SOP Cryosections of E14.5 mouse embryos

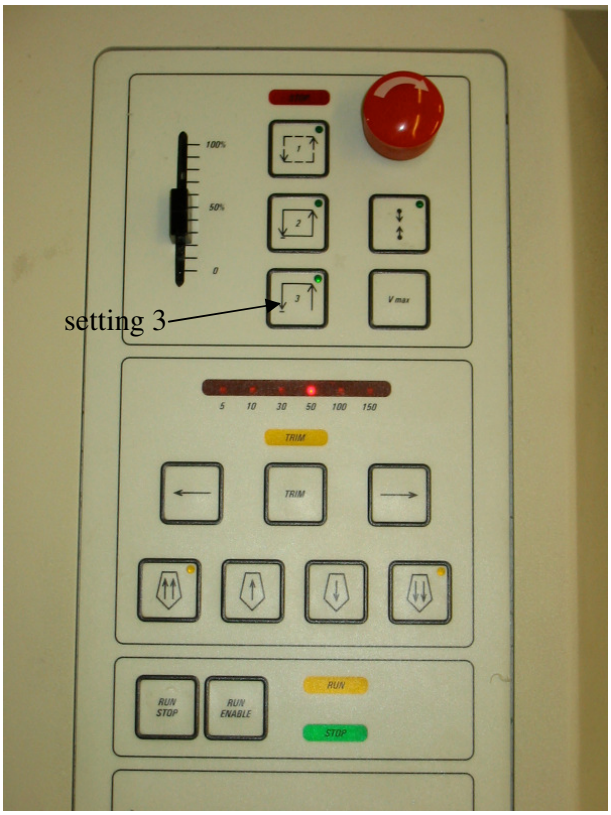
Materials

O.C.T. Sakura F4142
Disposable blades Accu-Edge 4689, low profile
One-sided razor blades
Fine and course brushes
Acetone
Forceps
Magnetic stirrers
Stir bars (25 x 5 mm)
Gilson Distriman repeat pipettor and disposable 12.5ml pipettes
Slide boxes for 25 slides (VWR 631-9140)
Silica gel desiccant from Sigma (S8394)
Electrical tape
Solutions and reagents for fixation, dehydration and acetylation (see at end)

Procedure

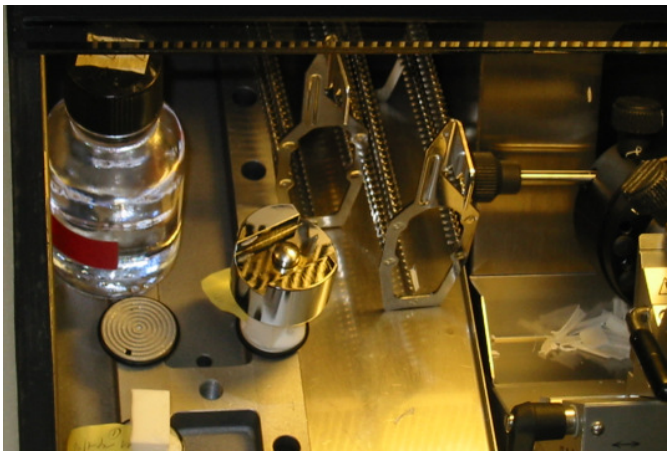
Before beginning make sure to

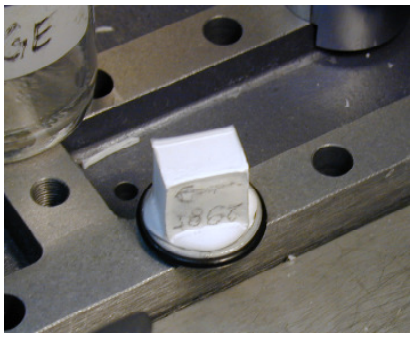
- adjust the temperature on the cryostat we use a CT (chamber temperature) of -12°C to -16°C and OT (object temperature) of –10°C to –14°C. The best temperature setting depends on local conditions, so you will have to experiment. Place in the cryostat a thermometer laying on the tray in front of knife holder.
- Also choose the desired stroke type on the control panel. For our purpose the up down stop mode (setting 3) works best.



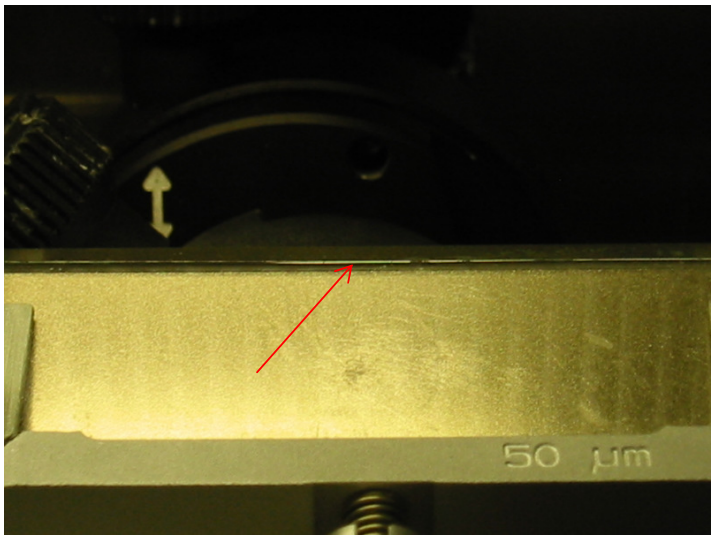
- equilibrate the temperature of the OCT tissue blocks by transfer from -80ºC storage to -20ºC one (minimum) to 3 days before sectioning.

(1) The day before you plan to section remove the tissue block from -20ºC and mount it onto the chuck: pour a small amount of OCT onto the chuck, place the tissue block on it, put it into the cryostat chamber and place the heat extractor on top of the block. The mounted tissue block can be left in the cryostat overnight. Be sure that it is mounted with correct specimen orientation, check the specimen data sheet for the orientation of the specimen. We cut through the embryo from left to right and from back to belly.

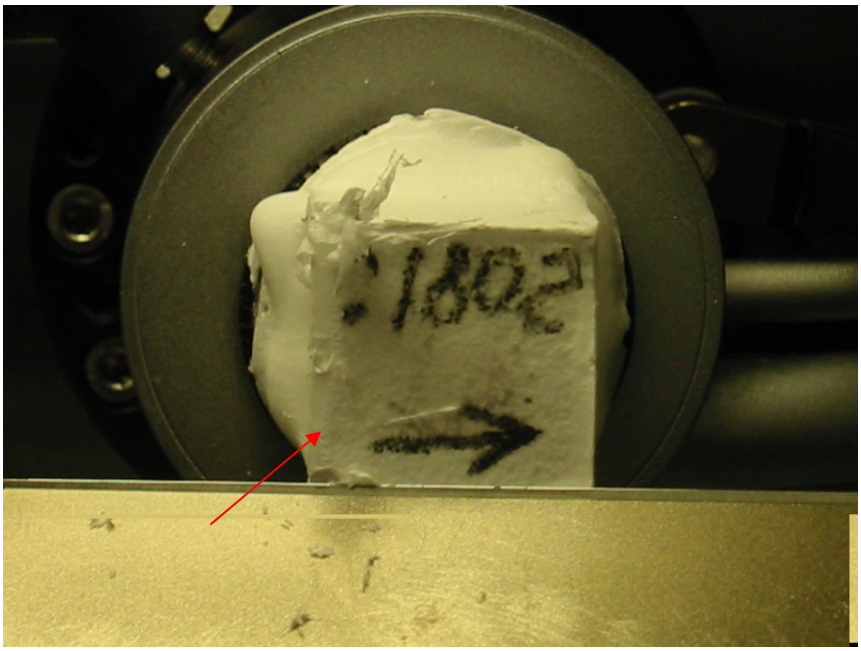




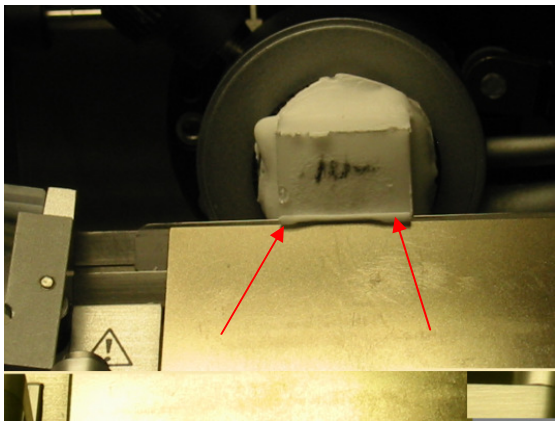
Sectioning process:
(1) Check that the anti-roll plate and the blade are correctly adjusted. Use the set screw to position the anti-roll plate so that you see a line of light reflection across the edge between the anti-roll plate and the blade. This shiny line needs not to extend along the whole length of the blade but it must be in the area that will cut through the specimen. Sectioning angels between 0 and 3 degrees are being used.



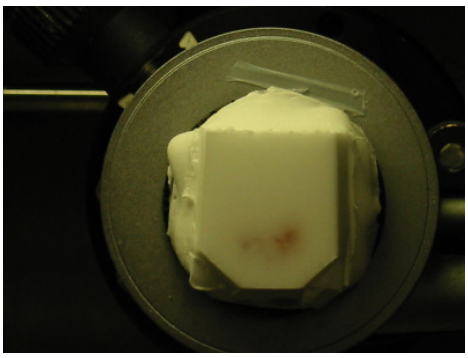
(2) Make sure that the anti-roll plate is clean and has no dents otherwise it will scratch the section. If necessary clean with acetone and a Kim-wipe.
(3) Use a fresh blade for each specimen but for trimming, the old blade should be used.
(4) Move the specimen holder all the way back to the starting position.
(5) Move the knife holder close to the specimen then bring the specimen block towards the knife using the manual advance wheel on the right side of the cryostat. Once you get close, switch to a small section thickness, e.g. 5 or 10 µm on the trim function.

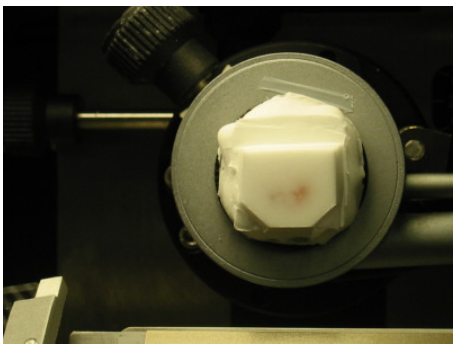


(6) If the blade starts to cut into the block straight and over the whole block face no further adjustment is necessary.
(7) However, if the blade asymmetrically cuts either the right or left side of the specimen or the upper or lower corner (illustration above) use the setting screws of the goniometer to adjust the chuck so that the blade sits parallel to the face of the tissue block.

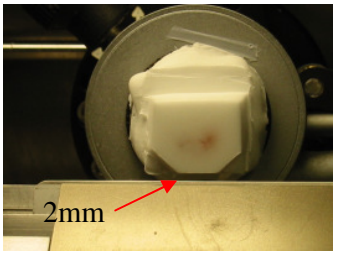


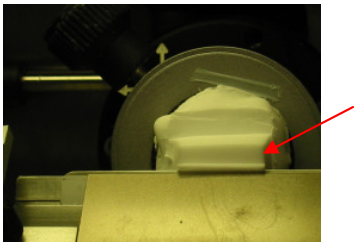
(8) Once the complete face of the block is cut away evenly when sectioning, trim the OCT until the tissue becomes visible. This can be done using 30 or 50 µm thickness on the trim function.
(9) Using a one-side razor blade cut the corners of the bottom edge of the block, and trim the sides of the block to center the specimen. The blade used for the previous specimen can be used up to this point. Now change either to a fresh position of the blade or replace blade. Before using a new blade, clean it with acetone and wipe dry. Always wipe away from edge so as not to dull the sharp edge.





(10) Next adjust the "cutting window" which defines the distance the cryostat will move with the selected sectioning speed through the specimen, but it will move faster outside of the defined window. To set the cutting window remove the anti-roll blade and move the block manually on the down stroke, until the edge of the knife is 1-2 mm away from the block, press the button (arrow figure below) on the control panel and move the knife through the specimen until 1-2 mm below the upper edge of the block press the button again. Put the anti-roll blade back and test by sectioning using the motorized function. Make sure that you see the shiny line.





(11) Check that you have the correct section thickness of 25 µm

(12) Set the sectioning speed, usually 20 - 40%. Note "cracks" appear on the sections if sectioned to rapidly.

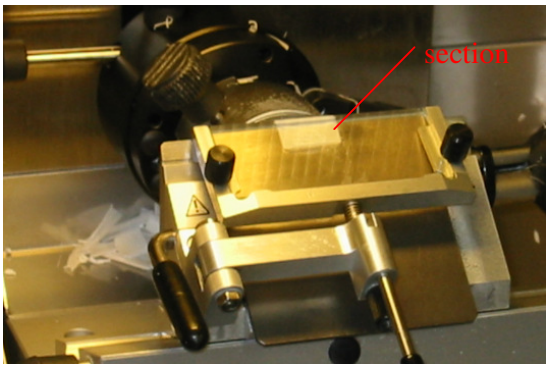
(13) Adjust temperature and sectioning speed, so that no bubbles or folds appear on sections. Proper adjustment of the temperature and sectioning speed will allow easier and better sectioning so it is important to practice by trial and error. Use thermometer located on tray.

(14) Use the big Leica brush to wipe the block each time as it moves towards the block, this removes dust and small OCT particles that can scratch the section.

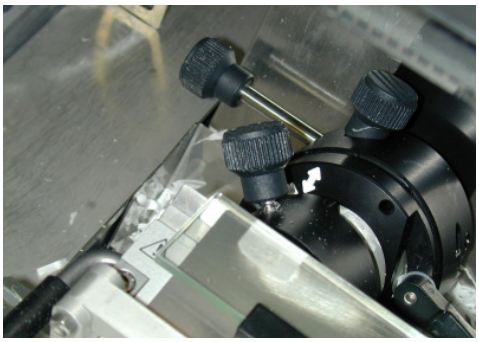
(15) If the section is very “wavy” or sticks to the anti-roll plate, the anti roll-plate is too warm. Cool the anti-roll plate by applying the heat extractor for 5 seconds to the top of the anti-roll plate, the next section should come out straight and flat to the surface of the knife holder block. You can also try to reduce CT and/or OT. If the section curls when the anti-roll plate is removed, the temperature in the cryostat is too cold. Check the temperature in the cryostat with a thermometer laying on the tray, try to keep this temperature constant –10°C, change OT and CT accordingly.

(16) Picking up the sections:

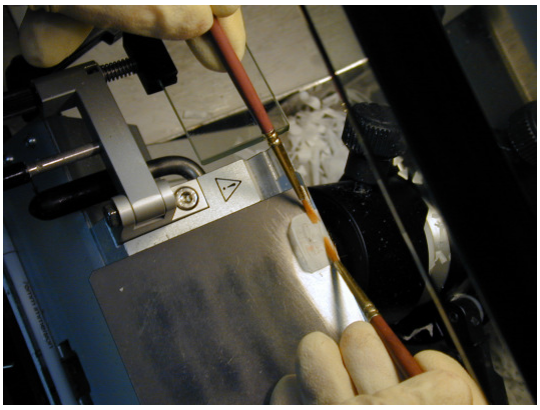
Section was cut.



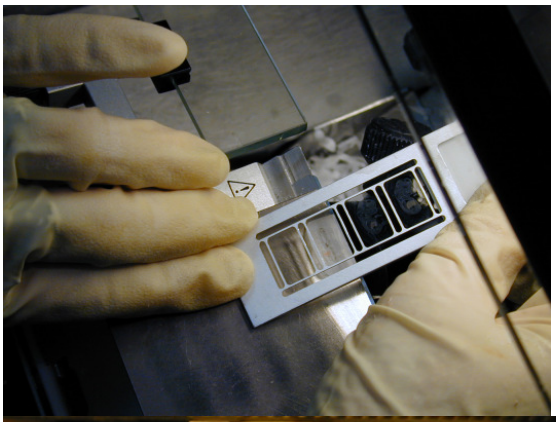
Section sits underneath anti-roll plate

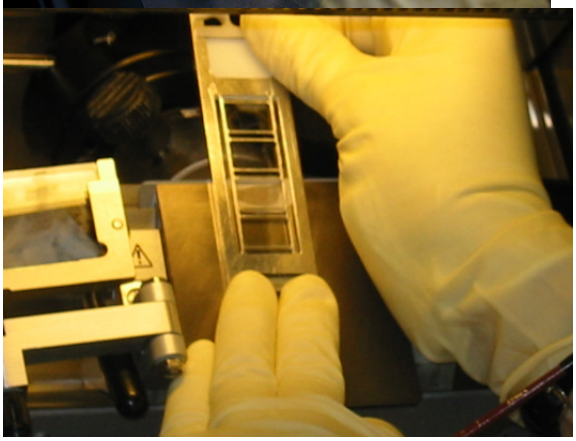


(17) Lift the anti-roll plate carefully. If the anti-roll plate is raised too quickly the section will not stay attached to the edge of the blade. Then take the two small brushes and gently press the edge of the section close to the blade making sure that it attached. If necessary you can straighten the sides of section with the brushes.



(18) The sections should not be attached to the knife holder block at the edge closer to you (front edge), they might even stick up a little. To pick up the section lower the microscope slide in the slide holder slowly to first catch the front edge of the section. Then slowly lower the slide towards the section that will attach itself to the warm slide.

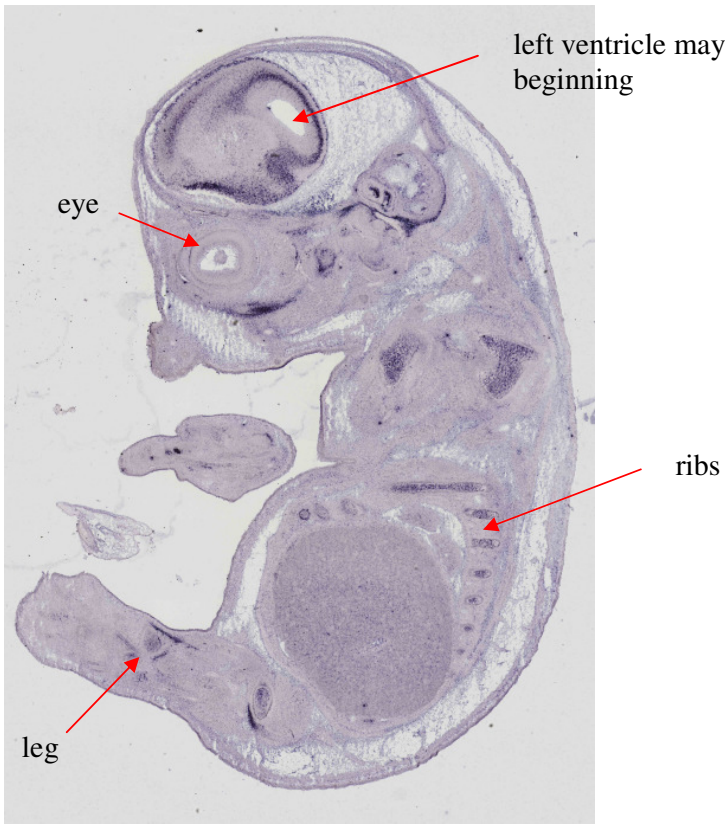




The above text is a general guide line, everybody cuts a little different so try what works best for you. The goal is to be able to get good sections without tears, bubbles and folds and to be able to collect all sections from a specimen.

The slide holder frame defines 4 areas on the slide. The position closest to the white part of the slide is position “a”, the next is “b”, followed by “c” and “d”. The first section is placed on the first slide in position “a”, the second section on the second slide in position “a”, the third section on the third slide in position “a” and so forth and the sixth section goes to position “a” on the sixth slide. The seventh section is placed on the first slide in position “b”, the eight on the second slide in position “b” etc.

The embryo is cut from eye to eye, with the first section depicted below (also consult E14.5 atlas for views of first sections on the right side of the embryo).



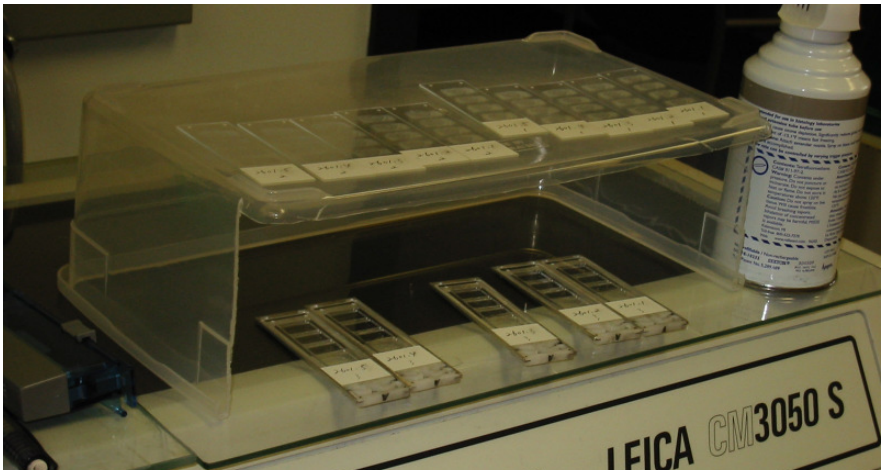
At the end of the sectioning process you will have six **sets** of slides, each set consisting of six slides. **Each set is destined for one RNA probe**.

(19) Label slides with pencil (specimen number, series and slide)

C1234.1 4

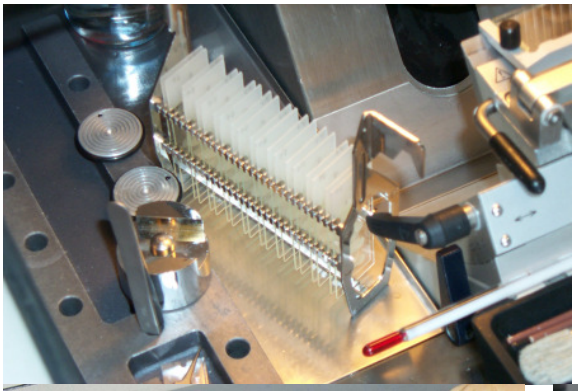
“C” is for Hannover, “1” is the set number going from 1 to 6;
“4” is the slide number going from 1 to 6

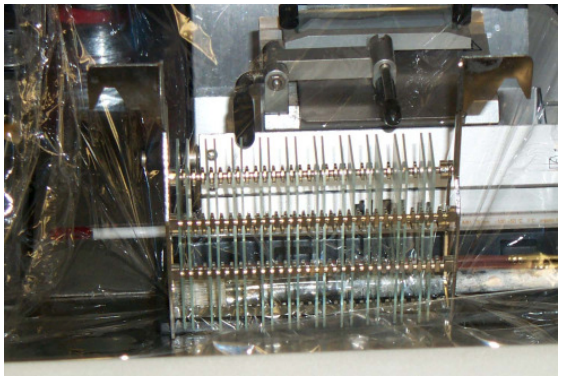
(20) After 24 sections are placed on six slides those are air dried for 30 minutes so that the tissue adheres to the slides.



(21) Slides are placed in a slide rack - back to back- with one space in between and are now ready for acetylation. Until a slide rack is full (20 slides), keep it inside the cryostat.

The full slide racks are wrapped in plastic foil (or zip-lock bag) and stored in the –20°C until acetylation, however the slides can also be acetylated right away.



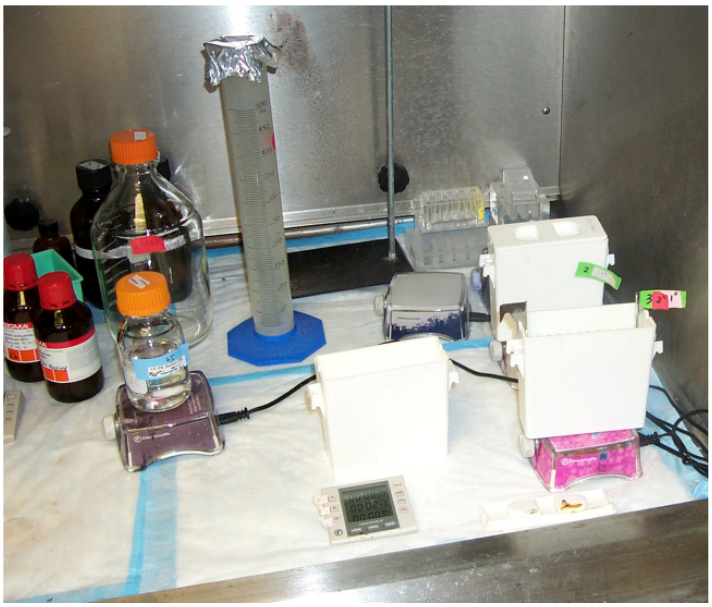


box with slide racks

Postsectioning procedures:

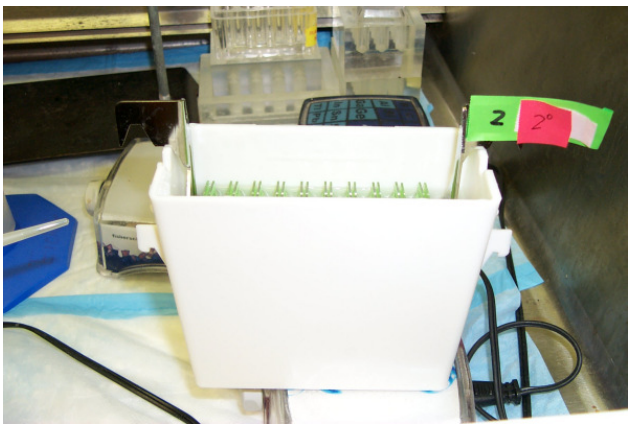
(1) Fixation is carried out in the Leica Autostainer at room temperature. The program is as follows:
PFA 4% 25 minutes (RT!)
NaCl (0.9%) two times 2 minutes

If you had left the slides at –20°C, get everything ready for fixation and after unwrapping the slide holder **immediately place slide holder into PFA – do not thaw slides**.



(2) Acetylation is carried out in a white Leica Autostainer container placed on a small magnetic stirrer. The containers accommodate 400ml of freshly diluted 1x TEA (see Stock Solutions for procedure) and a stir bar (25 mm and 5 mm thick). The solution has to be stirred vigorously. When a slide rack is ready after PFA fixation, 1ml of acetic anhydride (Sigma A-6404) is added to the TEA using a repeat Distriman

pipettor (Gilson), dispensing the acetic anhydride over the entire length of the container. The slide rack is the moved in and out of the container vigorously 10 times and a timer set to 5 minutes is started. After 5 minutes the rack is taken out and another 1ml of acetic anhydride is added, the timer is set for another 5 minutes. To carry out several acetylations in parallel it is useful to mark the timer used on the rack (here 2 on the green tape flag) we also mark on the flag whether a particular rack is being acetylated the first or second time (second time in the case shown below). Acetylation is an extremely critical step and determines background.



After acetylation the slides are returned to the Autostainer to go through an ethanol series and to be dried.

(3) The program for drying slides in an ethanol series (to remove water) in the autostainer is as follows:

1x PBS 2 min
0.9% NaCl 2min
30% EtOH 2min
50% EtOH 2min
70% EtOH 2min
80% EtOH 2min
95% EtOH 2 min
100% EtOH 2 min

100% EtOH 2 min
Autostainer oven 30°C for 3 min

(4) The slides are now dry and ready to be stored in small slide boxes in the presence of desiccant bags. The slide boxes are sealed with electrical tape, labeled (stage, specimen, set and slide number) and stored at
– 80°C.

Slide racks with acetylated slides in plastic shoe boxes and the lab bench is covered with disposable foil to keep the site clean. Slide boxes are labeled.

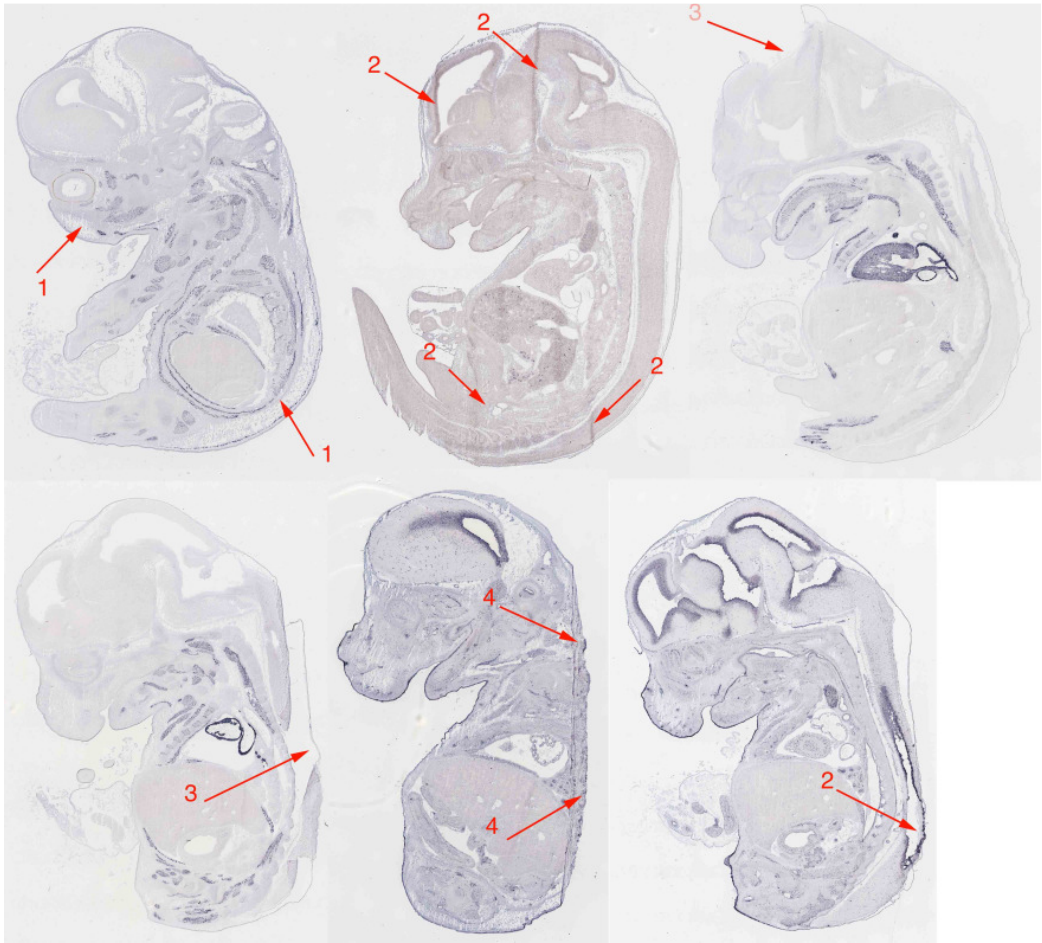
Slide boxes with desiccant bag from Sigma that need to be regenerated in vacuum at 120°C.

Slide boxes are stored in plastic boxes at –80°C that are numbered outside to indicate the content.

(5) Complete the "specimen/sectioning sheet", and also store the data in the section inventory Excel form. Record the specimen and set numbers as well as the section type, date and storage location in the inventory book.

Quality control

Before sorting the slides in the slide boxes, check the quality of the slides and note in quality inventory. For final ISH, 90% of the sections should be good. Below are some examples of problems that occur in sections.



1: lateral mesenchyme is spongy as a result of freezing. This is not a technical problem, but intrinsic to the process of freezing. Thus the lateralmost section sometimes look poor.
2: Folding that results from sections that are either not flat or picked up correctly.
3: Tears that can result when embedding specimen or damage during sectioning.
4: Tissue flipped back. This happens when the tissue detaches from the O.C.T. and when being picked up, that part of the section flipped back.

None of the above requires rejection of the set unless 3 or more sections of a set are damaged. Keep in mind that missing sections are also counting towards this threshold of 3. In addition, there may be artifacts upon ISH and this is why a quality control step is carried out after ISH. However, we do not want to waste money by using poor section sets for ISH.

Stock Solutions

10X PBS

- Measure out: 160 g of NaCl
  4 g of KCl
  28.8 g of Na_2HPO_4 anhydrous
  4.8g of KH_2PO_4
- Place all measured compounds into a 2 L beaker.
- Fill beaker with Milli-Q water to ~1800 mL mark.
- Stir solution until all compounds are dissolved.
- Adjust the pH to 7.40 with 10N NaOH.
- Pour solution into a 2 L cylinder.
- Fill cylinder to the 2000 mL mark with autoclaved Milli-Q water.
- Repeat to make another 2L of 10x PBS.
- Aliquot into 5x 800 mL volumes in 1 L bottles.
- Autoclave solutions.

20X PBS

- Measure out: 320 g of NaCl
  8 g of KCl
  28.8 g of Na_2HPO_4 anhydrous
  9.6 g of KH_2PO_4
- Place all measured compounds into a 2 L beaker.
- Fill beaker with Milli-Q water to ~ 1800 mL mark.
- Stir solution until all compounds are dissolved.
- Adjust the pH to 7.40 with 10N NaOH.
- Pour solution into a 2 L cylinder.
- Fill cylinder to the 2000 mL mark with autoclaved Milli-Q water.
- Aliquot into 5x 400 mL volumes in 500 mL bottles.
- Autoclave solutions.

5X PFA

- Measure out: 400 g of PFA
  10 g of NaOH pellets
- Pour measured compounds into an aluminum foil covered 2 L flask in hood.
- Measure 400 mL of Milli-Q water in a 500 mL flask.
- You may preheat flask and water in microwave, it should be ~ 60° C
- Pour water into 2 L flask in hood.
- Swirl by hand with aluminum foil covering opening in flask.
- Put in water bath shaker at 65° C and shake until mostly dissolved (the solution will not be clear but slightly cloudy)
- Add 250 mL of 20x PBS into flask, continue shaking for 30 – 60 minutes
- Cool to room temperature
- Adjust pH to 7.4
- Adjust volume to 1L
- Filter through a pleated filter to get a clear solution
- Aliquot 90ml in 100ml flasks

- Store at -20° C

9% NaCl

- Measure out 180g of NaCl
- Place in a 2L beaker, add 1900ml of Milli-Q water and dissolve by stirring
- Adjust volume to 2L
- Aliquot to 5 x 400 ml and autoclave
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10x TEA buffer stock for acetylation

In a 2L beaker weigh out 298.4 g of TEA (Sigma T-1377) add MilliQ H_2O to about 1800 ml and dissolve the TEA on a stirrer.
Adjust the pH with 12 N HCl (approximately 45 ml adding slowly thereafter) to 8.0 and adjust the volume to 2000 ml in a 2 L cylinder.
Check conductivity (~19 mS/cm)
Aliquot the 2 L to 5x 400 ml in 0.5L bottles.
When autoclaving, the solution sometimes turns yellow. Hence, instead of autoclaving, filter through a 0.2 µm sterile filter.
Store at room temperature protected from light by wrapping in aluminum foil and use within 4 weeks.

Important: do not use a TEA solution that has become yellow.