

# HIGH-RESOLUTION EPISCOPIC MICROSCOPY

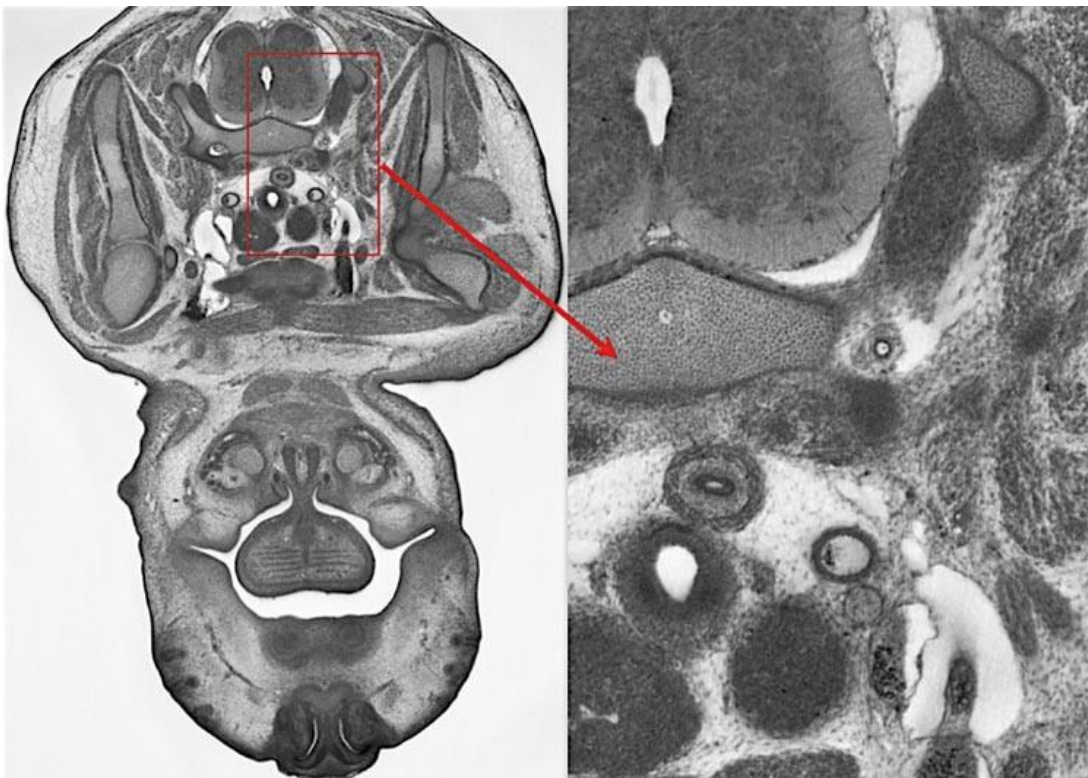
High-Resolution Episcopic Microscopy (HREM) is a technique for visualising tissue samples, such as mouse embryos, as a large stack of successive 2D images. The resolution of the images approaches that of conventional histology and, since they are perfectly aligned, they can be used to construct very detailed 3D models.

## 2D imaging technique

As with conventional histology, HREM imaging uses samples that have been embedded in a rigid matrix and sectioned with a microtome. However, the technique does not require sections to be individually stained or registered. Instead, each image is obtained from the exposed surface of the tissue during the sectioning process.

The embedding medium is a hard plastic resin, which enables sections as thin as 1  $\mu\text{m}$  to be accurately removed. The resin itself contains fluorescent dyes and, under fluorescent illumination, tissue at the cut surface of the block can be easily visualised against the bright background of the plastic. By sequentially imaging the block face during the sectioning process (a technique known as episcopic imaging), a comprehensive stack of accurately aligned images is acquired, documenting the 3D structure of the sample. The only practical constraints on the 2D image resolution are the choice of optics used to visualise the block surface and the capability of the camera capturing the images.

Because HREM does not require the collection, staining and registration of individual sections, samples can be imaged rapidly. For example, the E14.5 mouse embryo data on this site is based on 3  $\mu\text{m}$  sections, a process that takes around 8 hours and generates approximately 3,500 images for each embryo.



An HREM image taken transversely through the head and jaw of an E14.5 embryo. By combining relatively low magnification optics with acquisition of large images (around 4000 x 3000 pixels), we are able to image embryos at high resolution.

## 3D reconstruction

Building detailed 3D models from images of individual sections has always proved difficult, since accurate alignment of the images is required. In addition, the sections themselves often become distorted as they are cut, captured and stained.

Using HREM avoids these problems by sequentially imaging the face of the block, rather than imaging individual cut sections. As a result, the resolution of the 3D data is only constrained by the choice of section thickness, since this determines the frequency of images that will provide the 3D volume.

HREM images capture different tissues and structures as distinctive patterns and 'textures' of pixels, using a broad range of grayscale values. As a result, the data lends itself to simple, 3D *volume rendering* as is commonly used in medical imaging. It can also be used as the template for visualising individual structures traced through successive images, modelled by *isosurface rendering*.

## Advantages of HREM

HREM data can be analysed either as 2D image stacks or by 3D rendering. The 2D images are routinely used by the DMDD programme to identify detailed morphological phenotypes in developing mouse embryos. Thanks to the intrinsic alignment of the images, orthogonal and oblique views of the tissue sample can also be calculated from HREM data with relatively little loss of resolution. These alternative views provide valuable additional information about the morphology of a sample.

Since HREM sections can be cut as thin as 1  $\mu\text{m}$ , 3D models of HREM images typically have voxel resolutions of 1-8  $\mu\text{m}$ . At this resolution it is possible to identify features such as individual nerves and blood vessels, which may not be detectable using lower-resolution techniques such as optical projection tomography (OPT), micro-magnetic resonance imaging ( $\mu\text{MRI}$ ) or micro-computed tomography ( $\mu\text{CT}$ ).

## MORE ON HIGH-RESOLUTION EPISCOPIC MICROSCOPY

Mohun T.J., Weninger W.J. (2012)

Episcopic three-dimensional imaging of embryos.

53, Cold Spring Harbor Protocols (<http://cshprotocols.cshlp.org/content/2012/6/pdb.top069567.full>)

Geyer S.H., Mohun T.J., Weninger W.J. (2009)

Visualizing vertebrate embryos with episcopic 3D imaging techniques.

*Scientific World Journal* 16;9 , 1423-37 PubMed abstract (<http://www.ncbi.nlm.nih.gov/pubmed/20024516>)

Weninger W.J., Geyer S.H., Mohun T.J., Rasskin-Gutman D., Matsui T., Ribeiro I., Costa Lda F., Izpisúa-Belmonte J.C., Müller G.B. (2006)

High-resolution episcopic microscopy:

a rapid technique for high detailed 3D analysis of gene activity in the context of tissue architecture and morphology.

*Anat Embryol (Berl)*. 211(3) , 213-21 PubMed abstract (<http://www.ncbi.nlm.nih.gov/pubmed/16429276>)

# EMBRYO HARVESTING

## Consumables

### Essential

- 37 °C water bath
- PBSA or calcium-free Hanks saline
- Fine forceps (#5)
- Fine scissors (blade length no more than 1 cm)
- Stereomicroscope
- KCl stock solution (for example, 1 M) (facultative)

### Desirable

- Fine iridectomy scissors (blade length of a few mm)
- 37 °C heat pad
- Heparin

## Procedure

1. Working in PBSA or calcium-free Hanks saline at 37 °C, remove embryos from the yolk sac.
  - The addition of heparin to the PBSA or saline at this stage can help minimise blood clotting.

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2. Maintain embryos at 37 °C using a water bath or dry heat pad.

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3. Monitor using a stereomicroscope, flipping embryos periodically to minimise the pooling of blood on one side.

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4. Repeatedly clip the umbilical vessels to maintain blood flow for as long as possible.

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5. When the blood flow ceases, transfer the embryos to saline supplemented with at least 50 mM KCl.
  - This step increases the probability that the heart arrests in diastole.

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## HREM SAMPLE PREPARATION

### Dehydration and infiltration

In preparation for embedding and imaging, samples are dehydrated then infiltrated with a JB-4 dye mix. The exact procedure depends on the size and tissue density of the sample. The procedure below is intended as a guide, but the parameters are empirical, and some experimentation may be required with your own samples.

### Consumables

- JB-4 resin embedding kit
  - [Polysciences JB-4 Embedding Kit](#) (Polysciences, cat no. 0226A-800, Sigma, cat no. EM0100-1KT) containing Solution A (monomer), Solution B (accelerator) and Catalyst (Benzoyl Peroxide, Plasticised)
    - We usually use JB-4 for embedding. JB-4 Plus is supposed to polymerise with less heat and provide clearer blocks, but we have used both and they work equally well. We have noted that some batches of JB-4 appear slightly yellow, but this doesn't seem to have a noticeable effect on the data.
- Eosin B
  - [Sigma Aldrich Eosin B](https://www.sigmaaldrich.com/catalog/product/sial/861006?lang=en&region=GB) (<https://www.sigmaaldrich.com/catalog/product/sial/861006?lang=en&region=GB>)
- Acridine Orange
  - [Sigma Aldrich Acridine Orange](https://www.sigmaaldrich.com/catalog/product/sigma/a6014?lang=en&region=GB) (<https://www.sigmaaldrich.com/catalog/product/sigma/a6014?lang=en&region=GB>)
- Filters
  - [Sartorius 0.22µm polyethersulfone \(PES\) membrane filters](https://www.sartorius.co.uk/en/product/product-detail/180c2-e/) (<https://www.sartorius.co.uk/en/product/product-detail/180c2-e/>). PES filters need to be used or the membrane will break.

### JB-4 dye mix preparation

Final volume (ml)	80	100	150	200	300
Solution A (ml)	80	100	150	200	300
Catalyst (g)	1.00	1.25	1.88	2.50	3.75
Eosin B (g)	0.220	0.275	0.413	0.550	0.825
Acridine Orange (g)	0.045	0.056	0.084	0.113	0.169

1. Measure Solution A and place on a stirrer.
2. While mixing vigorously, add the catalyst slowly to avoid the formation of lumps.
3. Slowly add both dyes.

4. Stir for at least 4 hours (preferably overnight) at room temperature or below.
5. Filter the mix through 0.22 µm PES membrane to remove dust and any undissolved dye.
  - There should be very little or no undissolved dye to remove. If there are lots of undissolved dye grains you may need to start again. The mix will break standard filter membranes.

At this point, the JB-4 dye mix can be stored at 4 °C for 2-3 weeks. Any leftover mix can be used to prepare 50:50 JB-4/methanol for infiltration.

## Procedure

For an E14.5 mouse embryo (roughly 10mm by 5mm):

### Dehydration

1. Fix the sample overnight in Bouin's fixative, then transfer to PBS.
  - Other fixatives including 10 % formalin, 4 % paraformaldehyde and Dent's can be used. We prefer Bouin's fixative for whole embryos since it gives better preservation of the structure of soft mesenchymal tissue, which tends to collapse during dehydration.
2. Over the course of 1-2 days, wash samples with repeated changes of PBS for 2 hours per wash.
3. Dehydrate samples using a MeOH/H<sub>2</sub>O series ((10%), 20 %, 30 %, 40% 50 %, 60 %, 70 %) for 1-2 hours per step.
4. If using Bouin's fixative, wash the sample in 70 % MeOH containing 1% ammonia for three minutes.
  - This helps remove any residual yellow picric acid staining left from the Bouin's fix (facultative).
5. Complete dehydration using MeOH mixes (80 %, 90 %, (95 %), 100 %) for 2 hours each. Samples can be held overnight at any stage prior to 90 % if necessary (preferably in 70%)

### Infiltration

1. Immerse the sample overnight in a 50:50 mix of MeOH:JB-4 dye mix.
2. Briefly rinse in JB-4 dye mix to help minimise residual MeOH, then immerse in several ml of fresh JB-4 dye mix.
3. Leave to infiltrate at 4 °C, preferably with gentle rocking.
  - For example, infiltration of an E14.5 embryo usually takes 3 or 4 nights.
4. The sample is now ready to be embedded.

For small embryos or embryo tissue pieces, the length of dehydration and infiltration steps can be reduced drastically:

- For E9.5 embryos, dehydration steps need only be 30-60 minutes and infiltration takes only 4+ hours. -
- For denser embryo tissue such as an E14.5-E18.5 heart, we dehydrate in 1-hour steps and infiltrate 3-4 nights.
- For larger or much more dense samples such as adult tissues, successful infiltration can take more than a week and needs to be determined empirically.

## HREM EMBEDDING

Following infiltration, samples are embedded in polymerised JB-4 dye mix.

### Consumables

- JB-4 dye-mix, as prepared during the infiltration process.
- Solution B as provided in the JB-4 resin embedding kit (Polysciences, cat no. 0226A-800, Sigma, cat no. EM0100-1KT ) .

### Equipment

- A stereomicroscope with very bright illumination from the base. (Image 1)
  - This enables samples to be visualised during the embedding procedure, despite the dark red colour of the JB-4 mix.
  - As an example, we use a Schott KL 2500 LCD (<http://www.schott.com/lightingimaging/english/microscopy/products/kl/2500lcd.html>) fibre optic lamp, with a 250 watt bulb, providing a large bundle diameter up to 15 mm.
- Glass capillaries to manipulate samples, e.g. glass Pasteur pipette lengths that have been heated to produce blunt ends.
  - Although fine forceps can be used to manipulate samples, there is less danger of damage using glass capillaries.
- Moulds for embedding the samples. (Image 2)
  - Small commercial moulds (<https://www.emsdiasum.com/microscopy/products/preparation/molds.aspx>) are available from EMS (5 mm depth, catalog # 70176-30 and 70176-15).
  - PTFE block moulds are available at various sizes from Indigo Scientific (<http://www.indigo-scientific.co.uk/>) . These are not listed on the website, but can be ordered by emailing [sales@indigo-scientific.co.uk](mailto:sales@indigo-scientific.co.uk) (<mailto:sales@indigo-scientific.co.uk>)
- Plastic chucks to attach to the base of the samples. (Image 3)
  - Custom-made chucks are available from Indigo Scientific (<http://www.indigo-scientific.co.uk/>) . These are not listed on the website, but can be ordered by emailing [sales@indigo-scientific.co.uk](mailto:sales@indigo-scientific.co.uk) (<mailto:sales@indigo-scientific.co.uk>)
  - If samples are to be attached directly using the JB-4 mix, it is best to use chucks with both a central hole and cross grooves. This is more convenient than using traditional 2-step procedures in which an adhesive resin is used to attach the polymerised block to the chuck.

### Basic Embedding Procedure

1. At room temperature, add Solution B to cold JB-4 dye mix and mix vigorously. We recommend 0.6 ml of Solution B per 10 ml of JB-4 dye mix.
  - We use glass scintillation vials, with up to 20 ml of mix per vial, and add twice the manufacturer's recommendation of Solution B as the dyes appear to have some inhibitory effect on polymerisation.
2. Fill the mould with the polymerising mix and add the sample, ensuring no air bubbles are trapped on its surface.

3. Between approximately 10 and 30 minutes later, there will be an optimum period of a few minutes during which the viscosity of the mix increases. At this point, samples can be realigned and will be held in their new positions.
  - If Solution B is added to a room temperature JB-4 mix, polymerisation will occur more quickly and there will only be a very brief period of increasing viscosity. If the mix does not polymerise fully, or is taking longer to polymerise, check your components by testing polymerisation of JB-4 without dyes. Incomplete removal of methanol during sample infiltration also appears to inhibit polymerisation.
4. Adjust samples as necessary, but avoid excessive movement as this can cause unsightly variations in the background of HREM images.
  - Polymerisation proceeds from the bottom upwards. As a result, samples will be held more firmly at the bottom and can be easily damaged by realignments in very viscous mix.
5. Place a plastic chuck on top of the mould, ensuring that enough mixture is present to fill up the central hole in the chuck and the cross grooves.
  - Note that only some of this will polymerise, so it's essential that there is enough to attach the block adequately to the chuck.
6. Leave the samples to polymerise completely in an oxygen-free atmosphere. It is sufficient to cover the exposed surface of JB-4 with a thin layer of mineral oil.
7. Samples will polymerise in a couple of hours or less, but are best left overnight at room temperature.

Samples embedded using this method will rest on the bottom of the mould (i.e. the top surface of the block). As a result, after polymerisation, the sample will often protrude slightly through the block surface and this will be visible in the first HREM images.

## Embedding with a cushion

If it is important to obtain a complete image series (e.g. for complete 3D models), samples should be embedded after first forming a thin cushion of polymerised JB-4 at the base of the mould. (Since JB-4 polymerisation is oxygen-sensitive, it is extremely difficult to polymerise a layer thinner than 1-2 mm).

### Procedure

1. Add sufficient polymerising mix to the mould to obtain a layer 2-3 mm thick and completely cover with mineral oil.
2. Leave to polymerise for 30-60 minutes before following the Basic Embedding Procedure. (It is desirable to remove the mineral oil from the cushion before proceeding).

Note that the cushions will have a slightly convex surface, which can make stable positioning of samples difficult. One solution is to wait until increasing viscosity of the mix holds the sample in position. Alternatively, after filling the mould with polymerisation

mix, but before adding the sample, it is possible to flip the cushion upside down with fine forceps. This ensures that there is a flat surface for the sample to rest on. Note also that cushion thickness may vary from sample to sample, despite identical polymerisation conditions. Too thin cushion will not polymerise.

The cushion will be visible in the raw HREM images, since the interface between the cushion and the remainder of the JB-4 block generally appears much brighter than the rest of the plastic. This effect appears to get worse, the longer the time gap between polymerisation of the cushion and embedding the sample. It is not, therefore, advisable to leave cushions overnight before use.

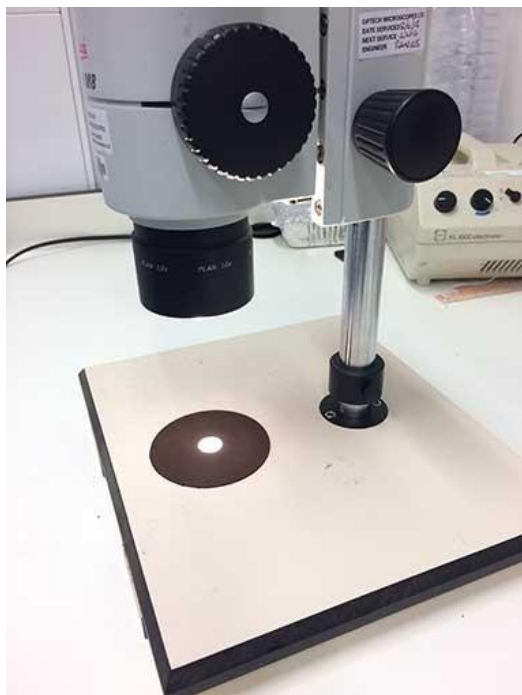


Image 1 - Stereomicroscope with fibre optic illumination from the base.

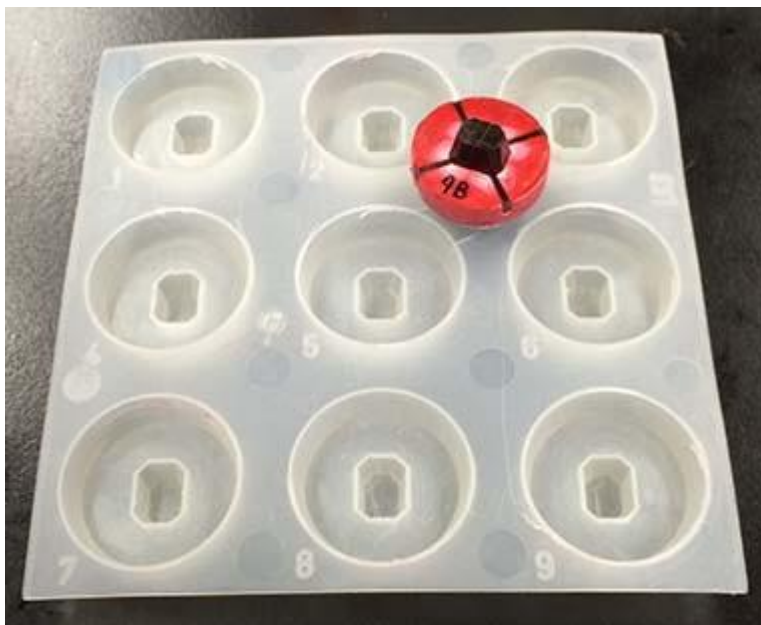


Image 2 - Small mould with polymerised block.



Image 3 - Larger PTFE mould with polymerised block and plastic chucks.

## PREPARING BLOCKS FOR IMAGING

After embedding, polymerised blocks are removed from moulds and trimmed. We recommend samples are baked to ensure optimal hardness. This stage is optional, but it can improve the stability of the sample during sectioning. Baking times are empirical and some experimentation may be required with your own samples

### Procedure

1. Invert the mould onto paper towels to remove mineral oil and unpolymerised JB-4 mix.

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2. Check that polymerised JB-4 is visible in both the central hole of the chuck and through the base of the chuck. (Image 1)
  - This is essential to avoid the sample block breaking off during sectioning. If necessary, a little fresh JB-4 embedding mix can be added to the top of the chucks and left to polymerise for a few hours. If the block does break off, use abrasive paper to ensure the surface of the chuck is completely flat (it is not flat from manufacture). Do the same with the base surface of the JB-4 block and then use SuperGlue contact adhesive to reattach it to the plastic chuck.

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3. Remove blocks from the mould and wipe dry.
  - For the small (5 mm) EMS flexible moulds, blocks can be popped out by firm pressure from underneath. Excess polymerised JB-4 must be removed from the side of the chuck using a blunt blade to enable the block to fit in the HREM block holder. (Image 2)
  - For the larger PTFE moulds, grip the plastic chuck with an adjustable wrench and pull each block vertically. Be careful not to apply too much pressure with the wrench - this can cause distortion of the chuck, weakening its attachment to the sample block.
  - If the blocks are very soft and jelly-like or rubbery immediately after removal from the mould, there is likely to be something wrong with your JB-4 mix or its components. Normal blocks will sometimes feel slightly springy when removed from the mould, especially if they are large in size. This is usually fixed by baking.

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4. Bake the samples at 95 to 100 °C to ensure the blocks are completely hard before sectioning.
  - For small (5 mm) samples, 12 hours is sufficient. Larger blocks benefit from longer times. For E14.5 mouse embryos (14-16 mm depth blocks), we bake for 24 to 48 hours. The blocks will become soft and pliable after 5 minutes of baking, but harden rapidly after removal from the oven. Baking has no effect on the sample or subsequent image quality, but baking for excessive periods can produce brittle blocks that can be prone to accidental chipping or cracking.

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5. Cool the block in a fridge for several hours until completely hard.

It can be convenient to trim off much of the JB-4 cushion prior to HREM imaging. This can be carried out either on the HREM apparatus or using a manual microtome. Trimming can be performed either before or after baking and cooling of blocks. To measure the approximate cushion thickness, view the block on its side and illuminated from below in a bright light beam.

Occasionally, blocks can break during trimming. Excessive baking can result in brittle blocks and these can sometimes shatter, especially if the section depth is unusually large. SuperGlue is very effective for repairing broken blocks.

Image 1



Polymerised JB-4 is visible in the central hole and through the base of the chuck.

Image 2



Before (left) and after (right) trimming excess

# HREM IMAGING

Once embedded, samples are sequentially imaged using an HREM microtome with optics and camera.

## Equipment

Stereo dissecting microscope with a strong fluorescence light source

HREM microtome with optics filters and camera

A commercially available HREM setup is shown in Image 1.

## Procedure

1. First check that the block is sufficiently hard for use.
  - For all blocks, a simple test is to see how easily the block surface can be distorted by pressure from a fingernail. A hard block will show a minimal, discrete indentation or scratch whilst a soft block will distort to give a broad groove. For larger blocks, also test the response to pressure across the sides of the block, especially near the chuck. Hardened blocks resist pressure whilst with soft blocks a slight compression is discernable. Blocks that have previously polymerised hard (or have been baked hard) can become slightly soft during prolonged storage, either at room temperature or in the cold. We are not sure what causes this and in the UK, this seems to be more of a problem in the summer months. It can be solved by baking the blocks again.

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2. Illuminate the sample from the base and use a stereomicroscope to view the specimen perpendicularly, through the block surface.

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3. Scratch a bounding box on the block surface, containing the entire sample.
  - Because of the stereo optics, for an accurately positioned box it is essential to choose one side to draw and then repeatedly rotate the specimen by 90°, drawing the same orientation mark each time. Otherwise the images are likely to clip the sample on at least one of its edges.

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4. Turn the block so that the microtome blade makes contact with a narrow part of the block first, rather than a wide block edge. This minimises the chance of block breakage and reduces any section *chattering*. (Image 2)

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5. Use the bounding box to set up the image magnification for the HREM system. The bounding box should fill the image frame to ensure the maximum possible magnification.
  - Ensure the surface of the block is at the level of the blade before setting the magnification.

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6. For the highest quality images it is advisable to use a new section of blade for each sample.
  - Blade quality can be variable and you may need to avoid regions that give excessive scratch marks on the block surface.
  - We use tungsten carbide Leica Disposable Blade1s4TC-65 (barcode: 14021626379). Although disposable

they still cost around £100 each, so we use them as much as possible.

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7. Trim the remainder of the cushion using the HREM microtome until the sample can just be visualised.
    - Samples can be detected in the live camera image, a few microns before they reach the block surface, appearing as a faint but distinctive shadow. Alternatively, with direct visualisation of the block surface under GFP excitation wavelength light, the top of the sample can be seen as a distinct dark region immediately prior to its appearance at the sectioned surface.
  8. Ensure the focus is accurate using the imperfections in the surface of the cut block.
    - Before imaging, it 's often hard to see the sample as it is slightly below the surface, which makes it difficult to check if the optics are focussed correctly. However, by looking carefully it 's possible to find a focus plane that reveals very slight irregularities in the polymerised block surface; this is a fairly accurate focus position.
  9. Establish optimum illumination and exposure settings.
    - These can vary from block to block, and cannot be standardised accurately. Remember that the cushion/block interface can appear much brighter than the rest of the block.
  10. Set the desired section thickness.
    - We do not recommend sectioning thicker than 6  $\mu\text{m}$  and usually choose to discard captured data from analysis rather than increasing section thickness. Typically we section E9.5 embryos at  $\sim 1\ \mu\text{m}$ , E14.5 hearts at  $\sim 2\ \mu\text{m}$ , E14.5 embryos, E18.5 hearts and adult tissues at  $\sim 3\ \mu\text{m}$ .
  11. Use a small fan to ensure that sections or section fragments do not remain on the block to obscure subsequent images.
  12. Whilst sectioning ensure there are no fluctuations in lighting, as this can cause exposure changes that will be evident in the image data.
    - In our experience LED light sources give reliably reproducible exposures even with triggering. The ambient lighting should be kept constant if possible as changes will be detected in the image stack.
  13. Review focus position
    - After a reasonable portion of the sample has been imaged (eg 20 – 30 sections) the run can be paused to enable fine adjustment of the focus position.
  14. Check images for uneven 'ripples '
    - When you are imaging a block, a tell-tale sign of softness is the appearance of uneven 'ripples ' across the image. This may not happen at the beginning of the sectioning since the top of the block is the least likely to be soft, but it will become much more evident as sectioning proceeds towards the base. If you see this with a sample, it is prudent to rebake the entire batch of samples.
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## Using HREM data

Image data on the DMDD website is captured at 12 bit resolution to maximise the information obtained. This enables us to apply subtle adjustments to grayscale mapping in order to give the best visualisation of different tissues. The precise adjustments vary between datasets and we try to achieve a consistent appearance of the final images. Data is scaled to obtain cubic voxels and the resulting images converted to 8 bit for display.

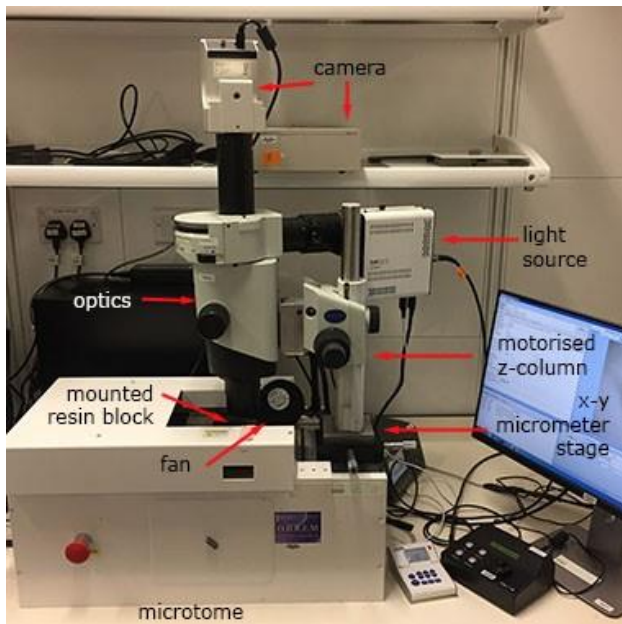


Image-1 - Typical setup of an HREM imaging system. For more information on HREM systems, visit Indigo Scientific (<http://www.indigo-scientific.co.uk/hrem-system>).

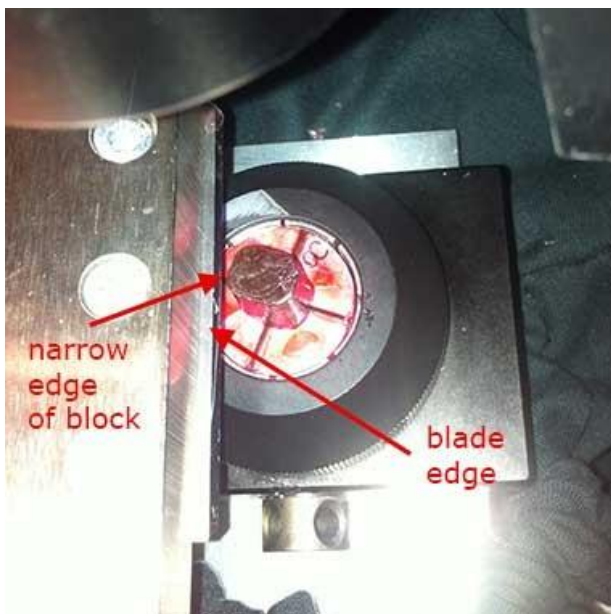


Image 2 - Block is turned so that the microtome blade makes contact with a narrow part of the block first, rather than a wide block edge.

## HREM FAQ

How long can I use JB4/dye mix?

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The manufacturer's advice for JB4 is to use it within a couple of weeks and addition of dyes doesn't seem to affect this. We have been keeping JB4 solution A for longer. We have not tested older mix. Any leftover old mix can be used for preparing 50:50 JB4/methanol or rinsing samples between the JB4/methanol step and infiltration with current JB4/dye mix.

Why is my JB4 polymerisation so slow?

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Normal polymerisation of JB4/dye mix taken directly from the fridge should begin within approximately 15 to 30 minutes. The mixture will begin to become viscous and if there is any surplus you should feel it begin to warm up. If the mix is taking substantially longer or does not polymerise fully at all check your components by testing polymerisation of JB4 without dyes. Incomplete removal of methanol during sample infiltration appears to inhibit polymerisation.

Why are my JB4 blocks soft?

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If the blocks are very soft and jelly-like or rubbery immediately after removal from the mould, there is likely to be something wrong with your JB4 mix or its components. Normal blocks will sometimes feel slightly "springy" when squeezed, especially if they are large in size. This can be cured by baking the blocks at 90 to 100 °C for several hours (conveniently, overnight). Blocks that have previously polymerised hard (or have been baked hard) can become slightly soft during prolonged storage, either at room temperature or in the cold. We are not sure what causes this and in the UK, this seems to be more of a problem in the summer months. It can be solved by rebaking the blocks. It appears that this may cause by humidity. Adding dessicant pouch in the box where the blocks are kept improve greatly this problem.

Why do my JB4 blocks break off their plastic chuck?

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This can happen when there is insufficient JB4 polymerised through the central hole and the crosshair gaps in the base of the chuck. Add a little fresh polymerisation mix to top up the base. If your sample has broken from the base, use abrasive paper to ensure the attachment surface of a fresh base is completely flat (- they are NOT flat from manufacture); do the same with the base surface of the JB4 block and then use SuperGlue contact adhesive to reattach to the plastic chuck.

How do I mend a broken JB4 block?

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Blocks can occasionally break during sectioning or trimming. Excessive baking can result in brittle blocks and these can sometimes shatter, especially if the section depth is unusually large. SuperGlue is very effective for repairing broken blocks.

Can I trim JB4 blocks?

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Yes. Bake the block for 5 to 10 minutes at 90 to 100 °C and it will become soft and pliable. It will remain like this for about 1 to 2 minutes after removal from the oven. During this time, it is possible to cut the block by firmly pushing a single side razor blade (or utility knife blade) through the block with a slight sawing motion. Be quick, as this rapidly becomes impossible as the block cools down and hardens!

Why do my images have a very bright region in the centre of the tissue?

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This is due to insufficient time of infiltration. The period is important not only for ensuring complete penetration by the methacrylate, but also for infiltration and tissue staining by the dyes. We do not fully understand why, but the latter process seems to be important for tissue visualisation and can take longer than the former. As a result, particularly dense tissue can show a very bright region in its centre with little or no imaging of the tissue. If this happens, you need to increase the infiltration period. (Note that prolonged infiltration times will also darken the overall grayscale range of the tissue in the image).

Why do my images have scratch lines?

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Straight scratch lines perpendicular to the microtome blade are caused by damage to the edge of the blade. These accumulate with use of the blade and are sometimes present even with a new blade. For optimal quality images and minimal scratch lines, we recommend using a new portion of blade for each sample.

Why do my images have ripple-like wavy lines?

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Irregular, wavy lines oriented roughly along the axis of the cutting blade are caused by soft JB4. This tends to happen when sectioning nearer the base of the block, probably because this region is more likely to suffer from inhibition of polymerisation by oxygen.