Total Retinal Blood Flow in a Nonhuman Primate Optic Nerve Transection Model Using Dual-Beam Bidirectional Doppler FD-OCT and Microsphere Method

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PURPOSE. We validated noninvasive Doppler-optical coherence tomography (OCT) blood flow measurements against the terminal microsphere method in a surgical induced optic nerve transection nonhuman primate model.

METHODS. In 6 nonhuman primates, total retinal blood flow (TRBF) was measured with a custom-built dual-beam bidirectional Doppler Fourier Domain (FD)-OCT. Peripapillary retinal nerve fiber layer thickness (RNFLT) was measured by Spectralis spectral-domain (SD)-OCT. Measurements were performed every 10 to 15 days before and after unilateral optic nerve transection (ONT) until RNFLT was reduced by more than 40% from baseline. Before the animals were killed, TRBF was measured using the microsphere technique.

RESULTS. A significant correlation between all arterial and venous Doppler OCT TRBF measurements was found in ONT and contralateral control eyes (both P < 0.01, n = 6). The Bland-Altman analysis showed a bias of 0.57 in the ONT group and 0.02 in the contralateral control group. Also, excellent agreement was observed between Doppler OCT and microsphere measurements (P < 0.01, r = 0.976, bias = 0.54). After ONT, TRBF and RNFLT decreased by −51% ± 42% and −44% ± 2% (n = 5), respectively. In the contralateral control eyes, TRBF and RNFLT were unchanged.

CONCLUSIONS. Very good accordance was found between TRBF measurements, obtained with dual-beam bidirectional Doppler FD-OCT and the microsphere method. It also was possible to monitor changes over time in TRBF after ONT with Doppler OCT. These findings highlight the accuracy and potential of noninvasive Doppler OCT to provide valuable information for detecting early changes in ocular disease in future.

Keywords: total retinal blood flow, Doppler OCT, microspheres, nonhuman primate, optic nerve transection

Doppler optical coherence tomography (OCT) was first introduced by Wang et al.1 two decades ago. Doppler OCT is a functional extension of OCT, capable of obtaining high-resolution OCT images in combination with quantitative blood flow and velocity information. In 2007, a first report of total retinal blood flow (TRBF) in one human subject was published,2 soon to be followed by other groups reporting larger sample sizes and different approaches for measuring total blood flow.3–8

So far several ocular diseases, such as age-related macular degeneration,9,10 glaucoma,11,12 and diabetic retinopathy,13–15 were shown to be linked with early changes in choroidal and retinal perfusion. To assess ocular blood flow changes, it is of special interest to have a validated system available, which is easily applicable in routine future clinical practice.

Previously, other techniques have been introduced, but did not find their way into clinical use because of various limitations. Color Doppler imaging, an ultrasound-based technique, is only able to assess large retrolubar vessels and no information on vessel diameters can be obtained, which is essential for the calculation of total blood flow.15,16 In single retinal blood vessels absolute blood flow can be assessed by combining Laser Doppler velocimetry (LDV)17 measurements with retinal vessel diameter measurements, as for example, by the means of the Dynamic Vessel Analyzer (DVA; Imedos Systems UG, Jena, Germany).18 Yet, this approach is very time-consuming, because of the multiple measurements needed to assess TRBF. The Laser Speckle Flowgraphy (LSFG) approach only provides relative blood flow numbers; thus, no absolute blood flow can be assessed and it is used mainly to investigate the optic nerve head.19,20 Clinically, the gold standards for retinal and choroidal vessel assessment in health and disease still are fluorescein (FL) and indocyanine green (ICG) angiography.21 Due to methodologic limitations, ways to extract absolute blood flow information from FL/ICG angiography have not found their way into the clinic.22 Also, the dyes used are a potential risk for allergic reaction and shock.23

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With the availability of functional Doppler OCT the assessment of TRBF has become possible. Various methods to extract blood flow information from Doppler OCT readings exist, yet none has been validated against a well-established technique, such as the microsphere method, still a gold standard for quantitative blood flow measurements in animals. This method often is used to validate other blood flow techniques.32–35

In this study, we sought to validate the blood flow measurements of our previously developed dual-beam bidirectional Doppler Fourier domain (FD)-OCT.32 Therefore, we chose an optic nerve transection (ONT) model in nonhuman primates (NHP) and made use of the microsphere method. Furthermore, we hypothesized, that a decreased retinal nerve fiber layer thickness (RNFLT) due to ONT leads to a decrease in TRBF because the loss of the retinal ganglion cell layer leads to a diminished blood supply need.

Comparing total blood flow values obtained from measurements with the two approaches may help validate the noninvasive measurements made by Doppler OCT, a promising approach for future real time, in vivo assessments of absolute retinal blood flow and its changes.

**METHODS**

**Animal handling and procedures** performed adhere to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved and monitored by the Institutional Animal Care and Use Committee at Legacy Research Institute (Portland, OR, USA). This study was embedded in another project, which has been published previously.33 Therefore, we also are able to report changes in TRBF over time after ONT.

**Animals and Anesthesia**

Six male adult rhesus monkeys (Macaca mulatta; age, 6.7 ± 1.3 years) were included in this study. All animals initially were anesthetized for measurement procedures with intramuscular ketamine (15 mg/kg; Henry Schein Animal Health, Dublin, OH, USA) and xylazine (1.5 mg/kg; Akorn, Inc., Decatur, IL, USA), as well as a subcutaneous injection of atropine sulfate (0.05 mg/kg; Butler Schein Animal Health, Dublin, OH, USA). Animals were then intubated with an endotracheal tube to breathe air supplemented with pure oxygen at a ratio of 9:1 (air:oxygen). This was achieved by a gas mixer, which mixed the gas from two corresponding compressed gas tanks. The ending oxygen concentration that the animals breathed in was approximately 30% to ensure sufficient oxyhemoglobin saturation. During anesthesia animals’ body temperature was kept at 37°C using a warming blanket. Heart rate, end tidal CO2, as well as arterial oxygen saturation were recorded continuously. Arterial blood pressure was recorded continuously after cannulating one superficial temporal artery with a 27-gauge needle coupled to a pressure transducer and recorder (BLPR2; World Precision Instruments). Anesthesia was maintained by intravenous pentobarbital (8–12 mg/kg/h) infusion by means of an anesthesia with 1.5% to 3% isoflurane in oxygen. The optic nerve was cut approximately 6 to 8 mm proximal of globe entry and care was taken not to severe the central retinal artery. After reattaching the lateral rectus muscle, facial muscles and skin incisions were sutured.

**IOP Assessment and Control**

Before manometric IOP control, IOP was measured by rebound tonometry (Tonopen XL; Reichert, Inc., Depew NY, USA) in both eyes. In both eyes, IOP was set to 10 mm Hg manometrically, for Doppler OCT measurements. Therefore, two 27-gauge needles were inserted temporally at the limbus into the anterior chamber of each eye. One was linked with a pressure transducer and recorder (BLPR2; World Precision Instruments), the second was connected to a custom-made manometer set at 10 mm Hg.34 Eyes were left to equilibrate for at least 10 minutes before TRBF was recorded.

**RNFLT Imaging Protocol**

Peripapillary RNFLT was measured in both eyes of each animal using a spectral-domain OCT system (SD-OCT; Spectralis; Heidelberg Engineering GmbH, Heidelberg, Germany). For this study, the average peripapillary RNFLT was obtained from a single circular tomogram (B-scan) consisting of 1536 A-scans. Nine to 16 individual sweeps were averaged in real time to comprise the final stored B-scan at each session. At the initial imaging session, the position of the scan on the ONH was centered and all subsequent scans were pinned (identical) to this location using the instrument’s eye-tracking software. A trained technician manually corrected the accuracy of the instrument’s native automated layer segmentations of the RNFLT. Spectral-Domain OCT data, including segmentations, then were exported for extraction of RNFLT values by custom software.

**Doppler OCT Retinal Blood Flow Measurement**

The Doppler-OCT setup used to measure absolute blood flow velocities in the NHP retina has been described in detail previously.32 However, some modifications were made to allow for measurement of TRBF in NHP (see Fig. 1). The system operates at a central wavelength of 840 nm with a bandwidth of 52 nm, which results in an axial resolution of 4.4 µm and a lateral resolution of 21 µm. The combined power of the two probe beams on the cornea is approximately 650 µW, which is below the limits of the American National Standard Institute for small-source ocular exposure to a laser beam within the measuring time.35

In brief, the vessel is illuminated by two probe beams separated by their polarization properties. By using measurements from two distinct directions, blood flow measurements become independent of the exact angle of incidence; that is, the Doppler angle. When the beams fall onto moving red blood cells (RBCs), the back reflected light’s frequency is Doppler-shifted. The resulting phase shift in each of the two channels is given by $\Phi = \frac{2\pi}{\lambda} \cdot R \cdot v$, where $\tau$ is the time between two subsequent charge-coupled device (CCD) camera recordings and $v$ is the projection of the RBCs’ velocity vector onto the direction of the probing beam’s wave vector $k$, and can be obtained for each channel separately from adjacent A-line recordings after Fourier transform of the signal detected by two identical spectrometers. Absolute blood flow velocity then can be calculated by:

$$v = \Delta \Phi \cdot \frac{\lambda_0}{4\pi \cdot \tau \cdot \cos(\theta)}$$

where $\Delta \Phi = \Phi_1 - \Phi_2$ is the difference in the phase shifts.
between the two beams, \( \lambda_0 \) is the light source’s central wavelength, \( n = 1.37 \) is the refractive index of blood at 840 nm,36 \( \beta \) is the angle between the velocity vector and the detection plane spanned by the two probe beams, and \( \Delta z \) is the angle between the probe beams at the ocular fundus. With a beam separation at the pupil of 2 mm and an average eye length of 18 mm in monkeys, \( \Delta z = 6.4^\circ \).

The previously presented dual-beam system has been extended by a custom-built fundus view based on a CCD camera with 640 \( \times \) 480 pixels and integrated by means of a dichroic mirror. This enables precise positioning and focusing of the OCT beams on the ocular fundus. In addition, a dove prism (PS992M-B; Thorlabs GmbH, Dachau/Munich, Germany) mounted in a motorized rotation stage (PRM1/MZ8; Thorlabs GmbH) was implemented into the sample arm of the OCT’s Michelson interferometer.4 This allows the application of a rectangular scanning pattern by rotating the detection plane. This is done in an effort to measure all retinal vessels entering or leaving the optic nerve head.

**Doppler Image Processing and Blood Flow Calculation**

A detailed description of Doppler image processing already has been reported by Werkmeister et al.37 In brief, phase tomograms were corrected for bulk motion first and, if required, for phase wrapping artifacts. Thereafter, single vessels were segmented and pixels with intensity values below an empirically set threshold were fitted according to a parabolic flow profile, while leaving original data untouched. Then, the instantaneous phase shift \( \Phi_i \), within the vessel cross-section was calculated for each channel separately and averaged over 10 to 15 pulse cycles giving the mean phase shift \( \Phi_i,\text{mean} \).

Using trigonometry, it can be shown that the only missing parameter for blood velocity calculation is the en face angle \( \beta ^{,8} \) which was manually extracted by superimposing the OCT data with an en face infrared fundus image (Spectralis; Heidelberg Engineering GmbH) that was acquired in combination with RNFLT assessments.

The obtained angle \( \beta \) together with the mean phase shifts \( \Phi_i,\text{mean} \) in both OCT channels then can be used to calculate the mean blood flow velocity \( v,\text{mean} \) in the vessel of interest based on Equation 1.

Average vessel diameters \( VDi,\text{mean} \) were extracted from OCT phase data by determining the axial dimension of the vessel in pixels, converting this value into micrometers by taking the depth range of the OCT system and the refractive index of blood into account and averaging the values for several tomograms to compensate for vessel diameter changes due to pulsatility. Blood flow \( Q \) in each individual vessel entering or leaving the ONH can be calculated as \( Q_i = v,\text{mean} \cdot A_i \), where \( A_i \) is the vessel’s cross sectional area given by \( A_i = VDi,\text{mean}^2 \cdot \pi / 4 \).

By summing up the blood flow of all individual arteries leaving or all veins entering the ONH, TRBF can be calculated as \( Q_{\text{tot}} = \sum_{i=1}^{A_i} Q_{A_i,\text{tot}} \) or \( Q_{\text{tot}} = \sum_{i=1}^{A_i} Q_{A_i,\text{tot}} \), respectively. In the following, blood flow data are presented as \( \mu\text{L/min} \). In Figure 2, a scanning laser ophthalmoscopy (SLO) fundus image and the phase tomograms obtained from both OCT channels at the

**FIGURE 1.** Optical setup of the dual-beam bidirectional Doppler FD-OCT. SLD, superluminescent diode; PC, polarization controller; BD, beam displacer; FC, fiber collimator; BS, beam splitter cube; PBS, polarizing beam splitter cube; DC, dispersion compensation; DP, dove prism; FV, fundus view; RM, reference mirror; DG, diffraction grating.
different locations of the rectangular scanning pattern around
the ONH are depicted.

**Calculation of Central Retinal Arteriolar Equivalent (CRAE), Central Retinal Venular Equivalent (CRVE), and Arteriovenous Ratio (AVR)**

To report changes in vessel diameters, the CRAE, CRVE as well as the AVR were calculated from previously extracted vessel diameters. This was done as described previously. Arteriovenous ratio was calculated as CRAE/CRVE. It describes the ratio of retinal arteries to retinal veins, a parameter frequently used to describe changes in vessel calibers in the course of diseases.

**Microsphere Method for Retinal Blood Flow Measurement**

At the day of the final blood flow measurements, both femoral arteries and a vein were cannulated with PE50 polyethylene tubes for drug administration, blood pressure (BP) recording, and reference blood sample collection. After cannulating the left ventricle via the right brachial artery and arterial BP and end-tidal pCO2 stabilized, the blood was heparinized (heparin, 500 IU/kg intravenous injection). Thereafter, depending on bodyweight 60 to 100 million fluorescence microspheres (PolySciences, Inc., Warrington, PA, USA) with a diameter of 10 μm suspended in 1 mL of 0.15 M NaCl were injected into the left ventricle over 25 seconds. Reference blood was drawn over a period of one minute from the cannulated femoral arteries, starting from the microspheres injection. Animals were killed using pentobarbital (Euthasol; Delmarva Laboratories, Inc., Midlothian, VA, USA). Enucleated eyes were immediately postfixed in paraformaldehyde for 48 hours. Under a stereomicroscope, the retinas were gently isolated from choroid with a brush and sliced off from the optic nerve with a blade. The whole retina was mounted on a glass slide and photographed under a fluorescence microscope equipped with an automated imaging system. The photographs were digitally postprocessed to create montages and microspheres within the whole retina were counted.

A Fuchs-Rosenthal counting chamber (Electron Microscopy Sciences, Hatfield, PA, USA) was used to determine the microsphere concentration in the reference blood. Total retinal blood flow was calculated as $Q_{tot, micro} = \frac{N_{tissue}}{C_{ref}}$, where...
Table 1. Mean Arterial BP and IOP Pre- and Post-ONT (n = 6)

<table>
<thead>
<tr>
<th></th>
<th>BP, mm Hg</th>
<th>Control Eye</th>
<th>ONT Eye</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-ONT</td>
<td>88.7 ± 1.6</td>
<td>12.7 ± 1.4</td>
<td>13.2 ± 1.6</td>
</tr>
<tr>
<td>Post-ONT</td>
<td>91.9 ± 1.5</td>
<td>12.8 ± 1.0</td>
<td>11.8 ± 0.9</td>
</tr>
<tr>
<td>P, t-test</td>
<td>0.15</td>
<td>0.97</td>
<td>0.18</td>
</tr>
</tbody>
</table>

N_tissue is the microsphere count of the total retina and C_ref is the number of microspheres per microliter reference blood per minute.43,44

Experiment Protocol of TRBF Measurements and Comparison

The current study was included within a project, which required multiple session measurements of IOP, RNFLT, and optic nerve head blood flow (BF) using LSFG. The end point of the project was set when the RNFLT loss was 40% or more from baseline level in the ONT eye for each animal. Every 2 weeks before ONT, five baseline IOP, RNFLT, and optic nerve LSFG blood flow measurements were performed in both eyes. Up to three baseline measurements of TRBF using Doppler-OCT also were acquired. After ONT, all the above measurements were repeated every 10 to 15 days until RNFLT declined by approximately 40% from baseline average.

The final TRBF measurement determined by using the dual-beam bidirectional Doppler OCT was compared to that measured by the microsphere method. Since the microsphere method is a terminal procedure, Doppler OCT blood flow was measured first as described above and was immediately followed by the injection of microspheres and collection of reference blood. The total duration from the beginning of Doppler OCT blood flow measurement to the completion of the microsphere method took approximately 20 minutes. During this period, the IOP was set manometrically at 10 mm Hg.

Statistics

All data are reported as mean ± SD. Statistical analysis was performed using Prism 6 (SoftPad Software, Inc., La Jolla, CA, USA). Paired t-test was used for paired comparisons. Pearson r was calculated for correlation analysis. P < 0.05 was considered the level of significance.

RESULTS

Blood Pressure, IOP, and RNFLT

Table 1 summarizes baseline characteristics. Average BP and IOP were measured after general anesthesia and before Doppler OCT measurements were performed. There was no significant difference in BP and IOP before and after ONT.33

Baseline RNFLT in pre-ONT eyes and contralateral control eyes was 109.2 ± 6.8 and 110.8 ± 6.8 μm, respectively. At the day of final measurements, which was 55 ± 0.5 days from ONT surgery, ONT eyes had a mean RNFL loss of 44% ± 2% from baseline. Contralateral control eyes did not change (0.1% ± 2.0%).

Total Retinal Blood Flow, Velocity, and Vessel Diameter Measured by Doppler OCT

Total retinal blood flow data presented in the following are derived from retinal arteries. At baseline TRBF as assessed with Doppler OCT, (n = 6) was 26.3 ± 3.8 μl/min (confidence interval [CI], 22.3–30.3 μl/min) for pre-ONT eyes and 24.6 ± 4.1 μl/min (CI, 19.2–29.3 μl/min) for contralateral control eyes. There was no significant difference at baseline between the two eyes (P = 0.48, paired t-test).

After ONT, mean TRBF changed significantly by ~51% ± 42% from baseline (P = 0.04, paired t-test, n = 5). Total retinal blood flow did not change significantly in contralateral control eyes (P = 0.85, paired t-test, see Fig. 3).

Retinal arterial velocity in ONT eyes decreased from 2.26 ± 0.65 to 1.45 ± 0.56 cm/s (P = 0.03). There was neither a significant change in contralateral control eyes, nor in retinal venous velocity in ONT and control eyes (see Table 2).

To assess changes in vessel diameters, the CRAE and CRVE of all retinal arteries and veins were calculated. No significant change was detected, except for CRAE in control eyes, which increased significantly from 113 ± 11.4 to 123 ± 13.3 (P = 0.02). There was no significant difference in the AVR between baseline and final day of examination in ONT and control eyes.

One eye was excluded from post-ONT analysis as the central retinal artery was accidently cut during ONT surgery.

Comparison of Doppler OCT Arterial and Venous TRBF

At baseline, there was no significant difference between retinal arterial and venous TRBF in pre-ONT and contralateral control eyes (paired t-test, P = 0.14 and 0.72, respectively).

Correlation analysis between arterial and venous TRBF in ONT eyes (n = 6), both measured with Doppler OCT at various time points, showed a high correlation (P < 0.001, Pearson r = 0.962). A significant correlation between arterial and venous TRBF also was found in the contralateral control eyes (P < 0.001, r = 0.994, n = 6).

Comparison of TRBF Measured With Doppler OCT and the Microsphere Technique

For validation purposes all Doppler OCT and microsphere TRBF data were plotted against each other to assess their correlation. Analysis shows very good agreement between the two measurement techniques (P < 0.0001, r = 0.953, n = 9, Fig. 4). This also is evident from the Bland-Altman plot.
Doppler OCT Validation in Nonhuman Primate Model

Table 2. Summary of Average Arterial and Venous TRBF Blood Flow Velocity, CRAE, CRVE, and AVR Measured During Baseline and at the Last Examination Day (SAC).

<table>
<thead>
<tr>
<th>Eye</th>
<th>Baseline</th>
<th>SAC</th>
<th>P Value, Paired t-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Velocity, cm/s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arterial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ONT</td>
<td>2.26 ± 0.65</td>
<td>1.45 ± 0.56</td>
<td>0.05</td>
</tr>
<tr>
<td>Control</td>
<td>2.39 ± 0.71</td>
<td>1.86 ± 0.69</td>
<td>0.30</td>
</tr>
<tr>
<td>Venous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ONT</td>
<td>1.45 ± 0.36</td>
<td>1.19 ± 0.56</td>
<td>0.69</td>
</tr>
<tr>
<td>Control</td>
<td>1.22 ± 0.34</td>
<td>1.40 ± 0.66</td>
<td>0.57</td>
</tr>
<tr>
<td>Diameter, µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRAE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ONT</td>
<td>117 ± 8.3</td>
<td>121 ± 10.3</td>
<td>0.10</td>
</tr>
<tr>
<td>Control</td>
<td>113 ± 11.4</td>
<td>123 ± 13.3</td>
<td>0.02</td>
</tr>
<tr>
<td>CRVE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ONT</td>
<td>189 ± 9.2</td>
<td>176 ± 19.3</td>
<td>0.25</td>
</tr>
<tr>
<td>Control</td>
<td>190 ± 8.9</td>
<td>191 ± 4.9</td>
<td>0.83</td>
</tr>
<tr>
<td>AVR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ONT</td>
<td>0.62 ± 0.06</td>
<td>0.7 ± 0.05</td>
<td>0.15</td>
</tr>
<tr>
<td>Control</td>
<td>0.6 ± 0.07</td>
<td>0.65 ± 0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>TRBF, µL/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arterial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ONT</td>
<td>26.3 ± 3.8</td>
<td>18.0 ± 2.8</td>
<td>0.04</td>
</tr>
<tr>
<td>Control</td>
<td>24.6 ± 4.1</td>
<td>22.2 ± 4.1</td>
<td>0.85</td>
</tr>
<tr>
<td>Venous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ONT</td>
<td>26.7 ± 4.2</td>
<td>18.9 ± 2.6</td>
<td>0.06</td>
</tr>
<tr>
<td>Control</td>
<td>24.4 ± 4.6</td>
<td>22.3 ± 3.4</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Data are displayed as mean ± SD. Baseline, assessments before ONT.

A major goal in ocular blood flow research is to have a tool at hand that can be used in a clinical setting. Two major concerns must be addressed, namely the extraction of absolute blood flow data from measurements at the ocular fundus and the validation of the results obtained by the different approaches.

Today, various technical approaches for visualization and quantification of retinal perfusion exist. However, to our knowledge none of these is used in a clinical setting so far. Fluorescein angiography (FA) visualizes retinal vascular anatomy, but there still is no method for absolute volumetric TRBF measurement. With the LDV approach, TRBF measurements are very time-consuming, as the probe beam must be positioned on all retinal blood vessels one by one and perfect patient cooperation and patience are required.45

Different approaches based on OCT technology have been realized to measure TRBF. Wang et al.46 reported a single beam Doppler OCT system relying on a circumpapillary double circular scanning pattern to ascertain the Doppler angle and enable calculation of TRBF. Blatter et al.4 presented a dual-beam swept source OCT with a rotating scanning scheme based on a dove prism, which allows for the extraction of absolute velocity of the vessels around the ONH. A recent development uses three laser beams to make blood flow measurements independent of angles as the velocity vector can be obtained in all three dimensions.47 However, the drawback of this approach is the high complexity of the alignment procedure currently hampering the application of such a system in a clinical setting. Also, swept-source Doppler OCT systems have become available recently. These setups allow fast acquisition speeds in the range of seconds as well as the assessment of higher blood flow velocities.6

In most of the Doppler OCT approaches vessel diameter data are obtained from the amplitude or phase image. Konduru et al.48 reported a fast and semi-automated approach for TRBF extraction from Doppler OCT recordings, which significantly reduces evaluation times and has a very good intergrader correlation, making TRBF evaluations reproducible. However, Rose et al.49 acknowledged a learning curve of graders. Alternatively, vessel diameters can be assessed when the Doppler OCT system is extended with a Dynamic Vessel Analyzer, which reads vessel diameters simultaneously at the exact same location as the OCT recordings are done.8

![Figure 4](https://joos.org/)

**FIGURE 4.** Correlation of TRBF measured with Doppler OCT and the microsphere method (n = 9).

Correlation analysis shows a significant association between RNFL thickness loss and TRBF loss (P < 0.0004, Pearson r = 0.57, Fig. 6). After ONT one animal reacted with an initial 31% increase in TRBF compared to baseline and then slowly decreased over time (-12%, +11%, +0.9%, -1.5%, -1.4%) gain/loss from baseline). This analysis is based on the data of five ONT eyes as one eye had to be excluded, since the central retinal artery was cut during surgery.

**Correlation RNFLT + TRBF (% loss)**

Correlation analysis shows a significant association between RNFL thickness loss and TRBF loss (P < 0.0004, Pearson r = 0.57, Fig. 6). After ONT one animal reacted with an initial 31% increase in TRBF compared to baseline and then slowly decreased over time (-12%, +11%, +0.9%, -1.5%, -1.4%) gain/loss from baseline). This analysis is based on the data of five ONT eyes as one eye had to be excluded, since the central retinal artery was cut during surgery.

**Discussion**

Retinal blood flow changes in health and disease have been a major focus of research over the last decades. Various approaches, such as ultrasound or laser Doppler velocimetry, were used to quantify retinal blood flow. With the evolution of OCT and its functional extension, Doppler OCT, a technique based on the optical Doppler effect has become available providing information on blood flow in addition to the structural B-scan images.
To our knowledge, all of the aforementioned methods have not been validated against a well-established technique in an in vivo model so far. Reported blood flow data in humans range from 26.6 to 78.1 μL/min, but of course similar numbers reported do not guarantee accurate TRBF readings.

Other validation approaches have been used. A tiltable rotating disc with a preset absolute velocity is used for calibration and absolute velocity measurements. Also, a flow phantom consisting of a glass capillary, which is perfused at a preset flow velocity with a scattering solution, such as diluted milk, serves as an ex vivo validation model. In vivo, vessel bifurcations were used to compare the sum of branch vessel blood flow with the source vessel blood flow, which must be identical since no blood is “lost” before and immediately after the bifurcation.

Total retinal blood flow is the sum of the flow of either all retinal arteries or all retinal veins. Due to the fact that the eye is an end organ, the blood entering the retina via the central retinal artery and optic nerve also drains into the optic nerve via the central retinal vein. Therefore, $Q_{4.0m}$ and $Q_{3.0d}$ must match. Further, the comparison to another technique, such as LDV, as well as the effect of various stimuli (flicker light stimulation, changes in oxygen partial pressure) on TRBF were used to draw conclusions on the validity of Doppler OCT measurements. However, all of the mentioned approaches are not as strong as the validation against an independent in vivo method.

Therefore, we decided to validate our system in a NHP model using the gold standard of blood flow validation, the invasive microsphere method. This animal model provides an anatomy and physiology almost identical to the human eye. Our dual-beam Doppler OCT setup uses two probe beams and orthogonal detection planes to acquire blood flow data. The use of two probe beams with a known separation angle at the ocular fundus makes measurements independent of the angle of laser light incidence and makes measurements susceptible for small eye movements. The implementation of a dove prism into the sample arm allows for rotation of the detection plane and, thus, for assessment of TRBF by assessing the blood flow in all retinal vessels entering or leaving the ONH.

The microsphere method is terminal and gives exact blood flow values of the tissue of interest. It already has been used in various animal models previously to assess the validity of other blood flow measurement techniques, such as magnetic resonance (MR), positron emission tomography (PET), computed tomography (CT), as well as ultrasound perfusion imaging techniques, laser Doppler velocimetry, and LSFG.

In this study, we found an excellent agreement of TRBF measurements assessed with dual-beam Doppler OCT and the microsphere method. The conformity of both approaches can be well appreciated in the Bland-Altman plot (see Fig. 5). One outlier is found in this Figure, which most is probably due to the long Doppler OCT measurement as alignment problems of the animal occurred, during which ocular blood flow may have changed. A limitation of this study is the small number of animals used in this experiment, a common limitation in monkey experiments.

As expected, we found a significant decrease in retinal blood flow after ONT due to reduced metabolic demands with the reduced number of retinal ganglion cells. Interestingly, this decrease in retinal blood flow was caused solely by the decrease in retinal blood flow velocity, but not by retinal vessel diameters. This is compatible with the hypothesis that increased vascular resistance in these animals is due to peripheral capillary dropout.

In conclusion, quantitative measurements of volumetric retinal blood flow show very good agreement with TRBF assessed with the microsphere method. Changes in TRBF occurring over time, as for example, in the course of neural degeneration in response to ONT, can be documented well with Doppler OCT.

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