Centre for Cellular Imaging
Sahlgrenska Academy

Updated: June 9, 2020

TOPOGRAPHY PREPARATION FOR CELL MONOLAYERS AND CELLS SETTLED ON SUBSTRATES

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

1. SETTLING THE CELLS ON SUBSTRATES

The cells have to be processed on a stable substrate that can be then mounted on a *specimen stub, which is in turn placed in specimen stage inside the microscope.*

If the cells are cultured directly on a substrate (glass, conductive silica, aclar, transwell inserts, metal implants etc.) proceed to step 2.

If the cells are cultured **in suspension**, begin by settling the cells onto poly-l-lysine-coated conductive silica wafer substrate. It is also possible to settle the cells on **glass coverslips** but since glass charges heavily in the microscope it will be harder to avoid coating the sample with metals which might not be suitable for all applications.

a. Clean the substrate: use ethanol and lens paper

b. Hydrophilise the substrate by applying glow discharge

c. Incubate the substrate by immersing it in 0.1 % w/v poly-L-lysine or applying the solution to the surface for about 10 minutes

d. Shake off excess solution and dry the substrate. You can gently blot excess liquid from the substrate by touching it with filter paper from the side but do not touch the main surface to avoid leaving any debris on it. Avoid leaving small droplets of solution to dry as these will leave marks where more cells will stick.

e. When the poly-L-lysine has dried, cover the substrate with culture medium with cells to sediment for 30 min to several hours (discuss with staff). For many bacteria you can do this on the bench. For other cells use the most optimal culturing conditions.

2. Remove culture medium and wash the substrates once and gently with PIPES buffer or PBS (RT for bacteria or 37°C for other cells).

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 at RT for bacteria or warmed up to 37°C for other cells. 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 30 min to 1h 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 thespecimen in a higher concentration of osmium tetroxide more than once.

   a. Incubate in 1% or 2% osmium tetroxide in 0.1M PIPES buffer or 0.1M cacodylate buffer at 4°C in the dark for 30 min-1h.

   b. Wash 5 x 3 min

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

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

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

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

9. 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

10. 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 a solvent with lower surface tension which is then evaporated from the sample.

Replace the EtOH with hexamethyldisilazane (HMDS) solution, which has very low surface tension. Incubate in HMDS for 2-3 minutes and then remove completely and allow the samples to air dry with incubation dish lids loosely on to prevent dust from settling on the specimens.

11. 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.

12. 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:……………………………………………

13. 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:**
  - 2.5% glutaraldehyde in 0.1M PIPES – from CCI EM staff

    OR
  - Karnovsky Fixative – from CCI EM staff

    2.5% glutaraldehyde, 2% formaldehyde, 0.02% sodium azide in 0.05M cacodylate buffer – stored at -20°C, from CCI EM staff

- **PIPES buffer or cacodylate buffer** (if using Karnovsky fix) for washes and osmium: 0.2M frozen stock from CCI EM staff, dilute with mq water as needed

- **1% osmium in 0.1M PIPES/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 PIPES/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 powder 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.

- **HMDS** (Hexametyldisilazan)

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