

The principle of tissue clearing

Biological tissue has fairly bad optical properties when it comes to whole-mount microscopy. This is mostly due to differences in refractive indices. The refractive index of a substance is defined as the ratio between light speed in vacuum and light speed in the medium (phase velocity of wave propagation). The higher the index, the more the substance slows down light. It is called “refractive” index because light that hits the interface between two substances with different indices will be refracted and propagate at a different angle in the second medium. Here are the refractive indices (n) of some substances of interest:

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|-----------------|------------|----------------------|------------|
| • Vacuum: | n = 1 | • Ethanol: | n = 1.361 |
| • Air: | n = 1.0003 | • 100% Glycerol: | n = 1.4739 |
| • Water: | n = 1.333 | • 80% Glycerol: | n = 1.4429 |
| • Glass: | n = 1.518 | • Methyl salicylate: | n = 1.5409 |
| • Immersion oil | n = 1.518 | | |

Because of refractive index differences between different parts of cells (proteins, water, lipids, etc.), light gets scattered a lot and tissue usually looks pretty opaque. In analogy, think of a water-oil emulsion. If you mix clear and transparent mineral oil with clear and transparent water and shake well, the mixture of tiny water and oil droplets will have very inconsistent refractive indices and will look opaque. Because of such optical properties, microscopic images will be fairly blurry if you just mount your specimen in saline or buffer. The way to remedy this is to clear the tissue by making the refractive properties more consistent. To illustrate the principle, think of white textiles or paper. They are white because they don't have chromophores that absorb light, but they are not transparent because the light has to pass through many layers of fibers and air, which have very different refractive indices. If the air is expelled, for example in a wet white shirt or a grease-soaked sandwich wrapper, the transparency increases substantially. There are basically two options to use the same principle to clear tissue. You can mount the tissue in glycerol or an aqueous solution of glycerol, or you can dehydrate the tissue in an ethanol series and mount in an organic solvent.

Glycerol vs Methyl salicylate

Glycerol: Using glycerol has two advantages. First, you don't have to go through the hassle of an ethanol series and you don't have to use stinky and hazardous organic chemicals. Second, for fluorescent stains, you can use anti-fading agents. Unfortunately, glycerol also has some disadvantages. Compared to organic solvents, its refractive index is not that high, and the tissue will clear less. Second (and more important), glycerol causes anisometric tissue shrinking that contorts the

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structure of the ganglion, particularly if it went through long processing protocols (e.g., immunohistochemistry) before. Therefore, if you really care about keeping the structure of the ganglion as close to its natural state as possible, you shouldn't use glycerol.

Methyl salicylate: This organic solvent, also known as wintergreen oil (used in chewing gums, Altoids, and topical pain relievers like IcyHot) has advantages over both glycerol and other organic solvents. Compared to glycerol, it has a higher refractive index that incidentally is very close to that of glass. This means that tissue clears very well and when using oil immersion objectives, there is pretty much no change in refractive indices in the whole light path, and therefore no problems with axial scaling caused by refraction. Second, if applied carefully (as described below), it causes very little anisometric shrinkage. The only real disadvantage is that the clearing and mounting procedure is much more elaborate. Compared to other organic solvents like xylene, toluene, or benzene, it is simply nowhere near as hazardous.

Permount: This is a toluene-based polymer adhesive that hardens and is intended for long-term storage of microscope slides. It preserves fluorescent stains well. In my opinion, it is nice to do that for non-fluorescent stains, but pretty unnecessary for fluorescent ones. Fluorescent stains will eventually bleach, anyway (either chemically or through imaging exposure). Once you took pictures or confocal scans, there is no real point in keeping the original specimen.

Clearing

The assumption here is that you start with a paraformaldehyde-fixed ganglion that is still pinned in a dish or to a piece of Sylgard and is sitting in phosphate buffer.

Clearing in glycerol: If you don't care too much about the general shape of the tissue and want to mount in glycerol, you can unpin the STG (the easiest way is to cut all nerves proximal to the pins), use forceps to transfer it to a microscope slide and mount in glycerol or any glycerol-based solution containing anti-fading agents. Put a drop of buffer on the microscope slide, place the ganglion in it, and use forceps or pins to straighten out the nerves (so they don't "hug" the ganglion). Then suck up the buffer with a piece of KimWipes and replace it with glycerol. Detailed instructions for mounting are described below

Clearing in methyl salicylate: If you do care about the structure of the ganglion and optimal transparency, you should clear the ganglion in methyl salicylate. Organic solvents are completely unpolar, so before mounting you have to dehydrate the tissue. Dehydration and clearing usually causes some amount of tissue shrinkage, but you can minimize this or at least make sure that shrinkage is fairly isometric by doing it carefully. Dehydrate slowly (30, 50, 70, 90, 100% EtOH, 5-10 min each). Ethanol is very hygroscopic, which means that you can't just use ethanol from a bottle. Fill a 200 ml glass bottle about halfway with large grain molecular sieves and then top up with 200 proof ethanol. Let it sit for

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a day and keep the bottle closed. The molecular sieve will retain any water in the solution, so avoid moving the bottle about too much. Always use a pipette or dropper, don't tilt the bottle! Even a tiny amount of water will mess up your prep when it is in methyl salicylate. For this reason, always apply fresh 100% ethanol before you add methyl salicylate. To do the transition between ethanol and methyl salicylate as gently as possible, you can use a "self-organizing concentration series". To this end, move the prep from 100% ethanol into a 50:50 EtOH:methyl salicylate mix. Make sure to use glass dishes from here on because methyl salicylate dissolves plastic. If you haven't done so already (e.g. for immunostaining in small volumes), cut out the piece of Sylgard the STG is pinned to and transfer it to a glass staining block (see picture, front) that you then fill with the EtOH:methyl salicylate mix. Place the dish immediately into a dry chamber to prevent the ethanol from drawing water. You can build a dry chamber easily out of a glass staining tank (see



picture, back). Fill the bottom with drierite dessicant and seal the lid with Vaseline. After 1-2h most of the ethanol should be evaporated, leaving almost pure methyl salicylate behind. If you do all this carefully, all you get is a little isometric shrinking. Then transfer the Sylgard piece with the STG to a Sylgard-coated glass petri dish and pin it down by inserting minute pins through the block into the Sylgard-coating. Put a drop of pure methyl salicylate on top. It is important that the ganglion never falls dry! For some reason, air exposure causes the tissue to shrivel. Now cut the nerves proximal to the pins that hold them. Don't use your nicest scissors for this and make sure to rinse of the methyl salicylate with ethanol after! You will notice that

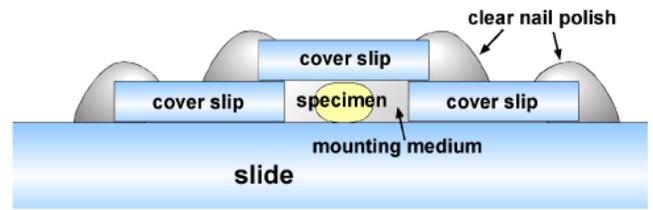
by this time you will actually have to play with the light to see the very clear ganglion. In addition, the tissue has hardened (and the nerves are not flopping around anymore!). To avoid air contact during transfer to the slide, you can use the "gurney method". Break a cover slip and select an appropriately sized shard. Place this shard under the STG (all under methyl salicylate!) and place it on the microscope slide. You can then carefully slide the STG off the shard and remove the latter.

Mounting

For microscopy, you want to make sure that you can use the best possible objective. Higher resolution objectives have fairly short working distances and you don't want to end up not being able to use one of those just because your cover slip is too far from the tissue. Most objectives are optimized for spherical corrections for a 170 μm cover slip (but some have a corrective collar) and if you deviate from that you will lose signal. Unless you have a very weak signal, you can compromise on that with a thinner cover slip to win working distance. Most companies have cover slips in different thickness ranges. I recommend ESCO No. 0, which reliably seems to be 100 μm thick. It's nice to have a range of different

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ones around, because you can also use them as spacers to keep the cover slip from touching and squishing the specimen (see figure below). Make sure that both the slide and the cover slips are dust-free. If they are not, wash with ethanol. Glue 2 coverslips on a microscope slide with nail polish from the top, leaving a gap between them large enough to accommodate the STG. Carefully position STG and nerve the way you want them. This is usually easier when it is sitting only in a small amount of mounting medium. When done, add more mounting medium. Placing the cover slip on top without creating air bubbles is easier when you use enough medium to ensure that it extends over the level of the spacers. The surface tension of the medium ensures that it is not overflowing. Carefully place the cover slip so one edge touches one spacer. Then lower it until it touches the medium and drop it. Hopefully, there will be no air bubbles. Now remove excess mounting medium with a little piece of KimWipes held to the edges of the cover slip. Use the back end of your forceps to press down on the cover slip edges



(where it touches the spacers) to make sure the cover slip is not still floating on excess medium. Finally, seal everything with nail polish. If using methyl salicylate, make sure it's clear nail polish; otherwise the color will dissolve into the medium. Methyl salicylate and other organic solvents will eventually eat through the nail polish. If you need to keep your slides for longer, apply fresh nail polish every couple of days.