Homogenization of capillary flow and oxygenation in deeper cortical layers correlates with increased oxygen extraction

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ABSTRACT

Our understanding of how capillary blood flow and oxygen distribute across cortical layers to meet the local metabolic demand is incomplete. We addressed this question by using two-photon imaging of microvascular oxygen partial pressure (PO_2) and flow in the whisker barrel cortex in awake mice at rest. Our measurements in layers I-V show that the capillary red-blood-cell flux and oxygenation heterogeneity, and the intracapillary resistance to oxygen delivery, all decrease with depth, reaching a minimum around layer IV, while the depth-dependent oxygen extraction fraction is increased in layer IV, where oxygen demand is presumably the highest. Our findings suggest that homogenization of physiological observables relevant to oxygen transport to tissue is an important part of the microvascular network adaptation to a local brain metabolism increase. These results will inform the biophysical models of layer-specific cerebral oxygen delivery and consumption and improve our understanding of diseases that affect the cerebral microcirculation.

KEYWORDS: cerebral cortex, capillary oxygenation, capillary blood flow, two-photon phosphorescence lifetime microscopy, partial pressure of oxygen

IMPACT STATEMENT

Homogenization of cortical capillary blood flow and oxygenation underpins an important mechanism, by which the microvascular network adapts to an increase in the local brain oxidative metabolism.

INTRODUCTION

Normal brain functioning is critically dependent on the adequate and uninterrupted supply of oxygen to brain tissue (Attwell and Laughlin, 2001; Raichle and Gusnard, 2002; Raichle et al., 2001). Significant efforts have been made over the years to investigate the regulation of cerebral blood flow (CBF), including auto-regulation of CBF in response to the changes in cerebral perfusion pressure (Aaslid et al., 1989; Ob et al., 1990) and the processes underlying neurovascular coupling (Anenberg et al., 2015; Cai et al., 2018; Girouard and Iadecola, 2006; Iordanova et al., 2015; Vazquez et al., 2010). Furthermore, neuronal and microvascular densities vary greatly between cortical layers (Blinder et al., 2013), suggesting a laminar variation of tissue metabolism (De Kock C. P. J. et al., 2007; Hyder et al., 2013). However, it is still not well understood *how blood flow and oxygenation are distributed through the microvascular network to support an adequate tissue oxygenation across the closely spaced, but morphologically and metabolically heterogeneous cortical layers*. Answering this question is important to improve our understanding of the normal brain physiology as well as brain diseases that affect cerebral microcirculation (Berthiaume et al., 2018; Iadecola, 2016, 2017; Pantoni, 2010; Zlokovic, 2011).

The recent development of tools for *in vivo* microvascular oxygen imaging enabled investigation of brain oxygen delivery and consumption within arteriolar, venular and capillary domains over large tissue volumes (Cao et al., 2017; Chong et al., 2015a; Hu et al., 2009; Lecoq et al., 2011; Parpaleix et al., 2013; Sakadžić et al., 2010, 2015; Vanderkooi et al., 1987; Wang et al., 2011; Yaseen et al., 2009). Distributions of microvascular blood flow and oxygen in mice at rest have been assessed in several studies using optical coherence tomography, two-photon microscopy and photoacoustic imaging (Cao et al., 2017; Chong et al., 2015b; Gutiérrez-Jiménez et al., 2018; Lecoq et al., 2011; Lyons et al., 2016; Moeini et al., 2018; Parpaleix et al., 2013; Sakadžić et al., 2011, 2014; Santisakultarm et al., 2012, 2014; Srinivasan et al., 2015). However, in most previous studies these variables have not been considered as a function of cortical depth and/or capillary branching order. Instead, measurements of intravascular oxygen concentrations were performed at limited cortical depths and typically under anesthesia, which could have significantly affected both brain CBF and metabolism (Alkire et al., 1999; Goldberg et al., 1966), or using samples of limited size that might

prevent detailed depth-dependent mapping of microvascular blood flow and oxygen with sufficient statistical power (Lecoq et al., 2011; Lyons et al., 2016; Parpaleix et al., 2013).

To this end, in the present work we applied two-photon phosphorescence lifetime microscopy to measure the absolute intravascular partial pressure of oxygen (PO_2) in a large number of arterioles, venules and capillaries, as well as the erythrocyte-associated transients of capillary PO_2 (EATs), capillary red blood cell (RBC) flux and microvascular structure as a function of cortical depth within the range of 0-600 μm . We used a new phosphorescent oxygen probe, based on a recently developed Pt tetraarylphthalimidoporphyrin (PtTAPIP) (Esipova et al., 2017). Compared to its predecessor PtP-C343 (Finikova et al., 2008), the new probe exhibits longer excitation and emission wavelength maxima, higher quantum yield and a larger two-photon absorption cross-section, facilitating simultaneous mapping of microvascular PO_2 and capillary RBC flux in cortical layers I-V in mice and making it possible to significantly extend both the sample size and imaging depth in comparison to the previous studies (Lecoq et al., 2011; Lyons et al., 2016; Parpaleix et al., 2013; Sakadžić et al., 2010, 2014). Moreover, our measurements were performed in head-restrained awake mice, and thus were free of the confounding effects of anesthesia on neuronal activity, CBF and brain metabolism. We found that in the whisker barrel cortex in awake mice the oxygen extraction fraction (OEF), measured at different cortical depths (i.e. depth-dependent OEF), reached its maximum at the depths of 320-450 μm , corresponding to cortical layer IV, where the neuronal and capillary densities are the largest, and, presumably, oxygen consumption is the highest. The increased OEF was accompanied by homogenization of the capillary PO_2 and RBC flux, as well as by a decrease in the intracapillary resistance to oxygen delivery (inferred from the EATs amplitude). These experimental results enabled quantification of parameters of importance for oxygen transport to tissue and put forward a potential mechanism, by which the microvascular networks at rest may adapt to the heterogeneous metabolic demands in different cortical layers. We anticipate that our results will improve our understanding of the normal brain function as well as of diseases that affect the cerebral microcirculation (Girouard and Iadecola, 2006; Iadecola, 2016; Müller et al., 2017; Pantoni, 2010; Wardlaw et al., 2013; Zlokovic, 2011). In addition, detailed knowledge of the distributions of capillary RBC flux and oxygenation

at different depths should inform the next generation of biophysical models of the layer-specific oxygen delivery and consumption (Gagnon et al., 2016; Mintun et al., 2001; Secomb et al., 2000).

RESULTS

Accumulated oxygen extraction fraction increases in deeper cortical layers

Figure 1. Experimental setup and data acquisition protocol. **a.** Schematic of our home-built two-photon microscope. The components are abbreviated as: mirror (M), electro-optic modulator (EOM), shutter (SH), galvo mirrors (GM), scan lens (SL), tube lens (TL), dichroic mirror (DM), objective lens (OL), and photomultiplier tube (PMT). **b.** Illustrative example of the phosphorescence decays for PO_2 recording at a single location. Each 300- μ s-long cycle includes a 10- μ s-long EOM-gated excitation, followed by a 290- μ s-long detection of phosphorescence decay. **c.** A representative phosphorescence intensity time course during PO_2 recording at a single location (blue curve). Each point in the time course represents the sum of the photon counts acquired during one 300- μ s-long excitation/decay

cycle in **b**. The red curve represents the binary segmented time course, with valleys and peaks representing RBC and blood plasma passages through the focal volume, respectively. **d**. An image of the brain surface vasculature, taken through the chronic cranial window using a CCD camera. **e**. The 3D representation of a Sulforhodamine-B labeled cortical microvasculature imaged over the region of interest outlined by the red dashed square in **d**. **f**. PO₂ measurements inside the microvascular segments at the imaging plane outlined by the orange dashed line in **e**. PO₂ values (in mmHg, color-coded) were spatially co-registered with the microvascular angiogram. **g**. Composite image shows the top view of the 3D projection of the PO₂ distribution in the microvascular network. The color bar serves for panels **f** and **g**. Scale bars: 200 μ m.

We used a home-built two-photon microscope (Sakadžić et al., 2010; Yaseen et al., 2015) (Fig. 1a) to measure the resting intravascular PO₂ in the whisker barrel cortex in head-restrained awake mice through a chronic cranial window. PO₂ imaging was performed within a 500×500 μ m² field of view (FOV) down to 600 μ m below the cortical surface. At each imaging depth, we selected the scan points for measuring PO₂ inside the microvascular segments (one point per segment), including arterioles, venules and capillaries (Fig. 1f). Point-based acquisition of PO₂ was conducted plane-by-plane from the cortical surface down to the cortical depth of 600 μ m with inter-plane separation $\leq$ 50 μ m. The intravascular Mean-PO₂ was measured in all diving arterioles, venules and in the majority of branching arterioles, venules and capillaries (6,544 vascular segments across n = 15 mice) within the FOV. In addition, capillary RBC flux, speed and hematocrit, as well as RBC-PO₂, InterRBC-PO₂, and EATs (please see the METHODS section for the details) were calculated in a large subset of capillary segments (978 capillaries across n = 15 mice). In each mouse, the measurements were grouped by the cortical layer and then averaged. Subsequently, the average measurements for each cortical layer were averaged over animals (please see the METHODS section for the details). Histograms of capillary Mean-PO₂, RBC flux, hematocrit and speed are presented in Supplementary Fig. 2.

Figure 2. Cortical layer-dependent distributions of the arteriolar and venular intravascular PO₂ and SO₂. **a.** Intravascular PO₂ in the diving arterioles (red symbols) and venules (blue symbols) across cortical layers I-V (11 arterioles, 14 venules, from n = 7 mice). **b.** SO₂ in the diving arterioles (red symbols) and venules (blue symbols) across cortical layers I-V, and the depth-dependent OEF (DOEF, black symbols). For each diving arteriole or surfacing venule in **a** and **b**, PO₂ was tracked from the cortical surface down to the cortical depth of 600 μm. Data are expressed as mean±SEM.

The average PO₂ values in the diving arterioles and surfacing venules across cortical layers I-V are shown in Fig. 2a. The PO₂ in the diving arterioles decreased from 99±4 mmHg in layer I to 84±3 mmHg in layer V; while the PO₂ in the surfacing venules exhibited a small increase starting from 43±3 mmHg in layer V to 49±4 mmHg in layer I. Similar trends were observed in the SO₂ values (Fig. 2b), but the amplitudes of SO₂ changes were different from those of PO₂ due to the sigmoidal shape of the oxygen-hemoglobin dissociation curve (Uchida et al., 1998). The SO₂ in the diving arterioles decreased slightly from 90.9±0.8 % in layer I to 87.1±1.0 % in layer V ($\Delta\text{SO}_{2,A} = 3.8 \pm 1.2 \%$), while the SO₂ change in the surfacing venules was larger, from 53.5±1.0 % in layer V to 61.8±0.8 % in layer I ($\Delta\text{SO}_{2,V} = 8.3 \pm 1.1 \%$). Consequently, the layer-specific difference between the SO₂ in the diving arterioles and that in the surfacing venules increased towards deeper layers, and the depth-dependent OEFs (DOEF) in layers IV (39.9±3.2 %) and V (38.7±3.3 %) were larger than in more superficial layers, e.g. layers I (32.0±2.7 %) and II/III (33.3±2.7 %; Fig. 2b).

The observed increase in the DOEF with cortical depth suggests that surfacing venules received more oxygenated blood in the upper cortical layers than in the deeper layers, and that SO₂ in the pre-venular

capillaries (or “downstream capillaries”) was higher for the capillaries joining venules in the upper cortical layers than for those in the deeper layers. We revisit this observation in the later section.

Capillary RBC flux and oxygenation homogenize in deeper cortical layers

Figure 3. Spatial variations of capillary RBC flux, speed, and Mean-PO₂ as a function of cortical depth. The panels a-c, d-f, and g-i show the dependence of the absolute values, standard deviations (STDs), and coefficients of variance (CVs) of capillary RBC flux, speed, and Mean-PO₂ on cortical layer, respectively. The absolute values, STDs, and CVs were calculated across capillaries. The analysis in a-i was made with 400, 356, 118, and 104 capillaries measured in cortical layers I, II/III, IV and V, respectively, across n = 15 mice. Data are expressed as mean±SEM. The asterisk symbol (*) indicates significant difference (Student's t-test, P<0.05).

To better understand how the distributions of the capillary RBC flow and oxygenation change in order to support the heterogeneous demand for oxygen across cortical layers, we assessed both the spatial distributions and the temporal fluctuations of the resting capillary RBC flux, speed and Mean-PO₂ in layers I-V. The spatial distributions for both the mean value and heterogeneity (quantified by STD and CV across capillaries) of capillary RBC flux, speed and Mean-PO₂ in cortical layers I-V are shown in Fig. 3. The RBC flux in layers IV (36 ± 4 RBC/s) and V (38 ± 6 RBC/s) were slightly lower than in layers I (41 ± 2 RBC/s) and II/III (41 ± 1 RBC/s; Fig. 3a). The decrease in the RBC flux in the deeper layers might be due to the redistribution of RBCs over a denser capillary network, especially in layer IV (Blinder et al., 2013; Sakadžić et al., 2014), causing the RBC flux to be lower in individual capillaries. Importantly, both the STD and CV of RBC flux were lower in layers IV-V than in layers I-III, reaching a minimum in layer IV (Fig. 3b,c); and the STD and CV of RBC flux in layer IV were significantly lower than their counterparts in layer I. This result suggests that a significant homogenization of RBC flux occurred in the deeper cortical layers, which is in line with the biophysical models that predict that capillary blood flow homogenization improves the efficiency of oxygen delivery to brain tissue (Jespersen and Østergaard, 2012). Similar trends in homogenization could be observed in the distributions of RBC speed (Fig. 3d-f) and capillary Mean-PO₂ (Fig. 3g-i), as well as in the distributions of capillary RBC-PO₂ and SO₂ (Supplementary Fig. 3).

Figure 4. Temporal fluctuations of RBC flux, speed, and Mean-PO₂ within individual capillaries in cortical layers I-V. Panels a-b, c-d, and e-f show the layer-dependent standard deviations (STDs) and coefficients of variance (CVs) of the temporal fluctuations of RBC flux, speed, and Mean-PO₂, respectively. The STD and CV of each observable for each capillary were calculated based on the 9-s-long time course. The analysis in a-f was made with 130, 140, 63, and 40 samples, collected in cortical layers I, II/III, IV, and V, respectively, across n = 7 mice. Each sample corresponds to a 9-s-long, 15-time-point measurement acquired in each capillary. Data are expressed as mean±SEM. The asterisk symbol (*) indicates significant difference (Student's t-test, P<0.05).

Next, we assessed the temporal fluctuations of RBC flux, speed and Mean-PO₂ within individual capillaries, extracted from the 9-s-long acquisitions (Fig. 4). The level of the temporal fluctuations (quantified by STD and CV) of capillary RBC flux (Fig. 4a,b) and speed (Fig. 4c,d) decreased only in layer V, while the temporal fluctuation of the capillary Mean-PO₂ was significantly attenuated in layers IV and

V (Fig. 4e,f). However, the mean amplitude of the temporal fluctuation of each observable was significantly smaller than that calculated across different capillaries (Fig. 3).

Figure 5. Correlations between the temporal fluctuations of capillary RBC flux, speed, hematocrit, and Mean-PO₂. **a.** Temporal evolutions (9-s-long traces with 0.6-s steps) of RBC flux and Mean-PO₂ from 30 representative capillaries. For each capillary, its Mean-PO₂ as a function of RBC flux is represented by a different line color; the consecutive time points are connected to illustrate the temporal trajectory of the variation. **b.** Boxplots of the pairwise correlation coefficients (r) between the temporal fluctuations of capillary RBC flux, speed, hematocrit (Hct), and Mean-PO₂. **c.** Boxplots of the Fisher z values calculated based on the r values in **b**. The analysis in **b** and **c** was made with 373 capillaries, collected in cortical layers I-V, across $n = 7$ mice. The double asterisk symbol (**) indicates significant difference (Student's t -test, $P < 0.001$).

Importantly, our measurements revealed that the Mean-PO₂, measured within individual capillaries as a function of time, was best correlated with RBC flux (Fig. 5). We calculated the Pearson correlations between the temporal fluctuations of RBC flux, speed, hematocrit and the temporal fluctuation of the Mean-PO₂. All these parameters were simultaneously measured in each assessed capillary ($n = 373$ capillaries). The correlation coefficient r between the temporal fluctuations of the RBC flux and Mean-PO₂ (median value = 0.71) was higher than between the RBC speed and Mean-PO₂ (median value = 0.37), and between hematocrit and Mean-PO₂ (median value = 0.29). The r values were converted to Fisher z values to compare

for statistical difference (Diamond et al., 2006). Mean-PO₂ was significantly stronger correlated with RBC flux than with RBC speed and hematocrit (Fig. 5c). The results of the pairwise correlations between the temporal fluctuations of capillary RBC flux, speed and hematocrit are presented in Supplementary Fig. 4.

Our depth-resolved measurements across capillaries revealed that the distributions of the RBC flux, speed and Mean-PO₂ became more homogeneous in layers IV and V compared to layers I-III. This increase in the spatial homogenization of the capillary flow and oxygenation (Fig. 3) was accompanied by an increase in the depth-dependent OEF (Fig. 2). We also observed attenuation of the temporal fluctuations of these parameters in layers IV and V compared to layers I-III (Fig. 4), although with much lower amplitudes than seen in the corresponding spatial variations (Fig. 3). Finally, the time-course cross-correlation analysis revealed that the Mean-PO₂ was best correlated with the RBC flux, as opposed to RBC speed and hematocrit (Fig. 5).

Intracapillary resistance of oxygen transport to tissue decreases in deeper cortical layers

Figure 6. Dependence of EATs on cortical layer. **a.** Intracapillary longitudinal PO₂ gradients. The PO₂ gradients measured in different capillaries (373 capillaries across n = 7 mice) are color-coded based on their Mean-PO₂ (color bar in mmHg). The black curve represents the average PO₂ gradient. The gray arrow denotes the mean half-distance between adjacent RBCs (~8 μm). The inset schematically illustrates the intracapillary PO₂ gradient. **b.** Boxplots of capillary Mean-PO₂, RBC-PO₂, InterRBC-PO₂, and EATs. **c.** Dependence of EATs on cortical layer. The analysis in

c was made with 130, 140, 63, and 40 capillaries measured in cortical layers I, II/III, IV, and V, respectively, across $n = 7$ mice. Data are expressed as mean $\pm$ SEM. The asterisk symbol (*) indicates significant difference (Student's t-test, $P < 0.05$).

We now turn to the analysis of EATs that reflect capillary modulation of PO_2 due to the passages of individual RBCs that have been observed in the peripheral and brain capillaries alike (Barker et al., 2007; Golub and Pittman, 2005; Lecoq et al., 2009). Larger EATs amplitudes have been associated with higher intracapillary resistance to oxygen transport to tissue from capillaries (Barker et al., 2007; Golub and Pittman, 2005; Hellums, 1977). Since cortical layers exhibit differences in neuronal and vascular density and possibly in oxidative metabolism, we examined whether the EATs amplitudes would differ across cortical layers. Benefitting from the improved sensitivity of the new oxygen probe, we were able to measure intracapillary longitudinal PO_2 gradients in a larger number of capillaries (Fig. 6a; 373 capillaries across $n = 7$ mice) and with greater imaging depth (down to the cortical depth of 600 μm) in comparison to the previous studies (Lecoq et al., 2011; Lyons et al., 2016; Parpaleix et al., 2013). Averaging over all the assessed capillaries, PO_2 (black curve in Fig. 6a) decreased from 55.0 ± 0.6 mmHg at the RBC center to 30.0 ± 7.8 mmHg, 30 μm away from the RBC center, where the PO_2 measurements were typically associated with the capillaries with low hematocrit. However, in most capillaries the half-distance between adjacent RBCs was much smaller than 30 μm . The mean half-distance between adjacent RBCs was estimated to be ~ 8 μm (denoted by the gray arrow in Fig. 6a), close to the previously reported 7 μm measured in anesthetized rats (Kleinfeld et al., 1998). Therefore, the estimated mean amplitude of EATs across all the assessed capillaries was 12.5 ± 0.4 mmHg, significantly smaller than that in the capillaries with low hematocrit (Supplementary Fig. 5). The median values of capillary Mean- PO_2 , RBC- PO_2 , InterRBC- PO_2 , and EATs were 39.4 mmHg, 49.2 mmHg, 36.3 mmHg, and 11.7 mmHg, respectively (Fig. 6b). Interestingly, we found a significant reduction in the EATs amplitude in cortical layers IV (9.9 ± 0.8 mmHg) and V (11.0 ± 0.8 mmHg) compared to layers I (13.4 ± 0.6 mmHg) and II/III (13.3 ± 0.3 mmHg; Fig. 6c), suggesting that the

intracapillary resistance to oxygen transport to tissue may be lower in deeper cortical layers, thus facilitating oxygen delivery to brain tissue in accordance with biophysical modelling (Hellums, 1977).

Low oxygen extraction along the superficial capillary paths contributes to an increase in the mean venular SO_2 towards cortical surface

Figure 7. Capillary flow and oxygenation in the upstream and downstream branches. **a-f.** Average capillary Mean- PO_2 , SO_2 , EATs, RBC flux, speed, and hematocrit, in the upstream (A1-A3) and downstream (V1-V3) capillary branches, across cortical layers I-III. Data are expressed as mean $\pm$ SEM. The single (*) and double (**) asterisk symbols indicate significant differences (Student's t-test) with $P < 0.05$ and $P < 0.001$, respectively. **g** and **h.** Correlations between capillary RBC flux and Mean- PO_2 (**g**) and SO_2 (**h**). Data points and regression lines from the A1-A3, V1-V3,

and all capillary segments (branching order unassigned) are color-coded red, blue, and gray, respectively. Linear regression slopes in **g**: V1-V3 slope = $0.37 \text{ mmHg} \cdot \text{s} \cdot \text{RBC}^{-1}$ ($R^2 = 0.61$), A1-A3 slope = $0.1 \text{ mmHg} \cdot \text{s} \cdot \text{RBC}^{-1}$ ($R^2 \approx 0.17$). Linear regression slopes in **h**: V1-V3 slope = $0.50 \text{ s} \cdot \text{RBC}^{-1}$ ($R^2 = 0.52$), A1-A3 slope = $0.03 \text{ s} \cdot \text{RBC}^{-1}$ ($R^2 \approx 0.04$). The analysis in **a-h** was made with 47 upstream and 50 downstream capillaries, across $n = 5$ mice. **i**. Correlation between the PO₂ ratio (the V1 capillary Mean-PO₂ to the adjacent PCV PO₂) and the V1 capillary Mean-PO₂. Histograms of the V1 capillary Mean-PO₂, and PO₂ ratio are at the top and on the right from the main panel, respectively. **j**. Histogram of the V1 capillary RBC flux. **k**. Correlation between the V1 capillary RBC flux and PO₂ ratio. The linear regression slope = $0.01 \text{ s} \cdot \text{RBC}^{-1}$ ($R^2 \approx 0.12$).

To better understand why SO₂ in the ascending venules increased towards the cortical surface (Fig. 2), we investigated the distributions of capillary flow and oxygenation along the capillary paths in the upper cortical layers. For this analysis, capillaries in the top 300 μm of the cortex (layers I-III) were grouped based on their branching orders into two main groups: 1) ‘upstream’ capillaries with branching orders A1-A3, which are closer to the arteriolar side of the network, and 2) ‘downstream’ capillaries with branching orders V1-V3, which are closer to the venular side of the network. This grouping provides sufficient statistical power to compare the RBC flow and oxygenation parameters in the beginning and in the end of the capillary paths while avoiding labor-intensive segmentation of the microvascular angiograms.

The average values of Mean-PO₂, SO₂, EATs, RBC flux, speed and hematocrit from the combined A1-A3 (upstream) and V1-V3 (downstream) capillaries are presented in Fig. 7a-f. The average Mean-PO₂ in the A1-A3 capillaries ($64.4 \pm 2.4 \text{ mmHg}$) in layers I-III was, as expected, significantly higher than that in the V1-V3 capillaries ($41.8 \pm 0.9 \text{ mmHg}$; Fig. 7a), suggesting that a large fraction of oxygen has been extracted along the capillary paths (i.e. from A1 to V1). Indeed, the average SO₂ in the A1-A3 capillaries ($77.6 \pm 1.7 \%$) was significantly higher than that in the V1-V3 capillaries ($67.3 \pm 1.2 \%$; Fig. 7b). Provided that the average SO₂ in the A1 capillary segments was $81.0 \pm 2.3 \%$, and that the SO₂’s in the arterioles (SO_{2,A}) and venules (SO_{2,V}) at cortical surface were $91.0 \pm 0.8 \%$ and $62.0 \pm 0.8 \%$, respectively (Fig. 2b), we estimated that the decrease in SO₂ from the pial arterioles to the A1 capillary segments in the upper 300 μm of the

cortex accounted for 34 % (or 1/3) of the total extracted oxygen. Here, the total extracted oxygen was calculated as the A-V difference in SO_2 at the cortical surface ($\Delta\text{SO}_{2,\text{A-V}} = 30\%$; Fig. 2b). Therefore, 66 % (or 2/3) of the oxygen extraction in awake mice took place after the arterioles. This is in contrast to our previous study in anesthetized mice, which reported that ~50 % of the oxygen delivered to brain tissue was extracted after the arterioles (Sakadžić et al., 2014). The average V1 capillary SO_2 in layers I-III was $65 \pm 1.9\%$, higher than that in the ascending venules, both in layer I ($62 \pm 0.8\%$) and layer II/III ($59 \pm 0.4\%$; Fig. 2b). Accordingly, capillaries that feed the surfacing venules in layers I-III apparently do so with more oxygenated blood, contributing to the increase in the venular SO_2 towards brain surface (Fig. 2b).

The RBC flux in the A1-A3 capillaries (97 ± 6 RBC/s) was ~2.7 times higher than that in the V1-V3 capillaries (36 ± 2 RBC/s; Fig. 7c). Since blood flow obeys the principle of mass conservation (i.e. the numbers of RBCs entering the capillary paths per unit time on the arteriolar side and exiting from the venous side must be equal), this result is in agreement with the previously observed ~3-fold-greater number of V1 than A1 capillaries in mouse cortex (Nguyen et al., 2011). Furthermore, the RBC speed in the A1-A3 capillaries (1.9 ± 0.2 mm/s) had a similar ratio (~3.2 times) to that in the V1-V3 capillaries (0.6 ± 0.1 mm/s; Fig. 7e), which is also consistent with the strong correlation between RBC flux and speed (Supplementary Fig. 6) (Desjardins et al., 2014; Kleinfeld et al., 1998; Lyons et al., 2016).

We found strong positive correlations between the capillary RBC flux and both Mean- PO_2 and SO_2 in the downstream (V1-V3) capillaries, but not in the upstream (A1-A3) capillaries (Fig. 7g,h), suggesting that a positive correlation between the RBC flux and oxygenation may be gradually building up along the capillary paths. In addition, we observed very heterogeneous distributions of both Mean- PO_2 (from ~11 mmHg to ~68 mmHg) and RBC flux (from ~30 RBCs/s to ~110 RBCs/s) in the V1 capillaries (Fig. 7i,j). Lastly, the V1 capillary RBC flux was correlated positively with the ratio of the V1 capillary Mean- PO_2 to the PO_2 in the adjacent post-capillary venules (PCV PO_2) (Fig. 7k). Therefore, while heterogeneous oxygen delivery is taking place along different capillary paths, the capillary paths with higher RBC flux are oxygenated, contributing more to the increase in the PCV oxygenation.

Finally, EATs observed in the A1-A3 capillaries (10.9 ± 1.9 mmHg) were noticeably smaller than in the V1-V3 capillaries (16.6 ± 2.3 mmHg; Fig. 7c), although this difference did not reach statistical significance ($P = 0.09$). In both this work (Supplementary Fig. 5) and a previous study (Lyons et al., 2016), EATs did not exhibit any obvious dependence on the Mean- PO_2 , RBC flux or speed, but they were correlated negatively with hematocrit. Nevertheless, the observed trend that the average EATs amplitude in the A1-A3 capillaries was smaller than that in the V1-V3 capillaries is unlikely to be related to hematocrit, as we did not observe significant difference between the upstream and downstream hematocrit values (Fig. 7f).

DISCUSSION

We have performed measurements of absolute intra-vascular PO_2 in arterioles, venules and a large number of capillaries (6,544 capillaries across $n = 15$ mice), as well as EATs, RBC flux, speed and hematocrit in a subset of capillaries (978 capillaries across $n = 15$ mice; Supplementary Fig. 2). These parameters were measured simultaneously in the whisker barrel cortex in head-restrained awake C57BL/6 mice and thus were free of the confounding effects of anesthesia on neuronal activity, CBF and brain metabolism.

A new two-photon-excitable phosphorescent oxygen probe was used in this study. This probe belongs to the class of dendritically protected oxygen probes (Lebedev et al., 2009), and it is built around a newly developed Pt tetraarylphthalimidoporphyrin (PtTAPIP) (Esipova et al., 2017). In comparison to its predecessor (PtP-C343) (Finikova et al., 2008), the new probe exhibits red-shifted two-photon excitation and emission maxima, higher phosphorescence quantum yield and larger two-photon absorption cross-section. Furthermore, in contrast to PtP-C343 the phosphorescence decay of the new probe exhibits well-defined single-exponential kinetics, which aids to improve accuracy of oxygen quantification. The above properties of the probe facilitated large-scale sampling of both capillary RBC flow and PO_2 deeper in the brain, e.g., $600 \mu m$ vs. $450 \mu m$ (Lyons et al., 2016; Sakadžić et al., 2014), and with higher signal-to-noise ratios, which, in turn, powered the statistical analyses beyond what was achievable with previous probes (Devor et al., 2011; Lecoq et al., 2011; Lyons et al., 2016; Parpaleix et al., 2013; Sakadžić et al., 2010,

2014). In addition, the very high phosphorescence quantum yield of PtTAPIP (Esipova et al., 2017) enabled detection of RBC passages in capillaries based on phosphorescence alone, without the need to enhance the signal from the blood plasma using additional chromophores (Lyons et al., 2016).

We first assessed oxygenation in the diving arterioles and surfacing venules and observed a decrease in PO_2 with cortical depth for both vessel types (Fig. 2a). The PO_2 decrease in the diving arterioles between layer I and V was about 15 mmHg, equivalent to the difference in SO_2 of 3.8 %. This modest oxygen extraction along the diving arterioles implies that much more oxygen was extracted from the rest of the microvascular network, including arteriolar branches, capillaries and potentially venules. Previous measurements performed in mice (Kisler et al., 2017; Moeini et al., 2018) and rats (Devor et al., 2011; Sakadžić et al., 2016) reported pronounced PO_2 gradients in the periarteriolar tissue around the cortical diving arterioles, implying extraction of oxygen from cortical diving arterioles. Specifically, a significantly steeper decrease in PO_2 with cortical depth was observed directly in diving arterioles in isoflurane-anesthetized mice by Kazmi et al. (Kazmi et al., 2013) and indirectly, based on the depth dependence of the extravascular (tissue) PO_2 measured immediately next to the diving arterioles, in α -chloralose anesthetized rats by Devor et al. (Devor et al., 2011) The most likely reason for these discrepancies is the higher suppression of CBF than $CMRO_2$ under different anesthesia regimes. In awake mice, Lyons et al. (Lyons et al., 2016) measured approximately constant PO_2 in the diving arterioles over cortical depth of 0-400 μ m. This result is in agreement with our current observation and implies a small loss of O_2 along the diving arterioles in awake mice.

In contrast to the moderate PO_2 decrease along the penetrating arterioles, the decrease in the venular PO_2 from layer I to V was much smaller ($\sim$ 6 mmHg), but resulted in a larger fractional decrease in SO_2 (8.3 %) than in the penetrating arterioles (Fig. 2b). An increase in PO_2 in ascending venules towards the cortical surface has been previously observed in isoflurane-anesthetized mice (Sakadžić et al., 2010). However, the PO_2 values, measured previously along the ascending venules in the fore-paw and hind-paw regions of the somatosensory cortex in awake mice, were reported to be nearly constant (Lyons et al., 2016). The difference between these observations may be attributed to the difference in the studied cortical regions

and/or to the lower measurement accuracy associated with the previous oxygen probe because of its lower brightness and inability to resolve small PO_2 changes. In addition, some discrepancies in the absolute baseline PO_2 in arterioles and venules at different depths were observed between our present and some previous studies (Kazmi et al., 2013; Lecoq et al., 2011; Lyons et al., 2016; Parpaleix et al., 2013; Sakadžić et al., 2010). Different anesthesia regimes, different cortical areas and, most importantly, use of anesthetized vs. awake animals would be the most obvious factors underlying the observed discrepancies. Furthermore, cortical temperature typically was not controlled in the previous studies, while the presence of a cranial window can cause reduction in the cortical temperature (Shirey et al., 2015), especially when imaging is carried out using a non-heated water-immersion objective. Temperature affects all physiological parameters and chemical properties, including O_2 diffusion and solubility, hemoglobin affinity to O_2 , CBF, $CMRO_2$ and the triplet decay time of the oxygen probe (Benesch et al., 1969; Croughwell et al., 1992; Finikova et al., 2008; Hanks and Wallace, 1949; Jones and Siegel, 1969; PRAY, 1952; Rosomoff and Holaday, 1954; Rossing and Cain, 1966; Soukup et al., 2002) – all of which may affect the experimental results.

A faster decrease in SO_2 with cortical depth in the ascending venules than in the penetrating arterioles resulted in a higher depth-dependent OEF in the deeper cortical layers, reaching the maximum in layer IV (Fig. 2b). This implied that oxygen extraction was higher in deeper cortical layers, which would be in agreement with the finding that layer IV has the highest neuronal and capillary density in mouse cortex (Blinder et al., 2013; Lefort et al., 2009; Patel, 1983; Wu et al., 2016), and that the cells in layer IV exhibit the highest cytochrome oxidase labeling activity, suggestive of the highest oxidative metabolism (Land and Simons).

The mean capillary RBC flux and speed decreased only slightly with cortical depth, without reaching statistically significant difference across layers I-V (Fig. 3a,d). This might be due to the redistribution of the blood flow over the denser capillary network in the deeper cortical layers, especially in layer IV (Blinder et al., 2013; Sakadžić et al., 2014; Wu et al., 2016), causing the RBC flux and speed to be lower in individual capillaries. The observed mean capillary RBC flux (41.5 ± 1.2 RBCs/s; Supplementary Fig. 2) is in good agreement with the value of 41.9 ± 1.8 RBCs/s, reported by Lyons et al. (Lyons et al., 2016),

and somewhat lower than ~48 RBC/s reported by Moeini et al. (Moeini et al., 2018) for somatosensory cortex in awake mice. The average capillary Mean-PO₂ increased slightly by ~7 mmHg from layer I to V (Fig. 3g), which is in agreement with the previously reported increase in capillary Mean-PO₂ in both awake (Lyons et al., 2016) and isoflurane-anesthetized mice (Sakadžić et al., 2014) across the upper 400 µm of the cortex. One potential explanation for this increase could be related to the finding by Blinder et al. (Blinder et al., 2013), who reported that, in the upper 600 µm of somatosensory cortex, the probability of penetrating arterioles giving off side branches increased with cortical depth, whereas the probability for the ascending venules decreased with depth. This suggests that the ratio between the number of the more oxygenated (upstream) capillary branches and that of the less oxygenated (downstream) capillary branches increases with depth, contributing to the higher capillary PO₂ at greater depth.

Importantly, we found that the distributions of capillary RBC flux, speed, and Mean-PO₂ undergo homogenization in cortical layers IV and V (Fig. 3). Both analytical and more accurate numerical models of oxygen advection and diffusion in microvascular networks predict that capillary blood flow homogenization facilitates oxygen delivery to tissue (Jespersen and Østergaard, 2012; Østergaard et al., 2013, 2014a, 2014b; Rasmussen et al., 2015). Indeed, homogenization of capillary blood flow, measured as capillary transit time heterogeneity and STD of capillary RBC flux during functional brain activation in healthy anesthetized animals, have been reported previously (Gutiérrez-Jiménez et al., 2016; Lee et al., 2015; Stefanovic et al., 2007). Interestingly, capillary flow homogenization was absent in the mouse model of Alzheimer's disease (Gutiérrez-Jiménez et al., 2018), while increased capillary blood flow heterogeneity was found in patients of Alzheimer's disease (Eskildsen et al., 2017; Nielsen et al., 2017), suggesting a possible link between disturbed capillary flow patterns, reduced oxygen supply to tissue, and the progression of neurodegeneration. Our observations are in agreement with the previously reported lower capillary transit time heterogeneity in cortical layers IV and V than in the upper layers in anesthetized mice (Merkle and Srinivasan, 2016). Furthermore, capillary Mean-PO₂ also exhibited a similar homogenization trend (Fig. 3h,i). This could be expected since capillary RBC flow is correlated positively with Mean-PO₂ (Lyons et al., 2016).

Temporal fluctuations of the resting state capillary RBC flux, speed, and Mean-PO₂, measured during 9-s-long periods, were found to be attenuated in the deeper cortical layers (Fig. 4). The temporal resolution of our measurements (0.6 s per single point) ensured that the blood flow and oxygenation fluctuations due to cardiac and respiratory cycles, as they occurred in awake mice, were averaged out, but it was sufficient to capture the dynamics related to the rate of oxygen consumption, which could be estimated as the time for tissue PO₂ to drop to zero after blood flow stoppage. As reported in cats, that time was at least several seconds (Acker and Lübbers, 1977; Whalen and Nair, 1975). The cause of the dampening of the temporal fluctuations with depth is unclear. It may be related to the anatomical and functional differences between cortical layers; it also can be due to the expected dampening along the vascular paths if the temporal fluctuations originate from the upstream arteriolar blood flow.

We observed strong positive correlations between the temporal fluctuations of capillary RBC flux and Mean-PO₂ (Fig. 5). This observation is in agreement with the previously reported positive correlation between the fluctuations of RBC flux and extravascular (tissue) PO₂ next to the capillaries in cancerous tumors in rats, although the latter occurred at a lower frequency range (Braun et al., 1999; Kimura et al., 1996). Importantly, the fluctuation of capillary Mean-PO₂ was found to be correlated positively with the fluctuation of RBC flux significantly stronger than with the fluctuations of RBC speed or hematocrit (Fig. 5b,c). This result could be expected, since most oxygen in the blood is bound to the hemoglobin inside RBCs (Pittman, 2011). We also observed strong positive correlation between the fluctuations of RBC flux and speed, but the fluctuation of hematocrit was poorly correlated with both of them (Supplementary Fig. 4). These correlations between the temporal fluctuations are in agreement with the trends measured using the populations of capillaries (Supplementary Fig. 6) as well as with the previous studies (Kleinfeld et al., 1998; Santisakultarm et al., 2012; VanTeeffelen et al.). Despite poor correlation between the RBC flux and hematocrit, we observed reasonably strong positive correlation between the temporal fluctuations of capillary Mean-PO₂ and hematocrit (Fig. 5). Similarly, capillary Mean-PO₂ and hematocrit, measured in the populations of capillaries, were also positively correlated shown in Supplementary Fig. 6 and in (Lyons et al., 2016) emphasizing the specific role of hematocrit in oxygen transport (Lücker et al., 2017).

Longitudinal capillary PO₂ gradients were measured to assess the intracapillary resistance to oxygen delivery to tissue and to calculate the capillary SO₂ (Fig. 6). For measuring EATs, we followed the previously developed methods (Lecoq et al., 2011; Lyons et al., 2016; Parpaleix et al., 2013). Compared to the previous studies performed with the old probe PtP-C343 (Lecoq et al., 2011; Lyons et al., 2016; Parpaleix et al., 2013), the higher brightness of the new probe enabled us to measure PO₂ gradients in a much larger number of capillaries (~1,000) over a greater cortical depth (down to 600 µm below the cortical surface) and using a shorter acquisition time (9 s) per measurement location. In agreement with Lyons et al. (Lyons et al., 2016), we observed EATs in capillaries (Fig. 6), although the magnitude was lower than previously reported. EATs did not appear to depend significantly on the RBC flux, speed or Mean-PO₂, but were correlated negatively with the hematocrit (Supplementary Fig. 5). Our measurements are in agreement with the previous observations (Lyons et al., 2016) as well as with the theoretical predictions (Hellums, 1977; Lückner et al., 2017). Importantly, we detected a significant decrease in EATs towards deeper cortical layers, dropping from 13.4 mmHg in cortical layer I to 9.9-11.0 mmHg in layers IV and V. Since EATs are directly related to the intravascular resistance to the diffusive oxygen transport from RBCs to tissue (Hellums, 1977), reduced EATs amplitude in the deeper layers represents another example of the adaptation of the microvascular network in order to facilitate local oxygen delivery in cortical regions with higher oxygen demand (Pries et al., 1994, 1998; Reglin et al., 2009, 2017).

The elevated SO₂ in the surfacing venules in layers I-III suggests that in the upper cortical layers (e.g. layers I-III) the downstream capillaries and post-capillary venules that feed the surfacing venules are more oxygenated compared to the surfacing venules in the deeper layers (e.g. layers IV and V). We therefore assigned branching orders to the capillary segments in cortical layers I-III and investigated the distributions of capillary RBC flow and oxygenation along the capillary paths. We found that the average SO₂ in the V1 capillaries selected across layers I-III was indeed higher than the SO₂ in the ascending venules in both layers I and II/III, confirming that high oxygen content in the superficial capillaries contributed to the increase in the venular SO₂ towards brain surface. This finding also suggests that the baseline oxygen extraction may be different between the cortical layers. If true, this may have implications on the interpretation of results

from different imaging modalities such as BOLD fMRI (Blockley et al., 2015; Griffeth and Buxton, 2011; Siero et al., 2011; Silva and Koretsky, 2002; Vazquez et al., 2006; Yu et al., 2014). Secondly, approximately 1/3 of the total oxygen extraction took place along the paths from the pial arterioles to the A1 capillary segments in layers I-III. This observation differs from our previous study in isoflurane-anesthetized mice, where we found, based on the measurements in the upper 450 μm of somatosensory cortex, that ~50 % of the oxygen delivered to brain tissue was extracted from arterioles (Sakadžić et al., 2014). The discrepancy might be due to the effect of anesthesia, which suppressed cerebral oxygen metabolism and possibly increased both CBF and arteriolar surface area due to vasodilation (Alkire et al., 1999; Goldberg et al., 1966; Ogawa et al., 1990). As a result of these perturbations, oxygen extraction could be shifted towards the upstream microvascular segments (Sakadžić et al., 2014). Based on the observed difference in oxygen extraction and the assumed difference in oxygen metabolism between cortical layers, we anticipate that in cortical layers IV and V, the fraction of the extracted oxygen from arterioles may be smaller than in layers I-III, but still significant.

Our data provided additional evidence that the distributions of capillary flow and oxygenation were highly heterogeneous and strongly positively correlated with one-another, in particular in the downstream capillaries (Fig. 7). This implies that the mixed venous blood oxygenation was, therefore, a result of the contributions from the wide distribution of capillary paths, carrying blood that was both more and less oxygenated than the blood in the postcapillary venules (Fig. 7). At the extreme ends of this distribution are capillary paths with very low and very high oxygenation and blood flow. The paths with very low oxygenation may be the first sites of coupling of microvascular dysfunction and progression of various brain pathologies (Erdener et al., 2017; Lückner et al., 2018). Indeed, it has been recently reported that only ~0.4 % of the cortical capillaries in healthy mice had ‘stalled flow’, but in transgenic mice of Alzheimer’s disease the fraction was as high as 2 % (Hernandez et al., 2016; Momjian-Mayor and Baron, 2005). As an example of such poorly oxygenated/perfused capillaries, we identified a capillary segment with PO_2 of ~15 mmHg and no detectable RBC flow during the 9-s-long acquisition (Supplementary Fig. 7). In contrast, the paths with very high oxygenation and blood flow (Supplementary Fig. 8) may be especially good sites for

implementing blood flow control in response to locally increased oxygen demand, since increasing their resistance to flow may quickly redistribute blood over the nearby capillary network. However, the prevalence of such highly oxygenated/perfused capillaries in the cortical microvascular network and the existence of mechanisms of their site-specific control are unknown (Hudetz et al., 1996). Further studies should address these questions in relation to both normal and pathological brain conditions.

One potential limitation of this study is that the assignment of the cortical layers as a function of the cortical depth was performed based on the literature data (Blinder et al., 2013; Lefort et al., 2009), as opposed to identification of layer-specific anatomical landmarks. Nevertheless, while slight shifts in the layer boundaries, which may have resulted from such an assignment, may have affected the exact values, it is unlikely that the observed general trends and significant differences in the observables between the layers would have changed. In addition, layer V in mouse barrel cortex spans the depth range of 450-700 μm (Blinder et al., 2013; Lefort et al., 2009), so that the range of 450-600 μm , interrogated in this study, likely overlaps the best with the upper part of layer V (i.e. layer V_a) (Blinder et al., 2013; Lefort et al., 2009). Another limitation is that capillaries as a vessel type and their branching order indices were identified based on their diameter, without taking into account the smooth muscle cell coverage and pericyte types (Attwell et al., 2016; Hall et al., 2014; Peppiatt et al., 2006). In principle, misclassifying vessel types and/or branching order indices could influence our analysis. However, based on the overwhelmingly larger number of capillaries compared to the non-penetrating arterioles and venules, our conclusions in general are unlikely to be different. Furthermore, identification of capillaries based on the biochemical staining of smooth muscle cells and pericytes (Hall et al., 2014; Hartmann et al., 2015) will add significant complexity to the study, especially considering a large sample size used in our analysis, but in the end may not provide more accurate results, as the debate on what is a proper classification of vessel-types based on staining is still ongoing (Hill et al., 2015).

Table 1. Summary of microvascular oxygenation and RBC flux changes with cortical depth. The arrows pointing up (↑) and down (↓) indicate increase and decrease of the measured observables from cortical layers I-III to layers

IV/V, respectively. The fields marked in blue represent the parameters reported in the literature. The fields marked in black represent our experimental measurements.

<i>Parameter</i>	<i>Change with depth</i>
Neuronal density (Blinder et al., 2013; Lefort et al., 2009; Wu et al., 2016)	↑
Capillary density (Blinder et al., 2013; Wu et al., 2016)	↑
Depth-dependent OEF	↑
Capillary RBC flux STD/CV	↓
Capillary PO₂ STD/CV	↓
EATs	↓

In conclusion, we have experimentally mapped the distributions of microvascular flow and oxygenation in the whisker barrel cortex in awake mice using two-photon phosphorescence lifetime microscopy. We have found evidence that oxygen was extracted differently in different cortical layers, and that the distributions of capillary blood flow and oxygenation were adjusted across layers in a way that facilitated oxygen delivery in the deeper layers (Table 1). Specifically, the depth-dependent OEF was measured higher in cortical layers IV and V, where the oxidative metabolism is presumably the highest in cortex. This increase was accompanied by the homogenization of capillary RBC flow and oxygenation, as well as by the reduction of intracapillary resistance to oxygen diffusion to tissue (inferred from the changes of EATs amplitude). In addition, we have found that arterioles in the superficial cortical layers (e.g. layers I-III) contributed significantly to the oxygen extraction from blood (34 %) even in awake mice. We anticipate that our results and analysis will help better understand the normal brain physiology, and the progression of brain pathologies that affect cerebral microcirculation. They will also inform more accurate biophysical models of the cortical layer-specific oxygen delivery and consumption, as well as improve the interpretation of the results from other brain imaging modalities.

MATERIALS AND METHODS

Animal preparation. We used $n = 15$ C57BL/6 mice (3-5 months old, 20-25 g, female, Charles River Laboratories) in this study. We followed the procedures of chronic cranial window preparation outlined by Goldey *et al.* (Goldey et al., 2014). A custom-made head-post (Mateo et al., 2011) allowing repeated head immobilization was glued to the skull, overlaying the right hemisphere (Supplementary Fig. 1). A craniotomy (round shape, 3 mm in diameter) was performed over the left hemisphere, centered approximately over the E1 whisker barrel. The dura was kept intact. The cranial window was subsequently sealed with a glass plug (Komiyama et al., 2010) and dental acrylic. After surgery, mice were given 5 days to recover before starting the habituation training. Mice were gradually habituated to longer periods (from 10 minutes to 2 hours) of head-restraint, resting on a suspended bed made of soft fabric, in a home built platform, under the microscope. They were rewarded with sweetened milk every 15 minutes during both training and experiments. While head-restrained, the mice were free to readjust their body position and from time to time displayed natural grooming behavior. All animal surgical and experimental procedures were conducted following the Guide for the Care and Use of Laboratory Animals and approved by the Massachusetts General Hospital Subcommittee on Research Animal Care (Protocol No.: 2007N000050).

Two-photon microscope. In this study, we employed our previously developed home-built two-photon microscope (Fig. 1a) (Sakadžić et al., 2010; Yaseen et al., 2015). Briefly, a pulsed laser (InSight DeepSee, Spectra-Physics, tuning range: 680 nm to 1300 nm, ~120 fs pulse width, 80 MHz repetition rate) was used as an excitation source. Laser power was controlled by an electro-optic modulator (EOM). The laser beam was focused with a water-immersion objective lens (XLUMPLFLN20XW, Olympus, NA = 1.0), and scanned in the X-Y plane by a pair of galvanometer scanners. The objective was moved along the Z axis by a motorized stage (M-112.1DG, Physik Instrumente) for probing different cortical depth. The phosphorescent oxygen-sensitive probe was excited at 950 nm. The emitted phosphorescence, centered at 760 nm, was directed towards a photon-counting photomultiplier tube (H10770PA-50, Hamamatsu) by a dichroic mirror (FF875-Di01-25×36, Semrock), followed by an infrared blocker (FF01-890/SP-25,

Semrock) and an emission filter (FF01-795/150-25, Semrock). During the experiments, the objective lens was heated by an electric heater (TC-HLS-05, Bioscience Tools) to maintain the temperature of the water between the cranial window and the objective lens at 36-37 °C. In addition, we attached the sensor of an accelerometer module to the suspended bed of the mouse platform to record the signals induced by mouse motion. Mice were continuously monitored during experiments by acquiring live videos with a CCD camera (CoolSNAPfx, Roper Scientific) using a LED illumination at 940 nm.

Intravascular PO₂ imaging. Before imaging, mice were briefly anesthetized by isoflurane (1.5-2 %, during ~2 minutes), and then the solution of the phosphorescent oxygen probe (0.1 ml at ~34 μM) was retro-orbitally injected into the bloodstream. Mice were recovered from anesthesia and then head fixed under the microscope. The imaging session was started 30-60 minutes after the probe injection and lasted for up to 2 hours.

The acquisition protocol is illustrated in Fig. 1b. We first recorded a CCD image of the mouse brain surface vasculature (Fig. 1d) under green light illumination to guide the selection of a region of interest for the subsequent functional imaging. The two-photon microscopic measurements were collected at different X-Y planes, perpendicular to the optical axis (Z). The imaging planes were separated in depth by ≤50 μm, spanning the depth range from cortical surface to 600 μm under cortical surface. At each imaging depth, we first performed a raster scan of phosphorescence intensity, which revealed the locations of the micro-vessels within a 500 × 500 μm² field of view (FOV). Then, we manually selected the measurement locations inside all the vascular segments captured within the FOV. At each selected location, the phosphorescent oxygen probe in the focal volume was excited with a 10-μs-long laser excitation at 950 nm gated by the EOM, followed by a 290-μs-long collection of the emitted phosphorescence. Typically, at each location, such 300-μs-long excitation/decay cycle was repeated 2,000 times (0.6 s) to obtain an average phosphorescence decay with sufficient signal-to-noise ratio (SNR) for an accurate lifetime calculation.

Imaging of EATs and capillary RBC flow. In a subset of capillaries, we repeated the 300-μs-long excitation/decay cycle 30,000 times at each measurement location, corresponding to a 9-s-long acquisition

per capillary (Lecoq et al., 2011). This long acquisition time allowed us to estimate EATs by grouping the phosphorescence decays as a function of their distance to their nearest RBC center, while ensuring a sufficient number of phosphorescence decays associated with each distance bin for averaging. The same dataset was used to calculate capillary RBC flux, as well as to estimate RBC speed and hematocrit averaged over the 9-s-long intervals. In addition, the same dataset was used to estimate temporal fluctuations of capillary RBC flux, speed, hematocrit, and capillary PO₂ during the 9-s-long periods.

Acquisition of microvascular angiograms. Microvascular angiograms were acquired by two-photon microscopic imaging of blood plasma labeled with dextran-conjugated Sulforhodamine-B (SRB) (0.15-0.2 ml at 5 % W/V in saline, R9379, Sigma Aldrich). The SRB solution was retro-orbitally injected into the blood stream under brief isoflurane anesthesia (1.5-2 %, during ~2 minutes). The microvascular stacks were acquired within a 700 × 700 μm² FOV, centered over the same region of interest as for PO₂ imaging. In each mouse, the angiogram and intravascular PO₂ were acquired separately on different days in order not to stress the animals by the combined long imaging session.

Calculation of PO₂. We rejected the initial 5-μs phosphorescence decay data after the 10-μs-long excitation gate, and used the remaining 285-μs decay to fit for the phosphorescence lifetime. The phosphorescence lifetime was calculated by fitting the average phosphorescence decay to a single-exponential decay function, using a standard non-linear least square minimization algorithm (Finikova et al., 2008; Sakadžić et al., 2010). The lifetime was converted to absolute PO₂ using a Stern-Volmer type calibration plot obtained in an independent oxygen titration experiment, conducted with the same 300-μs-long excitation/decay acquisition protocol as in our *in vivo* recordings.

Calculation of capillary RBC flux, speed, and hematocrit. The phosphorescence intensity was calculated by integrating the phosphorescence photon counts over each 300-μs-long excitation/decay cycle. Since the phosphorescent probe is confined to blood plasma, but not permeates RBCs, as all the dendritic oxygen probes (Lebedev et al., 2009), the variations of the phosphorescence intensity recorded in capillaries encoded the passing of RBCs through the optical focus (Lecoq et al., 2011; Parpaleix et al., 2013) (Fig.

1b,c). Following the previously described procedures (Lecoq et al., 2011; Parpaleix et al., 2013), the phosphorescence intensity time course was segmented using a binary threshold method. A representative phosphorescence intensity time course is shown in Fig. 1c, where a RBC and a blood-plasma-passage induced phosphorescence intensity transients are denoted by arrows. Subsequently, capillary RBC flux was calculated by counting the number of detected RBCs (i.e. valleys in the binary segmented curve) during the acquisition time. Hematocrit was estimated as the ratio of the combined duration of all valleys associated with the RBC passages to the duration of the entire time course. Finally, RBC speed for each RBC passage event was estimated as $v = \phi / \Delta t$, where Δt is the time for the RBC to pass through the focal zone, and ϕ is RBC diameter, assumed to be 6 μm (Unekawa et al., 2010). For each capillary, the mean RBC speed was calculated by averaging over all RBC passage events throughout the time course.

Calculation of capillary PO₂ gradients and EATs. The phosphorescence decays from the 9-s-long acquisition in each capillary were grouped by their distance to the nearest RBC center using 1- μm -wide bins. The distance to the nearest RBC center was estimated as $v \cdot \Delta t'$, where v was the speed of the nearest RBC, and $\Delta t'$ was the time interval between the phosphorescence decay event and the center of the nearest RBC passage (i.e. the center of the valley in the time course). The decays at each 1- μm -wide bin were subsequently averaged, and then PO₂ calculated using the procedure described above.

The phosphorescence decays at the RBC passages (i.e. valleys in the binary segmented time course) and in proximity to the mid-points between adjacent RBCs (corresponding to the central 40 % of the peaks in the binary segmented time course) were averaged to calculate RBC-PO₂ and InterRBC-PO₂, respectively. The erythrocyte-associated transients of capillary PO₂ (EATs) were calculated as $\text{EATs} = \text{RBC-PO}_2 - \text{InterRBC-PO}_2$. Mean-PO₂ was calculated by averaging all the phosphorescence decays acquired within a capillary, regardless of their distance to RBCs (Lecoq et al., 2011; Parpaleix et al., 2013; Lyons et al., 2016).

Calculation of SO₂ and depth-dependent OEF. The oxygen saturation of hemoglobin (SO₂) was computed based on PO₂ using the Hill equation with the parameters ($h = 2.59$, $P_{50} = 40.2$ mmHg) specific for C57BL/6 mice (Uchida et al., 1998). Here, h is the Hill coefficient, and P_{50} is the oxygen tension at which

hemoglobin is 50 % saturated. SO_2 in the penetrating arterioles and surfacing venules was calculated based on their Mean- PO_2 . SO_2 in capillaries was calculated based on the RBC- PO_2 (Lyons et al., 2016; Sakadžić et al., 2014).

The depth-dependent OEF (DOEF), in a given cortical layer, was calculated as $(\text{SO}_{2,A} - \text{SO}_{2,V}) / \text{SO}_{2,A}$, where $\text{SO}_{2,A}$ and $\text{SO}_{2,V}$ represent the layer-specific SO_2 in the diving arterioles and surfacing venules, respectively. Therefore, DOEF in a certain layer measures the OEF accumulated downstream from that layer and, as a special case, DOEF in layer I represents the global OEF in the interrogated cortical tissue territory.

Quantification of the temporal fluctuations of capillary Mean- PO_2 , RBC flux, speed, and hematocrit.

The phosphorescence decays recorded during the 9-s-long acquisition (30,000 repetitions of the excitation/decay cycle) in each capillary were divided into 15 groups using 0.6-s-long bins (2,000 repetitions for each bin). RBC flux, speed, hematocrit, and Mean- PO_2 were calculated with the 2,000 phosphorescence decays at each bin, yielding 15-point time courses of these 4 parameters for each assessed capillary. Subsequently, for each of the 4 parameters, we quantified the temporal fluctuation by computing the standard deviation (STD) and coefficient of variance (CV) from the 15-point data. Here, CV is defined as the ratio of STD to mean (Golub and Pittman, 2005).

Identification of capillary branching order. Capillaries were identified empirically based on morphology and vascular diameters (Cai et al., 2018; Sakadžić et al., 2014). Their branching order indices were assigned by visually inspecting the microvascular angiograms. Starting immediately after the pre-capillary arteriole (PCA), capillary segments were counted in the direction of blood flow and indexed as A_i ($i = 1, 2, 3, \dots$). Analogously, starting immediately before the post-capillary venule (PCV), capillary segments were counted in the opposite direction of blood flow and indexed as V_i ($i = 1, 2, 3, \dots$). In the analysis, only the first 3 upstream capillary segments (A_1 - A_3) and the last 3 downstream capillary segments (V_1 - V_3) were considered, as visually inspecting and confirming higher branching orders of capillaries would be more challenging. In addition, most of the capillaries selected were within the central part of the FOV of the

microvascular angiograms and in the cortical depth of $\leq 300 \mu\text{m}$, which were due to the difficulty in tracking the capillaries close to the boundaries of the FOV and at greater depth. PCAs and PCVs were identified based on their morphology, vascular diameters, and PO_2 values, and confirmed by tracing the vessels from the brain surface with the microvascular angiograms (Sakadžić et al., 2014).

Cortical layer-specific data analysis. The capillary RBC flow and PO_2 properties acquired in each animal were grouped into 4 groups based on the cortical depth: 0-100 μm , 100-320 μm , 320-450 μm , and 450-600 μm . These depth ranges approximately correspond to the cortical layers I, II/III, IV, and V, respectively, in the whisker barrel cortex in 3-month-old C57BL/6 mice (Blinder et al., 2013; Lefort et al., 2009). For each cortical layer in each mouse, the absolute values of capillary RBC flow and PO_2 properties were averaged, and the STD and CV computed. Subsequently, the measurements belonging to each cortical layer were averaged over mice.

Rejection of motion artefacts. Data affected by mouse motion were rejected based on the signal generated by the accelerometer attached to the fabric underneath the mouse. We excluded from analysis the phosphorescence decays acquired within the time intervals determined by an empirically defined threshold of the accelerometer signal amplitude. In addition, visual inspection of the phosphorescence intensity traces acquired in the microvascular segments was also used to find and reject motion artefacts. This was achieved by looking for the sudden changes of phosphorescence intensity or loss of contrast between RBC and plasma passing through the focal volume. Finally, long episodes of motion were captured by the live-videos recorded by a CCD camera during acquisition. When motion occurred, the acquisition was manually stopped and the corresponding measurements were excluded from the analysis.

Construction of the composite image. To construct composite images such as the one shown in Fig. 1g, tubeness filtering (Sato et al., 1998) and intensity thresholding were applied to segment the two-photon angiograms into binary images. Subsequently, PO_2 measurements were spatially co-registered with the segmented angiogram. The three-dimensional composite image was created by color-coding the experimental PO_2 values in the corresponding vascular segments (shades of gray). The color-coding was

performed by assigning the PO₂ (or Mean-PO₂ for capillary) value measured in the focal volume within a vascular segment to the whole segment. For some vascular segments without PO₂ measurements, such measurements were instead available for the segments joining them in each end. To such segments, we assigned the average PO₂ values of the connecting segments.

Statistical analysis. Statistical comparisons were made using Student's t-test (MATLAB, MathWorks Inc.). P value less than 0.05 was considered statistically significant. Specifically, in Figs. 3, 4, and 6c, pairwise t-test was performed between the parameters in layer I and those in each of other layers; in Fig. 5c, pairwise t-test was performed between the flux-PO₂ correlation and each of the other correlations. Further details about the statistical information of measurements are provided in the text and figure legends, where relevant.

Mean values and standard deviations of parameters needed to estimate the sample size were either assumed to be the same as measured previously in anesthetized animals or assumed empirically. Since multiple measurements were performed in the same animals, sample size (i.e., n=15 mice) was set based on anticipation that the most demanding one will be to detect 30% difference between mean EATs values (coefficient of variance = 0.3, power = 0.8, $\alpha=0.05$).

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DATA AVAILABILITY

All data generated or analyzed in support the findings of this study are within this paper.

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AUTHOR CONTRIBUTIONS

Conceptualization: B.L., S.S. and D.A.B.; **Data curation:** B.L. and S.S.; **Formal analysis:** B.L., S.S. and A.D.; **Funding acquisition:** S.S., A.D., D.A.B. and S.A.V.; **Investigation:** B.L. and S.S.; **Methodology:** S.S., S.A.V., D.A.B., A.D., T.V.E., M.A.Y., B.L., K.K., B.F., M.M. and I.S.; **Project administration:** S.S.; **Resources:** S.S.; **Software:** S.S., B.L., M.A.Y. and S.K.; **Supervision:** S.S.; **Validation:** S.A.V., L.Ø., A.D., D.A.B., F.L., M.D. and M.M.; **Visualization:** S.S., B.L. and S.K.; **Writing—original draft:** B.L.; **Writing—review and editing:** S.S., B.L., S.A.V., A.D., L.Ø., D.A.B., F.L., M.D. and M.M.

SUPPLEMENTARY INFORMATION

Supplementary results are available for this paper.

COMPETING INTERESTS

The authors declare that no competing interests exist.

SUPPLEMENTARY RESULTS

Supplementary Figure 1. Schematic diagram of a chronic cranial window and photograph of an awake head-restrained mouse. a. Schematic diagram of a chronic cranial window on mouse skull. **b.** Photograph of an awake head-restrained mouse on the training platform, snapshotted prior to imaging experiment using a CCD camera, under the two-photon microscope.

Supplementary Figure 2. Histograms of capillary Mean-PO₂, RBC flux, hematocrit and speed. **a.** Capillary Mean-PO₂ was measured in 6,544 capillary segments, across $n = 15$ mice. The Mean-PO₂ range is from ~5 mmHg to ~100 mmHg (mean value: 45.6 ± 1.4 mmHg). **b-d.** Capillary RBC flux, hematocrit and speed were measured in a subset of capillaries (967 segments). The RBC flux range (**b**) is from ~2 RBC/s to ~154 RBC/s (mean value: 41.5 ± 1.2 RBC/s); the hematocrit range (**c**) is from 8.6 % to 64.4 % (mean value: 37.2 ± 0.7 %); the speed range (**d**) is from 0.11 mm/s to 3.63 mm/s (mean value: 0.71 ± 0.03 mm/s).

Supplementary Figure 3. Distributions of capillary RBC-PO₂, SO₂ and their STDs and CVs as a function of cortical layer. **a-c.** Distributions of the absolute capillary RBC-PO₂, RBC-PO₂ STD and CV as a function of cortical layer, respectively. **d-f.** Distributions of the absolute capillary SO₂, SO₂ STD and CV as a function of cortical layer, respectively. The analysis in **a-f** was made with the measurements from 400, 356, 118 and 104 capillary segments measured in cortical layers I, II/III, IV and V, respectively, across n = 15 mice. Data are expressed as mean±SEM. The single (*) and double (**) asterisk symbols indicate significant differences (Student's t-test) with P<0.05 and P<0.001, respectively.

Supplementary Figure 4. Quantifications of the temporal fluctuations of capillary RBC flux, speed, hematocrit and Mean-PO₂. **a-d.** Correlations between the STDs and CVs of the temporal fluctuations of capillary RBC flux and Mean-PO₂ and their mean absolute values, calculated based on the 9-s-long measurements. The data points are represented by the blue dots. The linear regression lines of the correlations are in black. The computed slope (and R²) values of the linear regressions in panels **a-d** are: 0.1 (R² = 0.4), -0.002 s·RBC⁻¹ (R² = 0.17), -0.01 (R² = 0.001) and -0.003 mmHg⁻¹ (R² = 0.3), respectively. **e and f.** Pairwise correlations between the temporal fluctuations of RBC flux, speed and hematocrit (Hct). The panel **e** presents the correlation coefficients (r); the panel **f** presents the Fisher z values. The analysis in **a-f** was made with the measurements from 373 capillary segments in cortical layers I-V, across n = 7 mice. The double asterisk symbol (**) indicates significant difference (Student's t-test, P<0.001).

Supplementary Figure 5. Relations between capillary RBC hematocrit, Mean-PO₂, flux, speed and EATs. The analysis in **a-d** was made with the measurements from 373 capillary segments collected in cortical layers I-V, across n = 7 mice. Data are expressed as mean±SEM.

Supplementary Figure 6. Pairwise relations between capillary RBC flux, speed, hematocrit and Mean-PO₂. The analysis in **a-e** was made with the measurements from 978 capillary segments collected in cortical layers I-V, across n = 15 mice. Data are expressed as mean±SEM.

Supplementary Figure 7. Identification of a capillary segment having stalled RBC flow. **a.** A Sulforhodamine-B labelled mouse cortical microvasculature. The enlarged image includes the stalled capillary segment. The PO₂ measurement was performed on the location denoted by the blue dot. **b.** The average phosphorescence decay recorded on the measurement location in **a**. The 285-μs-long phosphorescence decay was used to calculate the phosphorescence lifetime. **c.** The associated phosphorescence intensity time course (3-s trace) acquired on the measurement location in **a**. **d.** A phosphorescence intensity time course (3-s trace) acquired in an arbitrary non-stalled capillary segment as a comparison.

Supplementary Figure 8. Identification of a suspected thoroughfare capillary. **a.** Upper panel: The maximum intensity projection of the vasculature stack (90-120 μm under cortical surface). Lower panel: The enlarged image includes the vascular paths of the suspected thoroughfare capillary. PO₂ measurements were performed on the segments labelled with numbers, and their values are shown next to the vascular image. **b.** Tracking of the arteriole (#1 in the lower panel in **a**) indicated by the red arrow and the venule (#4 in the lower panel in **a**) indicated by the blue arrow, from the cortical depth of 140 μm to the cortical surface.

The maximum intensity projection of the Sulforhodamine-B labelled microvascular stack (cortical depth: 90-120 μm) is shown in the upper panel of Suppl. Fig. 8a. The enlarged image (Suppl. Fig. 8a, lower panel) includes the vascular paths of the suspected thoroughfare capillary.

The vessel types of the vascular segments #1 and #4 were identified as arteriole and venule, respectively, based on their PO₂ values and the morphologies of their parent vessels by tracking them with

the three-dimensional angiogram to the cortical surface. The complete vascular path, starting from the arteriole (#1) to the venule (#4), consists of the vascular segments marked by the blue dots. PO₂ measurements were performed on the locations labelled with numbers, and their PO₂ values are shown at right. The three vascular segments from A to B were identified as capillaries, based on their diameters. The Mean-PO₂ of the pre-venule capillary (#2) was calculated to be 61 mmHg, much higher than the average capillary Mean-PO₂ (45.6±1.4 mmHg; Suppl. Fig. 2a). In addition, the vascular path from A to B consists of only three segments; and the physical length from A to B was estimated to be 85 µm. Therefore, this vascular path (A-B) is short in both number of capillary segments and physical length. We thus suspect that the capillary segment #2 is a thoroughfare channel that transported the highly oxygenated blood back to the venule, contributing to the increase in the venous oxygenation towards brain surface.