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

4  
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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 SO<sub>2</sub>  
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% SO<sub>2</sub>) 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 ([Boeckaert 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  $\text{SO}_2$ -dependent optical absorption spectra of  
107 haemoglobin. Changes in  $\text{SO}_2$  can be calculated by imaging blood vessels at two  
108 wavelengths: one wavelength where optical absorption is sensitive to variations in  
109  $\text{SO}_2$ , and at another wavelength which is insensitive to  $\text{SO}_2$  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  $\text{SO}_2$ . In vessels where  $\text{SO}_2$  is known, ODR can then be empirically  
113 calibrated to  $\text{SO}_2$  by assuming local arterial  $\text{SO}_2$  is equal to the  $\text{SO}_2$  of systemic  
114 arterial  $\text{SO}_2$  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  $\text{SO}_2$ , such as conjunctival or episcleral inflammation. Direct  
123 measurement of the partial pressure of oxygen ( $\text{pO}_2$ ) 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.

129

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  $25 \pm 2$  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). SO<sub>2</sub> 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). SO<sub>2</sub> 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 SO<sub>2</sub>  
198 samples ranging between 5% and 100% SO<sub>2</sub> 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<sub>2</sub> 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% FiO<sub>2</sub>) 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% FiO<sub>2</sub>) 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  $\pm 1$  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 SO<sub>2</sub> 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<sub>2</sub> (isobestic) and one waveband where  
325 optical absorption is sensitive to changes in SO<sub>2</sub> (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 ~60-100µm 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.

369

### 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  $0.90 \pm 0.08$  (mean  $\pm$  standard  
374 deviation) for bulbar conjunctival vessels and  $0.94 \pm 0.09$  for episcleral vessels. After  
375 eyelid closure, average ODR was  $0.90 \pm 0.08$  for bulbar conjunctival vessels and  
376  $0.93 \pm 0.08$  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  $0.916 \pm 0.011$  at hypoxia ( $p < 0.001$ ,  
384 paired t-test) (Figure 6b). Episcleral ODR increased on average, from  $0.881 \pm 0.019$   
385 (mean  $\pm$  standard error) at normoxia to  $0.938 \pm 0.018$  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  $3.4 \pm 1.4$  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<sub>2</sub>.

424

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

426 In episcleral vessels, vessel diameter increased and SO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>.

444

445 It is expected that when in equilibrium with ambient air (pO<sub>2</sub> ~160mmHg), bulbar  
446 conjunctival vessels will be close to 100% SO<sub>2</sub> because normal arterial blood (~95-  
447 97% SO<sub>2</sub>) corresponds to a typical pO<sub>2</sub> 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 ( $\sim 33\mu\text{m}$ ), 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<sub>2</sub> of bulbar  
559 conjunctival vessels will be in equilibrium with ambient air, resulting in a SO<sub>2</sub> 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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## 742 **Acknowledgements**

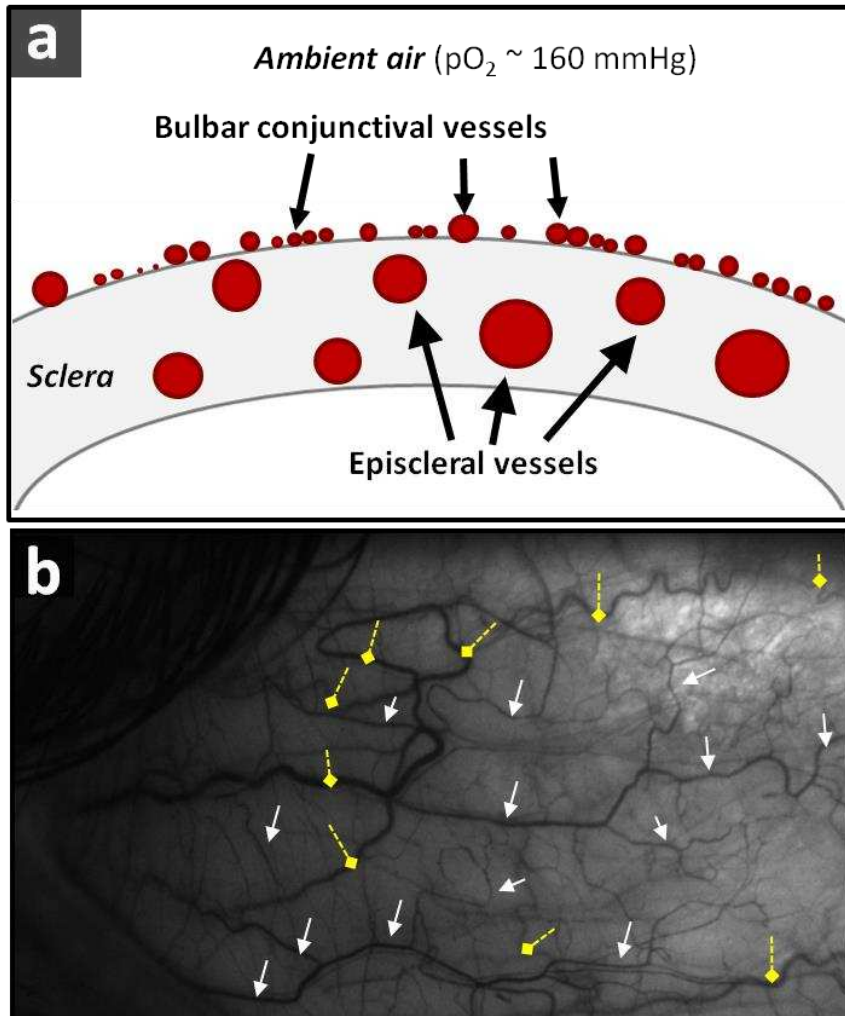
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746

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.



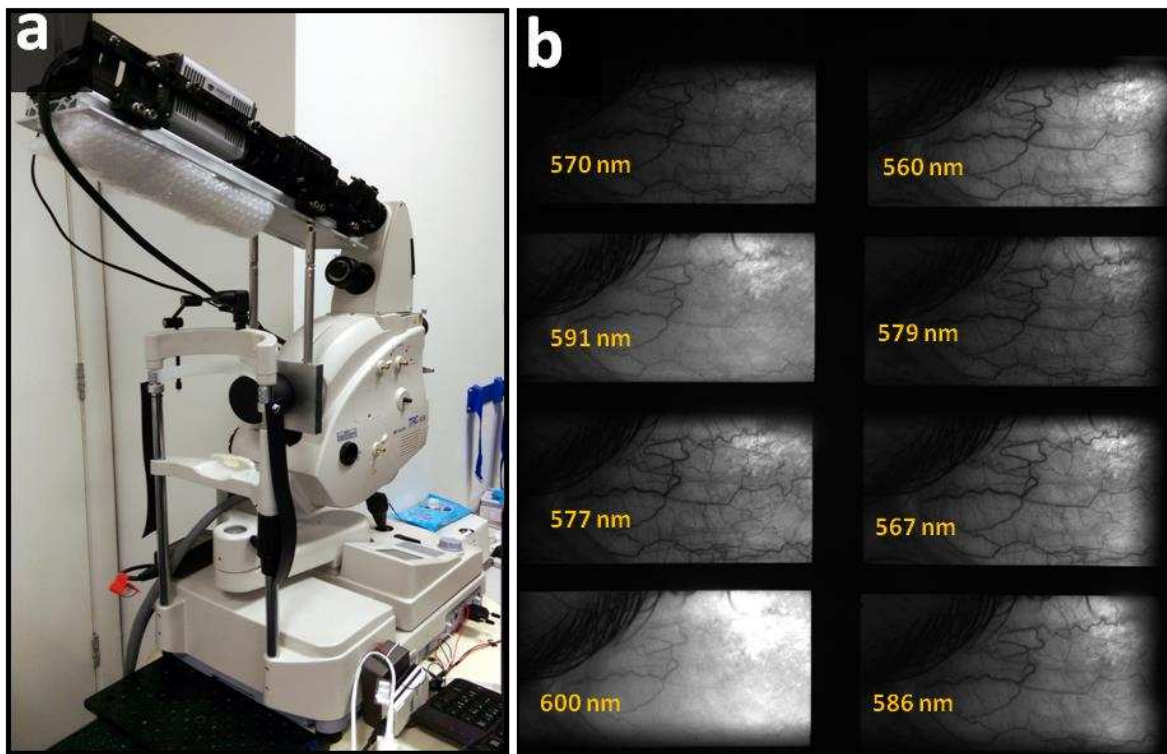
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766 **Figure 2 (a)** The imaging system: a commercial fundus camera with the Image Replicating

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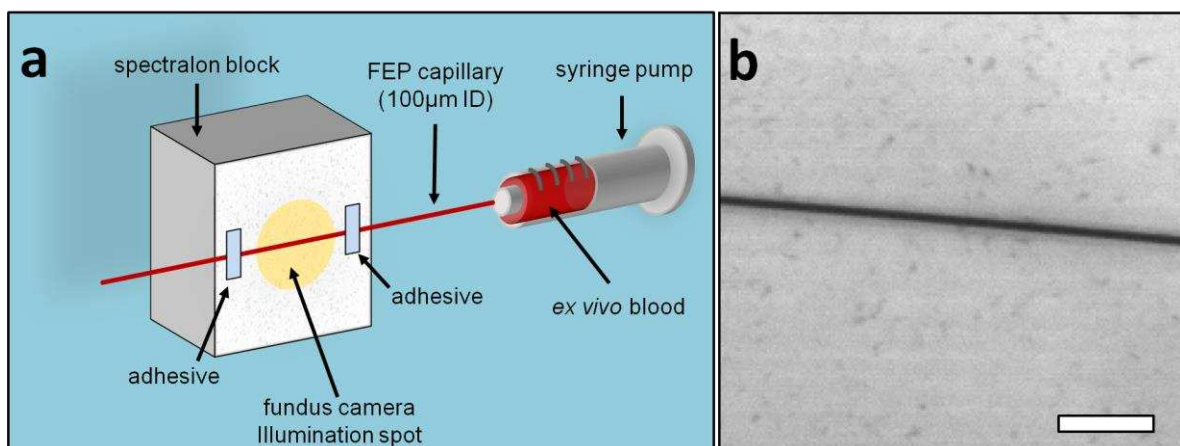
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774 **Figure 3. (a)** diagram of the scleral phantom. **(b)** 100µm capillary filled with blood;

775 scale bar represents one millimetre.

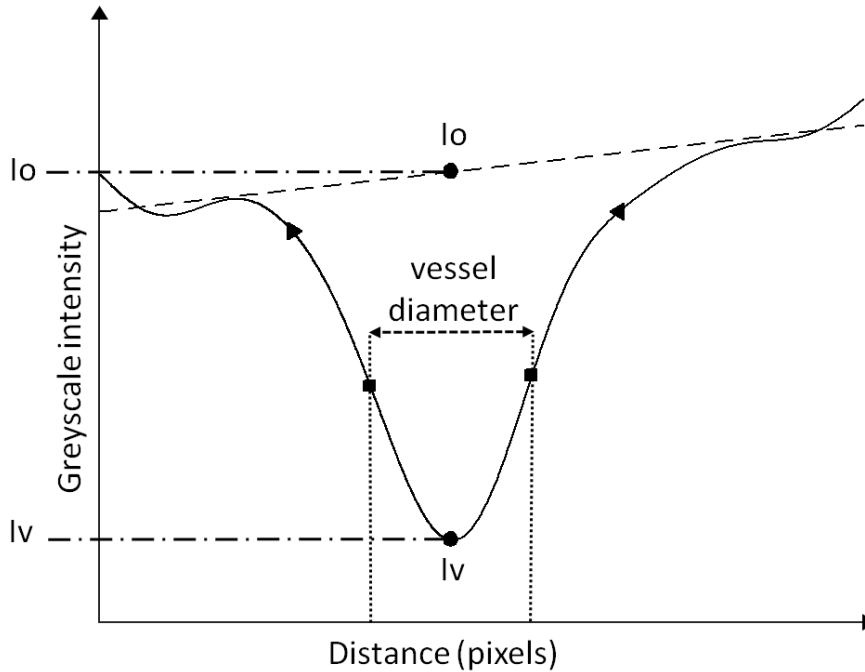


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779 **Figure 4.** Depiction of the vessel fitting algorithm applied to estimate vessel  
 780 diameter, the greyscale intensity in centre of vessel ( $I_v$ ), and the background  
 781 greyscale intensity ( $I_o$ ). Vessel boundaries are defined as 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*
<b>Conjunctival ODR</b> (mean ± SE)	10	64	0.846 ± 0.014	0.916 ± 0.011	<0.001
<b>Episcleral ODR</b> (mean ± SE)	7	24	0.880 ± 0.019	0.938 ± 0.018	0.03
<b>Conjunctival diameter (µm)</b> (mean ± SD)	10	64	80.1 ± 7.6	80.6 ± 7.0	0.89
<b>Episcleral diameter (µm)</b> (mean ± SD)	7	24	78.9 ± 8.7	97.6 ± 14.3	0.02
<b>Fingertip pulse oximeter SO<sub>2</sub> (%)</b> (mean ± SD)	10	N/A	97.1 ± 1.7	86.7 ± 4.3	<0.001

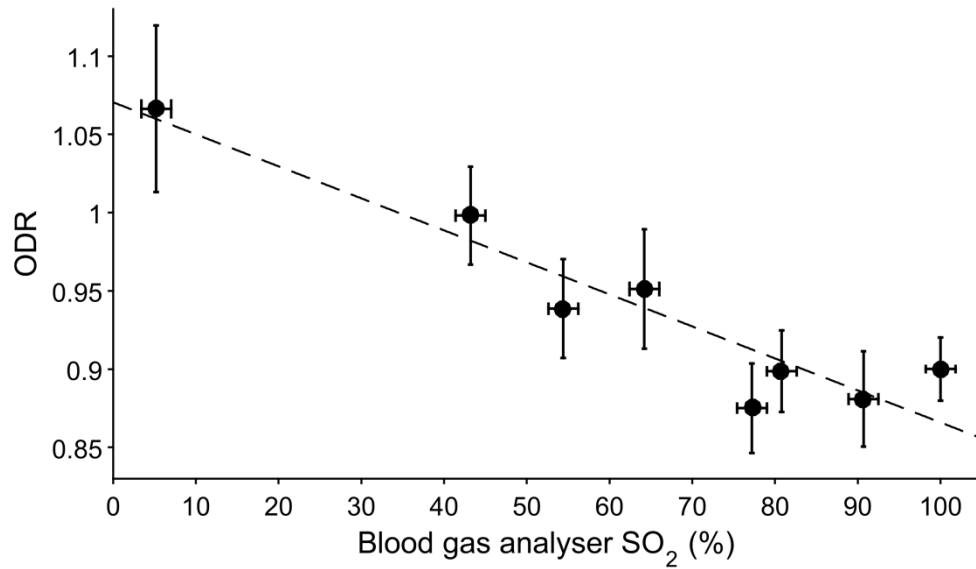
789 \*Pairwise t-test

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  $\text{SO}_2$  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 ( $R^2 = 0.89$ ).

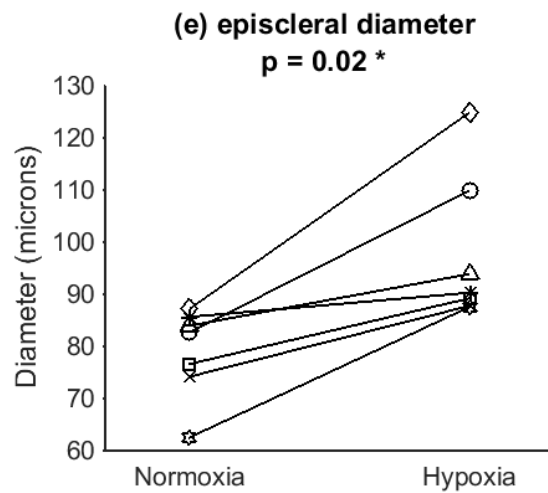
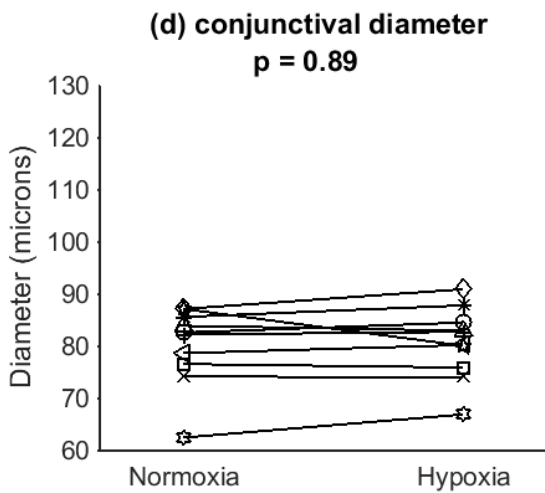
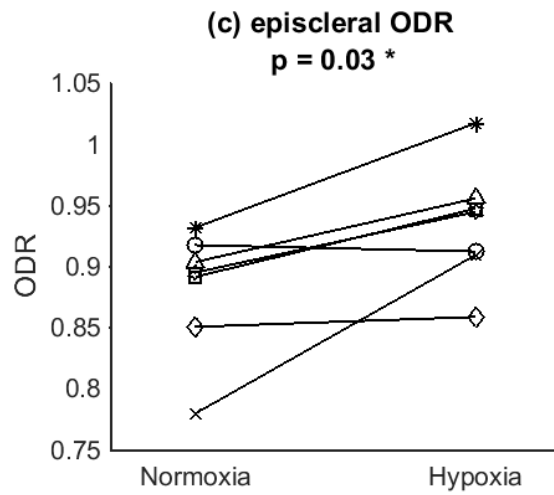
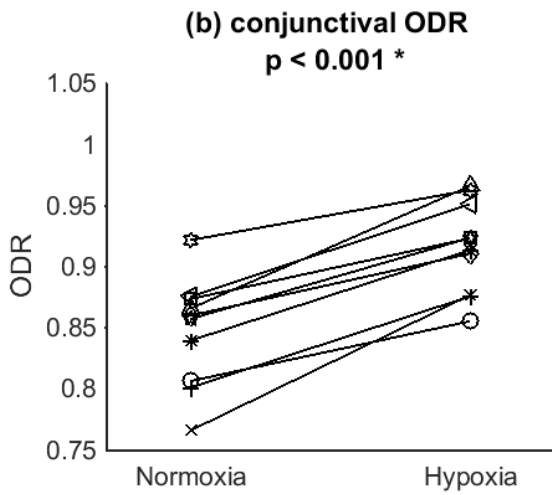
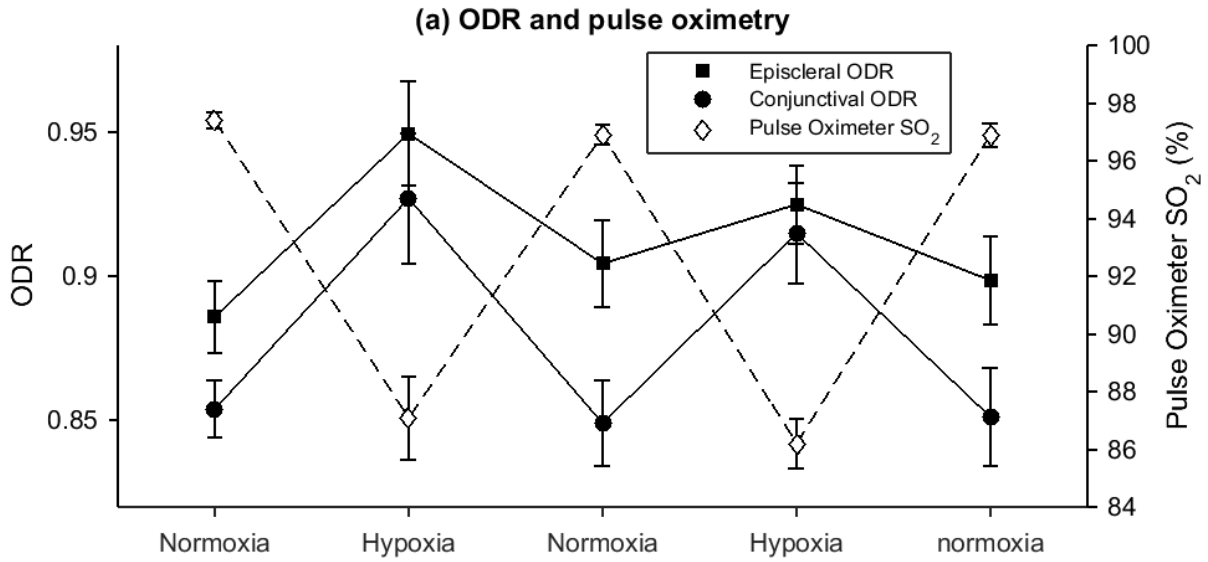


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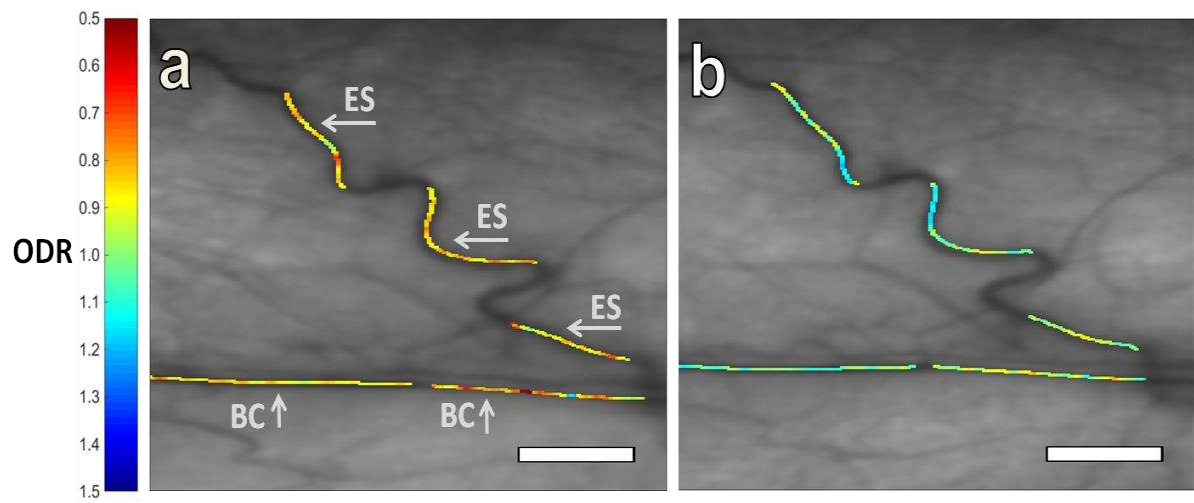
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800 **Figure 6. (a)** Average optical-density ratio (ODR) and pulse oximeter data  
801 throughout the normoxia/hypoxia sequence. Error bars are the standard error of the  
802 mean. Graphs **(b)-(e)** show pairwise change of average vessel diameter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

805



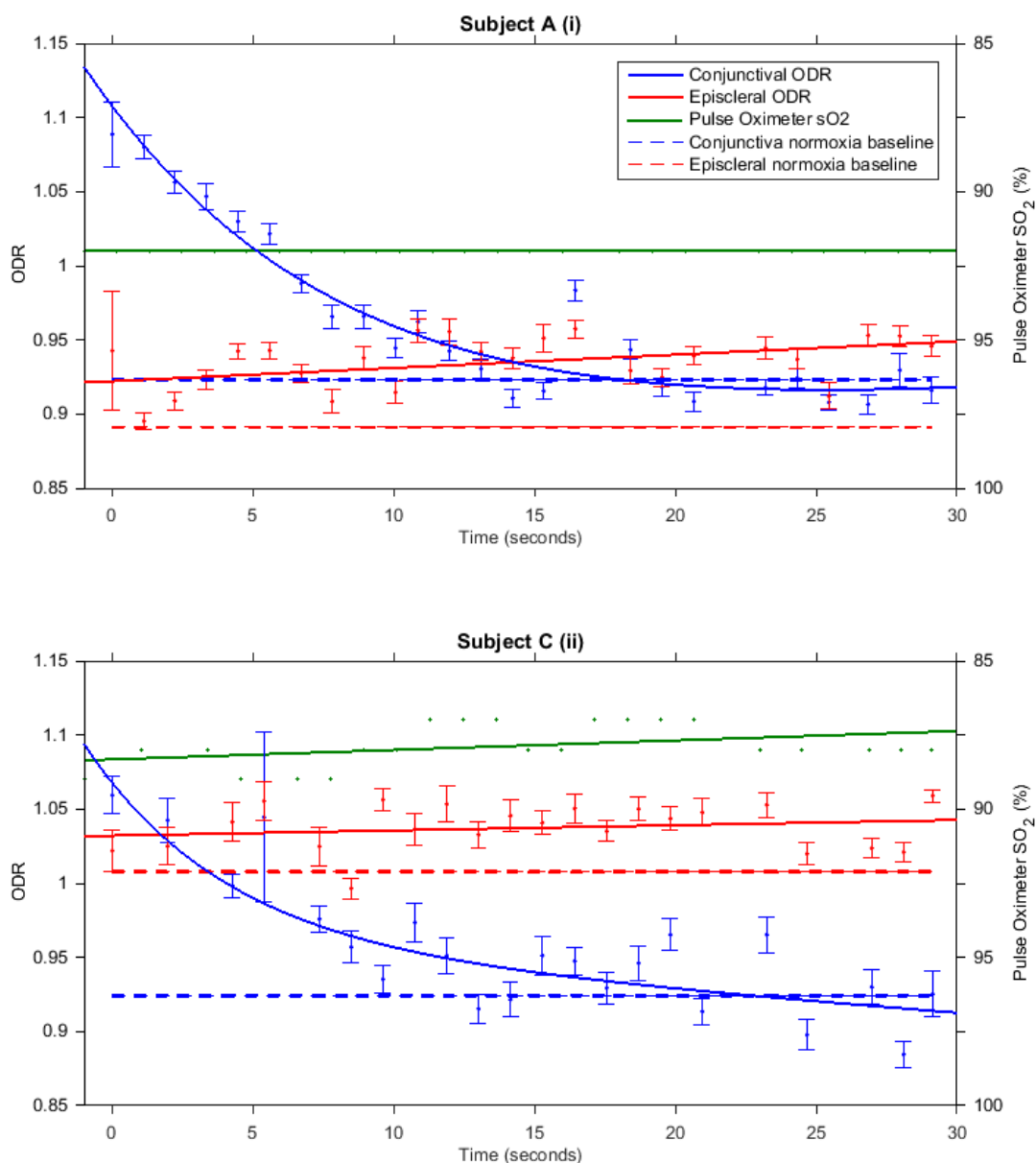
809 **Figure 7.** Optical-density ratio (ODR) map of vasculature at **(a)** normoxia and **(b)**  
810 hypoxia. ODR increases (i.e.  $\text{SO}_2$  decreases) with hypoxia. Episcleral vessels are  
811 labelled with (ES) and bulbar conjunctival vessels are labelled (BC). Scale bar  
812 represents 500  $\mu\text{m}$ .



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.

830

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

831