7

Preparation of Yeast Cells for Confocal Microscopy

Audrey L. Atkin

1. Introduction

Confocal scanning microscopy has been successfully used for immunofluorescence work in yeast. The major axis of a *Saccharomyces cerevisiae* haploid cell is approx 4 µm. Optical sections of approximately 2× 0.4 µm thickness from fluorescently-labeled yeast cells can be obtained using the laser scanning confocal microscope ***(1)***. This means that it is possible to look at optical sections corresponding to about one tenth of a yeast cell. For example, confocal scanning microscopy has been used to examine the distribution of actin in fixed cells prepared from reverting protoplasts, and to show that a monoclonal antibody raised to rat liver nuclear proteins recognized two protein components of the yeast nuclear pore complex, p95 and p110 ***(2,3)***.

Confocal scanning microscopy is especially useful for determining the cellular localization of proteins that may localize to more than one structure or compartment within the yeast cell. For instance, confocal scanning microscopy was used to show that Kap95p accumulated both at the nuclear envelope and inside the nucleus, and to look for potential nuclear localization sequences in eIF4E, a cap binding protein that cycles between the nucleus and the cytoplasm ***(4,5)***.

The distribution of more than one macromolecule can be determined by simultaneously labeling cells with different fluorochromes ***(6)***. The images are collected separately for each fluorochrome and then merged to determine if there is overlap in the subcellular distribution of macromolecules of interest ***(6)***. This approach was used to show that epitope tagged allele of *UPF1* is found primarily in the cytoplasm ***(7)***.

This chapter describes a method for the staining of fixed *S. cerevisiae* cells with fluorescently labeled antibodies (**Fig. 1**) and 4',6-diamidino-2-phenylindale (DAPI) for confocal microscopy (***7***; similar to a procedure

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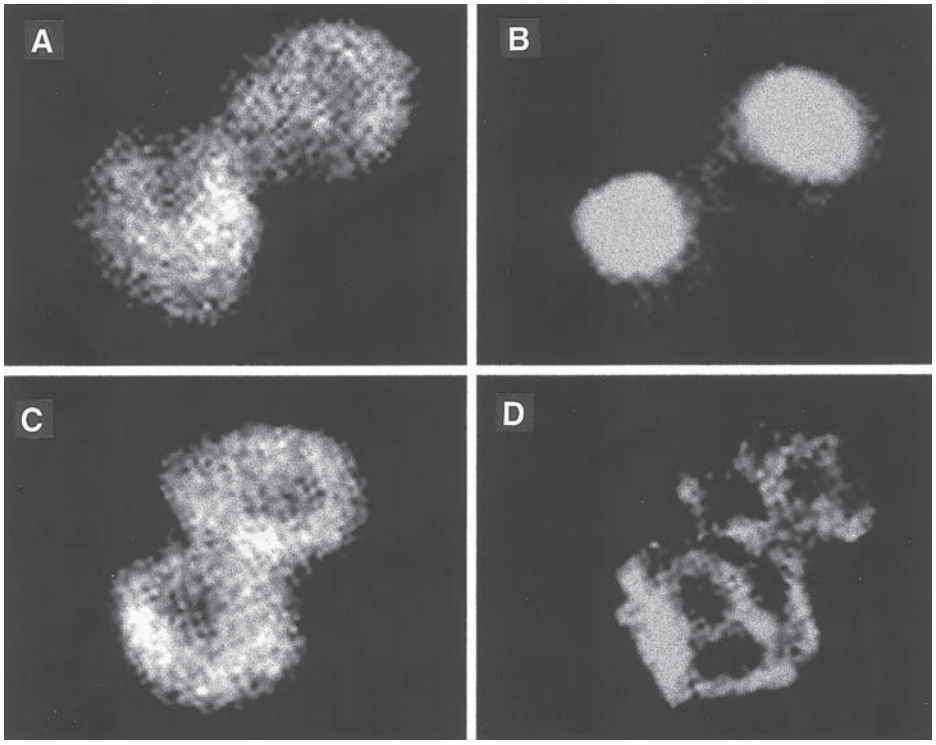

Fig. 1. Single optical sections of double-labeled yeast cells. The UPF1-3EP protein **(A,C)** is localized in the cytoplasmic compartment because it does not co-localize with the SEN1::βgal protein in the nucleus **(B)** or the KAR2 protein in the endoplasmic reticulum **(D)**, but it does co-localize with the L1 protein, of the large ribosomal subunit, in the cytoplasm (results not shown). *See* **ref. *7*** for more details.

described in **ref. *8***). This method can be adopted for other dyes for chromatin such as ToPro™ or propidium iodide have excitation and emission spectra that are better suited to the lasers supplied with confocal imaging systems (*see* Chapter 1).

2. Materials

1. 1% Polyethyleneimine: Mix 20 µL of 50% aqueous polyethyleneimine (Sigma P-3143) with 980 µL of dH_2O. Store at 4°C in the dark (*see* **Note 1**).
2. Glass slides
3. 37% Formaldehyde solution: (Sigma F-1635) prewarmed to 37°C
4. Phosphate-buffered saline (PBS): Potassium phosphate dibasic (7.0 g), potassium phosphate monobasic (1.4 g), sodium chloride (8.8 g), made up to 1 L with dH_2O (distilled water), and adjusted to pH 7.2. Sterilize by autoclaving. Store at room temperature.

5. Lyticase solution: Dissolve 1900 U of lyticase (Sigma L-4025) in 200 µL of PBS. Prepare immediately before use.
6. 1*M* Potassium phosphate (pH 6.5) stock solution. Combine 342.5 mL of 1*M* potassium phosphate monobasic, and 157.5 mL of 1*M* potassium phosphate dibasic. Sterilize by autoclaving. Store at room temperature.
7. Wash buffer 1 [1.2*M* Sorbitol, 0.1*M* potassium phosphate (pH 6.5), 1% 2-mercaptoethanol]: Mix 60 mL of 4*M* sorbitol and 20 mL of 1*M* potassium phosphate (pH 6.5) with 118 mL of dH_2O. Sterilize by autoclaving. Cool to room temperature. Add 2 mL of 2-mercaptoethanol. Store at room temperature.
8. Wash buffer 2 [1.2*M* sorbitol, 0.1*M* potassium phosphate (pH 6.5)]. Mix 60 mL of 4*M* sorbitol, and 20 mL of 1*M* potassium phosphate (pH 6.5) with 120 mL of dH_2O. Sterilize by autoclaving. Store at room temperature.
9. 10% Igepal CA-630 stock solution (*see* **Note 2**): Add 10 mL of Igepal CA-630 (Sigma I-3021) to 90 mL of dH_2O. Mix well (*see* **Note 3**). Store at room temperature.
10. Blocking buffer: Dissolve bovine serum albumin (0.1 g) in 10 mL of PBS. Add 50 µL of 10% Igepal CA-630. Store at –20°C in 1-mL aliquots.
11. Primary antibodies: Dilute the primary antibodies in PBS to 2× the desired final concentration immediately before use. Remove unwanted particles by centrifugation at 10,000*g* for 10 min at 4°C.
12. 0.05% Igepal CA-630/PBS: Add 5 mL of 10% Igepal CA-630 to 995 mL of PBS. Mix well. Store at room temperature.
13. Labeled secondary antibodies: Dilute the secondary antibodies in PBS to 2× the desired final concentration immediately before use. Centrifuge at 10,000*g* for 10 min at 4°C to remove unwanted particles.
14. 1 µg/mL DAPI solution: Prepare a 1 mg/mL DAPI stock solution by dissolving DAPI (1 mg; Sigma D-9542) in 1 mL of PBS. Prepare the 1 µg/mL working DAPI solution by diluting 1 µL of the DAPI stock solution in 999 µL of PBS. Store the stock and working DAPI solutions at 4°C.
15. 0.5*M* Carbonate buffer (pH 8.0): Add sodium bicarbonate (0.42 g) to 9 mL of dH_2O. Adjust the pH to 8.0 using 1*N* NaOH. Bring the volume up to 10 mL.
16. Glass coverslips, 18 × 18 mm, No. 1 thickness
17. Mounting medium: Dissolve ρ-phenylenediamine (30 mg; Sigma P-6001) in 4 mL of dH_2O. Add 6 mL of glycerol and 1 mL of 0.5*M* carbonate buffer (pH 8.0). Filter through a 0.2-µm filter to remove any undissolved chemical. Store in 0.5-mL aliquots at –70°C in the dark (*see* **Note 4**).

3. Method

3.1. Preparation of Slides

1. Prepare one slide for each sample.
2. Etch a 3/8-inch circle in the center of each slide with a diamond pen. Thoroughly rinse the slides with dH_2O and dry with a Kimwipe to remove the glass chips and any dust.
3. Outline the circles with a black permanent marker. Pipet 30 µL of 1% polyethyleneimine into each circle (*see* **Note 5**). Let the slide sit for approximately

5 min. Aspirate off the remainder of the solution. Rinse the slides thoroughly with dH_2O. Shake the slide dry and then place in a moist chamber (*see* **Note 6**).

3.2. Growth, Harvesting and Fixation of Yeast Cells

1. Grow cells to an OD_{600} of 0.4–0.6 in 10 mL of the appropriate medium (*see* **Note 7**). This can be done in two steps. First grow an overnight culture to saturation in 3 mL of medium. Second, inoculate 10 mL of fresh medium with different amounts of the overnight culture. Inoculation of 10 mL of selective medium with 5 µL, 10 µL, 50 µL, and 100 µL of the overnight culture the afternoon of the second day will generally ensure that at least one of the cultures will be at an OD_{600} of 0.4–0.6 the following day.
2. Add 1.4 mL of 37% formaldehyde per 10 mL-culture. Incubate for 5 min at 37°C with gentle shaking (*see* **Notes 8** and **9**).
3. Let the culture sit at room temperature for 1 h (*see* **Note 8**).
4. Pellet the yeast cells by centrifugation for 3 min at 3000*g*. Discard the supernatant. Be careful not to disturb the yeast cell pellet.
5. Wash the yeast cells three times in wash buffer 1. This is done by resuspending the yeast cells in 3 mL of wash buffer 1, pelleting the yeast cells by centrifugation at 3000*g* for 3 min, and discarding the supernatant.
6. Resuspend the yeast cells in 0.5 mL of wash buffer 1.

3.3. Spheroplasting of the Yeast Cells

1. Add 50 µL of freshly prepared lyticase solution to the yeast cell slurry.
2. Incubate for 9 min at 30°C with gentle shaking (*see* **Note 10**).
3. Pellet the cells immediately after the incubation by centrifugation for 3 min at 3000*g*.
4. Wash the cells two times with 3 mL of wash buffer 2.
5. Resuspend the cells in wash buffer 2 at approximately 5.0 A_{600}/mL (*see* **Note 11**).

3.4. Primary Antibody Incubations

1. Pipet 60 µL of cells onto each circle on the prepared slides in the moist chamber.
2. Let the cells settle for 30 min.
3. Rinse the slides twice by dipping the slides into coplin jars filled with PBS. These rinses will remove any cells that did not adhere to the slide.
4. Check the cells for density using a light microscope.
5. Immerse the slides into a coplin jar containing methanol that had been prechilled to –20°C, for 6 min (*see* **Note 12**).
6. Immediately transfer the slides to a coplin jar containing acetone that had been prechilled to –20°C, for 30 s (*see* **Note 12**).
7. Tilt the slides to drain on a paper towel. Allow the slides to air dry.
8. Place the slides back in the moist chamber.
9. Block the cells with 30 µL of blocking buffer. Do not let the cells dry up after this step.
10. Incubate 15 min at room temperature.

11. Add 30 µL of PBS containing the primary antibodies to the cells (*see* **Notes 13–19**).
12. Incubate overnight at room temperature (*see* **Note 14**).

3.5. Washes

1. Immerse the slides in a coplin jar containing 0.05% Igepal CA-630/PBS (*see* **Note 15**).
2. Immediately transfer the slides to a second coplin jar containing 0.05% Igepal CA-630/PBS. Incubate for 5 min.
3. Wash in PBS by transferring the slides to a coplin jar filled with PBS. Incubate for 5 min. Repeat this step a total of three times.
4. Drain the slides on a paper towel to remove excess PBS. Immediately place the slides in the moist chamber.

3.6. Secondary Antibody Incubations

1. Add 30 µL of blocking buffer to the cells on each slide.
2. Incubate for 5–10 min at room temperature.
3. Add 30 µL of PBS containing the secondary antibodies (*see* **Notes 16–19**).
4. Incubate for 1 h at room temperature in the dark (*see* **Note 20**).

3.7. Washes

1. Immerse the slides in a coplin jar containing 0.05% Igepal CA-630/PBS.
2. Immediately transfer the slides to a second coplin jar with 0.05% Igepal CA-630/PBS. Let sit for 5 min in the dark.
3. Wash three times in PBS for 5 min in the dark.
4. Drain the slides on a paper towel. Place the slides in the moist chamber.

3.8. DAPI Staining

1. Add 30 µL of a 1 µg/mL DAPI solution to the cells.
2. Incubate for 5 min at room temperature in the dark.
3. Rinse two times in PBS.
4. Drain off the excess liquid. Allow the slides to air dry in the dark.

3.9. Mounting

1. Place one drop of mounting medium on a 18 mm × 18 mm coverslip (*see* **Note 21**).
2. Carefully place the coverslip over the circle. Avoid trapping air bubbles under the coverslip. Cover the slide with filter paper and press gently on the coverslip to spread the mounting medium evenly. Be careful not to move the coverslip. Wick any excess mounting medium away with a Kimwipe.
3. Incubate the slides for 1 h at room temperature.
4. Place a drop of clear acrylic nail polish at each corner of the coverslip. Allow the nail polish to harden. Seal coverslip to the slide with additional clear nail polish.
5. The slides can be stored at –20°C in light tight boxes.
6. View the slides within 24–48 h (*see* **Note 22**).
7. Optical sections of 0.4 µm can be obtained by using a 60× objective lens, numerical aperture 1.4, with the pinhole closed (1 mm) and a laser emission of 0.1%

transmittance. Select laser excitation wavelength and emission filters appropriate for the fluorochrome and DAPI stain.

4. Notes

1. Phenylenediamine is very viscous and light sensitive. It can be pipetted using a tip that has had the end cut off.
2. Igepal CA-630 is chemically identical to Nonidet P-40. Nonidet P-40 is no longer commercially available.
3. Igepal CA-630 may require heat to dissolve in water.
4. The mounting medium is light sensitive. It is colorless and clear when freshly made. Discard this solution when it develops an orange color.
5. The spheroplasted yeast cells must be attached to the slides for efficient staining and washing. The slides can be prepared by coating the surface of the slide with polyethyleneimine. Polyethyleneimine prepares the surface of the slide so that the spheroplasted yeast cells will adhere to the slide. Polylysine is also commonly used for this purpose. A protocol for preparing the surface of slides with polylysine can be found in **ref.** ***8***.
6. The use of a moist chamber slows evaporation during the longer incubation steps. This is important to ensure that the cells stay moist during the staining process. A moist chamber can be prepared by lining a shallow, flat-bottomed container that has a tight-fitting lid with a sheet of 3 MM chromatography paper saturated with dH_2O.
7. The choice of yeast strain and growth conditions can affect the results obtained with immunofluorescence methods ***(8)***. Ideally diploid cells harvested in the exponential growth phase should be used. Diploid cells are usually larger than haploid cells and visualization of subcellular structures is easier in larger cells. Effective removal of the cell wall is essential for access of antibodies to the corresponding antigens within the cell. The yeast cell wall is easiest to remove from cells harvested during exponential growth. However, the growth conditions must be optimized for cellular components that are present transiently. Standard protocols for the growth and maintenance of yeast are available ***(9–11)***.
8. The fixation protocol presented in this chapter has worked well for a number of antigens ***(7)***. Cells are rapidly fixed by adding the fixative to the culture medium. This ensures that cellular structures are fixed before they can be potentially disturbed by harvesting. Several subcellular structures including actin in budding cells, as well as vacuolar and mitochondrial morphology, have been shown to be disturbed during the process of centrifugation ***(8)***. Optimal fixation times can vary between 30 minutes and 3 h. The best length of time for fixation must be determined for each antigen.
9. Optimal fixation protocols depend on the antigen and the location of the antigen. The goal of fixation is to preserve the ultrastructure of the cell as much as possible without destroying the antigenic determinants recognized by the antibody. Strong fixation generally gives better structural preservation, but leads to weaker antibody labeling. This is because the fixation protocol can inactivate an antigen,

the antibody may not be able to gain access to the antigen, the antigen can be extracted by the fixation procedure, or the 3° structure of the antigen may not be recognized by the antibody. Commercial formaldehyde is not always the best fixative for all antigens because these solutions are stabilized by the addition of 10–15% methanol, which is also a fixative. Paraformaldehyde has been successfully used as a fixative for yeast cells and a protocol is available ***(12)***. Additional fixation protocols have been described ***(13)***.

10. Spheroplasting helps to permeablize the yeast cells to reagents such as antibodies and DAPI. The time that it takes to spheroplast a yeast cell varies with the yeast strain and the growth conditions. Incubation times for spheroplasting must be optimized for each new yeast strain and each change in growth conditions. This is done by incubating the cells in the presence of lyticase for different times and monitoring cell wall removal by a dilution lysis assay ***(14)***. The dilution assay is done by mixing an aliquot of cells (~0.4 OD unit cells) with 1 mL of water. Mix a second aliquot of cells with 1 mL of PBS. Determine the OD_{600} of the two samples. Complete spheroplasting results in a greater than 10-fold drop in the OD_{600}.
11. After spheroplasting, the cells can be stored at 4°C for up to 16 h. Longer times at 4°C adversely affect the quality of the cells ***(8)***.
12. Methanol and acetone are inorganic solvents. Immersion of the fixed, spheroplasted cells attached to slides in methanol followed by acetone helps to lyse the yeast cells. This step can be omitted for staining with most antibodies. However, this step is essential for staining with some antibodies, such as the antibodies that recognize Mas2p, a mitochondrial matrix protein (Atkin, *unpublished data*). In some cases methanol and/or acetone can destroy the antigenicity of some antigens because they are also weak fixatives. When working with a new antigen or antibody preparation, try staining the cells with antibody after methanol/acetone treatment and in the absence of methanol/acetone treatment.
13. Determine the concentration of the primary (monoclonal or polyclonal, affinity-purified) antibodies needed to produce a suitable signal. The working concentrations can vary between 1:10 to 1:10,000. Test each new lot of primary antibody at 1:10, 1:100, 1:500, 1:1000, and 1:10,000 dilutions to determine which concentration gives the best signal relative to background.
14. Optimize primary antibody incubation time. Times can vary from 1 h to overnight.
15. The presence of the detergent Igepal CA-630 in the PBS helps to reduce nonspecific staining by the primary and secondary antibodies.
16. Secondary antibodies are directed against the IgGs of the species in which the first antibodies have been made. Many animals have had yeast infections, so the secondary antibodies should be affinity-purified. Affinity-purified, fluorochrome-labeled secondary antibodies can be purchased from Boehringer Mannheim Biochemicals. The working concentration of the secondary antibody needs to be determined for each new lot of antibody. The working concentrations can vary between 1:10 to 1:10,000. Optimal antibody concentrations will vary

according to the nature of the sample and the assay conditions. Test each new lot of secondary antibodies at 1:10, 1:100, 1:500, 1:1000, and 1:10,000 dilutions to determine which concentration gives the best signal relative to background.

17. The choice of fluorochrome conjugated to the secondary antibody is limited by the filter sets that are available for the microscope that will be used. Fluorescein, rhodamine, and Texas Red™-labeled secondary antibodies are available. Fluorescein emits a green light that is easy to detect; however, it is prone to rapid photobleaching. Rhodamine emits a red light. It is not as prone to photobleaching. However, it yields a higher background than fluorescein because it is hydrophobic. Secondary antibodies labeled with Texas Red are becoming more readily available. Texas Red also emits a red light. It is more resistant than fluorescein and rhodamine to photobleaching. Fluorescein-labeled secondaries can be combined with either rhodamine or Texas Red conjugates in double labeling experiments.
18. Controls are essential to help identify the source of any background problems. Stain cells with secondary antibody alone, and with no antibodies at all. If possible, a control of the immunofluorescence in a strain that has a null mutation in the gene encoding the protein of interest should be determined. Nonspecific background can be reduced by blocking in 5% serum derived from the same species as the labeled antibody, 3% bovine serum albumin (BSA) and 10% nonfat dry milk, diluting the antibody in blocking solution, adding 0.2% Tween 20 to all buffers and wash solutions, reducing the incubation time of the primary antibody or the labeled secondary antibody, and increasing the number and the duration of the washes.
19. Cells can be double labeled for comparing the intracellular localization of two cellular constituents within the same cell. Use primary antibodies from different types of animals or different immunoglobulin classes, and secondary antibodies for each primary antibody with different fluorochrome labels. Care must be taken to ensure that no cross-reaction is possible between the labeled detection reagents. Successful double labeling requires that each primary and secondary antibody combination produce intense staining with a clean background.
20. A dark chamber can be prepared by lining a small box with black paper.
21. Mounting medium includes specific antifade reagents. These reagents are antioxidants that minimize the concentration of free oxygen radicals ***(15)***. Free oxygen radicals attack the unexcited fluorochromes and damage them. ρ-Phenylenediamine is the antifade reagent in the mounting medium. Other commonly used mounting media are described elsewhere ***(16)***.
22. Formaldehyde fixation is not permanent ***(17)***. The crosslinks slowly reverse in aqueous buffers. The yeast cells should be stained and observed within a short time of fixation.

References

1. White, J. G., Amos, W. B., and Fordham, M. (1987) An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. *J. Cell Biol.* **105,** 41–48.

2. Kobori, H., Yamada, N., Taki, A., and Osumi, M. (1989) Actin is associated with the formation of the cell wall in reverting protoplasts of the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **94,** 635–646.
3. Aris, J. P. and Blobel, G. (1989) Yeast nuclear envelope proteins cross react with an antibody against mammalian pore complex proteins. *J. Cell Biol.* **108,** 2059–2067.
4. Iovine, M. K. and Wente, S. R. (1997) A nuclear export signal in Kap95p is required for both recycling the import factor and interaction with the nucleoporin GLFG repeat regions of Nup116p and Nup100p. *J. Cell Biol.* **137,** 797–811.
5. Ptushkina, M., Vasilescu, S., Fierro-Monti, I., Rohde, M., and McCarthy, J. E. G. (1996). Intracellular targeting and mRNA interactions of the eukaryotic translation initiation factor eIF4E in the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1308,** 142–150.
6. Paddock, S. W. (1994) To boldly glow. Applications of laser scanning confocal microscopy in developmental biology. *BioEssays* **16,** 357–365.
7. Atkin, A. L., Altamura, N., Leeds, P., and Culbertson, M. R. (1995) The majority of yeast UPF1 co-localizes with polyribosomes in the cytoplasm. *Mol. Biol. Cell* **6,** 611–625.
8. Pringle, J. R., Adams, A. E. M., Drubin, D. G., and Haarer, B. K. (1991) Immunofluorescence methods for yeast. *Methods in Enzymol.* **194,** 565–602.
9. Rose, M. D., Winston, F. and Hieter, P. (1990) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
10. Sherman, F. (1991) Getting started with yeast. *Methods in Enzymol.* **194,** 3–21.
11. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D,. Seideman, J. G., Smith, J. A., and Struhl, K., eds. (1993) *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley-Interscience, New York.
12. Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
13. Osborn, M. (1994) Immunofluorescence microscopy of cultured cells, in *Cell Biology: A Laboratory Handbook,* vol. 2 (J. E. Celis, ed.), Academic Press, San Diego, pp. 347–354.
14. Franzusoff, A., Rothblatt, J., and Schenkman, R. (1991) Analysis of polypeptide transit through yeast secretory pathway. *Methods Enzymol.* **194,** 662–674.
15. Johnson, G. D., Davidson, R. S., McNamee, K. C., Russell, G., Goodwin, D., and Holborow, E. J. (1982) Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. *J. Immunol. Methods* **55,** 231–242.
16. Pawley, J. B. and Centonze, V. E. (1994) Practical laser-scanning confocal light microscopy: obtaining optimal performance from your instrument, in *Cell Biology: A Laboratory Handbook,* vol. 2 (J. E. Celis, ed.), Academic Press, San Diego, pp. 44–64.
17. Reinsch, S. and Stelzer, E. H. K. (1994). Confocal microscopy of polarized MDCK epithelial cells, in *Cell Biology: A Laboratory Handbook,* vol. 2 (J. E. Celis, ed.), Academic Press, San Diego, pp. 89–95.