



ULTRASTRUCTURE PREPARATION FOR MAMMALIAN CELL MONOLAYERS

Note: The cells have to be immersed in reagent liquid during and between every step of processing!

1. Warm up reagents to 37°C: primary fixative and 0.1M buffer (same as in fixative).

2. Wash away the culture medium with 0.1M buffer warmed up to 37°C.

The cells must be kept in the incubator until just before this step is performed!

Remove culture medium and immediately add the buffer gently from the side of the dish. Immediately proceed to step 2.

3. PRIMARY FIXATION

In this step proteins are crosslinked by aldehydes in the fixative.

Remove the buffer and add 2.5% GA in 0.1M PIPES buffer or Karnovsky fixative warmed up to 37°C. Incubate for 30 min at RT. If you need to store samples for hours or days before processing, do it in fixative diluted x10 in 0.1M buffer and in the fridge.

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

4. Start dissolving TCH (1h) if you are going to use the OtO method (see point 6).

5. Wash 6 x 5 min with 0.1M PIPES buffer (or cacodylate buffer after Karnovsky fix)

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

6. 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 postfixation a black insoluble precipitate containing osmium is formed on the membranes, increasing sample conductivity and contrast.

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

BASIC VERSION:

Incubate in 1% osmium tetroxide in 0.1M PIPES or 0.1M cacodylate buffer for 15 min to 1h at 4°C in the dark.

OtO VERSION:

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%) in 0.1M PIPES or 0.05M cacodylate buffer for 15 min to 1h at 4°C in the dark.
- b. Wash 5 x 3 min
- c. Incubate in **filtered** 1% TCH in water at RT in the dark for 5 to 10 min
- d. Wash 5 x 3 min
- e. Incubate in 1% or 2% osmium tetroxide in water at 4°C in the dark for 15 min-1h.

7. Wash minimum 5 times x 3min with dH₂O

8. 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)

9. Wash 3 times with dH₂O

10. 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.




Dehydration at 4°C is recommended as it can minimize extraction of cell components by the solvent.



- 30% EtOH 5 min
- 50% EtOH 5 min
- 70% EtOH 5 min
- 85% EtOH 5 min
- 95% EtOH 5 min
- 100% EtOH 5 min
- 100% EtOH 5 min
- 100% EtOH 5 min
- 100% EtOH 5 min

11. Place pieces of filter paper under the substrates/cover slips to prevent them from sticking to the bottom of the dish.

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
 - 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 
 - used at 0.05-0.1M for washing
- **1% osmium:**
 - 1 part of 4% osmium in water
 - 1 part of mq water
 - 2 parts of 0.2M PIPES or cacodylate buffer
- **1% osmium- 1% potassium ferrocyanide** 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. Add 2.5 ml of 4% osmium tetroxide in water (from CCI EM staff)
- **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.
- **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 μ l 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.