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Abstract. Myoglobin is an essential oxygen-binding hemoprotein in skeletal and cardiac muscles that buffers intracellular oxygen (O_2) concentration in response to hypoxia or elevated muscle activities. We present a method that uses photoacoustic computed tomography to measure the distribution of myoglobin in tissue and the oxygen saturation of myoglobin (sO_2 -Mb). From photoacoustic measurements of mice in different oxygenation states, we performed calibration-free quantification of the sO_2 -Mb change in the backbone muscle *in vivo*. © 2015 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.21.6.061002]

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Myoglobin is a primary oxygen-carrying protein expressed in skeletal muscle fibers and cardiac myocytes.¹ It functions as an oxygen-storage unit, facilitating the diffusion of oxygen from the cell membrane to mitochondria. Quantification of oxygen saturation of myoglobin (sO_2 -Mb) is of great interest in pre-clinical and clinical applications. For instance, an increased capability of myoglobin to buffer oxygen in skeletal muscle is closely related to chronic heart failure.² Myoglobin may also be used as a cardiac biomarker in the blood stream to help diagnose a heart attack.³

Myoglobin has been demonstrated, along with hemoglobin, as a main muscle chromophore in the visible and near-infrared (NIR) spectral regions.⁴⁻⁶ Both myoglobin and hemoglobin have oxygen-bound and oxygen-unbound states that are spectrally distinct. Currently, optical spectroscopy,^{4,7} diffuse optical tomography (DOT),⁸ and functional magnetic resonance imaging (f-MRI)⁹ are mainly used to quantify sO_2 -Mb *in vivo*; however, all three techniques have limitations. Optical spectroscopy and DOT have low spatial resolution, and f-MRI is sensitive only to deoxygenated myoglobin.¹⁰

Photoacoustic tomography (PAT) can overcome the aforementioned limitations. The conversion of light to ultrasound provides this hybrid imaging technique with ultrasonically defined spatial resolution at depths beyond the optical diffusion limit¹¹ (~1 mm in skin). PAT directly measures specific optical absorption (J/cm^3), which is the product of the optical absorption coefficient (1/cm) and the local optical fluence (J/cm^2).^{12,13} Without invasive measurements or sophisticated modeling of light propagation to obtain the local optical fluence, we can quantify the absolute sO_2 by taking advantage of its dynamics.¹⁴

Photoacoustic (PA) signal amplitude is proportional to the initial pressure rise p_0 as given below:

$$p_0 = \Gamma \eta_{th} F \mu_a, \quad (1)$$

where Γ is the Grüneisen coefficient, η_{th} is the percentage of the specific optical absorption that is converted into heat, F is the

local optical fluence, and μ_a is the optical absorption coefficient. In muscle tissue, the major absorbers in the visible and NIR optical spectral regions are oxygenated myoglobin (MbO_2), deoxygenated myoglobin (Mb), oxygenated hemoglobin (HbO_2), and deoxygenated hemoglobin (Hb).⁶ Figure 1(a) shows the optical absorption spectra of the major chromophores found in muscle. Their distinct spectra give us an opportunity to differentiate each chromophore. The optical absorption coefficient at wavelength λ can be expressed as

$$\begin{aligned} \mu_a(\lambda, s) = \ln(10) C_T(s) [& f_{MbO_2}(s) \cdot \epsilon_{MbO_2}(\lambda) + f_{Mb}(s) \\ & \cdot \epsilon_{Mb}(\lambda) + f_{HbO_2}(s) \cdot \epsilon_{HbO_2}(\lambda) \\ & + f_{Hb}(s) \cdot \epsilon_{Hb}(\lambda)], \end{aligned} \quad (2)$$

$$f_{Hb} = 1 - f_{MbO_2} - f_{Mb} - f_{HbO_2}, \quad (3)$$

where s denotes the oxygenation state; C_T is the total concentration of myoglobin and hemoglobin; f_{MbO_2} , f_{Mb} , f_{HbO_2} , and f_{Hb} are the respective concentration fractions of MbO_2 , Mb, HbO_2 , and Hb relative to the total concentration C_T . ϵ_{MbO_2} , ϵ_{Mb} , ϵ_{HbO_2} , and ϵ_{Hb} are the respective molar extinction coefficients of the four chromophores. Between two different oxygenation states ($s = a$ and b), the ratio of the measured PA signal amplitudes (P) can be written as follows according to Eqs. (1) and (2).¹⁴

$$\frac{P(\lambda, a)}{P(\lambda, b)} = \frac{F(\lambda, a)}{F(\lambda, b)} \cdot \frac{C_T(a)}{C_T(b)} \cdot \frac{\mu_a(\lambda, a)}{\mu_a(\lambda, b)}. \quad (4)$$

Γ and η_{th} do not appear in the ratio because they do not change between different sO_2 states. For red and NIR light, the change in optical attenuation in the skin due to variations in sO_2 (e.g., from 90% to 30%) is less than 3%,¹⁵ mainly because of the high optical scattering and low volume fraction of blood in the skin. Therefore, we can neglect the change in local optical fluence due to variations in sO_2 , i.e.,

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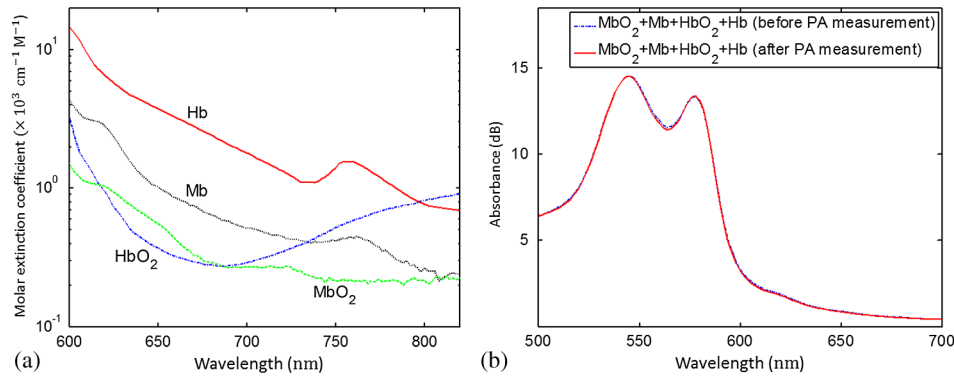


Fig. 1 Optical absorption properties of myoglobin. (a) Absorption spectra of major chromophores found in muscle, measured at wavelengths from 600 to 820 nm. (b) Absorption spectra of a mixture of MbO₂ (60.7%), Mb (24.8%), HbO₂ (6.9%), and Hb (7.6%), measured using a spectrophotometer before and after the photoacoustic (PA) experiment.

$F(\lambda, a) = F(\lambda, b)$.¹⁴ In addition, if we assume that the total concentration C_T does not change, i.e., $C_T(\lambda, a) = C_T(\lambda, b)$, the first two terms on the right-hand side of Eq. (4) can be removed. To solve the remaining six unknowns r_{MbO_2} , r_{Mb} , r_{HbO_2} in the a and b states, we need to measure the PA signals at a minimum of six wavelengths.

The source and the method adopted to prepare the solutions of pure MbO₂, Mb, HbO₂, and Hb were the same as reported in previous papers.^{4,7} The absorption spectra of the solutions were measured using a standard spectrophotometer (Varian Cary 50). The spectrophotometer measured the absorbance, which was the product of the molar extinction coefficient, molar concentration, and the path length of the cuvette. From the known molar extinction coefficients at various wavelengths and the path length of the cuvette, we derived the molar concentrations. To the mixture of MbO₂ and HbO₂, we added a moderate amount of sodium dithionite to partially deoxidize the sample, giving the spectrum shown in Fig. 1(b) (dashed line). From the spectrum, we calculated the concentration fractions of the four chromophores as a reference. After PA measurement, we repeated the spectral measurement [solid line in Fig. 1(b)]. We covered the cuvettes and the phantom with a piece of transparent film to avoid exposing the mixtures to air during the two spectrophotometer measurements (~ 5 min).

We first performed a phantom study to validate our method. We made an agar cylinder with four square vertical tunnels [Fig. 2(a)]. Each tunnel was 5-mm long, 5-mm wide, and

70-mm high, with a 3-mm spacing between tunnels. Initially, 0.11 mL 20% intralipid (deoxygenated) was injected in each tunnel. The reduced scattering coefficient of the intralipid solution was around 100 cm^{-1} at 700 nm.¹⁶ Three mixtures of MbO₂, Mb, HbO₂, and Hb with the same total concentration and different concentration fractions were then added into the first three tunnels, and the fourth tunnel was filled with pure water. The absorption coefficients of the first three samples at 700 nm were 0.23, 0.15, and 0.36 cm^{-1} , respectively. The samples in all tunnels had the same reduced scattering coefficient of 10 cm^{-1} at 700 nm.

To validate our model, we used a photoacoustic computed tomography (PACT) system equipped with a ring-shaped ultrasonic transducer array.^{17,18} The agar phantom was $\sim 5/6$ immersed in water and fixed at the center of the transducer array. The imaging plane was set at 2 mm below the sample's liquid surface. At the imaging plane, a 0.1-mm radial resolution and <0.25 -mm tangential resolution were achieved. For multi-wavelength imaging, we combined an optical parametric oscillator laser and a Ti-sapphire laser, each pumped by an Nd:YAG laser with a 10-Hz pulse repetition rate. After passing through a ground glass, the light beam was expanded and delivered onto the top of the phantom [Fig. 2(a)]. The laser fluence at the phantom surface was around 2.6 mJ/cm^2 . We imaged the phantom at 600, 620, 640, 734, 757, and 800 nm, with 60 times averaging at each wavelength. Figure 2(b) shows a representative image of the phantom acquired at 600 nm.

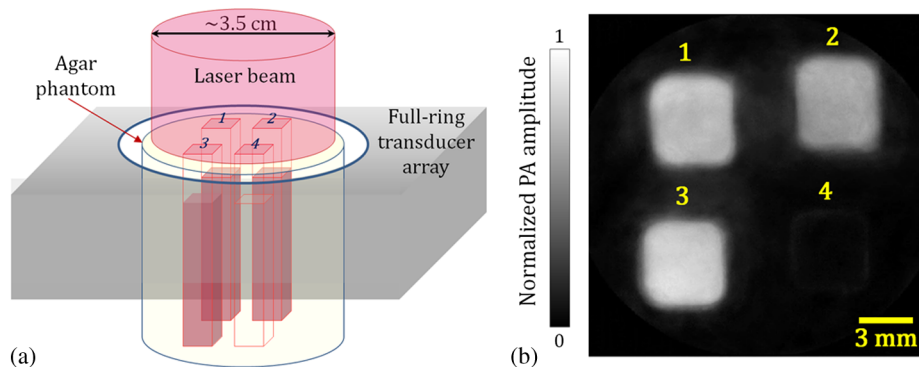


Fig. 2 Experimental setup and a representative PA image of the phantom. (a) Schematic of the phantom setup. (b) PA image of the phantom acquired at 600 nm.

Table 1 Summary of the phantom experimental results.

Concentration fraction	Tunnel #1		Tunnel #2		Tunnel #3		Tunnel #4 (water)
	True fraction (%)	Measured fraction (\pm standard error)	True fraction (%)	Measured fraction (\pm standard error)	True fraction (%)	Measured fraction (\pm standard error)	
f_{MbO_2}	60.7	59.5% ($\pm 2.4\%$)	78.5	71.4% ($\pm 2.3\%$)	0	2.6% ($\pm 1.4\%$)	—
f_{Mb}	24.8	22.1% ($\pm 1.7\%$)	0	2.3% ($\pm 1.3\%$)	85.5	77.6% ($\pm 1.9\%$)	—
f_{HbO_2}	6.9	8.3% ($\pm 2.0\%$)	21.5	24.6% ($\pm 2.4\%$)	0	3.3% ($\pm 1.2\%$)	—
f_{Hb}	7.6	10.1% ($\pm 1.5\%$)	0	1.7% ($\pm 0.9\%$)	14.5	16.5% ($\pm 2.1\%$)	—

From the images acquired at each wavelength, we first averaged the PA signals within each tunnel, and then subtracted the water absorption (PA signal amplitude in tunnel #4) from the first three tunnels before substituting the data into Eq. (4). The spectral unmixing results are summarized in Table 1. In this table, the true concentration fractions were calculated by using the molar extinction coefficient of each chromophore and the spectrophotometer measurements of the mixture, e.g., Fig. 1(b). The measured concentration fractions were in good agreement with the true values, with an average error of 10%. The accuracy is still sufficient to measure the $s\text{O}_2$ change *in vivo* from hyperoxia induced with 100% O_2 inhalation to hypoxia induced with 10% O_2 inhalation.^{14,19}

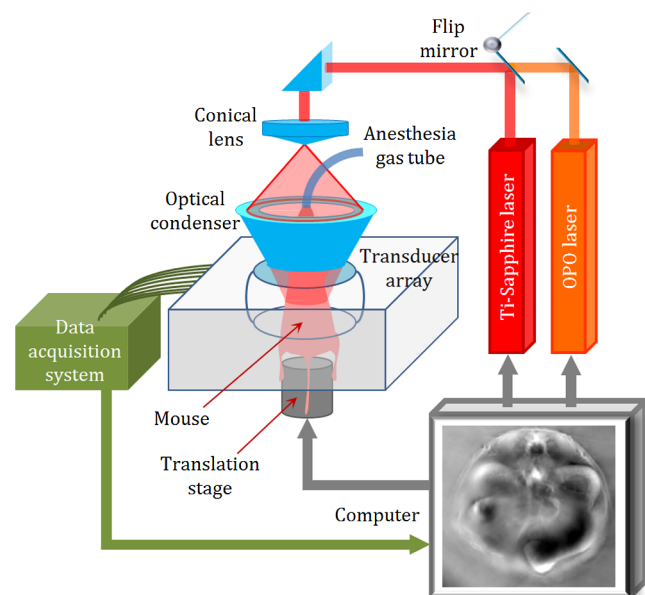
In the above phantom experiment, the first three samples with different oxygenation levels served as the different oxygenation states required for the ratiometric $s\text{O}_2$ calculation by using Eqs. (2) and (4). The estimated effective attenuation coefficients of the three samples were 2.65, 2.14, and 3.34 cm^{-1} at 700 nm, respectively. Therefore, the optical fluence at the imaging plane was different for each sample. The ratio between the light fluence in tunnel #1 and tunnel #2 was 0.91, and the ratio between the light fluence in tunnel #1 and tunnel #3 was 1.15. Because Eq. (4) assumes that the optical fluence remains constant between different oxygenation states, the actual difference in optical fluence between different samples (i.e., different oxygenation states) was the major source for the $s\text{O}_2$ measurement errors.

To further test the method, we noninvasively imaged a systemic $s\text{O}_2$ change in the backbone muscle of a mouse *in vivo*. All experimental procedures were carried out in conformity with the laboratory animal protocols approved by the Animal Studies Committee at Washington University in St. Louis. Before imaging, the hair on the mouse's body was removed with a depilatory. The mouse was then taped to a lab-made motorized animal holder, which was then mounted to the PACT system. Figure 3 shows a schematic of the experimental setup.²⁰ The $s\text{O}_2$ level in the backbone muscle was adjusted by challenging the animal with different oxygen concentrations in the inhalation gas. At first, the anesthesia gas was a mixture of pure oxygen and isoflurane, keeping the mouse in hyperoxia. We used 780 nm light with full-ring illumination to obtain a cross-sectional image of the mouse at the kidney level, as shown in Fig. 3. We then adjusted the light to the mouse back to form a half-ring illumination to image the backbone muscle at six wavelengths. The laser fluence on the mouse's dorsal surface was around 25 mJ/cm^2 .

We switched the six wavelengths one by one to image the mouse backbone region during hyperoxia. Then we decreased

the inhaled O_2 to 10% and increased N_2 to 90%, waited 3 min so that the mouse was in hypoxia, and repeated imaging with six wavelengths. Finally, we increased the inhaled O_2 back to 100% and repeated the measurements to compare the $s\text{O}_2$ -Mb values in two hyperoxic states. At each wavelength, we averaged 60 times.

To calculate the hemoprotein $s\text{O}_2$, we first smoothed the PA images with a 5×5 median filter. We then substituted the measurements into Eq. (4) and calculated the pixelwise concentration fraction of MbO_2 , Mb, HbO_2 , and Hb. Figure 4(a) shows the distribution of myoglobin in hyperoxia (state *a*), and Fig. 4(b) shows the distribution of hemoglobin in the same state. These two images clearly differentiate the regions of the spinal cord, backbone muscle, and kidney, which conform to the background structural image (shown in Fig. 3). The relative changes in myoglobin and hemoglobin $s\text{O}_2$ from hyperoxia (state *a*) to hypoxia (state *b*) are shown in Figs. 4(c) and 4(d), respectively. In Fig. 4(c), the $s\text{O}_2$ -Mb decreased primarily in the backbone muscle region when we reduced the inhaled O_2 concentrations. In Fig. 4(d), we observed that the hemoglobin $s\text{O}_2$ systemically decreases in both the backbone muscle and other internal organs. Moreover, with the same hypoxic challenge, the hemoglobin $s\text{O}_2$ decreased faster than

**Fig. 3** Schematic of the experimental setup for *in vivo* $s\text{O}_2$ measurement in backbone muscle.

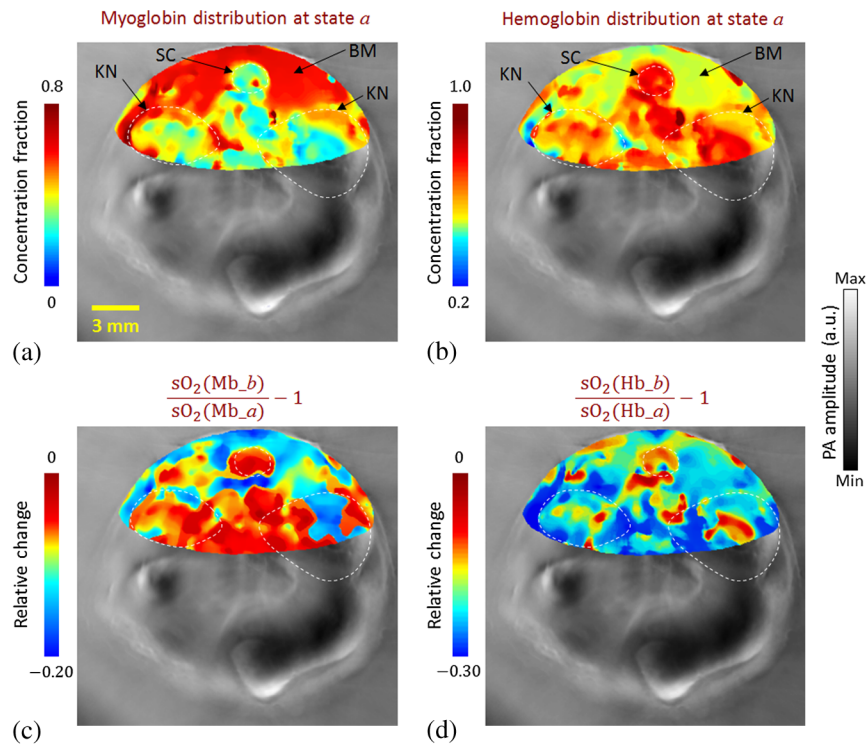


Fig. 4 *In vivo* experimental results. (a, b) Concentration fraction of (a) myoglobin and (b) hemoglobin in hyperoxia (state a) (shown in color) overlaid on the structural image (shown in gray). (c, d) The relative change in (c) myoglobin sO_2 and (d) hemoglobin sO_2 from hyperoxia (state a) to hypoxia (state b) (shown in color) overlaid on the structural image (shown in gray). BM, backbone muscle; KN, kidney; and SC, spinal cord.

the myoglobin sO_2 , probably due to the fact that myoglobin has higher oxygen affinity than hemoglobin.¹⁹

An accurate sO_2 measurement requires knowledge of the local optical fluence, which can be estimated through invasive measurements²¹ or sophisticated modeling of light transportation.²² To address this issue, we used a ratiometric method¹⁴ based on the dynamics in sO_2 , where knowledge of optical fluence was not required. We neglected the change in optical attenuation in tissue due to variations in sO_2 , mainly because of the high scattering and low volume fraction of blood in the skin. In future studies, a more accurate noninvasive sO_2 measurement in deep tissue can be achieved by taking into consideration of the light fluence changes in different sO_2 states. Direct validation of the *in vivo* sO_2 -Mb measurement in deep tissue is challenging. Invasive partial pressure of oxygen (pO_2) oximetry²³ or fluorescence lifetime-based pO_2 measurement²⁴ might be a surrogate validation. The spatial resolution of the sO_2 -Mb measurement is around 0.25 mm, which is finer than that of DOT (4 to 7 mm)²⁵ and f-MRI (1 to 5 mm).²⁶

In summary, we reported, for the first time, the PAT of myoglobin sO_2 based on the dynamics of PA signals at different oxygenation states. Using PACT, we demonstrated this method both in phantoms and *in vivo*. Because six wavelengths were used, the current *in vivo* measurements are not fast enough to monitor the dynamics of sO_2 -Mb changes. The imaging speed can potentially be improved by using faster wavelength switching. Noninvasive *in vivo* measurements of sO_2 -Mb might be useful in medical and surgical procedures.^{27,28} By taking advantage of the high spatial resolution of PACT, applications such as cardiac surgery and sports medicine might particularly benefit.

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