

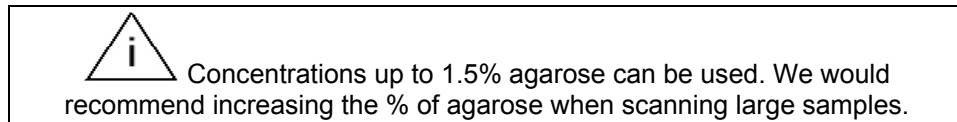
Appendix 1

Specimen Preparation

Specimens stored in PFA or other fixative should be thoroughly washed in PBS.
Specimens stored in ethanol or methanol should be rehydrated to PBS.

Embedding

Make up a 1% solution of Low Melting Point Agarose (gelling point 24-28°C) in deionised water.
40mls will be required for each 50mm x 25mm petri dish.

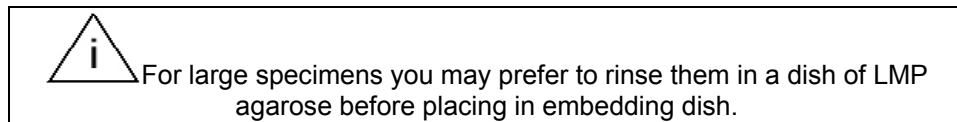


Cool to 60°C then filter through Whatman filter paper 113V.
Place in 32°C water bath or incubator.
When cooled to 32°C, fill deep petri dish with the agarose and place on the cold plate or ice.

If using ice it is vital that the surface is flat. Placing a glass or metal sheet on top of ice may help.

Transfer specimen(s) into petri dish with as little PBS as possible.

Place at edge of dish, using needle or forceps, gently move specimen to wash away excess PBS.
Move to centre of dish.



Monitor temperature, and when approaching gelling temperature, move specimen so that it is suspended in the middle of agarose and is horizontal.

It is important that the specimen is placed in the centre of the petri dish, as this will simplify trimming of the agarose block.

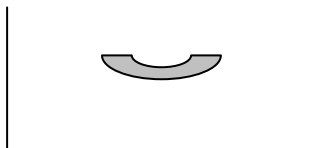
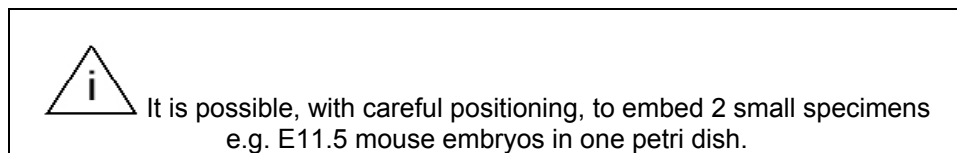


Fig. A1a Side view of specimen suspended in agarose

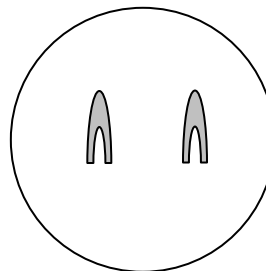


Fig. A1b Top view of petri dish with 2 specimens embedded

Leave until agarose has completely set.

Trimming Agarose Block



It is important that agarose is completely set before trimming.

Wear gloves when handling agarose blocks.

Agarose has to be trimmed into a block with the specimen in the centre.

To do this, insert a single edged blade into agarose perpendicular or parallel (depending on size and shape) to specimen, leaving as much agarose between specimen and blade as possible.



Fig. A1c Blade inserted

Leaving blade in position, dig out unwanted agarose between blade and side of petri dish. Any blunt instrument can be used for this. Repeat on opposite side. Depending on position of specimen you may have to use a scalpel blade to cut remaining two sides. On completion of this procedure, you will have a rectangular block.

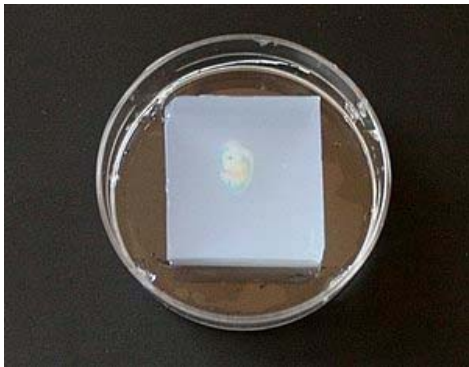


Fig. A1d Agarose block after trimming

Turn dish over and tap or prise block out.

Lay agarose block onto large petri dish lid or sheet of plastic which has been placed on top of line template (Appendix 3).

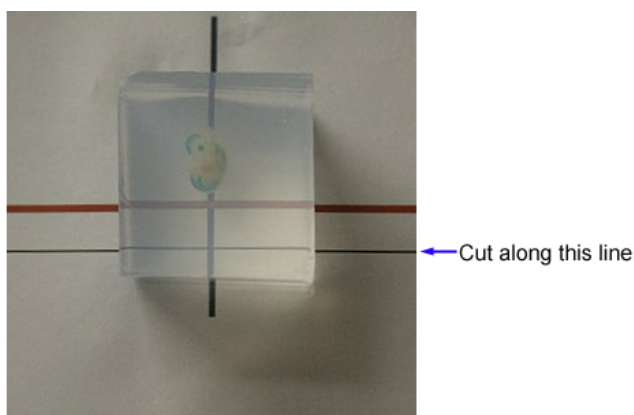


Fig. A1e Agarose block on template

Specimen is normally orientated so that long axis is directly above blue vertical line.



When reconstruction is performed the transverse orientation will be along this long axis.

When cutting the edge that will be attached to the mount, ensure that there is at least 5mm between specimen and end of block (the distance between the red & black line).

Place end of specimen on or above red line. Trim edge of block square on horizontal black line; it is important that this cut is completely straight. This is the edge which will be glued to the mount; if it is at an angle it could be difficult to align specimen in scanner.

Check edge is straight by placing block on edge to be attached to mount. The specimen should be suspended vertically in block. If not place onto template and re-trim.

Trim off excess agarose from opposite edge to face being glued, leaving a small amount of agarose ~3mm.

Trim all other faces of block so that the size is slightly bigger than the mount.

Attaching Specimen to Mount

Wipe grooved surface of mount with methanol to ensure it is clean.

Using tissue, dry the face of agarose that will be attached to the mount.

Brush a thin layer of superglue onto mount,



If too much superglue is used it will run down mount when block is attached. This could result in mount being glued to work surface or fingers.

Place agarose block onto mount, with specimen centred on mount.

Press gently to remove air bubbles and ensure complete contact, hold for ~20 seconds.

Allow glue to set for 5-10 minutes.



Fig. A1f Agarose Block Attached to Mount

Use a microtome or single-edged blade to trim agarose from four straight edges.

Start cut close to top of specimen and angle cut outwards so that it ends at the edge of mount.

Cut off the four corners at the same angle.

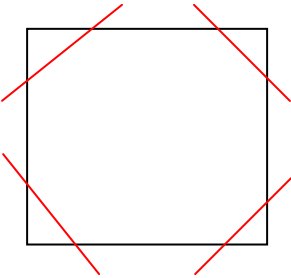


Fig. A1g Diagram of cuts to be made

Black lines cuts 1-4, Red lines cuts 5-8

You should now have eight angled sides, forming an octagon shape at the top of the plug.

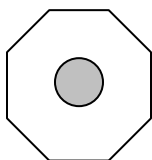


Fig. A1h Top view



Fig. A1i Side view



Fig. A1j Trimmed Agarose Block on Mount



Regularly check blade for glue during trimming process. Always trim using a clean, glue-free blade.

If any of the cut surfaces have nicks or glue on them, it is advisable to re-trim to remove damaged agarose as it may affect scanning image.

The cut edges do not have to be exactly all the same size and angle.

Dehydration and Clearing of Specimen

Using forceps, place mount into a screw top glass bottle filled with methanol. Change methanol until no water is remaining in agarose block. Gently swirl bottle. When no water can be seen, change in methanol one more time.

Replace the methanol with BABB.

Bottles must be left with lids off to allow remaining methanol to evaporate

BABB Recipe

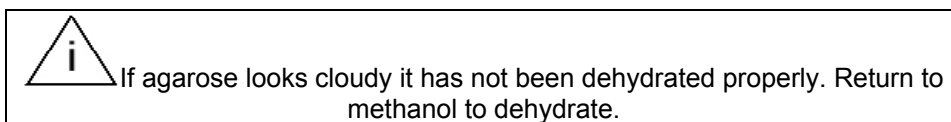
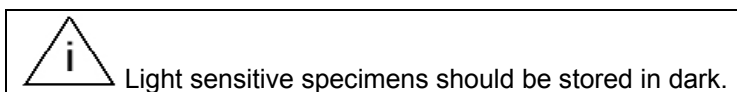
2 parts Benzyl Benzoate

1 part Benzyl Alcohol

Mix well and store at room temperature protected from light.

Specimens have to be cleared in BABB before they can be scanned. Length of time required in BABB is dependant on size of the specimen and block. Normally specimens are left overnight to clear. Small specimens may clear in a few hours.

For stains which are soluble in BABB e.g. X-Gal, incubation in BABB should be kept to a minimum.



The specimen can be scanned when it is transparent.

Specimens can be stored in BABB for a few days up to a few months, depending on stability of stain or signal.

Cleaning Mounts

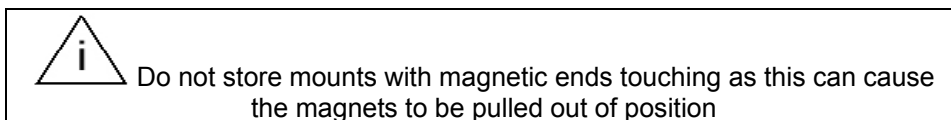
Remove agarose block.

Soak mount in methanol to remove BABB.

Ensure all glue has been removed from grooves: use scouring pad, pointed forceps or needle.

Rinse in methanol.

Air dry.



Appendix 2

Reattaching Agarose Block to Mount

If agarose block detaches from mount, it will have to be removed and re-glued.

Use a blade to remove agarose block from mount.

You will have to re-trim if there is glue attached to the agarose block or if the cut edge is no longer square.

Lay block on its side and cut off the minimum amount of agarose. Ensure the cut is straight; otherwise alignment of specimen could be affected.

Dry the surface to be glued on a tissue.

Glue onto a clean mount.

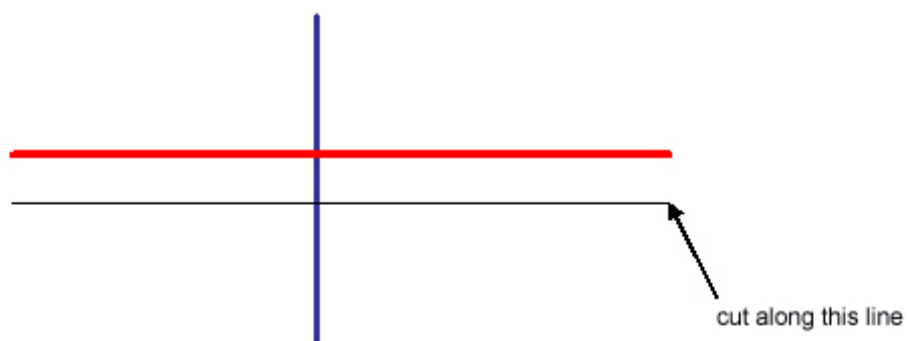


It is important that the faces of block are not damaged or dusty as this could affect quality of scanned image.

This page has been left blank intentionally.

Appendix 3

Trimming Template



Place end of specimen which has to be closest to mount on or above red line.
Place long axis of specimen on blue line.
Make a vertical cut along black line. This is the face to be glued to mount.

This page has been left blank intentionally.

Appendix 4

Removing Specimen from Agarose Post-OPT

Specimens that have been OPT scanned can be removed from agarose for further analysis e.g. H&E staining and immunohistochemistry after wax or cryosectioning.

Using a single-edged blade remove the agarose plug from the mount. Trim as much agarose from around specimen as possible.

BABB has to be completely removed from the agarose and the specimen before rehydration. Wash in 100% methanol, shaking gently at room temperature. Many changes of methanol will be required.

To monitor for the presence of BABB pour methanol wash into a beaker containing water. If the water turns cloudy, there is BABB present. Continue changing methanol until pour-off is clear in water.



Observe local health & safety policy when handling chemicals.
Dispose of waste chemicals according to local regulations.

Rehydrate agarose in 70%, 50%, 30% and 10% ethanol. A minimum of 60 minutes shaking gently at room temperature in each will be required. If specimen is floating after 60 minutes, replace ethanol and wait until it sinks. The time required for this will be dependant on size of agarose plug.

Incubate in 0.29M sucrose for at least 60 minutes at room temperature.

At this stage any remaining excess agarose can be trimmed off.

Incubate in 0.29M sucrose pre-heated to 57°C for 30-60 minutes.

Gently shake to see if agarose has melted/fallen away from specimen. You may have to incubate for longer (time is dependant on plug size), or increase temperature for a brief period.

If this is unsuccessful, pour sucrose and agarose plug into a petri dish.

Use fine forceps or a needle to carefully pull away agarose from specimen.

When agarose has been removed from specimen, wash a further twice in fresh 57°C sucrose.

The specimen can now be prepared for next procedure.

If long-term storage is required, place in 4% PFA or dehydrate and store in 70% ethanol.

This page has been left blank intentionally.