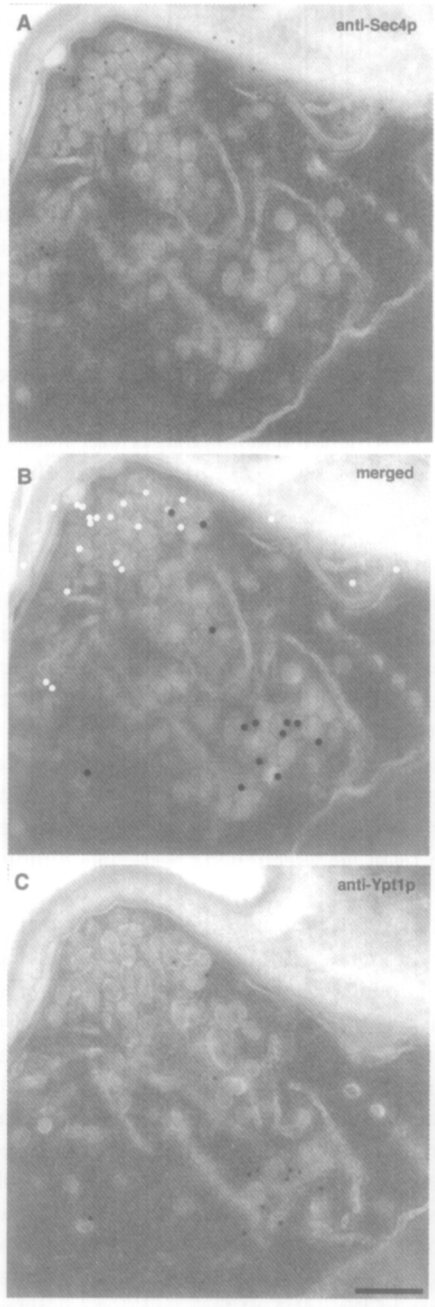
[36] I. L. van Genderen, G. van Meer, J. W. Slot, H. J. Geuze, and W. F. Voorhout, *J. Cell Biol.* **115,** 1009 (1991).

[37] M. Bendayan, *J. Histochem. Cytochem.* **30,** 81 (1982).

the other section prior to sectioning. To accomplish this, the first section is picked up by touching the grid to the section from above; the second section is picked up by submerging the grid in the water of the sectioning knife and picking the section up from below. These two different ways of picking up the sequential sections allow the adjacent face of both sections to be comparably exposed to subsequent antibody incubations and staining procedures. Once the two adjacent faces are recovered onto separate grids, they can be treated with the same reagents without fear of cross-labeling. We prefer to use the same secondary antibodies labeled with the same size gold particles (generally 10 nm) to minimize differences in detection of the two antigens of interest. The immunogold-localized section pairs are then stained with uranyl acetate and lead citrate as described later.

Each of the adjacent-face double localization sections is examined separately in the electron microscope. Subsequent analysis is facilitated greatly by the use of a digital camera. Low magnification images are obtained, and areas of the adjacent-face pair that are visible on both grids are identified and marked. High magnification digital images of cell sections having localization to proteins A and B are then acquired separately. The digital image pairs are merged using Photoshop software (Adobe Systems Inc., San Jose, CA) and examined for colocalization of anti-A and anti-B antibodies. It should be noted that this technique can eliminate considerable observer bias because the colocalization is not evident until the final merging step. We find it convenient to present images as triptychs, with the adjacent faces printed flanking a merged image in which the gold particles from each face represented in different symbols or colors (see Fig. 4).

The following comments should be kept in mind when examining adjacent-face double localization results. As with all the localization methods presented here, immunolocalization occurs after the cells have been chemically fixed, dehydrated, embedded in resin, and sectioned (postembedding immunolocalization). Therefore, although structures can be visualized throughout the cell section, only antigens that are within 5 nm of either side of the cell section surface are accessible to antibody. Accordingly, in the adjacent-face double localization technique, colocalization of two proteins will be restricted to an area of approximately 10 nm (approximately 5 nm on each section) sandwiched between two cell sections. Thus, it is possible to observe localization of an antigen on a cell section in which there is no visible structure only to find the structure clearly visible in the sequential section. This result suggests that in the first section the structure was just "grazed" during sectioning; proteins are present on the surface of the section but not enough of the structure is present to visualize. Conversely, it is possible to observe a structure that in previous experiments had localized a specific antibody, which now shows no localization of that antibody. In this case, observation of the adjacent section shows no structure, suggesting that the structure in the first section was not exposed at the surface of the adjacent face and instead extended in the opposite direction. Thus, in the "adjacent-face" double localization technique it is expected


A
anti-Sec4p
B
merged
C
anti-Ypt1p

that occasionally, colocalization will not be observed when expected for the trivial reason that the structure and/or antigen is not present within the narrow 10-nm area of the adjacent faces. Conversely, when colocalization does occur it can be inferred that the antigens are within 10 nm or less of each other.

Correlative Studies

It is often necessary to correlate protein localization with structural information. However, because immuno-EM uses weak fixation in combination with low contrasting stains, it does not produce the level of preservation and resolution needed for structural studies. Therefore, when a protein is found to be located, using immuno-EM, in a structure or organelle not previously identified or only minimally characterized, it becomes necessary to provide a more detailed characterization. Although tedious, this is done by splitting cultures into two and processing the cells for both immuno-EM and structural EM. The method applied most commonly to yeast for structural EM is the one developed by Byers and Goetsch[29]; this method produces well-fixed cells that are stained with OsO_4, the heavy metal that imparts considerable contrast to ultrastructure. Figure 5 illustrates the difference between the two methods both in terms of the appearance of fine structure and in the type of information each technique can provide.

Standard Protocol for Obtaining and Immunolabeling Yeast Thin Sections

Grid Preparation and Ultramicrotomy

Once cells have been embedded in resin, thin sections are cut and mounted on grids for use in immunoreactions and observation in the microscope. Thin sectioning, or ultramicrotomy, is difficult to learn and requires some time to acquire skill. Therefore, it is recommended that you use the local EM facility staff to do the sectioning or to train you. Regardless of who does the sectioning, the following observations and recommendations should be kept in mind.

FIG. 4. Adjacent-face, double localization (see text for details) of Ypt1p and Sec4p using two serial cell sections of a *sec8-4* temperature-sensitive mutant. (A) Image shows accumulation of late secretory vesicles; polyclonal anti-sec4p antibodies (rabbit) label the vesicles that accumulate in the bud. (C) The adjacent face localization of polyclonal anti-Ypt1p antibodies (rabbit) to the next serial section; note that Ypt1p is located on the secretory vesicles located in the mother cell. Both anti-Sec4p and anti-Ypt1p localizations were detected with a goat anti-rabbit IgG–10-nm gold secondary. (B) The merged image of (A) and (C) showing the double label of anti-Sec4p (white dots) and anti-Ypt1p (black dots). (B) Merged and processed image using Photoshop software (Adobe, Inc.); A and C were digitally adjusted for contrast and brightness only. Bars: 0.1 μm.

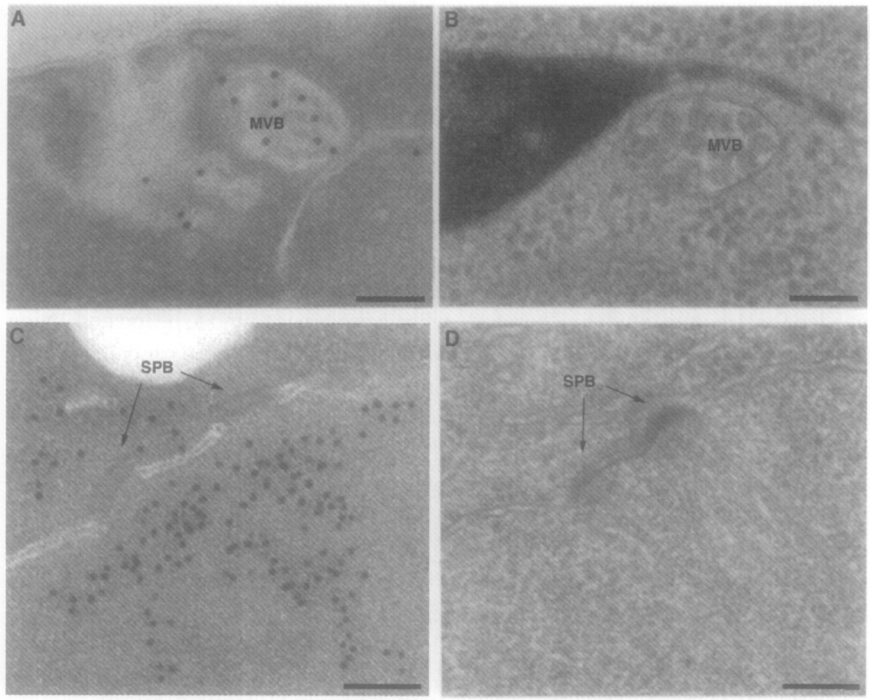


FIG. 5. (A and C) Wild-type cells processed for immuno-EM as described in the text. (B and D) Wild-type cells processed for structural EM using the method of Byers and Goetsch, *Methods Enzymol.* **194,** 602 (1991). (A and B) Images of late endosomes are shown. (A) Note that the endosome is well labeled (10 nm gold) with antibodies directed against the pheromone receptor, Ste2p, but that its fine structure is not well preserved and only low contrast profiles of internal membranes are evident. (B) In contrast, the late endosome is well preserved and is filled with well-stained, small vesicles; its morphology is similar to that of a multivesicular body (MVB). (C and D) Images of duplicate spindle pole bodies (SPB). (C) Note that the spindle is well labeled with antibodies directed against tubulin (10 nm gold) but that a microtubule fine structure is not evident. Note also that SPBs are slightly electron dense and that the nuclear membrane is negatively stained. (D) In contrast, the SPBs and microtubules are well preserved and contrasted and the nuclear membrane is positively stained. Bars: 0.1 μm.

1. Use a sharp, 35° diamond knife, (Diatome, EMS, Inc., Fort Washington, PA). LR White sections cut with a 35° knife angle appear to produce better cell morphology than those cut with a 45° knife.

2. For good resolution, sections should be silver to gray in interference color, approximately 50–70 nm thick.

3. Do not use heat pens or chloroform vapors to spread thin sections. LR White sections are hydrophilic and will, on their own, spread out on the water surface.

4. Prior to picking up sections, they should be grouped together using an eyelash attached (nail polish is a good adhesive) to a sharpened wood applicator

stick. Be sure there are enough sections to cover the grid. Sections can be picked up from above by gently pressing the grid down onto the sections. Press hard enough to slightly indent the water surface without breaking the surface tension. Do not submerge the grid. With the sections trapped beneath the grid, gently roll the grid off the water surface; do not pull the grid straight up off the water because this can damage sections. Reverse tweezers (Ted Pella, Inc., 510-EMX) are recommended because they always hold onto the grid and you do not have to slide an O ring up the tweezers.

5. Because phosphate buffer reacts with copper, grids made of nickel or gold are recommended. Nickel grids can become magnetized and, therefore, non-magnetizing forceps are useful. Also, magnetized grids can cause astigmatism during EM examination, requiring frequent correction. We routinely use nickel grids with little difficulty but have a demagnetizer available. Grids of 200–300 mesh provide adequate support for the LR White sections; coarser mesh grids will require a support film made of formvar or carbon.[7] We routinely use 300 mesh hexagonal mesh nickel grids purchased from Polysciences.

6. To prevent the loss of sections from grids during antibody incubations it is strongly recommended that the grids be made "sticky" prior to picking up sections; see recipe given later.[1] We prepare 100 sticky grids at a time and store them on hardened #50 Whatman (Clifton, NJ) filter paper in a covered glass petri dish (do not use plastic; static electricity will cause the grids to fly to the top and sides of the dish).

7. After picking up sections, grids are placed on hardened #50 Whatman filter paper in a glass petri dish. When placing the grid on the filter paper, it is important to let go of the grid when the water on the grid is absorbed into the filter paper. The movement of the water into the paper will pull the grid away from the tweezers; try not to drag the grid across the filter. If the grid jumps up the forceps due to capillary action, simply float the grid off on a clean drop of water and then pick up the grid and try again. Alternatively, grids can be left, held in the tweezers and covered, until the grid has dried, approximately 30 min, and then stored in a grid box. Sections mounted on grids and stored in a grid box should be used within a week. Sections up to 1 month old can be used; however, the quality of the ultrastructure, as well as the stability of the sections under the electron beam, is noticeably compromised with older sections.

Immunolabeling

Immunolabeling of yeast cell sections has been well described and our method is essentially unchanged from what is recommended by Wright and Rine.[6] However, for convenience, we have outlined here the immunoreactions for immuno-EM of yeast.

Immunolabeling of thin sections is done in a humidity chamber. Our humidity chamber is a large, covered plastic container that has several damp paper towels

placed in the bottom. Immunolocalizations are done in the chamber either directly on a clean sheet of Parafilm or in the wells of a spot plate (Coors, VWR, West Chester, PA). We prefer spot plates for incubations. All incubations are done at room temperature.

1. Two to three drops (approximately 200 μl) of block are delivered into the spot plate wells through a Millex-GS 0.22-μm filter (Millipore, Inc., Bedford, MA) unit attached to a disposable 10-ml syringe. Grids are wetted on each side by touching the surface of the block and then submerged by slowly slicing the grid into the blocking solution. This helps prevent damage to the sections as the grid breaks the surface tension. Sections are blocked in standard immuno-EM block (see later) for 15 min.
2. Grids are removed from the blocking solution, and excess block is absorbed by carefully touching the grid to a damp Kimwipe.
3. Grids are then gently submerged in 20 μl of diluted primary antibody. Grids are typically incubated for 1 hr at room temperature.
4. Grids are removed from the primary antibody and are washed for 5 min in each of three separate wells containing approximately 0.2 ml of wash solution (PBST, see later); 15 min total wash time. There is no need to blot grids dry during this step.
5. Grids are removed from the last wash, and excess PBST is absorbed by carefully touching the grid to a damp Kimwipe.
6. Grids are then submerged in 25 μl of secondary antibody. Incubate for 1 hr at room temperature. Typically, the secondary immunogold conjugate is diluted to between 1 : 50 and 1 : 100 in standard IEM block.
7. After secondary antibody incubation, the grids are moved through three washes of fresh PBST wash. Grids are then immediately rinsed by slowly slicing the grid (about 10 slices at approximately 1 slice/sec) through about 500 ml of high-purity water (Milli-Q or doubly distilled H_2O). Grids can now be placed to dry on Whatman #50 hardened filter paper in a glass petri dish or postfixed and stained as described later. For best results, it is recommended that postlocalization fixation and staining be done immediately following immunolocalization (see later).

Aldehyde Blocking Method: Used for Double Labeling on Same Section

In the aldehyde blocking method the first primary antibody and gold-labeled secondary are applied as described earlier. The cell sections are then washed in PBST and incubated in 4% formaldehyde, 0.1% glutaraldehyde, and 40 m*M* potassium phosphate, pH 6.7, for 15 min at room temperature. The immunolocalized, blocked cell sections are then washed five times (5 min each time) in 50 μl of PBST. Finally, the cell sections are incubated with the second, primary antibody followed by immunogold secondaries and postfixed and stained with uranyl acetate and lead citrate as described earlier. It should be noted that this technique does

not work for all antibodies. The immunoreactivity of the epitopes recognized by the second, primary antibody may be partially or completely eliminated using this blocking procedure. Thus, extensive control experiments may be necessary to determine the optimal concentration of aldehydes needed to adequately block unwanted adsorption but not block localization of the second, primary antibody.

Postlocalization Fixation and Electron-Dense Staining

1. To prevent the loss of antibody localization during low and high pH, electron-dense staining (uranyl acetate and lead citrate, respectively), sections are postfixed in a small amount (ca. 100 μl) of 8% glutaraldehyde for 15–30 min. This postembedding fixation cross-links the antibodies to the section. Post-embedding fixation also appears to provide more extensive fixation of minimally fixed cellular components, especially the endoplasmic reticulum (see earlier discussion).
2. After postlocalization fixation, grids are rinsed immediately by slowly "slicing" (about 20 times; approximately once per second) through 500 ml of high-purity water as described earlier.
3. Grids are then blotted with a Kimwipe and incubated in a drop of 2% aqueous uranyl acetate (2% uranyl acetate can be stored for 1 month at 4°, covered with foil. We store ours in a disposable 10-ml syringe and deliver it through a 0.2-μm syringe filter (Millex-GS, Millipore). Sections can be stain in uranyl acetate for 30 to 60 min at room temperature; longer staining tends to increase the electron density of the cytoplasm, thereby decreasing resolution. Uranyl acetate staining can be done on Parafilm or in a spot plate.
4. Following uranyl acetate staining, sections are washed by slowly slicing the grids 20–30 times through high-purity water as described earlier. After washing, grids are placed on #50 Whatman filter paper in a glass petri dish and allowed to dry for 15–30 min.
5. After drying, sections can be stained with lead citrate (also called Reynolds' lead[38]). Lead citrate imparts considerable contrast but can make the cytoplasm grainy (compare Figs. 3B to 3C). Also, lead staining, if not done carefully, can produce lead carbonate precipitate. Grainy lead staining can look very similar to gold particles 5 nm or smaller (see, for example, Fig. 2A). Additionally, prolonged incubation in lead citrate stain, pH 12, can damage sections. Therefore, it can be advantageous to examine sections prior to lead staining.

To avoid the formation of lead carbonate precipitate, lead staining is done in a semiclosed chamber in the presence of sodium hydroxide. The chamber is made by covering a glass plate (like those used for small gel boxes) with Parafilm on which a glass petri dish cover is placed. Under the cover, we place about 5 g of sodium hydroxide pellets. The first few drops of lead solution are delivered, using a syringe and a 0.2-μm filter, into the hydroxide pellets and then a droplet

[38] E. Reynolds, *J. Cell Biol.* **17,** 208 (1963).

of approximately 100 μl is placed on the Parafilm next to the pellets. The grid is placed in the droplet for 30 sec. During staining we keep the grid held in the tweezers and hold the petri dish cover slightly above the staining area to minimize exposure to air. One should also hold one's breath as CO_2 will cause the formation of lead carbonate. After 30 sec the grid is removed from the droplet and is washed immediately by slicing (described earlier) in fresh Milli-Q water or recently boiled and cooled doubly distilled H_2O. For more contrast, wash only 10 slices (approximately 1 sec/slice) but this will also impart a granularity to the cell section. Longer washes (>20 slices) will give less contrast, especially on thinner sections (<60 nm) but will impart a less grainy appearance. Washed grids are then placed on #50 Whatman filter paper in a glass petri dish and are allowed to dry overnight prior to examination in the electron microscope. Tweezers must be rinsed in water and wiped dry before staining the next grid.

Alternatively, lead acetate or vanadium can be used as electron-dense stains. Lead acetate stain can be used at near neutral pH and does not produce the granularity of lead citrate. Vanadium stain has little to no granularity but provides less contrast than either lead citrate or acetate. Vanadium staining[32] is particularly useful when using gold conjugates 5 nm and smaller (e.g., nanogold).

Sticky Grid Solution

4 ml 0.5% Formvar (Ted Pella, Inc.; final concentration of Formvar is 0.08% w/v)
21 ml of a 24 : 1 (v/v) dichloroethane : chloroform solution

Keep sticky grid solution tightly sealed. Grids can be placed in the solution, removed with tweezers, and placed, singularly, on #50 Whatman filter paper. Work with sticky grid solution and reagents in the hood; carefully wipe tweezers clean with a Kimwipe and ethanol.

Lead Citrate Stain

Lead citrate can be prepared using several different methods.[18] The method we use is as follows:

0.004 g Lead citrate (Polysciences, Inc.) per 1 ml staining solution needed (0.4% solution). We typically make 5 ml.
20 μl of 5 *N* sodium hydroxide per ml of 0.4% staining solution. NaOH should be no more than a week old.

Mix using vigorous vortexing until the lead citrate goes into solution. The lead solution can be taken up in a disposable syringe that is then fitted with a 0.2-μm filter. The lead solution can be stored in the syringe (remove filter) for 1–2 weeks.

Wash Buffer and Blocks

PBST: 140 m*M* NaCl, 3 m*M* KCl, 8 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 0.05% Tween 20, pH 7.4

Standard block: PBST, 0.5% (w/v) ovalbumin (Sigma), 0.5% (w/v) BSA (Sigma)

Fish gelatin (0.5% w/v) (BBInternational, gel#10) in PBS or PBST

Glycine (0.15%) block in PBS or PBST

Mannan (0.1 mg/ml) (Sigma) block in PBST or standard block. A 1-mg/ml stock solution can be made and stored at 4° with 0.1% (w/v) sodium azide (Ben Glick, University of Chicago)

[5] Electron Tomography of Yeast Cells

By EILEEN T. O'TOOLE, MARK WINEY, J. RICHARD MCINTOSH, and DAVID N. MASTRONARDE

Introduction

Electron microscopy (EM) is a useful method for the study of yeast cells and is complementary to a genetic analysis. EM provides high-resolution structure data about wild-type cells and can document structural defects in mutant strains.[1,2] A number of EM techniques have been used to study the complex three-dimensional (3D) arrangements of organelles in yeast. Examples include the use of freeze-fracture replicas to reveal the surface topology of nuclear envelopes and cytoplasmic membrane systems,[3–5] as well as the use of serial sections to reconstruct entire mating factor-arrested cells,[6] to describe the 3D geometry of the mitotic spindle,[7,8] and to document the 3D distribution of nuclear pore complexes in wild-type[9] and mutant strains.[10] More recently, electron tomography has been used to describe

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