## In vivo integrated photoacoustic and confocal microscopy of hemoglobin oxygen saturation and oxygen partial pressure

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Received December 15, 2010; revised January 29, 2011; accepted February 18, 2011; posted February 23, 2011 (Doc. ID 139708); published March 16, 2011

We developed dual-modality microscope integrating photoacoustic microscopy (PAM) and fluorescence confocal microscopy (FCM) to noninvasively image hemoglobin oxygen saturation ( $sO_2$ ) and oxygen partial pressure ( $pO_2$ ) in vivo in single blood vessels with high spatial resolution. While PAM measures  $sO_2$  by imaging hemoglobin optical absorption at two wavelengths, FCM quantifies  $pO_2$  using phosphorescence quenching. The variations of  $sO_2$  and  $pO_2$  values in multiple orders of vessel branches under hyperoxic (100% oxygen) and normoxic (21% oxygen) conditions correlate well with the oxygen—hemoglobin dissociation curve. In addition, the total concentration of hemoglobin is imaged by PAM at an isosbestic wavelength. © 2011 Optical Society of America OCIS codes: 170.3880, 170.5120, 170.1790, 180.5810.

Understanding of oxygen transport and consumption  $in\ vivo$  is of great significance to studies of angiogenesis and tumor growth. The oxygen partial pressure,  $pO_2$ , is proportional to dissolved oxygen concentration, and directly measures the oxygen available to cells. The percentage of hemoglobin saturated with oxygen,  $sO_2$ , quantifies the amount of oxygen carried by blood hemoglobin. Both  $pO_2$  and  $sO_2$  are important hemodynamic parameters for oxygen metabolism. Moreover, the relationship of  $pO_2$  and  $sO_2$  describes the binding affinity of hemoglobin for oxygen. The  $in\ vivo$  measurement of the oxygen-hemoglobin dissociation curve (OHDC) describes how our blood carries and releases oxygen under physiological and pathological conditions.

Photoacoustic microscopy (PAM), which can spectroscopically measure hemoglobin absorption [1], is ideal for high-resolution imaging of sO<sub>2</sub> in vivo. Other imaging modalities, such as optical coherence tomography and reflectance absorbance spectroscopy, have been used to map  $sO_2$  [2,3]. However, tissue scattering and the nonlinear relationship between signal intensity and absorption coefficients made their sO<sub>2</sub> quantifications problematic. Besides, two-dimensional reflectance absorbance spectroscopy also suffers from blood volume fluctuation. PAM, on the other hand, is 100% sensitive to optical absorption, fairly insensitive to scattering, and capable of volumetric imaging [4]. The use of phosphorescence lifetime quenching for measuring  $pO_2$  in vasculature has been well established [5–7]. In our studies, a generally used phosphorescent probe, Pd-meso-tetra (4-carboxyphenyl) porphyrin (PdT790, Frontier Scientific), was chosen for its peak absorption wavelength of 524 nm. The oxygen-sensitive phosphorescent probe was injected into the systemic vasculature and excited by light. The resulting phosphorescent emission was quenched by intravascular oxygen. As described by the Stern-Volmer equation, the phosphorescence decay time can be converted to the intravascular pO<sub>2</sub>. Here, we present a dual-modality microscope combining PAM and fluorescence confocal microscopy (FCM), designed for

imaging both blood  $sO_2$  and  $pO_2$  in vivo. By modulating the inspiratory oxygen concentration, the  $sO_2$  and  $pO_2$  responses can be correlated to study oxygenhemoglobin binding.

A schematic of the integrated photoacoustic and FCM (PA-FCM) system is presented in Fig. 1. Details about the PA-FCM system design and performance have been published previously [8]. The system employs a dye laser (CBR-D, Sirah) with tunable wavelengths in the range of 560–590 nm (Rhodamine 6G, Exciton), pumped by a 523 nm Nd:YLF laser (INNOSLAB, Edge-Wave). The 523 nm pump laser pulses excite the oxygensensitive phosphorescent probe; the wavelength-tunable dye laser pulses are used to image hemoglobin absorption at multiple wavelengths. The generated phosphorescent light and photoacoustic wave are collected by a photomultiplier tube module (H9307-03, Hamamatsu, bandwidth, DC-200 kHz) and a 75 MHz ultrasonic transducer (V2022 BC, Olympus NDT), respectively. The phosphorescent light passes through a dichroic mirror (DMLP605, Thorlabs) and an emission filter (FEL0650, Thorlabs). A 150  $\mu$ m diameter emission pinhole suppresses the out-of-focus phosphorescent light rays. To compensate the photoacoustic amplitude for laser fluence fluctuation, the laser pulses are sampled by a

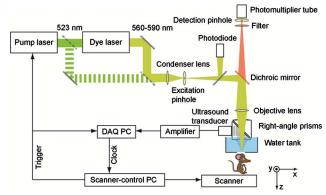


Fig. 1. (Color online) Schematic of the integrated photo-acoustic and confocal microscopy setup.

photodiode (SM05PD1A, Thorlabs). The amplified photoacoustic or phosphorescence signals are acquired and saved along with the laser fluence signals by a data acquisition instrument (CS 14200, Gage Applied).

Nude mouse (Harlan, body weight  $\sim 20\,\mathrm{g}$ ) ears were imaged to demonstrate the dual-modality microscopy of  $\mathrm{sO}_2$  and  $\mathrm{pO}_2$  in vivo. All experimental animal procedures were carried out in conformity with the laboratory animal protocol approved by the Animal Studies Committee of Washington University in St. Louis.

The PdT790  $(10\,\text{mg/ml})$  was conjugated with bovine serum albumin  $(60\,\text{mg/ml})$  in 0.9% NaCl solution to provide a uniform environment for bound phosphors [5]. A 0.1 ml volume of the phosphorescent probe solution was bolus injected into the systemic vasculature via the tail vein. To allow the probe to equilibrate in the blood, image acquisition started 10 min after injection.

Photoacoustic images at wavelengths of 570 and 578 nm were captured. From the photoacoustic amplitude, aided by the molar absorption spectra of oxygenated hemoglobin (HbO<sub>2</sub>) and deoxygenated hemoglobin (HbR), the relative concentrations of HbO<sub>2</sub> and HbR, and subsequently sO<sub>2</sub>, were calculated [1]. To measure the phosphorescence quenching, the phosphorescent light intensity was acquired for  $500\,\mu s$  at a sampling rate of  $20\,MHz$ . The relationship of pO<sub>2</sub> and the phosphorescence lifetime was assumed to follow the Stern–Volmer equation

$$\tau^{-1} = \tau_0^{-1} + k_a \cdot pO_2, \tag{1}$$

where  $\tau_0$  is the lifetime in the absence of  $O_2$  and  $k_q$  is the quenching constant. The constants  $\tau_0$  and  $k_q$  have been experimentally calibrated and published in the literature [5,9]. We used the quenching constants for pH = 7.4 and temperature = 23 °C ( $\tau_0$  = 711  $\mu$ s,  $k_q$  = 259 mm Hg<sup>-1</sup> s<sup>-1</sup>) [3]. A detailed description of the method used for computing sO<sub>2</sub> and pO<sub>2</sub> can be found in review articles [1,5].

To study the relationship of  $pO_2$  and  $sO_2$  in vivo, the blood  $pO_2$  and  $pO_2$  levels were modulated by switching the physiological state from systemic hyperoxia to normoxia in a mouse. Hyperoxia was induced by changing the inhalation gas to 100%  $O_2$ , and the mouse was returned to normoxia by changing the inhalation gas to air. Prior to imaging, the mouse was exposed to each oxygen concentration for 10 min to stabilize the hyperoxic and normoxic states.

First, to explore the mapping of  $pO_2$  and  $sO_2$ , we imaged a nude mouse ear under hyperoxia. Figure 2(a) shows a photoacoustic image of the mouse ear vasculature acquired at 570 nm, an isosbestic wavelength where HbO<sub>2</sub> and HbR have identical molar absorption coefficients. Thus the photoacoustic amplitude measures the total hemoglobin (HbT) concentration. By combination with another photoacoustic image acquired at 578 nm, a pixel-by-pixel map of sO<sub>2</sub> was computed. As shown in Fig. 2(b), the arterioles and venules are visualized in pseudocolors of red and green, based on the different  $sO_2$  levels. Figure 2(c) shows the time-integrated phosphorescence image, where sebaceous glands and blood vasculature can be seen as speckle and tree features, respectively. Autofluorescence from tissue often has a submicrosecond lifetime, while the phosphorescence

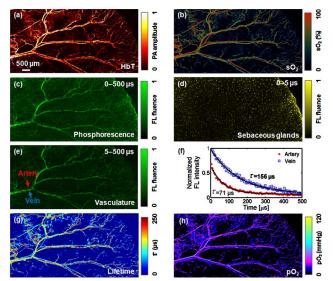


Fig. 2. (Color online) Imaging of the ear of a mouse in hyperoxia  $in\ vivo.$  (a) Photoacoustic image of total concentration of hemoglobin acquired at isosbestic 570 nm. (b) Photoacoustic image of  $sO_2$  acquired at 570 and 578 nm. (c) Confocal image of time-integrated phosphorescence excited at 523 nm. Sebaceous glands and blood vessels are separated in the confocal images by integrating the phosphorescence signal (d) before and (e) after  $5\,\mu s.$  (f) Phosphorescence decay curves from two regions of  $15\times15$  pixels each in the artery and vein indicated by the arrows in (e). (g) Confocal image of the phosphorescence lifetime. (h) Confocal image of pO2: PA, photoacoustic; FL, fluorescence; HbT, total hemoglobin concentration; and  $\tau$ , phosphorescence lifetime.

from the palladium porphyrin phosphorescent probe features  $\sim 100 \,\mu s$  decay time. We split the phosphorescence signal at  $5\mu s$  so that the images of sebaceous glands [Fig. 2(d)] and blood vasculature [Fig. 2(e)] are separated. Figure 2(f) plots example of phosphorescence decay curves measured in the artery and vein labeled with arrows in Fig. 2(e). The phosphorescence lifetime was determined by fitting the measured data to an exponential decay curve ( $R_2 = 0.98$  for arterial data and 0.99 for venous data). The shorter lifetime for the arterial data  $(71 \,\mu\text{s})$  compared with that for the venous data  $(156 \,\mu\text{s})$ shows phosphorescence quenching by dissolved blood oxygen. Pixelwise fitting produces a map of phosphorescence lifetime [Fig. 2(g)], which is further converted through Eq. (1) to a map of  $pO_2$  [Fig. 2(h)]. A comparison of Figs. 2(b) and 2(h) shows that the blood vessels with higher sO<sub>2</sub> values measured by PAM have correspondingly higher pO<sub>2</sub> values measured by FCM, which agrees with known physiology [10].

To closely investigate the  $pO_2$  and  $sO_2$  levels in response to oxygen variation,  $sO_2$  and  $pO_2$  in hyperoxia (100% oxygen) and normoxia (21% oxygen) were mapped. We selectively analyzed an ~1.5 mm × 1.5 mm area of a mouse ear that contained four microvascular branching orders, as shown in Fig. 3(a) (photoacoustic image) and Fig. 3(b) (phosphorescence image). Figures 3(c)–3(f) show the  $sO_2$  and  $pO_2$  mappings for hyperoxic and normoxic conditions. Our results suggest that switching from hyperoxia to normoxia elicited a decrease in both  $sO_2$  and  $pO_2$  levels. They further suggest that in the artery, the  $sO_2$  remained high (>80%) while

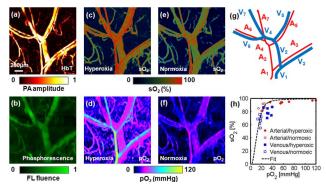


Fig. 3. (Color online) Photoacoustic and confocal microscopy of  $pO_2$  and  $sO_2$  levels in the ear of a mouse in response to switching from hyperoxia to normoxia. (a) Photoacoustic image of total concentration of hemoglobin acquired at 570 nm. (b) Confocal image of time-integrated phosphorescence in the same region excited at 523 nm. (c) Photoacoustic image of  $sO_2$  and (d) confocal image of  $pO_2$  in hyperoxia. (e) Photoacoustic image of  $sO_2$  and (f) confocal image of  $sO_2$  in normoxia. (g) Skeletons of arteries (Ai) and veins (Vi) shown in (a), where subscript i denotes the segmentation. (h) Plot of  $sO_2$  versus  $pO_2$  values in arteries and veins identified in (g). PA, photoacoustic; FL, fluorescence; and HbT, total hemoglobin concentration.

the pO $_2$  dropped significantly (from >100 mm Hg to ~30 mm Hg). In the vein, the decrease of sO $_2$  (from ~80% to ~70%) was correlated with a smaller decrease in pO $_2$  (from ~35 mm Hg to ~20 mm Hg). Our observation is in agreement with the sigmoidal shape of the OHDC. The precapillary arteriolar and postcapillary venular trees are drawn in red and blue, respectively, in Fig. 3(g). The vasculature in the imaged region is segmented by different branching orders, and the sO $_2$  and pO $_2$  values were averaged within each segment (the correlation coefficient between sO $_2$  and pO $_2$  values = 0.62). We compared the experimental data with the classic OHDC equation developed by Kelman [11]

$$sO_2 = 100 \times \frac{a_1 + a_2 x^2 + a_3 x^3 + x^4}{a_4 + a_5 x + a_6 x^2 + a_7 x^3 + x^4},$$
 (2)

where  $a_{1-7}$  are coefficients calibrated at the standard condition (temperature  $T=37\,^{\circ}\mathrm{C},~\mathrm{pH}=7.4,~\mathrm{and}~\mathrm{CO_2}$  partial pressure  $\mathrm{pCO_2}=40\,\mathrm{mm\,Hg}$ ), and

$$x = f(T, pH, pCO_2) \times pO_2, \tag{3}$$

$$f = 10^{[-0.024(T-37)+0.40(pH-7.40)-0.06(\log_{10}pCO_2-\log_{10}40)]}.$$
 (4)

The conversion factor f alters the scale of the  $pO_2$  axis in response to changes of temperature, pH, and  $CO_2$  partial pressure [11]. Although both the blood pH and  $pCO_2$  vary with the vasculature order and the physiological state [12], the OHDC maintains the sigmoidal shape. To demonstrate the nonlinear tendency for oxygen to bind to hemoglobin in the measured data, we applied a least-squares fitting of the  $pO_2$  and  $sO_2$  values to the above Kelman's equation with f being the fitting parameter. The fitted curve (f=1.83) rises steeply with increasing  $pO_2$  and reaches 90%  $sO_2$  at  $pO_2$  of  $32 \, \text{mm}$  Hg. The correlation coefficient between the fitted and measured  $sO_2$  values was 0.67.

In summary, we developed an integrated PA-FCM system to image  $sO_2$  and  $pO_2$  as well as the total concentration of hemoglobin vessel by vessel  $in\ vivo$ . The ability to extract noninvasively  $sO_2$  and  $pO_2$  information in individual vessels makes the dual-modality microscope system a potential tool for quantitative analysis of oxygen transport and consumption in tissues.

We thank C. Zhang for helping with data processing and manuscript preparation. This work was sponsored in part by National Institutes of Health (NIH) grants R01 EB000712, R01 EB008085, R01 CA134539, U54 CA136398, and 5P60 DK02057933. L. Wang has a financial interest in Microphotoacoustics, Inc., and Endra, Inc., which, however, did not support this work.

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