Microvascular oxygen quantification using two-photon microscopy

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An instrument is demonstrated that is capable of three-dimensional (3D) vasculature imaging and pO_2 quantification with high spatial resolution. The instrument combines two-photon (2P) microscopy with phosphorescence quenching to measure pO_2. The instrument was demonstrated by performing depth-resolved microvascular pO_2 measurements of rat cortical vessels down to 120 μm below the surface. 2P excitation of porphyrin was confirmed, and measured pO_2 values were consistent with previously published data for normoxic and hyperoxic conditions. The ability to perform 3D pO_2 measurements using optical techniques will allow researchers to overcome existing limitations imposed by polarographic electrodes, magnetic resonance techniques, and surface-only pO_2 measurement techniques. © 2008 Optical Society of America

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Because oxygen is vital to the metabolic processes of all eukaryotic cells, a detailed understanding of its transport and consumption is of great interest to researchers. Current methods for *in vivo* measurement of intravascular and tissue dissolved oxygen concentration (pO_2) at the cellular level are nonideal. Either they lack the three-dimensional (3D) spatial resolution desired, are invasive and disturb the local physiology, or they rely on hemoglobin spectrophotometry, which is not a direct measure of the oxygen available to cells. It has been suggested that combining two-photon (2P) excitation with the pO_2 quantification via phosphorescence quenching [1,2] could lead to a new method that is well suited for high resolution pO_2 measurement *in vitro* and *in vivo* [3].

The phosphorescence quenching technique relies on changes in the phosphorescence lifetime of a molecular probe as it is quenched by oxygen. pO_2 quantification based on this principle has been shown to be an effective and accurate method for measuring dissolved pO_2 both in tissue and vasculature [4–7]. The relationship between lifetime and pO_2 is described by the Stern–Volmer equation $1/\tau = 1/\tau_0 + k_q\, pO_2$, where τ is the measured phosphorescence lifetime, τ_0 is the lifetime of the phosphor under zero-oxygen conditions, and k_q is the quenching constant. Porphyrin-based oxygen probes are typically used for this type of application because porphyrin is known to be exclusively quenched by oxygen and its use *in vivo* has been well established in literature [1,2,4–8]. These types of probes have long lifetimes (~100 s of μs) and quenching constant values that make them well suited for measurement of physiological pO_2.

Combining 2P microscopy with oxygen determination via phosphorescence quenching would in principle have micrometer-scale resolution, have inherent 3D sectioning, and provide absolute pO_2 values. Although Mik *et al.* [8] demonstrated the proof of concept by measuring *in vivo* tissue pO_2 along the axial direction using a 2P excited phosphorescence lifetime technique, to our knowledge there have been no reports in the literature of combining 2P imaging with pO_2 sensing using phosphorescence quenching *in vivo*. This is most probably due to the low 2P action cross section of porphyrin [3] and the timing mismatch between the repetition rate of common 2P excitation sources (nanosecond) and the porphyrin lifetime (microsecond). In this Letter, we describe and demonstrate an instrument that combines 2P microscopy with pO_2 quantification using 2P excited phosphorescence quenching. The instrument is capable of simultaneous 3D imaging of microvasculature and image-guided measurement of intravascular pO_2.

3D image-guided pO_2 measurements are possible because of the simple introduction of an acousto-optic modulator (AOM) to a standard 2P microscope set up to serve as a temporal gate to the laser pulse train (Fig. 1). The AOM (NEOS Technologies 23080-2-LTD) is needed to overcome the timing mismatch between the phosphorescence lifetime of porphyrin *in vivo* (50–200 μs) and the time between pulses from our 2P excitation source (~13 ns). Light ($\lambda = 780$ nm) from a Ti:sapphire oscillator (Coherent Mira 900,

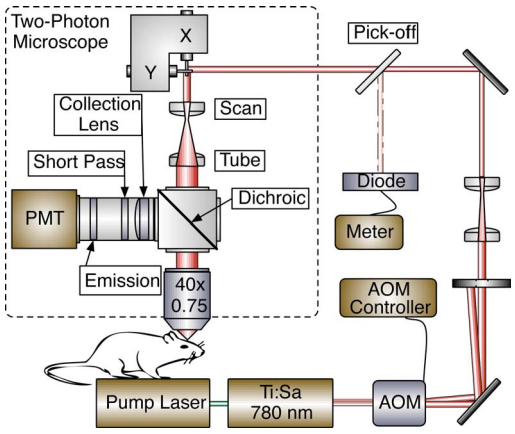

Fig. 1. (Color online) Instrumentation setup. AOM is added to a standard 2P microscope setup to facilitate pO_2 measurement.

10 W pump) is passed through the AOM under computer control. The first-order diffracted beam is directed to beam conditioning optics, to galvanometer scanning mirrors (Galvos model, Cambridge), and finally to an objective (40×0.7 NA water immersion). In imaging mode, a continuous voltage is applied to the AOM, and the pulse train from our excitation source is completely transmitted to the microscope. However, in pO_2 measurement mode, the AOM is made to gate the pulse train with a duty cycle of 1% and a repetition rate of 1 kHz. Fluorescence and phosphorescence signals are detected by a photomultiplier tube (PMT) (H7422P-50, Hamamtsu Corp.). Acquisition of phosphorescence decays was synchronized with gating of the AOM, enabling multiple excitation events to be quickly averaged to improve the signal-to-noise ratio (SNR) as needed.

To demonstrate the capabilities of this new instrument, we simultaneously mapped a region of vasculature and performed intravascular pO_2 measurements of rat somatosensory cortex while the fraction of inspired oxygen (FiO_2) was varied. Sprague-dawley rats were anesthetized for surgery with urethane (1.5, 0.3 g/kg supplements as needed) by intraperitoneal injection and an intravenous line inserted into the femoral vein. The skull and dura were removed over a 3 mm×3 mm area. A well was created around the exposed area, filled with agarose, then sealed with a coverslip that was secured to the stereotaxic frame with a metal coverslip holder as described elsewhere [9]. Dextran-conjugated fluorescein was intravenously injected at a concentration of 83 mg/kg for visualization of cortical vasculature. A solution of 28 mg/ml Oxyphor R2 (Oxygen Enterprises) and 20 mg/ml of BSA (BP671-10 Fisher Scientific) dissolved in physiologic saline was created, and 3 ml/kg of this solution was then intravenously injected. Sequences of vasculature images were recorded from the fluorescein emission at different depths. From these images multiple points within individual vessels were interactively selected for pO_2 measurements using our software. The selected points were translated to galvanometer positions and 2000 phosphorescence decay curves were recorded at each location and averaged together. Because of a transimpedance amplifier with limited bandwidth and an AOM driver with a slow turn-off time, the first 50 μs of data was discarded so as to ignore any artifacts from scattered excitation light. A single exponential decay model was fit to the average phosphorescence decay curve at each location to determine the phosphorescence lifetime. Using published values for the porphyrin quenching constant and unquenched lifetime [10], the lifetimes were converted to pO_2 values using the Stern–Volmer relationship. pO_2 measurements were taken at various depths. At each depth location the FiO_2 was varied and pO_2 data were acquired.

With *in vitro* experiments we verified that the presence of fluorescein had no effect on the porphyrin lifetime at the concentrations used *in vivo*. The phosphorescence lifetime was measured in the absence of oxygen and found to be unchanged, and to approximately match reported values (~500 μs) [4]. *In vitro* experiments also confirmed 2P excitation of porphyrin by observing that the phosphorescence intensity varied as the square of the excitation intensity [Fig. 2(g)].

Figure 2 shows typical results for cortical vessels under normoxic and hyperoxic conditions at depths of 120 and 100 μm. The figure depicts vasculature images at two locations with pO_2 measurement overlays for the two FiO_2 conditions. Vasculature imaging was done using a power of ~3 mW, and pO_2 measurements were taken with an average power of ~1.5–2 mW after the 1% duty cycling. Our combination of objective and laser wavelength resulted in an excitation focal volume of 0.5 μm (radial) by 1.3 μm (axial) in size. For intravascular pO_2 measurements, the focal volume moves downstream with the blood flow as it phosphoresces. Therefore, the spatial resolution of the pO_2 measurement is a function of the local flow rate and the lifetime of the probe at that location. For our experiments, we estimate the resolution to be ~10 μm or better. The measured pO_2 values are in good agreement with values reported in the literature for arterioles under similar conditions [11]. Shonat *et al.* report pO_2 values of ~40 mmHg in the normoxia case and ~60 mmHg in the hyperoxia case (60% FiO_2) for rat cortical arterioles under similar anesthesia.

Figures 2(c) and 2(f) show the nature of the fitting of the averaged phosphorescence signals for the highlighted points within each image. The highlighted pO_2 measurements in Figs. 2(a) and 2(b) had standard errors of ±1.7 and ±3.5 mmHg, respectively, whereas the standard error of the pO_2 measurements highlighted in Figs. 2(d) and 2(e) were ±1.3 and ±1.6 mmHg, respectively. The standard error can be improved as needed by simply averaging more phosphorescence decay events at the expense of temporal resolution. We found that ~2000 decays was sufficient to produce an error of <2 mmHg for the majority of our *in vivo* measurements down to a depth of ~300 μm across ten rats. For greater depths, more averaging is needed because of the SNR decrease caused by the loss of excitation intensity at the focal volume. The poor SNR that results from the low 2P cross section can also be mitigated to some extent by increased porphyrin concentration. We confirmed that for a given intravascular pO_2, the measured decay lifetime did not vary with the concentration of porphyrin in the blood over a range from 130–430 μM. Although these concentrations are relatively high compared to the conditions used to determine the reported [10] quenching constant and unquenched lifetime, these values do not seem to strongly vary with concentration. We estimate that we have a systematic error in our pO_2 calculation no greater than 10% as a result. To more accurately calculate pO_2 from the measured decay lifetime, one would need to perform a calibration of these parameters under similar high concentration conditions. As oxygen probes with higher 2P action cross sections become available, the SNR should improve. This will

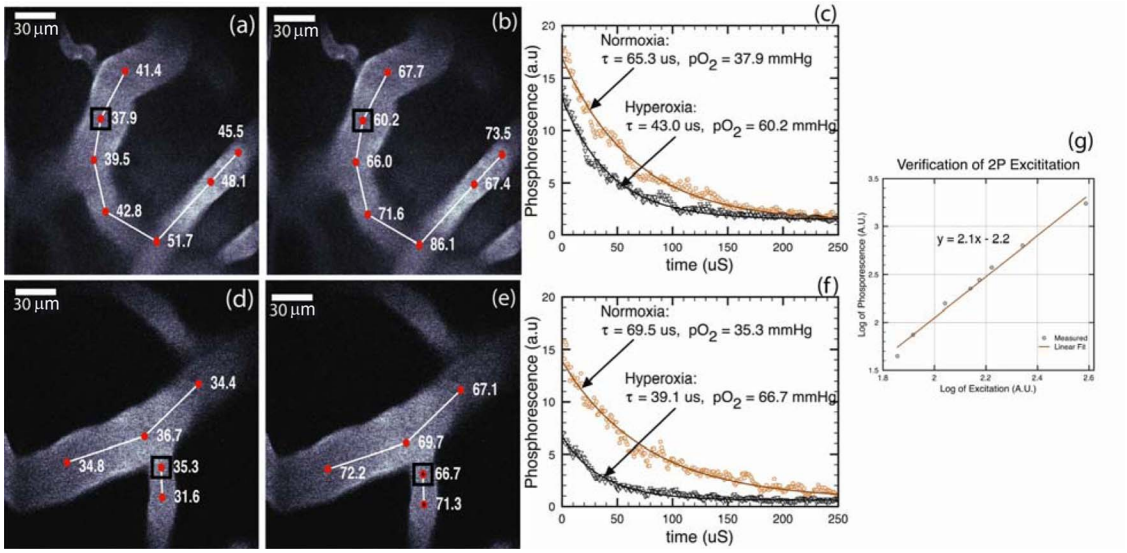

Fig. 2. (Color online) Image of arteriole network 120 μm below the surface under (a) normoxia and (b) hyperoxia. The phosphorescence decay data with best fit curves for the highlighted point in panels (a) and (b) are depicted in (c). Image of arteriole network 100 μm below the surface under (d) normoxia and (e) hyperoxia. The phosphorescence decay data with best fit curves for the highlighted point in panels (d) and (e) are depicted in (f). pO_2 values are in units of mmHg. (g) Verification of 2P excitation of porphyrin.

result in lower concentrations, lower average excitation power, and improved temporal resolution.

Figure 3 further demonstrates the unique capabilities of the instrument. The image shows a branching vessel 58 μm below the surface where one branch contains a highly localized occlusion. Red blood cell motion in the unoccluded branch shows up as an area of lower intensity since only the plasma is labeled. The pO_2 measurements indicate that oxygen is still able to reach the lower branch that is devoid of erythrocytes. However, the pO_2 values in that section are reduced and show a decreasing gradient as we move away from the unoccluded branch.

The instrument described in this Letter is distinct from other pO_2 sensing techniques in that it has the ability to provide image-guided depth-resolved pO_2 measurements at high resolution. Since only a simple modification to a standard 2P microscope setup is required, 3D pO_2 sensing capability can easily be incorporated into many existing 2P imaging setups.

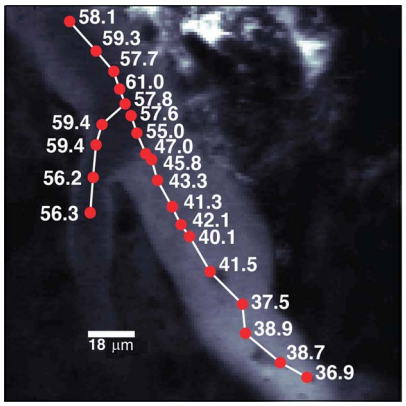

Fig. 3. (Color online) Image of branching cortical vessel 60 μm below the surface. A clear pO_2 gradient is visible owing to an occlusion in the lower branch of the vessel. pO_2 values are in units of mmHg.

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