Paraffin embedding and sectioning of mouse embryos

Materials:

Ethanol: 95%, 100%
70% Ethanol/saline (70%E/S)
PBS
Toluene
Surgipath infiltrating paraffin Cat # 01400 (4-5 lb/bag)
Surgipath embedding paraffin (EM400) Cat # 01300 (4-5 lb/bag)
Metal or plastic wax mold
Small or big Eppendorf tips blunted on flame for orientation of embryos
Marker for wax blocks
Plastic cups for melting paraffin
Hot plate for embedding
Microtome and blades
Slide warmer

I. Paraffin Embedding

Appropriately fixed embryos/tissues should be stored in 70%E/S at 4°C in scintillation vials, screw cap tubes, or 50ml conical tubes (depending on the size and number of embryos)

- On the day of embedding, first pour a sufficient amount of infiltrating paraffin and embedding paraffin into plastic cups. Melt paraffin in the vacuum oven for later use.

The duration of all the steps listed below depend on the size of the embryos. See table for details

1. Decant the 70% E/S from the storage vial. Be careful not to disturb the embryos.

2. Add 95% ethanol to dehydrate the embryos and rock on a nutator (see table).

3. Decant the 95% ethanol and add100% ethanol – rock on nutator (see table).

4. Clear with toluene – rock on nutator (see table)

5. Immerse the embryos in infiltrating paraffin for the desired time (see table)

6. Immerse the embryos in the embedding paraffin for the desired time (see table).

Reagents	Embryos(small)	Small tissue	Medium tissue	Large tissue
70-80% EtOH 95% EtOH 95% EtOH 100% EtOH 100% EtOH	1/2 - 1 hr 1/2 - 1 hr 1/2 hr 1/2 hr 10 min	1 hr – o/n 1 hr 1/2 hr 1 hr 1/2 hr	2 hr – o/n 1 hr 1 hr 1/2 hr 1/2 hr	4 hr – o/n 2 hr 1 hr 1 hr 1 hr
Toluene Toluene	1/2 hour	1 hr 1/2 hr	1 hr 1/2 hr	1 hr 1 hr till clear
Infiltrat.Paraffin Infiltrat.Paraffin		1 hr 1 hr	1 hr 2 hr	1 hr 2 hr +
EM400	I hr +	1 hr	1 hr	2 hr +

***Notes:*
1) The optimal parameters for tissue processing can be empirically adjusted. Please note that to obtain high quality sections, insufficient dehydration or over dehydration should be avoided. The same is true for clearing the tissues using toluene.

2) The solution volume:embryo ratio should be 20:1 or greater.

3) Extreme care should be taken when changing solutions to avoid damaging the embryos. Do not directly touch the embryos. Use a plastic pipet to aspirate as much solution as you can without allowing the embryos to dry out.

4) Clean, RNase-free utensils (i.e., wax cups), should be used throughout the whole procedure.

II. Tissue Sectioning

Set-up:

1. To begin with, turn on the tissue-embedding hot plate for pre-warming.

2. Metal mold(s) should be carefully cleaned with ethanol using Kimwipes.

3. After drying, fill the mold with embedding paraffin liquid and place it into the vacuum oven for pre-warming and dissipating of air bubbles. This should take about 10 min.

4. Transfer the embryos (from the embedding paraffin) to the pre-warmed wax mold. Leave it in the oven for an additional 5 min. **This is a critical step!**

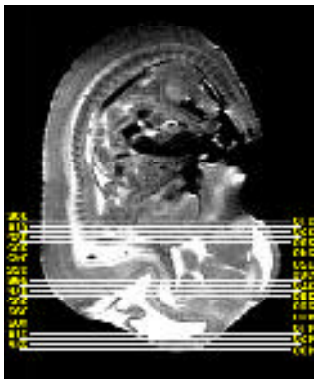
**Note: The correct alignment of the embryo(s) within the wax mold is necessary to achieve sections with the appropriate plane of symmetry (i.e., cross-sections, sagittal*

sections, etc.). Generally, cross (transverse) or sagittal sections are used for both in situ hybridization and immunohistochemical staining

A. Embryo Orientation:

For cross sections

Embryo Orientation: Embryos should be oriented to stand on their head in the mold with their longitudinal axis in an upright position and their body axis perpendicular to the bottom surface.



To do this:
1. Place the mold on the hot plate and gradually move it to cooler regions of the plate. After a few minutes remove the mold from the hot plate and place it on the cold plate.

Note: You will notice a thin, white, bottom layer of wax forms when the mold is placed on cool regions of the hot plate and a thicker layer of wax will quickly form when the mold is placed on the cold plate.

2. Orient the embryos (as described above) with two flame-polished plastic pipet tips that have been warmed on the hot plate. (use the tips designed for loading samples into SDS-PAGE gels).

***Note: Flame-polished tips will prevent the introduction of air bubbles into the wax. The embryos will stand firm with the support of the bottom wax layer. You must be really quick in positioning the embryos before the wax solidifies.*

- If you are embedding several embryos per mold, say 3 to 4, which is the routine in our lab, then you should pay particular attention to the nature of embryos. Their **genetic background** and **age** should be carefully noted. We recommend that you carefully place the embryos in positions that will mirror the actual positions you want the embryos, relative to each other, on the sections. Draw the embryo distribution on your notebook beforehand, taking note of their genetic background and age, so you will not forget which embryo is which later.

3. Once all of the embryos in the mold are standing on their heads in similar orientation (following the directions above) and at some distance from each other (at least 5mm apart), you can gently add a bit more paraffin

4. Apply a new, clean, marked plastic wax chuck on top of the mold

5. Add more paraffin to fill the chuck.

6. Once solidified, the wax mold can be stored at room temperature for at least an hour and then placed at 4°C to harden overnight.

7. The following day, detach the mold from the wax block by using your hand to gently separate the adhered wax connecting the plastic chuck to the mold.

8. The wax block should be marked and stored in a plastic bag at 4°C.

For sagittal sections

Embryo Orientation: Embryos should lie down on their sides at the bottom of the mold. The embryos should be gently tipped to lie in a balanced position with the sagittal plane parallel to that of the mold base.

1. Follow steps #1-8 under cross-sections but orient the embryos as described for sagittal sections.

B. Sectioning:

Preparation

1. Use 100% EtOH to clean the glass container of the water bath used for spreading the sections.

2. Fill the container, to 80% of the container height, with sterile ddH_2O and warm the water to 39 - 42°C. The suitable temperature differs among tissue blocks, but 40 - 42°C is a common starting point.

3. Turn on the microtome (Leica, Model RM2155). The power switch is located at the back of the machine. Release the lock handle on the right side.

4. We usually set the section thickness to 5μm on the control panel. Use the manual control mode by pressing Button 1.

5. Mount the wax block onto the chuck holder and loosen the angle control knobs (the one on top is for vertical control and the one on the right is for horizontal control) to adjust the relative position of the chuck holder with respect to the cutting blade.

6. Mount a **used** Leica 819 brand blade onto the blade base and fasten it using the L-shaped knife clamper. *The used blade is good for trimming the wax block.*

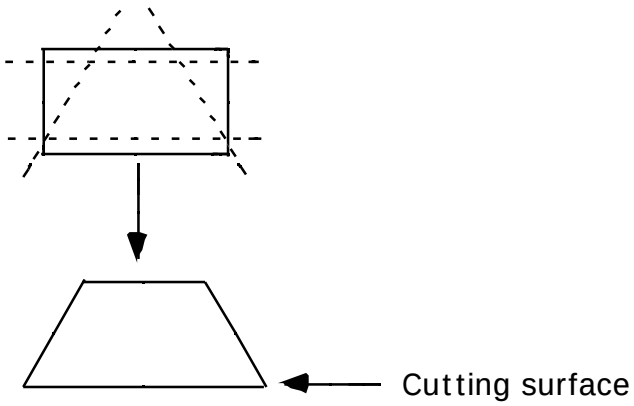
Block adjustments and tissue trimming:

1. Adjusting the relative position between the wax block and the blade:
Stand upright and look down over the wax block and the blade and make sure that the blade edge and block surface are parallel.

- If they are too far apart:
  1. Adjust the blade base using the control handles on both sides of the base (one is for lateral movement and the other is for back-and-forth gliding)
  ---OR---
  2. Position the chuck holder by pressing the knob at the left of the machine body to either advance it or to move it backward.

2. Checking the block/blade alignment:
Slowly cut the sections to check if the wax block on the chuck needs readjustment. Ideally, the cutting surface of the wax should be evenly cut.

3. Trimming the wax block:
Trim the wax block with a used blade to cut away extra wax above or below the embedded tissue. Mark the block by cutting a corner or alternate region. Trim the block to produce a trapezoid shape (see drawing). Cut sections until you have reached the desired tissue plane.



Setioning and collection of tissue sections:

1. Before cutting sections for collection, replace the **used** blade with a **new** one. You should start the cutting process from one end of the blade, thus maximizing the use of the whole blade edge (the blade is expensive, so use with care).
Once you are getting streaks on sections, it could mean that the blade is dirty and needs to be cleaned. Clean the cutting edge of the blades with xylene-soaked Q-tips. If steaks continue to appear in subsequent sections, it is time to change to a new blade.

2. Soak a sheet of Kimwipes in 1% Glycerol/ddH_2O. This is used to repeatedly wet the wax surface to facilitate sectioning. This step is necessary for a majority of wax blocks. However, some blocks will cut fine without wetting. In most cases, surface wetting makes a tremendous difference in the way the sections cut.

3. Cut sections with a steady and smooth movement of your right hand.

4. Cut continuous wax ribbons and carefully transfer the ribbon, with clean, sharp forceps, to the surface of the water bath.

5. Meticulously separate the sections with clean, blunt forceps.

6. Let the sections float on the water to allow full extension of the tissues. Thus, minimizing wrinkles in the sections.

7. Use a SuperFrost/Plus slide (Fisher, Cat # 12-550-15) to pick up (or scoop up) 1-3 sections (depends on the size of the sections) onto the surface of the slide.

8. Place the slide upright against a clean support on clean Kimwipes for a few minutes.

9. Transfer the slides onto a baked slide rack (stainless steel), wrapped in aluminum foil, on the slide warmer. The slides will be kept on the slide warmer overnight at 40°C.

10. The following day, label the slides and place them into slide boxes (each box can hold 100 slides/box). In addition, select one slide out of every ten to Hematoxylin-Eosin (H&E or HE) stain to examine the section quality. Additionally, these slides can show you your area of interest for further studies. For instance, if you want slides with the lung for *in situ* hybridization studies, H/E stained slides can guide you to the appropriate slides you should use.

****Notes:**
1) If the sections are intended for in situ hybridization, this entire protocol should be carried out very carefully to assure that all of the above steps are RNase-free.

2) Sections should be picked up in a similar manner each time, such that, all of the slides have sections with a consistent orientation. The sections should be picked up in

sequential order and stored in the slide boxes as such. They should be labeled with sequential numbers for future reference.

3) The ideal section should be perfectly intact, with no wrinkles, no artificial spaces, no streaks, and no missing parts of the embryos.

4) Be extremely cautious of the blades!!! Handle with care! Keep your fingers away from the edge. ***DO NOT CUT YOURSELF!!***

5) Rough estimates of time expenditure:

Tissue fixation and postfixation rinsing:	*2 days+*
Embedding:	*1 day + 1/2 night*
Sectioning of 1 wax block:	*0.5day ~2 day*
Hematoxylin-Eosin staining	*2.5 hours- 4 hours*

Hematoxylin-Eosin (HE) staining procedures

Presently, this is done in S-1343, Histology Lab.
Sequentially place the slide rack into the following glass boats:

Boat Label	Time	
1) xylene (1)	5 min	Deparaffinizing
2) xylene (2)	5 min	
3) 100% EtOH	5min	
4) 100% EtOH	5min	
5) 95% EtOH	4min	
6) 95% EtOH	4min	
7) 80% EtOH	4min	
8) Tap water	4 min	
9) Gill's Hematoxylin	2-3min	
10) Tap water	4min	
11) Acidic alcohol solution	5-10 seconds	Reducing background
12) Tap water	4min	
13) Scott's water	5min	Intensifying blue staining
14) Tap water	4min	
15) 80% EtOH	4min	
16) Eosin solution	10-20 seconds	
17) 95% EtOH	briefly	
18) 95% EtOH	briefly	

18) 95% EtOH	briefly
20) 100% EtOH	briefly
21) 100% EtOH	2-3 min
22) Coverslip Xylene (1)	5 min
23) Coverslip Xylene (2)	5 min

24) Seal the slides with Fisher's Permount or Shandon Mounting Medium

25) Air dry in the hood overnight

Note:

Acidic alcohol solution:	Sigma A 3179
Scott's water	Surgipath 02900
Eosin Y	Sigma HT110-1-16 (500mL) Surgipath 01600 (1gallon) Surgipath 01601 (1/2 gallon)