Araldite Embedding for Light Microscopy
Mouse Cochlea

Note: PBS is not used in this protocol – it might precipitate in the ethanol.

Intracardial Perfusion:

Use 2.5% Glutaraldehyde/1.5% Paraformaldehyde in 0.1M Phosphate buffer.

25% Glutaraldehyde	10 ml
4% Paraformaldehyde	37.5 ml
0.1M Phosphate buffer	52.5 ml
Total	100 ml

Cochlear Perfusion:

Immediately following intracardial perfusion, dissect as necessary to expose the cochlea; open both windows and flush fix through the scalae. To open oval window, remove stapes; if stapes breaks, make sure to dislodge footplate, or opening will still be sealed. For round window, use a right angle pick to make a slit in the membrane. IMPORTANT: bent portion of pick should be angling up the basal turn, so that the tip remains in the open channel, and does not poke through the osseus spiral lamina or basilar membrane of the hook. To flush scalae, a Pasteur pipet bulb on a 200ul pipet tip makes a good mini-pipet – the tip is about the same size as the windows. With the pipet tip apposed to either window, squirt fix into the scalae; watch other window for evidence of outflow to indicate that the fix really is circulating through. Gentle suction may alternatively be used, watching the other window to see that the fix is pulled in.

Post-fix:

Remove temporal bone(s) from head and put in fix at room temp on shaker overnight. (Tissue may stay in fix for more than one night, but it's best to osmicate as soon as possible after initial overnight post-fixation, for optimal preservation.)

Osmication:

15 minutes ddH2O rinse	Change water several times within this period. Flush scalae gently to wash out fix.
45 minutes 1% osmium tetroxide	DO THIS IN HOOD. Need only a small volume. Flush at 10-15 minute intervals. Check that color is developing along complete length of spiral – if there is a blockage, poke small hole at apex and flush there as well. Don't let it get too dark or you won't be able to orient the cochlea in the embedding mold – you must be able to see the turns.
15 minutes ddH2O rinse	Change water several times and flush. Dispose of rinse water with osmium waste.

Decalcification:

Decalcify in 0.12 M EDTA in 0.1M PB with 1% Glutaraldehyde. This will take about 7 days at 4°C using a stir bar or 2-3 days at room temperature on a shaker. (Most stir plates create heat, which might be undesirable for particular tissue.) Use a volume of at least 50 ml. The cochlea may be stored in EDTA after the decalcification period, but should be embedded as soon as possible for best morphology. Do not grasp temporal bone on cochlear spiral after decalcified – it is now very flexible, and the interior is easily damaged.

To make EDTA, mix on a stir plate:	**for 500 ml**	**for 1000 ml**
EDTA (Sigma, #ED4SS)	25 g	49.94 g
0.1M Phosphate buffer	400 ml	800 ml
25 % Glutaraldehyde	20 ml	40 ml

Adjust to pH=7.0 with full strength HCl, drop by drop. The EDTA will not completely dissolve until the pH is adjusted. Fill to volume with phosphate buffer.

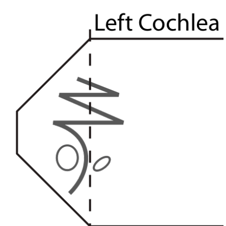
***Note:** There are many forms of EDTA – they have different molecular weights, however, and may not be substituted in this recipe. Any EDTA may be used for decalcification if the recipe is recalculated to produce correct molarity; some are acidic and require addition of NaOH instead of HCl to adjust pH.

Dehydration: flush scalae with each change

2 x 15 minutes ddH2O rinse	On shaker (30 minutes total)
15 minutes 70% ETOH	On shaker
2 x 15 minutes 95% ETOH	On shaker
4 x 15 minutes 100% ETOH	On shaker. With last change use fresh unopened ETOH (which has not absorbed water from air, so is truly 100%).
30 minutes Propylene Oxide	DO THIS IN HOOD. Use a covered glass dish, as propylene oxide (PO) dissolves some plastics, and is very volatile. Use a glass pipet to flush.

Embedding: (araldite recipe follows)

2 hours PO:araldite 1:1	On shaker. Need only small volume. Use disposable glass containers with sealable tops. Flush periodically.
Overnight PO:araldite 1:2	On shaker. Need only small volume. Use disposable glass containers with sealable tops. Do not try to flush (too thick).
2 hours 100% araldite	Under vacuum, to degas. Reset vacuum periodically. Need only small volume.



Transfer cochlea to a coffin mold (such as EMS #70905-01) filled with 100% araldite. Orient tissue as seen in diagram, with lower portion of basal and upper turns perpendicular to the plane of section. This yields very crisp images of these two areas in the midmodiolar sections, since the rows of

haircells are aligned with the line of sight. Submerge a tiny label (use pencil or print out with Times 8 condensed) so that the ID is included in the block. Allow tissue to settle in mold; check orientation and then place in 60º C oven for at least 2 days (may remain in oven longer).

Araldite:

This recipe is for medium hardness araldite. For harder, decrease DBP to 8 ml. For softer, increase DBP to 20 ml.

Araldite 502 (Electron Microscopy Sciences #10900)	50	ml
DDSA (EMS #13710)	42.5	ml
DBP (EMS #131000	13	ml
DMP-30 (EMS #13600)	1.75	ml

Measure volumes in disposable graduated beakers or pipets; add DMP-30 in hood. Combine and mix well. (Alternatively, weigh out the ingredients on a balance. Place a weigh paper and a disposable beaker on balance. Zero the balance. Pour in 50g of Araldite, then add 42.5g of DDSA, then 13g DBP; zero balance after each addition to simplify. Use a pipetter to measure DMP-30.)

Embedding Larger Bones:

For larger bones such as mouse skull with both cochleae, or guinea pig temporal bones, try longer processing times. Tissue may be left in 70% EtOH overnight for convenience. Suggested:

Water	2 X 10min
50% ETOH	1 X 30 min
70% ETOH	1 X 30 min
95% ETOH	2 X 30 min
100% ETOH	4 X 30 min
Propylene Oxide	1 X 30 min
Araldite:PO, 1:1	Overnight
Araldite:PO, 2:1	Overnight (can be longer)
100% Araldite	2 days
Vacuum	At least 2 hours, check vacuum every 30 min

Transfer to mold and harden in oven.

Sectioning tips:

Collect sections on a microscope slide as they are cut. Place each section on a drop of water; for a mouse cochlea embedded in a small coffin mold, 10 sections in 2 rows of 5 will fit onto one slide. Leave a little space between sections. Place slide on a slide warmer, where the heat helps to flatten the sections as they dry onto the slide. If a section dries out of position it is not too hard to loosen it with a razor blade and forceps and a little water.

Coverslip with permount and place a small weight on the coverslip to further flatten the sections. The weight should have a smaller footprint than the coverslip, to avoid becoming cemented to the coverslip itself by the permount that is pressed out around the edges.