

Lectin stain for retinal vasculature (flat-mounted retinas of rats or posterior eye cups of mice)

Retina Preparation (Mouse):

Equipment:

- Dissecting stereo microscope
- Goose-Neck with flexible Arms
- Dental Wax
- Dumont number 5 forceps (Dumont #5, Dumoxel, non-magnetic, stainless steel, 11 cm long, 0.1 x 0.06 mm tips [World Precision Instruments 14098])
- Dissection scissors (82mm long, Sharp tips, straight, 9.5mm, Titanium [World Precision Instruments 555640S]).
- Needle (26-30 G)
- Scalpel blades (no. 11 for mouse, no. 22 for rat)

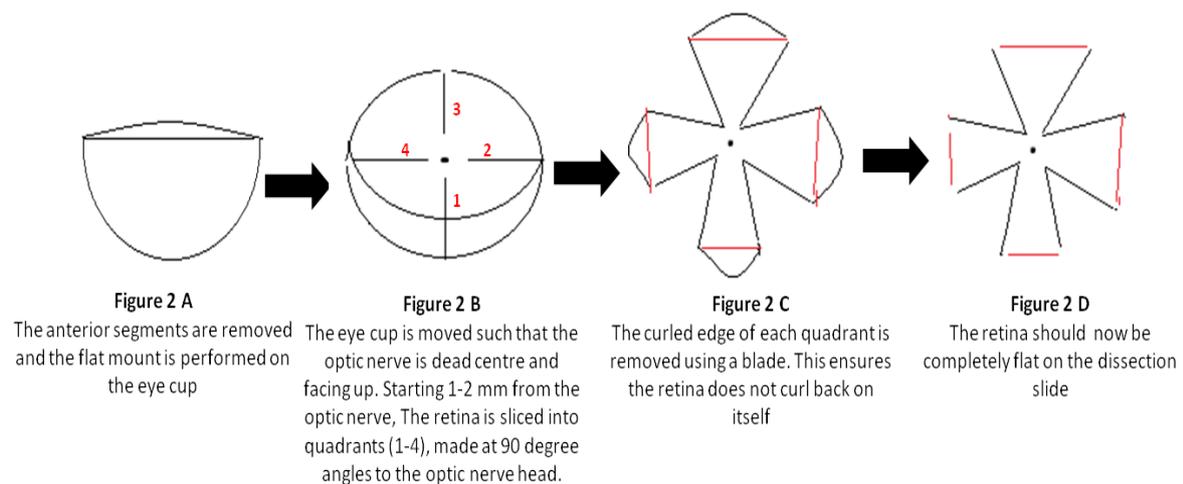
1. Remove the eye of mouse by putting a curved forceps right underneath the eye and pulling the eye gently up.
2. Fix eyes in 4% PFA (usually 4 hrs room temperature or overnight at 4°C)
3. Was in 1 X PBS twice.
4. For dissection use a dissecting stereo microscope with an external light source (I use Gooseneck Flexible Arms).
5. Get a sheet of dental wax and with the end of a paint-brush or pen push into the wax to make a circular depression.
6. Place a mouse eye within this depression and add a drop of PBS to keep moist.
7. Under the microscope, using a Dumont no. 5 forceps, rotate the eye such that you can see the back with the optic nerve pointing up. Grip the eye with the forceps and take a small micro-dissection scissors, carefully cut away all the tissue and muscle surrounding the back of the eye, cut off the optic nerve. This is very important as if there is any tissue remaining on the back of the eye the retina won't be perfectly flat when mounted.
8. Using the forceps gently rotate the eye such that the front (cornea and lens) is pointing towards you. While still maintaining a grip on the eye, take a 30 G needle and puncture the eye right at the front of the pars planar (White ring separating the posterior eye from the anterior)



Figure 1: The white stripe (Pars Planar) divides the back and front of the mouse/rat eye. The needle should pierce right on the front of the Pars planar (the anterior side of the eye).

9. Place dissection scissors within the puncture, and rotating the eye using the forceps cut around the eye, until the entire anterior segment (the entire bit in front of the pars planar) has been removed.

10. Gripping the back of the eye cup with a forceps, use another forceps to gently push out the lens.
11. Place the eye cup on a dry glass slide and under the microscope using the forceps manipulate the eye such that the hole left by the optic nerve (optic nerve head) is facing directly up and centre (Figure 2A)
12. While firmly gripping the eye cup using the forceps, take a no. 11 scalpel blade, place perpendicular to the optic nerve head and make a firm incision approximately 2 mm away from the optic nerve head slicing right through the edge of the eye cup (peripheral retina). (It is important you do not make this incision too near the optic nerve or you will completely sever each quadrant of the retina (Figure 2B (1)).
13. Rotate the glass slide 90 degrees and repeat the incision (Figure 2B (2)).
14. Rotate glass slide another 90 degrees and repeat the incision (Figure 2B (3)).
15. The glass slide is rotated a further 90 degrees and the final incision is made, this should divide the retina into quadrants (Figure 2B (4)).
16. Using the forceps to hold each retinal quadrant, carefully cut away (with the no. 11 blade) the very outer edge (the curved region) of each retinal quadrant to ensure that the edge of each retina does not fold back on itself when flatmounted (Figure 2C).



17. Each retina should now be divided into quadrants and be perfectly flat (Figure 2D). Further incisions can be made to flatten out the retina if necessary.

Retina Preparation (Rat):

1. Remove the eye from the rat by placing a curved forceps underneath the eye and cut the eye away from the ocular muscles and cartilage at the back of the rat eye. When this has been done pull the eye away from the socket using the forceps.
2. Wash the eye in 1X PBS
3. Fix eye in 4 % PFA for 2 hours at RT
4. 5-10 mins into PFA fixation protocol cut incision in the cornea of the rat eye (to ensure PFA gets in!)
5. Wash eye 2 X 1X PBS

6. Remove the anterior segments and lens of the rat eye (identical to steps 4-8 of mouse protocol, except a larger scissors is used (I just use a nail scissors).
7. Take a curved forceps and put it between the sclera and the retina (one point between sclera/retina the other outside sclera), hold very tightly. Then, take a Dumont no. 5 forceps and also place that loosely between the sclera and the retina again with one point between sclera/ retina the other outside the sclera (**DO NOT HOLD THE RETINA**). Slowly pull the curved forceps such that it causes the Dumont no. 5 forceps to slide along the eye, detaching the retina from the sclera (The action might be similar to grasping a piece of paper between your thumb and forefinger of one hand and gently pulling it through your grasp with the other hand. So that your thumb and forefinger slide along the paper). Finally, work the choroid off and pinch off where it connects to the optic nerve. It should just pop off and there is the retina
8. Flat-mount using a no. 22 scalpel blade (steps identical to steps 11-16 of mouse protocol).

Lectin Staining the retinal vasculature

Materials:

- Perm/Block buffer is PBS-containing 0.5% Triton-X100, 1% goat serum, and 0.1 mM CaCl₂ (11 mg/l). Add 0.05% sodium azide (0.5 g/l) if buffer is to be stored.
 - GS isolectin B4 (Sigma L21240): Stored in 1 ml aliquots
 - Normal Goat Serum (NGS): Sigma Goat serum G9023-5ml
 - Alexa-streptavidin (Alexa-488-streptavidin for green and Alexa-568-streptavidin or Alexa-594-streptavidin for red)
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1. Immediately after retina dissection, permeabilise/block each sectioned retina in Perm/Block buffer for 4 hrs at 37°C, 8 hrs at room temp or overnight at 4°C (use these times for all steps below except where specified differently).
 2. React with GS isolectin B4 diluted 20 µg/ml in Perm/Block buffer. If frozen lectin aliquot doesn't contain goat serum, add NGS to give a final concentration of 1%. React for 4 hrs at 37°C, 8 hrs at room temperature, or overnight at 4°C.
 3. Wash in Perm/Block buffer for 4 hours at 37°C, 8 hours at room temperature (2 changes of buffer/hour), or overnight at 4°C.
 4. Stain with Alexa-streptavidin diluted 1:500 in Perm/Block buffer in dark for 3 hrs at 37°C, 6 hrs at room temperature, or overnight at 4°C.
 5. Wash in Perm/Block buffer for 4 hours at room temperature (2 changes of buffer/hour)[keep in dark].
 6. Wash in PBS with only 0.1 mM CaCl₂ added (**no** Triton or goat serum)[can be kept in dark for up to a week]

Stain retinal whole-mounts in 96-well plates. Never use less than 100 µl for staining steps and ensure immersion after solution changes. For washes use 200 µl changes, to ensure removal of any dried solutes at liquid-air interface. Withdraw solutions by placing pipette tip at corners of wells; avoid touching specimens until mounting.

Retinal Flatmounting and coverslipping:

Materials:

- Cocktail sticks
 - Glass slides
 - Coverslips
 - Aqua-Poly/Mount non-permanent aqueous mounting medium, anti-fade (PolySciences, Inc 18606-20)
1. Using a cocktail stick, gently pick up the retina and place on glass slide. Ensure that the retina side is facing up (For mouse the underside will be black).
 2. Flatten out the retina on each slide, taking care to remove any debris, using pipette gently remove all liquid from the flat retina (this should ensure the retina straightens out).
 3. Place a drop of Aqua-Poly/Mount (or Vectashield) onto the top of each coverslip.
 4. Holding the coverslip at a 45 degree angle, gentle release down onto the retina. Ensure each retina is adequately covered with Aqua-Poly/Mount solution.
 5. I mount the left and right retinas from each animal on the same slide.
 6. Can add more mounting solution by placing solution around the edges of the cover slip – the solution will enter underneath the coverslip by capillary action.
 7. Leave to dry overnight in the dark.
 8. Image using a epifluorescent microscope with software capable of stitching segments of the retina together in a montage.

Microscope

- I use the Zeiss AxioVert 200M Fluorescent Microscope
- It is fitted with the Andor iXon 885 EMCCD for high resolution imaging providing frame rates of up to 10 frames per second. Align and merge optically split images in IQ and downstream analysis with IMARIS software
- I image my retina using the LED that reads RFP at 10X, after defining the edges and focal points of each retina I set up my montage to enable the microscope to stitch together the entire stained retinal flatmount using 16 segments.
- The avascular/neovascular regions of each retinal flat mount are quantified using Image J software.

