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Retinal vessel oximetry-calibration, compensation for vessel diameter and fundus pigmentation, and reproducibility

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Abstract. The purpose of this study was to measure the hemoglobin oxygenation in retinal vessels and to evaluate the sensitivity and reproducibility of the measurement. Using a fundus camera equipped with a special dual wavelength transmission filter and a color charge-coupled device camera, two monochromatic fundus images at 548 and 610 nm were recorded simultaneously. The optical densities of retinal vessels for both wavelengths and their ratio, which is known to be proportional to the oxygen saturation, were calculated. From 50-deg images, the used semiautomatic vessel recognition and tracking algorithm recognized and measured vessels of 100 μm or more in diameter. On average, arterial and venous oxygen saturations were measured at 98±10.1% and 65±11.7%, respectively. For measurements in the same vessel segments from the five images per subject, standard deviations of 2.52% and 3.25% oxygen saturation were found in arteries and veins, respectively. Respiration of 100% oxygen increased the mean arterial and venous oxygen saturation by 2% and 7% respectively. A simple system for noninvasive optical oximetry, consisting of a special filter in a fundus camera and software, was introduced. It is able to measure the oxygen saturation in retinal branch vessels with reproducibility and sensitivity suitable for clinical investigations.
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1 Introduction

The retina is a tissue with extraordinary high oxygen demand. Whereas the outer retina is supplied by the choroid, the inner neural retina is supplied by the retinal vasculature. Located in front of the photoreceptors, this is very sparse in order to minimize the disturbance of the visual function. Thus, the regulation of the oxygen supply to the retinal tissue is critical and the detection of alterations in the oxygen supply or consumption may have a diagnostic merit. Quantitative assessment of oxygen supply and oxygen utilization in the tissue requires the measurement of the blood flow in the supplying vessels on the one hand and the blood oxygenation on the other hand. Even though the blood flow can be calculated from velocity measurements by laser Doppler techniques[1,2] in conjunction with the measurement of the vessel diameter,[3] the measurement of the oxygen concentration is more difficult. The measurement of oxygen partial pressure (pO_2) could be done by an oxygen-sensitive electrode.[4–7] However, this is an invasive technique that cannot be applied in humans for routine diagnostic purposes. Alternatively, the pO_2 can be determined by the observation of phosphorescence quenching of palladium or ruthenium porphyrine as an oxygen marker.[8] This marker, however, has no permission for use in humans. Another indicator of oxygen supply is the oxygen saturation (SO_2) of the hemoglobin. Because of the different absorption spectra of oxygenated and deoxygenated hemoglobin, the SO_2 can be assessed noninvasively by spectral measurements.

Numerous techniques have been developed for the intravascular SO_2 measurement at retinal vessels referred to as optical oximetry.[9] First attempts date back to the 1960s.[10,11] Remarkable progress was made by Delori[12] and Schweitzer et al.[13] The latter approach may be the most accurate one because of the use of simultaneous measurements at 76 different wavelengths using a spectrometer. A major drawback of both techniques, however, is the limitation on measurements at one single cross section of one or two vessels. By contrast, a complete, two-dimensional mapping of the SO_2 in the retinal vascular tree is needed for clinical diagnostics. Thus, recently, different multispectra imaging techniques, intended to be used for oximetry, were introduced. Denninghoff et al.[14–16] used a scanning laser ophthalmoscope equipped with four or five lasers in an interlaced mode. The use of lasers restricted the investigators to available wavelengths and, thus, the cali-

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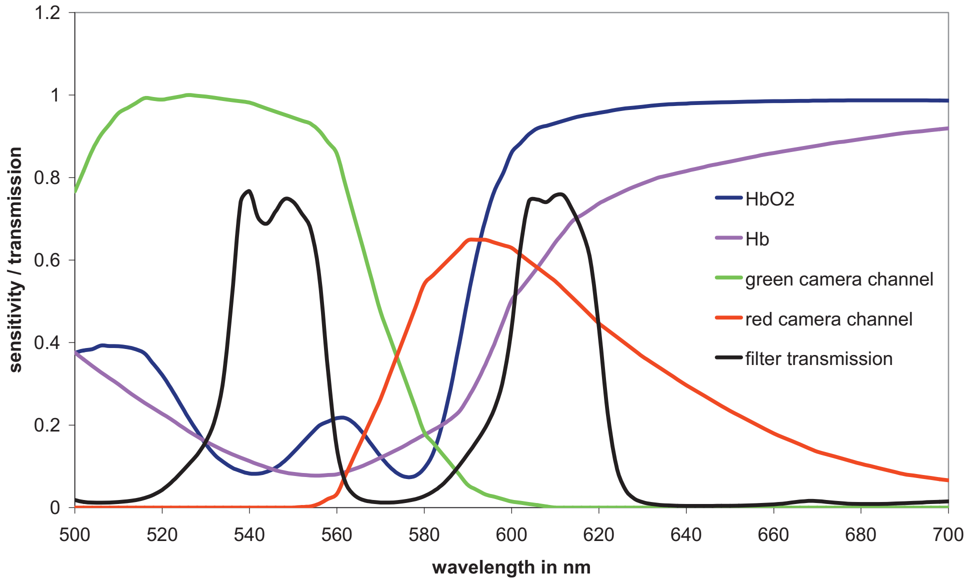

Fig. 1 Transmission spectra of reduced (Hb) and oxygenated (HbO_2) hemoglobin as well as the filter and the spectral sensitivity of the red and the green camera channels.

bration turned out to be difficult. Harvey et al.[17] are working at devices using either tunable filters or a series of Lyot filters. Whereas the first can provide images at any wavelength, but in a sequential mode, the latter gives six monochromatic images simultaneously at predefined wavelengths. The calculation of the SO_2 by statistic means (principal component analysis) is under development. An elegant method for the generation of hyperspectral images is the use of computer-generated two-dimensional holographic gratings. These are capable of producing a series of first- and second-order diffraction images.[18] However, the calculation of the spectral images from the diffraction patterns is laborious and perhaps dependent on the choice of initial conditions of the iterative process. By contrast, Beach et al.[19] found a linear dependence of the SO_2 in a retinal vessel on the ratio of the logarithmic contrast of the vessel versus its surrounding at two specific wavelengths [optical density ratio (ODR)]. Employing this method, the SO_2 can be determined from two images at two wavelengths only. Recently, Hardarson et al.[20] introduced a retinal oximeter based on that principle. The basic optical component of their instrument is an image splitter at the output port of a fundus camera.

In this study, we investigated a new, simple, filter-based technique for retinal vessel oximetry. For calibration purposes, the results were related to data recorded with a fundus spectrometer.[13] The influence of vessel diameter as well as fundus pigmentation on the blood oxygen saturation readings was compensated. The reproducibility of the SO_2 measurement was tested.

2 Methods

2.1 *Optical Setup of the Oximeter*

The concept of the optical density ratio by Beach et al.[19] needs two monochromatic fundus images for the determination of the retinal vessel oxygen saturation. One image has to be recorded at an isosbestic point of the hemoglobin. The other wavelength has to be chosen such that the absorption coefficients of oxygenated and deoxygenated hemoglobin have a great difference. This condition was met by a double bandpass filter specifically designed for oximetry. The filter was inserted into the illumination path of a fundus camera FF450 (Carl Zeiss Meditec, Jena, Germany) and transmitted light at the wavelength 548 ± 10 nm (full width at half maximum) and 610 ± 10 nm. The transmission outside the transmission bands was smaller than 3%. The images were recorded by a 3-charge-coupled device (CCD) digital camera HV-C20A (Hitachi Ltd., Tokyo, Japan): the image at the isosbestic wavelength of 548 nm is on the green channel of the camera and the 610-nm image is on the red channel (Fig. 1). The filter was designed such that the integral over the hemoglobin spectrum, weighted with the spectrum of the xenon flash of the fundus camera and with the spectral sensitivity of the green digital camera channel, within the borders of the 548-nm transmission range is independent of the hemoglobin oxygenation. Thus, the optical concept of the oximeter is very simple: It consists of a fundus camera equipped with a digital color camera and a specific filter.[21]

2.2 *Calculation of Oxygen Saturation and Data Processing*

The calculation of the oxygen saturation SO_2 was adopted from Beach et al.[19] but slightly modified

$$SO_2 = 100\,\% - (ODR - ODR_{a,100\%})/os. \tag{1}$$

Here, ODR is the corrected optical density ratio

$$ODR = \frac{\log \frac{I_{out}^{610}}{I_{in}^{610}}}{\log \frac{\eta I_{out}^{610}}{I_{in}^{548}}}, \tag{2}$$

where I_{in} and I_{out} are the intensities of reflected light inside and outside a vessel at the indexed wavelengths measured as gray values in the respective images and η is the ratio of the intensities measured at an ideal white reflector (Spectralon, Labsphere Inc., North Sutton, New Hampshire) at 548 and 610 nm. In Eq. (1), $ODR_{a,100}$ is an offset that Beach et al.,[19] introduced as the ODR at the arteriole during inhalation of pure oxygen but was set constant and determined experimentally here, and os is the oxygen sensitivity, another constant to be determined. Because we found linear dependences of the SO_2 value on the vessel diameter as well as on the fundus pigmentation (Sec. 3), we compensated for that by introducing of linear compensation terms into the oximetry equation, which finally resulted in

$$SO_2 = 100\,\% - (ODR - ODR_{a,100})/os - (a - VD) \times b + \left(c - \log \frac{I_{out}^{610}}{I_{out}^{548}}\right) \times d. \tag{3}$$

Here, VD is the diameter of the vessel in microns, measured from the image,[3] and $\log I_{out}^{610}/I_{out}^{548}$ represents the fundus pigmentation by melanin, which extinction decreases approximately linearly with the wavelength in the considered spectral range.[22] The constants a, b, c, and d were determined experimentally from measurements in 20 healthy volunteers (see Sec. 3).

A user-friendly software, easy to operate by clinicians and researchers, comprises the following algorithm. In an image, obtained by the camera and filter assembly described as the oximeter in Sec. 2.1, the operator has to mark the vessel of interest by a mouse click. The vessel is traced automatically applying the following procedure. The vessel walls are located as photometric edges in the vicinity of the mouse cursor in the green channel image. If edges were determined, the search was continued in their proximity. Once three or more edge segments were found, these were used to determine the direction in which the vessel is traced, even if single edge segments lack sufficient contrast, and the vessel is segmented according to changes of its direction at the fundus. For each vessel segment, 3 to 10 pixels in length, 6 pixels outside the vessel (3 on either side) and all pixels inside the vessel, excluding pixels representing the vessel wall, were considered. The grayscale values of the pixels inside and outside the vessel in the green as well as in the red camera channel are averaged and used as the respective intensities I in Eq. (3) for the calculation of the oxygen saturation. Finally, the SO_2 values are averaged over the vessel. To observe changes of the SO_2 along a vessel, the measurement can be restricted to an area of interest defined by the operator.

2.3 *In Vivo Investigations*

Two studies in healthy volunteers were performed. A first study, including 20 [6 male, 14 female, mean age: 28.6 years, standard deviation (SD): 10.9 years] subjects, was used for the calibration of the measurement. In this study, oxygen saturation was measured during oxygen breathing too. Two fundus images were taken from each subject, one before and one 6 min after the onset of the inhalation of pure oxygen through a mouthpiece. The statistical significance of the difference of mean values of SO_2 was tested by Student's t test for paired samples using the software SPSS 13.0 (SPSS Inc., Chicago, Illinois). The reproducibility of the oximetry device and procedure was tested in a second cohort of 10 (4 male, 6 female, mean age: 20.3 years, SD: 1.7 years) healthy subjects. Five oxymeter images of each subject were recorded under standard conditions (mydriasis, 50-deg. field). From each of the five images, the oxygen saturation was determined for 10 arterial and 10 venous vessel sections with a constant length of approximately one-half of the optic disc diameter. The standard deviation of the SO_2 values from the identical vessel sections over the five images was calculated as a measure of the reproducibility.

In all examinations, the subjects' pupils were dilated with Tropicamid (Mydriaticum Stulln, Pharma Stulln GmbH, Nabburg, Germany). All investigations in humans were approved by the institutional review board of the University of Jena. Informed consent was obtained from all subjects. All procedures conformed to the tenets of the Declaration of Helsinki.

3 Results

3.1 *Calibration*

For calibration of the oximeter, the constants $ODR_{a,100}$ and os [Eqs. (1) and (3)], had to be determined. For that purpose, ODR values have been obtained from 1087 segments of 80 arterioles and 1406 segments of 80 venules in oximetry fundus images (30-deg field) from 20 healthy volunteers. Furthermore, 2033 segments from 97 arterioles were measured during oxygen breathing. Reference data for the calibration were obtained from oxygen saturation measurements by fundus spectroscopy.[13]

The primary reading from the images is the ODR according to Eq. (2). To calculate the vascular oxygen saturation SO_2 from the ODR using Eq. (1), the parameters $ODR_{a,100}$ and os were determined as follows. The offset $ODR_{a,100}$ was determined as the ODR measured in 2033 arterial segments from the images taken under oxygen inhalation assuming an arterial SO_2 of 100% under that condition.[19] A mean ODR of 0.01208 with a standard deviation of 0.02413 was found. However, because the distribution of the ODR values was not symmetric, the median 0.01357 was used as $ODR_{a.100}$. Schweitzer et al.[13] found a mean arteriovenous SO_2 difference of 34%. This was used to adjust the oxygen sensitivity os in Eq. (1) to $0.0023/\%SO_2$.

3.2 *Compensation for Vessel Diameter and Fundus Pigmentation*

Reviewing the SO_2 data obtained from Eq. (1), we found dependence on the vessel diameter as well as on the iris color. Whereas those dependencies were marginal for the arterioles, they were considerable for the venules. The SO_2 values were approximately linearly dependent on the vascular diameter [Fig. 2(a)]. Thus, it was compensated by a linear term in Eq. (3) resulting in diameter-independent SO_2 values [Fig 2(b)].

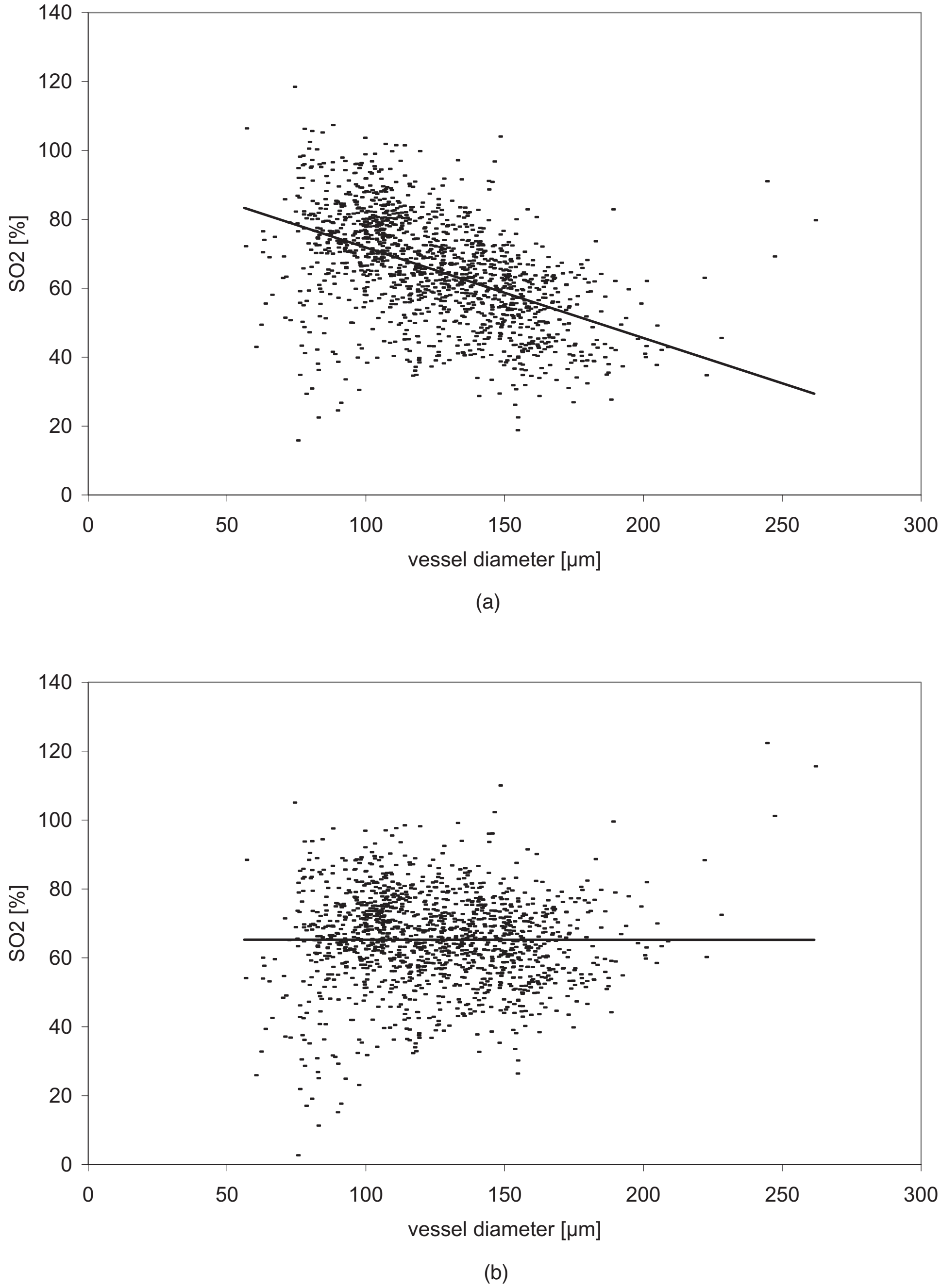

Fig. 2 Dependence of venous SO_2 on the vessel diameter before (a) and after (b) compensation (same measurements).

Because iris color is a good estimate of the fundus pigmentation that cannot be measured directly, we calculated mean and standerd deviation of the SO_2 separately for different iris colors and found venous SO_2 values of $74.1 \pm 6.6\%$ for blue irises, $65.4 \pm 11.1\%$ for green irises, and $61.7 \pm 9.9\%$ for brown irises. Although only the difference of the venous saturation of the blue and green eyes was significant, we added another linear term for the compensation of the fundus pigmentation in Eq. (3). The dependence of the venous SO_2 on $\log I_{out}^{610}/I_{out}^{548}$, a measure of fundus pigmentation, with and without compensation is shown in Fig. 3. All constants, inserted in Eq. (3), are summarized in Table 1.

3.3 *Reproducibility*

To check the reproducibility of the SO_2 measurement, the standard deviation of measurements in 5 consecutive images was determined for 10 arterial and 10 venous vessel sections in each of the subjects. For the arterioles, a mean standard deviation of $2.52\% \, SO_2$ (SD: 1.23, range: 0.59 to 6.72) was found and for venules $3.25\% \, SO_2$ (SD: 1.78, range: 0.63 to 10.82). The reproducibility was independent from the vessel diameter and the fundus pigmentation and was stable from the posterior pole to the mid periphery as was assessed in 50-deg images.

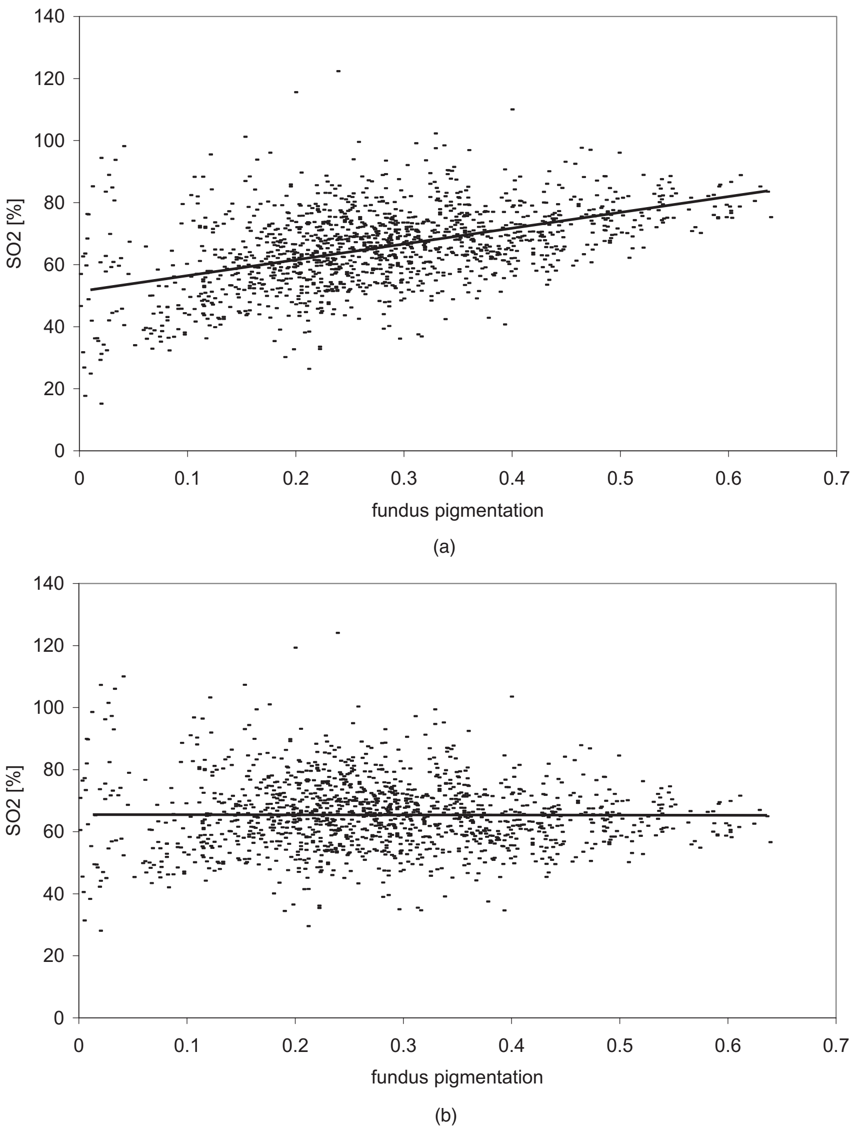

Fig. 3 Dependence of venous SO_2 on fundus pigmentation before (a) and after (b) compensation (same measurements).

3.4 *Oxygen Saturation in Healthy Subjects*

Retinal vessel SO_2 was measured in all gaugeable vessels of 20 healthy subjects before and during pure oxygen respiration (calibration cohort) and 10 more healthy subjects (reproducibility cohort). Reliable oxygen saturation values were measured in vessels of 100 μm or more in diameter in the 50-deg image of the fundus camera. By reducing the field to 30 or 20 deg, smaller vessels can be assessed accordingly. The SO_2 readings showed to be independent from the field size, provided that the fundus camera flash energy is set accordingly. This is in accordance with the theory [Eq. (2)] that the oxygen saturation depends on reflection ratios, not on absolute reflection measurements. Figure 4 shows an example of the retinal vessel oxygenation of a healthy subject as pseudocolor presentation. The mean values and standard deviation are given in Table 2. The standard deviation reflects the intra- and interindividual variability that is higher than the standard deviation of the repeated measurement at the same vessel reported in Sec. 3.3.

Pure oxygen respiration (Table 2) resulted in a highly significant ($p < 0.0005$) global increase of the venous oxygen saturation by 7% and a slight increase of the arterial saturation by 2%.

4 Discussion

The retinal vessel oximeter, presented here, is based on the ODR concept by Beach et al.[19] and is somewhat similar to an implementation published recently by Hardarson et al.[20], Its main advantage is the simple optical concept just employing a color CCD-camera in conjunction with a special optical filter. This enables the oximetry measurement in the full field of the fundus camera (tested here up to 50 deg) without any distortion of the results by different shading of the two monochromatic images as may occur in image splitters.

As for all dual wavelength oximeters, the SO_2 calculated by this instrument from the measured ODR depend on the calibration. Thus, the absolute SO_2 values are difficult to

Table 1 Parameters for the calibration of the oximetry equation [Eq. (3)].

Parameter	Arteriole	Venule
$ODR_{a,100}$	0.01357	
os	$0.0023/\%SO_2$	
a	109 μm	125 μm
b	$0.0667\%SO_2/\mu m$	$0.2626\%SO_2/\mu m$
c	0.265	0.272
d	$15.149\%SO_2$	$51.055\%SO_2$

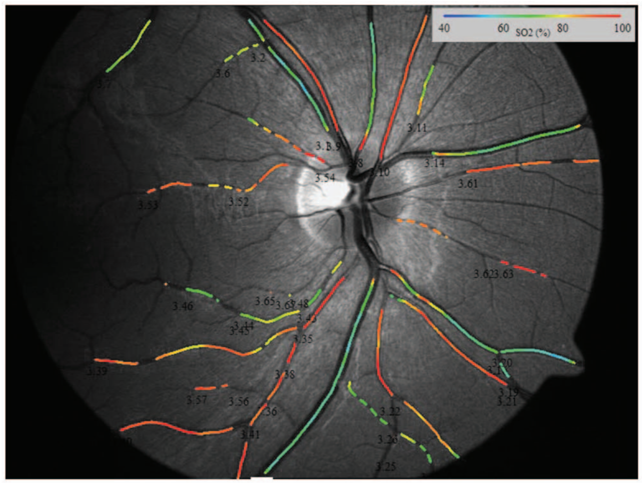

Fig. 4 Pseudocolor representation of retinal vessel SO_2 in a healthy subject. Blank vessel sections were too narrow to be measured.

compare with the results of others. Different calibration is simply the reason why we found a mean venous oxygenation of 65% whereas Hardarson et al.[20] found 52%. Our calibration is based on our experience with the ocular fundus spectrometer.[13] In this study, a spectrograph and an intensified CCD camera at the exit port of a fundus camera were used to measure retinal vessel reflectance spectra with a 2-nm resolution. Least-square fitting of a model, describing the backscattering of light from a retinal vessel taking the hemoglobin oxygenation into account, to the spectra gives absolute oximetry readings. The major drawback of this spectroscopic technique was the restriction to measurements at single points at a vessel. In that previous study,[13] measurements in 30 healthy subjects showed an arteriovenous oxygen saturation difference of 34%, which is in agreement with the value known from the brain.[23]

The pseudocolor map of SO_2 values (Fig. 4) clearly shows distinct saturation in arterioles and venules. On the other hand, it shows some limitations of the method. Oxygen saturation readings are incorrect at the optic disc because of the completely different structure and color of the background tissue. Furthermore, there are venous segments with a locally increased SO_2 reading as, for examples in the vein at the four o'clock position proximal to the optic disc. Although increased venous oxygen saturation near the optic nerve head was found in previous studies[13,24] and may be explained by short diffusion lengths between arterioles and venules as well as by arteriovenous shunts, the strictly localized increase, found here, also may reflect artifacts. We have to bear in mind that local SO_2 readings may be disturbed by abnormalities of the fundus reflection such as juvenile reflexes of the inner limiting membrane, as seen in Fig. 4, retinal nerve fiber reflex, or pathologic features and are generally subject to a relatively strong noise of the reflection images. Thus, reliable SO_2 values always need averaging over a sufficient vessel length or over readings from multiple images. Due to the large number of measured vessel segments, we were able to observe the dependence of the oxygen saturation on the vessel diameter and the fundus pigmentation. That enabled us to introduce linear correction terms into the oximetry equation accordingly. This, however, needs a measure of the pigmentation. Here, the logarithmic ratio of the fundus reflectance besides the vessel at both wavelengths (548 nm and 610 nm) was employed in agreement with the melanin extinction spectrum.[22]

Whereas the absolute SO_2 readings are subject to a somewhat arbitrary calibration and, thus, are difficult to compare with the results of others, the reproducibility of the measurement is of major importance for the estimation of the accuracy of the method and can be compared. The mean standard deviation of the repeated SO_2 measurements at the same vessel section in five consecutive images of the same subject was slightly lower in arterioles (2.52%) than in venules (3.25%). These values are superior to those reported by Hardarson et al.[20] (3.7% and 5.3%, respectively). Furthermore, their reproducibility data were obtained from measurements at large branch vessels only whereas ours were averaged over all vessels down to a diameter of 100 μm. Schweitzer et al.[13] achieved a reproducibility of 4.6% for arterioles and 4% for venules as the standard deviation over 19 repeated measurements in 1 subject using fundus spectrometry. Smith et al.[25] reported a repeatability of typically $\pm 5\%$. A better reproducibility than ours was reported only by Delori et al.[12] who found a standard deviation of $2.1\%SO_2$ between two consecutive measurements of the same vessel. Their technique, however, scanned across a single vessel with three wavelengths and, thus, gives the oxygen saturation for one point at one vessel only. In contrast, the method reported here provides SO_2 values for any vessel of a sufficient diameter

Table 2 Oxygen saturation in retinal arterioles and venules (calibration cohort).

	Normoxia		Hyperoxia	
Breathing Conditions	Arterioles	Venules	Arterioles	Venules
Mean (%)	98	65	100	72
SD (%)	10.1	11.7	10.2	9.6

Mean values and standard deviation over all gaugeable vessels of 20 healthy subjects.

within the visual field of a fundus camera and, therefore, has the advantage to be an imaging technique, which is regarded to be very helpful in clinical diagnostics.

The measurement of the oxygen saturation is sensitive to the change of the respiratory conditions of the subject under investigation. During the inhalation of pure oxygen, a mean increase of SO_2 by 2% in the arterioles and by 7% in the venules was found. This increase, however, was lower than that reported by others. Hardarson et al.[20] found a 5% increase in arterioles and an increase by 24% in venules. Similar values were reported by Delori et al.[12] (23% increase in venules, no change in arterioles) and Schweitzer et al.[24] (increase of 3% in arterioles and 23% in venules). The reason why we found lower changes here is probably the fact that we delivered the oxygen by a mouthpiece instead of a full face mask. Although we advised our probands not to breath through the nose, there might have been a certain fraction of room air inspired resulting in a lower degree of hyperoxia. This is a weakness of the current study. The effect on the calibration, however, was negligible because the arterial hemoglobin oxygen saturation hardly changed during the oxygen respiration. The remarkable change in the venous saturation is due to an increased oxygen supply by an increased oxygen partial pressure.

In summary, we were able to demonstrate a very simple setup for retinal vessel oximetry that can easily be adapted to any fundus camera. It needs three components: a special dual wavelength bandpass filter, a standard digital color camera and a software module. The software, calculating the SO_2 readings for single vessels or vessel sections, however, has to be calibrated as described in Sec. 3.1. This technique was able to measure vessel oxygenation within a large field and its reproducibility turned out to be very high. The system clearly detected SO_2 changes due to oxygen inspiration.

Hypoxia, as a thread of retinal nerve fiber and ganglion cells as well as a stimulus of neovascularization, is an issue in frequent eye diseases such as glaucoma, diabetic retinopathy, age-related macular degeneration, and retinal vascular occlusion. Thus, the determination of oxygen supply to the tissue and oxygen use may contribute to our understanding of these diseases and may help in early diagnosis. This, however, needs the measurement of the blood flow and the hemoglobin oxygenation. Whereas different systems for the measurement of the flow are currently employed in the clinics, here we describe an oximetry technique ready for clinical application. This indicates its possible usefulness in diagnostics as well as guidance and control of therapy of the diseases mentioned.

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