**Sectioning frozen adult heads**
**Adapted from L. Vosshall (2002) by M. Sniffen / R. French, 2006**

***Drosophilan eye pigments autofluoresce (rhabdomere containing region of photoreceptor neurons). If proceeding to immunofluorescence staining, you can use w- flies if you want to avoid this problem.*

## EMBEDDING

**Tools:**
- Heisenberg fly collar (Genesee Scientific #48-100)
- Embedding molds (Richard Allan #58952, or similar)
- Forceps
- Optimal Cutting Temperature freezing medium (O.C.T.)
- 95% ethanol
- Dry ice bath with some ethanol for quicker freezing.
- Clean, sharp razor blade
- Plastic wrap
- 15 or 50 ml polypropylene screw-cap tube

### Embedding

1. Collect adult flies and anesthetize them with $CO_2$.
2. Kill 5-12 adult flies by immersing them in 95% ethanol for 10-20 seconds. Remove flies from the ethanol and place them on a Kimwipe to air dry. (OMIT STEP 2 if sectioning to collect antennae.)
3. Fill an embedding mold with O.C.T. or other freezing medium. Immerse flies in liquid O.C.T. and allow them to equilibrate for 10 minutes at room temperature.
4. Align heads of the flies in a fly collar. The heads should be on top of the blades and the bodies below. Gently nudge each fly into the collar using your forceps. If a decapitation occurs, remove the body. (Optional: You can save the loose head by placing it in the correct location on the collar, but it may not be vertically aligned in the frozen O.C.T. exactly with the others.) Orient all heads in the same direction, and be sure there is at least one head's height distance between each head.
5. When the collar is fully loaded (12 heads max), cover the heads with a thin stripe of O.C.T.
6. Check the liquid O.C.T. in your embedding mold. Pop or push any air bubbles to the edges.
6. Quickly invert the collar onto the center of the embedding mold, so that the fly heads are immersed in the O.C.T, clear of any air bubbles.
7. Immediately place the mold (with collar on top) directly onto dry ice / ethanol. (Note: Be sure the freezing medium never comes into contact with ethanol. Once it does, it will never freeze solidly enough to cut well.)
8. When samples are frozen, the O.C.T. will be completely opaque and white. (Wear latex gloves when handling the frozen sample to prevent melting.)
9. Dry the ethanol off of the embedding mold and pop the frozen sample out of the mold, being careful to avoid any contact of ethanol with the sample.
10. Carefully use a razor blade to pry the sample block off of the fly collar.
11. Working on a piece of plastic wrap (to protect your table top), carefully trim the block. It is not necessary for the total width at the top to be more than 3x the width of a head. Make the two long edges parallel.
    Option 1: A rectangular block with the heads aligned along the top edge. [Fig. 1a]
    Option 2: A flat-topped pyramid, with the heads aligned along the top edge. [Fig. 1b]

12. Wrap the block in a piece of plastic wrap and place that in a screw-top 50 ml centrifuge tube to prevent dehydration or freezer burn. Blocks can be stored at –80ºC for up to 1 week for in situ hybridization or 1 month of antibody staining.

** Option: Cut a block of frozen O.C.T. without heads to the same shape and size as your other block(s). This "test block" can be used at the start of your sectioning session to optimize the cryostat settings before you begin working with your precious sample blocks.

## SECTIONING

**Tools:**
Cryostat
Low-profile blades
Microscope slides (Superfrost/Plus, Tespa-treated, or VectaBond treated)
Fine paint brush (Ted Pella #11806)
PAP pen
4% paraformaldehyde in PBS (Ted Pella #18505)
Humidified chamber
Coplin jar(s)

### Sectioning

** Training is required for all cryostat users. You must receive training from the local cryostat administrator, in order to become familiar with the guidelines of use.

1. Remove your sample blocks from the freezer and allow them to equilibrate in the cryostat chamber for 20-30 minutes. Set Block Temperature = 14-16 C, and Chamber Temperature = 20 C.
2. Place a blade into the blade holder. Allow it to cool before you begin cutting.
3. Adjust the blade angle (on some cryostats, the blade is immobile, and you can only adjust the sample angle) to 0-5º for the least compression in your sections. This angle is variable, so find what works best for you.
4. Place a clean, room temperature cryostat chuck inside the chamber. Quickly place a drop of O.C.T. on the center of the chuck then center your sample block on top of the drop and press down. Allow the sample to freeze firmly to the chuck.

** Optional: Begin with a "test block" to optimize the cryostat settings. Once you get your test block ribboning well, proceed with cutting your sample tissues.

5. Attach the chuck with your sample to the sample holder. Rotate and adjust the angle of the chuck to align the long axis of your sample block parallel with the edge of the blade.
6. If needed, retrim your sample block using a clean razor blade. Be sure the edges of the long axis are as parallel as possible, to ensure better ribboning of your sections.
7. Set your section thickness. 10-14 $\mu$m is standard for Drosophilan brain tissue.
8. Cut a series of subsequent sections and allow them to remain attached to one another (top-to-bottom) forming a long "ribbon" of sections. (Fig. 2) A few basic troubleshooting tips:
   - Speed: The slower the cutting speed, the better the morphology of the sections.
   - Compression: If your sections are losing their width by being compressed after passing the blade, try adjusting the blade to a lower angle.
   - Curling: If your sections don't lie flat for ribboning, but instead curl up and separate after each section is cut, your sample block is too cold. Increase the Block Temperature slightly and/or allow the sample block to equilibrate to a higher temperature.

- The glass plate: Be sure the edge of the glass plate on your cryostat is smooth and unblemished. Any nicks in this edge can catch your sections and make ribboning extremely difficult. Try to slide the blade and glass plate assembly across to a point where only an unblemished part of the glass plate can touch your sample. Handle the glass plate gently, as these are fragile and somewhat expensive to replace… but can make sectioning much easier when working well!

10. Pick up each ribbon on a room temperature slide. Carefully label your slides with sample information. Be sure to number sequential slides from each sample block.
11. Immediately after each full slide of sections has been collected, draw around the sections with a PAP hydrophobic pen and apply a puddle of fix (4% high-grade paraformaldehyde/PBS). Fix for 7 minutes in a humidified chamber at room temperature.
12. Wash slides in PBS 3 x 5-10 minutes at room temperature.
13. Proceed immediately to your staining protocol. (If necessary, you can store slides overnight in PBS at 4ºC before proceeding with staining, but avoid if possible, as this may reduce tissue adhesion.)

*Do not allow tissues to dry out once washes have begun. Drying out can destroy the epitope.
*Optional: For longer post-fixation storage, put slides through a methanol dehydration series (25%, 50%, 75%, 100%) then store in the freezer. Note, however, that methanol may destroy some epitopes.

14. CLEAN UP the cryostat and surrounding workspace.

Figure 1a

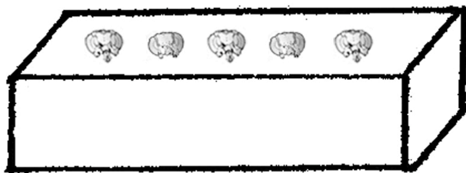

Figure 1b

Figure 2