

ULTRASTRUCTURE PREPARATION FOR TISSUES

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 needs to be 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)
- **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.

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 cause precipitates in the presence of osmium tetroxide!

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

4. POSTFIXATION IN OSMIUM TETROXIDE

This step ensures that lipids, for example the phospholipids forming membranes, are preserved and are not extracted during dehydration. During the fixation 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 is particularly useful for imaging with the SEM, for example for array tomography. However, it can also adversely affect membrane structure 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 dH₂O, 30 min in total

Unwashed osmium can cause precipitates in the presence of uranyl acetate!

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 dH₂O**8. DEHYDRATION**

In order 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 |
| - 80% EtOH | 10 min |
| - 90% EtOH | 10 min |
| - 100% EtOH | 10 min |
| - 100% EtOH | 10 min |
| - 100% Acetone | 10 min |
| - 100% Acetone | 10 min |

9. RESIN INFILTRATION

Infiltrate with Hard Plus resin at RT, on rotor.

Extend the recommended step lengths 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

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. 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).

11. Continue resin infiltration

- 100% resin without accelerator 30 min

- 100% resin without accelerator 30 min

- 100% resin with accelerator 30 min

We recommend a minimum of four 100% resin changes but many specimens might require more. Extend step number and length for larger pieces and denser tissues. You can leave the samples on the rotor overnight.

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

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

13. Polymerise for at least 16h at 60°C

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Solutions:

- 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: 0.038 g aliquotes 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.
- Aqueous uranyl acetate (UA) 2% stock (in black bottle, ask CCI EM staff).
Very light sensitive! Keep out of light.
- 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 has to 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 needs to be **warmed up to RT**.