

EM Method for Yeast Cells

1. Prepare 5 OD units of cells in logarithmic growth (e.g., 10 mls of $OD_{600} = 0.5$). Spin at 2500 rpm in IEC centrifuge for 10 minutes. The steps below are performed at room temperature.
2. Resuspend (R/S) cell pellet with 1 ml of ddH₂O. Transfer to microfuge tube. Spin in microfuge at 5000 rpm for 1 minute. Remove supernatant. Wash cells once again with ddH₂O.
3. Wash cells with 50 mM KP_i (pH 6.5), 0.5 mM MgCl₂.
4. Fixation: R/S cell pellet in 1 ml of 50 mM KP_i (pH 6.5), 0.5 mM MgCl₂, 2.0% glutaraldehyde. ImmunoEM: use same buffer with 3% (freshly prepared) formaldehyde + 0.05% glutaraldehyde.
5. Fix for 30 minutes. Use rotator to gently mix cells. Or, place at 4°C overnight.
6. Wash cells with 1 ml ddH₂O. Pretreat with 1 ml 0.2 M Tris (pH 9), 20 mM EDTA (pH 8), 100 mM β-mercaptoethanol for 15 minutes. Wash cells once again with 1 ml ddH₂O.
7. Wash cells with phosphate-citrate (PC) buffer:
2.4 g KH₂PO₄
0.8 g sodium citrate
90 mls ddH₂O.
Adjust pH to 5.8 with H₃PO₄
QS to 100 mls with ddH₂O.
8. Digestion: R/S cells in 500 μl PC buffer. Add 50 μl of 10 mg/ml Zymolyase 100T (diluted in ddH₂O; sonify briefly). Place on rotator for 1-2 hours at room temperature. For overnight fixation, spin down cells, wash with PC buffer, and repeat the digestion step. For ImmunoEM, use 5 μl of 10 mg/ml Zymolyase 100T. Monitor digestion with phase microscope at 400X.
9. Wash cells 2X with 0.1 M NaAcetate, pH 6.1.
10. Postfixation: R/S pellet with 50 μl of 0.1 M NaAcetate, pH 6.1. Add 50 μl of 4% OsO₄. Incubate for 15 minutes. Work with OsO₄ in fume hood.
11. Wash cells 2X with ddH₂O. If pellet does not disperse well, do mild and brief bath sonication during the first ddH₂O wash.
12. UA staining: R/S pellet in 100 μl of 1% uranyl acetate. Incubate 1 hour in the dark.
13. Wash cells 2X with ddH₂O.
14. Dehydration: Wash cells 2X with 95% EtOH. Wash cells 2X with 100% EtOH. Do each wash step for 5 minutes or more. Place on rotator during wash steps. Secure tops of tubes.
15. Embedding: R/S pellet in 0.5 ml of Spurr's resin. Usually vortexing works well, but re-pipetting may be needed. Infiltrate 30 minutes to 1 hour. Place on rotator to mix gently.

Spurr's Resin: 5.0 g VDC + 2.0 g DER + 13 g NSA + 0.2 g DMAE. This is the "hard" recipe. Mix at room temperature, seal, and store at -20°C. Place at 25°C for a few minutes before use.
16. Spin at top speed for 1 minute in microfuge. Remove SN. Repeat steps 15 and 16.

17. Cut off the end of a yellow tip and lift out pellet (with residual Spurr's resin). Transfer to the bottom of a Beem capsule. Fill Beem capsule with Spurr's resin. Use a paper label as wide as Beem capsule (write on only one side of label).
18. Close tightly. Eliminate air bubbles; close cap while pressing on lip opposite hinge. Wear gloves.
19. Polymerize 8 hours to overnight at 65°C in vacuum oven.

Post-Staining EM Sections

1. Submerge grid in drop (50 μ l) of 1:1 mixture of 4% uranyl acetate and acetone. Incubate at room temperature for 5 minutes.
 - A range of 1-4% uranyl acetate in ddH₂O is standard. Lower UA concentrations are milder, but will give lower contrast. For heavier staining, dissolve 4% UA in 50% acetone or 50% MeOH. For low (1%) UA, the time can be extended to 10-15 minutes. Store UA in brown glass bottle.
 - For ImmunoEM, post-staining may be done before or after immunolabeling, depending on the antibody, etc. If you post-stain after binding colloidal gold, keep incubation with Reynold's lead brief (1 minute) to avoid loss of gold staining in the high pH solution. Some antibodies will not tolerate post-staining, either before or after immunolabeling. In this case, the sample must be visualized from en bloc stain.
2. Rinse with gentle stream of ddH₂O from squirt bottle for 1 minute. Or, transfer through 3 drops of ddH₂O. Use a self-closing, antipillary forceps. Touch grid to filter paper to blot away ddH₂O.
3. Submerge in freshly prepared Reynold's lead for 5 minutes at room temperature. Some do this in a covered dish containing wet filter paper and a separate container with several NaOH pellets.

Reynold's lead: 6 ml ddH₂O in glass tube (blue-cap)
 0.35 g NaCitrate•2H₂O
 0.27 g Pb(NO₃)₂
 Vortex for 1 minute
 Add 1.6 ml 1M NaOH while vortexing
 Add 2 mls ddH₂O to 10 mls (Store at 4°C in dark for maximum of 12 hours)
4. Rinse with 10 mM NaOH using gentle stream from squirt bottle for 30 seconds. Then rinse with ddH₂O for 30 seconds. Or, transfer through 3 drops of 10 mM NaOH and 3 drops of ddH₂O.
5. Drain droplet of ddH₂O from grid on filter paper, and air dry (on forceps or filter paper).
 - To destain, submerge grid in 1-10% AcCOOH for 1 or more minute(s) at room temperature.

Developing Kodak EM Film

1. Use diluted D19 for 4 minutes with intermittent agitation. Dilute stock, D19:H₂O = 1:2.
2. Wash with water for 2 minutes. Set up wash bath with running water. Use safelight facing ceiling.
3. Rapid fix for 4 minutes. Wash with water for 2 minutes.
4. Permawash for 1 minute. Wash with water for 2 minutes.
5. Rinse with photoflow for 15-30 seconds. Air dry in dust-free area.