Centre for Cellular Imaging
Sahlgrenska Academy

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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 PBS or 0.1M PIPES buffer.

2. **Wash away the culture medium** with PBS or PIPES 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.

   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 dH_2O

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 dH_2O

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.

12. RESIN INFILTRATION

Infiltrate with Agar 100 resin at RT, on rotor.

Extend the minimum recommended step lengths and apply more 100% resin changes if necessary. Check with CCI EM staff what is best for your sample type.

- 25% resin without BDMA in EtOH	30 min
- 50% resin without BDMA in EtOH	30 min
- 75% resin without BDMA in EtOH	30 min
- 100% resin without BDMA in EtOH	30 min

13. 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.

14. 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).

15. Continue resin infiltration.

- 100% resin without BDMA in EtOH	30 min
- 100% resin without BDMA in EtOH	30 min
- 100% resin without BDMA in EtOH	30 min

16. EMBEDDING

Embed in a resin-filled BEEM or gelatin capsule with a label pushed down towards the middle. The resin must contain BDMA!

Invert the coverslip on top of a resin-filled BEEM or gelatin capsule with cells facing towards the resin and gently squeeze both towards each other. Invert again, capsule bottom up. If using free coverslips, place the coverslip-capsule assembly on a flat and stable support (for example plastic Petri dish) lined with aluminum foil.

17. 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

- **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.

- **Agar 100 resin** hard premix from Agar Scientific (AGR1140): 
  - **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 the hardener mix. The accelerator is also stored in the fridge and must be warmed up to RT before use.