**Protocol for Human Eye Preparation for Posterior Scleral Sample Inflation and LSM Imaging of the Lamina Cribrosa and Peripapially Sclera Using SHG & TPF:**

*DM, last updated: 12/20/17*

**Day 0: Preparation**

* Human Donor tissues are received from active eye bank protocols such as the National Disease Research Interchange (NDRI) or Eversight Eye Bank once a valid donor has been identified. We usually request 1 eye pair each week we intend to experiment.
* You should ensure that a time for imaging exists within 48 hours after patient death. NDRI will tell you over the phone when that was and you should immediately check the Wilmer Microscopy Calendar to ensure a time for imaging is available. These imaging sessions are very long (4-6 hours) and thus usually require imaging in the evening when most other researchers have retired for the day.
* Cause of death usually will not affect our experiments but ocular globes should be intact with at least 10 mm of optic nerve left for sampling and the patient should not have diabetes.
* Inform the front administration office of Latrobe that you are expecting a perishable package the next day and to check up on it with the Keswick mail delivery service and make sure it gets here before noon. They are often very bad at getting things here early so there is no point in rush shipping for the 8:30 delivery spot. They will not distribute the package to Latrobe until after 10:30 AM.
* Check and make sure there is 2% PFA in the perfusion lab fridge to use the day before eyes arrive. Mix more using the Quigley lab protocol if there isn't as this is required for preserving nerve samples and the specimens after testing.
* (optional) If planning a treatment experiment (where you image before after chemical treatment), prepare necessary buffers and agents beforehand that will last more then a day. (See Barbara's treatment protocol for ChABC and sGAG degradation as an example)
* Make sure you have: custom gimble-mounted ocular inflation chamber, attached polyethylene lines with male/female connectors, polycarbonate posterior scleral mounting rings, O-ring seals, large 30 ml syringe, and Permabond 910 tissue adhesive. I usually leave these supplies at the Quigley lab during times the donor protocols are active, but they should be returned to the Nguyen Soft Tissue Lab at Homewood after these phases.

**Day 1: Posterior Sclera Specimen Preparation**

* Eyes will arrive usually at 10:30 AM the following morning from the call. You will receive an email that a perishable package has arrived in Latrobe and request that it be picked up at the administration front office desk immediately. Pick it up and take it to the Nguyen Tissue lab. Open the cooler package briefly with a box cutter to ensure that there are 2 human eyes.
* Reseal the package and carry it the JHMI with ice intact to keep the specimens chilled at near 2C. If independent transportation is not available the Hopkins shuttle can be taken at 11:00 AM to arrive at JHMI at 11:30 AM.
* Specimens should be removed from the cooler package and containers for the right and left eye labeled with tape and sharpie and kept in the 2C fridge until worked with.
* Put one eye in the 2C Fridge and the other on the table under the scope in the perfusion room
* Use the general supplies scalpel and tweezers kept behind the work desk near the sink to clean the remaining extraorbital tissues around the optic nerve head.
* Take a nerve sample: Wrap the eye in wet gauze, hold it in place, and place rolled wax underneath the optic nerve.
* Use a razor blade to cut a sample 5 mm posterior to the surface of the sclera, mark the superior orientation, note LE/RE, and note the orientation (back/front) and place in a small glass vial of 2% PFA in the fridge to fix before cutting further as these samples are usually too squishy to work with before fixation
* Refer to the human eye log book and enter known info from the NDRI info sheet that came with your specimens. Label with latest number HXXXX LE/RE and your name and date.
* After about 1 hour the nerve sample will be fixed. Slice off the most posterior part of the length, leaving a sample disc that is about 2 mm thick. Make a single small cut with the razor blade through the disk in the superior orientation. Make two small cuts with a razor blade through the disk in the nasal orientation.
* Place the labeled bottle with the cut disc nerve sample in the fridge in the human nerve sample cardboard holder container and inform Quigley lab there is a new nerve sample that is not graded. They grade these in batches every few months to assess the degree of vision loss in the donor.
* Measure the axial length, nasal-temporal length, and superior-inferior length of the eye: Use calipers 3 times and record the results. Take the mean of these measurements to estimate the true dimensions of the eye. Measurements should be taken when the eye is under uniform pressure and without wrinkled. Use a syringe if necessary to inflate the eye if it is low on pressure after shipping.
* Mark the superior direction on the eye using the marker
* Using the scope cut through the prelaminar tissue until LC is exposed, investigate under the scope after each cut. Prelaminar tissues are white and when you get near the LC the tissue will start to turn gray and beams will appear. You want the white to be gone and the beams to be visible all across the LC. Try to take a very level cut of the LC and the surrounding PPS for best imaging.
* Take out the second eye and repeat previous steps
* If the cut is good then pick a polycarbonate ring and glue the specimen LC-side up and centered in the best-fitting plastic ring. Allow 10 minutes to dry while applying pressure to make the seal good. Apply periodic drops of buffer to the LC to keep moist but do not let it get near the glue for 5 minutes.
* Once dry, make a perimeter cut about 2 mm anterior to the equator and remove the cornea, choroid, retina, lens, and aqueous humor.
* Cut the exposed sclera into strips and glue them down along the periphery with enough glue to seal. Apply pressure for 10 minutes with the LC-side down and placed in a dish of buffer to keep hydrated. Use extra glue to ensure the seal is good.
* Allow it to dry an additional 10 minutes unattended LC-side down in a plastic dish to keep the posterior-side hydrated.
* Open the holder, fill 4/5 of the way with PBS, and insert the posterior scleral eye holder. Replace screws, tightening in a clockwise fashion gently.
* Flush the lines and reservoir of air by attaching a syringe full of PBS to on line. Tilt the holder upward so the draining line is at the top of the reservoir and open the second line to drain into the sink. Push the PBS through the system using the syringe till no air bubbles are visible under the clear holder. Close the draining line.
* Pressure test by gently applying pressure using the syringe. If eye inflates smoothly under syringe pressure with no leaks then remove the ring.
* Select a reservoir cup large enough to contain the ring and sample and fill with enough buffer to cover it. Put cover over it.
* Repeat previous sample preparation steps with the second eye
* (optional) Begin incubating specimens in a trizma buffer solution in the warm room if this run requires chemical treatment, if not, skip this step.
* If it passes the pressure test carry the holder and extra PBS to the confocal room.

**Day 1: Posterior Sclera Imaging**

* Start the first sample equilibrating at 45 mmHg on the counter by connecting the pressure lines on the sample holder to the adjacent water column, and sliding the open top up to 45 mmHg pressure
* Hold the pressure at 45 mmHg to see if any leaks start at the higher pressure as you set up the equipment and align the specimen. Periodically check to see if the water line is falling and leaking into the holder reservoir. If the eye has a bad leak it cannot be used. If the eye has a slow leak (falls a few mmHg over a few minutes) you can top-fill it during equilibration and image anyway, though your imaging errors will be a bit higher. Ideally it will not leak at all.
* Go through the start-up protocol as specified in the hand-out next to the LSM 710: replace condenser with inverter arm, align arm, turn of parfocality and stage motor, record the time and lamp time, and load the inverted setup file, then start up Zen, set the #3 imaging objective to 10x M27 W, and replace the 20X with this objective. You must be trained in this imaging protocol to use the machine. Currently either Rhonda or Mary Ellen Pease give training and are familiar with this imaging protocol.
* Insert the sample onto the stage under the objective and dip the objective into the reservoir to ensure continuous water. Top-fill the the holder until the specimen surface is immersed.
* Lower the pressure to 10 mmHg and record the time
* Click reuse on past SHG/TPF settings for a Human eye image. You can use any CZI file contained in my past imaging sessions (see data backup drives for original Zeiss CZI files where imaging settings are stored). Make sure two channels containing SHG & TPF are being recorded
* Locate the LC using DAPI and the eye scope and center objective over area.
* Put dark cover on and put out the lights and scan area using SHG and TPF
* Alter gain/power until a good balance of contrast is found without oversaturation.
* Set tiled images to 2×2 and 15% overlap
* Correct for tilt iteratively using the screws on the side of the sample holder until the stitching looks close to correct.
* Use the tilt-correcting gears on the sides of the holder to level the specimen as best possible so the most areas come into focus in the LC at once as is possible
* Sometimes automatic rotation calibration works for these images but usually not. I ended up guessing the rotation offset value in the tiled Z-stack settings and arriving at a suitable value (usually 1-3 degrees if you aligned the sample well, Ask mel for questions about this process). You can guess in increments of 0.5 degrees until you see the 4 images align at all 4 image borders, then switch to increments of 0.1 for fine adjustment. Stitching will then combine these images before export. Try stitching early if you want to see how it will look in the final export.
* Determine the best range of 350-450 um to image over. Ideally you want to see all of the LC beams and all of the visible edges of the PPS. Use 0.6x magnification unless the specimen is very small in which case you can use 0.65x. You do not want more then 70 images in your volume. If the volume needs to be particularly large due to an uneven PPS then you can change the spacing between slices from 3 um to 5 um to bring down the imaging time.
* After 30+ minutes at 10 mmHg, image the specimen with 2 consecutive Z-stacks.
* Record the current Z position of the bottom of the stack.
* Set pressure to 5 mmHg and record the time.
* Repeat imaging steps after 30+ minutes, record Z position
* Set pressure to 7.5 mmHg and record the time.
* Repeat imaging steps after 30+ minutes, record Z position
* Set pressure to 20 mmHg and record the time.
* Repeat imaging steps after 30+ minutes, record Z position
* Set pressure to 45 mmHg and record the time.
* Repeat imaging steps after 30+ minutes, record Z position
* Stitch all volumes and export both duplicates as .czi files to an external drive to take the data back to the Nguyen Lab
* If this is a single eye shut down using protocol sheet for shut-down.
* If this is a full eye pair, get the 2nd out of the freezer and repeat all previous imaging steps for the new eye.
* Put eyes(s) in 2% PFA and fix in their polycarbonate holders. Place in screw-topped containers in the fridge, label, and transport to Nguyen Lab fridge for long-term storage.
* (Optional) If you are doing a treatment protocol, testing the effects of chemical treatment, then you will want to incubate the samples after the first imaging session at 37C with the chemical agent. We used 4 hours with ChABC which degraded about 90% of sGAGs in the LC and sclera. After incubation with the chemical agent, repeat all imaging steps above to determine changes in structure and pressure-strain response from chemical treatment.