Blue-green spectral minimum correlates with oxyhemoglobin saturation in vivo

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Abstract. An imaging multi-spectral retinal oximeter with intravitreal illumination is used to perform the first in vivo test of the blue-green minima shift oximetry method (BGO) in swine [K. R. Denninghoff, R. A. Chipman, and L. W. Hillman, Opt. Lett. 31, 924–926 (2006); J. Biomed. Opt. 12, 034020 (2007)]. A fiber optic intravitreal illuminator inserted through the pars plana was coupled to a monochromator and used to illuminate the retina from an angle. A camera viewing through the cornea recorded a series of images at each wavelength. This intravitreal light source moves the specular vessel glint away from the center of the vessel and directly illuminates the fundus behind most blood vessels. These two conditions combine to provide accurate measurements of vessel and perivascular reflectance. Equations describing these different light paths are solved, and BGO is used to evaluate large retinal vessels. In order to test BGO calibration in vivo, data were acquired from swine with varied retinal arterial oxyhemoglobin saturations (60–100% saturation). The arterial saturations determined using BGO to analyze the multispectral image sets showed excellent correlation with co-oximeter data ($r^2=0.98$, and residual error $\pm 4.6$% saturation) and are similar to results when hemoglobin and blood were analyzed using this technique.

Keywords: biomedical optics; backscattering; image understanding; reflectometry; image evaluation.

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1 Introduction

Measuring retinal vessel oxygen saturation levels could successfully detect relative hypoxia prior to vision-threatening complications, and therapy could be started early, potentially preventing disease progression. Retinal oximetry measurements may provide additional benefits by allowing the provider to give targeted therapy and to determine the response to treatments using the same technique.

In an effort to address this need, retinal vessel oximetry was pioneered in 1963 by Hickham et al. prior to the development of the clinical pulse oximeter in the early 1970s. Improvements to this fundus photography technique were made by Cohen and Laing and Tiedeman et al. The latter identified the influence of fundus reflectivity on these measurements and attempted to compensate for this and calibrate their measurements. In addition to photographic retinal oximeters, systems have been developed that can scan monochromatic beams across the retinal vasculature and collect the light that is scattered back out of the eye. The earliest scanning oximeter was developed by Delori, and recent advancements have been made by Smith et al. and Schweitzer et al. who used a diffraction grating that measures the reflected optical spectrum of a retinal blood vessel in 2-nm increments from 510 to 586 nm. Schweitzer’s group also published a method using three isobestic wavelengths and a measurement wavelength in this region to measure oxygen saturation in reflection. Neff et al. applied a multispectral imager to retinal oximetry, illuminating with white light and capturing two or four images simultaneously through bandpass filters. A similar two-wavelength retinal oximeter has been evaluated by Stefansson. Central vessel glints, variations in fundus reflectivity, oxyhemoglobin intermediates, and red blood cell scattering significantly alter the measurement of optical signals in the eye and subsequent calculation of retinal blood oxygen saturations limiting the clinical use of these methods. We have reported an invasive technique, intravitreal illumination, which allows for rejection of the cen-

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tral vessel glint and improved oximetry by displacing the ves-
sel glint from the trans corneal solid collection angle in the
swine eye. We recently described the blue-green oximetry (BGO) tech-que, this approach takes advantage of the shift in the spectral minimum from around 470 to 510 nm as oxyhe-
moglobin increases from 0 to 100% of the hemoglobin present. BGO does not rely on the simplifications required by
the methods used to date in retinal oximetry and is more ac-
curate and more easily calibrated than the Lambert-Beer law-
based methods when used in hemoglobin solutions or with
intact red blood cells. However, until now this new oxim-
ery method has not been tested in vivo.

In the following, we use intravitreal illumination of the
retina in swine to evaluate BGO in vivo.

2 Surgical Procedure and Animal Preparation

Intravitreal surgery was performed on four young (approximately five months old) female American Yorkshire domestic
swine, and a series of experiments were performed using in-
travitreal illumination to measure the retinal blood oxygen
saturation. The experimental methods procedure detailed be-
low was performed on each swine. The experimental protocol
was approved by the University of Arizona Institutional Ani-
mal Care & Use Committee.

The swine was administered a general anesthetic mixture
consisting of air, nitrogen, oxygen, and isofluorane. Swan-
Ganz catheters were placed through the femoral vein and ar-
tery to monitor blood pressure and arterial oxygen saturation
\( (S_aO_2) \) and mixed venous oxygen saturation \( (S_vO_2) \). The first catheter tip was placed in the aorta just distal to the heart, and
the second catheter tip was placed in the inferior vena cava
just proximal to the heart. A peripheral oxygen-saturation
monitor was also placed in the rectum of the swine, and a lid
speculum was placed in the operative eye.

A 4-mm infusion cannulum was placed through the pars
plana and sutured in place. The infusion cannulum was at-
tached to a balanced salt solution gravity feed system to main-
tain intraocular pressure at \( \sim 14 \) cm of water. A contact vit-
rectomy lens (Bausch & Lomb, F36202.08, Rochester) was
placed onto the cornea using an index-matching (refractive
index: \( n_d = 1.337 \)) viscoelastic coupling agent.

3 Data Acquisition

Following the surgical preparation of the animal, light from a
scanning monochromator (Oriel Spectral Luminator, Irvine) was
coupled into a fiber optic intravitreal illuminator inserted
through the pars plana into the vitreous illuminating the
retina. The spectral resolution of the monochromator was
\( \sim 10 \) nm. A 12-bit scientific-grade CCD camera (Hamamatsu
Orca-AG) with a 3.3X macrozoom lens was used to image the
retina. The peak wavelength of a 10-nm wavelength band was
stepped from 420 to 700 nm, and an image of the target area
was acquired at each illumination wavelength. All the retina
were interrogated using the wavelengths 420, 430, 440, 460,
480, 490, 500, 510, 521, 532, 540, 545, 548, 552, 555, 558,
561, 565, 570, 575, 580, 590, 600, 615, 630, 645, 660, 680,
and 700 nm. In two animals, additional wavelengths in the
blue-green region were 475, 488, 506, and 514 nm. In order
to minimize the 2–17% vessel-diameter change across the car-
diac cycle, each camera exposure was triggered using a
cardiac monitor (Digicare Life Windows, Boynton Beach). By
minimizing the effect of vessel pulsation, the associated
change in optical path length during the cardiac cycle should
also be minimized. Some studies indicate that systemic blood
oxygen saturation can vary with cardiac cycle; also this effect
is avoided by capturing spectral images at the same point in
the cardiac cycle. Finally, exposure time was adjusted to
\(< 40 \) ms (\( \sim 1/25 \) of the cardiac cycle) in an attempt to mini-
bize the blood vessel size variation during exposure.

The multispectral image sets were obtained over a 5
\( \times 5 \) mm region of the illuminated fundus containing a se-
lected vein/artery pair. A dark image (obtained with the shut-
ter closed) was subtracted from each image on acquisition to
correct for dark current and external light sources.

The measurement series began by setting the inspired res-
piration mixture to 100% oxygen, 0% medical-grade air. The
animal was allowed to stabilize for \( 8 \) min and a multispectral
retinal image set was acquired. The oxyhemoglobin saturation
\( (S_O_2) \), blood partial pressure of oxygen \( (P_O_2) \), blood partial
pressure of carbon dioxide \( (P_CO_2) \), and hematocrit were
measured from small blood samples collected from the two
Swan-Ganz catheters using an ISTAP Personal Clinical Ana-
lyzer (Heska Corporation). The measurement cycle (8-min
stabilization, spectral data acquisition, and blood gas analysis)
was repeated at 10% decrements, until a pulse oximeter read
\( \sim 50\% \).

4 Data Calibration and Analysis

The data were calibrated for the spectral distribution of our
illuminator by measuring the output port of an integrating
sphere illuminated with the fiber optic illuminator. Then, the
multispectral images were normalized by dividing the image
intensities by this calibration data set. The inner wall of the
integrating sphere is coated with Spectralon\textsuperscript{®}, a common cali-
boration standard. This procedure ensured accurate pig’s eye
spectral reflectivity by minimizing any spectral power varia-
tions from the illumination system or the detector system.

The fundus multispectral image sets were registered to cor-
correct for eye motion during data collection, and images were
aligned to within a fraction of a pixel using a bicubic spline
interpolation in conjunction with a maximum mutual informa-
tion merit function algorithm.

5 Oxygen Saturation Calibration Standard

Absolute calibration of the retinal arterial oxyhemoglobin
saturation \( (S_aO_2) \) measurement relies on the assumption that
large arterial oxygen saturation values are constant throughout
the body and that the \( S_O_2 \) in the aorta will be the same as the
\( S_aO_2 \). This assumption is supported by the observation
that the oxygen uptake from a vessel is based on the surface
area-to-volume ratio and the retinal arterial vessels that we
analyzed are all much larger than the capillaries where oxygen
exchange primarily occurs.

6 Light Path Analysis

The multispectral retinal images were analyzed to determine
the spectral reflectivity of the retina, the spectrum of scatter-
ing from blood vessels, the transmission of blood vessels, and
the level of integrated scatter within the globe. These light
fluxes were then applied to evaluate the oxygen saturation of a large artery and vein using BGO. Our initial motivation was to obtain the single-pass scattered light spectrum through vessels by (i) illuminating behind the vessels to provide a strong single-pass signal and (ii) move the vessel glint to the side of the vessel to minimize its interference with the transmitted signal. In the process of this experiment, we learned the significance of the light scattered from the blood column.

Figure 1 shows the set of light paths associated with these intravitreal illumination experiments. The source flux from the light pipe illuminates the fundus from the side, displacing reflected glints from the center of the vessel image well to the side of the vessel. The iris established the solid angle of the flux collected from the fundus. This method simplifies the collected flux from the vessels. Let \( \Psi \) be the flux incident on a point of interest in the fundus from the light pipe. The globe is also full of singly and multiply scattered light, similar to the light that is not absorbed by the blood in the vessel, the vessel wall, and other structures, and scattered out of the collection angle by blood in the vessel, the vessel wall, and other structures. The light scattered from the vessel \( S_3 \) is related to the incident flux

\[
S_3 = \Theta (\Psi + \Psi_i) \tag{2}
\]

by \( \Theta \), the portion of the total incident flux that is scattered from the blood in the vessel, the vessel wall, and other structures into the collection angle. Thus, the total flux reflected from or through the center of the vessel into the collection angle, \( R_v \), is

\[
R_v = S_2 + S_3, \tag{4}
\]

the sum of the transmitted and scattered components.

Finally, side illumination produces distinct vessel shadows in the retinal images, clearly displaced from the vessels. The flux collected from the vessel shadows \( S_3 \) is

\[
S_4 = R_v (T_{i,v} T_{a} + \Psi_i). \tag{5}
\]

This shadow is the result of the interaction of the scattered and transmitted light, the retinal reflectivity, and the power terms.

These equations, despite being approximations, provide insight into the underlying scattering processes as follows. The ratio of the vessel flux to the adjacent avascular fundus reflectance is

\[
\frac{R_v}{S_1} = \frac{R_v (\Psi + \Psi_i) T_{i,v} T_{a} + \Theta (\Psi + \Psi_i)}{R_v (\Psi + \Psi_i)} = T_{i,v} \frac{\Theta}{R_v}, \tag{6}
\]

Equation (6) is independent of the flux terms, providing a solution expressed in transmission and scatter coefficients.

We have approximately determined \( T_{i,v} T_{a} \) from another experiment measuring the forward transmission of light through a 100-\( \mu \)m flow cell in a spectrophotometer. The effective path length near the center of 130-\( \mu \)m cylindrical vein is similar in length to the flow cell. The blood hemoglobin concentration, effective path length, and oxyhemoglobin saturation were set close to the expected values for the swine eye, and the data used to approximate \( T_{i,v} T_{a} \). The spectrophotometer values of \( T_{i,v} T_{a} \) were compared to the measured value of \( R_v/S_1 \) at each wavelength, recognizing that this approximation neglects scattering and absorption by vessel walls. We found the following relation held,

\[
\frac{R_v}{S_1(130-\mu m \text{ vein})} - (T_{i,v} T_{a})(\text{spectrophotometer}) \approx \frac{\Theta}{R_v}. \tag{7}
\]

The value of \( T_{i,v} T_{a} \) was <2% of the total \( \Theta/R_v \) at all wavelengths tested from 450–700 nm, demonstrating that, for the wavelengths and the angle of incidence used in these swine,
the assumption that \( R_r \approx T_s T_a \) is reasonable. Thus, the approximation

\[
\frac{R_v}{S_1} \approx \frac{\Theta}{R_t}
\]  

(8)

is valid. Equation (8) is useful because it is a ratio involving transmission and reflectance without any absolute flux terms and is relatively easily determined from the \textit{in vivo} experimental images. The perivascular reflectance, \( R_v \), was a source of calibration error in previous attempts to measure \textit{in vivo} retinal oxygen saturation.1,26

The data obtained from this experimental design provide measures of the spectral behavior of the perivascular retinal reflectance \( R_r \). Referring to the light-path analysis from Fig. 1, combining and simplifying Eqs. (1) and (4) yields

\[
S_1 - S_4 = R_t (\Psi + \Psi_t) - R_t (\Psi T_a T_s + \Psi_t) = R_t \Psi (1 - T_s T_a).
\]  

(9)

When \( T_s T_a \ll 1 \), as seen when light is highly absorbed by hemoglobin (450–600 nm),1,29 then Eq. (9) further simplifies,

\[
S_1 - S_4 \approx R_t \Psi.
\]  

(10)

Because the power from the light pipe is normalized at each wavelength, the relative reflectance of the perivascular fundus is given by \( S_1 - S_4 \) at wavelengths significantly absorbed by hemoglobin.

7 Results

The spectra of the measured components, \( S_1 \), \( R_v \), and \( S_4 \) from a retinal vein are shown in Fig. 2. Each spectrum is a complex combination of the interaction of the source flux \( \Psi \) and the reflected flux \( \Psi_t \) with a specific region of the retinal anatomy. These signals are similar to the retinal reflectance spectra reported by others.1,4 We used the spectra from the 460–521 nm region to calculate the values \( -\log(R_v/S_1) \) and \( S_1 - S_4 \).

Figure 3 shows the spectra of the perivascular retina \( -\log(S_1) \) and an adjacent retinal artery \( -\log(R_v) \), and the spectra of \( -\log(R_v/S_1) \). Fluctuations in power in the two empiric signals are removed by dividing \( R_v \) by \( S_1 \). Because the reflected flux \( \Psi_t \) is an integrated signal from the globe, it contains a bulk average of the arterial, capillary, and venous hemoglobin along with pigment signals from the eye. Dividing \( R_v \) by \( S_1 \) significantly reduces signal noise (removing \( \Psi_t \)) while shifting the minimum to the right for arteries and to the left for veins (removing \( \Psi_t \)).

For the BGO technique to be useful, the analysis must be consistent with changes in vessel diameter, illumination angle, blood concentration, and oxyhemoglobin saturation. Figure 4 shows the monochromatic image of a region of a swine retina at 460 nm. One can see the vessel shadows adjacent to and
Fig. 3 The spectra shown above were taken from a retinal artery [−log(Rv)] and the adjacent perivascular retina. Note that fluctuations in the power terms seen in these two signals are attenuated when dividing Rv by S1. The reflected flux Ψ_r is an integrated signal from the globe and has a bulk average of the arterial and venous hemoglobin signals. Dividing Rv by S1 removes Ψ_r, shifting the minimum to the right for arteries and to the left for veins.

Fig. 4 Shown above is a monochromatic image of the retina at 460 nm that shows the vessel shadows adjacent to and just left of the vessels. The shadow is most apparent where the left-hand bifurcation of the large vein crosses over the artery that is also to the left of the large vein in the image. The minima of the spectra of −log(Rv/S1) from the branches of the bifurcated vein and the large confluent vein to the right of the large artery in the image are shown above. Note that the minima are very similar when scans are taken from markedly different sized veins with different angles of illumination.
just left of the vessels. The shadow is most apparent where the left-hand bifurcation of the large vein crosses over the artery that is also to the left of the large vein in the image. The minima of the spectra of $-\log(R_v/S_1)$ from the branches of the bifurcated vein and the large confluent vein to the right of the large artery in the image are also shown in Fig. 4. Local differences in retinal metabolism and autoregulation will cause small variations in the oxyhemoglobin saturation found in two veins that feed a larger vein. The confluent vein should have a bulk average of the two feeder vein oxyhemoglobin saturations. The minima shown in Fig. 4 are very similar when scans are taken from markedly different-sized veins with different angles of illumination and the large vein minimum is between the two feeder vein minima as expected.

Figure 5 shows how the spectra of $-\log(R_v/S_1)$ for the artery and the vein shown in Fig. 4 have minima that are significantly different from each other and behave as predicted by the BGO technique. The minimum of the arterial fit is 502 nm and the vein is 489 nm, demonstrating sensitivity to oxygen saturation.

In Fig. 6, we show the relative optical density and retinal reflectivity from a swine retinal artery calculated using Eqs. (8) and (10). The BGO minimum shown here is in the region of the spectrum expected for an artery, and the reflectivity of the perivascular fundus is nearly neutral. By selecting the capillary-poor perivascular region and using our light-path analysis to remove the scattered light component, we are able to determine the retinal reflectance ($R_v$). Because 460–521 nm light that traverses the retinal pigment epithelium is nearly all absorbed by melanin, the return signal $R_v$, shown in Fig. 6, is nearly all flux that has been scattered back to the detector from the inner retina. This result is consistent with recently published work with a retinal microsurgery isolated in vivo inner retina, showing that the perivascular inner retina has very little absorption and is nearly spectrally neutral.

On the basis of these observations, we fit a parabola to the plot of $-\log(R_v/S_1)$ for the retinal arteries in four swine and calculated the minima. A calibration plot was generated by comparing these minima to the oxyhemoglobin saturations in the swine arterial circulation. The quality of the calibration plot was evaluated by calculating the standard deviation of the residuals. Figure 7 shows the calibration plot for this in vivo analysis using BGO to analyze 22 measurements in 4 swine with different arterial oxyhemoglobin saturations, vessel diameters (58–130 μm) and hematocrits (24–45%). The slope of 0.33 nm/percent saturation, the intercept of 469 nm and the standard deviation of the calibration residual error of ±3.4% saturation are similar to BGO in hemoglobin and blood. The difference in calibration intercepts from the hemoglobin calibration line to the blood calibration line in transmission is related to the slope of wavelength-dependent scattering and is discussed at length elsewhere. The opposite shift in intercepts seen when BGO is used in vivo is likely due to the change in the sign of the slope of wavelength-dependent scattering when a signal is collected in reflection in the retina rather than in transmission with a spectrophotometer. Using the calibration line from Fig. 7 to convert minima to saturations, the large vessels shown in Fig. 4 have a retinal venous oxyhemoglobin saturation of 64% (489 nm) and retinal arterial oxyhemoglobin saturation of 98% (502 nm).

Fig. 5 The spectra of $-\log(R_v/S_1)$ for the artery and vein shown in Fig. 4 are shown here. The artery and vein have minima that are significantly different from each other and behave as predicted by the BGO technique.
We performed multiple measures on vessels in the same region moving up one pixel at a time for five lines. The standard deviation of the minima residuals was 0.33 nm or 1% saturation. Though this is not exactly the same as repeated measures at the same position, it is a more accurate test of the sensitivity of the method to error because changes in oxyhemoglobin saturation over time and systematic movements are significant confounders likely more important than the changes that occur over a five-pixel range while one frame of data is being collected.

One longstanding problem with retinal oximetry has been the poorly conditioned nature of the equations. Small errors in data lead to far larger errors in the oxygen saturation determinations. We evaluated the conditioning of the BGO technique theoretically by assuming a 1% error in each optical density and rereducing the swine reflectance data. A 244-value Gaussian distributed pseudorandom number set with a zero mean and a standard deviation of 0.01 was generated. Using the spectra from two swine arterioles and a venule with different oxyhemoglobin saturations (96.7, 85, 54%) and three wavelengths (475, 488, 514 nm), five (460, 475, 488, 500, 514 nm) and 10 wavelengths (460, 475, 480, 488, 490, 500, 506, 510, 514, 521 nm), we measured the standard deviation of the calculated minimum when the 244 errors where added to the spectra. The greatest residual error was seen using the 96.7% oxyhemoglobin saturation data with three wavelengths (±2.7% saturation), and the lowest error when 54% oxyhemoglobin saturation data with 10 wavelengths were used (±0.9% saturation).

9 Comment on Intravitreal Illumination

Intravitreal illumination is highly invasive, requiring a puncture through the pars plana. It must be performed in a sterile environment by a highly trained practitioner. A vitrectomy is also necessary if one seeks to freely move the light probe through the vitreous chamber. As such, we are not proposing intravitreal illumination as the basis for a widely applicable retinal oximetry test. We do emphasize that the unique light paths provided by intravitreal illumination are particularly useful for developing models of light propagation in the posterior chamber. These model equations take advantage of the reduced specular reflections occurring anterior to the blood column in the vessel and also remove the possibility of light double passing through blood vessels. Using these model equations with information available from other sources allowed us to solve for the spectrum of the perivascular inner retina without using Spectralon, as we had previously done.

Because the back-reflected light that traversed the vessel and returned to the detector was shown to be negligible when BGO wavelengths were used, removing double-pass light was
shown to be unnecessary. Our work here indicates that the exclusion of central glints or specular reflections from anterior to the vessel remains an issue, but an on-axis illumination system that allowed for the removal of the glint would lead to a similar set of model equations.

## 10 Arterial Calibration of Venous Measurements

The use of arterial signals to calibrate venous measurements using BGO as done here assumes that the arterial profiles and the vein profiles have the same components. The retinal arteries contain blood, are anatomically in analogous circumstances with the same light paths, have vessel walls that scatter light, are in the same size range, and can have the oxyhemoglobin saturation manipulated so that it reaches the range seen in veins. It will be very difficult to generate a better model for the retinal venous circulation than the retinal arteries. However, we know that, aside from oxyhemoglobin saturation, there is a physiologic anemia on the venous side of the circulation, retinal veins are often larger than retinal arteries and venous vessel walls are thinner than corresponding arterial vessel walls. Our results address some of these concerns directly and others by inference.

The sizes and the hematocrits of the blood in the arterial vessels we analyzed varied substantially without causing a significant change in the minima we measured based on these parameters. This makes it unlikely that the differences in size between the arteries and veins or physiologic changes in hematocrit from the arterial to venous circulation will have a significant impact on calibration. We remain uninformed about how different vessel wall thicknesses may influence the backscattered light, but the arterial and venous saturations determined using BGO are reasonable.

## 11 Conclusions

BGO, described previously in hemoglobin and blood, is demonstrated here in vivo in an anesthetized pig using a specialized optical system. BGO is sensitive to oxygen saturation and is robust to errors at the 1% level. The relative contribution of single-pass light, double-pass light by inference, and backscattered light in reflectance retinal spectroscopy was separately measured and reported. The shift in BGO calibration line intercepts caused by the slope of wavelength-dependent scattering described by us previously using blood in transmission was present in a system using reflected light, demonstrating a similar but opposite shift in the calibration line intercepts. More work is needed to evaluate varied blood concentration, vessel diameter, blood flow, vessel wall scattering, and techniques for excluding the vessel glints when on axis illumination is used for BGO.

## References