

Laboratory for Advanced Electron and Light Optical Methods

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Staining Semithin and Ultrathin Sections

A. Purpose:

This section describes the procedures for cutting and staining semithin and ultrathin sections of resin-embedded samples.

B. Procedure:

1. Semithin Sections

- a. Trim block face to approximately 1 mm² and cut off one corner so that orientation can be determined for later re-trimming.
- b. Cut semithin sections (approximately 0.5 µm thick) with a glass knife. Place a drop of distilled water approximately 1 cm in diameter on a clean glass slide. Pick the sections up with a wire loop. Invert the loop to place 2-4 sections into the drop of water on the slide. It is critical that the sections are turned upside down from the way they were floating in the glass knife boat so that when viewed with a compound light microscope, they will have the same orientation as the block face viewed with a dissecting microscope (the former reverses the image side-to-side, while the latter does not).
- c. Label the slide with the block number (e.g., G97-42a).
- d. Put the slide with the water droplet containing sections on a hot plate adjusted to approximately 60-65° C and wait until all the water evaporates.
- e. Locate the sections on the slide and circle the bottom of the slide with a felt-tip marker to indicate their position.
- f. Place 1-2 drops of 1% toluidine blue O in 1% sodium borate¹ onto the sections and place the slide on the hot plate. Wait until the edges of the drop of stain begin to turn golden (approximately 20-30 sec) and then quickly rinse the sections with a stream of distilled water from a squeeze bottle to wash off the excess stain. If the stain dries down completely, the sections will still be useable. When washing, direct the stream slightly above the sections with the slide tilted at about a 45° angle above a catch basin for the excess stain. If the stream is pointed directly at the sections, they may come off the slide from the water pressure. If the sections seem to come off the slide too easily when washing, they are probably too thick or the hot plate temperature is too low. Replace the washed slide on the hot plate after gently drying the bottom of the slide with a piece of paper towel and wait for the residual water to evaporate from the slide surface.
- g. Remove the slide from the hot plate and add 1-2 drops of Polymount mounting medium to the top of the sections adhering to the slide. Gently lower a 22 mm X 22 mm coverslip (#1.5) onto the droplet of Polymount over the sections.
- h. Examine the stained semithin sections with a compound light microscope. Look for evidence of hard materials in the block face that need to be avoided when trimming for cutting ultrathin sections with a diamond knife.

- i. Draw a cartoon of the block face with the cut-off corner on a block sheet and sketch in the area of interest that should appear in the ultrathin sections. Remember that the block face for ultrathin sectioning should be approximately 0.5 mm^2 (about what you can see in the field of view with a 40 X objective on the compound light microscope). The block may be longer than 0.5 mm, but should not be wider than 0.5 mm, because it is hard on the diamond knife edge. Do not trim the block face any smaller than indicated because there will not be enough viewable area with our conventional 200-mesh grids and there will not be enough edge support for the areas that are viewable.
- j. If the block has a surface (e.g., skin) that is to be photographed with the electron microscope, make sure that there is sufficient empty resin beyond the surface to provide support over a grid bar or two so that the edge will not curl when the electron beam is put on it.

2. Ultrathin Sections

- a. Mount the block in a chuck and examine it with the dissecting microscope and compare the block face to the cartoon drawn previously from the semithin sections. Trim the block face as shown in the cartoon. The final block face should be approximately $0.5\text{-}0.75 \text{ mm}^2$. It is also permissible to trim the block face so that it is $0.5\text{-}0.75 \text{ mm} \times 1 \text{ mm}$, as long as the $0.5\text{-}0.75 \text{ mm}$ -wide side is the side presented to the diamond knife edge. If there is the potential for splitting of the sample surface from the resin block (e.g., skin surfaces, the surface of filters with adherent cells, etc.), orient the block so that the splittable surface is perpendicular to the direction of cutting.
- b. Cut ultra-thin sections approximately 80-90 nm-thick for standard work (clearly gold in color by diffuse light examination while floating in the knife boat). After stretching them by waving a xylene-soaked applicator stick over them, they should be very light gold to dark silver in color.
- c. Pick up 5-6 sections with a **freshly-cleaned* 200-mesh copper grid** for standard work (stainless steel or nickel grids for cytochemical procedures, formvar-coated slot grids or large-mesh grids for other purposes). Pick the sections up by orienting them into a block with an eye-brow lash tool and then submerging a clean grid and bringing the shiny side up underneath the floating sections. Try to get them into the center of the grid.
 - *Clean the grids, just before use, by dipping them sequentially into 0.1 N HCl (8-10 sec), 95% ethanol (three quick dips) and 100% acetone (3 quick dips). Insert a fresh piece of Whatman #1 filter paper between the forcep jaws to absorb acetone wicked up between the forcep jaws and, with the filter paper still in place, touch the grid down to a fresh piece of filter paper in a plastic petri dish. Finally, push the grid from between the forcep jaws with the filter paper onto a clean area on the filter paper in the petri dish. Never put a grid down into a wet spot on the filter paper because this will produce dirt on the grid.
- d. Blot the grid with sections from the bottom with a clean piece of Whatman #1 filter paper to remove water on the grid. Run a clean piece of the filter paper down between the forcep jaws to absorb the water drawn between the jaws by capillary action. Place the grid down on a clean piece of filter paper in a plastic petri dish, pushing it out of the forcep jaws with the filter paper piece used to remove the capillary water.
- e. Stain the grid with **methanolic uranyl acetate**² for approximately 5 min: Place a droplet of methanolic uranyl acetate on a piece of fresh Parafilm tacked down at the corners into a plastic petri dish. Always use a clean Pasteur pipet to remove the stain from the top of the stain bottle. If it is taken from the bottom, large crystals of uranyl acetate will contaminate the sections (the saturated solution in the bottle will have precipitated stain in the bottom). Touch the shiny (section) side of the grid to the droplet of stain and then insert the grid into the droplet shiny side up so that it winds up sitting on the parafilm, section-side up, beneath the droplet of stain.
- f. Rinse the grid by picking it up with forceps and gently inserting it into a 50 or 100 ml polypropylene Tri-pour beaker containing fresh deionized water. Wait about 2 sec, jog the grid up and down about 10 times in the water and then remove the grid and insert it into a

- second beaker of water. Jog the grid up and down in the water about 10 times and then place the grid into a third beaker of water and jog it up and down 10 times again. Remove the grid from the beaker, insert a piece of clean Whatman #1 filter paper between the forcep jaws to absorb the stain and water drawn up by capillary action and touch the grid dull-side down to a piece of filter paper in a plastic petri dish. Next, touch the shiny side of the grid down to a dry place on the filter paper in the dish. Finally, push the grid off with the filter paper already between the forcep jaws onto a clean, dry area of the filter paper in the petri dish. It is imperative that the grid be dry when placed onto the filter paper in the petri dish and that it not be put into a wet spot on the filter paper. To do otherwise will result in contamination of the sections.
- g. Stain for approximately 8 min in **Reynolds' Lead Citrate**³. Remove the lead citrate stain from the 4° C refrigerator just before use. Use a clean Pasteur pipet to obtain a droplet of stain solution from below the surface of the staining solution in the 50 ml volumetric flask. Place the droplet of the stain onto a piece of Parafilm tacked to the bottom of a plastic petri dish. Make sure that the lead citrate stain does not come into contact with the uranyl acetate stain, as they will immediately co-precipitate, causing masses of stain dirt everywhere. Keep the dish covered and well away from your breath, since CO₂ will cause formation of lead carbonate that will be seen on sections as a black, granular precipitate. Barely open the petri dish lid, touch the dry grid section-side (shiny side) down on the droplet of stain and then invert the grid and bury it in the stain droplet as described above, with the sections up. Immediately cover the petri dish to decrease exposure to CO₂. While the grid is staining, rinse all three rinsing beakers with deionized water and re-fill them for the next rinsing step.
 - h. After about 8 min, quickly remove the grid from the stain and insert it into a beaker of fresh deionized water. This is very important, since the stain will attract CO₂ from the air and produce lead carbonate dirt on the sections.
 - i. Jog the grid up and down in the water about 10 times, go to the next beaker of clean water and repeat, and then go to the final beaker of water and repeat. Dry the grid as described for uranyl acetate staining above (Step 2.F).
 - j. Put the stained grid into a grid box and fill out the block sheet so that it can be relocated and fill out the small file sheet associated with the grid box as well. After staining the grid, put a check mark after the grid number on the small file sheet associated with the grid box as a reminder that it has been post-stained.
 - k. The filter paper and Parafilm that has stain on it should be put in the waste box at the staining station, the rinse beakers rinsed out with deionized water and left upside down to dry at the deionized water sink, and all pipets should be put into the waste glass containers.
 - l. The uranyl acetate staining mixture is left at room temperature and the lead citrate stain is kept in the refrigerator at approximately 4° C.
 - m. **Use caution when working with these stains. They are both heavy metals, and thus are toxic to humans.**

Recipes:

¹ **Toluidine Blue O** for Staining Semithin Sections of Samples Embedded in Acrylic or Epoxide Resins

Purpose: This stain is used to stain semithin (approximately 0.5 µm-thick) sections for examination with a compound light microscope. All cellular features will be stained blue, but different areas stain with different intensity. The stain may be stored in a syringe and pushed through a 0.45 µm-pore size filter to reduce the potential for stain dirt on slides with stained sections.

Procedure for stain preparation:

Mix 0.25 g sodium borate with 25 ml distilled water. Add 0.25 g Toluidine Blue O powder to the sodium borate solution and let it sit overnight at room temperature. Store the stain at room temperature. It has a shelf life of months, or until excess precipitate is seen on stained sections.

² **Methanolic Uranyl Acetate** for Staining Samples Embedded in Spurr Resin

Purpose: Uranyl acetate attaches to nucleic acids, phospholipids and other cellular constituents with phosphate groups. This confers electron opacity to the areas that bind uranyl acetate and helps provide contrast to specimens viewed with the transmission electron microscope. Spurr resin does not stain well with aqueous uranyl acetate, so an alcoholic stain must be used. Uranyl acetate serves as a mordant for the subsequent lead citrate staining procedure, resulting in better staining with Reynolds' lead citrate. It is important to remember that uranyl acetate is a toxic heavy metal and both liquid and dried uranyl acetate constitute hazardous materials.

Procedure:

Add approximately 1 g of uranyl acetate powder to 10 ml of absolute methanol in a glass container. Sonicate the solution until little precipitate is visible. Wrap the container with aluminum foil or store it in a light-tight box. Let the solution sit overnight before use to let any undissolved crystals settle to the bottom (the solution is supersaturated and will normally have some precipitate in the bottom of the bottle). The solution may be used until it has evidence of excessive precipitate, though changing it every month is usually a prudent measure.

^{2a} **Aqueous Uranyl Acetate** for Staining Samples Embedded in Acrylic Resins or Epon-substitutes

Purpose: Aqueous uranyl acetate stains are used for acrylic resin sections (e.g., LR White or LR Gold). Aqueous uranyl acetate is also used in staining DNA spreads.

Procedure:

Add 1 g uranyl acetate powder to 10 ml distilled water (producing a 10% aqueous solution of uranyl acetate) and sonicate and store as described above for methanolic uranyl acetate. It appears that adequate staining is achieved on the intended materials with solutions at this concentration.

³ **Reynolds' Lead Citrate** for Staining Samples Embedded in Acrylic and Epoxide Resins

Purpose: Reynolds' lead citrate (E.S. Reynolds. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17:208-212) used as a section stain following uranyl acetate staining imparts further electron density to sections of cells, yielding more specimen contrast in the transmission electron microscope.

Procedure:

1. Put into a 50 ml volumetric flask the following, in the order listed:

30 ml distilled water

1.33 g $\text{Pb}(\text{NO}_3)_2$ (Analytical reagent grade, freshly opened, if possible)

1.76 g $\text{Na}_3(\text{C}_6\text{H}_3\text{O}_7) \cdot \text{H}_2\text{O}$

2. Stopper and shake the flask vigorously for about one min. Allow it to stand with occasional shaking for about 30 min.
3. Add 0.32 g of NaOH pellets, or 8 ml of 1 N NaOH. Bring the fluid volume in the volumetric flask to 50 ml with distilled water. Mix by inversion.
4. The precipitate will dissolve and the staining solution is ready for use.

The stain solution will have a pH of approximately 12.0. If tightly stoppered, the stain will have a shelf life of approximately 3 mo. Discard it when any precipitate is seen.

Store at approximately 4° C in a tightly stoppered volumetric flask.