Yeast Immunofluorescence

Prepare Cells:
1. Grow yeast in 25 mls YPD to OD$_{600}$ = 0.2. I usually grow cultures at 30°C overnight at ~225 rpm. Use fresh starter culture.

2. Add 2.5 mls of 30% paraformaldehyde stock to 25 mls of culture while swirling. Swirl gently for 10 minutes at room temperature.

   Prepare 30% paraformaldehyde stock as follows:
   A. Add 2.0 g paraformaldehyde to 5 mls of ddH$_2$O in a Pyrex tube (16 X 150 mm).
   B. Heat slowly with burner to boiling. Vortex intermittently.
   C. Add 0.1 ml 1M NaOH while vortexing.
   D. Cool to room temperature. Filter (0.45 µm). Keep for one day.

3. Spin in IEC centrifuge for 5 minutes at 2500 rpm at room temperature.

4. Resuspend pellet with 1.0 ml of KP$_i$/pFA fixative at room temperature. Transfer to microfuge tube. Rotate gently at room temperature for 30 minutes.

   KP$_i$/pFA fixative: 9 parts 50 mM KP$_i$, pH 6.5 + 1 part 30% stock paraformaldehyde.

5. Spin ~15 seconds to pellet yeast in microfuge. Do step 11 now.

6. Wash yeast 3 times for 5 minutes each with 1 ml 50 mM KP$_i$, pH 6.5 at room temperature. Add 50 mM NH$_4$Cl to last wash (fresh 1/100 dilution of 5M stock).

7. Pretreatment of cells.
   A. Resuspend cell pellet in 0.5 ml of freshly prepared Pretreatment Buffer at room temperature.

      Pretreatment Buffer: 100 mM Tris•HCl, pH 9.0
      50 mM DTT
      5 mM EDTA

   B. Incubate at room temperature for 5 minutes.

   C. Spin down cells. Wash 1X with 1 ml of ddH$_2$O. Wash 1X with SPC buffer.

8. Digest yeast cell wall. Resuspend yeast with 0.5 ml SPC buffer. Add 20 µl of freshly made 10 mg/ml Zymolyase 100T in SPC buffer. Or, add 20 µl of 50 mg/ml freshly made Zymolyase 20T in SPC buffer. Incubate at room temperature on rotator for 10 minutes.

   SPC buffer: 18.22 g sorbitol (1 M)
   0.87 g K$_2$HPO$_4$ (anhydrous) (50 mM)
   0.34 g citric acid (monohydrate) (16 mM)
   Adjust pH to 5.8, and volume to 100 mls.

9. Add 0.5 ml of ice cold SPC buffer + protease inhibitor cocktails (PICs) to stop digestion.

10. Wash yeast cells. Spin ~15 seconds in microfuge to pellet yeast. Resuspend in 1 ml of ice cold SPC buffer + PICs. Spin again. R/S pellet in 50 µl of SPC buffer + PICs per slide. Can prepare up to 5 slides (i.e., can resuspend in up to 250 µl buffer).
**Prepare Slides:**
11. Use clean 15-well slide(s). Start with 25 µl of 2 mg/ml poly-D-lysine (Sigma P-1024) per slide. Pipet on and then off, leaving a thin film of polyK on slide well. Hold at room temperature for 1 minute. Wash slide with 50 mls of ddH₂O using a gentle broad stream. Air dry.
12. Add 3-5 µl cells in SPC buffer + PICs per well and hold at room temperature for 5 minutes.
13. Remove cells and add SPC buffer + PICs. Do one well at a time.
14. Aspirate off wash solution with a Pasteur pipette (fire polish end). Leave a thin film of SPC + PICs buffer. Plunge into -20°C MeOH. Move slide up and down quickly, in short strokes, 10 times. Hold at -20°C for 5 minutes.
15. Transfer slide to acetone at room temperature for 30 seconds. Flick dry in air for 30 seconds. Mark around wells with wide black Sharpie. Dry slides may be stored at 4°C for 1-4 weeks.

**Probe Slide:**
16. Block for 30 minutes at room temperature with a drop of PBS/2% Milk/0.1% Tween 20 plus protease inhibitors (PBS/M/T/PICs). Place slide in plastic Petri dish on wet filter paper.
   
   PBS/2% Milk/0.1% Tween 20:
   A. Stir 1 g powdered milk in 44 mls ddH₂O for >10 minutes.
   B. Add 5 mls 10X PBS.
   C. Add 0.5 mls 10% Tween 20 (wt./vol., 0.2 µm filtered).
17. Incubate with 1° antibody. Dilute 1° Ab in PBS/M/T/PICs. Aspirate off blocking solution. Add 3-5 µl of the 1° Ab per well. Incubate 1-2 hr at room temperature, or overnight at 4°C.
18. Wash 5 times at room temperature with PBS/M/T/PICs. Do 2 quick washes. Do one 15 minute wash. Do 2 washes for 5 minutes each.
19. Incubate with 2° antibody for 1-2 hr at room temperature. Dilute Cy2-antirabbit or Cy3-antimouse at 1/200. Do this step in the dark.
   
   • Optional: Pre-adsorb 2° antibody with fixed and blocked yeast (FBY). Suspend FBY into diluted 2° Ab. Incubate 30 minutes at room temperature. Microfuge 2 X 2 minutes to remove FBY.
20. Wash with PBS/M/T/PICs as described above. Do washes in the dark.

**Mount and View Slide:**
21. Do three quick washes with PBS.
22. Mount in 1 mg/ml p-phenylene diamine/glycerol mountant. Use 1.0 µl per well. Place a single large cover slip over wells carefully. Use 6 very small drops of nail polish at edge of cover slip to seal it. Or, use a thin strip of tape at each end to secure coverslip. DAPI can be included in the mountant at 0.1 µg/ml. Or, DAPI can be added in the next to the last wash at 1 µg/ml.

   For 125 X final magnification on Zeiss (100X objective plus 1.25 X Optivar), 300 pixels = 27.20 µm, and 1 inch = ~27 µm at 300 dpi print out. For 200 X final magnification (100X objective plus 2 X Optivar), 300 pixels = 17.00 µm, and 1 inch = 17 µm at 300 dpi print out.

   **In general:** final magnification X (0.088255) = pixels/μm
   
   3399 ÷ final magnification = μm per inch (at 300 dpi)