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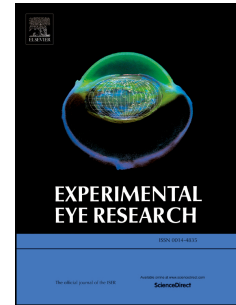
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L.E. MacKenzie, T.R. Choudhary, A.I. McNaught, A.R. Harvey



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1 ***In vivo* oximetry of human bulbar conjunctival and**
2 **episcleral microvasculature using snapshot**
3 **multispectral imaging**

4
5 **L.E. MacKenzie,¹ T.R. Choudhary,^{2,3} A.I. McNaught,^{4,5} and A.R. Harvey.¹**

6 1. School of Physics and Astronomy, University of Glasgow, Glasgow, United
7 Kingdom.

8 2. Institute of Biological Chemistry, Biophysics and Bioengineering, Heriot-Watt
9 University, Edinburgh, United Kingdom.

10 3. EPSRC IRC "Hub" in Optical Molecular Sensing & Imaging, MRC Centre for
11 Inflammation Research, Queen's Medical Research Institute, University of
12 Edinburgh, Edinburgh, UK

13 4. Department of Ophthalmology, Cheltenham General Hospital, Gloucestershire
14 Hospitals NHS Foundation Trust, Gloucestershire, United Kingdom.

15 5. Department of Health Professions, Plymouth University, Plymouth, United
16 Kingdom.

17
18 **Correspondence:** Andrew Harvey, School of Physics & Astronomy, Kelvin Building,
19 University of Glasgow, Glasgow, G12 8QQ, United Kingdom;

20 Andy.Harvey@glasgow.ac.uk

21

22 Abstract

23

24 Multispectral imaging (MSI) is now well established for non-invasive oximetry of
25 retinal blood vessels, contributing to the understanding of a variety of conditions
26 affecting the retinal circulation, including glaucoma, diabetes, vessel occlusion, and
27 auto-regulation. We report the application of a unique snapshot MSI technique to
28 enable the first oximetric imaging of the blood vessels of the anterior segment, i.e.
29 the episcleral and bulbar conjunctival microvasculature. As well as providing a new
30 capability of oximetry of the scleral vasculature, this technique represents ocular
31 oximetry that is complimentary or alternative to retinal oximetry. We report the
32 oxygen dynamics of these microvascular beds and assess how acute mild hypoxia
33 effects the blood oxygen saturation (SO_2) of bulbar conjunctival and episcleral
34 microvasculature.

35

36 A retinal-fundus camera fitted with a custom Image-Replicating Imaging
37 Spectrometer enabled oximetric imaging of bulbar conjunctival and episcleral
38 microvasculature in ten healthy human subjects at normoxia (21% Fraction of
39 Inspired Oxygen [FiO_2]) and acute mild-hypoxia conditions (15% FiO_2). Eyelid
40 closure was used to block oxygen diffusion between ambient air and the sclera
41 surface. Four of the ten subjects – those that presented suitable vasculature for
42 direct comparison between bulbar conjunctival and episcleral vessels - were imaged
43 for 30 seconds following eyelid opening. Vessel diameter and Optical Density Ratio
44 (ODR: a direct proxy for oxygen saturation) of vessels was computed automatically.
45 Oximetry capability was validated using a simple phantom for the scleral vasculature,

46

47 Average episcleral diameter increased from $78.9 \pm 8.7\mu\text{m}$ (mean \pm standard
48 deviation) at normoxia to $97.6 \pm 14.3\mu\text{m}$ at hypoxia ($p = 0.02$). Diameters of bulbar
49 conjunctival vessels showed no significant change from $80.1 \pm 7.6\mu\text{m}$ at normoxia to

50 80.6 ± 7.0µm at hypoxia (p= 0.89). Acute mild hypoxia resulted in a decrease in SO₂
51 (i.e. an increase in ODR) from normoxia levels in both bulbar conjunctival (p <0.001)
52 and episcleral vessels (p= 0.03).

53

54 Hypoxic bulbar conjunctival vasculature rapidly re-oxygenated in an exponential
55 manner, reaching normoxia baseline levels, with an average ½ time to full
56 reoxygenation of 3.4 ±1.4 seconds. This reoxygenation occurs because the bulbar
57 conjunctival vessels are in direct contact with ambient air. This is the first study to
58 characterise and also to image the oxygen dynamics of bulbar conjunctival and
59 episcleral microvasculature, and to directly observe the rapid reoxygenation of
60 hypoxic bulbar conjunctival vessels when exposed to air.

61

62 Oxygen diffusion into the bulbar conjunctiva must be taken into account to provide
63 meaningful oximetry because bulbar conjunctival vessels will be highly oxygenated
64 (close to 100% SO₂) when exposed to ambient air.

65

66 Oximetry of bulbar conjunctival vessels could potentially provide insight into
67 conditions where oxygen dynamics of the microvasculature are not fully understood,
68 such as diabetes, sickle-cell diseases, and dry-eye syndrome. Further, *in vivo*
69 oximetry of individual capillaries and groups of flowing red blood cells could be
70 achieved with a high magnification slit lamp adapted for MSI.

71

72 **Keywords:** multispectral imaging, oximetry, hypoxia, bulbar conjunctiva, episclera,
73 oxygen saturation, microvasculature, oxygen diffusion,

74

75 1. Introduction

76 Multispectral imaging (MSI) is well established for non-contact oximetry of blood
77 vessels (D J Mordant et al., 2011a; David J Mordant et al., 2011b) which has
78 enhanced the understanding of a variety of retinal conditions, such as diabetes
79 (Hammer et al., 2009; Hardarson and Stefánsson, 2012; Isenberg et al., 1986),
80 glaucoma (Boeckeaert et al., 2012; Mordant et al., 2014; Olafsdottir et al., 2011), and
81 vessel occlusion (Eliasdottir et al., 2014), as well as auto-regulation response to
82 flicker stimulation (Hammer et al., 2011) and acute mild hypoxia (Choudhary et al.,
83 2013). However, oximetry of capillaries in the retina is beyond the technical
84 capabilities of MSI-enabled retinal fundus cameras. The anterior segment provides
85 two alternative ocular microvascular beds that are easily accessible for multispectral
86 imaging and which could be used to probe ocular blood oxygen saturation and
87 potentially provide new physiologically-relevant information; the bulbar conjunctival
88 and episcleral microvascular beds. This is the first study to use MSI to non-invasively
89 measure the oxygen saturation of bulbar conjunctival and episcleral
90 microvasculature with high spatial and temporal resolution, revealing rapid oxygen
91 diffusion from ambient air into bulbar conjunctival vessels.

92
93 The episcleral microvasculature is located within the scleral tissue, with few
94 episcleral vessels visible near the scleral surface. In contrast, the bulbar conjunctival
95 microvasculature is semi-mobile above the sclera, and presents many arterioles,
96 venules, and capillaries for imaging (Meighan, 1956). Groups of individual red blood
97 cells can be observed to flow in bulbar conjunctival capillaries if imaged with high
98 magnification (Jiang et al., 2014). The bulbar conjunctiva may be unique in that it is
99 the only microvascular bed in the human body which is directly exposed to ambient
100 air. Figure 1a shows generalised vessel positions with respect to the sclera. Figure
101 1b shows a representative image of bulbar conjunctival and episcleral vasculature in

102 a single subject. However, despite potential for new oximetry information and ease of
103 imaging, no MSI oximetry studies of either the bulbar conjunctival or episcleral
104 microvasculature have been published to date.

105

106 MSI oximetry is based on the SO_2 -dependent optical absorption spectra of
107 haemoglobin. Changes in SO_2 can be calculated by imaging blood vessels at two
108 wavelengths: one wavelength where optical absorption is sensitive to variations in
109 SO_2 , and at another wavelength which is insensitive to SO_2 variations (i.e. isobestic).
110 From images of vessels, the optical density (OD) of vessels at each wavelength can
111 be calculated, allowing the calculation of optical density ratio (ODR); ODR is directly
112 proportional to SO_2 . In vessels where SO_2 is known, ODR can then be empirically
113 calibrated to SO_2 by assuming local arterial SO_2 is equal to the SO_2 of systemic
114 arterial SO_2 as measured by pulse oximetry (Beach et al., 1999), or by using
115 reference values from previous studies. (Hardarson et al., 2006).

116

117 To the best of our knowledge there are no reported MSI oximetry studies of the
118 bulbar conjunctival or episcleral microvasculature. Instead, insights into the oxygen
119 dynamics of microvasculature have generally been indirectly inferred from vessel-
120 diameter or blood-flow measurements (Jiang et al., 2013; Shahidi et al., 2010;
121 Wanek et al., 2013), however these parameters may be affected by factors other
122 than changes in SO_2 , such as conjunctival or episcleral inflammation. Direct
123 measurement of the partial pressure of oxygen (pO_2) of the palpebral conjunctival
124 microvasculature has been achieved with Clark-type electrodes (Chapman et al.,
125 1986; Iguchi et al., 2005; Isenberg et al., 2002; Kwan and Fatt, 1971; Mader et al.,
126 1987), however these electrodes have insufficient spatial discrimination for
127 localisation of oximetry to blood vessels and crucially, block oxygen diffusion
128 between ambient air and blood vessels under study.

130 In this study, we report the use of a retinal fundus camera modified for Snapshot
131 Multispectral Imaging (SMSI) to non-invasively quantify the oxygen dynamics of both
132 bulbar conjunctival and episcleral microvasculature in ten healthy human subjects.
133 The high temporal resolution of the SMSI system (10ms exposure, 1Hz image
134 acquisition rate) enables observation of fast biological processes (Fernandez
135 Ramos et al., 2014). We observe rapid oxygen diffusion from ambient air into bulbar
136 conjunctival vessels due to the unique location of the bulbar conjunctiva (i.e. directly
137 in contact with ambient air); such observations are not possible with time-sequential
138 MSI or Clarke-type electrodes because these techniques lack sufficient temporal and
139 spatial resolution respectively.

140

141 **2. Material and methods**

142 **2.1. Subject recruitment**

143 This study was approved by the Ethics Committee of the University of Glasgow,
144 College of Medical, Veterinary and Life Sciences. All volunteers provided written
145 informed consent before participation and all procedures were performed in
146 accordance with the tenets of the Declaration of Helsinki. Ten healthy volunteers
147 (age 25 ± 2 years, six males and four female) were recruited. Subjects reported no
148 history of ocular, respiratory, or vascular disease. Volunteers that regularly wore
149 contact lenses or who were suffering from allergic conjunctivitis were excluded
150 because this may induce fluctuating bulbar conjunctival vasodilatation (Gartner,
151 1944; Cheung et al., 2012; Jiang et al., 2014) .

152

153 **2.2. Imaging system**

154 The imaging system consisted of a commercial retinal fundus camera (Topcon
155 TR50-DX; Topcon, Itabashi, Tokyo, Japan), fitted with an Image Replicating Imaging

156 Spectrometer (IRIS) and a cooled sCMOS camera (Zyla 5.5; Andor, Belfast, United
157 Kingdom). IRIS is discussed in detail elsewhere (Harvey et al., 2005; Alabboud et
158 al., 2007; Gorman et al., 2010; Fernandez Ramos et al., 2014); but in brief, IRIS
159 simultaneously spectrally de-multiplexes a white-light image into eight distinct
160 narrowband spectral images onto a single detector without rejection of light.
161 Orthogonal-polarization imaging was used to minimise specular reflections from the
162 sclera and blood vessels (van Zijderveld et al., 2014). Fundus-camera flash and
163 image acquisition were synchronized using a custom graphical user interface written
164 in LabVIEW, and images were saved in uncompressed Tiff format. Image acquisition
165 was limited to 1Hz by the fundus camera flash refresh rate with an exposure time of
166 10ms. This imaging set-up and a representative multispectral IRIS image of the
167 sclera are shown in Figure 2.

168

169 The curved scleral surface presents a challenge for imaging because it causes the
170 position of blood vessels to vary with respect to the imaging plane of the fundus
171 camera, potentially up to ~12mm from the anterior segment to the extreme lateral
172 side of the sclera. To insure sharp focus over an extended scleral region, the 'small
173 aperture' setting of the fundus camera was selected. This resulted in an estimated
174 depth-of-field (DOF) of ~10mm; DOF was estimated by imaging a USAF test chart
175 (USAF 1951 Chart; Applied Image Group-Imaging, Rochester, New York, USA) as it
176 was moved through prime-focus on a linear-translation stage. A 35-degree field-of-
177 view was selected to provide a field of view at the object plane of approximately 85 x
178 45mm. This combination of settings enabled the imaging of bulbar conjunctival and
179 episcleral vessels over an extended scleral region with an optimal, sharp focus.

180

181 **2.3. Scleral phantom**

182 For assessment of the validity of our oximetry technique, a simple sclera-mimicking
183 phantom was manufactured (see Figure 3). Similar phantoms have previously been
184 used to validate retinal oximetry (David J Mordant et al., 2011). The phantom
185 consisted of a transparent Fluorinated Ethylene Propylene (FEP) capillary of 100µm
186 inner diameter (Zuess inc., Belfast, Northern Ireland), placed in contact with optical-
187 grade *Spectralon* (Spectralon® Diffusion Material; Labsphere inc, North Suttan, New
188 Hampshire, USA); *Spectralon* has similar spectral reflectance characteristics to the
189 sclera (Bashkatov et al., 2010; Labsphere Inc.). To simulate *in vivo* blood circulation,
190 *ex vivo* whole horse blood (40% hematocrit) (E&O labs, Bonnybridge, Scotland,
191 United Kingdom) was flowed through the FEP capillary under feed from a syringe
192 pump (KDS260, Linton Instrumentation, UK). SO₂ of the blood was reduced by
193 adding measured quantities of Sodium Dithionite (EMD Millipore, Fisher Scientific,
194 Loughborough, UK) to 5ml samples of blood according to the procedure described in
195 Briely-Sabo and Bjornerud (Briley-Saebo and Bjornerud, 2000). SO₂ blood samples
196 was measured prior to imaging using an optical blood gas analyser (GEM OPL,
197 Instrumentation Laboratory, Bedford, Massachusetts, USA). A total of eight SO₂
198 samples ranging between 5% and 100% SO₂ were imaged in the FEP capillary.
199

200 **2.4.1. Experimental procedure for *in-vivo* imaging**

201 ~~Subjects positioned their head in the standard fundus-camera chin-rest; head-straps~~
202 ~~were used to restrain the subject and minimise any motion. The fundus camera~~
203 ~~objective lens was positioned approximately five centimetres from the subject's~~
204 ~~sclera. In this configuration, the fundus camera illumination formed a circle~~
205 ~~approximately four centimetres in diameter. Subject gaze was controlled by the~~
206 ~~subject fixating on the fundus camera external fixation target (a movable red LED).~~
207 ~~For each subject, the scale of images was calibrated by imaging a millimeter scale~~
208 ~~located in the nominal plane of the sclera at prime focus. This yielded an average~~

209 image scale of 13.5 microns per pixel, enabling conversion of vessel diameter in
210 pixels to diameter in microns.

211

212 Subjects positioned their head in the standard fundus-camera chin-rest; head-straps
213 were used to restrain the subject and minimise any motion. The fundus camera
214 objective lens was positioned approximately five centimetres from the subject's
215 sclera. In this configuration, the fundus camera illumination formed a circle
216 approximately four centimetres in diameter. Subject gaze was controlled by the
217 subject fixating on the fundus camera external fixation target (a movable red LED).
218 For each subject, the scale of images was determined by imaging a millimeter scale
219 placed in front of the sclera at prime focus. All subsequent images were acquired at
220 this focal position. This enabled a calibration of the size of each pixel on the detector
221 to the real size of an image; on average, one pixel corresponded to ~13.5 microns.
222 From this, the measured vessel diameter in pixels was calibrated to diameter in
223 microns."

224

225 Scleral regions of each subject were selected for imaging so as to maximise the
226 number of bulbar conjunctival vessels meeting the inclusion criteria (see Section
227 2.6.1). Bulbar conjunctival and episcleral vasculature was distinguished by moving
228 the gaze of a subject; this moved the position of the bulbar conjunctiva above the
229 sclera, altering the relative position of bulbar conjunctival and episcleral vessels.
230 However it was not possible to classify individual vessels as arterioles and venules
231 because of the diverse morphology of bulbar conjunctival vasculature and the limited
232 number of episcleral vessels available for imaging (see Section 4.4). Scleral regions
233 were chosen for imaging so as to maximise the number of bulbar conjunctival
234 vessels meeting inclusion criteria whilst including some episcleral vessels for
235 analysis (see Section 2.6.1). Once selected, the same blood vessels in a single

236 scleral region of a single eye for each subject were consistently imaged and
237 analysed throughout the experiment.

238

239 Throughout the imaging protocol, the scleral region exposed to air was kept constant
240 by the subject constantly gazing at the stationary fixation target and peripheral
241 arterial SO_2 was recorded throughout the experiment using a fingertip pulse oximeter
242 (AUTOCORR; Smiths Medical ASD Inc., Rockland, MA, USA) interfaced to a
243 computer using a custom LabVIEW interface.

244

245 **2.4.2. Repeatability**

246 To assess repeatability of ODR measurement, eight consecutive images of the same
247 scleral region were acquired in a period of approximately ten seconds for each
248 subject. Gaze fixation was maintained for 2.5 minutes with their eyelid open prior to
249 imaging to expose the target vasculature to ambient air.

250

251 **2.4.3. Effect of eyelid closure**

252 Eyelid closure was used to control oxygen diffusion; eyelid closure places a tissue
253 barrier between the scleral surface and the ambient air, drastically decreasing the
254 rate of any oxygen diffusion from ambient air to this scleral surface. To assess if eye
255 closure affects the ODR of vessels, subjects were imaged before and after a period
256 of eyelid closure. As before, subjects continually gazed at the fixation target for 2.5
257 minutes to expose the target vasculature to ambient air prior to imaging; subjects
258 then closed their eyelids for a further 2.5 minutes. After 2.5 minutes of eyelid closure
259 subjects opened their eyelid and synchronised imaging occurred

260

261 **2.5.4. Acute mild hypoxia**

262 To assess the effects of acute mild hypoxia on ODR, subjects were imaged at
263 normoxia and acute mild-hypoxia. For normoxia measurement, subjects inhaled
264 room air (21% FiO₂) for 2.5 minutes whilst fixating on the red LED fixation target,
265 after which they were imaged. To induce acute mild hypoxia, subjects closed their
266 eyelids and breathed a hypoxic air mixture (15% 2.5 minutes of inhalation of hypoxic
267 air mixture (15% FiO₂) supplied via a hypoxic-air generator (Everest Summit II
268 Hypoxic Generator; Hypoxico, Inc., New York, NY, USA) (Spurling et al., 2011). The
269 hypoxic-air generator was calibrated before use and the air supply was monitored
270 with an in-line oxygen analyzer (AD300 oxygen analyser; Teledyne Analytical
271 Instruments, City of Industry, California, USA). Hypoxic air generators have been
272 previously used for a study into retinal response to acute mild hypoxia (Choudhary et
273 al., 2013).

274

275 After 2.5 minutes of hypoxic-air inhalation, subjects opened their eyelids and
276 synchronised imaging occurred. Synchronisation of imaging with events, such as
277 eyelid opening, was accomplished with a five-second oral countdown and with an
278 accuracy of ± 1 seconds. Subjects were then returned to normoxia by breathing room
279 air. This process was repeated in the following sequence: normoxia 1, hypoxia 1,
280 normoxia 2, hypoxia 2, normoxia 3; this sequence provides a robust time-sequential
281 modulation in SO₂ and associated ODR change that is highly distinct from normal
282 physiological variations.

283

284 **2.5.5. Exposure of hypoxic vasculature to ambient air**

285 A sub-group of four subjects (3 male, 1 female) were selected for further study.
286 These subjects presented bulbar conjunctival and episcleral vessels suitable for
287 analysis within single scleral region, allowing concurrent imaging - and thus
288 comparison of oxygen dynamics - between bulbar conjunctival and episcleral

289 vessels. Hypoxia was induced as described in section 2.5.3. However, when
290 subjects opened their eyelids, a synchronised 1Hz frame-rate imaging sequence was
291 subsequently recorded for the 30 seconds, enabling observation of any rapid
292 diffusion processes. This was repeated twice per subject.

293

294 **2.6. Image analysis**

295 **2.6.1 Vessel section inclusion criteria**

296 The following inclusion criteria were applied to ensure that only appropriate vessel
297 sections were selected for analysis: (1) vessel sections had to be greater than 5
298 pixels ($\sim 67\mu\text{m}$) in diameter to ensure that the contrast is not significantly affected by
299 the modulation-transfer function of the imaging system. (2) vessel section had to
300 have no other vessel sections within 12 pixels of either side of the vessel to be
301 analysed; the presence of small vessels was accepted due to the high number of
302 small bulbar conjunctival vessels; (3) vessel sections had to be at least 30 pixels
303 long ($\sim 405\mu\text{m}$); (4) vessels close to vessel intersections, regions of scleral glare,
304 specular reflections, or images with poor focus were excluded; (5) episcleral vessels
305 had to be of high apparent contrast with respect to the scleral tissue and not show a
306 significant decrease in contrast along the analysed vessel section length (i.e. not
307 appear to go deeper in the sclera tissue); (6) vessel sections had to meet all these
308 inclusion criteria for all images in each section of the study.

309

310 **2.6.2. Vessel tracking**

311 Image processing was implemented *post hoc* using custom algorithms implemented
312 in MATLAB. Raw IRIS images were cropped and co-registered to create a
313 multispectral data cube. Vessels were tracked semi-automatically using manually
314 identified control points. Repeated semi-automatic tracking demonstrated negligible
315 variation in ODR (a standard deviation of $<0.5\%$ in 10 repeated measurements).

316 Fully automatic tracking was not implemented because inter-image registration of
317 bulbar conjunctival vessels is affected by the relative motion of bulbar conjunctival
318 and episcleral vasculature (Crihalmeanu and Ross, 2012).

319

320 **2.6.3. Oximetric analysis and vessel diameter measurement**

321 Our oximetric analysis is based on two-wavelength oximetry developed by Beach et
322 al (Beach et al., 1999). For two-wavelength oximetry, the optical-density (OD) of
323 blood vessels at two spectral wavebands is calculated: one waveband where optical
324 absorption is insensitive to changes in SO_2 (isobestic) and one waveband where
325 optical absorption is sensitive to changes in SO_2 (contrast). The 570nm IRIS
326 waveband was utilised as the isobestic reference and the 560nm waveband was
327 used as the oxygen sensitive waveband (Prahl, 1999). Each waveband has a full
328 spectral-width of approximately 7nm (Fernandez Ramos et al., 2014). Simple
329 modelling based upon the Beer-Lambert law of optical absorption shows that the OD
330 of blood vessels of ~60-100 μ m at 560nm and 570nm wavebands is expected to be
331 between 0.15 and 1; near-optimal for oximetry (van Assendelft, 1970).

332

333 A vessel-fitting algorithm was used to estimate vessel diameter (in pixels) and optical
334 transmission of vessels (see Figure 4). Vessel diameter at 570nm was estimated
335 according to the method described by Fischer et al., (Fischer et al., 2010), where the
336 vessel boundaries are defined as the points in the vessel profile with the maximum
337 rate of change in grayscale intensity. This provided reputable fitting for both bulbar
338 conjunctival and episcleral vessels. Using this fitting algorithm, greyscale intensity in
339 the centre of each vessel (I_v) was calculated and the background greyscale intensity
340 at the centre of the vessel (I_o) was estimated by a linear fit to the background. OD
341 was then calculated for each wavelength by:

$$OD_\lambda = -\log_{10} \left(\frac{I_v}{I_o} \right). \quad (1)$$

342 ODR, defined as $ODR = OD_{560}/OD_{570}$, was then calculated for each vessel; ODR is a
343 direct proxy for SO_2 ; if SO_2 increases, ODR decreases. ODR is approximately
344 independent of vessel diameter and concentration of hemoglobin.

345

346 If two or more reference SO_2 values are known, then ODR can be empirically
347 calibrated to SO_2 (Beach et al., 1999). However, no calibration is possible for this
348 study because no empirical measurements of SO_2 in either bulbar conjunctival or
349 episcleral vasculature have been reported in the literature, so we report results
350 simply in terms of ODR.

351

352 **3. RESULTS**

353 **3.1 Sclera phantom**

354 A total of eight *ex vivo* blood samples of various oxygenations were imaged and
355 analysed in the scleral phantom. Some variation in ODR was seen as blood flowed
356 along the capillary. Overall, ODR was found to decrease with increasing SO_2 and the
357 data was well fitted by a linear trend ($R^2 = 0.89$) (see Figure 5), validating the use of
358 our MSI technique for oximetry of vessels in a scleral-like configuration. Repeatability
359 of scleral phantom ODR measurements was $<0.5\%$ (standard deviation of 10
360 consecutive images).

361

362 **3.2 Repeatability of *in vivo* ODR**

363 The repeatability of *in vivo* ODR measurements is summarised in Table 1. The
364 greater repeatability of ODR measurement of bulbar conjunctival vessels (0.96%)
365 compared to episcleral vessels (1.55%) when calculated as an average across
366 vessel type is probably due to the larger number of bulbar conjunctival vessel
367 sections analyzed (57 in total) compared to episcleral vessel sections (22 in total);
368 the larger number of vessels analysed reduces the sensitivity to fluctuations in ODR.

370 **3.4. Eyelid closure during normoxia**

371 Eyelid closure during normoxia resulted in no statistically significant change in ODR
372 of either bulbar conjunctival or episcleral vessels. When the eyelid was open with
373 constant gaze for 2.5 minutes, the average ODR was 0.90 ± 0.08 (mean \pm standard
374 deviation) for bulbar conjunctival vessels and 0.94 ± 0.09 for episcleral vessels. After
375 eyelid closure, average ODR was 0.90 ± 0.08 for bulbar conjunctival vessels and
376 0.93 ± 0.08 for episcleral vessels ($p = 0.99, 0.72$ respectively; paired t-test).

377

378 **3.5. Acute mild hypoxia**

379 Table 2 and Figure 6 summarise measurements of ODR, vessel diameter, and
380 fingertip pulse oximetry at normoxia and hypoxia. Figure 6a shows ODR and pulse
381 oximeter data throughout the whole normoxia/hypoxia sequence. Bulbar
382 conjunctival ODR increased with hypoxia (indicating a reduction in SO_2) from $0.846 \pm$
383 0.014 (mean \pm standard error) at normoxia to 0.916 ± 0.011 at hypoxia ($p < 0.001$,
384 paired t-test) (Figure 6b). Episcleral ODR increased on average, from 0.881 ± 0.019
385 (mean \pm standard error) at normoxia to 0.938 ± 0.018 at hypoxia ($p = 0.03$, paired t-
386 test) (Figure 6c). Figure 7 shows an overlaid ODR map of vessels at normoxia and
387 hypoxia.

388

389 Bulbar conjunctival vessel diameter did not change significantly between normoxia
390 and hypoxia ($p = 0.89$, paired t-test), however increases in vessel diameters were
391 apparent in some subjects, whereas decreases in diameters were seen in others
392 (Figure 6d). Diameters of episcleral vessels were observed to increase from $78.9 \pm$
393 $8.65\mu\text{m}$ (mean \pm standard deviation) at normoxia to $97.6 \pm 14.3\mu\text{m}$ at hypoxia
394 (Figure 6e) ($p = 0.02$, paired t-test).

395

396 3.6. Exposure of hypoxic vasculature to ambient air

397 For all eight datasets (four subjects, each imaged twice) ODR of hypoxic bulbar
398 conjunctival vessels rapidly decreased upon eyelid opening (indicating an increase in
399 SO_2), tending asymptotically to an ODR corresponding to ODR measured at
400 normoxia. The variation in ODR was well-fitted by an exponential-decay function
401 representing re-oxygenation of the conjunctival vessels plus a linear component,
402 reflecting the incoming hypoxic blood supply:

$$OD = a * e^{-bt} + ct + d \quad (2)$$

403 Where t is time and a, b, c, d , are empirically calculated constants. The half-time to
404 full reoxygenation ($T_{1/2}$) can then be calculated by:

$$T_{1/2} = -\frac{\ln(2)}{b}. \quad (3)$$

405 $T_{1/2}$ varied on both an intra and inter-subject basis (see Table 3) but averaged over
406 all measurements $T_{1/2}$ was 3.4 ± 1.4 seconds (mean \pm standard deviation). Figure 8
407 shows this reoxygenation process in two representative subjects.

408

409 Episcleral vessel ODR remained higher (i.e. lower SO_2) after eyelid opening than at
410 normoxia levels and was well fitted by a linear trend. Pulse oximeter SO_2 followed a
411 similar trend to episcleral ODR.

412

413 4. Discussion

414 4.1. Validation of oximetry technique using scleral phantom

415 Results from the scleral phantom measurement validated the ability of the spectral
416 imaging technique to characterise ODR for oximetry for blood vessels. Some
417 variation in ODR was observed when blood flowed through the capillaries; this
418 variation is likely to be due to non-homogenous SO_2 due to non-uniform
419 deoxygenation by discrete crystals of Sodium Dithionite added to blood (Briley-
420 Saebo and Bjornerud, 2000). Further variation in ODR may be caused by the

421 aggregation of blood cells, which alters the optical path of light through blood.

422 Nevertheless, the results shown in Figure 5 clearly support that ODR decreases be
423 with SO_2 .

424

425 **4.2. Effects of acute mild hypoxia**

426 In episcleral vessels, vessel diameter increased and SO_2 decreased at acute mild
427 hypoxia conditions. This is similar to auto-regulation of retinal vessels during acute
428 mild hypoxia (Choudhary et al., 2013). In bulbar conjunctival vessels, SO_2 also
429 decreased with hypoxia, but average vessel diameter did not change significantly.
430 This confirms that the increase in ODR observed is due a decrease in SO_2 and not
431 due a secondary effect due to change of vessel diameter.

432

433 **4.3. Consequences of oxygen diffusion**

434 Our study is the first to directly show that oxygen diffusion from air results in rapid
435 reoxygenation and saturation of hypoxic bulbar conjunctival vessels. This
436 measurement would not be possible with Clark electrodes, which are limited to a
437 single point measurement and crucially, block oxygen diffusion between ambient air
438 and the tissue in measurement. Hill and Fatt (1963) did however use a Clarke
439 electrode to demonstrate that the bulbar conjunctiva would uptake oxygen from a
440 limited pO_2 reservoir via diffusion, concluding that oxygen diffusion from ambient air
441 to the exposed bulbar conjunctival vessels occurs constantly (Hill and Fatt, 1963).
442 This study is the first to directly observe how this oxygen diffusion alters bulbar
443 conjunctival SO_2 .

444

445 It is expected that when in equilibrium with ambient air ($pO_2 \sim 160\text{mmHg}$), bulbar
446 conjunctival vessels will be close to 100% SO_2 because normal arterial blood (~ 95 -
447 97% SO_2) corresponds to a typical pO_2 of 80-100mmHg; much less than 160mmHg

448 (Verma and Roach, 2010; Williams, 1998). The average ODR was of exposed
449 bulbar conjunctival vessels was consistently ~0.95 (see Figure 6a), indicating a
450 constant equilibrium as expected.

451

452 In retinal oximetry, ODR is often empirically calibrated to SO_2 by assuming retinal
453 arterial SO_2 to be equal to the systemic arterial SO_2 as measured by a pulse
454 oximeter. Our results show that the oxygen dynamics of episcleral vessels are
455 similar to pulse oximetry, so this calibration approach would be valid for episcleral
456 vessels *if* arteries and veins could be accurately identified. However, this calibration
457 approach would not be valid for bulbar conjunctival vessels because our results
458 show that the oxygen dynamics of bulbar conjunctival vessels do not reflect the
459 oxygen dynamics of systemic arterial SO_2

460

461 **4.4. Challenges of bulbar conjunctival and episcleral oximetry**

462 In the retina, oximetry results are often reported independently for arterioles and
463 venules. However, in this study we report results for generalised vasculature and not
464 separately as arterioles and venules for several reasons. (1) Bulbar conjunctival
465 arterioles and venules could not be reliably distinguished from morphology alone due
466 to the significant variation in bulbar conjunctival vessel morphology (Meighan, 1956).
467 (2) Bulbar conjunctival vessels will be highly oxygenated when exposed to ambient
468 air due to oxygen diffusion from air, and thus could not be distinguished on the basis
469 of ODR. (3) The relatively low number of episcleral vessels that met inclusion criteria
470 did not allow sufficient comparison to identify arteries and veins by either vessel
471 morphology or ODR. Reliable discrimination between episcleral arteries and veins
472 could however be achieved with fluorescence angiography (Ormerod et al., 1995).

473

474 Rattlesnaking - a false apparent change in ODR along the length of a vessel section
475 - is a common artefact in two-wavelength oximetry. Rattlesnaking was observed in
476 both bulbar conjunctival and episcleral vessels and can be seen in Figure 7.

477 Rattlesnaking may be caused by a number of factors such as nearby vessels,
478 variations in scattering properties of background tissue, and groups of erythrocytes
479 flowing in vessels. In small vessels, rattlesnaking may be enhanced in magnitude by
480 the small numbers of red blood cells flowing through narrow vessels.

481
482 In this study, only the short-term repeatability of oximetry measurements was
483 assessed. In future, quantification of repeatability of measurements over the course
484 of an entire day is desirable to assess longer term variations including fluctuating
485 diurnal variation in vessel diameter and temperature of bulbar conjunctival vessels
486 (Duench et al., 2007).

487

488 **4.5. Influence of light scattering by scleral tissue**

489 Optical scattering of light by tissue may influence ODR and vessel diameter
490 measurement. We assume negligible scattering for the bulbar conjunctival
491 vasculature, which lies within a thin (~33 μ m), transparent bulbar conjunctiva (Efron
492 et al., 2009). However, episcleral vessels are embedded in scleral tissue; this will
493 affect our measurement in two ways. Firstly, the sharpness of vessel boundaries
494 may be decreased, which may produce a small increase systematic and random
495 errors in the measurement of vessel diameter for episcleral vessels. The relative
496 change in vessel diameter measured will however be relatively unaffected by
497 scattering. Secondly, scattering from overlying tissue will act to reduce contrast of
498 vessels, generally acting to reduce the changes in ODR observed. Secondly,
499 scattering from overlying tissue will act to reduce contrast of vessels, generally
500 acting to reduce the changes in ODR observed. Scattering will also be increased if

501 vessels dilate; this will reduce the apparent change in ODR of episcleral vessels
502 which were observed to dilate significantly (see Figure 6e). In the scleral phantom,
503 the FEP plastic of the capillary will contribute to scattering. The challenge of light
504 scattering by tissue and within blood and the absence of reliable SO_2 values for
505 calibration, makes absolute oximetry in bulbar conjunctival and episcleral vessels
506 challenging, however, as we describe here, changes in SO_2 can be robustly
507 characterised with ODR and can provide useful biological insight.

508

509 **4.6. Future work**

510 There are good prospects of achieving an absolute oximetry, with minimal
511 requirement for calibration by incorporating the modified Beer-Lambert law (Delpy et
512 al., 1988; Pittman and Duling, 1975) into multi-waveband optical transmission
513 models. Absolute oximetry would be of particular use because there have been no
514 reference values for SO_2 of the bulbar conjunctival or episcleral microvasculature
515 reported in the literature, so two wavelength oximetry cannot be accurately
516 calibrated.

517

518 With appropriate flash illumination, imaging at 100Hz or greater could be achieved
519 and oximetry in smaller bulbar conjunctival vessels and capillaries could be enabled
520 by adapting a slit lamp for high-magnification multispectral imaging. This could
521 enable the potential for non-contact oximetry of groups of red blood cells in humans
522 *in vivo*. Individual red blood cell oximetry has previously been achieved *ex vivo* using
523 SMSI (Fernandez Ramos et al., 2014) and invasively *in vivo* in anaesthetised mice
524 by photoacoustic microscopy (Wang et al., 2013). SMSI offers faster image
525 acquisition and a simpler image system compared to PAM.

526

527 **4.7. Vascular conditions that may affect anterior segment vessel SO_2**

528 Understanding SO_2 of bulbar conjunctival and episcleral vessels may provide insight
529 into a range of conditions. For example, diabetic retinopathy is known to result in
530 increased retinal vessel SO_2 (Hammer et al., 2009; Hardarson and Stefánsson,
531 2012), however, previous studies have shown that oxygen tension in diabetic
532 subjects is lower than in healthy controls (Isenberg et al., 1986). Further, diabetes is
533 associated with increased bulbar conjunctival vessel diameter (Cheung, Anthony T.
534 W. Ramanujam et al., 2001), capillary loss (Owen et al., 2008), and decreased
535 vessel reactivity (Fenton et al., 1979). Snapshot multispectral-imaging oximetry could
536 also provide direct *in vivo* measurement of resultant hypoxia in bulbar conjunctival
537 vasculature from contact lens wear (Heitmar et al., 2012; Sweeney, 2013).
538 Furthermore oximetry of the bulbar conjunctival vessels may be of interest in
539 studying the recovery of ocular burns using oxygen therapy (Sharifipour et al., 2011),
540 recovery of circulation after surgical or traumatic wound healing, and possibly in the
541 study of ischemic conditions such as dry-eye syndrome (Menezo and Lightman,
542 2004). High intra-ocular pressure (IOP) is associated with narrowed episcleral veins
543 and increased diameter of episcleral arteries (Nanba and Schwartz, 1986), but it is
544 not known if this may alter episcleral SO_2 .

545

546

547 **5. Conclusions**

548 This is the first study to quantify changes localised in SO_2 of bulbar conjunctival and
549 episcleral microvasculature. Oximetry was achieved using SMSI and was validated
550 using a sclera-mimicking phantom.

551

552 *In vivo*, acute mild hypoxia resulted in a repeatable reduction in SO_2 of both bulbar
553 conjunctival and episcleral microvasculature. Episcleral vessels were observed to
554 dilate due to acute mild hypoxia, whereas bulbar conjunctival vessels did not show

555 statically significant dilation under hypoxia. Hypoxic bulbar conjunctival vessels were
556 observed to rapidly reoxygenate due to oxygen diffusion when exposed to ambient
557 air. Episcleral vessels were not observed to reoxygenate due to overlying episcleral
558 tissue. This oxygen diffusion means that after exposure to air, the pO_2 of bulbar
559 conjunctival vessels will be in equilibrium with ambient air, resulting in a SO_2 close to
560 100%. SMSI is currently the only oximetry technique with sufficient spatiotemporal
561 resolution to measure this rapid oxygen diffusion in individual vessels. However we
562 have shown that the role of oxygen diffusion in the bulbar conjunctiva must be
563 considered for any future oximetry studies to provide meaningful results.

564

565 SMSI oximetry of the bulbar conjunctival and episcleral microvasculature may be of
566 interest in investigating oxygen dynamics in a variety of microvasculature conditions
567 where hypoxia may play a role, such as diabetes, (Isenberg et al., 1986; Hammer et
568 al., 2009; Hardarson and Stefánsson, 2012), sickle-cell disease (Isenberg et al.,
569 1987), dry-eye syndrome (Menezo and Lightman, 2004), contact lens wear (Heitmar
570 et al., 2012; Sweeney, 2013), high intra-ocular pressure (Nanba and Schwartz,
571 1986), traumatic or surgical wound healing, and ocular-burn recovery (Sharifipour et
572 al., 2011). Further, high-magnification MSI of the bulbar conjunctiva could enable
573 non-invasive *in vivo* oximetry of individual red blood cells.

574

575

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741

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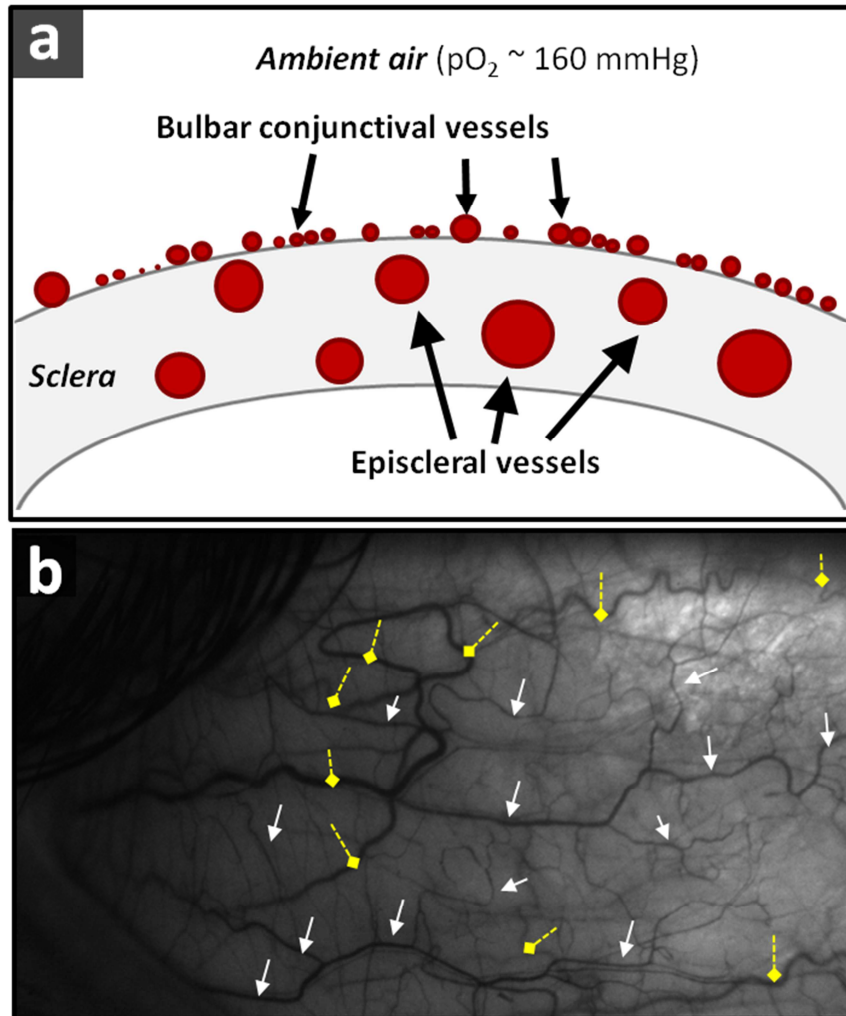
745 A.R. Harvey, None.

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747 **Figure 1 (a)** Simplified diagram showing position of bulbar conjunctival and episcleral
748 vasculature with respect to the sclera and ambient air. **(b)** Representative image of
749 vasculature observed when imaging the sclera. Bulbar conjunctival vessels are marked with
750 white arrows and episcleral vessels are marked with yellow dashed diamond arrows.



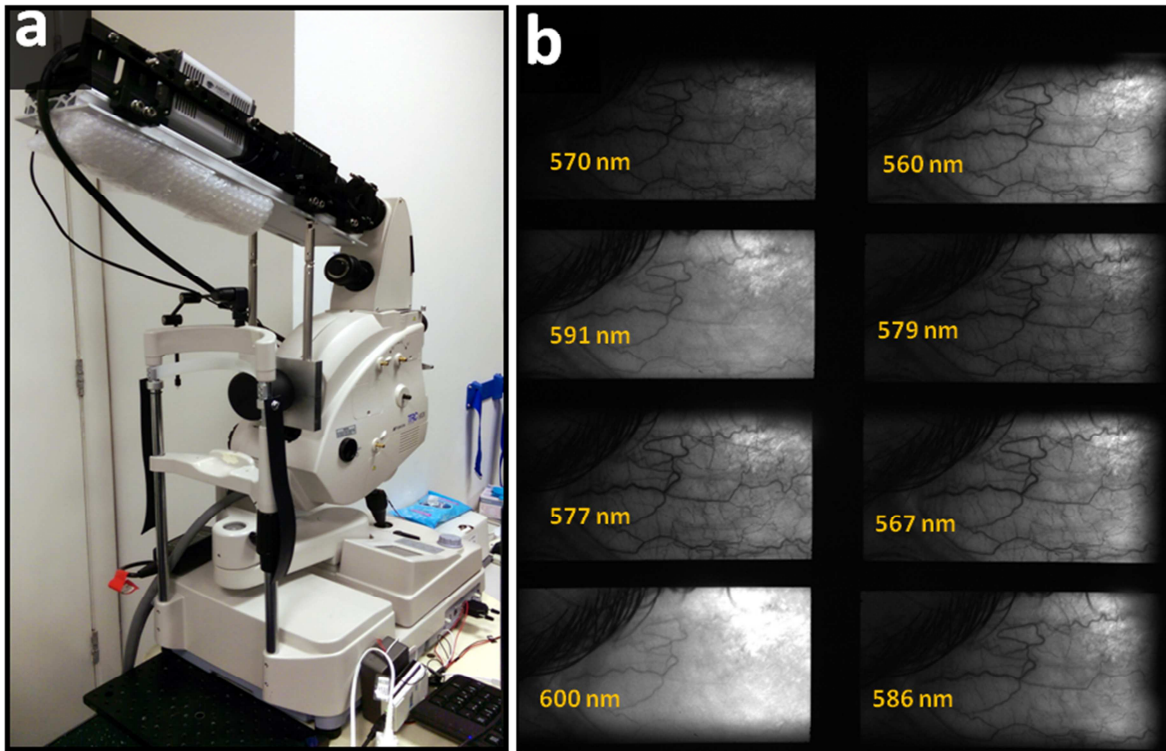
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766 **Figure 2 (a)** The imaging system: a commercial fundus camera with the Image Replicating

767 **Imaging Spectrometer (IRIS) fitted to the upper imaging port. (b)** A representative 8-band

768 IRIS image of bulbar conjunctival and episcleral vasculature.



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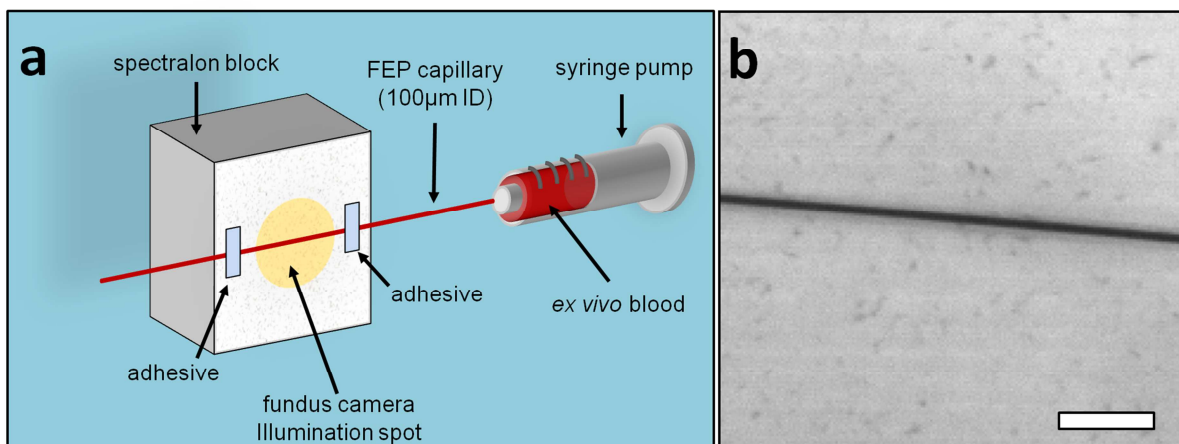
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774 **Figure 3. (a)** diagram of the scleral phantom. **(b)** 100µm capillary filled with blood;

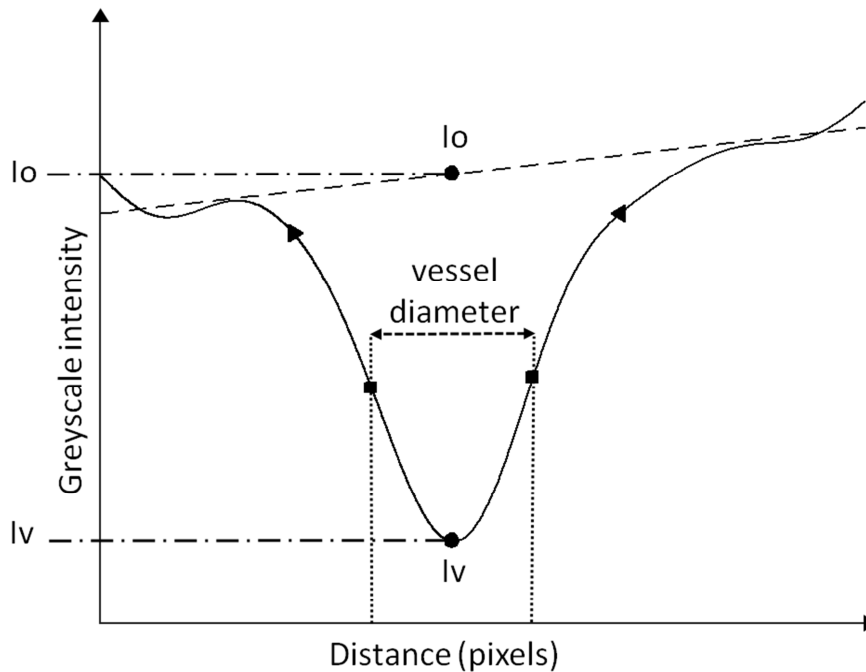
775 scale bar represents one millimetre.



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777

779 **Figure 4.** Depiction of the vessel fitting algorithm applied to estimate vessel
 780 diameter, the greyscale intensity in centre of vessel (I_v), and the background
 781 greyscale intensity (I_o). Vessel boundaries are defined as the points of maximum rate
 782 of change of greyscale intensity in the vessel profile.



783

784 **Table 1.** Repeatability of optical-density ratio (ODR) measurements for conjunctival
 785 and episcleral vessels.

Parameter	Bulbar Conjunctival vessels	Episcleral vessels
Number of subjects	10	7
Total number of sampled vessel sections	57	22
ODR repeatability: individual vessels*	2.27%	2.28%
ODR repeatability**	0.96%	1.55%

*standard deviation of 8 repeated measurements of individual vessels, averaged across all subjects

** standard deviation of the average ODR of vessels when averaged by vessel type, then averaged across all subjects

786

787 **Table 2.** Average optical-density ratio (ODR), diameter of vessels, and pulse

788 oximeter data at normoxia and hypoxia.

Parameter	Number of subjects	Number of vessel sections analysed	Normoxia	Hypoxia	p-value*
Conjunctival ODR (mean ± SE)	10	64	0.846 ± 0.014	0.916 ± 0.011	<0.001
Episcleral ODR (mean ± SE)	7	24	0.880 ± 0.019	0.938 ± 0.018	0.03
Conjunctival diameter (µm) (mean ± SD)	10	64	80.1 ± 7.6	80.6 ± 7.0	0.89
Episcleral diameter (µm) (mean ± SD)	7	24	78.9 ± 8.7	97.6 ± 14.3	0.02
Fingertip pulse oximeter SO₂ (%) (mean ± SD)	10	N/A	97.1 ± 1.7	86.7 ± 4.3	<0.001

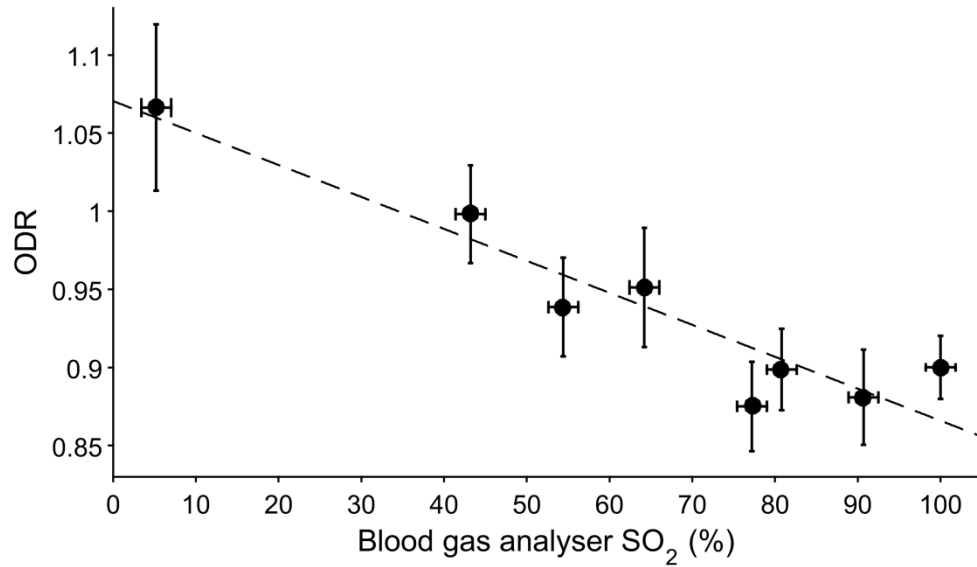
789 *Pairwise t-test

790 SE = standard error

791 SD = standard deviation

792

793 **Figure 5.** Phantom validation; optical-density ratio (ODR) was measured to be
794 inversely proportional to SO_2 as measured by a blood gas analyser (BGA). Vertical
795 error bars represent standard deviation of ODR as measured along the length of the
796 FEP capillary, horizontal error bars represent the blood gas analyser manufacturers
797 quoted error of $\pm 1.8\% \text{SO}_2$. Dashed line is fitted linear trend ($R^2 = 0.89$).

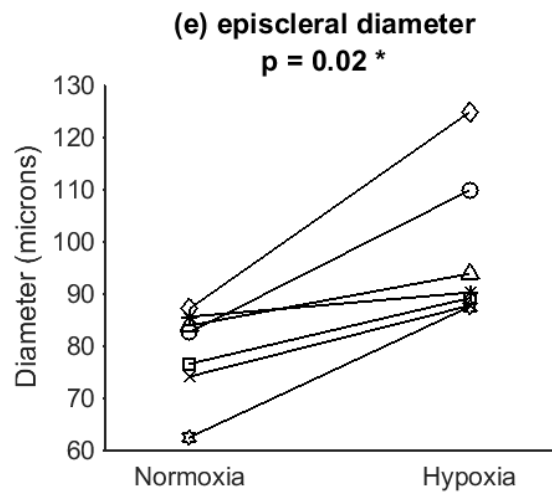
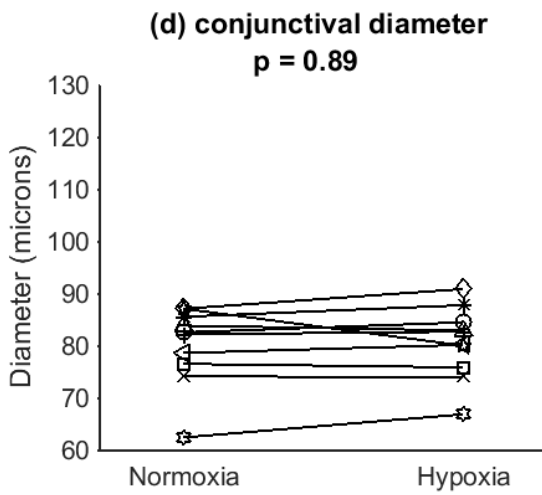
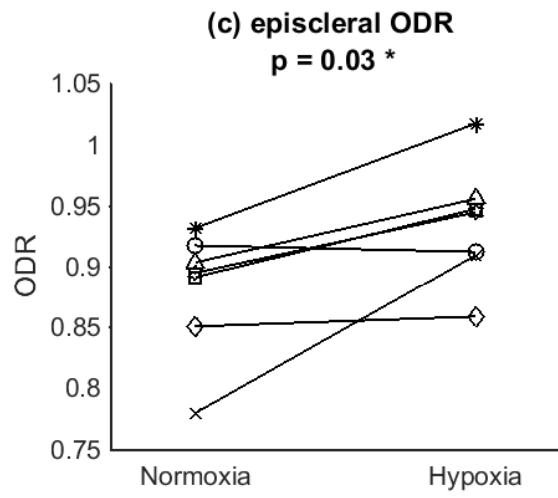
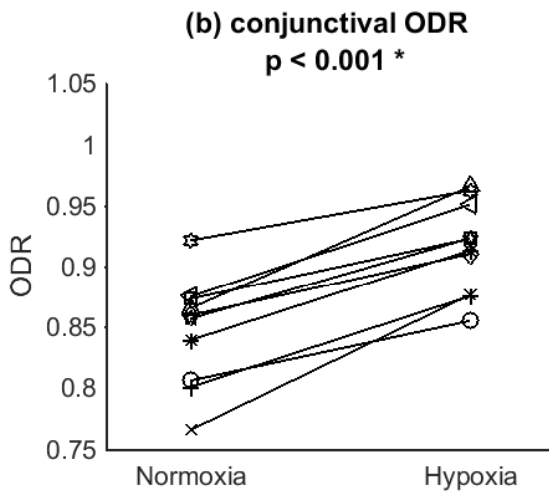
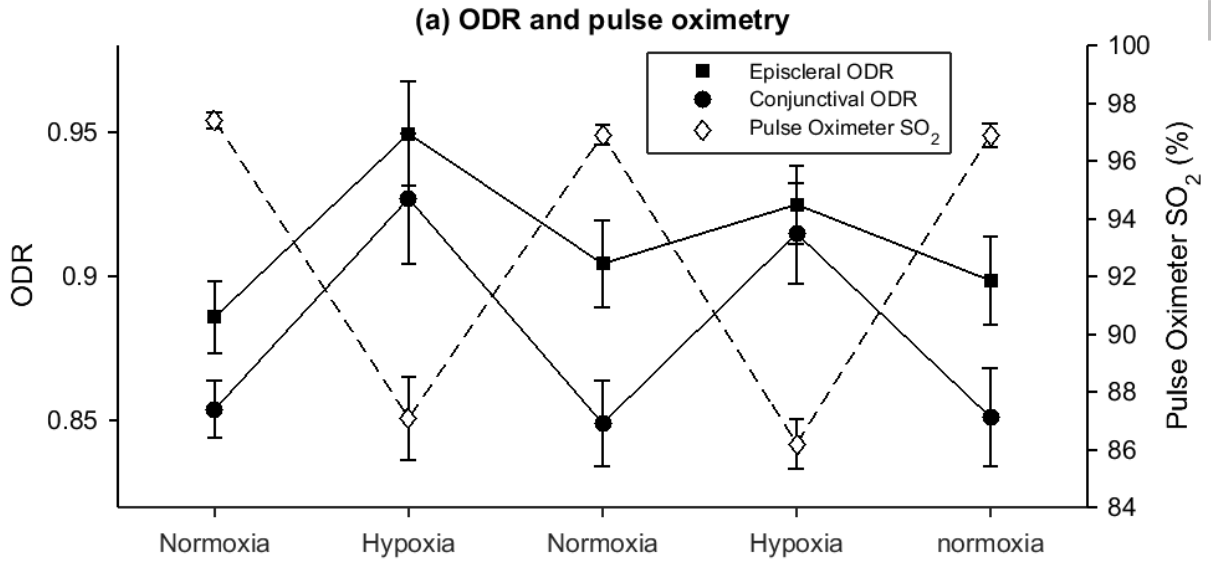


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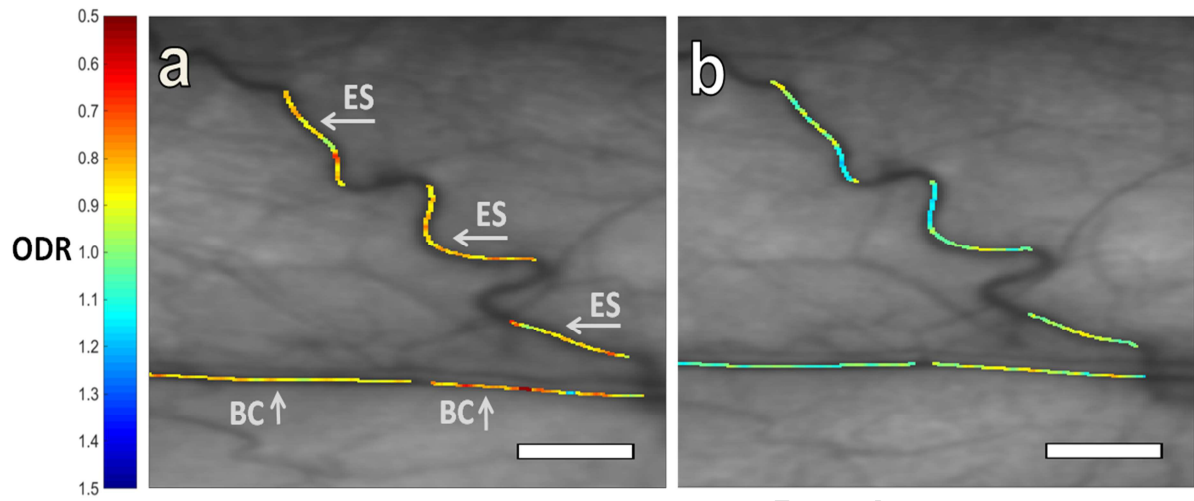
799

800 **Figure 6. (a)** Average optical-density ratio (ODR) and pulse oximeter data
801 throughout the normoxia/hypoxia sequence. Error bars are the standard error of the
802 mean. Graphs **(b)-(e)** show pairwise change of average vessel diameter and
803 average ODR for each subject at normoxia and hypoxia. Statistically significant
804 results are denoted with an asterisk (*).

805



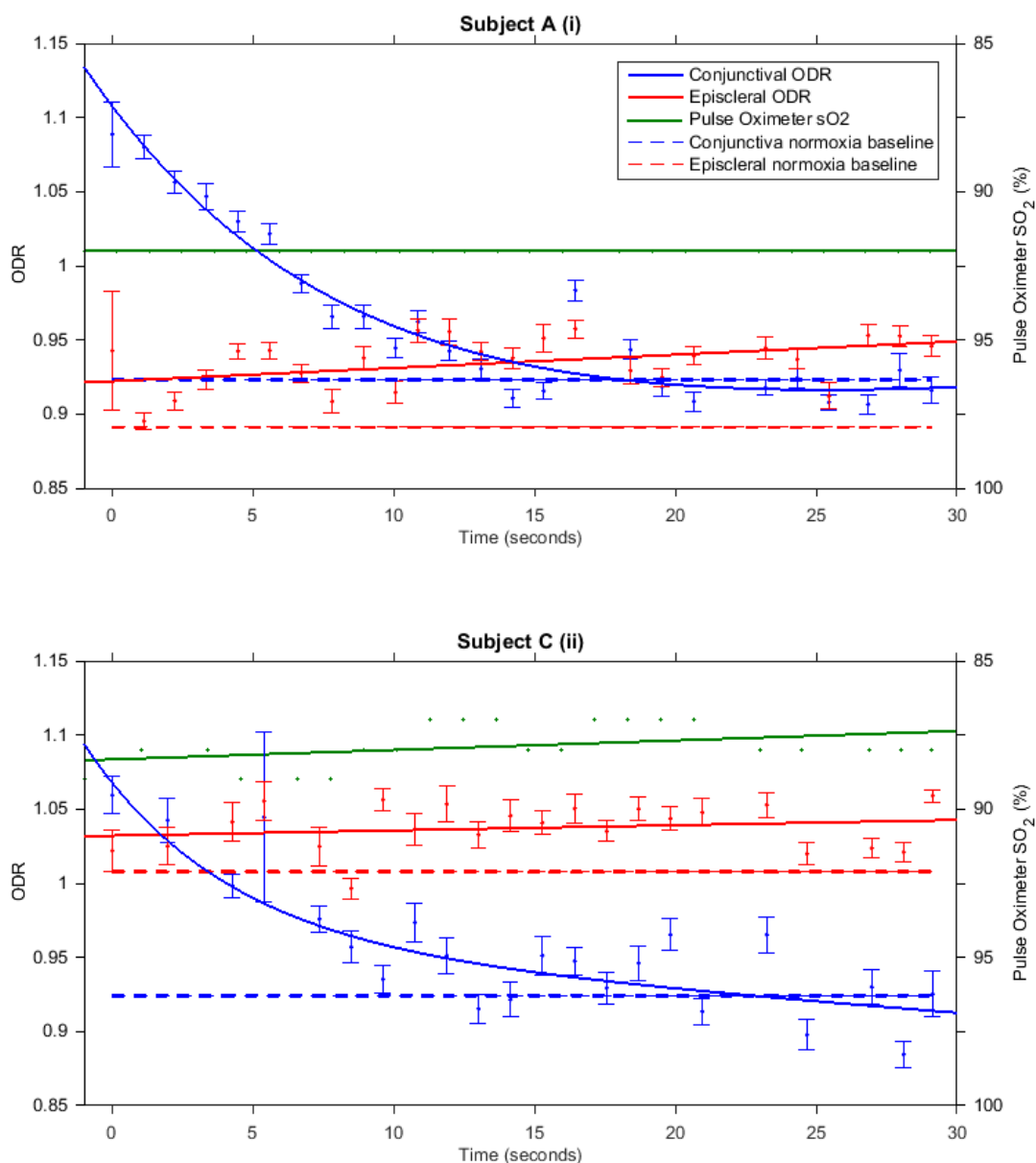
809 **Figure 7.** Optical-density ratio (ODR) map of vasculature at **(a)** normoxia and **(b)**
810 hypoxia. ODR increases (i.e. SO_2 decreases) with hypoxia. Episcleral vessels are
811 labelled with (ES) and bulbar conjunctival vessels are labelled (BC). Scale bar
812 represents 500 μm .



813

814

816 **Figure 8.** Optical-density ratio (ODR) of hypoxic vasculature versus time after eyelid
 817 opening (i.e. exposure to ambient air) in two representative subjects. Bulbar
 818 conjunctival ODR (blue fitted line) decreased exponentially upon eyelid opening
 819 before reaching normoxia baseline levels (blue dashed line). Episcleral ODR (red
 820 fitted line) remained higher than normoxia levels (red dashed line). This indicates
 821 that hypoxic bulbar conjunctival vessels rapidly reoxygenated by oxygen diffusion
 822 when exposed to ambient air whereas hypoxic episcleral vessels (embedded in
 823 episcleral tissue) did not reoxygenate. Error bars represent the standard error of the
 824 mean. The green fitted line is pulse oximeter data ($\pm 2\%$ SO_2 uncertainty quoted by
 825 the manufacturer not depicted for clarity).



828 **Table 3.** Calculated values of '1/2 time to reoxygenation' ($T_{1/2}$) for 4 subjects,

829 repeated twice per subject.

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Subject	Data set	$T_{1/2}$ (seconds)
A	(i)	6.6
	(ii)	4.1
B	(i)	3.0
	(ii)	2.9
C	(i)	2.1
	(ii)	3.4
D	(i)	2.2
	(ii)	3.2
Average		3.4
Standard Deviation		1.4

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Highlights

- First non-invasive multispectral imaging oximetry study of bulbar conjunctival and episcleral microvasculature.
- Rapid oxygen diffusion occurs when hypoxic bulbar conjunctival vessels are exposed to air (i.e. when the eyelid is open).
- Bulbar conjunctival vessels be highly saturated with oxygen (i.e. close to 100% SO_2) when exposed to air.
- Potential applications include studies of hypoxia due to contact lens wear, diabetes, sickle-cell disease, ocular burn recovery, intra-ocular pressure, and post-surgical wound healing.