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Spectroscopic oximetry in the eye: a review

ABSTRACT

Introduction: Non-invasive measurement of blood oxygen saturation via spectroscopic imaging has facilitated insights into the development and progression of a variety of ocular conditions, including retinal vascular occlusion, diabetic retinopathy and glaucoma. Major developments since the late 90s have been enabled by advancements in imaging technology, computational image analysis, and experimental methods.

Areas covered: We review the theory of spectroscopic oximetry, the ocular blood vessels targeted for oximetry, imaging systems used for oximetry, and oximetry validation methods. Important physiological and clinical insights provided by oximetry in the eye are detailed.

Expert commentary: Oximetry has revealed physiological norms and auto-regulatory effects in the retina, choroid, episcleral, and bulbar conjunctival blood vessels. Retinal oximetry has provided crucial insight into the development of diabetic retinopathy and glaucoma, and has enhanced the evaluation and treatment of retinal vessel occlusion. Commercially available retinal oximetry systems have enabled oximetry in the clinic. The development more sophisticated phantoms that resemble *in vivo* environments has oximetry validation in diverse oximetry applications. New insights into ocular physiology and disease are likely to be gleaned from future studies.

Keywords: oximetry, retina, autoregulation, diabetes, glaucoma, vessel occlusion, choroid, bulbar conjunctiva.

1. INTRODUCTION

Oximetry - the measurement of blood oxygen saturation (OS) – can be achieved non-invasively by measuring the spectrum of light absorbed by blood. This is achieved by imaging the eye at two or more wavelengths of light, and calibrating the OS to absorption of light by either (1) assuming standard normal OS values or (2), by combining information from many wavelengths into a sophisticated physical model incorporating the known spectral properties of blood with other biological and optical parameters.

Since the late 1950s, retinal oximetry has progressed from a photographic technique [1–3] through to the cutting edge of digital imaging technology with automated computational analysis.[4,5] Over the decades, retinal oximetry has revealed valuable insights into retinal physiology and metabolism under conditions such as flicker illumination,[6,7] dark adaptation,[8] hyperoxia,[4,9,10] and acute mild hypoxia.[11,12] Further, retinal oximetry has provided insights into the development and progression of ocular diseases such as diabetic retinopathy,[13–17] glaucoma,[18–22] and retinal vessel occlusion.[23–26] Recently, oximetry has allowed the understanding of the fundamental physiological oxygen dynamics of the bulbar conjunctival circulation,[27] the episcleral vessels,[27] and the choroidal circulation.[28]

From a patient's perspective, the experience of oximetry imaging in the eye is typically indistinguishable from conventional retinal-fundus imaging because oximetry systems are typically based on a modified conventional retinal-fundus cameras[4,11,16] or multi-wavelength scanning laser ophthalmoscopes.[10,29] As such, a great deal of oximetry innovation has arisen from improved image recording, computational image analysis, and the development of complimentary functional oxygen-sensitive stress interventions that allow insight into OS under altered metabolic conditions. These intervention tests include subjecting subjects to hyperoxia,[6,7] acute mild hypoxia,[11,12] or altered light-exposure conditions.[6–8] As well as physiological and clinical insights, these intervention tests help validate the accuracy of oximetry measurements.

Invariably, advances in oximetric-imaging technology have provided new insights and applications. In particular, the development of commercially available retinal oximetry devices with automated analysis – such as the *Oxymap T1* (Oxymap ehf, Iceland)[4] and *Imedos (*Imedos Systems UG, Germany*)*[16] retinal oximetry systems - have enabled oximetry in the clinic, allowing insights into treatment of individual patients[24] and also enabling clinicians to build large datasets with high statistical power. Additionally, the development of oximetry systems with capability for imaging with greater temporal[11] and spatial resolution[29] enable development of novel techniques and fresh insights by investigating OS of smaller blood vessels or by studying OS dynamics on a shorter timescale. For example, snapshot multispectral imaging technology has enabled measurement of rapid oxygen diffusion in the bulbar conjunctiva[27] and adaptive optics have enabled oximetry in small retinal vessels, which are not typically studied due to their small diameters.[29]

This review focuses on the current technology and methods of spectroscopic oximetry, new applications in spectroscopic oximetry of the eye, and the clinically useful ophthalmological insights that have been consequently enabled.

2. PRINCIPLE OF SPECTROSCOPIC OXIMETRY

Spectroscopic oximetry is enabled by the distinct absorption spectra of oxygenated haemoglobin (O_2Hb) and deoxygenated haemoglobin (Hb) as shown in Figure 1: partial oxygenation results in an absorption spectrum that is a weighted average of these two spectra. This difference in optical absorption can be clearly seen by eye for a low-volume sample of blood: highly oxygenated blood appears bright red to the eye, whereas deoxygenated blood is much darker in appearance. Whilst this difference in optical absorption is readily observed in *ex vivo* blood samples, it is highly challenging to accurately quantify in the complex optical environment of the eye due to the influence of optical scattering[30] and the uncertain absorption of light by variable background pigmentation.[9,31] There are two approaches to spectroscopic oximetry measurement and analysis: two-wavelength oximetry and multi-wavelength oximetry.

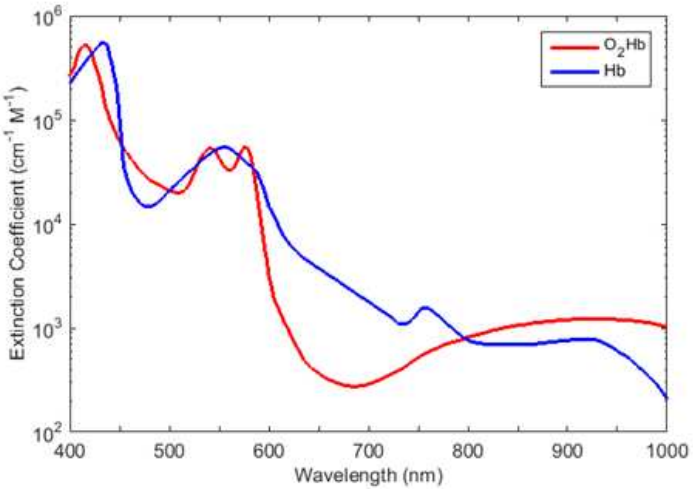

Figure 1. The absorption spectra of oxygenated haemoglobin (O_2Hb) and deoxygenated haemoglobin (Hb) at visible and near infra-red wavelengths.

2.1. Two-wavelength oximetry

In two-wavelength oximetry, blood vessels are imaged at one OS-sensitive contrast wavelength, and another – typically OS-insensitive (isobestic) - wavelength. The optical transmission of blood vessels (T_λ) is measured at each wavelength, and the optical density (OD), defined as: $OD_\lambda = -log\,(T_\lambda)$, is computed. From this, the optical density ratio (ODR), defined as $ODR = \frac{OD_{\lambda\,contrast}}{OD_{\lambda\,isobestic}}$, is calculated. If an isobestic wavelength is used, then for a blood vessel of a given diameter, ODR is theoretically directly proportional to blood oxygen saturation (verified experimentally in 1959)[1], and so ODR can be empirically related to OS by measuring ODR of blood vessels at two OS levels followed by calibration against a blood vessels of known oxygenation (i.e. using pulse oximetry or blood gas measurement). The theory of two-wavelength oximetry is based upon the simple Beer-Lambert law of light transmission, which neglects the effects of optical scattering that is incorporated into the modified Beer-Lambert law.[31] For visualisation purposes, ODR or OS is often overlaid as a colour map on images of blood vessels (see Figure 2 for an example).[4]

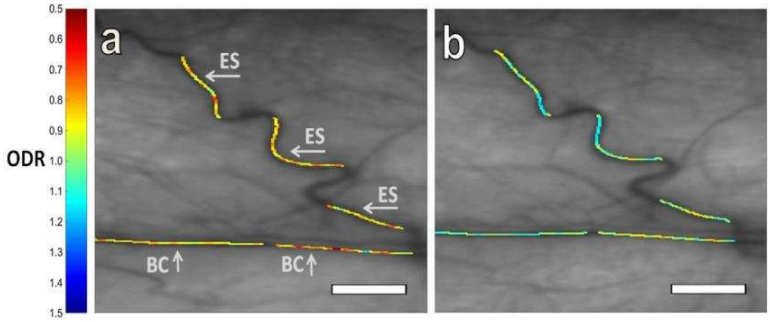

Figure 2. ODR colour-map of bulbar conjunctiva (BC) and episcleral (ES) vessels at (a) normoxia, and (b) acute mild hypoxia. Reproduced with permission from MacKenzie et al., (2016).[32]

The optimal transmission of a blood vessel for accurate oximetry is around 37%, [33] but, because the optical transmission of blood vessels varies according to several factors – including vessel

diameter - an optical transmission of between 10 % and 70 % has been found to work well.[33] For retinal oximetry, the combination of 570 nm (isobestic) and 600 nm (contrast) wavebands has been commonly adopted for retinal oximetry.[4,11,31] However, the optimal wavelength combinations used for any oximetry study are dependent upon the calibre of vessels of interest and the efficiency of optical imaging and detection systems.

Care is required in calibration of two-wavelength oximetry. In early retinal oximetry studies retinal arterial OS was calibrated by comparison with *ex vivo* brachial-artery blood samples and blood gas measurement.[1–3] Whilst useful for establishing relations between retinal arterial OS and systemic arterial OS, *ex vivo* blood gas measurement is undesirable or not feasible and has been rendered unnecessary by the advent of fingertip pulse oximetry which allows non-invasive estimation of systemic arterial OS. However, calibrating arterial OS alone can lead to artefacts in estimated venous OS. For example, it has been shown that ODR is dependent on blood vessel diameter and background pigmentation; so two-wavelength oximetry requires calibration correction factors that are dependent upon both background pigmentation and blood vessel diameter.[9,31] It has recently been postulated that the laminar flow from multiple tributary branching veins, which leads to non-inform blood oxygenation within a vein may also degrade estimation of OS in retinal veins, which is based upon homogeneous blood oxygenation. This significance of this factor requires experimental testing and validation.[34]

Two-wavelength oximetry is the basis for the *Oxymap* and *Imedos* commercial retinal imaging devices which have found considerable use in clinical applications (see Section 6).

2.2. Multispectral oximetry techniques

Multispectral oximetry is broadly defined as any oximetry technique that incorporates information from three or more spectral wavebands to calculate OS.

2.2.1. Three-wavelength oximetry

The simplest form of multispectral oximetry, three-wavelength oximetry, [35] utilises two isobestic wavelengths relatively close to each other on the wavelength spectra combined with a wavelength providing oximetric contrast. From the two isobestic two wavelengths it is possible to quantify the scattering of light by blood, and thus to appropriately alter estimation of blood-vessel transmission via the modified Beer Lambert law.[35] Three-wavelength retinal oximetry was applied to the retinal imaging with a scanning laser device by Delori in the 1980s and 1990s.[36,37] However, three wavelength oximetry was somewhat difficult to apply because of the requirements of three wavebands where blood exhibits similar optical scattering properties; this limited the range of useful wavebands to which it can be applied.[33] As such, three-wavelength oximetry has tended to be superseded by multispectral oximetry using four or more bands.

2.2.2. Multispectral oximetry models

Multispectral oximetry models enable estimation of OS by incorporating the optical transmission of blood at a number of wavebands to isolate the absorption of light by blood and estimate or compensate for other optical parameters, e.g. the scattering of light by blood and tissue, or the optical absorption by melanin pigmentation. Typically, the transmissions of blood vessels are measured and the experimental transmission compared to a theoretical model incorporating these parameters. This enables direct estimation of OS without need of a reference value; i.e. multispectral algorithms are

"calibration free". Thus, multispectral oximetry algorithms can provide quantitative oximetry in blood vessels where OS levels have not yet been measured by other means (e.g. in the spinal cord)[38] or in blood vessels that may be expected to be very different from physiological norms (e.g. studying angiogenesis in tumour development)[39,40]. However, validation of estimated OS from multispectral oximetry algorithms is normally desirable, and provides vital information for the wider field of oximetry (see Section 5).

The number of wavebands incorporated into a multispectral imaging system varies greatly. Some oximetry studies incorporate many wavebands (e.g. between 25 and 76 wavebands) into a 'hyperspectral' model (see Table 1). Hyperspectral imaging offers the advantage a comprehensive measurement of blood vessel transmission, but as a result of this, hyperspectral imaging systems are typically burdened with a long acquisition time (i.e. > 1 minute) and high data volumes for a single data set. Consequently, challenges arise from subject eye motion and alterations of OS of vessels during hyperspectral measurement. Additionally, some wavebands may be sub-optimal for oximetry; for example, blue wavelengths suffer from lower instrumental signal-to-noise ratios and high absorption by melanin pigmentation, and auto-fluorescence from blood or other tissue. Imaging spectrograph devices offer excellent spectral resolution and have been employed for spectroscopy of the eye,[41] but have only found limited usage because there are no significant advantages associated with over-sampling the absorbance spectra of blood for oximetry.

Multispectral oximetry models incorporating a few (typically < 10) key wavelengths have achieved high quality oximetry, whilst reducing acquisition time required for spectral data acquisition. Of particular note is the development of 'snapshot' multispectral imaging techniques where several wavebands are acquired simultaneously; this avoids artefacts associated with misregistration of time-sequentially recorded images.[42] The simplest form of snapshot multispectral imaging involves to spliting the imaged light with an optical beam-splitter and then filter each resultant image separately with bandpass filters, or by using a dichroic mirror, [4,42] however, this becomes optically inefficient for large numbers of bands and problematic to implement with a single detector. Several snapshot multispectral imaging devices that require only a single detector have been developed, including use of a lenslet array[43] and the Image Mapping Spectrometer[44] using degmented mirrors; however to date neither of these approaches have been applied extensively to oximetry. A snapshot imaging system which has found significant use for oximetry is the Image Replicating Imaging Spectrometer (IRIS).[11,45] This optically efficient device enables video-rate imaging at eight distinct wavebands optimised for retinal oximetry. The high temporal resolution and 8 wavebands afforded by IRIS has enabled several new oximetry applications, including direct observation of oxygen release by red blood cells.[46]

Table 1. Retinal oximetry studies utilising multispectral imaging oximetry algorithms.

Study	Wave-range	Known parameters	Estimated parameters	Key reported OS (i.e. normal OS at normoxia - unless otherwise stated)
Schweitzer et al., (1999)[47]	510 – 586 nm 76 wavebands	ε, εmel	OS, c, d, η	A: 92.2 ± 4.1 % V: 57.9 ± 9.9 %
Drewes et al., (1999)[48]	629, 678, 821, & 899 nm.	ε	OS, S, c, d	A: 101 % V: 65 %
Smith et al., (2000)[49]	488, 635, 670, 752, & 830 nm	ε	OS, S, c, d, η	V: 42 - 56 %
Alabboud et al., (2007)[50]	500 – 700 nm 27+ wavebands	ε, S	OS, d, c, η	A: 96 % V: 55 %
Mordant et al., (2011)[51]	500 - 650 nm 300 wavebands	ε, S	OS, d, c, η	A: 104 % V: 35 %
Salyer et al., (2006)[52]	420 – 700 nm 29 wavebands	ε	OS, S	Arterial OS correlated well with *ex vivo* blood OS measurements
Khoobei et al., (2007)[53]	522 – 586 nm 7 wavebands	ε	OS	A: 92 % V: 76 %
Arimoto et al., (2010)[54]	510 – 600 nm 45 wavebands	ε	OS	N/A: relative OS
Furukawa et al., (2012)[54]	510 – 600 nm 7 wavebands	ε	OS	N/A: relative OS
Gao et al., (2012)[44]	510 - 586 nm 8 wavebands	ε	OS	N/A: relative OS

Key**:** A = arteries, V = veins. SO_2 = oxygen saturation, ε = extinction coefficient of Hb and O_2Hb, εmel = extinction coefficient of melanin, S = scattering contribution; c = concentration of haemoglobin, η = single/double pass contribution factor; d = diameter of vessels; K = contrast reduction factor.

3. BLOOD VESSELS TARGETED FOR OXIMETRY

3.1. Retinal oximetry

To date, the retinal vasculature has been the primary target for oximetry investigations in the eye. The retina is a site of extremely high metabolic demand, and problems with retinal blood flow can lead to a number of serious ocular conditions, including: retinal vascular occlusion[23–26] and diabetic retinopathy.[13–17] Further, in ocular conditions characterised by cell loss e.g. glaucoma, estimation of oxygen utilisation has provided new insights.[18–22]

Choroidal oximetry

A recent development in oximetry is the targeting of choroidal vessels for oximetry in subjects with very low retinal pigmentation. The lack of retinal pigmentation allows light to traverse through the retina and and dense choroidal vasculature. Kristjansdottir et al., (2013)[28] conducted an imaging study of OS of choroidal blood vessels in healthy subjects; results were reported in terms of ODR because ODR to OS calibration coefficients for choroidal vessels are not known and high levels optical scattering prevent the use of existing oximetry models. For example, some choroidal vessels have a negative optical density; i.e. they appear brighter than surrounding tissue. To date, a calibration scheme for choroidal oximetry calibration has not been derived. However, this study revealed a very low artery-vein difference of 4% in the choroid (instead of the typical ~30% artery-vein difference in retinal vessels).[11,28] Further studies of the choroidal circulation are required to understand what further physiological and clinical insights choroidal oximetry could bring.

3.2. Bulbar conjunctival oximetry

A recent oximetry study by MacKenzie et al., (2016)[32] revealed that bulbar conjunctival blood vessels have oxygen dynamics that are remarkably different from any other blood vessels in the eye. The bulbar conjunctival blood vessels are situated within the thin conjunctival membrane on the outer surface of the sclera, and consequently are in direct contact with ambient air. This unique position of the bulbar conjunctival vessels leads to oxygen diffusion occurring from air into the bulbar conjunctival blood vessels. Consequently, all bulbar conjunctival blood vessels exposed to air will be highly oxygenated, with little to no artery-vein difference. Study of bulbar conjunctival oxygen dynamics could potentially yield insights into microvascular oxygen dynamics and related parameters. This may be of particular interest for the study of oxygen dynamics in diabetes, where microvascular vessel wall hardening is known to occur.[32]

3.3. Episcleral oximetry

Oximetry of the superficial episcleral blood vessels embedded into the sclera has recently been achieved for the first time by MacKenzie et al (2016).[32] This study observed that when episcleral vessels are exposed to a temporary hypoxic intervention, episcleral vessels dilated and episcleral OS decreased. This auto-regulatory behaviour is similar to the autoregulation observed in retinal vessels under similar hypoxic conditions.[11] Because episcleral vessels can be imaged on the surface of the sclera, it has been suggested that episcleral vessels could be used as a proxy for retinal OS in situations where retinal imaging is not feasible; e.g. in patients with cataracts. Alternatively, episcleral vessels could be used as a comparison point with retinal vessels to study ocular autoregulation. Further

studies of episcleral vessels is required before their utility for clinically relevant oximetry can be ascertained.[32]

3.4. Oximetry elsewhere in the body

It is noteworthy that oximetry techniques originally developed for oximetry in the eye have benefited oximetry applications elsewhere in the body. For example, multispectral imaging oximetry has been employed to investigate diverse applications, including murine brain vascular oximetry,[55] diabetic foot-ulcer development,[56] bowel laproscopy,[57] skin blood flow,[58] the study of skin damage due to beta radiation exposure,[59] labial, periodontal, and sublingual microvasculature,[60] cancer tumour development in murine models,[39,40] and oximetry of the rat spinal cord dorsal vein for studying the development of multiple sclerosis disease models.[38]

4. SPECTROSCOPIC IMAGING MODALITIES FOR THE EYE

The majority of oximetry studies in the eye are conducted with retinal-fundus cameras adapted for two wavelength or multispectral imaging. Dual wavelength imaging can be enabled by splitting a broadband retinal image with a dichroic beam-splitter, spectrally filtering the resultant images, and then recording with one or more detectors.[4,31] Alternatively, wavebands may be recorded using separate channels of a three-colour RGB CCD detector with additional spectral filtering to ehance spectral discrimination.[9,38] The majority of multispectral and hyperspectral imaging studies have employed time-sequential filtering of broadband retinal images, utilising either multiple bandpass filters, a liquid-crystal tuneable filter (LCTF),[21,50,51,53,61,62] or an acousto-optic tuneable filter (AOTF) for spectral discrimination.[54,63,64] LCTFs and AOTFs offer the advantages electronically-controlled optical waveband switching in short timescales (~ 50 ms and 25 µs respectively), with a wide-variety of accessible wavelengths.[65] The Image Replicating Imaging Spectrometer (IRIS) has enabled video-rate oximetry which has been utilised observe oxygen release by *ex vivo* red blood cells upon exposure to sodium dithionite.[46,66]

Dual-wavelength scanning laser ophthalmoscopes (SLOs)[10,29,67,68] offer several advantages for retinal oximetry compared to fundus cameras, including improved control of stray light, reduced fundus light exposure levels, wide-field retinal scanning,[10] no requirement for pupil dilation,[68] and the option to incorporate adaptive optics to compensate for eye motion and improve imaging of small retinal blood vessels.[29] Recently this has enabled retinal oximetry of infants without mydriasis.[52] However, despite these advantages, SLOs are limited to available laser wavelengths, which are not optimal for oximetry,[33] so further development and testing of SLO oximetry systems is required.

Slit lamps have not yet been utilised for oximetry, but could be modified for multispectral imaging. The high mangification and resolution of slit lamps could enable oximetry of blood vessels as small as individual capillaries and groups of red blood cells in the bulbar conjunctiva.[27]

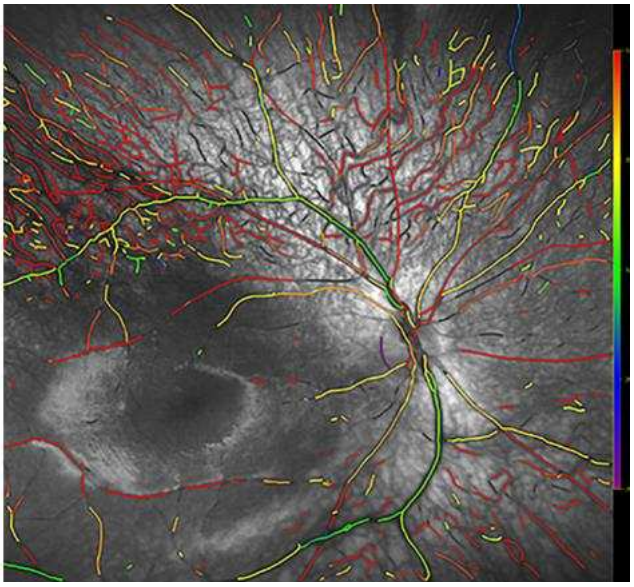

Figure 3. Psuedo-colour OS map of retinal vessels in an infant obtained via imaging with a scanning laser ophthalmoscope. Red indicates 100% OS, purple 0% OS. Figure reproduced from Vehmeijer et al., (2016) under a Creative Commons BY license[68]

Optical Coherence Tomography (OCT) is emerging as a potential retinal oximetry technology, but to date OCT oximetry has been applied only to murine models. In OCT, light backscattered from tissue is collected, and by coherence gating, is processed to form a 3D volumetric image of tissue. The ranging function of OCT provides the enhanced possibility of good control of the light paths defining optical absorption and with a reduced influence of scattering Spectroscopic Optical Coherence Tomography (S-OCT) has recently emerged as technology capable of recording 3D maps of OS with high spatial resolution. [69–71] Initially, S-OCT systems utilised the near infra-red illumination wavelengths commonly used by OCT systems. Unfortunately, near infra-red wavelengths suffer from weak optical absorption contrast between Hb and O_2Hb, resulting in sub-optimal oximetry. Consequently, newer S-OCT oximetry systems have improved oximetry capability by switching to visible wavelength illumination; visible wavelengths provide improved spectral contrast for oximetry. [72,73] However, S-OCT requires long acquisition times of up to 20 minutes for data acquisition. As such S-OCT oximetry studies have been conducted in murine models only. [74–76]

Photoacoustic imaging has recently emerged as a hybrid imaging modality that combines optical spectral contrast with the tissue depth penetration of ultrasound. In photoacoustic imaging, high intensity pulsed laser light (typically < 10 ns duration per pulse) is incident on the target tissue, heating the blood by less than 0.1°C and leading to rapid expansion and contraction which generates an ultrasound pulse with an amplitude proportional to the absorption of light by blood.[77] Ultrasound imaging of the emission therefore enables deep-tissue volumetric mapping of optical absorption. However, the high laser powers required for photoacoustic signal generation and requirement for ultrasonic transducer coupling to the eye make photoacoustic imaging a less attractive prospect for oximetry in the human eye compared to oximetry with fundus cameras. So far all photoacoustic studies of the eye have been limited to murine models. A good overview of photoacoustic ophthalmic imaging has been provided by Liu and Zhang (2016).[78]

5. VALIDATION AND TESTING OF OXIMETRY MEASUREMENTS

5.1 Oxygen-sensitive challenges and interventions

Oxygen-sensitive physiological challenges and interventions alter OS in subjects, allowing the oximetry capability of measurement systems to be assessed, and to provide useful calibration points for oximetry.[1,3,31,38,74] Further, these challenges and interventions provide physiological insights into metabolism.

5.1.1. Hyperoxia

Perhaps the most commonly used oxygen sensitive intervention is short duration hyperoxia (i.e. an excess of O_2), where a subject breathes a high O_2 (typically 100% O_2) air mixture. Hyperoxia greatly increases the partial pressure of oxygen (pO_2) in blood and increases both venous and arterial OS.

Using the *Oxymap* system, Hardarson et al., (2006)[4] reported that retinal arterial OS is increased from 96 ± 9% OS (mean ± SD) at normoxia to 101 ± 8 % OS at hyperoxia. Retinal veins experience a greater increase in OS from 55 ± 14 % to 78 ± 15%.[4] Using the *Imedos* system, Hammer et al., (2008)[9] reported that retinal arterial OS increased from 98 ± 10% (mean ± SD) at normoxia to 100 ± 10 % at hyperoxia and that retinal venous OS increased from 65 ± 12% at normoxia to 72 ± 10 % at hyperoxia.[9] Also using the *Oxymap* system, Klefter et al., (2014)[79] reported that retinal arterial OS increased from 95.1 ± 5.0% (mean ± SD) at normoxia to 96.6 ± 6.4% at hyperoxia and that retinal venous OS increased from 62.9 ± 6.7% at normoxia to 70.3 ± 7.8% at hyperoxia. Notably retinal and veins constricted in diameter by 5.5% and 8.2%, respectively at hyperoxia compared with normoxia. In all cases the increase in retinal venous OS was greater than the increase in retinal arterial OS.

A hyperoxia intervention was used by Krisjandottir et al., (2013)[28] to show that all choroidal vessels are highly oxygenated at normoxia; this could only have been achieved with a hyperoxia intervention because two-wavelength oximetry calibration has not yet been demonstrated for choroidal vessels.[28]

5.1.2. Hypoxia

An alternative OS-sensitive intervention is acute mild hypoxia, where systematic OS is decreased by subjects inhaling a hypoxic air mixture (typically ~ 15% O_2) for several minutes. The 10 - 15 % OS decrease induced by acute mild hypoxia studies is similar in magnitude to the decrease in OS experience in high-altitude airplane travel [80] and thus can be considered safe in healthy subjects for short durations.

Choudhary et al., (2013)[11] investigated the auto-regulatory effects of acute mild hypoxia in retinal vessels. OS was observed to decrease for both arterioles and venules but artery-vein (AV) OS difference remained constant. Both retinal arteries and veins were observed to dilate under hypoxia, with arteries showing a larger increase.[11] This provided new insight into the interplay between the vascular and choroidal oxygen supplies in the retina and auto-regulatory responses.

MacKenzie et al., (2016)[32] utilised a similar hypoxia intervention to study bulbar conjunctival and episcleral oxygen dynamics. Episcleral vessels were observed to behave similarly to retinal vessels under acute mild hypoxia, but episcleral arteries and veins were not distinguished. However, hypoxic bulbar conjunctival vessels were observed to re-oxygenate to high OS via oxygen diffusion when

exposed to ambient air. This effect would not have been possible to observe without the hypoxic intervention.[32]

Severe graded hypoxia to as low as 9% O_2 was used by Yi et al., (2015)[74] to investigate the metabolic response of the rat retina to hypoxia, finding that the metabolic demand of the retina is increased at hypoxia.[74]

5.1.3. Retinal light exposure

Retinal light exposure is known to alter retinal metabolic demand, thus inducing an autoregulation effect, and thus altering retinal OS. Using the *Oxymap* system, Hardarson et al., (2009)[8] found that retinal OS of both arteries and veins is higher in for an eye not exposed to light than for an eye exposed to light.[8] With the *Imedos* system, Hammer et al., (2011)[6] found that retinal venous OS increased by an average of ~ 5% after ~ 100s of 12.5 Hz flicker stimulation. In contrast, retinal arterial OS decreased slightly on average by ~ 1%. The diameter of both arteries and veins increased significantly, indicating increased blood flow. A later study by Hammer et al., (2012)[7] that compared diabetic patients to healthy controls found that venous OS of diabetic patients increased less under flicker stimulation than the change observed in healthy controls.[7] Similarly, venous dilation was less for diabetic patients than healthy controls. This suggests that retinal blood flow regulation is impaired in diabetic patients.[7]

5.2. Eye-mimicking *ex vivo* oximetry phantoms

Oximetry can be validated using *ex vivo* blood of various OS levels in *in vitro* phantoms that mimic blood vessels within the eye. Various strategies to phantom design and construction have been implemented.

Lemaillet et al., (2009)[81] developed a multi-layered dynamic eye phantom, incorporating flowing blood to simulate *in vivo* blood flow. Flowing blood is known to have different reflectance and scattering parameters than static blood due to alignment of red blood cells in laminar flow conditions.[82,83] The phantom consisted of: a plano-convex lens to mimic the human lens, a choroid mimicking layer, and a layer mimicking the retinal pigment endothelium. A 100 µm capillary was supplied with flowing bovine blood from a reservoir, and OS was modified by adding sodium hydrosulphide to deoxygenate blood, with additional oxygen supplied via a fuel cell to the blood reservoir. It was noted that construction of this phantom was highly time consuming.[81]

Mordant et al., (2011)[62] constructed a simple model eye consisting of straight quartz capillaries of various inner diameters (50, 100, and 150 µm) filled with static human blood (see Figure 4). A convex lens mimicked the human lens and distilled water mimicked the vitreous humour. A *w*hite diffuse reflectance material - *Spectralon*™ - mimicked the scleral back-reflectance.[30,84] Blood OS was varied by placing samples in air mixtures with varied fractions of O_2 and OS was confirmed with a blood gas analyser.[62] This phantom was used to validate later in vivo oximetry studies.[51]

MacKenzie et al., (2016)[32] constructed a simple sclera-mimicking phantom for bulbar conjunctival and episcleral oximetry. This phantom consisted of a 100 µm FEP plastic capillary above a *Spectralon*™ backing. The capillary was filled with flowing *ex vivo* equine blood and OS was varied by addition of sodium dithionite[66]; OS was confirmed with a blood gas analyser. Whilst not sophisticated, this phantom was sufficient for oximetry validation.[32]

Recently, Ghassemi et al., (2015)[85,86] have reported a bio-mimicking phantom designed from real retinal blood vessel patterns. These phantoms are 3D printed from a photoreactive resin in a pattern based up a 3D sectioned image of retinal vessels. A commercially available O_2Hb solution was used in place of whole blood. Yeast was used to consume oxygen in the solution, with OS decreasing over time.[85] Bio-mimicking phantoms may be advantageous in future because they could simulate realistic blood flow patterns and thus reproduce features like laminar flow seen in branching retinal veins.[42]

Corcoran et al., (2014)[87] created an advanced, wide-field spherical eye with 3D-printed phantom retina, an optics based on a rigorous schematic eye model. The retina incorporated embedded image resolution test targets and simulated retinal tissue layers. This style of advanced phantom has not yet been applied to oximetry, but could be highly beneficial in future studies that more closely resemble *in vivo* applications.[87]

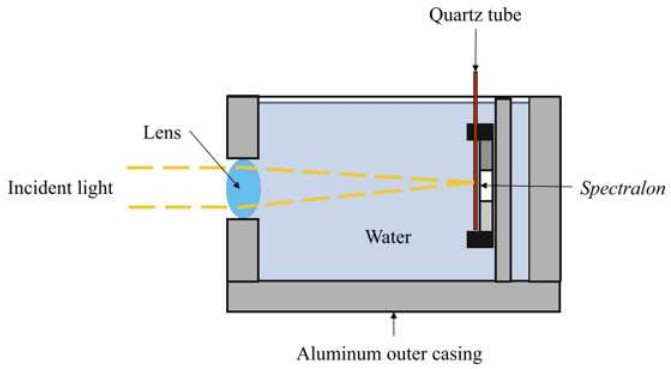

Figure 4. Diagram of a model eye phantom used by Mordant et al., (2011)[62] to validate oximetry capability of a hyperspectral imaging system. Figure reproduced with permission.

6. CLINICAL INSIGHTS

6.1. Diabetic retinopathy

Diabetic retinopathy is an important potentially blinding eye condition, which can affect patients in the working-age population. Diabetic retinopathy causes loss of capillaries in the retinal circulation, and visual loss can occur following the resultant retinal ischaemia, production of vaso-proliferative substances e.g. vascular endothelial growth factor (VEGF), and then misdirected growth of new vessels, which are fragile, and ultimately result in vitreous haemorrhage and/or retinal detachment. Diabetic visual loss can also follow leakage from retinal vessels close to the macula causing macular oedema. Research has explored the use of retinal oximetry to detect the effects on arterial and venous oxygen saturation of diabetic retinopathy. Early work by Beach et al. detailed acute changes in retinal oxygen utilisation with hyperglycaemia.[13] Later work, using dual-wavelength oximetry has demonstrated that higher venous oxygen saturation is associated with established diabetic retinopathy,[17] which, in some studies shows a dose-related relationship with increasing severity of proliferative diabetic retinopathy.[15,16] These observations might represent the formation of arterio-venous shunts in the retinal circulation to circumvent the capillary loss, or, alternatively, might

simply represent reduced oxygen utilisation by pathologically atrophic inner retinal cellular elements. Elevated venous saturation in patients with diabetic retinopathy may be a clinically useful measure of underlying retinal ischaemia, and may become an indicator of the need for retinal photocoagulation treatment. Interestingly, application of retinal photocoagulation has not been shown to result in any significant changes in venous retinal OS.[15]

6.2. Retinal vessel occlusion

Retinal venous occlusion is an important cause of visual morbidity in older patients with general vascular risk factors, e.g. hypertension. The pathological effects of a central retinal vein occlusion may include retinal ischaemia, production of VEGF, and growth of new vessels, which can cause visual loss from vitreous haemorrhage, or neo-vascular glaucoma. Retinal oximetry research has been directed at detecting OS abnormalities. Such abnormalities might provide early evidence of retinal ischemia, and thus enable treatment to be applied at an earlier stage; hopefully reducing the risk of the patient developing neo-vascular glaucoma. Dual-wavelength oximetry research studies in central retinal venous occlusion have consistently shown lower levels of venous OS, consistent with generalised retinal ischaemia.[24, 25] Studies in partial, or branch, retinal occlusion have shown variable results, with sometimes increased, and sometimes reduced venous saturations. This may reflect the different, localised impact of a venous occlusion, and/or the maturity of the occlusion, as well as the impact of local homeostatic mechanisms.[23,26] Central retinal artery occlusion has been noted to result in severe retinal hypoxia, with low arteriovenous difference, suggesting cell death.

6.3. Glaucoma

Glaucoma is a common cause of blindness in older patients, and a family history of glaucoma is an important risk factor. The diagnosis of glaucoma rests on detection of characteristic changes at the optic nerve head which are associated with visual field loss. Many, but not all, patients with glaucoma will have elevated intraocular pressure. The pathological change, which ultimately results in visual loss is retinal ganglion cell axon loss, which essentially results in atrophy of the cellular elements of the inner retina. Oximetry studies have investigated the changes in oxygen consumption within the retinal circulation that are associated with the cellular loss. Research work using both dual wavelength and multispectral imaging has demonstrated elevated venous saturation, with some studies showing increasingly elevated venous saturation in more severely affected eyes.[18–22] These studies suggest a role for retinal oximetry as a means to estimate inner retinal oxygen consumption in glaucoma and other optic neuropathies. Further research is required to investigate whether interventions to lower the intraocular pressure might demonstrate reversible changes in oxygen utilisation, which could be a favourable prognostic outcome from the intervention.

7. EXPERT COMMENTARY

Oximetry in the eye has revealed a great deal about physiological norms and autoregulation in the retina. However, there is still considerable insight to be gleaned in application of oximetry to choroidal, episcleral, and bulbar conjunctival blood vessels. OS norms of these vessel beds require further study, and may provide insights into disease development and treatment.

The advent of commercially available retinal oximetry systems, i.e. the *Oxymap* and *Imedos* systems have enabled retinal oximetry in the clinic, which has allowed studies of many retinal diseases with increasing statistical power. Clinical studies hold the potential to provide deeper insight into disease development and treatment of conditions such as diabetic retinopathy,[13–17] glaucoma,[18–22] and retinal vessel occlusion.[23–26]

The use of oxygen-sensitive interventions such as hyperoxia, hypoxia, and retinal light exposure have been able to compliment oximetry measurements on provide comparative intervention tests. In the retina a flicker intervention has enabled comparisons of the metabolic response of healthy control subjects and patients with diabetic retinopathy.[6] Notably, oxygen-sensitive interventions have enabled insight into the fundamental physiology of oxygen supply to the choroidal vessels,[28] episcleral circulation, and bulbar conjunctival circulation.[27] There is much potential in investigating these new targets for oximetry.

The development of new technology and innovation in image processing is also helping to drive new oximetry applications that were not previously possible. With improved techniques, oximetry is now possible in small retinal vessels,[29] in the choroid,[28] in the episcleral and bulbar conjunctival vessels,[32] and in the retina of infants.[68] New applications will invariably find new insights into the eye which are fundamentally and clinically important.

One of the fundamental challenges for oximetry is validation of measurements. Arterial OS can be compared to invasive blood gas measurements or pulse oximetry. However, venous calibration is extremely challenging, and is influenced by blood vessel diameter and fundus pigmentation.[9,31] Multispectral algorithms offer a 'calibration free' oximetry method, but accurate validation is still highly challenging due to the complex optical environment of the eye. Validation with *ex vivo* blood is also complicated by numerous factors including blood aggregation, red blood cell death, alignment of red blood cells under flow, and imperfect phantoms.[12,51,81]

Five-year view

In the last five years, oximetry has revealed the fundamental physiology of OS in episcleral vessels,[27] the bulbar conjunctival vessels,[27] and the choroidal circulation.[28] The physiology understanding of these vessel beds has been enabled by the development of hyperoxia and hypoxia OS-sensitive interventions.

Application of oximetry to the eye is continuing to yield fresh insight into the physiology of the eye and ocular disease development, and treatment. Commercially available two-wavelength oximetry systems have enabled oximetry in the clinic and studies of diseases such as diabetic retinopathy, vessel occlusion, and glaucoma have resulted. In the next five years, oximetry will likely be applied to increasingly diverse disease applications.

Imaging technology and imaging analysis is also continuing to develop, pushing new boundaries in spatial and temporal resolution. This can enable oximetry in new applications, such as the study of small retinal vessels,[29] oximetry in infants,[68] in *ex vivo* red blood cells.[46] The advent of 3D printing has allowed the development of bio-mimicking phantoms[87] based on real blood vessel

network patterns,[85] enabling the study of blood flow in realistic yet controlled scenarios. The fast paced development of 3D printing makes this field of phantom development particularly promising.

Key issues

- Spectroscopic oximetry is most widely applied to the study of the retinal circulation, but oximetry of the bulbar conjunctival, episcleral, and choroidal circulations has also recently emerged.
- Oxygen-sensitive interventions such as hyperoxia, hypoxia, and retinal flicker illumination provide oximetry validation, clinical insights, and have revealed fundamental physiology of the choroid, the episcleral vessels, and the bulbar conjunctival vessels.
- Commercial oximetry systems have enabled clinical studies in individual patients and in larger group studies, enabling deeper understanding of the development and treatment of diseases such as diabetes, retinal vessel occlusion, and glaucoma.
- Development of new imaging capabilities, advanced image processing concepts, and ever-improving phantom development are continuing to push new oximetry applications and enable fresh insights into the physiology of the eye.

7. CONCLUSIONS

Oximetry has enabled many insights in the fundamental physiology and oxygen dynamics of the eye. In particular, oximetry has enabled the study of retinal autoregulation, retinal disease development, and retinal disease treatment. Recently, the fundamental oxygen dynamics of the bulbar conjunctiva, the episcleral vessels, and the choroid have been revealed by oximetry combined with complimentary OS-sensitive hyperoxia and hypoxia interventions.

Commercially available retinal oximetry systems have enabled oximetry in the clinic, allowing studies of retinal OS in many diseases and treatments. In particular, diabetic retinopathy, retinal vascular occlusion, and glaucoma. Oxygen sensitive interventions - such as retinal flicker illumination, hypoxia, and hyperoxia - have provided means to validate oximetry, establish physiological norms, and probe auto-regulation responses.

The predominant imaging systems in the field of retinal oximetry are based upon modified retinal-fundus cameras, however SLO-based systems show considerable promise with a comparatively larger field of view, the potential for improved spatial and depth resolution and accuracy. Further, SLOs require no mydriasis. Yet, existing SLO systems are limited to commercially favoured laser wavelengths, and as such may provide sub-optimal oximetry measurements. Confocal SLOs offer potential for improved accuracy through improved control of stray light and the longer effect path lengths enable good oximetric contrast with a wide range of low-cost infrared lasers, although optical efficiency is necessarily lower. Further development of SLO oximetry systems is a particularly fertile area.

New oximetry applications are currently emerging, namely oximetry of the small retinal vessels, retinal oximetry in infants, and oximetry of the bulbar conjunctiva, episcleral, and choroidal vessels. These new applications for oximetry will enable researchers to understand the OS in physiological norms and disease across multiple ocular blood vessel beds.

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