Axonal transport of mitochondria: developing an in vivo imaging assay for glaucoma research.

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GLAUCOMA RESEARCH FOUNDATION

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Introduction:

The earliest reported evidence to support the idea that the pathophysiology of glaucomatous damage to retinal ganglion cell axons is obstruction of axonal transport within the optic nerve head included observations of swollen and distorted mitochondria accumulated within the non-human primate lamina cribrosa, particularly along its posterior boundary. More recent evidence similarly suggest that abnormalities of mitochondrial function and transport are among the earliest events in glaucoma pathogenesis (Chrysostomou V, et al 2013 & Osborne NN, et al 2016). Nevertheless, nearly all of these studies were based on histological material (i.e., a temporally static view).

Purpose:

To develop a reliable in vivo assay of mitochondrial transport in the Brown-Norway rat eye. Such an assay would benefit future studies that seek to investigate the role of this critical function during the early sequence of pathophysiological events of glaucomatous axonal damage, as well as to assess novel therapeutic interventions.

Methods:

- Subjects: Adult Brown-Norway rats (*Rattus norvegicus*; Charles River Laboratories Inc. Wilmington, MA).
- All injections used DMSO as the solvent and vehicle.
- Anterograde transport studies utilized intravitreal injections performed by inserting the needle through the sclera superiorly, approximately 1 mm behind the limbus, at an angle of 45° to avoid contact with the relatively large crystalline lens of the rodent eye.
- Retrograde transport studies utilized stereotaxic injections into the right, or left superior colliculus (SC) depending on experiential assignment.
- CSLO images of *in vivo* retina and post-mortem brain structures were obtained (Spectralis HRA + OCT; Heidelberg Engineering GmbH, Heidelberg, Germany).
- The infrared and BluePeak™ blue laser (488 nm) autofluorescence imaging modes were used with 100 images averaged using the automatic real-time (ART) eye tracking software.
- Custom rigid gas-permeable contact lenses (3.5 mm posterior radius of curvature, 5.0 mm optical zone diameter, and +5.0 diopter back vertex power) were used to maintain corneal hydration and clarity during imaging.
- In order to quantify in vivo labeling results obtained across tracker concentration and time course, all CSLO-FL images were exported from the instrument as raw TIFF files without contrast manipulation and analyzed with ImageJ. The parameter Integrated Pixel Density (IPD, mean pixel intensity per unit area) was measured for a region of interest that was 80% of the total area of the image centered on the optic nerve head (to exclude perimeter of all images).
- For post mortem histological studies tissue was post-fixed in a solution of 4% paraformaldehyde and permeabilized with 1% Triton for three hours.
- Primary antibodies incubated for 16 hours in 1:200 RBPMS (PhosphoSolutions), 1:1,000 Neurofilament (Convance), and 1:250 GS (Abcam).
- Secondary antibodies incubated for 16 hour with 1:300 (Alexa488 & Alexa633 Thermofisher).
- Whole mount retina images where taken using a Leica DMi8 Confocal Microscope with 20x and 63x objective lenses.

Results Anterograde Transport:

The MitoTracker dyes we evaluated, MitoTracker Green, MitoTracker Orange, and MitoTracker Orange-Redox, each have their own potential advantages and also clear limitations. With MitoTracker Green **(Figure 1)** only axon bundles arising near the injection site are labeled consistently despite varying concentration between 1 and 150µM and imaging at 4 hours, 24 hours, 72 hours and 5 days post-injection.

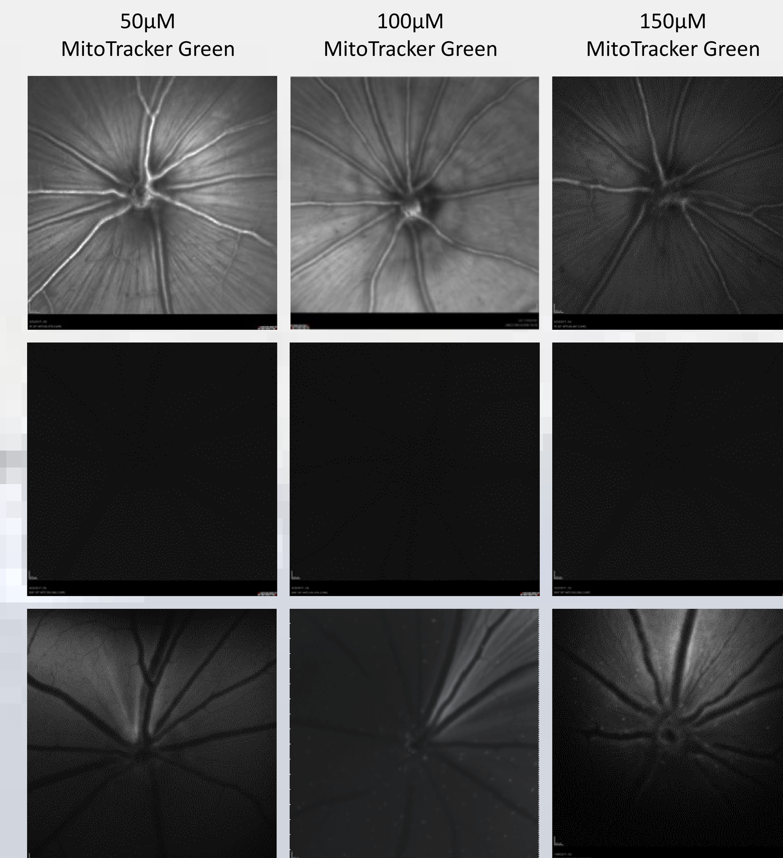

Figure 1: Results of MitoTracker Green labeling 24hrs after dye injection. CSLO infrared reflectance images (CSLO-IR, top row) provide orientation to the Brown Norway rat ocular fundus. CSLO Fluorescence (CSLO-FL) images taken at baseline and at 24 hours after injection of MitoTracker Green dye into the vitreous body. These representative images show spatially limited and somewhat inconsistent labeling at all three concentrations of MitoTracker Green dye (50 µM, 100µM and 150µM). These CSLO-FL images were obtained using 80% sensitivity setting on the HRA camera.

The MitoTracker Orange results **(Figure 2)** show transient, non-specific labeling at higher concentrations (i.e., label not limited to RGCs/axons; see **Figure 4** for post mortem histological assessment)

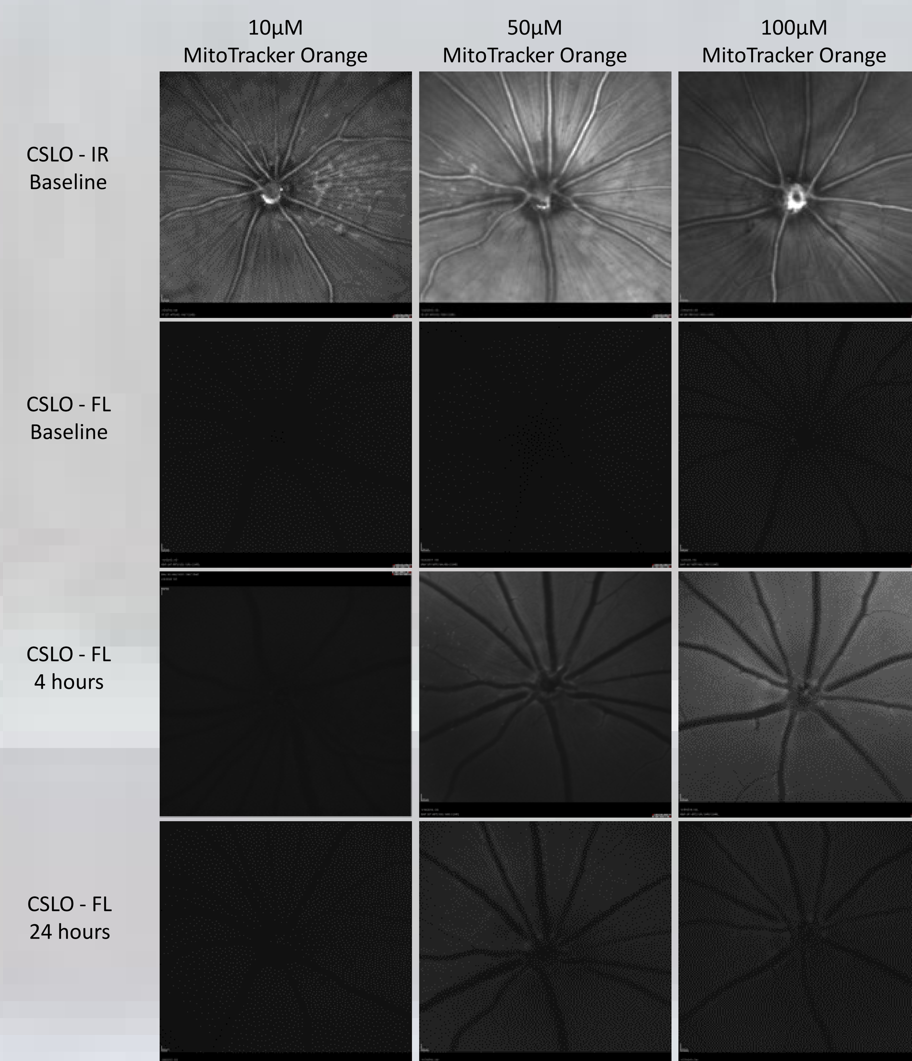

Figure 2: Results of MitoTracker Orange labeling over seven days after dye injection. CSLO-IR provides orientation to the Brown Norway rat ocular fundus. CSLO-FL images taken at baseline, 4 hours, and 24 hours after injection of dye into the vitreous body. These representative images show non-specific labeling at higher concentrations of MitoTracker Orange dye (i.e., label not limited to RGCs/axons; see below for post mortem histological assessment). CSLO-FL images were obtained using 80% sensitivity.

While 100 µM MitoTracker Orange concentration provided the largest overall fluorescent signal, it did not persist in the retina any longer than did the lower concentrations. At all concentrations studied, a significantly increased "background" signal was observed, which included non-RGC/axonal label and non-specific (non-mitochondrial) binding (see Fig. 4), thus complicating reliability and practicality for use in a high-throughput assay of RGC injury models. After 36 hours, there was no change in signal for any of the concentrations piloted.

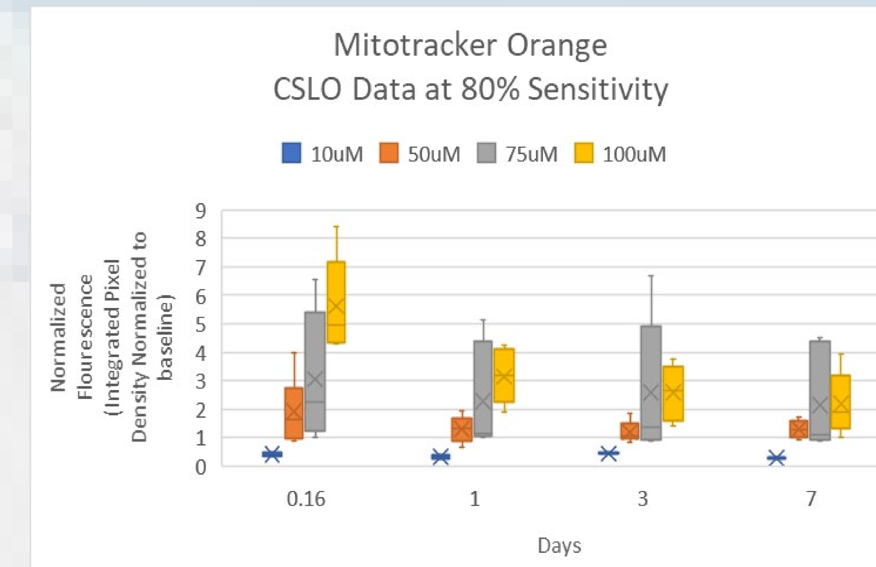

Figure 3: CSLO Fluorescence as a function of time after intravitreal injection of MitoTracker Orange. Normalized fluorescence (integrated pixel density normalized to baseline) of the CSLO-FL image as a function of time after MitoTracker Orange (six eyes per concentration) were injected into the vitreous body. After data was normalized to baseline, the fluorescence signal did not increase to a level that would support a reliable live animal imaging assay.

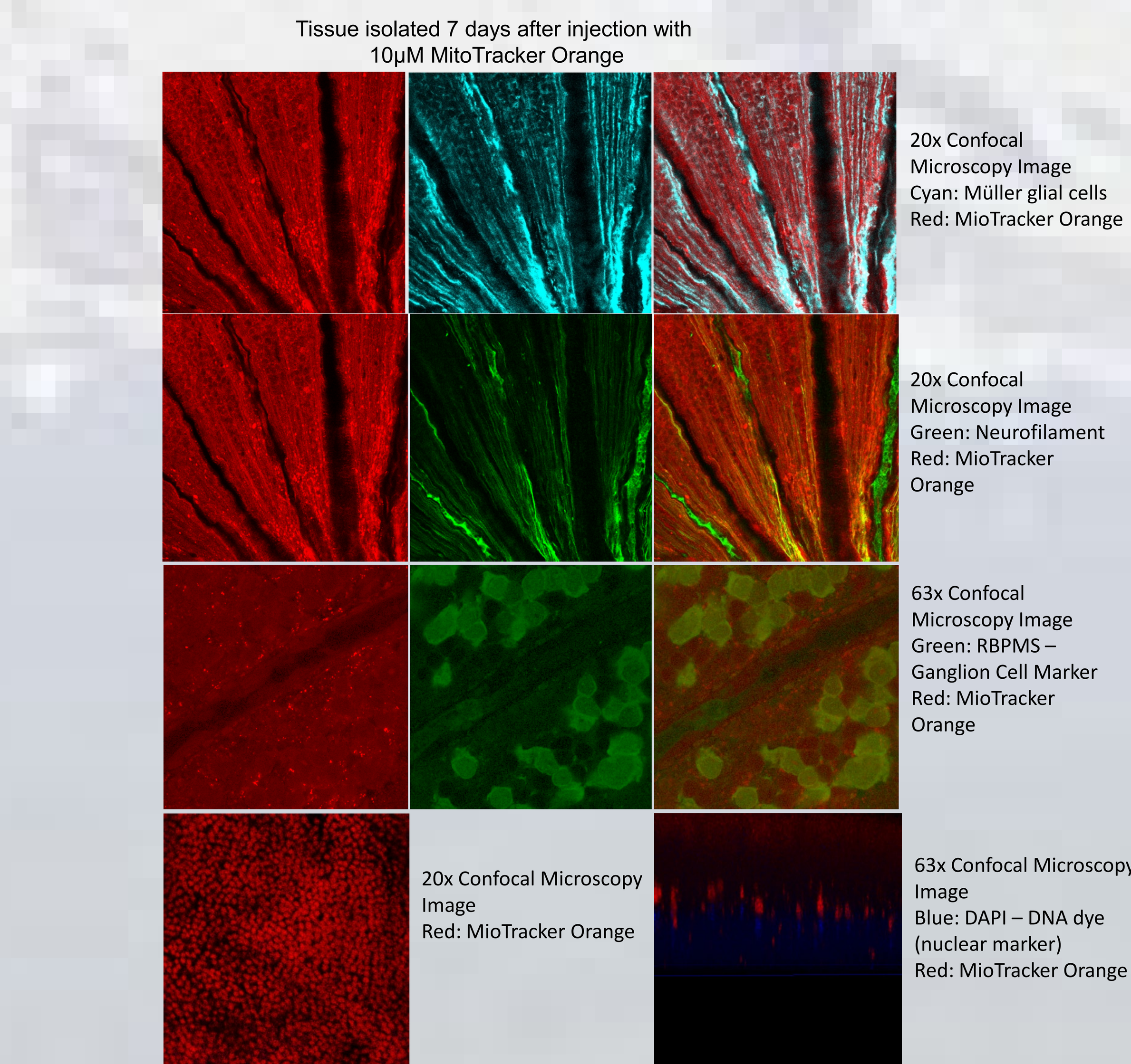

Figure 4: Confocal Microscopy Images show co-localization of MitoTracker Orange and various antibody markers. Left panel shows MitoTracker channel only, right panel shows all channels with assigned pseudocolor. Row 1 - Images showing MitoTracker Orange in red and glutamine synthetase (GS) marker of Müller Glial Cells in cyan, Row 2 - MitoTracker Orange in red and Neurofilament RGC/axon marker in Green, Row 3 - MitoTracker Orange in red, and RNA-binding protein with multiple splicing (RBPMS), marker RGC soma shown in green. Row 4 - MitoTracker Orange in red, and DNA dye (nuclear marker) DAPI in blue. These images were obtained from tissue isolated 7 days after injection of 10µM MitoTracker Orange. These images show that MitoTracker Orange ostensibly binds to mitochondria inside Retinal Ganglion Cells, Müller Glial Cells and photoreceptors, although the specific sub-cellular / organelle binding sites in each case require confirmation by electron microscopy, which is one of our primary goals for next steps and future directions.

MitoTrackerH2TMRos is a non-fluorescent version of MitoTracker Orange while in a reduced state, however, once it enters an actively respiring cell, the dye is oxidized and becomes fluorescent allowing for measurement of oxidative activity. Four replicates of anterograde transport labeling were performed using a final concentration of 100µl MitoTrackerH2TMRos. **Figure 5** shows that even at this high concentration, very little labeling of the retina was observed (i.e., fluorescence intensity did not rise above pre-injection baseline level of background at any time point studied).

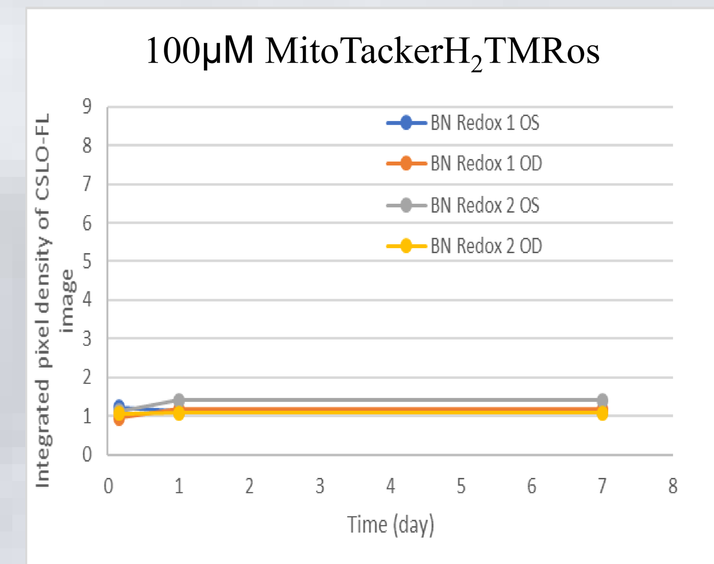

Figure 5: CSLO Fluorescence as a function of time after intravitreal injection of MitoTracker H_2TMRos. Normalized fluorescence (integrated pixel density normalized to baseline) of the CSLO-FL image as a function of time after MitoTracker H_2TMRos (four eyes per concentration) were injected into the vitreous body. After data was normalized to baseline, the fluorescence signal did not increase above background.

Results Retrograde Transport:

Retrograde transport studies utilized stereotaxic injections into the right, or left superior colliculus (SC) depending on experiential assignment **(Figure 6)** .

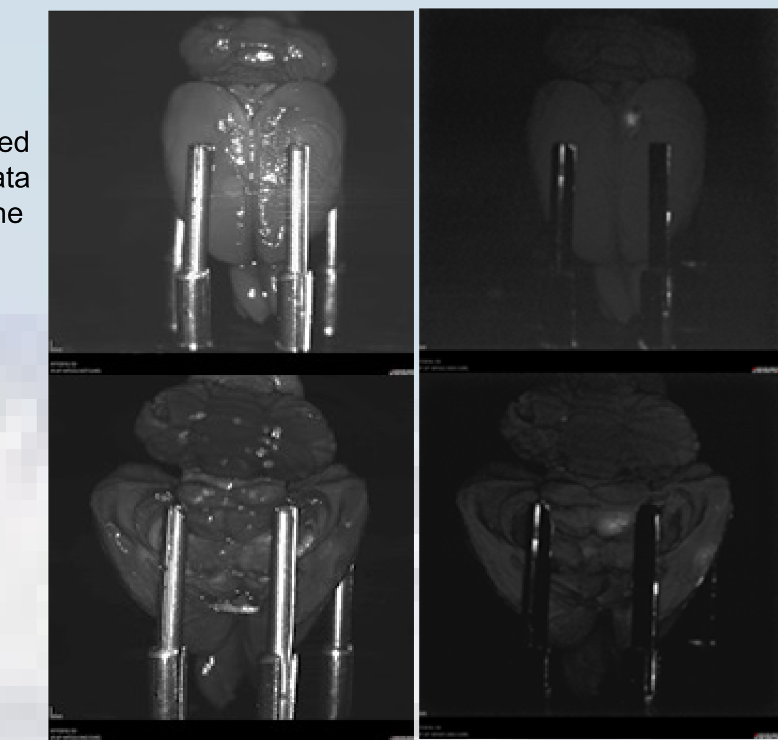

Figure 6: Labeling of the left superior colliculus by 100µM MitoTracker Orange. Left column – post-mortem IR reflectance image of the dorsal cortical surface (top row) and midbrain (bottom row, with cortices splayed to reveal midbrain). Right column - CSLO-FL of the dorsal aspect of the cortex (top) and midbrain surface (bottom).

MitoTracker labeling was observed by CSLO-FL in both eyes 4-hours after injection (**Figure 6**) in six different animals. This is both faster than would be predicted for mitochondrial bulk transport rate and unexpected insofar as only 3-5% of axons were predicted to be labeled by MitoTracker in the contralateral eye by active retrograde axonal transport mechanisms given that only 3-5% of RGC axons cross at the optic chiasm to project to the contralateral hemisphere in pigmented rats. This fascinating finding suggests an alternative route of delivery, perhaps via subarachnoid space, glymphatics, or another systemic pathway. However, with the limitations created by the need to normalize the signal to detect the fluorescent marker, we were unable to continue with the aim of creating a viable assay that would be reliable under experimental conditions with this dye.

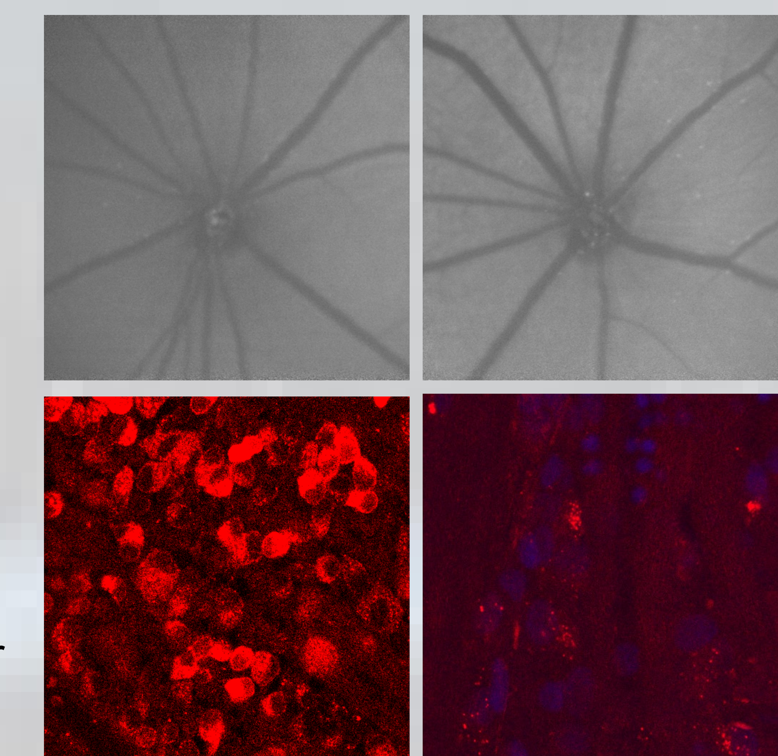

Figure 6: Ganglion Cell labeling in both eyes 4 hours after injection of 100µM MitoTracker Orange in the left superior colliculus. Confocal microscopy images of whole, flat-mounted retinas from the same animal shown in Figure 10. Each retina was dissected from the eye cup under 4% paraformaldehyde then mounted on a microscope slide. Labeling by MitoTracker Orange is localized in ganglion cells in both the right eye (OD, left panel) and the left eye (OS, right panel).

Conclusions:

The Mitotracker dyes are not selective enough to support an assay of axonal mitochondrial transport in the live rat retina using commercial CSLO instrumentation. Future experiments will seek to determine if labeled mitochondria are traveling the entire length of the axon, from the superior colliculus to the retinal ganglion cell soma, or if there is a non-mitochondrial, sulfhydryl-rich molecule to which MitoTracker also binds and undergoes transport back to the eye. These experiments will be carried out by attaching an EM contrast agent, such as a gold nanoparticle, to the MitoTracker dye, that will allow for verification that the dye is being transported by mitochondria and not another sulfhydryl rich organelle.

While we were able to carry out anterograde and retrograde transport studies, multiple limitations led us to conclude that without further development of methodology to overcome critical barriers, these MitoTracker dyes are not good candidates for use in a reliable assay of axonal mitochondrial transport.