Centre for Cellular Imaging
Sahlgrenska Academy

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ULTRASTRUCTURE PREPARATION FOR TISSUES

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

1. **PRIMARY FIXATION**

   During this step proteins and, to a lesser extent, other cell molecules, become crosslinked by formaldehyde and/or glutaraldehyde molecules. Small mammals can be fixed by perfusion, whereby the fixative is introduced via the vascular system. Other samples need to be fixed by immersion and the specimen dissected no thicker than 1 mm in at least one direction.

   - **Perfusion-fixed** samples need to be brought to the EM facility in Karnovsky fixative (at least x10 the volume of the tissue) and dissected after washing if needed
   - **Immersion fixation:** dissect tissue in modified Karnovsky fixative without letting it dry out. Then incubate in Karnovsky (at least x10 the volume of the tissue) for 1h at RT, if possible, on rotor, then at 4°C for at least one more hour or until processed.

   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.

2. **Wash** 5x at RT on rotor:
   - 10 min 0.05M cacodylate buffer
   - 10 min 0.05M cacodylate buffer
   - 20 min 0.05M cacodylate buffer with 0.02 M glycine (0.038g in 10 ml)
     glycine should neutralize any remaining aldehydes
   - 10 min 0.05M cacodylate buffer
   - 10 min 0.05M cacodylate buffer

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

3. **Start dissolving TCH at 60°C in the dark if using OtO method (see point 4).**

4. **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.

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

   BASIC VERSION:

   Incubate in osmium tetroxide/potassium ferrocyanide (1%/1%) in 0.05M cacodylate buffer at 4°C for 1-2h in the dark.

OtO VERSION (for increased membrane contrast):

This variation of the protocol will produce membranes with stronger contrast, which may be particularly useful for atomic contrast imaging with the SEM, for example for array tomography. However, it may also adversely affect membranes by making them appear more "fuzzy".

a. Incubate in osmium tetroxide/potassium ferrocyanide (1%/1% or 2%/1.5%) in 0.05M cacodylate buffer for 1-2h (in the dark).

b. Wash 5 x 3 min

c. Incubate in **filtered** 1% TCH in water at RT for 15 min

d. Wash 5 x 3 min

e. Incubate in 1% or 2% osmium tetroxide in water at 4°C in the dark for 1h.

5. Wash 5 times with dH_2O, 30 min in total
Unwashed osmium can react with uranyl acetate and produce precipitates!

6. 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 1-2 hours at 4°C (in the dark)

7. Wash 3 times with dH_2O

8. 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

9. RESIN INFILTRATION

Infiltrate with Hard Plus resin at RT, on rotor.

*Extend the **minimum** recommended step lengths and apply more 100% resin changes for larger pieces and denser tissues. You can leave the samples on the rotor overnight. Check with CCI EM staff what is best for your sample type.*

- 25% resin without accelerator in acet. 30 min
- 50% resin without accelerator in acet. 30 min

This step should be extended for larger pieces and denser tissues, even for as long as overnight.

- 75% resin without accelerator in acet. 30 min
- 100% resin without accelerator 30 min

10. 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.

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. Continue resin infiltration.

You can slowly centrifuge the tissue pieces through the resin (speed setting ten on small centrifuge) to facilitate better infiltration. Lift the tissue pieces gently with a toothpick after centrifuging so that they do not remain stuck to the tube bottom.

- 100% resin without accelerator 30 min
- 100% resin without accelerator 30 min
- 100% resin with accelerator 30 min

13. EMBEDDING

Embed in a resin-filled BEEM or gelatin capsule or silicon “coffin” mould with a label.
The resin must contain accelerator!

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

14. RESIN CURING

Polymerise for at least 16h at 60°C

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Solutions:

- 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

  The fixative is **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

- glycine: 0.038g aliquots from staff, dilute 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.

- Aquaeous 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.