Centre for Cellular Imaging
Sahlgrenska Academy

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TOPOGRAPHY PREPARATION FOR TISSUES

Note: The samples have to 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. **Start dissolving TCH** (1h) if you are going to use the OtO method (see point 6).

3. **Wash** 6 x 5 min with 0.05 M cacodylate buffer
   Insufficiently washed aldehydes can react with osmium tetroxide and produce precipitates!

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 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 cacodylate buffer for 1-2h at 4°C in the dark.

OtO VERSION:

If you want to avoid coating the specimen surface with metals (see pt.12) or to increase conductivity of any specimen throughout (not only on the surface), you can incubate the specimen in a higher concentration of osmium tetroxide more than once.

a. Incubate in 1% or 2% osmium tetroxide in 0.1M cacodylate buffer at 4°C in the dark for 1-2h.

b. Wash 5 x 3 min

c. Incubate in **filtered** 1% TCH in water at RT in the dark 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 30 min-1h.

You can apply further cycles of TCH followed by Osmium incubation if required (OtOtO etc).

5. **Wash** minimum 5 times x 3min with dH_2O

6. **DEHYDRATION**

In order not to collapse in the vacuum conditions inside the microscope, the specimen needs to be dehydrated. Solvent concentration is increased gradually so that water is removed gently, without causing shrinkage.

- 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

7. **DRYING**

This step is crucial for preserving the fine ultrastructural details on specimen surface. Ethanol has relatively high surface tension and simply left to evaporate would inflict surface damage upon leaving. Therefore, it has to be replaced with liquid CO_2 which has lower surface tension which is then evaporated from the sample.

This step is carried out by the CCI EM staff in a critical point dryer.

8. **MOUNTING ON STUBS**

The mounting has to ensure specimen stability on the stub and conductive continuity.

- Start with labeling the stub underside with prep number and sample code.
- Use carbon tab first and silver glue if you have samples that will not adhere flatly and tightly against the carbon tab.
- If you have used silver glue, let it dry overnight, otherwise it will start degassing in the coater and the microscope, degrading vacuum. Store the samples with silica beads during glue drying to prevent them from absorbing humidity.

9. COATING WITH METAL

This step is applied to prevent charge buildup on the surface and to improve focal point of the beam i.e. decrease the size of area from which signal is collected and therefore improve resolution. Too thick coating in insufficient vacuum conditions can on the other hand create surface artefacts such as gold granules and crusts.

Always leave some uncoated samples as control!

Coating protocol name: ……………………………………………………………………………………………

Metal: ……………………………………… Extra pumping time:……………………..

Thickness: ………………………………… Pump hold: …..………………………………

Current: …………………………………… Stage:……………………………………………

10. SAMPLE STORAGE

It is optimal to store the specimens in an air-tight container with silica beads to eliminate dust and humidity.

SOLUTIONS:

- **Primary Fixative:** Karnovsky Fixative – from CCI EM staff

- **cacodylate buffer** for washes and osmium: 0.2M frozen stock from CCI EM staff, dilute with mq water as needed

- **1% osmium in 0.1M cacodylate buffer**:
  - 1 part of 4% osmium tetroxide in water (from CCI EM staff)
  - 1 part of MQ water
  - 2 parts of 0.2M PIPES or cacodylate buffer

- **2% osmium in 0.1M cacodylate buffer**:
  - 1 part of 4% osmium tetroxide in water (from CCI EM staff)
  - 1 part of 0.2M PIPES or cacodylate buffer

- **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.

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

MAIN GOALS OF SAMPLE PREP FOR TOPOGRAPHY SEM:

- preserve fine surface details
- render the specimen conductive and stable under the beam and in vacuum inside the microscope