Optical Coherence Tomography Angiography in Mice: Quantitative Analysis After Experimental Models of Retinal Damage

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PURPOSE. We implemented optical coherence tomography angiography (OCT-A) in mice to: (1) develop quantitative parameters from OCT-A images, (2) measure the reproducibility of the parameters, and (3) determine the impact of experimental models of inner and outer retinal damage on OCT-A findings.

METHODS. OCT-A images were acquired with a customized system (Spectralis Multiline OCT2). To assess reproducibility, imaging was performed five times over 1 month. Inner retinal damage was induced with optic nerve transection, crush, or intravitreal N-methyl-d-aspartic acid injection in transgenic mice with fluorescently labeled retinal ganglion cells (RGCs). Light-induced retinal damage was induced in albino mice. Mice were imaged at baseline and serially post injury. Perfusion density, vessel length, and branch points were computed from OCT-A images of the superficial, intermediate, and deep vascular plexuses.

RESULTS. The range of relative differences measured between sessions across the vascular plexuses were: perfusion density (2.8%–7.0%), vessel length (1.9%–4.1%), and branch points (1.9%–5.0%). In mice with progressive RGC loss, imaged serially and culminating in around 70% loss in the fluorescence signal and 18% loss in inner retinal thickness, there were no measurable changes in any OCT-A parameter up to 4 months post injury that exceeded measurement variability. However, light-induced retinal damage elicited a progressive loss of the deep vascular plexus signal, starting as early as 3 days post injury.

CONCLUSIONS. Vessel length and branch points were generally the most reproducible among the parameters. Injury causing RGC loss in mice did not elicit an early change in the OCT-A signal.

Keywords: mice, optical coherence tomography angiography, experimental disease models

Retinal ganglion cell (RGC) loss is a defining characteristic of optic neuropathies, including glaucoma. Clinical estimates of RGC loss rely on surrogate measures such as retinal nerve fiber layer or neuroretinal rim thickness for structural changes. However, while distal from the purported origin of damage, it is possible that the inner retina, where RGC somas and their axons reside, could show alterations in metabolic demand. As a result, possible changes in vascular perfusion could manifest as early signs of damage before current clinical measures are impacted. Likewise, perfusion changes in the deep capillary plexus, which provides oxygen delivery to the outer plexiform layer where photoreceptors synapse, could be early indicators of outer retinal damage.

Optical coherence tomography angiography (OCT-A) is a noninvasive imaging technique in which temporal light intensity variations at each scanned pixel are used to derive functional information on retinal perfusion. The major assumption behind the principles of OCT-A is that the only components undergoing motion in the retina are blood cells coursing through vessels; consequently, nonperfused areas are not theoretically detected by OCT-A. This temporal flow signal from OCT-A is analyzed axially, allowing acquisition of flow maps at different retinal depths and improved visualization of retinal perfusion. Its advantages over conventional use of fluorescein angiography are that it is noninvasive and permits axial differentiation of the vascular plexuses. OCT-A currently does not provide reliable quantitative information on blood velocity or flow; rather, it indicates only the presence or absence of flow. The ability to quantify different parameters in OCT-A images, important for monitoring disease progression, often requires postprocessing analysis. Previous studies have focused on quantitative measures of vessel density, space between vessels, and perfusion density from two-dimensional projected images.

There is evidence that changes in blood oxygenation can be detected by OCT-A. Therefore, it is plausible that if the metabolic needs of the retina change, specifically due to cell death, blood flow to those regions would be affected. Prior to longitudinal OCT-A studies in mice with models of injury, it is necessary to determine the normal variability of measurements in healthy mice. To date there have been no published reports that have used experimental models of retinal damage to determine the quantitative effects of these parameters in the various retinal vascular beds.
The purpose of this study was to implement OCT-A in mice with the following specific objectives: (1) develop quantitative parameters from OCT-A images, (2) measure the reproducibility of the parameters, and (3) determine the impact of experimental maneuvers, including three different models of acute RGC loss and one model of outer retinal damage, on OCT-A measurements. We also measured loss of the OCT-A signal after respiratory arrest as a positive control experiment.

**METHODS**

**Animal Preparation**

Animal procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Ethics approval was obtained from the University Committee on Laboratory Animals at Dalhousie University. Three groups of animals were used for the main part of this study: (1) for assessing variability, female C57BL/6 mice (n = 7) (strain code 475; Charles River Laboratories, Saint-Constant, QC, Canada); (2) for RGC damage, female Thy1-GCaMP3 mice (n = 7) (stock no. 017893; The Jackson Laboratory, Bar Harbor, ME, USA) in which a fluorophore is expressed under the control of the Thy1 promoter, permitting in vivo imaging and quantification of RGCs longitudinally; and (3) for outer retinal damage, male and female BALB/c mice (n = 2) (strain code 028; Charles River Laboratories). An experiment in a single female C57BL/6 was performed to assess OCT-A changes after respiratory arrest. All mice weighed approximately 20 g each and at the time of initial imaging were on average (SD) 4 (1) months old.

Approximately 10 minutes before imaging, the left pupil was dilated (one drop of 1% tropicamide and one drop of 2.5% phenylephrine hydrochloride; Alcon Canada, Inc., Mississauga, ON, Canada). Animals were anesthetized with 3% inhalant isoflurane (Baxter Corp., Mississauga, ON, Canada) in 1.5 L/min of oxygen for induction and maintained at 1.5% to 2% volume with 0.8 L/min oxygen flow via a nose cone. Ophthalmic gel and a custom-made polymethyl methacrylate plano contact lens (Cantor and Nissel Ltd., Brackley, UK) were used to maintain corneal hydration and reduce the likelihood of cataract formation while the animal was anesthetized.

**Image Acquisition**

The retina was imaged with a customized imaging device for rodents (Spectralis Multiline OCT2; Heidelberg Engineering GmbH, Heidelberg, Germany) with an A-scan rate of 85,000 Hz, an axial resolution of 3.9 μm, and a transverse resolution of 5.7 μm in the mouse. The device uses an 820-nm wavelength diode laser for infrared imaging, a 488-nm excitation wavelength solid-state laser and a >500-nm emission filter for fluorescence imaging, and an 870-nm average wavelength superluminescent diode for OCT imaging. An auxiliary 25 diopter lens (Heidelberg Engineering GmbH) was attached to the camera to allow imaging of the mouse retina. For this device, the OCT-A signal is based on probability values calculated with a proprietary algorithm.

Mice were positioned in a modified stereotaxic frame and on a heating pad. The imaging device was attached to a custom-made arm that allows the operator to direct the light path to the center of the pupil. Motion artifacts were minimized by real-time eye tracking in the device software, and all follow-up images were acquired in the same position with image registration. Each imaging session comprised imaging with the following scan patterns: (1) for retinal thickness measures, a circular peripapillary B-scan subtending 12°; (2) for OCT-A images, four volumes acquired superior, temporal, inferior, and nasal to the optic nerve head with the center of each volume between 600 and 700 μm from the center of the optic nerve head; and (3) for RGC visualization, in GCaMP mice only, fluorescence confocal scanning laser ophthalmoscope (CSLO) imaging of RGCs by focusing axially where the fluorescence signal was strongest, namely the ganglion cell layer. Each OCT-A volume was 512 A-scans by 512 B-scans (10° × 10°) high-resolution mode, approximately 450 × 450 μm, each an average of six B-scans.

For the variability study, animals were imaged twice within the same experiment on the first day (sessions 1 and 2), the following day (session 3), 1 week later (session 4), and 1 month later (session 5), for a total of five scans of each region. For the models of RGC loss (see below), animals were imaged with the scan patterns described above at baseline and weekly post injury, while for light-induced damage (see below), animals were imaged at 3 days and then weekly post injury.

**Models of Injury**

**Respiratory Arrest.** After inhalant anesthesia, the animal was positioned for imaging as described above in the Animal Preparation and Image Acquisition sections. One OCT-A volume was acquired and used as the reference scan. The animal remained in position and was given 0.3 mL (240 mg/mL) sodium pentobarbital (Euthansol; Intervet Canada Corp., Kirkland, QC, Canada) by intraperitoneal injection. Monitoring of the animal occurred until respiratory arrest was observed, at which point repeated scanning of OCT-A volumes began. Scanning was repeated at frequent intervals until it was assumed cardiac arrest had occurred, that is, when flow had ceased in large vessels visible in the live infrared image.

**Optic Nerve Transection and Crush.** Mice (n = 3) were anesthetized as described above in the Animal Preparation section. Under an operating microscope, the left eye was rotated downward and held in place with a 90° conjunctival suture. To expose the optic nerve, an incision was made in the skin near the supraorbital ridge, after which the intraorbital subcutaneous tissues were dissected. The optic nerve dura was cut longitudinally, and the optic nerve was transected completely within the sheath. The ophthalmic artery, located beneath the nerve, was kept intact. The incision was closed and the fundus examined to confirm no ischemic damage.

The same approach was used for optic nerve crush (n = 2). Instead of transection, the nerve was crushed with self-closing forceps for 3 seconds approximately 1 mm from the globe.

**N-Methyl-D-Aspartic Acid (NMDA) Injections.** The left eye was dilated topically with one drop of 1% tropicamide and continuously rehydrated with lubricant eye drops. Mice (n = 2) were anesthetized as described in the Animal Preparation section. Under an operating microscope, the left eye was rotated downward and held in place with a 90° conjunctival suture. To expose the optic nerve, an incision was made in the skin near the supraorbital ridge, after which the intraorbital subcutaneous tissues were dissected. The optic nerve dura was cut longitudinally, and the optic nerve was transected completely within the sheath. The ophthalmic artery, located beneath the nerve, was kept intact. The incision was closed and the fundus examined to confirm no ischemic damage.

The same approach was used for optic nerve crush (n = 2). Instead of transection, the nerve was crushed with self-closing forceps for 3 seconds approximately 1 mm from the globe.

**Light-Induced Retinal Damage.** Prior to light exposure, mice (n = 2) were dark-adapted overnight. Both eyes first were diluted with one drop each of 1% atropine (Valeant Canada Ltd., Laval, QC, Canada) and 1% cyclopentolate (Valeant Canada Ltd.); the mice were then placed individually in standard transparent cages with food and water. Animals were exposed to 22,000 lux of white fluorescent light in a well-ventilated, air-conditioned room for 12 hours. During exposure, mice were regularly monitored to ensure they did not develop dry eye and that they maintained adequate mydriasis.
drops were reapplied when necessary). Efforts were made to keep the mice from hiding or falling asleep by introducing new stimuli in the cages.

**Image Segmentation and Reconstruction**

OCT layer segmentation was performed with the device segmentation algorithm (Heidelberg Eye Explorer; Heidelberg Engineering GmbH), after which, each volume was checked for segmentation errors and manually corrected when required. Thickness measures of retinal layers were calculated as follows: (1) inner retinal thickness: between inner limiting membrane and outer border of the inner plexiform layer and (2) outer retinal thickness: between outer border of the inner plexiform layer and retinal pigment epithelium.

Transverse projection images were calculated for three different vascular volumes or slabs: superficial vascular plexus (SVP), intermediate capillary plexus (ICP), and deep capillary plexus (DCP). The reference layer, distance from reference layer, and thickness parameters used for each slab are shown in Table 1. The projection images correspond to an integral over the thickness of the slab and clipped at the contrast setting value set by the user (Equation 1), where \( p \) is the pixel value in the OCT-A volume, \( c \) is contrast, and \( \text{tp} \) is the transverse projection pixel value. Projection images were exported as TIFF files.

\[
\text{tp}_{x,y} = \min \left( \frac{c \int p_{x,y,z}dz}{c} \right)
\]

**OCT-A Image Analysis**

The OCT-A projection images were analyzed (MATLAB; The MathWorks, Natick, MA, USA) by first converting them to grayscale. They were then resized to 840 × 840 pixels, and a Gaussian filter (\( \sigma = 1, \ b = 7 \)) was applied for noise removal, followed by contrast enhancement. A locally adaptive image-thresholding algorithm was then used to create a binarized image (Fig. 1) where regions with the presence of flow are shown in white and the regions with absence of flow are shown in black. Large vessels were identified in the SVP and then removed from the ICP and DCP images to avoid shadowing artifacts. The vessel skeleton image was calculated by thinning all objects to lines, and spurious edges that were less than 30 pixels to the nearest branch point were removed. Skeletonized images were used to measure vessel length and map the branch points. Perfusion density was computed as the ratio of white pixels (area of flow) over the total image area in the binarized image. Vessel length was taken as the total length of the path in the vessel skeleton image. The number of branch points was calculated as the total number of intersections within the vessel skeleton image. Perfusion density, vessel length, and branch points were calculated and exported from MATLAB to be used for statistical analyses (Fig. 1).

OCT-A image analysis included separating the signal from the background and calculating the mean and SD signal (\( S \)) and background (\( B \)) values in each image. Noise was measured in

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**Table 1. Parameters Used to Create Projection Images for Each Vascular Slab**

<table>
<thead>
<tr>
<th>Vascular Slab</th>
<th>Reference Layer</th>
<th>Distance From Reference Layer, ( \mu \text{m} )</th>
<th>Thickness, ( \mu \text{m} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVP</td>
<td>Inner limiting membrane</td>
<td>17</td>
<td>40</td>
</tr>
<tr>
<td>ICP</td>
<td>Inner limiting membrane</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>DCP</td>
<td>Retinal nerve fiber layer</td>
<td>97</td>
<td>40</td>
</tr>
</tbody>
</table>

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**Figure 1.** Representative OCT-A image of the ICP and the major image-processing steps taken to calculate perfusion density (ratio of white pixels/area of flow over the total image area in the binarized image); vessel length (total length of the path in the vessel skeleton image); and branch points (total number of intersections within the vessel skeleton image). The OCT-A image was first binarized, followed by a combination of noise removal (filtering) and vessel enhancement (gap filling). Large vessels (yellow arrow) in the SVP were isolated and removed from the ICP and DCP images.
Quantification of In Vivo Fluorescence Images

Fluorescence CSLO images (1536 × 1536 pixels) were quantified by calculating the grayscale intensity ratio. This approach was taken as an alternative to individual cell counting in selected areas for objectivity because the density of axon labeling can interfere with individual cell counting. Regions were categorized as either over large vessels or within the remainder of the retina. The normalized fluorescence intensity was calculated by the mean grayscale signal in the retina, excluding large vessels, divided by the mean grayscale value in the large vessels (Equation 5). The minimum $I_{\text{normalized}}$ value was assumed to be 1 when retinal tissue was the same grayscale intensity as the vessels.

$$I_{\text{normalized}} = \frac{I_{\text{vessels}}}{I_{\text{background}}}$$  

Statistical Analysis

Analyses were performed with statistical software (R: https://www.R-project.org; provided in the public domain by R Foundation for Statistical Computing, Vienna, Austria) and RStudio (http://www.rstudio.com; provided in the public domain by RStudio, Inc., Boston, MA, USA). Unless otherwise indicated, all results are expressed as mean (SD), and statistical significance was assumed when $P < 0.05$. For measuring variability, reproducibility coefficients (range within which 95% of reproducibility measures occurred) were reported as $1.96 \times \sqrt{\frac{1}{2}} \times \text{SD}$, and relative difference was calculated as a percent of the difference between sessions divided by the mean. One-way ANOVA was applied to test the significance of signal intensity and image quality measures between plexuses, and the paired $t$-test was used for comparing retinal thickness measures between time points.

RESULTS

In all cases, each OCT-A volume was acquired within 5 minutes. The mean (SD) signal difference observed across all plexuses between static tissue and perfused regions was 62.9 (2.6), and the average noise measured in the static tissue was 4.8 (0.6), resulting in a signal-difference-to-noise ratio of 3.2 (0.2). Signal intensity and image quality showed that SBR, SNR, and CNR all decreased progressively and significantly from the SVP to DCP ($P < 0.01$; Table 2).

Customized Quantitative Measurements

An example OCT-A image of the ICP from a C57BL/6 mouse shows the workflow developed for quantitative analysis of the OCT-A images (Fig. 1). Additional steps requiring optimization included identifying small objects (<50 pixels) classified as noise and removing them. Small gaps or spaces within an area of perfusion were also considered artifacts and filled, so that flow density and vessel skeletonization were more accurate. The algorithm required approximately 10 seconds per image to execute with a 3.4-GHz CPU (Intel Core i5; Intel, Santa Clara, CA, USA); 68% of that time was for completing the geodesic distance transform to remove spurious/artifact edges of the vessel skeleton. The processing time was reduced by nearly half when the algorithm was executed using a parallel pool.

Variability of Intersession OCT-A Imaging

OCT-A images obtained on the same day within the same experiment (sessions 1 and 2) were very similar, with low, within 5% (Table 3), relative difference for all parameters; however, variability was slightly higher between sessions separated by 1 month (Table 3). The reproducibility coefficients for perfusion density within the SVP, ICP, and DCP were 0.01%, 0.03%, and 0.03%, respectively. For vessel length, the respective values were 647, 406, and 169 pixels and for branch points, 6, 8, and 10. The distribution of values for each parameter, vascular plexus, and imaging session are shown in Figure 2. The repeated measures ANOVA showed that for each of the plexuses and parameters, there was no significant difference between sessions ($P > 0.09$).

Impact of Injury Models on OCT-A Measurements

Within 1 minute of respiratory arrest, there was a significant attenuation of the OCT-A signal (Fig. 3). Two minutes after respiratory arrest, there was an almost complete loss of signal in all three vascular plexuses. Surprisingly, in the absence of flow, there was a wide range of pixel brightness that varied between the plexuses, presumably due to contrast enhance-

### Table 2. Signal Intensity and Image Quality Measures of OCT-A Images*

<table>
<thead>
<tr>
<th>Plexus</th>
<th>SBR (CI)</th>
<th>SNR (CI)</th>
<th>CNR (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVP</td>
<td>5.49 (0.46)</td>
<td>6.38 (0.25)</td>
<td>5.16 (0.28)</td>
</tr>
<tr>
<td>ICP</td>
<td>2.95 (1.15)</td>
<td>3.59 (0.18)</td>
<td>2.25 (0.16)</td>
</tr>
<tr>
<td>DCP</td>
<td>2.65 (0.08)</td>
<td>3.00 (0.16)</td>
<td>1.87 (0.15)</td>
</tr>
</tbody>
</table>

* Values are expressed as mean (95% CI).

### Table 3. Reproducibility of Imaging on the Same Day, Session 1 and Session 2 (S1 vs. S2), and a Month Apart, Session 1 and Session 5 (S1 vs. S5)*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SVP, % (CI) S1 vs. S2</th>
<th>SVP, % (CI) S1 vs. S5</th>
<th>ICP, % (CI) S1 vs. S2</th>
<th>ICP, % (CI) S1 vs. S5</th>
<th>DCP, % (CI) S1 vs. S2</th>
<th>DCP, % (CI) S1 vs. S5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfusion density</td>
<td>2.8 (1.0)</td>
<td>3.7 (0.8)</td>
<td>5.1 (1.1)</td>
<td>7.0 (1.5)</td>
<td>5.0 (1.4)</td>
<td>6.5 (1.9)</td>
</tr>
<tr>
<td>Vessel length</td>
<td>2.1 (0.7)</td>
<td>2.8 (0.6)</td>
<td>1.9 (0.7)</td>
<td>3.9 (0.9)</td>
<td>2.9 (0.8)</td>
<td>4.1 (1.3)</td>
</tr>
<tr>
<td>Branch points</td>
<td>3.4 (1.4)</td>
<td>5.0 (1.5)</td>
<td>1.9 (0.8)</td>
<td>2.7 (0.7)</td>
<td>2.5 (0.8)</td>
<td>3.0 (1.0)</td>
</tr>
</tbody>
</table>

* Values indicate the relative difference between sessions (95% CI). S, session.

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ment used by the device software in the presence of a low signal.

Four months after optic nerve transection there was a loss of RGCs, represented by a 70% decrease of relative fluorescence labeling in the ganglion cell layer (Supplementary Fig. S1). The mean inner retinal thickness decreased from 73.2 (3.2) µm at baseline to 60.1 (1.6) µm at 4-months post transection ($P = 0.057$), whereas the outer retinal thickness was 154.0 (6.4) µm at baseline and 163 (3.8) µm at 4 months post transection ($P = 0.120$) (Supplementary Fig. S2). However, there was no measurable change in the perfusion measurements in either plexus for up to 4 months post transection (Fig. 4; Supplementary Fig. S3). Specifically, all calculated parameters were within the range of values measured in the variability studies above.

Similar results were obtained following optic nerve crush: 71% decrease in relative fluorescence (Supplementary Fig. S1), inner retinal thickness decrease from 73.2 (3.2) µm at baseline to 62.0 (0.1) µm at 4 months post crush ($P = 0.017$), outer retinal thickness of 159.8 (2.7) µm at baseline and 159.9 (2.3) µm at 4 months post crush ($P = 0.97$) (Supplementary Fig. S2), and no appreciable decrease in calculated perfusion parameters outside of normal variability (Fig. 5; Supplementary Fig. S3).

For animals that received an intravitreal NMDA injection, the inner retinal thickness was 75.7 (4.6) µm at baseline and 52.8 (0.8) µm at 7 weeks post injection ($P = 0.107$), and the outer retinal thickness was 156.4 (6.6) µm at baseline and 163.4 (7.9) µm at 7 weeks post injection ($P = 0.08$) (Supplementary Fig. S2). However, there was no detectable change in the OCT-A images or parameters in any of the three plexuses at any measured time point (Fig. 6; Supplementary Fig. S3) in spite of a 40% decrease of relative fluorescence, indicating substantial RGC loss (Supplementary Fig. S1).

Finally, light-induced retinal damage resulted in loss of photoreceptors, confirmed with OCT structural imaging (Fig. 7), resulting in an outer retinal thickness decrease from 121.7 (8.7) µm at baseline to 58.7 (6.3) µm 10 days after initiation of damage ($P = 0.002$) (Supplementary Fig. S2). This change in thickness loss was accompanied with a progressive loss of the OCT-A signal (Fig. 7). Loss of signal first occurred in the DCP at day 3 after light exposure, and was confirmed by perfusion density and vessel length measurements outside normal variability limits (Supplementary Fig. S3). Changes also
FIGURE 3. In vivo OCT-A imaging of a mouse retina before and after respiratory arrest (RA). For each time point, images were acquired at the same transverse location with sufficient axial depth to include (1) SVP, (2) the ICP, and (3) DCP. Times shown after respiratory arrest are in mm:ss (m = minutes, s = seconds). Scale bar: 100 μm.

FIGURE 4. Longitudinal in vivo imaging of a mouse retina before and after optic nerve transection. For each time point, images include (1) fluorescence image with confocal scanning laser ophthalmoscopy showing labeled GCaMP cells and the fluorescence ratio (top right corner); (2) OCT-A images of SVP; (3) the ICP; and (4) DCP. Peripapillary OCT B-scan at the same location at baseline and at 4 months post optic nerve transection. Retinal thicknesses (inner and outer) were 71 and 158 μm, respectively, at baseline and 59 and 166 μm at 4 months post optic nerve transection. Scale bar: 100 μm.
occurred in the ICP and SVP, although they were not consistent. However, inner retinal thickness was not significantly different from 69.5 (1.8) μm at baseline to 67.5 (2.2) μm 10 days after light-induced retinal damage ($P = 0.394$).

**DISCUSSION**

In this study, we developed quantitative parameters to aid interpretation of longitudinal changes in retinal perfusion obtained with OCT-A imaging in mice. We also quantified the amount of variability with repeated imaging in control animals. Across all 3 vascular plexuses, perfusion density had generally the highest variability among the 3 parameters, while measurements were generally the least variable in the SVP. This quantification technique also enabled us to noninvasively gauge changes in retinal perfusion after various models of retinal damage. We showed no changes in any of the OCT-A parameters that exceeded the variability limits in control animals after optic nerve transection, optic nerve crush, and NMDA injection. In contrast, light-induced outer retinal damage showed a clear loss of the OCT-A signal in the outer retina. A positive control experiment in an animal with respiratory arrest demonstrated a rapid and progressive signal loss in the smaller vessels first, particularly in the ICP.

We expected that RGC loss would result in a diminished OCT-A signal in the SVP and ICP due to energy requirements of the nerve fiber layer, RGC cell bodies, and synapses\textsuperscript{15} that cannot be met by other vascular beds.\textsuperscript{16} The retinal vascular supply in mice is arranged such that there is a common arterial supply for the three vascular plexuses,\textsuperscript{17} with some vessels directly connecting the superficial and deep plexuses, located within the ganglion cell layer and the outer plexiform layer, respectively.\textsuperscript{18} The intermediate vascular plexus has been shown to form last during development\textsuperscript{19} and consists of shorter capillary segments between the inner plexiform layer and inner nuclear layer.

Surprisingly, there was no detectable change in the OCT-A images in either of the three imaged plexuses for up to 4 months after optic nerve transection or optic nerve crush despite substantial loss of RGCs. These findings could be partially explained by the neuroinflammatory response following RGC apoptosis, such as glial cell activation,\textsuperscript{20–23} thus requiring sustained oxygen supply. However, Nadal-Nicolás and colleagues\textsuperscript{24} showed that glial activation remained elevated in rats for only 2 months after optic nerve crush and transection, whereas our time point after these injuries extended to 4 months. Intravitreal NMDA injection causes near complete loss of synaptic activity in the inner plexiform layer\textsuperscript{25–27}; however, like the optic nerve damage models, and in spite of extensive RGC loss, there still was no detectable change in OCT-A images in either of the three vascular plexuses imaged. This was especially surprising, and we cannot rationalize why a change in retinal connectivity would not significantly affect retinal perfusion; however, the spatial and temporal properties of the retinal vascular supply may explain these findings. From a spatial perspective, the three-dimensional network of vessels supplies oxygen to multiple layers.

**FIGURE 5.** Longitudinal in vivo imaging of a mouse retina before and after optic nerve crush. For each time point, images include (1) fluorescence image with confocal scanning laser ophthalmoscopy showing labeled GCaMP cells and the fluorescence ratio (top right corner); (2) OCT-A images of SVP; (3) the ICP; and (4) DCP. Peripapillary B-scan at the same location at baseline and at 4 months post optic nerve crush. Retinal thicknesses (inner and outer) were 74 and 162 μm, respectively, at baseline and 62 and 161 μm at 4 months post optic nerve crush. Scale bar: 100 μm.

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FIGURE 6. Longitudinal in vivo imaging of a mouse retina before and after NMDA injection. For each time point, images include (1) fluorescence image with confocal scanning laser ophthalmoscopy showing labeled GCaMP cells and the fluorescence ratio (top right corner); (2) OCT-A images of SVP; (3) the ICP; and (4) DCP. Peripapillary OCT B-scan at the same location at baseline and at 7 weeks after NMDA injection. Retinal thicknesses (inner and outer) were 79 and 161 μm, respectively, at baseline and 52 and 169 μm at 7 weeks after NMDA injection. Scale bar: 100 μm.

FIGURE 7. Longitudinal in vivo imaging of a mouse retina before and after light-induced retinal damage (LIRD). For each time point, OCT-A images include (1) SVP; (2) the ICP; and (3) DCP. Peripapillary OCT B-scan at the same location at baseline and 42 days after LIRD. Retinal thicknesses (inner and outer) were 71 and 124 μm, respectively, at baseline and 64 and 45 μm at 42 days, respectively, after LIRD. Scale bar: 100 μm.
and each plexus is not terminal; thus, blood must traverse the superficial and intermediate plexuses to provide continued supply to the deep plexus. From a temporal perspective, the metabolic needs of the tissue will trigger changes in vascular tone to regulate blood flow. Therefore, if the outer retina remains metabolically functional, then it would require continued oxygen supply; there is evidence that autoregulation occurs in the retinal vasculature of rodents. Nonetheless, our results from three models of RGC loss (i.e., optic nerve transection, optic nerve crush, and NMDA injection) sharply contrast those in glaucoma patients where there is a dropout of capillaries in the SVP that corresponds to areas of both structural and functional damage, however, whether changes in retinal perfusion precede or follow clinically measured neuroretinal or nerve fiber layer loss remains to be determined.

The implementation of OCT-A quantification and reproducibility in healthy rodents has been described previously by other groups, but most studies have either analyzed the superficial plexus to include the ICP or incompletely determined inter- and intrasession variability. We analyzed OCT-A images based on the recognized vascular plexuses in mice, an approach that should be more suited for assessing changes in the individual vascular plexuses. Previous studies that utilized OCT-A in animal models of damage focused on choroidal neovascularization, retinal neovascularization, oxygen-induced retinopathy, and diabetic retinopathy, which are direct insults to the retinal vasculature or perfusion, and thus potential cell loss would be a consequence of decreased oxygenation. OCT-A changes have also been evaluated following acute intraocular pressure elevation; however, these studies did not determine the degree or presence of RGC loss. The approach in this study was to initiate cell loss and investigate subsequent effects on OCT-A findings. Our findings have shown metabolic changes that are a result of cell loss are not always reflected by changes in perfusion.

While the OCT-A signal cannot be used to make inferences of blood flow rate or velocity, the algorithm used for this work calculates signal intensity based on the probability of flow, a more accurate measure that reflects perfusion. This approach provides high-resolution, high-contrast images that make discrimination of signal from noise more approachable when binarizing the images. Therefore, all images were binarized for analysis so that regions were classified either as having flow or no flow, rather than a gradient of high flow to no flow. This respects the limitations of current OCT-A technology, which does not quantify flow, and processing algorithms, in which signal intensity and contrast settings may differ at follow-up. It could be postulated that in order to detect a change in OCT-A signal, a large change in blood flow would be necessary.

For this study, we used change in relative fluorescence in GCaMP transgenic mice as a method of measuring RGC loss. Since normalizing the fluorescence signal in the images creates a floor effect, our method could have underestimated actual RGC loss, characterized previously by cell quantification in models of acute optic nerve damage. Our goal was to study the time course of OCT-A changes in the same animal after inner retinal damage and to provide evidence of massive RGC loss for which exact quantification was not necessary.

In summary, we developed quantifiable parameters of OCT-A imaging and demonstrated that it is feasible to undertake longitudinal OCT-A imaging in mice with high reproducibility. These findings can be used to gauge when true changes in retinal perfusion have occurred and may provide new insights into the disease pathogenesis and changes in vascular perfusion.

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**References**


