

Blood oxyhemoglobin saturation measurements by blue-green spectral shift

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Abstract. Previous work describing a resilient method for measuring oxyhemoglobin saturation using the blue-green spectral shift was performed using cell free hemoglobin solutions. Hemoglobin solution and whole blood sample spectra measured under similar conditions in a spectrophotometer are used here to begin evaluating the impact of cellular scattering on this method. The blue-green spectral shift with changing oxyhemoglobin saturation was preserved in these blood samples and the blue-green spectral shift was relatively unaffected by physiological changes in blood pH (6.6, 7.1, and 7.4), path length through blood (100 and 200 μm), and blood hematocrit (19 to 48%). The packaging of hemoglobin in red blood cells leads to a decreased apparent path length through hemoglobin, and an overall decrease in scattering loss with increasing wavelength from 450 to 850 nm. The negative slope of the scattering loss in the 476 to 516-nm range leads to a +3.0 nm shift in the oxyhemoglobin saturation calibration line when the blue-green spectral minimum in these blood samples was compared to cell free hemoglobin. Further research is needed to fully evaluate the blue green spectral shift method in cellular systems including *in vivo* testing. © 2007 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2745312]

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1 Introduction

The use of Lambert-Beer's law to model the optical behavior of hemoglobin solutions and solve for oxygen saturation requires knowledge of the optical density per unit path length per unit concentration at two wavelengths.^{1,2} An important assumption required by these models is that the change in optical density with changing oxyhemoglobin saturation is the weighted average of the optical density of pure oxyhemoglobin and deoxyhemoglobin.¹⁻⁴ These models also assume that the optical density is unaffected by physiological changes in carbon dioxide concentration, pH, or other compounds known to change the configuration of the hemoglobin structure such as 2,3 biphosphoglycerate.^{5,6} Recent work showing that the pH of hemoglobin and blood can be measured using near-IR wavelengths to interrogate the specimen independent of oxyhemoglobin saturation^{7,8} and published extinction coefficients for oxyhemoglobin and deoxyhemoglobin that vary significantly (by as much as 20%) make the use of these parameters without calibration controls problematic.³

Steinke and Shepherd developed and empirically verified a model equation for red blood scattering and absorption losses from forward transmitted collimated light with the form:^{9,10}

$$T_c = \exp[-d(\Sigma_a + \Sigma_s)], \quad (1)$$

where T_c is the transmitted light striking a narrow angle detector, $\Sigma_a + \Sigma_s$ is the total cross-sectional loss, Σ_a is the absorption cross section, Σ_s is the scattering cross section, and d is the path length through a plane of blood. Converting Eq. (1) into optical density space gives:

$$\text{OD}_T = d(\text{OD}_A + \text{OD}_S), \quad (2)$$

where OD_T is the optical density due to the absorption and scattering of light $[-\log(T_c)]$; OD_A is the optical density of a hemoglobin solution having the same oxyhemoglobin saturation, hemoglobin concentration, and path length as the blood sample; and OD_S is the apparent optical density due to scattering.⁹ Note that Eqs. (1) and (2) use the same path length (d) for the scattering loss (OD_S) and the absorption loss (OD_A). In addition, many subsequent investigators attempting to measure the oxyhemoglobin saturation of blood using this equation have modeled scattering loss as a constant with changing wavelength when data is obtained in a narrow range. Combined, these two assumptions facilitate three or more wavelength approaches to the oximetry of blood *in vivo*.^{3,11-15} However, when *in vivo* oximetry has been attempted using these approaches, the combination of scattering with the above assumptions related to the behavior of hemo-

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globin is associated with significant difficulty making calibrated measurements.^{3,4,12-21}

Our group has recently described a new method for measuring oxygen saturation in hemoglobin solutions that is insensitive to path length, pH, or concentration changes.²² The wavelength band required for this analysis is narrow, leading to the hope that this simple method for measuring blood oxyhemoglobin saturation might also be insensitive to scattering effects facilitating *in vivo* measurements. In the following, we report the results of our work designed to measure the impact of red blood cell scattering on forward transmission from 450 to 850 nm. In addition, we evaluate how this scattering loss influences the blue-green spectral shift seen with changing oxyhemoglobin saturation.

2 Methods

We obtained blood from two healthy volunteers according to an Institutional Review Board approved protocol. The blood was anticoagulated using citrate dextrose solution A with the pH of the anticoagulant adjusted using dilute hydrochloric acid or dilute sodium hydroxide as needed so that the blood pH after anticoagulation was 6.6, 7.1, or 7.4. The donated blood was stored on ice while aliquots of the blood were centrifuged at 4000 rpm for 8 min, followed by aspiration of the plasma from the packed red blood cell mass. Because blood samples anticoagulated and stored undergo red blood cell lysis over time, we evaluated the plasma from each centrifuged sample for hemoglobin content using the spectrophotometer. If lysis occurred, the sample was rejected, a new sample was obtained from a donor, and the blood preparation procedure repeated.

These blood samples were placed in a blood oxygenation system of our own design, described in detail elsewhere,²³ and either plasma or packed cells obtained by centrifugation of an aliquot of the same blood sample was used to set the desired blood hemoglobin concentration. Each sample was set to the desired blood oxyhemoglobin saturation using a gas mixture that was warmed and humidified at 39°C. The gas mixtures included a fixed concentration of carbon dioxide (partial pressure of CO₂=40 mm Hg) and various mixtures of oxygen and nitrogen.²³ The blood samples were then pumped at a rate of 650 μL/min through either a Starna 100-μm or Starna 200-μm flow cell in a Cary 100 Spectrophotometer. The Cary Spectrophotometer was standardized using a saline solution in the respective 100- or 200-μm cuvette to set 100% transmission and then blocking the beam to set 0% transmission. The spectra of the blood samples (OD_T) were measured at 2-nm increments with a 2-nm bandwidth. The spectrum was measured for each sample with a pH of 6.6, 7.1, or 7.4, a path length of 100 or 200 μm, and hemoglobin concentrations from 6.6 to 14.7 g/dL (hematocrit from 19 to 48%).

We measured the spectra of cell free hemoglobin solutions (OD_A) with the same hemoglobin concentration ([Hb]), path length, pH and oxyhemoglobin saturation as the blood (OD_T). Hemoglobin samples were prepared from fresh packed human red blood cells in the following manner. Anticoagulated blood was centrifuged at 8000 rpm for 8 min, and the buffy coat removed along with the plasma by aspiration. Either distilled water was added or 0.02% saponin was added to the packed red blood cells followed by centrifugation and filtration to

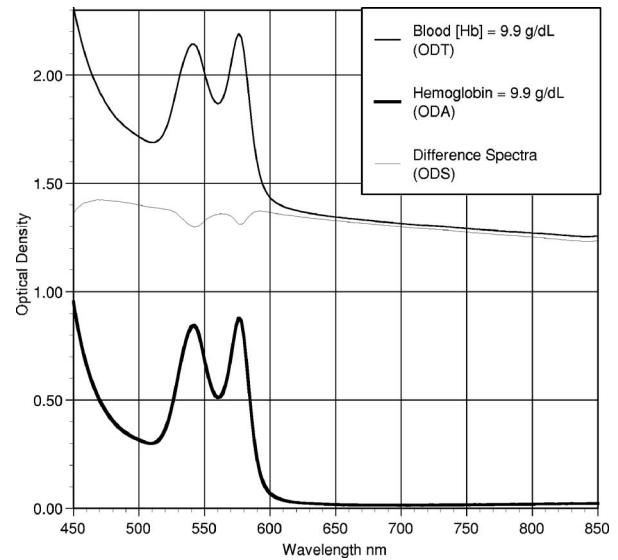


Fig. 1 Spectra of blood (OD_T) and hemoglobin (OD_A) each with an oxyhemoglobin saturation of 98% and the difference spectra (OD_S). Note the small negative hemoglobin signature in the difference spectra which may be caused by red blood cell packaging.^{16,24,25} The wavelength dependence of scattering by red blood cells in forward transmission is nearly linear and has a negative slope.

remove the red blood cell membranes leaving concentrated hemoglobin solutions.² The hemoglobin solutions were each set to the desired concentration using 0.9 N saline and the oxyhemoglobin saturation was set using the same oxygenation system that we used for the blood samples described above and elsewhere.²³

Each time a sample of blood was pumped through the cuvette in the spectrophotometer or a hemoglobin sample was placed in a cuvette, an aliquot was evaluated using a Nova Biomedical pHOX Co-Oximeter that measured the oxyhemoglobin saturation (package material reports an accuracy to less than ±0.5% saturation), the concentration of carboxyhemoglobin, and the concentration of methemoglobin. The blood and hemoglobin solutions were prepared from the same source and the concentrations of hemoglobin species in the samples were comparable. Neither donor was a smoker and the sum of the carboxyhemoglobin and methemoglobin was less than 4% of the total hemoglobin present in each sample.

3 Results

The spectra of a blood sample (OD_T) with an oxyhemoglobin saturation of 98%, [Hb]=9.9 g/dL, path length of 100 μm, and a pH of 6.6, the spectra of a hemoglobin solution (OD_A) with the same parameters and the difference spectrum (OD_S=OD_T-OD_A) are shown in Fig. 1. The similarity between the spectrum of the blood (OD_T) and hemoglobin (OD_A) is obvious. The difference spectrum (OD_S=OD_T-OD_A) is nearly linear with wavelength having the form $b\lambda+c$ in the blue green region from 470–516 nm. We note, however, that there remains a small negative hemoglobin signature in the difference spectra shown in Fig. 1. This negative hemoglobin signature was present in all of our difference spectra independent of saturation, blood hemoglobin concentration, path length, or pH. Recent work on scattering and the relative absorption of

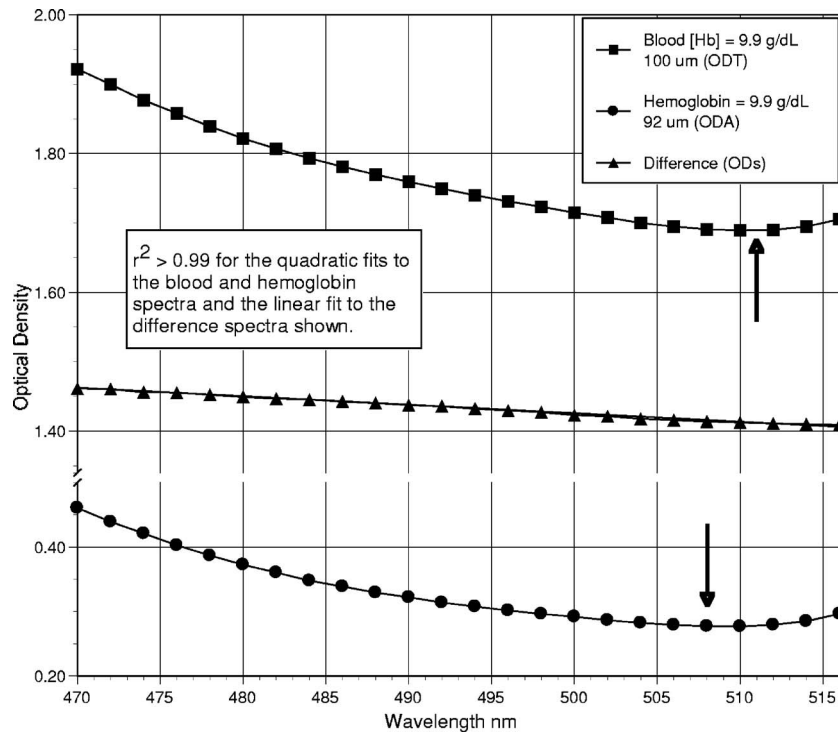


Fig. 2 Spectra of the blood sample (OD_T), the hemoglobin with path length corrected for RBC packaging (OD_A), and the scattering loss (OD_S) all in the blue green region used for oximetry. Note that the blood and hemoglobin spectra are quadratic and the scattering is highly linear in this region ($r^2 > 0.99$). The negative slope as the scattering changes with wavelength causes a 3.0 nm rightward shift from the hemoglobin minimum in the blood indicated by the arrows above.

red blood cell (RBC) packaged hemoglobin versus cell free hemoglobin solutions indicates that the effective path length through blood is less than the equivalent path length through hemoglobin.¹⁶ Other work done modeling the components of the scattering and absorption cross sections reported a similar spectrum for the scattering component in this region.^{24,25} Taken together, these results suggest that the assumption by Stienke and others that the value of d in Eq. (1) is the same for the scattering cross section Σ_s as it is for the absorption cross section Σ_a may be inaccurate.¹⁶ Another possible explanation for this negative hemoglobin signature is that the changing refractive index with wavelength due to the contribution of the imaginary component causes a small but measurable change in the scattering properties of the red blood cells. Nevertheless, for our purposes, we wish to separate the contribution of hemoglobin absorption from scattering as much as possible. Based on the red blood cell packaging study¹⁶ and our findings, we analyzed the data shown in Fig. 1 in the following manner. We used the empirical spectrum of hemoglobin in Fig. 1 along with Lambert-Beer's law to calculate the expected spectra with decreasing path length for this sample. We then calculated the difference spectra for these various hemoglobin path lengths and the blood spectrum from Fig. 1. Our goal was to minimize the residual hemoglobin signal in these difference spectra. We found that the hemoglobin signature was minimized for this sample pair by using a hemoglobin path length of 92 rather than 100 μm . Figure 2 shows the resulting highly linear difference spectrum ($r^2 > 0.99$) along with the parabolic blood and hemoglobin

spectra in the region used for the blue-green minimum shift oximetry technique.²²

Based on this result, we redefined Eq. (2) as follows:

$$OD_T = d(aOD_A + OD_S), \quad (3)$$

where a is the ratio of the effective path length through hemoglobin to the cuvette path length for the blood sample. Substituting the linear relationship between this simplified scattering loss and wavelength into Eq. (3), we write

$$OD_T = d(aOD_A + b\lambda + c). \quad (4)$$

Using eight paired hemoglobin and blood samples, we measured the optical densities at three wavelengths (476, 488, and 514 nm) with oxyhemoglobin saturations of 0.967, 0.88, 0.806, 0.722, 0.654, 0.591, 0.259, and 0.067. We then used Eq. (4) and the spectra of each sample pair to solve uniquely for a , b , and c . The data were quite consistent with values for $a = 0.94 \pm 0.039$, $b = -1.0 \pm 0.13 \text{ nm}^{-2}$, and $c = 2.0 \pm 0.075 \mu\text{m}^{-1}$ (all errors shown are the standard deviation from the mean).

Figure 3 shows the spectra of samples of blood with the same path length, pH level, and different oxyhemoglobin saturations in the region from 476 to 516 nm. Note that the minimum of each spectrum marked with an arrow correlates with the oxygen saturation. Figure 4 shows the blood oxyhemoglobin saturation and the spectral minima of all 32 blood samples that we studied with a pH of 6.6, 7.1, or 7.4, a path length of 100 or 200 μm , and blood hemoglobin concentra-

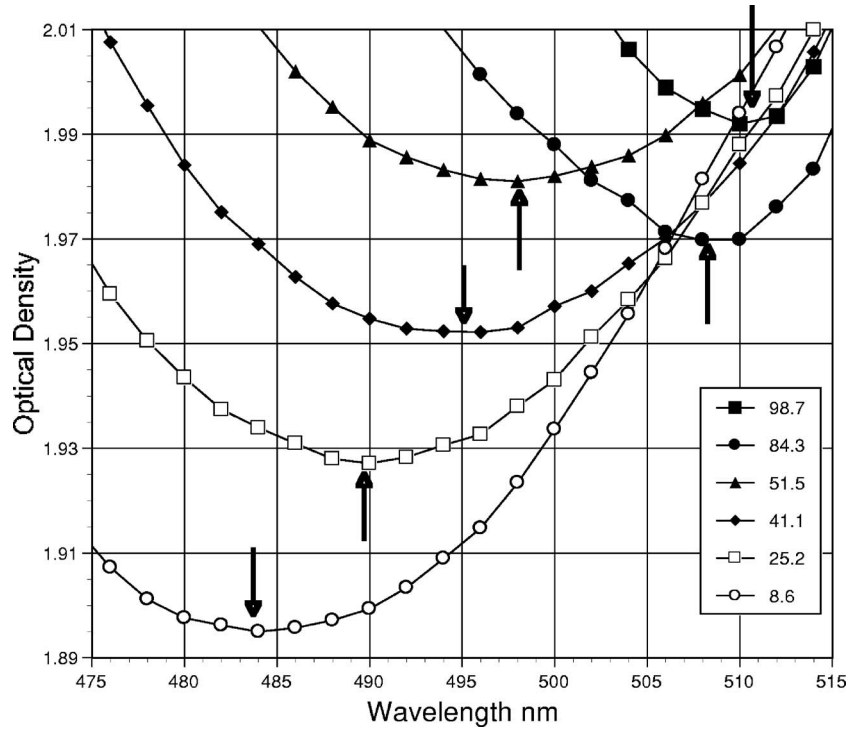


Fig. 3 Spectral minimum of these blood samples indicated by arrows correlates with the blood oxyhemoglobin saturation.

tions from 6.6 to 14.7 g/dL. These minima were measured using the parabolic fit technique described earlier²² without any compensation for path length or blood hemoglobin concentration. These spectral minima correlated strongly with the

oxygen saturation measured using the cooximeter and the standard deviation of the residuals using the cooximeter measurements as the true values was $\pm 3.9\%$ saturation. When compared to the previously reported oxyhemoglobin blue

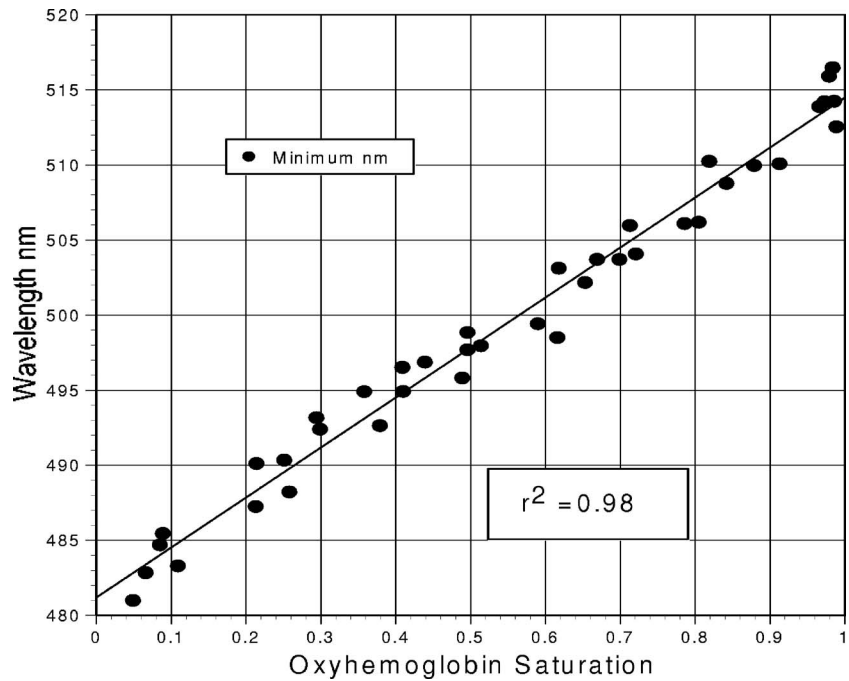


Fig. 4 Spectral minima of 32 blood samples with path lengths of 100 or 200 μm ; pH levels of 6.6, 7.1, or 7.4; and hemoglobin concentrations from 6.6 to 14.7 g/dL (corresponding to a hematocrit from 19 to 48% in our two donors). The spectral minima correlate strongly with the oxygen saturations measured using the cooximeter. The standard deviation of the residuals using the cooximeter measured values as the true saturation is $\pm 3.9\%$ saturation.

green shift using the same analysis techniques,²² the slope of the minimum versus oxyhemoglobin saturation plot is the same but the wavelength intercepts are changed.

We analyzed our data empirically using the fit equation for the blue-green minimum shift method on hemoglobin solutions $OD_A = A\lambda^2 + B\lambda + C^1$ and the best fit to the scattering $OD_S = b\lambda + c$ from the preceding. Substituting these two empirical equations, one reported previously and the other from the data already presented into Eq. (2) gives the following model description of the optical density of blood in the 476 to 516-nm region:

$$OD_T = A\lambda^2 + (B + b)\lambda + C + c. \quad (5)$$

This equation is another parabola with a new minimum λ_m given by setting the differential equal to zero and solving for λ_m or $\lambda_m = -0.5(B + b)/A$. Thus, the expected change in the minimum when compared to hemoglobin is a shift of the quantity $-b/A$. Since the slope b of the scattering line in Fig. 2 as well as in the data sets above were negative ($-1.0 \pm 0.13 \text{ nm}^{-2}$), the shift in this transmission model with scattering is to the right (positive). In the group of blood samples analyzed here (hematocrits from 19-48%) shown in Fig. 3, the total shift to the right when compared to the hemoglobin signature published earlier²² was +3.0 nm.

4 Conclusions

The scattering of whole blood from 460 to 516 nm in this transmission system is linear and decreases with increasing wavelength. The blue-green minimum spectral shift with changing oxygen saturation seen in hemoglobin solutions is preserved in scattering whole blood at physiologic concentrations when spectra are measured in transmission, but the minima are shifted to the right by scattering.

The blue green spectral shift oximetry technique uses wavelengths that are also used in white light *in vivo* oximetry where reflected light is used because highly absorbed wavelengths require that the path length is short.^{22,26} Scattering in reflection spectroscopy is not expected to behave in the same manner as it did here in transmission. Because the blue-green minimum shift technique was altered in this simple transmission blood scattering system, changes when the minimum is measured in reflection should be studied, and back scattering of light carefully quantified.

References

1. D. L. Drabkin and J. H. Austin, "Spectrophotometric studies: V. Technique for analysis of undiluted blood and concentrated hemoglobin solutions," *J. Biol. Chem.* **112**, 105-115 (1935).
2. O. W. van Assendelft, *Spectrophotometry of Haemoglobin Derivatives*, Kononklijke Van Gorcum and Company N. V. and Carles C. Thomas, The Netherlands and Springfield, IL (1970).
3. S. J. Matcher, C. E. Elwell, E. C. C. M. Cope, and D. T. Delpy, "Performance comparison of several published tissue near-infrared spectroscopy algorithms," *Anal. Biochem.* **227**(1), 54-68 (1995).
4. J. G. Kim, M. Xia, and H. Liu, "Extinction coefficients of hemoglobin for near-infrared spectroscopy of tissue," *IEEE Eng. Med. Biol. Mag.* **24**(2), 118-121 (2005).
5. W. A. Eaton, E. R. Henry, J. Hofrichter, and A. Mozzarelli, "Is cooperative oxygen binding by hemoglobin really understood?" *Nat. Struct. Biol.* **6**(4), 351-358 (1999).
6. J. W. Severinghaus, "Simple, accurate equations for human-blood O₂ dissociation computations," *J. Appl. Physiol.* **46**(3), 599-602 (1979).
7. N. A. Rosen, W. E. Charash, and E. F. Hirsch, "Near-infrared spectrometric determination of blood pH," *J. Surg. Res.* **106**, 282-286 (2002).
8. B. R. Soller, P. O. Idwasi, J. Balaguer, S. Levin, S. A. Simsir, T. J. Vander Salm, H. Collette, and S. O. Heard, "Noninvasive, near infrared spectroscopic-measured muscle pH and PO₂ indicate perfusion for cardiac surgical patients undergoing cardiopulmonary bypass," *Crit. Care Med.* **31**(9), 2324-2331 (2003).
9. J. M. Steinke and A. P. Sheperd, "Diffusion model of the optical absorbance of whole blood," *J. Opt. Soc. Am. A* **5**, 813-822 (1988).
10. J. M. Steinke and A. P. Sheperd, "Comparison of Mie theory and the light-scattering of red blood-cells," *Appl. Opt.* **27**(19), 4027-4033 (1988).
11. M. H. Smith, "Optimum wavelength combinations for retinal vessel oximetry," *Appl. Opt.* **38**(1), 258-267 (1999).
12. M. H. Smith, K. R. Denninghoff, A. Lompadó, and L. W. Hillman, "Effect of multiple light paths on retinal vessel oximetry," *Appl. Opt.* **39**, 1183-1193 (2000).
13. F. C. Delori, "Noninvasive technique for oximetry of blood in retinal-vessels," *Appl. Opt.* **27**(6), 1113-1125 (1988).
14. J. S. Tiedeman, S. E. Kirk, S. Srinivas, and J. M. Beach, "Retinal oxygen consumption during hyperglycemia in patients with diabetes without retinopathy," *Ophthalmology* **105**(1), 31-36 (1998).
15. D. Schweitzer, M. Hammer, J. Kraft, E. Thamm, E. Konigsdorffer, and J. Strobel, "In vivo measurement of the oxygen saturation of retinal vessels in healthy volunteers," *IEEE Trans. Biomed. Eng.* **46**(12), 1454-1465 (1999).
16. J. C. Finlay and T. H. Foster, "Effect of pigment packaging on diffuse reflectance spectroscopy of samples containing red blood cells," *Opt. Lett.* **29**(9), 965-967 (2004).
17. J. B. Hickam, R. Frayser, and J. C. Ross, "A study of retinal venous blood oxygen saturation in human subjects by photographic means," *Circulation* **27**(3), 375-385 (1963).
18. A. J. Cohen and R. A. Laing, "Multiple-scattering analysis of retinal blood oximetry," *IEEE Trans. Biomed. Eng.* **23**(5), 391-400 (1976).
19. K. R. Denninghoff, M. H. Smith, R. A. Chipman, L. W. Hillman, P. M. Jester, C. E. Hughes, F. Kuhn, and L. W. Rue, "Retinal large vessel oxygen saturations correlate with early blood loss and hypoxia in anesthetized swine," *J. Trauma: Inj., Infect., Crit. Care* **43**(1), 29-34 (1997).
20. K. R. Denninghoff, M. H. Smith, A. Lompadó, and L. W. Hillman, "Retinal venous oxygen saturation and cardiac output during controlled hemorrhage and resuscitation," *J. Appl. Physiol.* **94**(3), 891-896 (2003).
21. M. H. Smith, K. R. Denninghoff, A. Lompadó, J. B. Woodruff, and L. W. Hillman, "Minimizing the influence of fundus pigmentation on retinal vessel oximetry measurements," *Proc. SPIE* **4245**, 135-145 (2001).
22. K. R. Denninghoff, R. A. Chipman, and L. W. Hillman, "Oxyhemoglobin saturation measurements by green spectral shift," *Opt. Lett.* **31**(7), 924-926 (2006).
23. K. R. Denninghoff and M. H. Smith, "Optical model of the blood in large retinal vessels," *J. Biomed. Opt.* **5**(4), 371-374 (2000).
24. V. Tuchin, D. Zhestkov, A. Bashkatov, and E. Genina, "Theoretical study of immersion optical clearing of blood in vessels at local hemolysis," *Opt. Express* **12**(13), 2966-2971 (2004).
25. N. T. Clancy and M. J. Leahy, "Effect of glucose on the optical properties of arterial blood using Mie theory simulations," in: M.-A. Mycek, Ed., *Diagnostic Optical Spectroscopy in Biomedicine III Proc. SPIE* **586**, 213-219 (2005).
26. D. A. Benaron, I. H. Parachikov, S. Friedland, et al., "Continuous, noninvasive, and localized microvascular tissue oximetry using visible light spectroscopy," *Anesthesiology* **100**(6), 1469-1475 (2004).