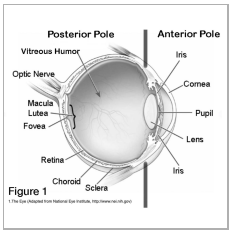

Figure 1

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We use this protocol and it's working

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UAB-VU BIOMIC Preparation of Left Fresh Frozen Eye

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VU Biomolecular Multimodal Imaging Center / Spraggins Research Group

Human BioMolecular Atlas Program (HuBMAP) Method Developmen

Jamie Allen
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ABSTRACT

Scope:
This protocol describes the process of procuring donor eyes from Advancing Sight Network, the processing and freezing of the left eye, and the sectioning of tissue for the HuBMAP Eye project.

Glossary of Terms (Figure 1):

1. **Anterior**- Refers to the front part of the eye – right side of Figure 1 – the light shines in from this aspect
2. **Anterior segment** - includes iris, cornea, pupil aqueous humor, and the conjunctiva (not shown).
3. **Cornea**- Clear, and continuous with sclera; allows light to enter into the eye to the retina, at the back of the fundus.
4. **Choroid**- Vascular bed of blood vessels to nourish the posterior half of the retina.
5. **Fovea**-Small depression in the retina, in the back of the eye, specialized for detailed and color vision.
6. **Fundus**- A term used for the lining of any organ. The ocular fundus consists of the neurosensory retina, RPE (retinal pigmented epithelium) and the choroid.
7. **Iris**- Controls the amount of light entering the eye.
8. **Lens**- Changes shape to allow viewing of close or distant objects.
9. **Macula lutea** – yellow spot due to xanthophyll pigments, containing the fovea
10. **Optic nerve**- Transfers visual information from the retina to the brain.
11. **Posterior**- refers to the back part of the eye – left side of Figure 1.
12. **Posterior segment -** All parts of the eye that are located behind the lens. Usually referring to the retina between the optic disc and the macula.
13. **Pupil**- Opening formed by the iris, varies in size with ambient light.
14. **Retina**- lining the fundus in the back of the eye; receives light signals and convert them into neural signals and send those signals through the optic nerve back to the brain.
15. **Sclera**- White part of the eye that maintains its shape, for optical alignment and muscle attachment.

MATERIALS

Reagents:

1. Tissue marking dyes black, red, yellow, Cancer Diagnostics, CAN MD2000
2. Liquid Nitrogen
3. Fish gelatin, Sigma, G7041-100G
4. Poly-L-Lysine solution, 0.1%, Sigma, P8920

Materials:
1. Workspace for GLP and BSL 2/2+
2. -80°C freezer
3. 18 Gauge needle BD Vacutainer™ Syringe Needle, Fisher, 23-021-020
4. Dewar
5. Narrow Mouth Bio-Tite™ 40mL (1 oz.) 48mm Specimen Containers, Fisher 13-711-86
6. Peel-A-Way Embedding molds, 22x30mm, Polysciences, 18646B
7. Stereo Microscope, Nikon, SMZ 1000
8. Allis tissue forceps, Jarit 135-095
9. Bitran Freezer Bag, Fisher, 19240150
10. Cryostat, Leica, CM3050-S
11. Superfrost Plus Gold slides, Fisher, 15-188-48
12. Indium-Tin-Oxide (ITO) coated slides, Delta Technologies, CG-801N-S115
13. Vacuum packing unit, FoodSaver
14. Absorbent bench underpad, VWR, 56616-031
15. Dremel Rotary Tool, Dremel, 3000
16. Dremel Multi Vise, Dremel, 2500-1
17. Dremel Rotary Shield, Dremel, A550
18. Dremel 545 Diamond Wheel, Dremel, DCW0025
19. Dremel 402 mandrel, Dremel, 402
20. Zeiss Axio Z1, Zeiss, 430038-9001-000
21. Zeiss Colibri LED light source, Zeiss, 423052-9710-00
22. Optimal Cutting Temperature Polymer, Fisher SH75-125D
23. Foodsaver V2244 vacuum sealer
24. Food-grade vacuum freezer bags (many manufacturers)

Reagent Preparation:
1. 15% Fish Gelatin (made day of use)
   Warm 100 mL of milliq H_2O
   Add 15g fish gelatin
   Gently mix until gelatin is completely dissolved
   Keep cool until use

2. Poly-L-Lysine Coating Slides
   Dilute the poly-L-lysine solution 1:10 in water and dilute 1:10 again
   Place ITO slides in diluted poly-L-lysine solution for 5 minutes
   Remove slides and allow to air dry
   Place ITO slides in diluted solution again, for 5 minutes
   Use an air line to blow off access solution
   Place in 37^{o}C oven to completely dry

SAFETY WARNINGS

1. Use Biosafety 2 precautions with human samples when cutting the globe and sectioning in the cryostat.
2. Use (at minimum) a chemical fume hood when cutting tissue with a dremel.

Positioning and Marking Globe

1 Whole donor globes on wet ice are received within less than 6 hours' time of death (TOD) from the Advancing Sight Network (500 Robert Jemison Rd Birmingham, AL 35209).

2 Laterality is verified using extraocular anatomy.

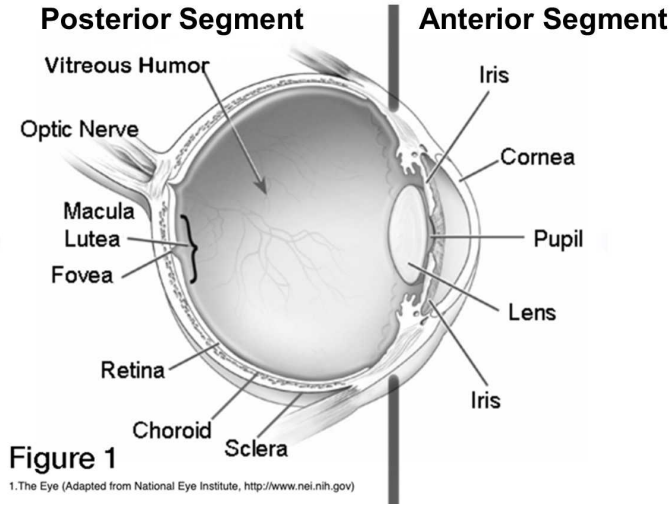


Figure 1. Division of the anterior and posterior segment during tissue processing. The anterior segment contains the cornea, iris, ciliary body, and lens. The posterior segment contains the retina, fovea, choroid, sclera, and optic nerve head.

3 Tissue orientation is preserved using tissue marking dyes in black, red and yellow (identifying the superior rectus muscle, approximate location of fovea, and optic nerve head respectively)

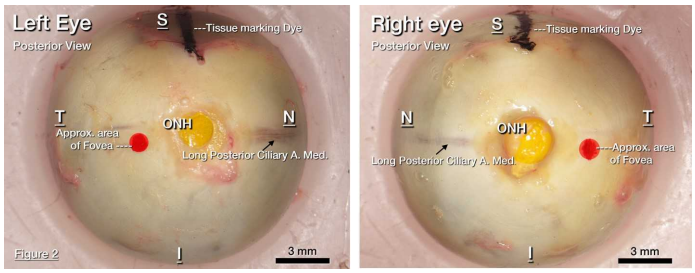
3.1 

Figure 2: Posterior view of excised ocular globes, as if one is standing behind the patient. The superior portion of the eye is marked in black. The yellow marker demonstrates the location of the optic nerve head (ONH). The red marker demonstrates the approximate location of the fovea. Superior, inferior, nasal and temporal (S, I, N, T) refer to the 4 ocular quadrants. The long posterior ciliary artery divides the superior and inferior hemisphere.

Freezing of Globe

4 Breach the vitreous cavity to create a pressure release upon quick freezing as follows:

4.1 Insert an 18 gauge needle 5 mm slightly tangential to the sclera. The puncture should be made into the black tissue mark in the posterior to anterior direction.

Insert just to the point where the tip of the needle penetrates the sclera and breaks into the vitreous humor.

Figure 3. Superior aspect of the globe. In the left panel, the black tissue marking ink divides the superior quadrant of the globe into anterior and posterior portions. In the right panel, the needle is inserted into the sclera, parallel to the tissue mark from the posterior to anterior direction. Once removed, a lumen is formed to allow internal expansion of vitreous exiting during flash freezing.

4.2 A slight leakage of the vitreous fluid will occur when the needle is retracted.

5 Create a double-stacked cryomold by carefully removing the bottom of a Peel-A-Way embedding mold using a razor blade or similar. It will stack neatly on top of a second cryomold. This allows for sufficient height within the molds to fully embed the tissue.

Figure 4: Example of a double-stacked, filled cryomold

6 The molds are filled with 5 mL of cold 15% fish gelatin. Liquid nitrogen is used to semi-freeze the fish gelatin until semi-solid and tacky for sample adherence to the cryomold. Use forceps to suspend the mold within the liquid nitrogen, ensuring that it does not flow over top of mold.

6.1 Using a stereo microscope, place the whole globe into the anatomically labeled double-stacked cryomold matching the tissue dye orientation. The globe is oriented so that the superior edge (black tissue mark) faces upwards within the cryomold and the cornea faces the shorter, 25-mm, end of the rectangular mold.

6.2 Fill the mold with 15% fish gelatin.

6.3 Quickly freeze embedded globe with liquid nitrogen. Use forceps to suspend the mold within the liquid nitrogen, ensuring that it does not flow over top of mold.

6.4 Place in Britran Freezer bag and store in -80°C freezer to await shipment.

Globe Dissection

7 In order to obtain reproducible sections with minimal warping, the embedded globe is dissected into two halves: the anterior segment and the optic nerve/macula regions. All cutting must be performed in an enclosed container within a HEPA-equipped fume hood to prevent contact with donor tissue. Proper personal protective equipment including face shield, gloves, Tyvek IsoClean Sleeves, and lab coat must be worn at all times in accordance with BSL2 certification.

8 First, the anterior segment (containing cornea, iris, lens, ciliary body, limbus) is removed with a Dremel rotary cutting tool.
The Dremel 545 Diamond Wheel and rotary shield are attached to the cutting tool
The tool is placed in a fixed position using a Dremel Multi Vise that is modified to have a weighted stand as shown in Figure 5:

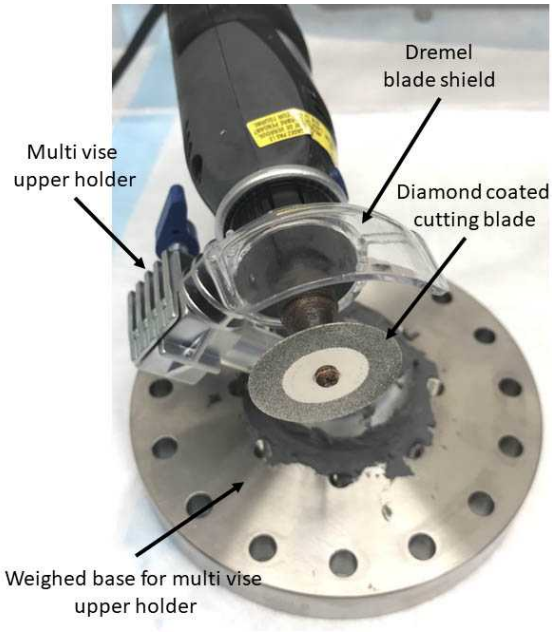


Figure 5: Dremel apparatus

9 The stationary Dremel cutting tool apparatus is placed below a rectangular acrylic shield and wrapped in an absorbent disposable bench under-pad. This protects the tool from contamination with tissue.
Dry ice is placed below to aid in keeping the sample frozen while cutting as shown in Figure 6:

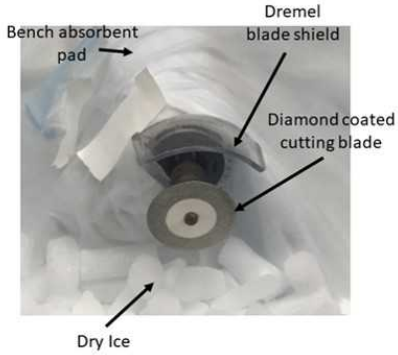


Figure 6: Dremel cutting tool prepared for cutting frozen tissue

10 The embedded globe is cut by carefully rotating the block on the cutting wheel along the indicated black line (Figure 7) to separate the anterior and posterior segments of the globe as shown in Figure 8:

Figure 7. Example of embedded eye in double stacked mold, black line indicating where to cut with Dremel tool to separate the anterior and posterior segments.

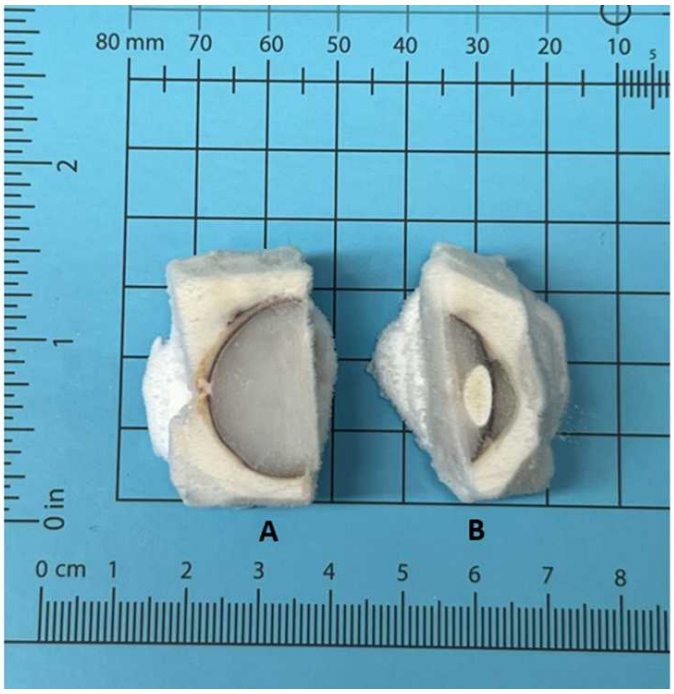


Figure 8. Halved ocular globe with scale measurements
A. Posterior segment containing neural retina with optic nerve and macular
B. Anterior segment containing ocular lens, cornea, iris and associated supporting anatomy.

11 Place both halves back into cryomold. Making sure to preserve the orientation by placing them back in the original position in which the whole globe was removed. Wrap in foil and store in the -80°C.

Sectioning the Globe Blocks

12 Before sectioning, the globe halves are removed from the -80°C freezer and left in the cryostat chamber for 30 minutes to equilibrate to -20°C.

13 Mount the posterior globe block onto a cryostat chuck using polyethylene glycol (Optimal Cutting Temperature Polymer) ensuring good attachment.
Note the orientation (nasal-to-temporal cross-sectional cut) markings on the mold that match the optic nerve head and fovea. Use these and the inked landmarks to mount the block half with the nasal side towards the cutting face (Figure 9).

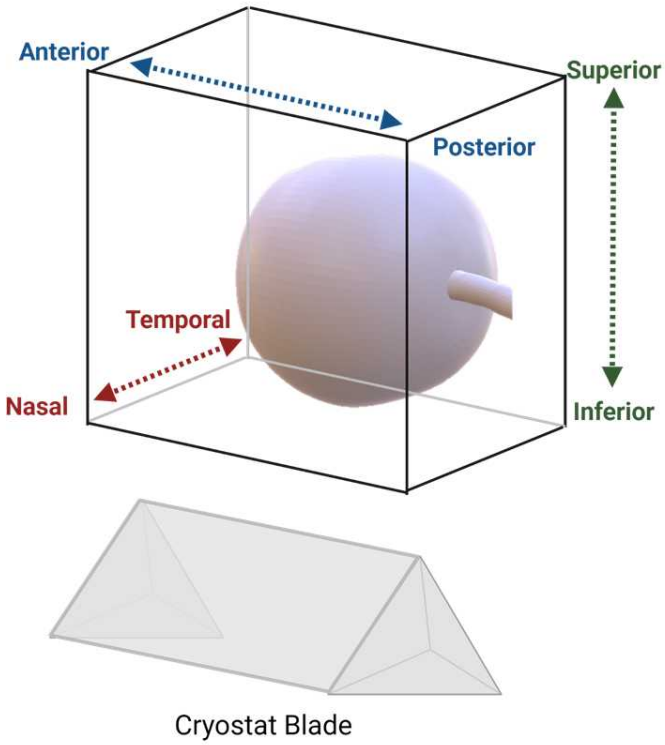


Figure 9. Schematic of tissue block mounting orientation for the posterior segment during cryosectioning relative to the sectioning plane (ie cryostat blade).

13.1 The tissue is trimmed by sectioning relatively thickly (up to 20 µm) until the yellow dye is seen (and extension of dura mater along the length of the nerve).

Locate the optic nerve head by noting how the optic nerve protrudes and invaginates through the RPE layer. The optic nerve vein and artery should be located approximately 800µm from the beginning of the optic nerve head.

13.2 Sections through the optic nerve were then collected for all analysis modalities: Imaging Mass Spectrometry, CODEX, lipidomics and proteomics. Sections for other modalities may be collected as needed.

*Note: sections placed on slides must be thaw mounted onto slides until dry. Sections for IMS are vacuum desiccated for a minimum of 30 minutes. Proceed directly to step 15.

13.3 Continue sectioning an additional ~1.5 mm to reach edge of the macula. Confirm the macular location using a light microscope to look for the Henle fiber layer (a layer of neuronal and glial processes).

Collect sections for the same modalities as in step 13.2. When finished sectioning, remove block from chuck and place what is left back into the mold.

*Note: sections placed on slides must be thaw mounted onto slides until dry. Sections for IMS are vacuum desiccated for a minimum of 30 minutes. Proceed directly to step 15.

14 Mount the anterior segment block onto a cryostat chuck with the temporal side facing the observer and the nasal side on the mounting surface (Figure 10).

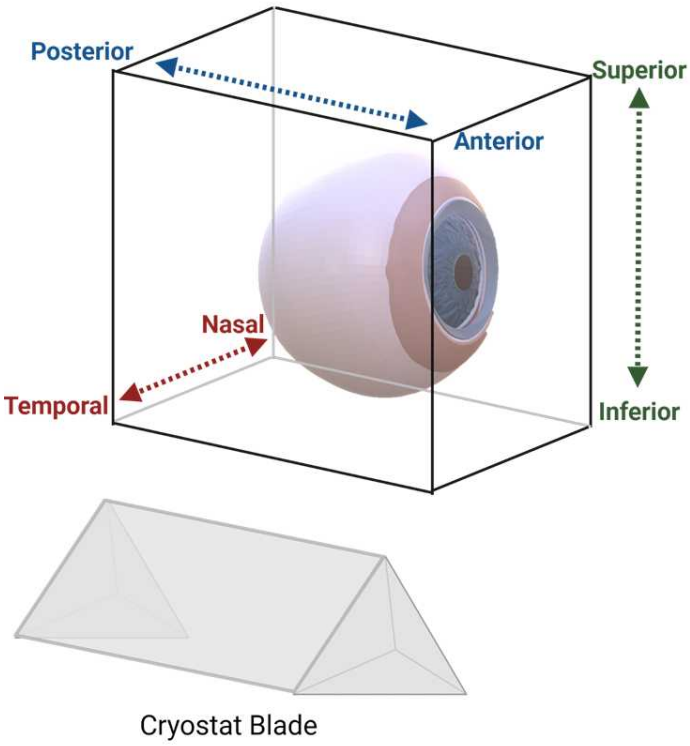


Figure 10. Schematic of tissue block mounting orientation for the anterior segment during cryosectioning relative to the sectioning plane (ie cryostat blade).

14.1 Trim the block until the ocular lens is visible as this indicates the central region of the anterior segment. Use the appearance of the iris and the pupil (gap in the iris) to help aid in the start of section collection. Collect suitable sections of the entire anterior segment with minimal wrinkling or warping.

*Note: sections placed on slides must be thaw mounted onto slides as flat as possible until dry. Sections for IMS are vacuum desiccated for a minimum of 30 minutes. Proceed directly to step 15.

14.2 When all sections are collected, place the block in the cryomold with the remaining posterior block.

Autofluorescence and Storage

15 Autofluorescence microscopy images are collected on all sections before analysis. If slides are frozen, place them in a vacuum desiccator for 20 minutes to come to room temperature and completely dry.

Protocol

NAME
Autofluorescence Microscopy Data Acquisition

CREATED BY
Angela Kruse

PREVIEW

When finished, slides placed in slide mailers and sealed in food-grade vacuum freezer bag using a food-grade vacuum sealer. Slides are then stored at -80°C to await analysis.

16 Sections for IMS analysis are now ready for matrix application (via Sublimation) or other omics processing.

Protocol

NAME

Matrix Sublimation via In-House Developed Sublimation Apparatus

CREATED BY

Katerina V Djambazova

PREVIEW