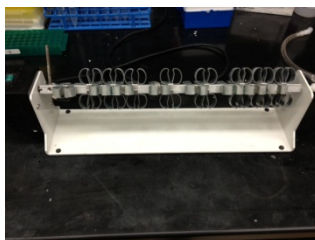


Cryostat Procedure

Cryoprotection:

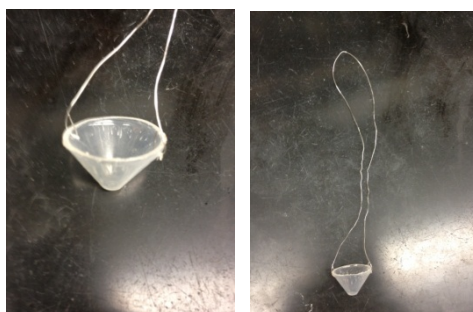
- Rinse sample(s) 3x5min in PBS if in paraformaldehyde.
- Under microscope, remove extra fat and connective tissue from mouse or rat sclera.
- Make the following sucrose solutions in smaller beaker with stir bar:
 - 10% Sucrose: 1g Sucrose + 10ml PBS
 - 20% Sucrose: 2g Sucrose + 10ml PBS
 - 30% Sucrose: 3g Sucrose + 10ml PBSStir solutions until sucrose completely dissolved.
- Distribute sucrose solutions into smaller vials matching the number of tissue samples. Make sure to label the vials with sucrose percentage and the identity of tissue.
- On rotator, leave samples in 10% sucrose for one hour. Below is an example of a rotator.



- After one hour, remove sample from 10% sucrose and proceed to place samples into 20% sucrose for one hour on rotator.
- Finally, place sample(s) into 30% sucrose overnight on rotator.

Freezing:

- Make sample mold by cutting off the bottom of a Corning 50ml conical tube. Using a Bunsen burner heat a needle over flame. Use the hot needle to pierce two holes on either side of plastic cone. Attach a wire into holes to create a handle.



- After sample has incubated in 30% sucrose overnight, acquire liquid nitrogen. Fill container approximately half-way.

- O.C.T. (Optimal Cutting Temperature) Compound will be used as the embedding medium. Pour a small amount into conical mold. Add the sample. Orient in direction needed and fill remainder of mold with O.C.T.
- Keeping mold even, lower into tube with liquid nitrogen. Do not push the sample into the liquid nitrogen. Hold the mold right above the surface.
- Hold until all the O.C.T. is frozen. Remove from liquid nitrogen.
- Remove frozen sample from mold. Wedge tweezers between plastic mold and sample to remove sample from mold.
- Place frozen sample into tin foil and leave in freezer until ready to use. Make sure to label the sample with tissue identity.

Sectioning:

- Email: ucsbmicroscopy@lifesci.ucsb.edu to set up a training time with Mary Raven for cryostat use. Machine is located on the 5th floor of Bio II in the Microscopy Facility.
- After the training, you will be allowed to sign up online to use the machine.
- Place paintbrushes in cryostat, attach blade, and screw glass cover (pictured below) into place about 20 mins. prior to sectioning, this allows all material to equilibrate to the chamber temperature of -20 degrees.



- Set dial to cut sections 20 μ m thick unless otherwise stated.
- Attach sample to circular cryostat block (pictured below) by covering block with Neg50 and placing sample on top. Place the largest side of the conical sample on the flat surface of the block so the pointed tip is facing outward. Equilibrate for approximately 10 mins.



- Attach block into center of metal circle block below. Using tall, black handle on left, screw block into place. The center, black handle should also be tightened before cutting sections. Loosening this handle allows you to change the angle of the block face.



- Begin moving block forward using the dials on left side or the handle on the right side of cryostat. Be very careful once sample gets close to blade.
- Once sections have begun to be cut, make sure block is cutting evenly. You can make changes by adjusting the angle of the block face or the angle of the metal platform. You can also change the temperature.
- Put glass cover down, lightly pressing on either side and cut sections.
- If sections are not going underneath glass cover, the solution is usually to bring the glass cover out further from blade, closer to block.
- Label slides with series number and slide number. The blue rectangles below each represent a slide. The five slides would make up one series. The numbers on the slides represent the number of sections. The first section cut would go onto series 1, slide 1. The next section would go onto series 1, slide 2, and so on. This pattern would continue on to series 2. A mouse eye averages about 5 series, with 5 slides each, or 125 sections.

Slide: #1	#2	#3	#4	#5
1	2	3	4	5
6	7	8	9	10
11	12	13	14	15
16	17	18	19	20
21	22	23	24	25

- To pick up a section from the metal platform, first make sure it's flat. Use paintbrushes to flatten out edges if necessary. Then breathe on the back side of a slide and place it face down on top of the section. The section should then transfer from the metal platform to the slide.
- After series is finished, keep slides cold in cryostat while you finish sectioning in slide box.
- After whole sample is sectioned, store slides in freezer.

Staining:

Hematoxylin & Eosin (H&E):

- Place slides flat in a slide box with moist paper towel for one hour. This will air dry them without dehydration.
- Put Copeland jar in sink, with hose inside lightly turned on. Rinse slides in running water.
- With glass pipette, add Mayer's Hematoxylin stain to slides, covering all sections. Leave on for 2min.

- Rinse in jar in the sink with running water for 2min.
- Dip in Copeland jar of 95% ethanol 10 times.
- With glass pipette, add Eosin stain for 45sec.
- Dip in Copeland jar of 95% ethanol 10 times. It can be the same 95% ethanol jar as before; there is no need to add fresh ethanol. You will need fresh ethanol for the next round of slides though, because the excess Eosin stain is left in the jar after this step.
- Add slides to Copeland jar of 100% ethanol for 10min.
- Switch slides into Copeland jar of xylenes for 10min.
- Using Permount, mount slides with 1.5mm cover slips. Slides may be far apart so the larger rectangular coverslips are ideal.
- Store in slide box at room temperature. There is no need to seal cover slip with nail polish.

Immunocytochemistry:

- Place slides flat in a slide box with moist paper towel for one hour.
- Using Liquid Blocker Pen, draw a rectangle around the all sections on each slide. Throughout the procedure, place liquid inside rectangle. The amount of liquid may vary due to the size of the rectangle drawn.
- If using 1⁰ antibody Ki67 alone or with another 1⁰, start with the steps below:
 - After air drying, hydrate sections with about 350µl PBTA for 10min.
 - Wick away PBTA by tilting slide and absorbing liquid with Kim Wipe. Dry all sides of rectangle.
 - Add 350µl (or same amount of PBTA added) of 2N HCL for 30min to slides.
 - Dip slides into Copeland jar of PBTA 5 times to rinse off 2N HCL. Then dry all sides of liquid blocker rectangle and add 300µl of PBTA to slides for 10min.
- If using an antibody that doesn't require an acid treatment, like STEM 121 or NESTIN only, start here. If using the acid treatment in the step above, continue on to the following step.
- Add 300µl NDS (1:20) to each slide. Let sit for 15min.
- Wick away NDS with Kim Wipe by tilting slide and absorbing liquid. There is no need to rinse NDS. Dry all sides of liquid blocker rectangle with Kim Wipe.
- Add 1⁰ antibodies for 1 hour.
- Rinse antibodies by dipping in Copeland jar of PBTA 5 times. This should be fresh PBTA, do not reuse PBTA from previous rinse. Then dry all sides of rectangle and add 300µl of PBTA for 15min.
- Wick away PBTA using same method as before, making sure to dry sides of rectangle. If necessary, redraw rectangle with Liquid Blocker Pen.
- Add 2⁰ antibodies to slides for 1 hour.
- Rinse antibodies by dipping slides in Copeland jar of fresh PBTA 5 times. Dry all slides of rectangle and add 300µl of PBTA to slides for 15 mins.
- Wick away PBTA using same method as before.
- Add Hoescht stain (1:5000) for 10 mins.
- Mounting slides:
 - Use n-propyl-gallate /glycerol and #0mm cover slips to mount slides.
 - Tilt slide and remove bulk of liquid, there is no need to rinse slides before mounting. It is easier if there is still liquid left on slide. Remove liquid from each slide as you mount. It will be more difficult to mount the slides if you dry all the slides at once.

- Add drop of n-propyl gallate/ glycerol and place #0mm cover slip on top. It may be necessary to use more than one cover slip per slide to make sure all sections are covered.
- Use vacuum to suction any residual n-propyl-gallate / glycerol on sides of cover slip.
- Use nail polish to seal cover slip.
- Store in 4⁰ refrigerator in cardboard slide box.