**Protocol for Thickness Measurement of the Preserved Posterior Scleral Samples after Inflation Testing**

*DM, last updated: 12/13/16*

* Several days after fixing the posterior scleral samples in PFA, come back to reimage. Make a reservation on the scope for around 3 hours.
* Cutting LC Sections with the microtome:
  + Take eye 1 sample, fixed in 4% PFA from the fridge and get out a razor blade and forceps.
  + Cut a 4x5 mm block around the LC out from the holder and glue it retina-side down with permabond 710 to the microtome cutting block. Orient the long side parallel to the closest side of the blade. Spread the glue thinly and evenly and try to glue the LC smoothly and. Allow to dry for 15 minutes. Place drops of PBS periodically on the LC to keep it hydrated as it dries
  + Carry the block with the specimen to the microtome machine and insert it into the cutting block holder. Wheel the microtome machine close to a counter with running water and fill the bath with normal water till the sample is covered. Now power on the machine and place a new cutting blade in the holder and turn on the overhead light near the magnifying glass. Place the machine in single cut mode, set the section thickness to 75 um by pulling up on the switch, scroll the wheel counter-clockwise relative section thickness wheel to reverse the blade height till it is cutting just above the sclera and at a 20-degree angle from parallel. (cutting angle should already be set if you don’t adjust it as no one else uses the machine anymore)
  + Make sure the cutting block is oriented as levelly as possible relative to the blade and machine! You can judge this by looking at all sides relative to the metal holder around the block (IMPORTANT!!!)
  + Start above the specimen and gradually begin to cut 75 um deeper each time. Keep going until you fully bit into the specimen. Usually the LC is deeper then the PPS so cut a few slices into the PPS and through away these sections as the cut begins to level and near the LC.
  + Once it is close to the LC, begin to cut 75 um sections by pressing up on the direction switch, start by turning the speed wheel to 10 to increase the speed, slow it down to 5 as it approaches the sample, allow it to slowly cut through, after it does immediately reverse the blade back after the cut to keep the new section from folding. If completely cut through the section will float on the water bath, but usually a corner or edge is still attached. Use forceps to gently tug it free. Keep track of the original orientation! It helps if the cut LC block is rectangular and superior is oriented toward the blade.
  + Now insert a glass slide under the water and place it on the flat surface behind the cutting block. Move the section with the forceps to the glass slide and maintain a superior orientation away from you. Do NOT rotate or flip the section! Now lift the slide out of the water, use the paintbrush to gently pin the section flat as you lift it out.
  + Dry the bottom and edges of the slide with kim wipes and place on an absorbent paper towel.
  + Smooth the section flat gently with the paintbrush and rotate till the cut edges are mostly parallel with the edges of the slide. Make sure the posterior side of the section is always upward and they are oriented superior-side toward the top of the slide. Place a cover slip over the sample, gently, taking care not to squash it to much, by rolling it on from one side, dry the edges, and seal with nail polish.
  + Allow to dry and mark the superior direction and label the slide with the eye and section number.
  + Continue this process till you have cut all the way through your sample to the dried glue. You should have about 8-10 sections, 5-7 of which will contain LC depending on how well the LC was cut and prepared.
  + Put the slide book aside and repeat all the previous steps for eye 2 as well. Now you have 2 sets of slides for imaging.
* Imaging the specimen 1: this is a non-inverted imaging, but otherwise the same as in the inflation experiments.
  + Boot up the LSM using the protocol on the wall - All users that do this should be trained by either Mel or Rhonda from JHMI:
    - Turn on the Chameleon laser power switch
    - Check the gas pressure on the wall
    - Place the imaging in progress sign on the door
    - Sign in on the lab notebook with your lab name/time
    - Remove the condenser by unscrewing the hex screw and detaching the signal cord
    - Remove the 20x C-Apochromat objective lens and take out the inverter arm from the case on the work desk. Attach the 10x C-Apochromat Dipping lens 0.45W to the inverter and align the arm and objective in-plane using the desk edge
    - Carefully carry the inverter arm and 10x objective over the the LSM 710 and screw the base of the inverter arm into the spot where the 20x objective was removed.
    - Check to make sure the BIG Chroma SHG cube with 390-410 bandpass filter and elastin 470-550 bandpass filter is installed on the lower-right filter cube spot.
    - Boot up the CPU and instrument using the protocol and switch order prescribed on the wall. When the info panel on the left boots up turn of parfocality and disable automatic objective Z-motion.
    - Look at the inverter arm from the side and attempt to line up the objective in-plane with the back of the arm (there will be a rotation imaging error otherwise)
    - Log into the Quigley account on the computer using the password: “ratman”. Open the Software StandSelect.exe. select System XXXXX – without\_cond\_stage\_backwasds.mdb. Use the 2402 kB one (not the 1 kB shortcut one) and click open. Zeiss will now use inverted mode.
    - Boot up Zen. Select start, There should be one warning: “attenuator calibration data is not for this system, use it?” Press “yes”. Record the starting lamp number on the log book near your sign in.
    - Click on “maintain” click on 3 – 20x/change objective, select C Apochromat 10x/0.45 W M27 from favorites, select objective
  + Imaging:
    - Place the first slide of eye 1 into the inverted slide holder, oriented with the superior side toward the back. (see figure).
    - Place a drop of iodized water on the slide over the sample site and dip the dipping lens into it, taking care that the lens is completely immersed in the drop connected to the cover-slip. Make sure there are no bubbles between them! Orient the superior side toward the back of the machine.
    - Reacquire the imaging settings by loading a previous dual SHG-collagen and TPF-elastin imaging run and clicking “reuse settings”. Lower the gain and signal power a bit from the last run to prevent oversaturation (if this eye signal is brighter then the last)
    - First, using DAPI locate mode, locate the top edge of the PPS block cut and rotate the slide holder until it completely bisects the view. This will ensure all slides are close to the same orientation since the PPS edge cut is the same relative to all sections. Then, taking care not to move the slide again, locate the LC and center objective over the LC area near the CRAV.
    - Put dark cover on and put out the lights
    - Do a live scan of the area to center further. Then move to the brightest, central Z-slice. Move the objective center until the LC/PPS interface edges to be about equidistant from the center of the images. Maximize the zoom to 0.6x to include as much PPS as possible.
    - Bring up a previous picture of the LC that clearly shows the LC-PPS border and try to center the 2x2 stitched image of the slide section on the same spot to have comparable images.
    - Alter gain/power until a good balance of contrast is found without oversaturation. You can use the extreme labeling histogram program to try and minimize the amount of blue (completely dark) pixels and red (oversaturated pixels). The SHG should have oversaturation on the PPS but not elsewhere. The Elastin signal should look good everywhere.
    - Correct for relative rotations by imaging the brightest center of the Z-stack, observing if the tiles align, and iteratively changing the rotation calibration. In generally it will be between -5 to 5 degrees and varied based on the orientation of the inverter arm. You will know the rotation calibration is close to correct when features in the tiles start to align on the edges. Once they align on all vertical and horizontal interfaces you have the correct value.
    - Select the range to image over by setting the z starting and ending positions. You can tell these not from when you start to be able to see something but from when features suddenly come into focus (you start seeing changes). There is no need to include the backscattered and fuzzy “shadows” of the signal above and below otherwise your volume will be too big and over 100 um thick. You want to select the best range of 75 um or less. It can be less if the section was squashed by cover-slipping and generally varies from 60-75 um.
    - Image the Z-stack once and then remove the slide from the holder. Duplicate Z-stacks are NOT needed.
  + Repeat the previous steps for all section slides in order until the signal of the LC disappears at deeper slices.
  + Repeat previous steps for imaging setup and imaging for Eye 2 slides. It will likely need a different gain and excitation setting to be optimized compared to Eye 1.
  + Place slides in the 2C fridge for a few weeks in case reimaging is needed. They will expire in about 2 weeks.
  + Stitch all images and export to a flash drive or online data transfer.
  + Shut down the machine using the protocol on the wall. If someone is waiting after you, put the machine in standby, if not, do a complete shut down.