Attached please find protocols for fixation and processing of mouse embryos and fetuses for morphological analysis.

Please note:

1. The fixative must be prepared just prior to use.

2. The fixative must be prepared with RNase-free stock solutions.

3. The samples should be fixed for 16 – 24 hours in 20X the volume of the tissue.

4. Rinse in PBS and dehydrate to 70% ethanol as described (in 20x the volume of the tissue).

5. Ship on ice in 70% ethanol as quickly as possible.

6. Weigh and measure crown-to-rump lengths prior to fixation and transmit these values with the samples.

7. Send non-transgenic littermates and/or control animals of the same age (weighed and measured as well).

8. Pups between E17 and birth should be divided into two segments, between the thorax and abdomen with a sharp razor blade, leaving the head attached to the thorax. Divide the thorax from the abdomen by cutting just below the inferior extent of the rib cage. Bleed out the animal by blotting until blood is no longer seen. Strip off the diaphragm to allow the fixative to enter the chest cavity and immerse in fixative. Keep E17 and E18/19 pups on ice at all times to prevent premature attempts at breathing.

9. Pups between postnatal day 1 and 4 are problematic. If your institution allows it, decapitate the animal upon inspiration. The lungs will fill with air upon decapitation/inspiration and remain filled, i.e. expiration should not occur. Immediately invert the animal and bleed out by blotting until blood is no longer seen. Subdivide as described above and immerse thorax, abdomen and head in fixative.

10. Pups 5 days postnatal and older should be anesthetized, exsanguinated by severing the inferior vena cava and descending aorta, and the lungs inflation fixed as described in the attached protocol. The trachea / esophagus / heart / thymus / lungs should be removed *in toto* and immersed in fixative.

FIXATION & CRYOPROTECTION OF TISSUE FOR *IN SITU* HYBRIDIZATION & IMMUNOHISTOCHEMISTRY

A. Solutions.

1. D_2H_2O (DEPC-treated and autoclaved).
   Add 1 ml of diethylpyrocarbonate (DEPC) to 1 liter of D_2H_2O, stir vigorously for 30 min, let sit overnight, and autoclave for 30 minutes at 15 lb/sq. in. on the liquid cycle.

2. PBS (phosphate-buffered saline), DEPC-treated and autoclaved as above.

10X stock solution, 1 liter, pH 7.2 to 7.4*			20X stock solution		
80	g	NaCl	160	g	NaCl
2	g	K Cl	4	g	KCl
11.5	g	Na_2HPO_4-$7H_2O$	23	g	Na_2HPO_4-$7H_2O$
2	g	KH_2PO_4	4	g	KH_2PO_4

   Obtain RNase free reagents from SIGMA where possible.
   Dilute stock solution with DEPC-treated, autoclaved, D_2H_2O.
   From Sambrook, Fritsch & Maniatas, 1987. "Molecular cloning. A Laboratory Manual."

3. 4% paraformaldehyde in DEPC-treated, autoclaved, D_2H_2O
   Must be made up fresh, i.e. on the same day as fixation.

   (a) Recipe #1 - for small volumes.

10	ml of 16% paraformaldehyde stock solution, EM grade*
4	ml of 10X PBS (prepared as above)
26	ml of D_2H_2O (treated as above)
40	ml total (pH 7.2-7.4)

   (b) Recipe #2 - for larger volumes.

   Add 8 g to 100 ml of DEPC-treated, autoclaved water (ddH_2O). Heat to 60°C in a fume hood. Add a few drops of 1 N NaOH to help dissolve. When the solid has completely dissolved, let the solution cool to room temperature, and add 100 ml of 2X, DEPC-treated PBS and adjust pH to 7.4. This solution should be prepared fresh. You may have to filter solution if a precipitate persists. We have also frozen batches of this solution at -20°C for subsequent use.

   These are basic recipes. You will have to make more if fixing a large number of animals. Figure 5-10 ml per embryo (or 10X the volume of the specimen). You may fix the tissue in sterile 50 ml tissue culture tubes.

   *Obtain from Electron Microscopy Sciences, #15710, 10 X 10 ml per box, 1-800-523-5874.

4. Cryoprotectant - 30% sucrose in DEPC-treated PBS and filter sterilized.

   Add 30 g of RNase-free sucrose to a sterile, DEPC-treated container. Add DEPC-treated 1X PBS to 100 ml mark. Stir vigorously with DEPC-treated, autoclaved, stir bar. Filter through sterile 0.2 µm Nalgene filter set (filter and bottle) and store in refrigerator. Obtain RNase free-sucrose from SIGMA (#S-0389). Glassware and stir bar may be baked at 150° to 180°C overnight instead of DEPC-treating.

B. General Protocol for Fixed, Cryoprotected Tissues

1. Place embryos or tissue* in ice-cold 4% paraformaldehyde in PBS.
2. Leave at 4°C overnight (16 hours maximum).
3. Replace solution with PBS at 4°C X3, 5-10 minutes each, then with 30% sucrose in PBS.

4. Leave at 4°C overnight.
5. Drain and replace solution with a 2:1 mixture of 30% sucrose in 1X PBS: OCT.
6. Leave at 4°C overnight.
7. Drain, blot off excess liquid, and immerse embryos or tissue in OCT or M1-embedding medium (LIPSHAW) in molds. Freeze mold on surface of liquid nitrogen (LN_2), in crushed dry ice, or in LN_2-cooled isopentane. Mark orientation of specimen on mold.
8. Store tissue in precooled vials or plastic freezer bags (FISHER, #01-815-20), and store at -70°C.

Adult lung - Better morphology will be obtained if lung is fixed via rapid tracheal infusion for 1 min at 25 cm of pressure height (see below).

C. Immersion fixation of mouse embryos, fetuses, and neonatal animals (postnatal day 1 - 5).

1. Kill pregnant dam by any acceptable technique. We use cervical dislocation.

2. Deliver pups by hysterotomy, weigh and measure crown-to-rump length, and place into cold fixative:

   (a) Pups younger than 13 days of gestation may be fixed within the uterus, then dissected free, weighed and measured, and processed further.

   (b) Pups between fetal day 13 and 16 may be dissected free of the uterus and immersed whole in cold fixative.

   (c) Pups between gestational day 17 and postnatal day 5 may be killed by decapitation. The body may be further subdivided by transection below the diaphragm. Strip off the diaphragm and immerse the head, thorax, and abdomen in cold fixative.

3. Fix for 16 to 24 hours and cryoprotect at 4°C for 24hours. Alternatively, the tissue may be processed into paraffin as outlined below (section F).

D. Inflation fixation of adult mouse lung.

1. Anesthetize the animal.

2. Arrange the animal on the dissection tray, securing the limbs. Wash fur with 70% alcohol. Make a midline incision through the animal's skin exposing the abdomen, thorax, and neck.

3. Expose the posterior abdominal aorta, and kill the animal by exsanguination. This will reduce hemorrhage in the lung and bleeding from the external jugular vein in the neck.

4. Expose the trachea and remove the ventral thoracic wall. Place 2 ligatures around the trachea, below the larynx.

5. Make a slit in the intercartilaginous space between the larynx and the first cartilaginous tracheal ring. Cannulate the trachea with a small blunt-ended needle and secure with the bottom ligature. The needle should be attached to the tube extending from the infusion apparatus before insertion into the trachea.

6. Infuse the lung for 1 minute at 25 cm fluid height.

   (a) Infusion height is measured as the distance between the lungs and the meniscus of the fixative in the reservoir. The volume of fixative used to fill the adult mouse lung will be approximately 1 to 1.4 cc. If more fixative flows into the lung during the 1-minute infusion period, then there will be a visible leak present:

      (1) either the needle has gone through the posterior wall of the trachea and the lung will not fill, or

(2) the lung is nicked during removal of the anterior thoracic wall. In this case, the fixative will fill the lung and then flow out through the damaged area.

7. Withdraw the cannula and tie off the trachea with the upper ligature.

8. Dissect the lung out of the thorax as a unit including the heart, thymus, trachea, larynx, and esophagus.

9. Immerse in cold fixative for 16 to 24 hours.

10. Cryoprotect or process into paraffin as outlined below.

E. For frozen sections:

1. After fixation, rinse in 3 changes of cold PBS for 10 minutes each.

2. Transfer to a cold, filter-sterilized, 30% sucrose/PBS solution for 24 to 72 hours.

3. Transfer to a 2:1 mixture (2 parts:1 part) of 30% sucrose/PBS and embedding medium (OCT) for an additional 24 hours.

3. Blot off excess liquid and immerse tissue in OCT embedding medium in molds. Freeze mold on dry ice, on the surface of LN_2, or in LN_2-cooled isopentane. Store at -70°C.

4. Tissue may be shipped overnight in fixative. However, if transport takes longer than 24 hours, it is best to ship the material in the cryoprotectant (i.e., 30% sucrose/PBS solution on ice).

F. For paraffin sections:

1. After fixation, rinse in 3 changes of cold PBS for 10 minutes each.

2. Dehydrate through a graded series of ethanol solutions as follows:

10 minutes in 30% EtOH
10 minutes in 50% EtOH
10 minutes in 70% EtOH, X 3 washes

3. Transfer to 70% EtOH. Tissues can be shipped or stored indefinitely in 70% EtOH.

4. Alternately, tissues can be paraffin embedded using routine histological protocols, and can be shipped en bloc or cut and sections mounted on silane-coated slides.

G. References

1. Buckingham, K.W. and W.E. Wyder (1981). Rapid tracheal infusion method for routine lung fixation using rat and guinea pig. Toxicol. Pathol. 9:17-20.
2. Barthel, L.D. and P.A. Raymond (1990). Improved method for obtaining 3-µm cryosections for immunocytochemistry. J. Histochem. Cytochem. 38:1383-1388.