Modified Jan 2012

Fixation and Pouring Boats

(Jeff Long Protocol)

Kathy Barton's lab has tried FAA, paraformaldehyde, and glutaraldehyde. They get the best signals in FAA and I like it because it's a quick fix.

(Day 1) Tissue fixation and ethanol dehydration

A. Fixation

1. Fixative Mix:

	Percent in mixture	For 100 ml
Ethanol (100% bulk)	50%	50 ml
Acetic Acid	5%	5 ml
Formaldehyde	3.7%	10 ml of 37%
Water	41.3%	35 ml

2. Place 10-15 ml of fixative in 20 ml glass scintillation vials. Note time: **At the end the tissue should be exposed to fixative for 3-4.5 hours. Try to be consistent each time you fix your tissue.**

3. Cut a cluster of flowers at the apex of the floral stem that includes stages 1-14, and immediately immerse in fixative. Cut so that 1-2 mm of floral stem is present—this helps to orient the tissue when sectioning. Cut off older flowers if you are not interested in these stages. Place about 30 pieces of tissue in each vial.

4. The tissue will float in the fixative. Place tissue/fixative in a vacuum for 15 minutes. Pull vacuum very slowly to prevent tissue damage. This step pulls the air out of the tissue, allowing the fixative to penetrate better. Usually, air bubbles will come out of the solution and get trapped in the trichomes, causing the tissue to float. Release vacuum, again very slowly.

5. Repeat step 4 until all tissue is sunk. Usually this happens after the second round. Incubate at RT for 2 hours. During this time make up the ethanol solutions for the dehydration series.

6. Pull vacuum again for 15 minutes. As always, go slowly.

7. Rinse out fixative with some 50% EtOH.

B. Dehydration (all steps at RT)

At this stage itself, it is a good idea to put the paraplast plus chips into 2 X 1 L glass bottles in the 60C wax oven. It takes a long time to melt (overnight). The tissues must be completely dehydrated before taking into xylene.

____ 50% EtOH 2 X 30 minutes
____ 60% EtOH 1 X 30 minutes
____ 70% EtOH 1 X 30 minutes (the tissue can be stored for 3-4 months at this stage according to Schneitz, Barton and Meyerowitz labs). PAUL says NO
____ 85% EtOH 1 X 30 minutes
____ 95% EtOH + 0.1% Eosin O/N or longer (Eosin stains tissues red). Eosin is 50 ml water and 0.05 g Eosin Y). But if you are in a hurry just go for 2 hours and continue on.

(Day 2) Clearing

____ 100% EtOH. 1 X 60 minutes (Tissue destains slowly so don't leave O/N)
____ 100% EtOH 2 X 30 minutes

C. Clearing

The tissue must be permeated with sylenes because paraffin is not miscible in ethanol. The tissue does not destain (Eosin) in xylenes.

____ 25% xylene:75% ethanol 1 X 30 minutes
____ 50% xylene:50% ethanol 1X 30 minutes
____ 75% xylene:25% ethanol 1X 30 minutes
____ 100% xylene 2 X 30 minutes (fill vial 1/2 full last time)

D. Infiltration

It is important that the infiltration is done slowly in order to preserve the morphology of the tissue.

____ Add 20 paraplast plus chips to vial and incubate ON at RT.

(Day 3) Infiltration cont'd

____ Place vial at 42C and wait until all chips are melted (1 hr)
____ Place vial at 60C for several hours (at least 4 hours but o/n is OK)
____ Change xylene/wax for 100% wax.

After this you need at least 6 wax changes spaced at least 6 hours (takes 2 or 3 days). The paraplast will solidify very rapidly. So, get everything ready when you need to change wax, keep everything close, and work quickly. The wax in the vials should not solidify in order to not alter the morphology of the samples.

(Day 4) Infilration cont'd

____ Morning Wax change 1
____ Evening Wax change 2

(Day 5) Infiltration cont'd

____ Morning Wax change 3
____ Evening Wax change 4

(Day 6) Infiltration cont'd

____ Morning Wax change 5
____ Evening Wax change 6

(Day 7) Making the wax molds.

These wax molds can be kept in the fridge for two years or so. Avoid temperature changes though. I've had some samples go off if they are super old.

Need: -set up items right beside wax oven on cart or table. You need to work quickly for this procedure.
-hotplate with crystallization dish filled with water at 60C. Make a tin-foil cover
-thermometer
-dissecting needle for positioning tissue (keep it on the hotplate so that the tip is warm)
-petrie plates
-stack of boxes at same level as waterbath, right beside

1. Confirm that temperature of water is between 60 and 65C. Above 68C the structure of the wax changes. Below 60, the wax forms a skin on the top and its very difficult to position the tissue properly.

2. Float petrie plate on water bath. Working very quickly, swirl tissue and pour contents into the petrie dish. Immediately, top up with molten wax as if you are pouring an AT plate (a nice fat plate). If you are slow to pour and some tissues gets trapped in the solidifying wax in the vial, top up with wax and put back in oven and wait until it remelts. Should a bad skin form, cover dish with tin-foil and wait until it heats up a little more and the skin melts.

3. Using the dissecting needle, position the tissues so they are as well separated as possible.

4. Carefully remove the petrie plate to the cooling stack. When it is solid, put it somewhere safe. Label the top and the bottom!!!! Store at 4C for 1-2 years.