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# *In vivo* absorption spectroscopy for absolute measurement

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**Abstract:** In *in vivo* spectroscopy, there are differences between individual subjects in parameters such as tissue scattering and sample concentration. We propose a method that can provide the absolute value of a particular substance concentration, independent of these individual differences. Thus, it is not necessary to use the typical statistical calibration curve, which assumes an average level of scattering and an averaged concentration over individual subjects. This method is expected to greatly reduce the difficulties encountered during *in vivo* measurements. As an example, for *in vivo* absorption spectroscopy, the method was applied to the reflectance measurement in retinal vessels to monitor their oxygen saturation levels. This method was then validated by applying it to the tissue phantom under a variety of absorbance values and scattering efficiencies.



**OCIS codes**: (300.1030) Absorption; (170.6510) Spectroscopy, tissue diagnostics.

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## 1. Introduction

Absorbance spectroscopy, based on the Beer-Lambert law, is effective for measuring the quantity of a particular chemical in a given medium [1–3]. The absorbance of a substance is determined by the product of its absorption coefficient and the length of the optical path through which the light travels.

However, three challenges must be overcome to enable absorption spectroscopy for biological samples *in vivo*. First, intensity-based measurements are likely to provide unreliable results for *in vivo* biological applications because the intensity of light is susceptible to the sample conditions and device configurations. For laboratory use, the absorbance can be measured by using the reflected or transmitted intensity normalized by the reference intensity; however, this normalization may be difficult for *in vivo* measurements because it is not possible to prepare a reference tissue without the target chemical. Second, the length of the optical path should be measured accurately so that the Beer-Lambert law can be applied [4]. Third, the measurable value is a purely optical parameter, which must be transformed or normalized to an objective value by using a statistical estimation approach.

For example, in optical oximetry [4–8], the reference intensity is estimated from the adjacent capillary area, where the absorbance of hemoglobin is less than that of the vessel. This procedure is usually troublesome and difficult to automate properly. Then, to determine the absorbance, the optical path length is estimated from the best-resolved image of the vessel at each measured point. Additionally, the oximetry provides only the absorbance, which must be transformed to an oxygen saturation value by using a regression curve that incorporates many test subjects.

The complexities of these estimating procedures have made it even more difficult to perform *in vivo* spectral analysis. In this study, we have proposed an effective absorption spectroscopy method for *in vivo* quantitative chemical analysis. The complexities of conventional absorption spectroscopy are attributable to the intensity-based measurement, which is susceptible to individual variability *in vivo*. Therefore, to overcome these drawbacks, we have proposed an effective method utilizing the reflectance spectrum along the wavelength axis instead of the intensity axis. This method can be realized by using a spectrometer and intensity thresholding.

## 2. Method

The basic approach to obtaining the absorption spectrum of a scattered and diluted substance [4] involves measuring the light intensities at sampled wavelengths before and after the light passes through the substance. The intensity ratio of the incoming and outgoing light is called the absorbance, which is given as follows:

$$-\log\left(\frac{I}{I_0}\right) = \alpha L + S = \varepsilon c L + S. \tag{1}$$

Here, $\alpha$ is the absorption coefficient of the substance. $\varepsilon$ and $c$ are the molar absorptivity and the concentration of the absorbing species, respectively. $S$ is an additional attenuation term, representing the scattering effect of the substance. The molar absorptivity is usually given or can be measured under ideal conditions [9], and hence, only the optical path $L$ must be measured to obtain the concentration of the target species.

Figure 1 shows an example of the molar extinction coefficient spectrum of hemoglobin [9]. As examples of *in vivo* medical observations, brain functional mapping and retinal functional monitoring are thought to be significant [4–8]. Cellular activity can be estimated by monitoring the blood vessels. Although the oxygen saturation levels in an artery are nearly 100% for most parts of the human body, the venous saturation levels generally indicate the state of activity or disorder in the adjacent tissue. Therefore, we describe the measurement of blood oxygen saturation levels in the brain and, in particular, in the ocular fundus.

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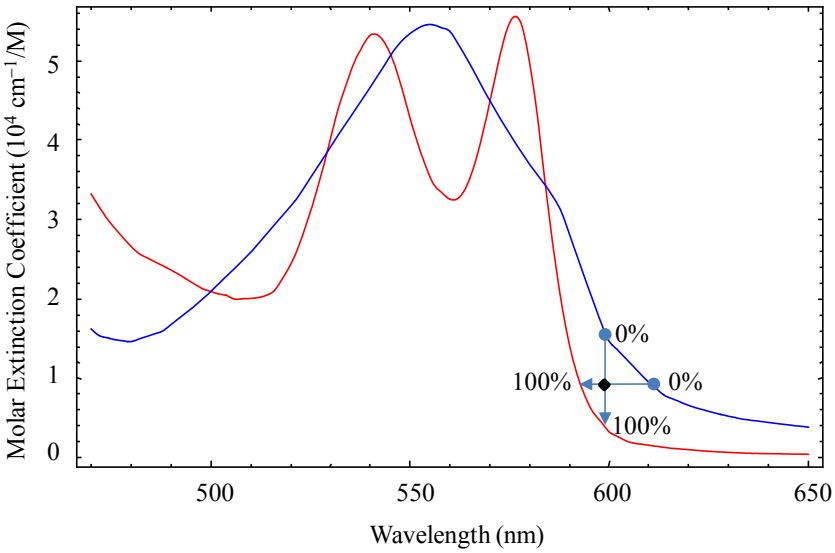


Fig. 1. Molar extinction coefficient spectra of oxy-hemoglobin (red) and deoxy-hemoglobin (blue). The blue line and red line represent the spectra for 0% and 100% oxygen saturation, respectively.

The concept of the proposed method is shown in Fig. 1 in contradistinction to the conventional spectrum analysis. The conventional method estimates the molar extinction coefficient at one wavelength (as shown by the vertical line) using the reflectance and the optical path length. The proposed method estimates the wavelength at which the molar extinction coefficient is equal to a particular value. Therefore, the proposed method finds the two wavelengths where different spectra have the same molar extinction coefficient (as indicated by the horizontal line).

Now, we discuss the difference between the vertical method and the horizontal method. If the spectral analysis could be performed *in vitro*, both methods would be the same. However, *in vivo* spectrum analysis presents three difficulties, as discussed in the introduction.

On the other hand, the horizontal method may greatly reduce these difficulties because it does not require the value of the molar extinction coefficient. It only involves finding the two wavelengths where the molar extinction coefficients of each wavelength are the same.

Figure 2 shows the reflectance spectra of target vessels with three thicknesses ranging between 25 and 100 μm. These spectra were calculated from the molar extinction spectrum shown in Fig. 1 by applying the Beer-Lambert law. The reflectance spectra in Fig. 2 can be resolved to have the same molar extinction coefficient as those in Fig. 1 by applying the Beer-Lambert law, if the diameters of the vessels are given.

In the intensity-axis method (corresponding to the black vertical lines in Fig. 2), the reflectance spectra must be transformed to the molar extinction coefficient, which has a linear relationship with the concentration, in order to determine the oxygenated levels of hemoglobin; this is because the raw reflectance data is nonlinear with respect to the optical path length. Therefore, the concentration cannot be obtained without accurately determining the diameters of the vessels, which would be difficult for *in vivo* measurements.

In contrast, consider the point of view of the proposed wavelength-axis method (corresponding to the black horizontal lines), as shown in Figs. 2(d)–2(f). In this lateral view of the spectrum, the concentration ratio is found to be uninfluenced by the thickness of the samples. Although it is not surprising that samples with equal absorption show the same reflectance at any given thickness, it is a very useful property for *in vivo* measurements in that the concentration can be determined directly from the raw reflectance data without the need for measuring the sample thickness. Even if the equation for the molar extinction coefficient is

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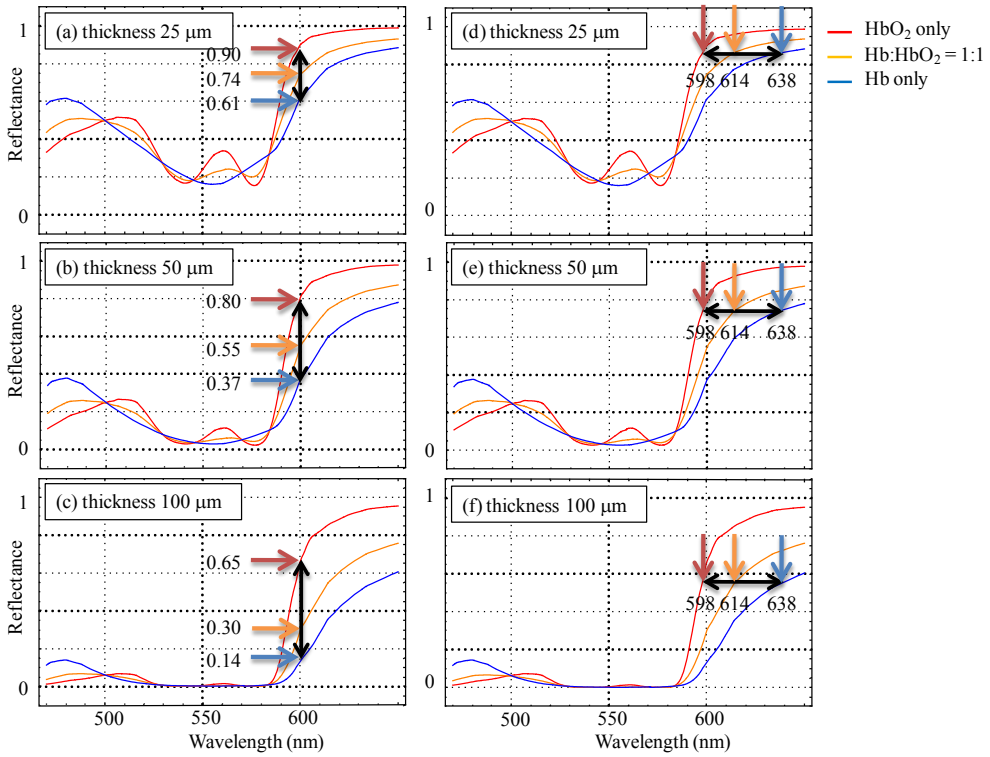


Fig. 2. Calculated reflectance spectra for a mixture of oxy- and deoxy-hemoglobin for three thicknesses of the sample cell (25, 50, and 100 μm). The red, orange, and blue lines indicate oxygen saturation levels of 100%, 50%, and 0%, respectively. (a)–(c) At a fixed wavelength of 600 nm, conventional measurements of the intensity give different reflectance ratios (owing to the differences in the reflectances for different oxygen saturation levels) for the three different sample thicknesses. Specifically, the relative position of the orange arrow shifts toward that of the blue arrow as cell thickness increases. (d)–(f) In contrast, at a fixed reflectance, the lateral measurements of wavelength give a constant wavelength ratio that is independent of the sample thickness. Here, the assumed reflectance was set by reference to oxy-hemoglobin at a wavelength of 598 nm.

not considered, it is evident that the reflectance values should be equal if they have the same molar extinction coefficient at each wavelength.

If the diffused reflection model is considered to closely represent actual tissue, the reflectance spectrum would be greatly influenced by scattering and absorption; this assumption is consistent with the model known as the Kubelka-Munk theory [10], which gives a result that is different from that obtained by using only the Beer-Lambert law. However, the proposed method can be applicable even in this case without considering the nonlinearity, because the nonlinearity is not observed along the wavelength axis but along the intensity axis.

Another advantage of the proposed method is that the concentration can be directly estimated from the raw data of the reflectance spectrum without the need for translation to a molar extinction coefficient spectrum. This is because the ratio of wavelength positions at a fixed reflectance (as shown in Figs. 2(d)–2(f)) is the same both in the reflectance spectrum and in the molar extinction coefficient spectrum.

In the next section, the quantitative development of the proposed method is discussed.

## 3. Analysis

Figure 3 shows the range of the molar absorptivity spectra of a blood vessel. The oxygen saturation level in this vessel is assumed to be $x$ ($0 \le x \le 1$), and the molar absorptivity

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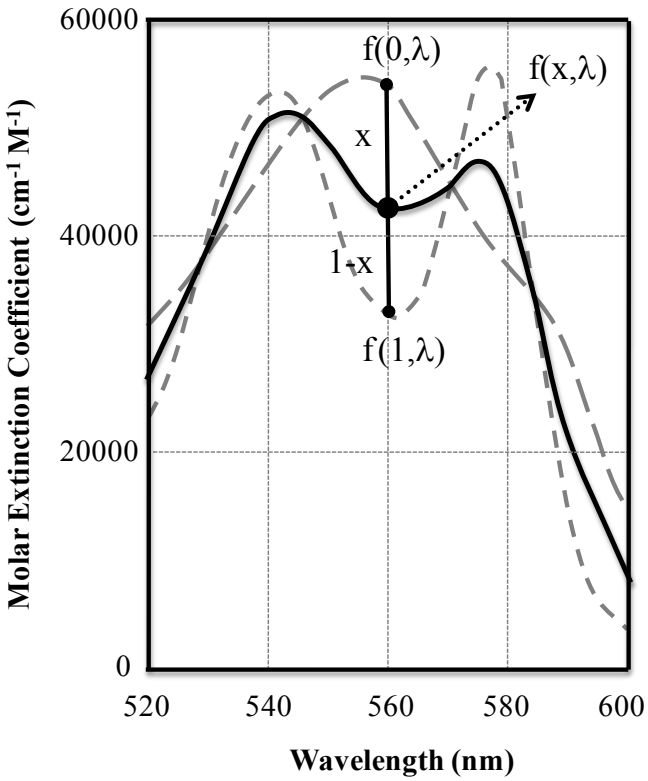


Fig. 3. Molar extinction coefficient spectra of oxy-hemoglobin ($HbO_2$, indicated by the curve with the double peak structure and the line with the short dashes), and of deoxy-hemoglobin (Hb, indicated by the curve with the single peak and the line with the long dashes) in the visible light range. The solid curve represents a spectrum measured at an unknown saturation level.

spectral curve is denoted by $f(x, \lambda)$. The dashed lines indicate the molar absorptivity spectra of oxy-hemoglobin ($HbO_2$, oxygen saturation 100%) and deoxy-hemoglobin (Hb, oxygen saturation 0%) in the visible light range, represented as $f(1, \lambda)$ and $f(0, \lambda)$, respectively.

In this case, the following relationship holds:

$$f_{(x,\lambda)} = x f_{(1,\lambda)} + (1-x) f_{(0,\lambda)}. \tag{2}$$

Here, the molar absorptivity of $HbO_2$ is recorded at any wavelength $\lambda_a$. The wavelength of the measured vessel is set to $\lambda_m$ so that the molar absorptivities at the two wavelengths are the same. Then the following equation is satisfied because the molar absorptivities at this position are equal:

$$f_{(1,\lambda_a)} = f_{(x,\lambda_m)}. \tag{3}$$

Equation (2) is substituted into Eq. (3), and the following expression is obtained:

$$f_{(1,\lambda_a)} = x f_{(1,\lambda_m)} + (1-x) f_{(0,\lambda_m)}. \tag{4}$$

Consequently, $x$ can be obtained as

$$x = \frac{f_{(1,\lambda_a)} - f_{(0,\lambda_m)}}{f_{(1,\lambda_m)} - f_{(0,\lambda_m)}}. \tag{5}$$

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Oxygen saturation levels $x$ can be obtained because the molar absorptivity curves $f(1, \lambda)$ and $f(0, \lambda)$ are known, and the wavelengths $\lambda_a$ and $\lambda_m$ are observed values. As a result, the oxygen saturation level $x$ can be solved for from the molar absorptivity spectral curves of oxy-hemoglobin and deoxy-hemoglobin, which are shown in Fig. 1. Thus, this method can provide the oxygen saturation level without requiring knowledge of the value of the molar extinction coefficient at that location.

As an example, Table 1 summarizes the results of retinal oxygen saturation levels calculated using Eq. (5). If the two wavelengths that have the same molar absorptivity are $\lambda_a$ and $\lambda_m$, the corresponding oxygen saturation level may be found at the intersection of the row and the column corresponding to $\lambda_a$ and $\lambda_m$, respectively. Although the arterial oxygen saturation can be calibrated by pulse oximetry, it was reported that the saturation in retinal arteries is considerably lower than 100% even in normal subjects [11]. Some correction between the saturation of the retinal artery and the pulse oximetry would be needed in the future. As the saturation level of the artery was between 98 and 100% in our case, Table 1 was made assuming that the arterial saturation is 100%, which may be adequate for general use.

**Table 1. Oxygen saturation levels at observed wavelengths $\lambda_a$ and $\lambda_m$ for an artery and a vein of equal intensity**

| $\lambda_m$ \ $\lambda_a$ | 590 | 591 | 592 | 593 | 594 | 595 | 596 | 597 | 598 | 599 | 600 | 601 | 602 | 603 | 604 | 605 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 590 | 100 | | | | | | | | | | | | | | | |
| 591 | 85 | 100 | | | | | | | | | | | | | | |
| 592 | 74 | 88 | 100 | | | | | | | | | | | | | |
| 593 | 64 | 78 | 90 | 100 | | | | | | | | | | | | |
| 594 | 55 | 69 | 81 | 91 | 100 | | | | | | | | | | | |
| 595 | 46 | 61 | 73 | 84 | 92 | 100 | | | | | | | | | | |
| 596 | 38 | 53 | 66 | 77 | 86 | 94 | 100 | | | | | | | | | |
| 597 | 30 | 46 | 59 | 71 | 80 | 89 | 95 | 100 | | | | | | | | |
| 598 | 21 | 38 | 52 | 65 | 75 | 83 | 91 | 96 | 100 | | | | | | | |
| 599 | 11 | 29 | 44 | 57 | 68 | 77 | 84 | 90 | 94 | 100 | | | | | | |
| 600 | 2 | 21 | 37 | 50 | 61 | 71 | 78 | 84 | 89 | 95 | 100 | | | | | |
| 601 | | 16 | 32 | 46 | 57 | 67 | 75 | 81 | 86 | 92 | 97 | 100 | | | | |
| 602 | | 12 | 29 | 43 | 54 | 64 | 72 | 78 | 83 | 90 | 95 | 98 | 100 | | | |
| 603 | | 8 | 25 | 39 | 51 | 61 | 69 | 75 | 80 | 87 | 92 | 95 | 97 | 100 | | |
| 604 | | 3 | 20 | 35 | 47 | 57 | 66 | 72 | 77 | 84 | 90 | 93 | 95 | 98 | 100 | |
| 605 | | | 16 | 31 | 43 | 54 | 63 | 69 | 75 | 81 | 87 | 91 | 93 | 96 | 98 | 100 |
| 606 | | | 11 | 26 | 39 | 51 | 60 | 66 | 72 | 79 | 85 | 89 | 91 | 94 | 97 | 99 |
| 607 | | | 6 | 22 | 36 | 48 | 57 | 64 | 70 | 77 | 84 | 87 | 90 | 93 | 95 | 98 |
| 608 | | | 0 | 17 | 32 | 44 | 54 | 61 | 68 | 75 | 82 | 86 | 88 | 92 | 95 | 97 |
| 609 | | | | 12 | 27 | 40 | 51 | 59 | 65 | 73 | 81 | 84 | 87 | 90 | 93 | 96 |
| 610 | | | | 6 | 22 | 36 | 47 | 55 | 62 | 71 | 79 | 83 | 85 | 89 | 92 | 95 |
| 611 | | | | 1 | 18 | 32 | 44 | 52 | 59 | 69 | 77 | 81 | 84 | 87 | 91 | 93 |
| 612 | | | | | 13 | 28 | 40 | 49 | 57 | 66 | 75 | 79 | 82 | 86 | 89 | 92 |
| 613 | | | | | 7 | 23 | 36 | 45 | 53 | 63 | 72 | 77 | 80 | 84 | 88 | 91 |
| 614 | | | | | 1 | 18 | 32 | 41 | 50 | 60 | 70 | 75 | 78 | 82 | 86 | 89 |
| 615 | | | | | | 15 | 29 | 39 | 47 | 58 | 68 | 73 | 76 | 81 | 85 | 88 |
| 616 | | | | | | 12 | 27 | 37 | 46 | 57 | 66 | 72 | 75 | 80 | 84 | 87 |
| 617 | | | | | | 9 | 24 | 34 | 43 | 55 | 65 | 70 | 74 | 78 | 83 | 86 |
| 618 | | | | | | 6 | 21 | 32 | 41 | 53 | 63 | 69 | 72 | 77 | 81 | 85 |
| 619 | | | | | | 2 | 18 | 29 | 39 | 51 | 61 | 67 | 71 | 76 | 80 | 83 |
| 620 | | | | | | | 15 | 26 | 36 | 48 | 59 | 65 | 69 | 74 | 79 | 82 |
| 621 | | | | | | | 12 | 24 | 34 | 47 | 58 | 64 | 68 | 73 | 77 | 81 |
| 622 | | | | | | | 10 | 21 | 32 | 45 | 56 | 62 | 66 | 71 | 76 | 80 |
| 623 | | | | | | | 7 | 19 | 29 | 43 | 54 | 60 | 65 | 70 | 75 | 79 |
| 624 | | | | | | | 4 | 17 | 27 | 41 | 53 | 59 | 63 | 69 | 74 | 77 |
| 625 | | | | | | | 2 | 14 | 25 | 39 | 51 | 57 | 62 | 67 | 72 | 76 |

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When oximetry is applied to an ocular fundus, the molar absorptivity of melanin should be included in addition to that of hemoglobin. However, the relative difference in the wavelengths $\lambda_a$ and $\lambda_m$ would be negligible because there is little dependence on wavelength in this wavelength region for melanin.

## 4. Experimental setup

This method was applied to the *in vivo* measurement of blood circulation in the retina. The experimental system was composed of a combination of imaging and illumination components shown in Fig. 4. For the imaging component, a fundus camera was employed. The illumination components consisted of a 150-W halogen lamp (Ushio Inc., Tokyo) as a white light source and a wavelength-variable filter with a wavelength range of 500–700 nm and a spectral bandwidth of 7 nm (Varispec, CRi Inc., Massachusetts). Wavelengths outside the measured range were filtered using a colored glass filter. The light intensity was less than that used with an ordinary fundus camera because the colored glass filter and the wavelength-variable filter were placed in front of the white light source. These conditions were confirmed to satisfy the eye safety regulations of JIS C 6802 in Japan and IEC 60825-1 worldwide [12].

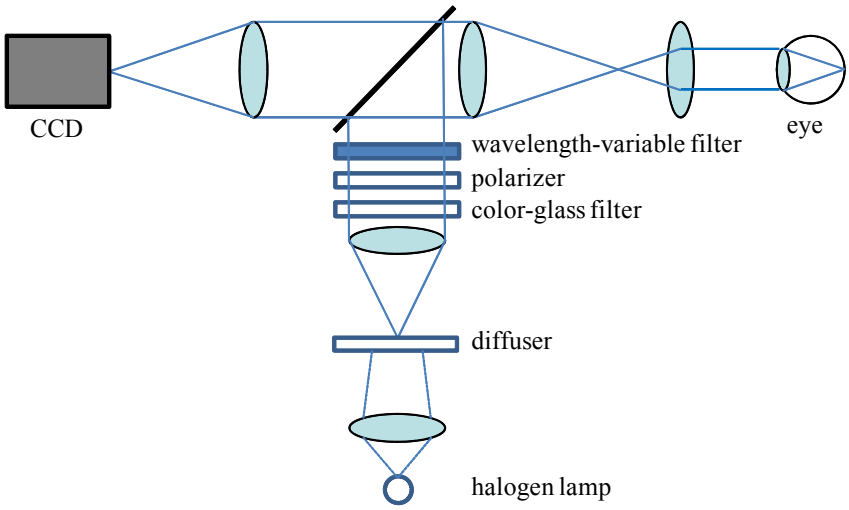


Fig. 4. Spectral imaging system for the retina. An ocular fundus camera was created for imaging the human retina. The spectroscopic light source was constructed mainly from a halogen illumination lamp as the white light source. A wavelength-variable filter was included.

## 5. Results and discussion

The absorption spectra of human retinal vessels were measured for wavelengths between 570 and 650 nm. The sampling points are shown in Fig. 5. The vessels were chosen such that they have the same intensities at the isosbestic point for arteries and veins (584 nm). Although we should be mindful of the variation in optical scattering with the sampling locations on the tissue, this scattering effect would have less influence in application to the retina because most of the light in the 570–650 nm wavelength region is absorbed by the normal retinal pigment epithelium. The centers of the retinal vessel were avoided because they often appear as bright lines due to specular reflection. The vessels were considered to be of approximately the same diameter for the same intensities at the isosbestic point. In this regard, the proposed method does not address the fundamental limitation of the length of the optical path. However, vessels having the same intensities can be selected using a much simpler method than the retinal spectroscopy that is the mainstream approach [4] because our method does not require knowledge of the background intensity nor accurate vessel diameter values. Automatic analysis would not be so difficult if combined with the image processing of the vascular extraction method [13].

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Here, we should mention the difference in hematocrit between the vein and the artery. In the retinal vasculature, the hematocrit remains unchanged during passage of the blood in and out of the eye at the optic nerve head. The retinal vasculature features very tight junctions between the cells comprising the vessel wall. Furthermore, the eye does not have a lymphatic system into which lost fluid could drain. What about the general case of the peripheral vasculature? Using the following reasoning, we have concluded that a small difference does not affect the oxygen saturation. In chemical analysis, the concentration is adjusted to contain the same number of molecules in a certain volume of the sample cell. For instance, one can obtain the same result, corresponding to the quantity $c \times L$ in Eq. (1), by using a lower concentration and a longer sample cell of correct length. This method of selecting vessels of the same intensity has the effect of maintaining the same number of red cells. The venous hematocrit is usually about 2% higher than the artery [14]. Therefore, a 2% smaller venous vessel must be chosen to find the same intensity as the arterial vessel at the isosbestic wavelength by assuming the venous hematocrit to be 2% higher than the arterial one. However, one should consider carefully the choice of the measured points in applying this method to one's cases.

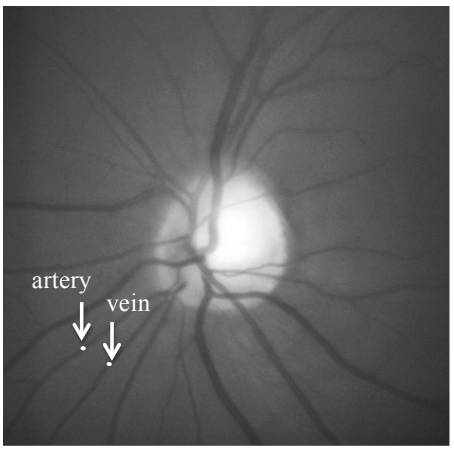


Fig. 5. Sampled points for retinal oximetry with the same absorbance at the isosbestic wavelength, 584 nm.

Figure 6 shows the detected reflectance spectra of the artery and the vein, sampled at the points shown in Fig. 5. The dashed line shows the threshold intensity chosen as an example. In this method, the reflectance spectra do not need to be transformed to the molar extinction coefficient because the absolute value of the vertical axis is not used. So, although any photodetector can be used, the threshold level should be selected by considering the signal-to-noise ratio of the system. The wavelengths in the sampled artery and the sampled vein that achieved this intensity were $\lambda_a$ = 597 nm and $\lambda_m$ = 612 nm; therefore, by substituting these values in Eq. (5) or Table 1, the oxygen saturation level at the measured point was obtained to be 48.4%, which may be confirmed to be reasonable on the basis of previously published results [4,5,8,9]. Calculation of the oxygen saturation value in Eq. (5) is simple, as shown below. The molar absorptivities at each wavelength were taken from the theoretical curve or the *in vitro* measurement results shown in Fig. 1:

$$\begin{aligned} x &= \frac{f_{(1,\lambda_a=597)} - f_{(0,\lambda_m=612)}}{f_{(1,\lambda_m=612)} - f_{(0,\lambda_m=612)}} \\ &= \frac{5094-8591}{1364-8591} \\ &= 0.4839. \end{aligned} \tag{6}$$

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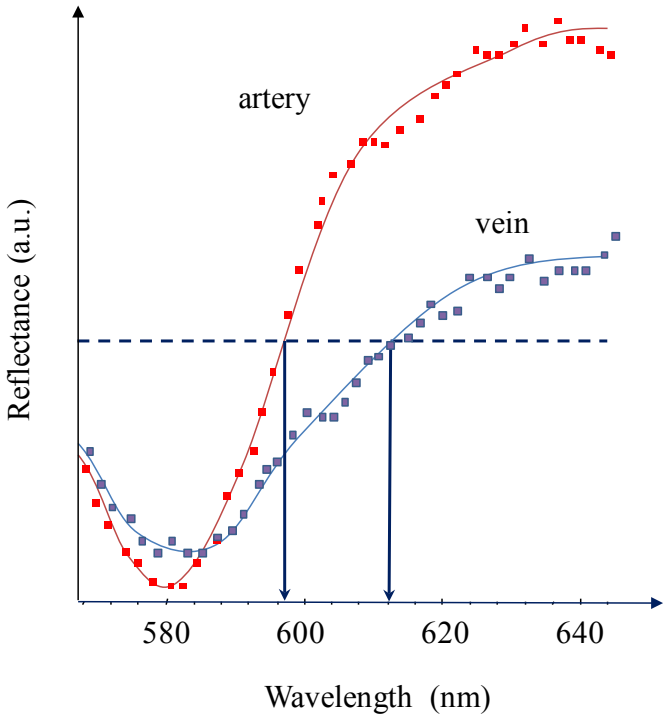


Fig. 6. Reflectance spectra of the retinal vessels, sampled at the points shown in Fig. 5.

As shown in Eq. (5), the intravascular oxygen saturation level $x$ is obtained from $\lambda_a$ and $\lambda_m$, which are the wavelengths where the absorbance of the measured vessel and $HbO_2$ saturation are equal. As shown in Table 1, there are many wavelength pairs $\lambda_a$ and $\lambda_m$ corresponding to the same $x$ value. Regardless of the choice of $\lambda_a$, the oxygen saturation level can be obtained correctly. For example, in the case shown in Fig. 6, we can choose another pair such as $\lambda_a$ = 600 nm and $\lambda_m$ = 626 nm, $\lambda_a$ = 594 nm and $\lambda_m$ = 604 nm, and so on. The estimated saturations were 49% and 47% respectively, which were within the margin of measurement error. However, for the hemoglobin measurement at around 600 nm, the measurement error can be minimized if the longer wavelength $\lambda_m$ is employed to reduce the Rayleigh scattering effect that becomes obvious at shorter wavelengths. Furthermore, although the absorbance decreases with an increase in the wavelength, measurements can be performed in the range of 590–600 nm without using a high-sensitivity detector.

Thus, we have shown that this method can be applicable to *in vivo* spectroscopy. The absolute value can be solved for directly from the reflectances without converting them to absorbance values, a step that requires knowing the concentration and the optical path length of the vessels. Unfortunately, accurate figures for the oxygen saturation in the human retina have not been determined [4,5,8,9]. Therefore, we have validated the accuracy of this method using the tissue phantom as described below.

In general, certain preconditions must be met for this method to be applicable. (1) Optical properties other than the absorbance of target chemicals, such as the scattering coefficient of the tissue and wavelength dispersion, should be maintained virtually constant within the wavelength range measured. (2) Ideal spectra before and after the chemical reaction should be obtained before applying the method. In addition, although the measurement of time-series data at the same position does not require the third precondition, one more requirement should be considered if the observation point is spatially separated from the reference, as in the above-mentioned demonstration. (3) The sample should have an isosbestic point before and after the chemical change to enable the verification of the equivalent amount. These three

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requirements are not very difficult to satisfy for most *in vivo* spectroscopic measurements, including retinal oximetry.

If these conditions are satisfied, the proposed method may be applicable under conditions where the Beer-Lambert law does not hold. Conventional absorption spectroscopy is not applicable to samples with high concentrations because of nonlinear optical processes [15,16]. However, the proposed method would be less affected by the nonlinearity of reflectance because this method is independent of intensity. If the intensity changes nonlinearly depending on the chemical object to be measured, the ratio of the intensity changes of two measured points would be constant as long as condition (1) is satisfied. This advantage would be powerful for analyzing the biological or medical samples *in vivo*.

To test what would be a difficult case for the conventional intensity-based method, we applied the proposed method to samples having different efficiencies of scattering and absorbance. We have developed two kinds of artificial blood to validate our spectroscopy, which give 100% and 0% saturation spectra of hemoglobin. They were prepared by dissolving the following dyes in polyvinyl alcohol (PVA). Acid red 52, chlorophenol red sodium salt, eosine, erythrosine, phloxine, sunset yellow, erioglaucine, and sulforhodamine B were mixed with a ratio by weight of 9:51:2:7:17:8:4:2 for the deoxy-hemoglobin. Acid red 52, erythrosine, and acid yellow 23 were mixed with a ratio by weight of 24:18:58 for oxy-hemoglobin. The choice of dyes was made to have the isosbestic wavelength between 580 nm and 585 nm. These mixtures do not function as blood, but their spectra were prepared to be similar to hemoglobin within the 500–650 nm wavelength range. Figure 7 shows the absorbance spectrum and the photo of the artificial blood we prepared. Our method was tested based on this artificial blood absorbance, not on the absorbance of real blood shown in Fig. 1. The isosbestic wavelength for both kinds of artificial blood was 580.9 nm.

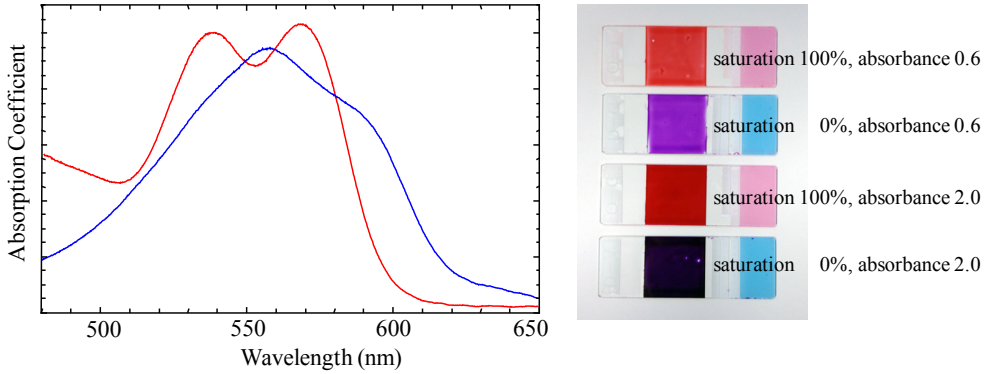


Fig. 7. The absorption coefficients and the photos of the artificial blood, which were similar to oxy-hemoglobin (red line) and deoxy-hemoglobin (blue line) in the 500–650 nm wavelength range.

The sample substrates were prepared for modeling the case of the functional measurement of the brain or retina. The artificial blood was applied to the glass slide with the spin-coating method, and the scattering substrate was placed in contact with the artificial blood. The absorbance of the artificial blood was adjusted to be 0.6, 0.9, 1.5, and 2.0 by controlling the concentration. The scattering efficiencies were changed with three substrate materials: a mirror as a non-scattering substrate, a plastic paraffin film (Parafilm, Pechiney Plastic Packaging Co., Chicago) as a weakly scattering substrate, and laboratory paper (KimWipes, Kimberly-Clark Co., Irving) as a strongly scattering substrate. The reflectance spectra were measured using the multichannel dispersion spectrometer (USB4000-VIS-NIR, Ocean Optics Inc., Florida).

Figure 8 shows the reflectance spectra of the artificial blood, which has an absorbance of 0.6 on each scattering substrate. The wavelength for 100% oxygen level was assumed to be fixed at 591.5 nm, which had a reflectance of 0.5 on the mirror substrate. The corresponding

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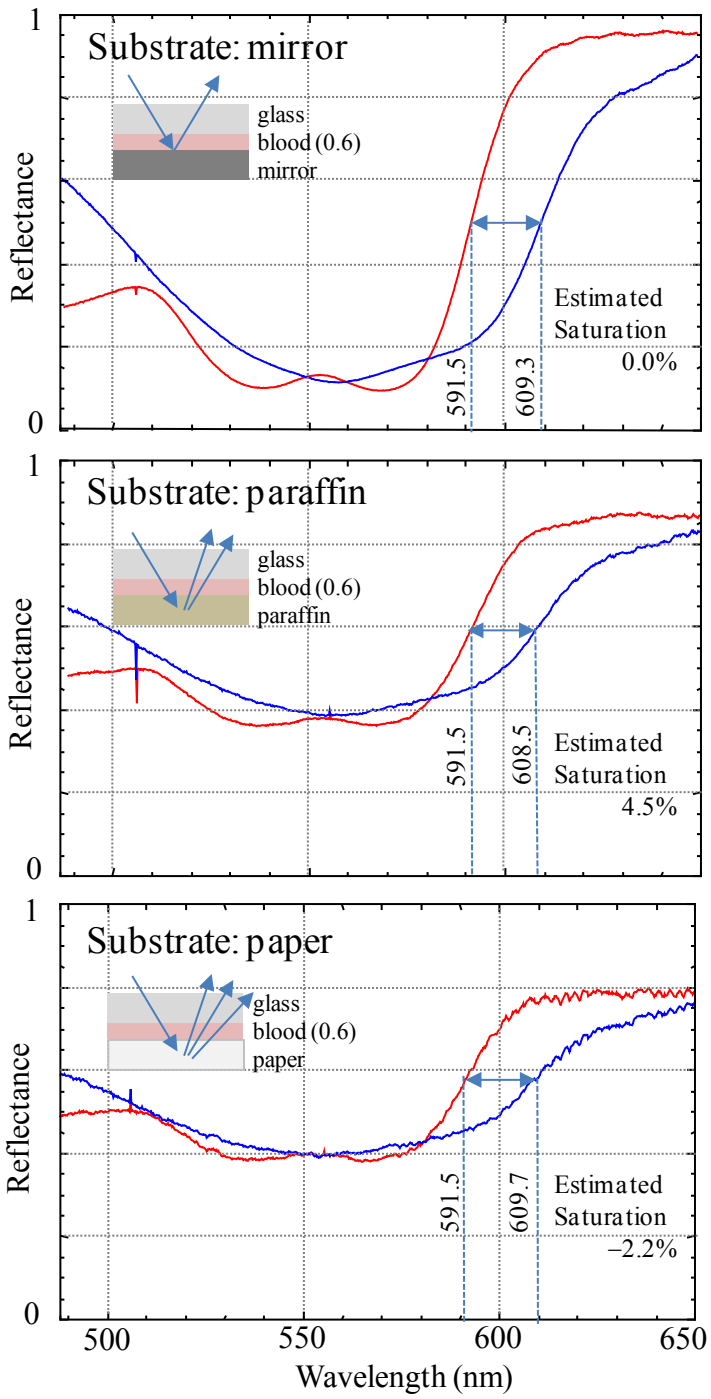


Fig. 8. The reflectance spectra of the artificial blood whose absorbance is 0.6 on each scattering substrate: the mirror (no scattering), the paraffin (weak scatterer), and the paper (strong scatterer).

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wavelengths for 0% oxygen level were 609.3 nm, 608.5 nm, and 609.7 nm. Since the correct wavelength, which can be solved from Fig. 7, is 609.3 nm for 0% oxygen level, the respective errors of the oxygen level for the substrates were estimated to be 0.0%, 4.5%, and −2.2% from Eq. (5).

In the same procedure, the other absorbances were also measured, and the results were arranged in Table 2. Despite the differences of the scattering and the concentration, the oxygen saturation can be estimated correctly with an accuracy range of 7%. We did not need the information of the sample absorbance and the scattering efficiency of the sample to apply this method, while the reflectance spectra measured were greatly influenced by them.

As mentioned in the introduction, there seems to be three difficulties with *in vivo* absorption spectroscopy. The proposed method solves the first difficulty by assuming that two adjacent wavelengths are subjected to the similar scattering process. The conventional methods use the reference intensity, which is obtained by measuring a tissue that does not include the target chemical. This method is complicated and is likely to be affected by individual errors. The second difficulty is overcome by finding the two equal-brightness points on the image at the isosbestic wavelength. In this regard too, conventional methods such as scattering simulation [5,9] and measurement of the vessel diameter from a microscopic image [17] are complicated and are likely to be affected by individual errors. The third difficulty is also solved because the proposed method is a direct measurement method and does not require calibration. The conventional method provides only the absorbance, which must be transformed or normalized to an objective value such as oxygen saturation level or chemical concentration by using a statistical estimation approach [4–7] or *in vitro* calibration. Thus, this method may be expected to reduce difficulties associated with *in vivo* measurements.

Pulse oximetry is a successful example of applying absorbance spectroscopy to the medical field. By using the pulse of the artery, the transmittance change of arterial blood can be converted to the corresponding saturation level through the statistically calibrated constant [18]. However, it has still been difficult to apply this method to situations that do not involve pulsed organs, such as venous blood, capillaries, or chemicals distributed in an organ's cells. The proposed technique could provide a complementary approach for analyzing biological functions.

**Table 2. Estimated saturation of the artificial blood for various absorbance values and substrates**

| Number | Absorbance | Substrate | $\lambda_a$ (fixed) | $\lambda_v$(measured) | Saturation |
|---|---|---|---|---|---|
| 1 | 0.6 | mirror | 591.5 | 609.3 | 0.0 |
| 2 | 0.6 | paraffin | 591.5 | 608.5 | 4.5 |
| 3 | 0.6 | paper | 591.5 | 609.7 | –2.2 |
| 4 | 0.9 | mirror | 591.5 | 609.1 | 1.1 |
| 5 | 0.9 | paraffin | 591.5 | 610.3 | –5.7 |
| 6 | 0.9 | paper | 591.5 | 609.9 | –3.4 |
| 7 | 1.5 | mirror | 591.5 | 609.5 | –1.1 |
| 8 | 1.5 | paraffin | 591.5 | 610.1 | –4.6 |
| 9 | 1.5 | paper | 591.5 | 609.1 | 1.1 |
| 10 | 2.0 | mirror | 591.5 | 609.9 | –3.4 |
| 11 | 2.0 | paraffin | 591.5 | 610.5 | –6.9 |
| 12 | 2.0 | paper | 591.5 | 609.7 | –2.2 |

## 6. Conclusion

In this research, we have proposed an effective method for performing *in vivo* quantitative chemical analysis. In *in vivo* spectroscopy, there are individual differences in the tissue scattering and the sample concentration, such as in the hematocrit. Therefore, in conventional spectroscopy, the statistical calibration curve is assumed to be for an average level of scattering, and the average concentration is prepared by making measurements on many subjects. However, there would be many individuals who depart from the average parameters. The proposed method can give the absolute value, independent of the individual differences of

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tissue scattering and sample concentration. This method is also expected to greatly reduce the difficulties encountered during *in vivo* measurements. While this method was developed to solve difficulties in hemoglobin photometry, it may be applicable to other *in vivo* analyses such as brain functional mapping and biological secretory activity.

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