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Localization of Proteins and Organelles Using Fluorescence Microscopy

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Abstract

This chapter describes the different methods used for localization of proteins and organelles in *Pichia pastoris*. A series of plasmids and a modified immunofluorescence protocol for localization and co-localization of proteins and organelles are described. Also included are protocols for the labeling of different subcellular organelles with vital stains.

Key Words: Protein localization; protein colocalization; fluorescence microscopy; organelle markers; green fluorescent protein variants; peroxisomes.

1. Introduction

In considering the best option for localization of proteins and organelles, it is necessary to consider the advantages and disadvantages of each technique.

Indirect immunofluorescence is a rapid and convenient technique for the detection and localization of endogenous proteins, if an antibody against the protein of interest is available. Different proteins can be localized and colocalized without resorting to plasmid constructions, yeast transformation or having to contend with the availability of suitable selectable markers for the introduction of DNA constructs into the host strain. The antibody specificity is very important in making unambiguous conclusions about the location of a given protein. Additionally, colocalization experiments require primary antibodies from two different species.

In the absence of specific antibodies, the construction of a fusion protein with a fluorescent or epitope tag is the only option to detect the protein, but it requires many considerations and controls. First, the tagged protein must be

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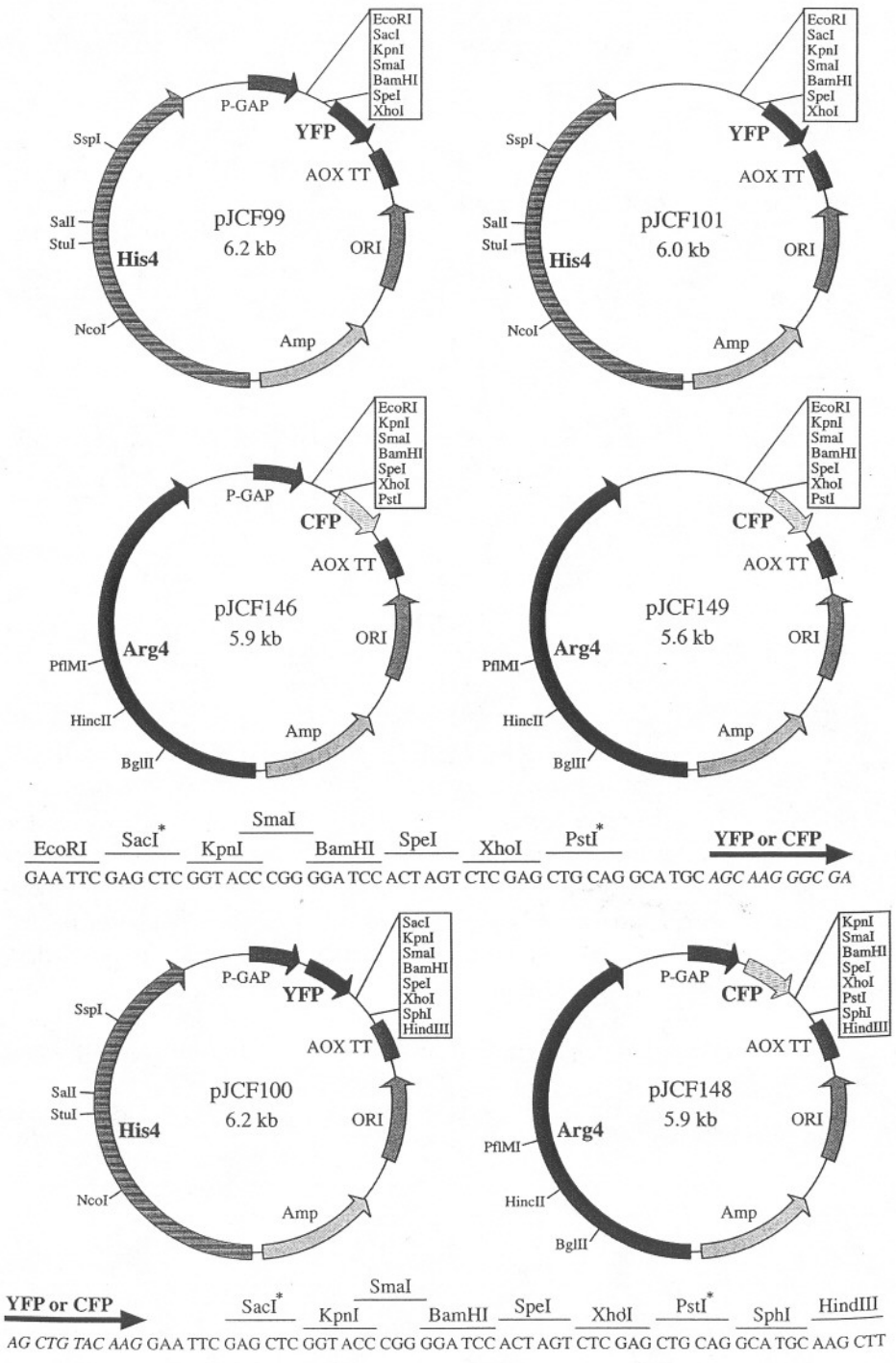

shown to be functional by complementation of a strain lacking the gene for this protein. Second, before the DNA constructs are made for the fusion protein, one needs to consider the selectable markers available in the strain into which the fusion protein is to be introduced. Third, the choice of the appropriate tag and its location in the fusion protein (N-terminal or C-terminal) has to be made. There are many possibilities for this, such as the use of the green fluorescent protein (GFP) from *Aequorea victoria*, or one of the green, blue, cyan, or yellow variants (GFP, BFP, CFP, YFP), or the use of small epitope tags (e.g., HA, Myc, Protein A, Flag, His). GFP and its derivatives offer the possibilities of (i) using live cells, (ii) controlling the level of expression of the fusion protein (e.g., the use of a stronger promoter for detection of a weak signal), (iii) modifying the protein sequence (e.g., use of truncated or mutated forms), and (iv) using CFP-YFP or GFP-BFP combinations in fluorescence resonance energy transfer experiments (FRET: the energy emitted by one fluorophore has the potential to excite a nearby second fluorophore so that the physical proximity of the two proteins can be studied) ***(1)***.

1.1. Protein Localization and Colocalization

1.1.1. Fusion Proteins With GFP, BFP, CFP, or YFP

To obtain good images, the first step is to use a bright fluorophore. Many variants of GFP exist. Currently, the brightest GFP molecules are the enhanced GFP (EGFP) and its color variants (EBFP, ECFP, and EYFP). Typically, we fuse these fluorescent proteins to the extreme C termini of proteins whose localization we wish to investigate, and we express these fusions from native (pJCF101 or pJCF149 in **Fig. 1**) or constitutive promoters (e.g., GAP promoter; pJCF99 or pJCF146 in **Fig. 1**). It is important to confirm that the fusion protein complements a deletion strain lacking the protein of interest and that its expression is as close to normal as possible, because overexpression can cause protein mislocalization. If the fusion protein is unable to complement the deletion strain or to be expressed correctly, we resort to fusions at the N termini of the proteins (pJCF100 or pJCF148 in **Fig. 1**).

Usually CFP-YFP or GFP-BFP combinations are correctly separated using appropriate bandpass filters (*see* **Note 1**) and a conventional fluorescence microscope. Maps of the plasmids used in this laboratory for the localization and colocalization experiments are shown in **Fig. 1**. One set of plasmids has the fluorescent protein, YFP, and the *HIS4* marker, and the second set of plasmids has the fluorescent protein, CFP, and the *ARG4* marker.

Fig. 1. *(Opposite page)* Plasmid maps (*indicates that these sites are present only in vectors where they are shown).

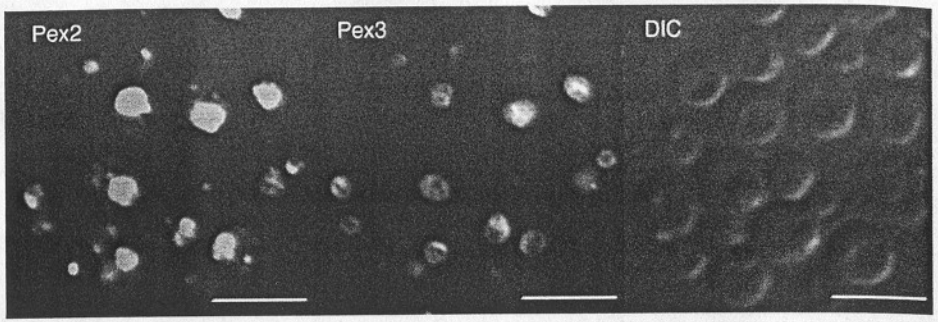

Fig. 2. Colocalization of Pex2 and Pex3 on the peroxisome membrane of *P. pastoris*. The Δ*pex2* cells harboring Pex2-myc were incubated in methanol medium for 6 h and subjected to immunofluorescence. The mouse primary antibody to the myc epitope was visualized using Alexa Fluor 546 goat anti-mouse IgG (Molecular Probes), while the rabbit primary antibody to Pex3 was visualized with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes). DIC: Nomarski optics. Bar, 5 μm.

1.1.2. Immunofluorescence

Despite the great developments in the use of protein fusions with fluorescent proteins, indirect immunofluorescence is still a powerful tool to detect the subcellular location(s) of a given protein and to determine its colocalization with other proteins (*see* **Fig. 2**). We have applied the method developed by Rossanese et al. *(2)*, with some modifications, to the localization of peroxisomal membrane proteins. Preservation of the subcellular organelles such as peroxisomes is greatly improved by postfixing the cells with acetone. The acetone treatment might affect the protein structure and might result in some loss of recognition by antibodies. In that case, we recommend trying the method without the acetone fixation. For certain kinds of colocalization experiments, one might want to retain the GFP or red fluorescent protein (RFP) signals during the preparation of samples for fluorescence microscopy using antibodies. The RFP and GFP signals can be preserved during indirect immunofluorescence by fixing the cells with paraformaldehyde instead of formaldehyde (*see* **Note 2**).

1.2. Organelle Localization

1.2.1. Dyes (FM4-64, DAPI, MitoTracker)

Many dyes have been employed to trace organelles (*see* **Fig. 3**). FM4-64 [*N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenyl-hexatrienyl) pyridinium dibromide] is a vital stain for the vacuolar membrane. FM4-64 stains the vacuole red by endocytosis of the dye *(3)*. DAPI (4′, 6-Diamidino-2-phenylindole) stains the nucleus blue by passive diffusion across the membrane and binding to AT regions of the DNA *(4)*. Mitotracker Green FM (Molecular Probes) is a

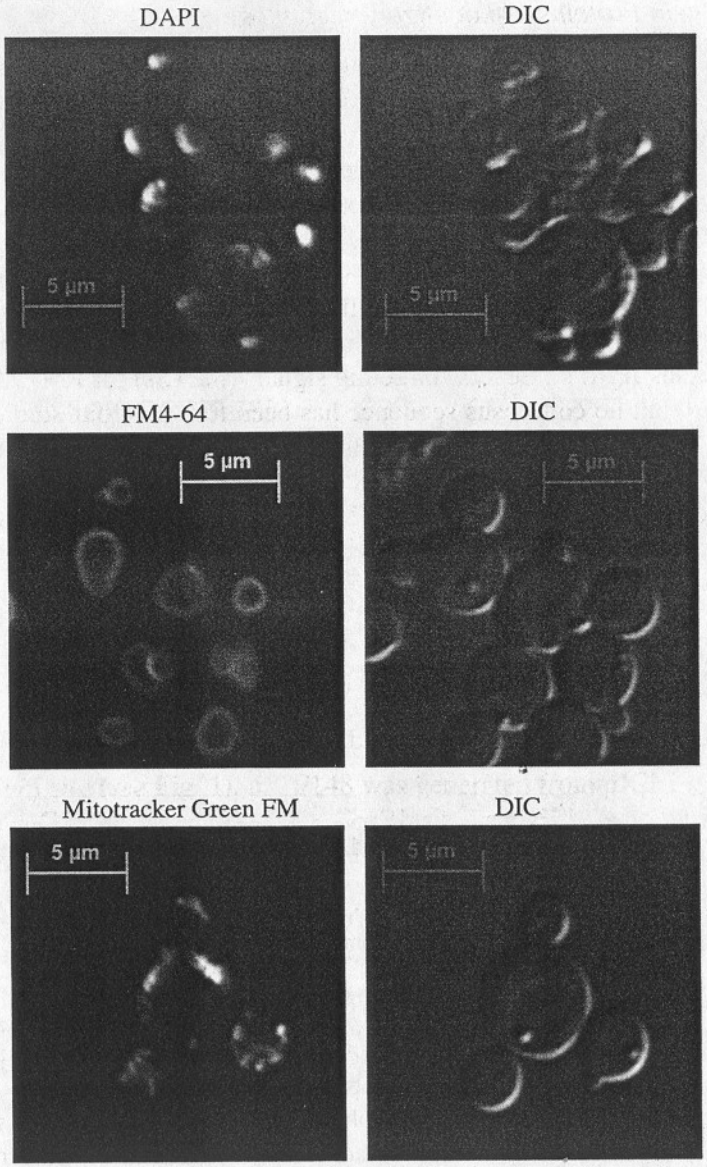

Fig. 3. Localization of the nucleus using DAPI, the vacuole using FM4-64 and the mitochondria using Mitotracker Green FM. DIC: Nomarski optics.

green, fluorescent mitochondrial stain, which appears to localize to mitochondria regardless of mitochondrial membrane potential. The MitoTracker Green FM probe is essentially non-fluorescent in aqueous solutions and only becomes fluorescent once it accumulates in the lipid environment of mitochondria.

1.2.2. Fusion Proteins to Localize Organelles

We have developed a number of fusion proteins in which a peroxisomal targeting signal (PTS) is appended to different fluorescent proteins to follow the biogenesis or the degradation of the peroxisomes. We have used three different targeting signals: (i) Peroxisomal Targeting Signal 1 (PTS1) present at the C terminus of the protein and composed of three amino-acids Ser-Lys-Leu (SKL) *(5)*, (ii) PTS2 present at the N terminus of the protein and whose minimal consensus sequence is (R/K)(L/I/V)-X_5-(H/Q)(L/A) (we add an amino acid sequence of MERLSQLRKHLA to the N terminus of monomeric RFP1, mRFP1) *(6)*, and (iii) Membrane PTS (mPTS) derived from peroxisomal membrane proteins (PMPs). Several targeting signals (mPTSs) for PMPs have been determined, but no consensus sequence has been found. In our studies on peroxisomal membrane localization, the first 40 amino-acids of PpPex3p serve as the mPTS that is fused to GFP variants *(7)*.

These different targeting signals have been used to study each import pathway and to colocalize other proteins with peroxisomes in wild-type or peroxisome biogenesis-deficient *(pex)* cells.

2. Materials

2.1. Strain

The host strain most commonly used is PPY12 (*his4*, *arg4*) (*see* **Note 3**).

2.2. Dyes

1. FM4-64 (Molecular Probes), dissolved in dimethyl sulfoxide (DMSO) at 1 mg/mL stock.
2. DAPI (Sigma) 1 mg/mL stock in H_2O.
3. MitoTracker Green FM (Molecular Probes) 1 m*M* stock in DMSO.

2.3. Mounting Solution

There are several commercially available mounting media containing an anti-fading reagent. We use 0.1% *p*-phenylenediamine (Sigma) in 90% glycerol. Dissolve *p*-phenylenediamine in phosphate buffered saline (PBS) to make 1% solution, adjust to pH 9.0, and add glycerol. Store at –80°C in the dark.

2.4. Immunofluorescence

1. Multi-well Teflon-coated glass slide.
2. Cover glass.
3. Fixation buffer: 50 m*M* potassium phosphate (pH 6.5), 1 m*M* $MgCl_2$, and 4% formaldehyde.
4. Wash Buffer: 100 m*M* potassium phosphate (pH 7.5) and 1 mM $MgCl_2$.

5. Zymolyase 20T (Seikagaku-Kogyo): dissolve in H_2O at 10 mg/mL and store at –20°C. Store as aliquots and do not freeze-thaw more than once.
6. 0.1% polylysine (Sigma; cat. no. P 8920).
7. PBS-Block: PBS (137 m*M* NaCl, 2.7 m*M* KCl, 1.4 m*M* KH_2PO_4, 10 m*M* Na_2HPO_4) (pH 7.4), 1% skim milk, 0.1% bovine serum albumin (BSA), 0.1% *n*-octyl-β-D-glucopyranoside (Calbiochem; cat. no. 494459).
8. Secondary antibodiesAlexa Fluor 488 and 546 conjugated to goat anti-rabbit IgG, and goat-anti mouse IgG (Molecular Probes).
9. Mounting medium: see Mounting Solution.

2.5. P. pastoris Vectors

CFP and YFP were respectively amplified by PCR from pECFP-C1 and pEYFP-C1 (Clontech). pECFP-C1 and pEYFP-C1 encode enhanced cyan and yellow-green variants of the *Aequorea victoria* GFP. Plasmids pJCF99 and pJCF100 were derived from the plasmid pIB2, which expresses genes from the constitutive GAP promoter and has the *HIS4* marker *(8)*. The YFP was located at the *Sph*I site in pJCF99 and at the *Eco*RI site in pJCF100 (*see* **Fig. 1**). pJCF101 was derived from pIB1, which has no promoter and is a useful vector for expressing genes containing endogenous promoters that function in *P. pastoris*. pIB1 also has *HIS4* as a selectable marker *(8)*. The YFP was located at the *Sph*I site in pJCF101 (*see* **Fig. 1**). pJCF135 and pJCF136 (not shown) were derived from pIB1 and pIB2, respectively, by replacement of the *HIS4* marker by *ARG4*. pJCF146 was generated from pJCF136 by insertion of the CFP gene into the *Sph*I site (*see* **Fig. 1**). pJCF148 was generated from pJCF135 by insertion of the CFP fragment into the *Eco*RI site, pJCF149 was derived from pJC135 by cloning the CFP fragment at the *Sph*I site (*see* **Fig. 1**).

3. Methods

3.1. Mounting Live Cells Without Agar

1. Use 1 to 2 mL of log phase cell culture.
2. Centrifuge for 5 min at 2000*g* at room temperature.
3. Remove the supernatant and resuspend in 250 to 500 µL of fresh media.
4. Mount the cells by spotting 2 µL on a dust-free slide and then pressing the cover glass firmly in place.
5. Seal the cover glass using Cytoseal 60 (Richard-Allan Scientific) (*see* **Note 4**).

3.2. Mounting Live Cells With Agar

1. Make a 1% low-melting agarose (Apex Fine Chemicals) solution in the desired medium (SM, SD, or YPD).
2. Melt the agarose at 55°C and keep liquid at 37°C.
3. Resuspend the cells from **Subheading 3.1., step 3** in 50 to 100 µL of fresh medium.
4. Spread 2 µL of cells on the coverslip.

5. Drop 5 µL of the low melting agarose solution on the slide.
6. Immediately place the cells on the coverslip on top of the drop of agarose on the slide.

3.3. Fixing and Mounting the Cells

1. Centrifuge 1 to 2 mL of log-phase cell culture (OD_{600} of 1–2/mL) as above.
2. Resuspend the cells in 100 µL of 4% paraformaldehyde (electron microscopy grade) in 0.1*M* potassium phosphate buffer (pH 7.5).
3. Incubate at room temperature for 15 min.
4. Centrifuge the cells and remove the supernatant.
5. Wash the cells once in 1 mL of 0.1*M* potassium phosphate buffer (pH 7.5).
6. Resuspend the cells in a small volume of 0.1*M* potassium phosphate buffer (pH 7.5) (*see* **Note 5**).
7. Sonicate the cells briefly using a water-bath sonicator to dissociate them from each other.
8. Mount the cells without agar.

3.4. FM4-64 Staining of the Vacuole in Live Yeast Cells

1. Inoculate 10 mL of YPD with a fresh *P. pastoris* colony.
2. Incubate with a vigorous shaking overnight at 30°C.
3. Use the overnight culture to inoculate a 2 mL culture in the desired medium (SM, SD or YPD [*see* **Note 6**]) in a 50-mL Falcon tube to starting OD_{600} of 0.1/mL.
4. Add 20 µL of 1 mg/mL of FM4-64 and the medium should turn purple when mixed.
5. Incubate with vigorous shaking at 30°C until the culture reaches an OD_{600} of 1 to 2/mL and the medium turns yellow.
6. Centrifuge the cells 5 min at 2000*g* at room temperature.
7. Discard the supernatant, and wash the cells with the desired medium.
8. Centrifuge the cells 5 min at 2000*g* at room temperature.
9. Discard the supernatant, and resuspend the pellets of cells in the desired medium.
10. Observe under the fluorescence microscope using the Rhodamine filter set (*see* **Note 1**).

3.5. DAPI Staining of Nuclei in Live Yeast Cells

1. Inoculate 10 mL of YPD with a fresh *P. pastoris* colony.
2. Incubate with vigorous shaking overnight at 30°C.
3. Use the overnight culture to inoculate a 2 mL culture in the desired medium (SM, SD or YPD [*see* **Note 7**]) in a 50-mL Falcon tube to starting OD_{600} of 0.1/mL.
4. Grow the cells up to an OD_{600} of 1.0/m.
5. Add 5 µL DAPI to the final concentration of 2.5 µg/mL.
6. Grow the cells for 30 min.
7. Centrifuge the cells 5 min at 2000*g* at room temperature.
8. Resuspend cells and wash with 1X PBS.
9. Centrifuge the cells 5 min at 2000*g* at room temperature.
10. Finally resuspend the cells in 1X PBS and observe under the fluorescence microscope using the DAPI filter set.

3.6. DAPI Staining of Fixed Cells

1. After the antibody incubation for immunofluorescence (*see* **Subheading 3.8.3., step 7**) add 20 µL of 1 µg/mL DAPI in 1X PBS over the cells (*see* **Note 8**).
2. Incubate for 2 min.
3. Wash 3× with 1X PBS.
4. Add mounting solution and place the coverslip over the cells (eliminate excess).
5. Seal the edge of the coverslip with Cytoseal 60.

3.7. MitoTracker Green FM Staining of Mitochondria (see Note 9)

1. Inoculate 10 mL of YPD with a fresh *P. pastoris* colony.
2. Incubate with vigorous shaking overnight at 30°C.
3. Use the overnight culture to inoculate a 2 mL in the desired medium (SM, SD, or YPD [*see* **Note 7**]) in a 50-mL Falcon tube to starting OD_{600} of 0.1/mL.
4. Grow the cells up to an OD_{600} 1.0/mL.
5. Add 0.2 µL MitoTracker Green FM to a final concentration of 100 n*M*.
6. Incubate for 1 h at 30°C.
7. Wash cells 2× with fresh medium.
8. Examine the cells under the fluorescence microscope using the FITC or GFP filter set.

3.8. Immunofluorescence

3.8.1. Fixing and Spheroplasting of Cells

1. Collect the cells of 10-mL culture at an OD_{600} of 0.25 to 1.0/mL by centrifugation at room temperature.
2. Resuspend the cells in 5 mL of freshly prepared fixation buffer.
3. Fix the cells for 1 h at room temperature in a 15-mL tube on a rotator.
4. Collect the cells by centrifugation for 3 min at 1000*g*.
5. Aspirate the supernatant completely.
6. Resuspend the cells in 5 mL of freshly prepared wash buffer and centrifuge again as above. Repeat the wash again.
7. Resuspend the cells in wash buffer to an OD_{600} of 10/mL (*see* **Note 10**).
8. Add 0.6 µL of 2-mercaptoethanol to 100 µL of cell suspension.
9. Add 10 µL of 10 mg/mL Zymolyase 20T to the cell suspension (*see* **Note 11**).
10. Incubate the cell suspension for 10 to 20 min with mixing end-over-end or on a rotator (*see* **Note 12**) at room temperature.
11. Centrifuge the spheroplasts for 2 min at 400*g*.
12. Gently resuspend the spheroplasts in 100 µL of wash buffer and centrifuge again.
13. Resuspend the spheroplasts in 200 µL of wash buffer (*see* **Note 10**).

3.8.2. Adhesion and Permeabilization of Cells

1. Add 20 µL of 0.1% polylysine (Sigma) to each well of multi-well slide glass (*see* **Note 13**).
2. Remove the solution by aspiration after 5 min.

3. Wash each well three times with water and air dry. (Alternatively, wash the slides with running water and air dry).
4. Add 20 µL of the spheroplast suspension to each well.
5. After 3 min, blot off excess liquid with a piece of filter paper.
6. Postfix the spheroplasts by immersing the dried slide glass in 40 mL of pre-chilled 100% acetone in a 50-mL Falcon tube for 5 min at –20°C.
7. Remove the slide glass, blot off excess solvent, and allow dry. You can store the slides at 4°C (*see* **Note 10**).

3.8.3. Antibody Incubation

1. Incubate each well to re-hydrate and block with a drop of PBS-Block for 30 min.
2. Aspirate the solution and add 15 µL of primary antibodies mixture in PBS-Block.
3. Incubate in a humid chamber for 1 to 2 h at room temperature or overnight at 4°C.
4. Wash each well eight times with PBS-Block.
5. Add 15 µL of secondary antibodies mixture in PBS-Block.
6. Incubate the slide in a humid chamber for 30 to 60 min. Keep the slide glass in the dark.
7. Wash each well eight times with PBS-Block and completely remove the liquid by aspiration after the final wash.
8. Add suitable mounting medium to each well, cover the slide glass with a cover glass, and seal with nail polish or equivalent (e.g., Cytoseal 60).
9. Observe under a fluorescence microscope or store the slide at 4°C (*see* **Note 10**). The signals are stable for at least 1 wk.

4. Notes

1. The bandpass filters we use are the Endow GFP filter set (41017), Blue GFP filter set (31021), Yellow GFP BP filter set (41028), Cyan GFP V2 filter set (31044 V2), and Rhodamine filter set (C2915) from Chroma Technology Corp.
2. We fix the cells with the fixation buffer containing 4% paraformaldehyde (EM grade), and convert the cells to spheroplasts in the wash buffer containing 1.2 *M* sorbitol. The spheroplasts are permeabilized by treating the cells with 0.5% Triton X-100 in the wash buffer for 5 to 15 min and applied to the wells. The later steps are essentially the same as above except the sealing. Organic solvents affect the structure of the fluorescent proteins so that one should avoid using acetone or methanol to fix the cells, and sealants that contain organic solvents.
3. In some cases, autofluorescence may be a problems. *ade*$^-$ strains are particularly fluorescent. Also, dead cells yield strong fluorescence with the GFP and Rhodamine filters.
4. The sample is good for about 1 h until carbon dioxide production causes the coverslip to bow outward.
5. If you keep the suspension at 4°C, the signals of fluorescent proteins are stable for a few weeks.
6. pH problems: the medium should turn purple with the FM4-64. If it turns yellow, it is because the pH of the medium is too acidic (less than pH 5.5). Adjust the pH of the medium to between 6.0 and 6.8 with KOH.

7. Avoid growing the cells in YPD for the fluorescence microscopy experiment, because it gives a high background.
8. You can add DAPI directly in the mounting solution (100 ng/mL final concentration), but the background could be higher.
9. The dye will stain live cells but is not well-retained after aldehyde fixation. MitoTracker Green FM can be used to stain mitochondria in fixed cells as well.
10. You can interrupt the process at this point.
11. The original method used recombinant yeast lytic enzyme (ICN Biomedicals), and most labs use Oxalyticase or Zymolyase 100T for immunofluorescence, but Zymolyase 20T also works.
12. We suggest doing this process as a time course at least for the first time because it is the most critical part of the procedure. In our hands, 10 to 20 min incubation gives good results.
13. We usually spin the poly-lysine and water for 10 min at 14,000*g* before the preparation of the slides to remove any dust in the solution, and clean the slides with 100% ethanol or acetone.

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