

Loretta's "Super Quick" EM Prep for Chemically Fixed Yeast

Reference: Byers, B. and Goetsch, L. (1991) Preparation of yeast cells for thin section electron microscopy. *Methods Enzymol.* **194**: 602-608.

1. Glutaraldehyde Fixation

- A. A single sample should be approximately 1 or 2×10^8 cells. Loretta will use 10 ml of 1 or 2×10^7 cells/ml, Mark prefers 20 ml of 5×10^6 cells/ml. Cells can be fixed from YEPD media, but they will look better if grown in YM-1 (a Hartwell media, it's buffered and the cells grow a little slower). We'll use YM-1 when YEPD grown cells aren't working.
- B. Cells are washed twice in an equal volume of water. If need be, there can be osmotic support, such as sorbitol (important for mating factor arrested cells for instance). This entire protocol is done in glass tubes, most of which are disposed of after use. All spins are done in a clinical table top centrifuge at speeds that would be used for yeast cells.
- C. Bring up pellet from second rinse in 2.5 ml of fixative, which is 2.5% glutaraldehyde in 40 mM phosphate buffer ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 6.5) and 0.5 mM MgCl_2 . Purchase "Biological Grade" 50% glutaraldehyde (Polyscience cat. # 00377), cleaner glutaraldehyde doesn't seem to work as well. For 10 mls of fix: 9.1 ml dH₂O, 0.5 ml 50% glut., 0.4 ml 1M PO_4 , pH 6.5 , 5 ul 1M MgCl_2 . Sometimes a faint precipitate will appear when making the fix, it can be removed by filtration or centrifugation without affecting the solution. This solution can be stored for months at 4 degrees C. If at all discolored, make new fixative solution.
- D. Cells should sit in fix at room temperature for a few hours. They can then be stored at 4 degrees C for at least a month before further processing. Our general tactic is to grow cells, do the experiment and fix on one day, store the cells overnight at 4 degrees C and process the samples the next day, or after other tests are run that show that the experiment worked prior to working up the EM samples.

2. Spheroplasting (Once you start this, you are committed to several hours work):

- A. Wash cell pellets stored in fix twice in 4 to 5 ml 0.1M PO_4 -citrate buffer, pH 5.8 (diluted from 0.2M stock)
- B. We almost always do the "optional" pre-treatment that makes it easier to spheroplast the cells. It was developed for meiotic cells, but works well on mitotic cells. Resuspend cells in 5ml of 0.2M Tris pH 9.0 , 0.02M EDTA + 0.1M bME for 15 min. ONLY at room temperature. For 10 ml of pre-treat: 2 ml 1M Tris, pH 9.0 , 0.4 ml 0.5M EDTA, 70 ul b-ME
- C. Wash cells twice in 4 to 5 ml 0.1M PO_4 -citrate buffer, pH 5.8 (diluted from 0.2M stock)
- D. Resuspend pellet in 2 ml of 10% glucosylase (DuPont) in 0.05M 1M PO_4 -citrate buffer, pH 5.8 , to spheroplast. Incubate in 37 degree C bath for at least 2 hours, resuspending occasionally. Monitor spheroplasting in the

microscope. Cells will get darker under phase optics, and if the cells are smashed between a coverslip and slide (press and twist coverslip with your thumbs while applying a lot of weight) little, if any, residual cell wall material should be observed in well spheroplasted cells. The cell wall bits are translucent and can be shaped like parts of an egg shell that have come off the cell. Spheroplasting can be aided by spiking with fresh glucylase after a few hours, or with some zymolyase. We have used 50 to 100 ul of the zymolyase stock we keep for dissociating tetrads. Good spheroplasting is key to having good images and it is hard to over do it on these fixed cells.

- E. During the spheroplasting, get your SPURR resin mixing. Recipes below. Mix it slowly so it does not get air bubbles in it.
- F. After spheroplasting, wash 2x in 0.1M NaOAc, pH 6.2, using 5ml per wash.

3. **Post-fixation and Staining:**

- A. Post-fix cells with 2% Osmium tetroxide. This is done by gently resuspending the cells in 5 drops (from a Pasteur pipette) of 0.1M NaOAc, pH 6.2 and then adding 5 drops of 4% OsO₄ (available pre-made, or preferable get this from your EM facility) **IN THE HOOD!!** Incubate a room temperature for 15 minutes **ONLY**. The cells turn a dark brown or black and were adhere very well to glass. From this point on do not vortex or attempt to resuspend the cells until the very end. Simply add solutions to the pellets and incubate.
- B. Add 3 to 5 ml of 0.1M NaOAc, pH 6.2. Pellet and decant carefully into osmium waste.
- C. Put 3 to 5 ml of 0.1M NaOAc, pH 6.2 or water on pellets, spin and decant to wash. Some people will store at 4 degrees C at this point with the buffer still in the tube, overnight only.
- D. Put 0.5 ml of 1% uranyl acetate on pellets. Incubate in the dark for 30 min. at room temperature. Your EM facility should have a stock that is either 1% or 2% in water, dilute as need be. UA is not very soluble, so gently take what you need for the top of the containing without disturbing the sediment (you don't want it in your samples). UA is also weakly radioactive. There should be a disposal container for it.
- E. At the end of the UA staining, spin and decant UA. Put a few mls of water on the pellets, spin and decant.

4. **Dehydration and Embedding:**

- A. Put 4 ml or more of 95% EtOH on the cell pellet. Let stand 10 minutes at room temperature, spin and gently decant. Blot the tube on paper towels to get out as much ethanol as possible.
- B. Put 4ml or more of 100% EtOH on the cell pellet. Let stand 10 minutes at room temperature, spin and gently decant. Blot the tube on paper towels to get out as much ethanol as possible. Do this **TWICE**.
- C. After decanting second 100% EtOH rinse, air dry for 20 to 30 min.
- D. Put 4 ml of SPURR on the pellet and let sit at room temperature for 30 min to 1 hr (or overnight, 4 degrees C). Spin for at least 15 minutes to

form as tight a pellet as possible. We have done the entire incubation while spinning in the centrifuge.

- E. Decant the SPURR solution and blot of excess. Allow time for the SPURR to drain. Put about 0.5 ml of fresh SPURR on the pellet and gently resuspend with Pasteur pipette that has been broken off to give a large hole. Draw-up resuspended material and put it in large BEEM capsule (possibly available from your EM lab) and insert label (small piece of paper with identification written in pencil). Top off capsule with fresh SPURR. (Avoid air bubbles). BEEM capsules can be overloaded with cells such that they are brittle. Filling the point of the capsule with cells is OK, but a little heavy. If the yield of cells from this protocol is good, not too much loss, you should be able to make two or three capsules.
- F. Spin the BEEM capsules in swinging bucket rotor to pellet the cell material at bottom of capsule. This usually takes at least 15 minutes. Loretta had cork adaptors that held the capsules in a swinging bucket. We put the capsules inside a 15 ml. disposable conical tube that has glass wool in it to cushion the capsule.
- G. Bake capsules at 65 degrees C overnight, if not rock hard, bake longer (you may have to drive off more ethanol).

5. Sectioning and Stains:

- A. Sectioning of the samples is done using standard techniques. Cut a very small block face since yeast cells are so small. We can get many (50+) serial sections on a slot grid. We use standard Formavar-coated grids that have be carbon coated as well.
- B. Sections are routinely stained with lead citrate for just 10 to 15 seconds, and we often stain with uranyl acetate as well.

6. Solutions:

0.2M PO₄-citrate

4.63g KH₂PO₄

1.76g sodium citrate
(Dihydrate)

Water to 200mL, pH should
be 5.8

0.1M sodium acetate

4.08g NaOAc (3xH₂O) in 300mL
volume.

pH to 6.1 with glacial acetic acid

7. Phosphate Buffer

27.2g KH₂PO₄ in 200mL (1M)

34.8g K₂HPO₄ in 200mL (1M)

Mix 68.5mL of KH₂PO₄ solution with 31.5mL of K₂HPO₄ solution.

The pH should be 6.5, adjust as needed.

YM-1 Media

Yeast Extract

5g

Peptone

10g

Yeast Nitrogen Base (<u>with</u> amino acids)	6.7g
Adenine	10mg
Uracil	10mg
Succinic Acid	10g
NaOH	6g
Water	900mL

pH should be 5.8, adjust as needed.

- Autoclave above solution, separately sterilize 100mL of 20% glucose, add to above media.

SPURR Resin Recipes: Units are grams, weigh out the solutions into a beaker that will be disposed of when done. Make 10 grams for each sample (more than you'll need). Mix very slowly so bubbles are not incorporated. The stir bar can be cleaned by soaking in ethanol.

4-Vinycyclohexene Dioxide (VIN)	20	10	5	2.5
DER 736 Epoxy Resin	8	4	2	1
Nonenylsuccinic Anhydride (SUC)	52	26	13	6.5
2-Dimethylaminoethanol (DAE)	0.8	0.4	0.2	0.1
TOTAL:	80.8	40.4	20.2	10.1