Visible light optical coherence tomography measures retinal oxygen metabolic response to systemic oxygenation

Ji Yi1, Wenzhong Liu1, Siyu Chen1, Vadim Backman1, Nader Sheibani2, Christine M. Sorenson2, Amani A. Fawzi3, Robert A. Linsenmeier1,5,4 and Hao F. Zhang1,3

The lack of capability to quantify oxygen metabolism noninvasively impedes both fundamental investigation and clinical diagnosis of a wide spectrum of diseases including all the major blinding diseases such as age-related macular degeneration, diabetic retinopathy, and glaucoma. Using visible light optical coherence tomography (vis-OCT), we demonstrated accurate and robust measurement of retinal oxygen metabolic rate (rMRO2) noninvasively in rat eyes. We continuously monitored the regulatory response of oxygen consumption to a progressive hypoxic challenge. We found that both oxygen delivery, and rMRO2 increased from the highly regulated retinal circulation (RC) under hypoxia, by 0.28 ± 0.08 μL min⁻¹ L⁻¹ (p < 0.001), and 0.20 ± 0.04 μL min⁻¹ L⁻¹ (p < 0.001) per 100 mmHg systemic PO2 reduction, respectively. The increased oxygen extraction compensated for the deficient oxygen supply from the poorly regulated choroidal circulation. Results from an oxygen diffusion model based on previous oxygen electrode measurements corroborated our in vivo observations. We believe that vis-OCT has the potential to reveal the fundamental role of oxygen metabolism in various retinal diseases.

Keywords: oxygen metabolism; retinal circulation; visible light optical coherence tomography

INTRODUCTION

Retinal cell degeneration is ubiquitous in the early stages of essentially all the major blinding retinal diseases (e.g., loss of pericytes in diabetic retinopathy (DR), retinal ganglion cells in glaucoma, retinal pigment epithelial (RPE) cells in age-related macular degeneration). Because the highly metabolic retinal cells rely on sufficient oxygen supply to maintain their functions, abnormality of retinal oxygen metabolism naturally could cause cell degeneration. This aberrant oxygen metabolism can further create prolonged impact on retinal function through oxygen-sensitive signaling pathways, such as hypoxia-induced factor-mediated pathways. Therefore, when accurately measured, the retinal oxygen metabolic rate (rMRO2) could potentially be a sensitive biomarker for early-stage diagnosis or an indicator for progression of several retinal diseases.

Currently there is no single non-invasive technique to measure rMRO2 in vivo. Direct measurement of intraretinal oxygen tension (PO2) using microelectrodes is considered as the gold standard in quantifying oxygen metabolism; however, the procedure is invasive and difficult to practice. Two-photon phosphorescence imaging has also been applied to quantify intraretinal PO2, but it relies on contrast agents. Magnetic resonance imaging can measure temporal changes of PO2 but only qualitatively in humans. Alternatively, rMRO2 can be measured by quantifying the consumption of oxygen derived from the retinal circulation (RC), which requires concurrent quantification of both blood flow and hemoglobin oxygen saturation (sO2). Several techniques such as laser Doppler velocimetry, multi-wavelength fundus photography and laser scanning ophthalmoscopy, and photoacoustic ophthalmoscopy have been demonstrated to measure one of the two parameters, but not both together. Optical coherence tomography (OCT) is another major technique for three-dimensional (3D) retinal imaging. Different OCT methods such as phase-sensitive optical Doppler coherence tomography and optical microangiography have been introduced to measure retinal blood flow, but rMRO2 is still inaccessible without other independent sO2 measurements.

Here we demonstrate that visible light optical coherence tomography (vis-OCT) can quantify rMRO2 in vivo through concurrent measurement of blood flow and sO2 from RC. We have previously demonstrated that sO2 can be measured in vivo through spectral analysis of OCT signals acquired within the visible light spectral range. The key advantage of vis-OCT is the 3D imaging capability allows one to recover optical spectra specifically from blood vessels and eliminate the confounding signal from other retinal layers. We first validated the blood flow and sO2 measurements both in vitro and in vivo. Then, as a proof of principle, we investigated the metabolic response to progressive hypoxia challenges. The experimental results were cross-validated by an oxygen...
diffusion model derived from direct oxygen tension measurements in rat outer retina using microelectrodes.

MATERIALS AND METHODS

Visible light optical coherence tomography imaging system

Vis-OCT (Figure 1) uses a supercontinuum light source (SuperK, NKT photonics) with a working bandwidth from 520 nm to 630 nm. The illuminating power on the cornea was measured to be 0.8 mW, which is within the ANSI laser safety standard (Supplementary laser safety standard calculation). Briefly, we used a free-space interferometry configuration to minimize the dispersion, where the probing beam was collimated and split by a pair of galvanometer mirrors to scan the focal point across the retina. The two beams from the reference and sample arms recombined at the beam splitter and were collected by an optical fiber. The fiber delivered the light to a home-built spectrometer that collected the interference spectral fringes by a line-scan CCD camera (spl2k, Basler). The full-width-half-maximum (FWHM) of the spectral coverage was ~85 nm, giving us 1.7-μm axial resolution. The lateral resolution was estimated to be 15 μm in the retina. Two scanning protocols were performed. Protocol 1 raster-scanned a 20 × 6 squared retinal area with 256 × 256 pixels in each direction at a 25 kHz A-line rate. Protocol 2 used dual circle scanning that scanned two concentric circles centered at the optic disk with 4096 pixels in each circle at a 70 kHz A-line rate. The dual circle scan pattern was repeated eight times and the results were averaged to remove motion artifacts and pulsatile flow pattern. Protocol 1 was used for sO2 measurement and microvascular imaging. Protocol 2 was used for blood flow measurement, which requires much denser scanning. For visualizing the microvasculature, the mean intensity projection of a slab at depth range from 150 μm to 200 μm with respect to the retinal surface was taken to capture the microvasculature. The system schematic (Supplementary Fig. S1), scanning, data acquisition protocols, and data processing (Supplementary Fig. S2) are explained in detail in the Supplementary Information.

Quantification of rMRO2 by vis-OCT

To quantify rMRO2 (gas volume of oxygen consumed per unit time, mL min⁻¹), we measured the overall oxygen difference between the major arterioles and venules in RC. The extracted oxygen from RC primarily supports inner retinal metabolism but could also supply part of the outer retina. Two parameters from the RC: total retinal blood flow \( F \) (μL min⁻¹) and sO2 (%) were measured, and the rMRO2 was calculated according to:

\[
rMRO2 = 1.34 \times C_{Hb} \times F \times (s_{a}O2 - s_{v}O2),
\]

where \( C_{Hb} \) is the total hemoglobin concentration (g mL⁻¹); 1.34 is the oxygen-binding capacity of hemoglobin (mL g⁻¹); \( s_{a}O2 \) and \( s_{v}O2 \) are arterial and venous sO2 with the subscript of \( a \) and \( v \) denoting arterial and venous blood, respectively. All major retinal arterioles branch (and venules merge) at the optic disk such that the global rMRO2

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**Figure 1** Principle of vis-OCT retinal imaging. (a) Illustration of vis-OCT system. A laser beam with a bandwidth from 520 nm to 630 nm was focused onto the retina. The reflected light interferes with the reference light, and the interference spectral fringes were collected by a home-built spectrometer. The interference spectral fringes were used to reconstruct the echo reflected from different depths of the retina. As the focal point was scanned across the retina by galvo mirrors, the 3D retinal structure was obtained. Further post-processing calculated the rMRO2 and produced the retinal microangiogram. (b) Example of a wide-field view of retinal microvasculature imaged by vis-OCT. (c–d) Magnified view of the highlight squared areas in the panel b. The arrows highlight the smallest capillary vessels. Bar: 200 μm.
derived from the RC can be calculated by measuring the flow and sO2 from major vessels around the optic disk.

**Blood flow measurement by phase sensitive Doppler OCT algorithm**

In phase sensitive Doppler OCT, interferometric spectral signals from two adjacent A-lines were used to extract blood velocity. The blood velocity can be expressed as

\[
v = \frac{f_{\text{sample}} \cdot \lambda_0 \cdot \Delta \phi}{4 \pi \cdot n \cdot \cos(\theta)},
\]

where \(f_{\text{sample}}\) is the OCT A-line scanning frequency; \(\lambda_0\) (nm) is the center wavelength of the OCT light source; \(\Delta \phi\) (radians) is the phase shift between adjacent A-scans after inverse Fourier transform; \(n = 1.38\) (dimensionless) is the refraction index of the sample; and \(\theta\) (radians) is the Doppler angle: the angle between the blood vessel and probing light (Supplementary Fig. S3). Phase wrap was present when the blood velocity was high. With the knowledge of flow direction (inward or outward from the optic disk), we corrected the phase wrapping by adding or subtracting \(2\pi\) to or from the original phase shift. The Doppler angle \(\theta\) was calculated by the relative displacement of each vessel between two circular scan images (Supplementary Information).

Because of the higher axial resolution than lateral resolution in vis-OCT, we directly measured the vessel height \(H\) axially from B-scan intensity images from the circular scanning protocol. The upper and lower boundaries of the vessels were manually selected.

After correcting the Doppler angle, the diameter of the blood vessel, \(\text{Dia}\), is

\[
\text{Dia} = H \times \sin(\theta).
\]

The total blood flow is equal to the product of the velocity and the vessel cross-sectional area

\[
\text{Flow} = v \times \frac{\pi}{4} \text{Dia}^2.
\]

**Oxygen saturation rate (sO2) measurement by spectroscopic OCT analysis**

A Gaussian window with FWHM \(k_w = 0.32 \mu m^{-1}\) (17-nm bandwidth at 585-nm center wavelength) swept the interferometric spectrum to select sub spectral bands. After the inverse Fourier transform on each sub-band, spatially resolved spectra can be obtained at each 3D voxel. The ability to selectively collect light reflected from a particular depth was the key to avoid the background reflectance that has hampered the accuracy of fundus photography-based methods. The spectral signal from the vessel bottom along the vessel center lines was used to fit the following analytical model based on a modified Beer’s law:

\[
I^2(\lambda) = I_o^2(\lambda) R(\lambda) \exp[-2d\mu_{\text{HBO}_2}(\lambda) \times sO_2 - 2d\mu_{\text{Hb}}(\lambda) \times (1 - sO_2)],
\]

where \(I_o(\lambda)\) is the incident intensity on the retina. We ignored the optical attenuation induced by the cornea, lens, and vitreous chamber due to their high transmission within the visible spectral range and,

Figure 2: Validation of blood flow and sO2 measurements *in vivo* using vis-OCT. (a) Illustration of scanning pattern for flow measurement. Two concentric circular trajectories around the optic disk scan cross all the major retinal blood vessels. (b) Pulsatile flow pattern from arteries and the simultaneous recorded EKG signal. (c) Fourier transform of the pulsatile flow pattern from all arterioles showing the heart rate. (d) Correlation of arterial sO2 measurement by vis-OCT (x-axis) and spO2 measured by a pulse oximetry (y-axis). Measurements from different rats were labeled with different markers (n = 4 rats). Each vis-OCT sO2 was averaged from all the major arteries. Solid line plots the linear regression from all the data. Dashed line plots the ideal (unity slope) correlation line. (e) Variations in arterial (n = 5) and venous (n = 5) sO2 responding to the changing oxygen content of the inhaled air. Error bar = SEM.
thus, took the source spectrum as $I_0$; $R_0$ is the reference arm reflectance; $d$ (mm) is the vessel diameter; $n(\lambda)$ (dimensionless) is the reflectance at the vessel wall, whose scattering spectrum can be modeled as a power law under the first-order Born approximation $n(\lambda) = A\lambda^{-s}$, where $A$ (dimensionless) is a constant. The optical attenuation coefficient $\mu$ (mm$^{-1}$) combines the absorption ($\mu_a$) and scattering coefficients ($\mu_s$) of the whole blood, which are both wavelength- and oxygenation-dependent. The subscripts $\text{Hb}$ and $\text{HbO}_2$ denote the contributions from deoxygenated and oxygenated blood, respectively. A linear regression to the logarithmic spectra returned the value of $s\text{O}_2$.

**Animal preparation**

All the experimental procedures were approved by the Northwestern University IACUC and conformed to the Association for Research in Vision and Ophthalmology Statement on Animal Research. Two anesthesia protocols were used in this work. For data shown in Figure 2, we initially anesthetized adult Long-Evans rats with 4.5% isoflurane for 10 mins, followed by 2.5% isoflurane during the imaging session. Isoflurane provided deeper anesthesia and better animal stability during the experiment, which is preferable for technology validation purposes. However, isoflurane is known to introduce vasoconstriction which would affect physiological studies. Thus, for data shown in Figures 3 and 4 that involved vessel size measurements, we anesthetized rats with a mixture of ketamine and xylazine (ketamine: 0.05 mg/mL; xylazine: 0.4 mg/mL, in saline) intraperitoneally. The initial injection used a dose of 10 mL kg$^{-1}$ body weight, followed by a 0.5-mL intraperitoneal injection every half an hour after the initial injection. A pulse oximeter was attached to the left rear paw of the animal to monitor its arterial $\text{sO}_2$ and heart rate (Supplementary Table). The body temperature was maintained between 36.5°C and 37.5°C using a heating pad (homeothermic blanket system, Stoelting Co.) underneath the animal. A home-made three-lead electrocardiogram (ECG) was used to record cardiac cycles. The animal was placed on a custom-made animal holder for imaging. We applied 0.5% Tetracaine Hydrochloride ophthalmic solution to the rats’ eyes for local anesthesia and applied 1% Tropicamide Hydrochloride ophthalmic solution to dilate the pupil. Commercial artificial tears were applied to the rats’ eyes every other minute to prevent corneal dehydration. The inhaled gas was a mixture of oxygen and nitrogen. The ratio of the two gases was controlled by a gas proportioner (7300 Series, Matheson). The ventilated gas was changed to a hypoxic mixture according to the protocols as shown in the section on “Results and discussion”. After the hypoxia challenge, the animal was allowed to recover and stabilize under normal air (21% $\text{O}_2$) for 5 min before being released.

**Analytical model of oxygen consumption in the outer retina**

Oxygen consumption is defined as volume of oxygen consumed by tissue per unit time (gas volume consumed per unit time). The units of $r\text{MRO}_2$ and oxygen consumption are the same, but the distinction is that we refer to oxygen consumption as what the inner and/or outer retina actually uses, whereas $r\text{MRO}_2$ is what the RC supplies.

Because the outer retina is avascular, oxygen is supplied solely through diffusion. An analytical model can be applied based on Fick’s second law according to the PO$_2$ at both sides of the outer retina and the geometry of the outer retina. This model has been validated by microelectrode measurements on different species, including rats, cats, and macaques.

Oxygen diffusion in the outer retina can be described by a one-dimensional three-layer diffusion model based on Fick’s second law:

$$QO_2 = D\frac{\partial^2 p}{\partial x^2},$$

where $QO_2$ is the oxygen consumption normalized by tissue weight (mL $\text{O}_2$ - min$^{-1}$ - 100 g$^{-1}$); $D$ is the diffusivity of oxygen in tissue $(1.97 \times 10^{-5}$ cm$^2$ s$^{-1}$); $k$ is the solubility of oxygen $(2.4 \times 10^{-5}$ mL O$_2$/ (mL retina$\times$mmHg)); $P$ is PO$_2$ (mmHg); and $x$ is the distance from the choroid. The photoreceptor outer segments, which do not consume oxygen, occupy layer 1 in the model, between $x = 0$ and $x = L_1$. The oxygen-consuming inner segments are between $x = L_1$ and $x = L_2$.

### Figure 3

Retinal vasculature diameter variation under hypoxia. (a–b) Mean intensity projection images around the optic disk under normoxia and hypoxia, respectively. The insets show pseudo-colored microvasculature images. (c) Comparison of average diameters of major arterioles (A) and veins (V) under normoxia and hypoxia ($n = 33$ from six rats). Error bar = SEM. (d) Magnified view of the inset in the panel a. (e) Magnified view of the inset in the panel b. (f) Comparison of the arteriole diameters highlighted in both panels d and e under normoxia and hypoxia. Bar: 200 µm. **$p < 0.01$ from two sample t-test.**
The third layer is the outer nuclear layer (photoreceptor cell bodies), which do not consume oxygen, and is between $x = L_2$ and $x = L$, where $L$ is the thickness of the outer retina. The averaged $QO_2$ in outer retina ($Q_{o2,O2}$) under light adaption has been characterized by fitting this model to microelectrode data.

From the parameters of the model, the fraction of the outer retina supplied by the RC ($F_r$) can be derived as

$$F_r = \frac{\left(\frac{L_1 + L_2}{2L}\right)}{P_r} + \frac{(P_t - P_r)}{(Q_{o2,O2} / (DkL^2))},$$

where $P_r$ and $P_t$ are the $O_2$ partial pressures at the choroid and at $L$, respectively. For the implementation of the model in the present work, $L_1$, $L_2$, and $L$ were manually measured from circular scan OCT B-Scan images at ten discrete locations (Supplementary Fig. S4). The averaged values were used in the simulation. It has been experimentally validated that the averaged $PO_2$ in inner retina and $P_r$ are well maintained during hypoxia challenge, whereas $P_t$ is linearly proportional to arterial oxygen tension ($PaO_2$) as

$$P_t = 0.64PaO_2 + 1.66.$$  \hspace{1cm} (8)

Under normoxic light-adapted conditions, $F_r$ is usually less than 10%; that is, most of the outer retina is supplied by CC rather than RC. However, because $P_t$ decreases in hypoxia, $F_r$ increases, and part of rMRO2 supplies the outer retina, which could be calculated as:

$$O_2(\text{RC} \rightarrow \text{Outer retina}) = Q_{o2,O2} \times F_r \times m.$$  \hspace{1cm} (9)

**Figure 4** Retinal oxygen consumption derived from retinal circulation in responding to systemic oxygen tension variation. (a) Progressive hypoxia challenge procedure. The oxygen content in the inhaling gas was reduced gradually in six steps from 21% to 9%. Arterial and venous sO2, blood flow, and blood vessel diameter were directly measured at each step. Arteriovenous sO2 difference, oxygen delivery, oxygen extraction fraction of retinal tissue, and rMRO2 were further calculated. (b) and (c) are sO2 and oxygen tension ($PO_2$) changes under reduced oxygen content (red-arterial, blue-venous), respectively. The corresponding progression of (d) arteriovenous sO2 difference, oxygen delivery, oxygen extraction fraction of retinal tissue, and rMRO2 were further calculated. (b) and (c) are sO2 and oxygen tension ($PO_2$) changes under reduced oxygen content (red-arterial, blue-venous), respectively. The corresponding progression of (d) arteriovenous sO2 difference, oxygen delivery, oxygen extraction fraction of retinal tissue, and rMRO2 were further calculated.
where $m$ is the weight of the outer retina. We measured the total weight of the rat retina to be 12 mg and the outer retinal weight $m$ to be 6 mg. Table 1 lists the values of characteristic parameters. Some of the parameters necessary to calculate this were measured by us, and some were taken from the literature. Detailed methods to solve the diffusion equation and the simulation of the PO$_2$ profile are provided in Supplementary Information.

**Statistics**

Statistical analysis was performed using STATA (StataCorp LP). Two sample T-test was used to test the significance in Figure 3c. Piecewise linear regression was used in Figure 4d–4f. The location of the discontinuity between fitted line segments were determined by minimizing the total square errors (least square error). A fixed effect statistical model was used for the linear regression in Figure 4g–4i to evaluate the fitted slopes assuming the slopes were the same in different subjects. The intercepts of the fitted line in Figure 4g–4i were averaged from all the animals.

**RESULTS AND DISCUSSION**

We imaged the 3D structure of the rat retina and quantified rMRO$_2$ using vis-OCT (Figure 1a). A focused broadband laser was scanned across the retina to provide transverse ($x, y$) discrimination. Reflectance at different depths ($z$) was reconstructed through interference between the reflected light and the reference light. Different contrasts were utilized to calculate rMRO$_2$. Blood flow is the product of the vessel cross-section area and velocity, where the vessel diameter was measured from the tomographic image and the velocity was measured based on the phase variation from the moving blood cells. The distinct optical absorption spectra of oxy- and deoxy-hemoglobin provided the contrast for sO$_2$ measurement.

Because of the strong attenuation of light in blood within the visible light spectral range, a shadow was cast underneath the vessels (Supplementary Fig. S4). We used an *en face* slice in the outer retina as a "screen" to capture this shadow effect and to create a "2D print" of the microvasculature (Figure 1b–1d). We were able to clearly visualize the large retinal vessels as well as the details of the microvasculature. This method does not require a high-density raster scan protocol as reported previously, and yet it provides robust label-free microangiography.

**Testing the accuracy of sO$_2$ and blood flow measurements**

First we evaluated the accuracy of flow and sO$_2$ measurement *in vitro* using vis-OCT (Supplementary Information). For flow measurement verification and calibration, a turbid aqueous solution (1% Intralipid) was pumped through a capillary tube by a syringe pump, and then the vis-OCT flow measurements were calibrated against the pump flow settings. For sO$_2$ measurement verification, bovine whole blood samples with controlled sO$_2$ were pumped through a capillary tube and we compared the vis-OCT sO$_2$ quantification with the results derived from blood analyzer readings. The accuracies were within 4.7% and 4% relative errors for flow and sO$_2$ measurements, respectively (Supplementary Figs. S5 and S6).

To accomplish flow measurement *in vivo*, we adopted a dual-circle scanning protocol around the optic nerve head (ONH). Because retinal blood vessels run radially from the ONH, each circle scanned cross all of the arteries and veins, which allowed us to capture the total retinal blood flow (Figure 2a). We repeatedly performed eight dual-circle scans at an A-line rate of 70 kHz. The high-speed scanning allowed us to capture the pulsatile profile of the blood flow (Figure 2b). EKG signals were recorded simultaneously to provide correlation between measured retinal blood flow and cardiac cycles. The pulsatile flow pattern from an artery coincided well with the EKG profile, with a slight delay (~0.1 s) between the peaks of the flow and the QRS complex. This delay was likely caused by the time needed by the sequence of atrioventricular node discharge, ventricular contraction, and the pressure propagation from heart to head. We took a Fourier transform of the pulsatile profile and the distinct peaks from all the arterial flows were consistent, indicating that the frequency of the cardiac cycle was 4.36 Hz (261.6 min$^{-1}$ heart rate). We averaged the pulsatile flow readings over the eight dual-circle scans for each vessel and calculated the total arterial and venous flows. The two total blood flows agreed with each other within a measurement precision ($\pm 0.38$ µL min$^{-1}$ averaged from $n = 4$ rats) (Supplementary Fig. S7), verifying the consistency between the inward (venous) and outward (arterial) blood flows. The value of total averaged blood flow was 6–8 µL min$^{-1}$ ($n = 4$ rats), which agrees well with reported values using the same anesthesia protocol.

Two experimental tests were used to examine the accuracy of our *in vivo* sO$_2$ measurement. In the first test, we gradually changed the oxygen content in the ventilated air from 21% to 10% in six steps. After each adjustment of O$_2$ content, the animals were allowed to adapt to the changed air and re-stabilize for 2 mins. The systemic peripheral arterial oxygenation (sPO$_2$) was monitored by a pulse oximeter attached to a rear leg of the rats. At each inhalation condition, we measured arterial sO$_2$ by vis-OCT and compared the averaged values with the pulse oximeter sPO$_2$ readings (Figure 2d). The linear correlation ($R^2 = 0.839$) established the responsiveness of our retinal sO$_2$ measurements to systemic sO$_2$ changes. We observed a slightly lower sPO$_2$ value than the vis-OCT sO$_2$, which may be due to the mild ischemia caused by the mechanical pressure from the sensor clip designed for human use. In the second test, we changed the inhaled oxygen from 21% to 100%, then back to 21%, and finally to 10% (Figure 2e). Arterial sO$_2$ was roughly unchanged from 21% to 100%, but dropped significantly at 10% oxygen (0.95 ± 0.02 at 21% oxygen, 0.95 ± 0.01 at 100% oxygen, 0.96 ± 0.01 at recovery 21%, and 0.59 ± 0.03 at 10% oxygen); on the other hand, the venous sO$_2$ changed with every change in oxygen content (0.75 ± 0.03 at 21% oxygen, 0.86 ± 0.01 at 100% oxygen, 0.74 ± 0.02 at recovery 21%, and 0.48 ± 0.01 at 10% oxygen). The arterial sO$_2$ changed much less than venous sO$_2$ during hyperoxia because the arterial blood was already well oxygenated under normal air breathing.

**Testing the longitudinal monitoring of blood flow, sO$_2$, and rMRO$_2$**

The advantage of using vis-OCT to measure rMRO$_2$ is the label-free and non-invasive nature, which allows us to perform longitudinal monitoring of blood flow, sO$_2$, and rMRO$_2$ from the same object. To demonstrate that, we performed a time-course study, in which five
measurements were taken from the same subjects over two weeks (Supplementary Fig. S8). The standard deviations of four functional parameters measurements were all within 11% of the mean values (7.4% for arterial \( sO_2 \), 6.4% for venous \( sO_2 \), 9% for blood flow, and 11% for \( rMRO_2 \)).

Retinal metabolic response to systemic hypoxia

Having characterized the accuracy of blood flow and \( sO_2 \) measurements, we studied how systemic oxygen tension affects \( rMRO_2 \) during hypoxia. Although previous studies have shown hemodynamic (increased retinal blood flow) and anatomical vascular changes (increased vessel diameter) under low oxygen supply\(^{41}\), a comprehensive observation of how inner retinal oxygen consumption reacts to limited oxygen supply was never reported using a non-invasive approach. In addition, the oxygen supply to the outer retina relies mostly on the choroidal circulation (CC) and little on the RC under light-adapted conditions\(^{34,35}\), but how their roles change in supplying the outer retina during hypoxia is still unknown.

We first investigated the retinal vascular anatomical changes under hypoxia (Figure 3). We observed that the major arteries and veins dilated under hypoxia. The average vessel diameter increased by 35% in arteries (59.7 ± 1.5 \( \mu \)m during normoxia; 80.8 ± 2.0 \( \mu \)m during hypoxia), and 16% in veins (77.4 ± 2.0 \( \mu \)m during normoxia; 90.2 ± 2.3 \( \mu \)m during hypoxia). Under normoxia, the arteries were curved due to a constrictive vascular tone; under hypoxia, as a comparison, straighter arteries indicated relaxation of vascular smooth muscle. In addition, we also observed dilation in smaller arterioles (Figure 3d–3f), which allows more blood flow into the deep retinal capillary network in the outer plexiform layer (OPL).

In order to progressively track the auto-regulatory response, we used a “step-down” hypoxia challenge protocol, in which the inhaled oxygen content was reduced from 21% (normoxia) to 9% (hypoxia) in six steps (21%, 19%, 16%, 14%, 11%, and 9%) as shown in Figure 4a. Multiple measurements were taken at each step and the progressing oxygen content of 9%.

As expected, both arterial and venous \( sO_2 \) decreased with reduced oxygen supply (Figure 4b). The venous \( sO_2 \) decreased almost linearly, while the arterial \( sO_2 \) decreased more rapidly when the oxygen content was below 14%. Because \( PO_2 \) is a direct stimulus to arteriovenous \( sO_2 \) difference and the RC has a large arteriovenous \( sO_2 \) difference and is well regulated\(^{43}\). To determine whether this balancing mechanism could quantitatively account for the increased oxygen extraction from the RC, we compared our experimental results with a simulation model built upon oxygen electrode measurements in the retina\(^{34} \). The model quantifies the oxygen consumption in the outer retina, and theoretically predicts the percentage of oxygen supply from RC and CC. It has been experimentally shown that the oxygen consumption does not change significantly (i.e. averaged \( PO_2 \) does not change significantly) in the inner retina during hypoxia challenge under light adaptation\(^{34,35}\). Thus by calculating the oxygen supplied from RC to outer retina, the model can predict the change of \( rMRO_2 \).

The \( PO_2 \) profile across the retina is illustrated in Figure 5b and 5c. When the systemic \( PO_2 \) drops, the choriocapillaris \( PO_2 \) also drops and increases the oxygen gradient from inner retina to outer retina\(^{34–36} \). The amount of oxygen delivered from the inner retina to the outer retina can

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**Table 2 Summary of the slopes of the linear fitting against arterial \( PO_2 \)**

| Parameter          | Slope       | Robust SE | \( p \) value | (95% confidence interval)     |
|-------------------|-------------|-----------|---------------|-----------------------------|
| Oxygen extraction fraction (OEF) | -0.0013995 | 0.00274   | <0.001        | [-0.019501 to -0.008489]    |
| Oxygen delivery (OD)   | -0.0028637 | 0.00828   | 0.001         | [-0.0045277 to 0.0011997]   |
| \( rMRO_2 \)         | -0.0020111 | 0.003709  | <0.001        | [-0.0027563 to -0.0012658]  |
then be calculated by Equations 7–9, in which the averaged outer retina oxygen consumption and PO2 has been previously characterized34. Notably, Equation 9 reveals an expected linear relationship between \( rMRO_2 \) and arterial PO2, based on which we linearly fitted our experimental data. When we compared the simulated change of \( rMRO_2 \) with the experimental \( rMRO_2 \) as in Figure 5d, the trends with decreased arterial PO2 were almost identical (slope \( \frac{52}{0.0023} \) in the simulation and \( \frac{52}{0.0020} \) in the in vivo experiment). This implies that the apparent increase in the detected oxygen consumption during hypoxia was due to the additional oxygen provided to the outer retina, and that for the range of arterial PO2 in our experiments, inner retinal oxygen consumption actually did not change.

Discussion
It is well known that RC and CC behave differently under systemic hypoxia5. This has been experimentally shown by direct micro-electrode measurements by Linsenmeier and Braun in cats35. They showed that the PO2 in the inner retina was well maintained under hypoxia challenge under light adaption; while the PO2 dropped in the chorioiopacillaris. This difference in PO2 results from the fact that blood flow in the RC increases during hypoxia (as we have verified noninvasively), while blood flow in the CC remains unchanged. Although the balance of oxygen supply between RC and CC is somewhat predicted by the diffusion model, noninvasive measurement of consumption of oxygen derived from RC has never been reported. Here we used a step-wise hypoxia challenge to experimentally observe the \( rMRO_2 \) response to systemic PO2 under light adaption. The non-invasive nature of our approach allows us to continuously monitor the same animal through the entire hypoxia challenge. Counterintuitively, we found that \( rMRO_2 \) increased with lower systemic PO2, which was explained by the oxygen diffusion model. In contrary, Wanek et al. reported a reduced \( rMRO_2 \) under hypoxia challenge using fluorescent microsphere to measure blood flow and two photon phosphorescence life time to measure \( sO_2 \)44. Although the reduced arterial \( sO_2 \) and arteriovenous \( sO_2 \) difference in our study were consistent with their findings, we observed monotonically increased blood flow under hypoxia; whereas in their results the blood flow increased at moderate hypoxia and returned to the normal level at severe hypoxic challenge. This appears to be the major discrepancy. If we decreased inspired oxygen further, we would expect that eventually the RC would fail to compensate and see the result observed by Wanek et al.

Translating vis-OCT from rodents to humans requires a few additional considerations. The first one is whether the visible light illumination will induce metabolic change during imaging. It has been shown that transitions between dark and light adaptation cause transient changes in retinal blood flow and oxygen metabolism45–47;
however, constant visible light exposure under light adaptation does not [38,49]. This indicates that vis-OCT is not likely to affect retinal oxygen metabolism when eyes are already light-adapted before imaging. Another practical concern is that the scanning visible light beam may distract eye fixation during imaging. However, this can be overcome by integrating an additional near infrared OCT channel for alignment purpose, where the visible light channel can only be turned on at the time of data acquisition for a short period of time.

The ability to quantify rMRO2 can potentially provide valuable insight into the pathogenesis of various retinal diseases, particularly DR and glaucoma. A key element is to understand the causal relationship between retinal cell degeneration and hemodynamic dysregulation. For example in DR, it is known that endothelial and pericyte disruption occurs in early-stage DR, but how the associated hemodynamic changes are unknown. Some studies have shown increased retinal blood flow [36,51] and suggested that the higher blood flow and high glucose level causes hyperperfusion, which further damages the endothelium and pericytes [51]. On the other hand, contradictory data exist and show that decreased blood flow is one of the earliest changes in diabetic retina [52,53]. The hypothesis is that the loss of pericytes in the early phase of the disease reduces oxygen consumption, which may paradoxically lead to increased oxygenation of the retina. This might create a hyperoxia, resulting in vasoconstriction and reduced blood flow [31]. Similarly, in glaucoma, there exists degeneration of retinal ganglion cells. Although altered blood flow and vasculature were observed in glaucoma, their causal relationship to ganglion cell death remains unknown [55]. Through measuring rMRO2, we will have the opportunity to correlate metabolic function and blood flow, which could potentially reveal the connection between hemodynamic dysregulation and retinal cell degeneration. With improved understanding of retinal metabolic function, improved approaches to early disease detection and therapeutic strategies can be designed.

The ability to quantify oxygen metabolism will expand the applications of OCT to a much broader scope. Vis-OCT provides a convenient approach that allows both blood flow and sO2 quantification with one set of measurements, which otherwise would either be invasive or requires different imaging systems.

CONCLUSIONS
We demonstrated the capability of vis-OCT to accurately measure rMRO2 and to monitor rMRO2 response subject to systemic oxygen modulation. This method allowed us to monitor retinal function via its oxygen consumption. We experimentally measured the response of rMRO2 to systemic PO2 variations and observed increased oxygen consumption supplied from the RC under hypoxia. This is explained by the balanced oxygen supply between retinal and CC. This method presents a noninvasive way of studying the role of oxygen consumption in various diseases whether the dysregulation of oxygen metabolism is important.

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