Centre for Cellular Imaging
Sahlgrenska Academy

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ULTRASTRUCTURE PREPARATION FOR CELL PELLETS

Note: The samples must be immersed in reagent liquid during and between every step of processing!

1. PRIMARY FIXATION AND PELLETING

During fixation proteins and, to a lesser extent, other cell molecules, become crosslinked by formaldehyde and/or glutaraldehyde molecules. Pelleting cells in fixative can help consolidate the pellet so that it does not become fragmented during processing.

In all three variations of this step use fixative at 37°C and keep the cells in an incubator until just before fixation.

The fixative is mildly photoactive (light-sensitive) - it is best to incubate samples in the dark.

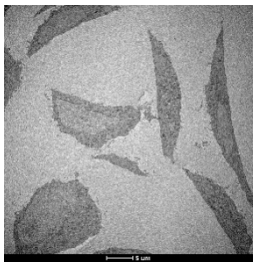
If the samples cannot be processed immediately, they should be stored in primary fixative diluted x10 in 0.1M cacodylate buffer at 4°C.

FOR CELLS CULTURED IN SUSPENSION

Take out the cells from incubator and immediately add 25% glutaraldehyde stock in water at a volume which will make up a final concentration of approximately 2.5% in the suspension, for example 1µl added to 9 ml of suspension. Gently mix for about a minute and pellet at a maximum of 300 g for 2 min, ideally in a 1.5 ml Eppendorf tube(s). Immediately remove the supernatant and add 2.5% glutaraldehyde in 0.1M PIPES or cacodylate buffer. Keep the sample in fixative on the rotor at 4°C for further 30 min to 1h depending on the pellet size. You can try gently lifting the pellet so that it is moving during incubation on rotor. Optionally you can fragment it if it is too big*.

FOR CELLS IN ADHERENT CULTURE

Option 1: Fix and scrape cells

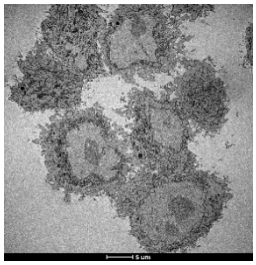


This method preserves the original cell shape and organelle arrangement.

Remove culture medium and rinse the cells quickly with PBS pre-warmed to 37°C. Remove PBS and immediately add 2.5% glutaraldehyde in 0.1M PIPES or cacodylate buffer and let the monolayer fix for 15 min at room temperature. Remove the fixative and wash sample with the same buffer as per fixative. In the meantime prepare 4% agarose (or 10% gelatin for array tomography) in the buffer and when it cools down to 40°C cover the cells with a thin layer. Keep the dish with cells in a water bath at about

40°C to prevent the agarose/gelatin from setting and use a rubber policeman to scrape the cells off the dish surface. Immediately transfer them to a tube, pellet, place in the fridge and when the agarose/gelatin is set, cut out small fragments with cells for further processing. The fragments should be no bigger than 1mm^3 and ideally thinner than 1 mm in one direction.

Option2: Trypsinise and fix



The unfixed cells will become round after trypsinisation and the shape will be preserved by fixation.

Remove culture medium and rinse the cells quickly with PBS pre-warmed to 37°C. Remove PBS and immediately add trypsin, just enough volume to cover the cells. Keep the trypsin on for 2 minutes and tap the side of the dish to dislodge the cells, ideally in a 1.5 ml Eppendorf tube(s). Then immediately add 2.5% glutaraldehyde in 0.1M PIPES or cacodylate buffer and pellet the cells (more than 300 g can be used if necessary for fixed cells). Remove the supernatant and replace with fresh fixative. Keep the sample in fixative on the rotor at 4°C for further 30 min to 1h depending on the pellet size. You can try gently lifting the pellet so that it is moving during incubation on rotor. Optionally you can fragment it if it is too big*.

***** *If a pellet after fixation appears like it might disintegrate during processing, embed it in 4% low melting point agarose in PIPES (or other non-toxic buffer). Ask staff to help you. Agarose embedding can be done at any point after fixation (best to do a couple of washes first) but before dehydration! If the pellet is large and easy to spin down, you can also choose to spin down after each step.*

2. **Wash** 5x in 0.1 M or 0.5M buffer (the same as the one used in fixative):
   - 10 min
   - 10 min
   - 20 min buffer with 0.02 M glycine (0.038g in 10 ml)
   glycine should neutralize any remaining aldehydes
   - 10 min
   - 10 min

   Insufficiently washed aldehydes can react with osmium tetroxide and produce precipitates!

3. **POSTFIXATION WITH OSMIUM TETROXIDE**

   This step ensures that lipids, for example the phospholipids forming membranes, are preserved and are not extracted during dehydration. During the postfixation a black electron-dense insoluble precipitate containing osmium is formed on the membranes, increasing their contrast.

   Incubate in 1% osmium tetroxide in 0.1M buffer for 30 min, in the dark.
   Unwashed osmium can react with uranyl acetate and produce precipitates!

4. **Wash** 6 times x 5min with dH_2O

5. **TERTIARY FIXATION AND CONTRASTING IN URANYL ACETATE**

   Uranyl acetate can to some extent stabilise proteins and nucleic acids and renders them more electron dense, increasing specimen contrast.

   Incubate in 0.5% aqUA overnight or in 2% aqUA for 2 hours at 4°C in the dark

6. **Wash** 3 times with dH_2O

7. **DEHYDRATION**

   To facilitate infiltration of the sample with hydrophobic epoxy resin the specimen needs to be dehydrated. Solvent concentration is increased gradually so that water is removed gently, without causing shrinkage.

   Dehydrate on rotor if possible. Dehydration at 4°C is recommended as it can minimize extraction of cell components by the solvent.

   - 30% EtOH 10 min
   - 50% EtOH 10 min
   - 70% EtOH 10 min
   - 85% EtOH 10 min
   - 95% EtOH 10 min
   - 100% EtOH 10 min
   - 100% EtOH 10 min

   Acetone-resistant plasticware or glassware needs to be used for the next steps!

   - 100% Acetone 10 min
   - 100% Acetone 10 min

8. **RESIN INFILTRATION**

   *Extend the **minimum** recommended step lengths and apply more 100% resin changes for larger pellets and cells with cell walls such as yeast. You can leave the samples on the rotor overnight. Check with CCI EM staff what is best for your sample type.*

   Infiltrate with resin, on rotor:

   - 1:3 resin without accelerator:acetone min 30 min
   - 1:1 resin without accelerator:acetone min 30 min
   - 3:1 resin without accelerator:acetone min 30 min
   - 100% resin without accelerator 20 min

   You can slowly centrifuge the pellets through the 100% resin (speed setting ten on small centrifuge) to facilitate better infiltration. Lift the pellets gently with a toothpick after centrifuging so that it does not remain stuck to the tube bottom.

9. **Prepare resin containing accelerator** (240 µl per 10 ml of premix) for the last change and embedding (a little less than 1ml per BEEM capsule). Let it mix for at least 30 min.

10. **Continue infiltration with resin**

    You can centrifuge the pellets as above at the beginning of each new resin change.

    - 100% resin without accelerator 20 min
    - 100% resin without accelerator 20 min (or overnight)
    - 100% resin with accelerator 20 min

11. **Prepare capsules and labels for embedding**.

    Include preparation number (ask staff) as well as sample and replicate codes, for example C500, s1 bl.A (preparation number C500, sample 1, replicate block A).

12. EMBEDDING

Embed in a BEEM or gelatin capsules filled with resin containing accelerator, with a label.

If using pointy BEEM capsules, use a toothpick to remove the small air bubble which usually forms at the bottom.

13. RESIN CURING

Polymerise for at least 16h at 60°C

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Solutions:

- **Trypsin:** frozen aliquots from CCI EM staff

- **Primary fixative:** 
  - Karnovsky fixative
    2.5% glutaraldehyde, 2% formaldehyde, 0.02% sodium azide in 0.05M cacodylate buffer – stored at-20°C, from CCI EM staff

    OR
  - 2.5% glutaraldehyde in 0.1M PIPES buffer

  The fixatives are **mildly photoactive (light-sensitive)** - it is best to incubate samples in the dark.

- **cacodylate buffer** for washes and osmium: stored as 0.2M, frozen, from CCI EM staff 
  - for washing samples use 0.5 M or 0.1M cacodylate buffer
  - for diluting fixative (for storing samples) use 0.1M cacodylate buffer

- **PIPES buffer** for washes and osmium: stored as 0.2M, frozen, from CCI EM staff
  - for washing samples use 0.1M
  - for diluting fixative (for storing samples) use 0.1M

- **glycine:** 0.038g powder aliquots from staff, dissolve in 10 ml 0.05M cac. buffer

- **1% osmium- 1% potassium ferrocyanide (osmium ferricyanide)** solution (10 ml):

  1. Mix the following 3 ingredients: 
  - 2.5 ml MQ water
  - 5 ml 0.2M cacodylate buffer
  - 0.1g potassium ferrocyanide (pre-weighed aliquots from CCI EM staff)

  2. Immediately before use add 2.5 ml of 4% osmium tetroxide in water (from CCI EM staff)

  Osmium ferricyanide is light sensitive! Incubate samples in the dark.

- **1% aq. TCH** (thiocarbohydrazide): 0.1g aliquots from staff 

  Dissolve 0.1 g in 10 ml of mq water at 60°C for **1h** (use oven or water bath). Check the solution several times and mix it gently to help the crystals dissolve. Cool down to room temperature and filter before use. Protect the solution from light and do not store it for a long time before use.

- **Aq. uranyl acetate (UA) 2%** stock (in black bottle, ask CCI EM staff).
  Very light sensitive! Keep out of light! 

- **Ethanol:** use wash bottles for 30-95% concentrations. Use an original supplier EtOH bottle for the 100% changes.

- **Acetone:** use the supplier's bottle for acetone-water dilutions and use dry acetone (w. molecular sieve) for 100% acetone incubations and for diluting resin. 
- Hard-Plus Resin 812 from EMS: 
    - 812 resin+hardener mix made by CCI EM staff is stored in the fridge and must be **warmed up to room temperature before use** to avoid condensation.
    - For the last 1-2 infiltration steps and polymerisation add 240 µl of accelerator to 10ml of the 812+hardener mix. The accelerator is also stored in the fridge and must be **warmed up to RT** before use.