

Journal of Biomedical Optics

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Abstract. The determination of oxygen levels in blood and other tissues *in vivo* is critical for ensuring proper body functioning, for monitoring the status of many diseases, such as cancer, and for predicting the efficacy of therapy. Here we demonstrate, for the first time, a lifetime-based photoacoustic technique for the measurement of oxygen *in vivo*, using an oxygen sensitive dye, enabling real time quantification of blood oxygenation. The results from the main artery in the rat tail indicated that the lifetime of the dye, quantified by the photoacoustic technique, showed a linear relationship with the blood oxygenation levels in the targeted artery. © 2012 Society of Photo-Optical Instrumentation Engineers (SPIE). [DOI: 10.1117/1.JBO.17.5.057004]

Keywords: hypoxia; oxygen sensing; oxygen sensitive dye; photoacoustics; lifetime-based sensing.

Paper received Jan. 4, 2012; revised manuscript received Mar. 16, 2012; accepted for publication Mar. 29, 2012; published online May 4, 2012.

1 Introduction

Oxygen plays a key role in the energy metabolism of living organisms. Hypoxia is a condition where the body or a particular region of a body has less than adequate oxygen; while hypoxemia refers to a low level of oxygen in the blood. Depending on the severity of the hypoxia or hypoxemia, it may lead to complications such as unconsciousness, seizures, coma and even death.^{1,2} Hypoxia is also a very important factor in tumor biology as it is very prominent in tumor tissues. It is a common characteristic of locally advanced solid tumors and has been revealed to promote angiogenesis, tumor aggressiveness, local recurrence and metastasis.³⁻⁵ Hypoxia is also known to impede the effectiveness of different therapies such as photodynamic therapy, radiotherapy, and chemotherapy.⁵⁻⁷

Several methods have been developed to monitor oxygenation *in vivo*. One of the most commonly used techniques involves polarographic electrodes.⁶ However, this technique is invasive in nature and also has the tendency to consume oxygen from the surroundings. Other non-invasive methods such as magnetic resonance imaging (MRI),⁸ positron emission tomography (PET),⁹ and phosphorescence¹⁰⁻¹⁴ have also been explored previously. Although these techniques have a fairly high degree of success, there are drawbacks. PET is expensive, with limited availability, and utilizes ionizing radiation, which can be harmful to the body. MRI is also fairly expensive and cannot be used for patients with implants or pacemakers. Neither can be very conveniently used. They are also not suitable for continuous online monitoring of oxygen concentration. The use of phosphorescent oxygen indicator dyes is highly attractive, due to its high sensitivity and ability for continuous monitoring of rapid kinetic changes in the surrounding oxygen level, and also due to the relative simplicity and low-cost of optical techniques. These dyes can be used to determine the oxygen level quantitatively, by monitoring the change in the

upper (excited) state lifetime or the change in the absolute phosphorescence intensity. Monitoring the upper state lifetime of the dye is more desirable because it eliminates errors due to variations in dye concentration, absolute signal calibrations, autofluorescence, light scattering, etc., or, alternatively, the requirement for a good reference dye.

Although the standard optical phosphorescence and fluorescence techniques are highly sensitive and reliable, they suffer from low penetration depth due to the strong optical attenuation in tissues, especially blood. Moreover, the autofluorescence from tissues also poses potential problems. In comparison to conventional optical fluorescence techniques, photoacoustic imaging (PAI), combining the merits of both light and ultrasound, can lead to much better penetration depth, of up to a few centimeters, while preserving high spatial resolution.¹⁵ PAI does not require the use of ionizing radiation, as do some of the other imaging modalities.

Oxygenated- and deoxygenated-hemoglobin, the two major chromophores in the blood, have distinct optical absorption spectra in the spectral range from visible to near-infrared. Therefore, PAI, when performed in a spectroscopic way by using multiple wavelengths, could evaluate the blood oxygen saturation based on the endogenous contrast between the two forms of hemoglobin, without using any contrast agent.^{16,17} However, due to the strong optical attenuation of biological tissue, which is also a function of laser wavelength, the distribution of light fluence in a sample is strongly heterogeneous in most cases, especially when the imaged sample has a large scale and a complicated morphological structure. Inaccurate prediction of light fluence in the sample, at the wavelengths for spectroscopic imaging, strongly affects the accuracy of PAI in quantifying blood oxygen saturation based on the endogenous optical contrast. Thus in most deep tissue applications, the hemoglobin-based spectroscopic photoacoustic measurements of blood oxygenation can achieve only limited accuracy.

Here, we demonstrate the technique of photoacoustic lifetime (PALT) measurement using an oxygen sensitive dye to quantify the oxygen concentration in biological tissues *in vivo*. Unlike the

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spectroscopic PAI measurements based on the endogenous contrast, this technique can evaluate the oxygen concentration in not only blood, but also other media, such as water, as well as blood-devoid tissues. Moreover, since the PALT measurement is focused on the exponential decay curve that gives the lifetime of the indicator agent, rather than the absolute photoacoustic signal intensity from the indicator agent, the PALT based measurement of oxygen saturation is not affected any more by the heterogeneous optical properties and the inhomogeneous light fluence in the background.

The technique of PALT measurement of indicator dyes was first developed in our lab.¹⁸ A porphyrine based dye, Pt(II) octaethylporphine, was used for the purpose of *in vitro* oxygen sensing. However, this particular dye has an absorption at 532 nm, which would severely limit the penetration depth for any *in vivo* measurements, as a result of the strong light scattering and absorption in the visible spectrum region. Moreover, the dye was not soluble in aqueous solution, and therefore not readily applicable for biological applications. This technique was further developed with an oxygen sensitive water-soluble methylene blue dye¹⁹ that has an absorption peak at 660 nm, enabling improved imaging depth in optically scattering tissues. However, often the methylene blue dye, in its free form, gets degraded into an inactive leuco (white) form in the body, by the ubiquitous cellular coenzymes, NADH and FADH.^{20,21} Both the previous studies on oxygen sensing using methylene blue and PtOEP had been limited to *in vitro* applications.

To validate the feasibility of this technique for *in vivo* measurements of oxygenation, we used Pd-tetra-(4-carboxyphenyl) tetraabenzoporphyrin dendrimer (G2) (from Oxygen Enterprises Ltd., Philadelphia, PA) as an oxygen sensing dye.¹⁰⁻¹⁴ The G2 dye is based on a dendrimer with a Pd-tetraabenzoporphyrin core. The G2 dye and a newer variant of the same dye have previously been used for oxygen mapping in tumors *in vivo*.^{10,22} It has a ground state absorption maximum at 630 nm. Once the dye is excited to an intermediate triplet state, it has a relatively long lifetime ($\sim 250 \mu\text{s}$) before relaxing to the ground state by a parity-forbidden transition mechanism. However, in the presence of oxygen, the lifetime of this dye at its excited triplet state gets shortened. In PALT measurements, we first excite the dye with a pump beam at 630 nm and then use a probe beam at 950 nm to measure its transient absorption in the excited triplet state. The excited triplet state absorption at 950 nm depends on the phosphorescent lifetime and the delay between the pump and the probe beams. By changing the time delay between the pump and the probe beams, an exponentially decaying photoacoustic amplitude curve can be obtained. Evaluating the upper state lifetime of the indicator dye by a series of photoacoustic measurements facilitates a quantitative assessment of the oxygen concentration in the medium containing the dye.

2 Methods

The schematic of the experimental setup is shown in Fig. 1. A tunable dye laser (ND6000, Continuum) pumped by the second harmonic of a pulsed neodymium-doped aluminum garnet (Nd:YAG) laser (Powerlite, Continuum) was used as a pump beam to provide pulsed light, with a pulse width of 5 ns, at a wavelength of 630 nm. The probe beam at 950 nm, with pulse duration of 5.5 ns, was generated using an optical parametric oscillator (Vibrant B, Oportek) pumped by the second harmonic of another Nd:YAG laser (Brilliant B, BigSky). Both the

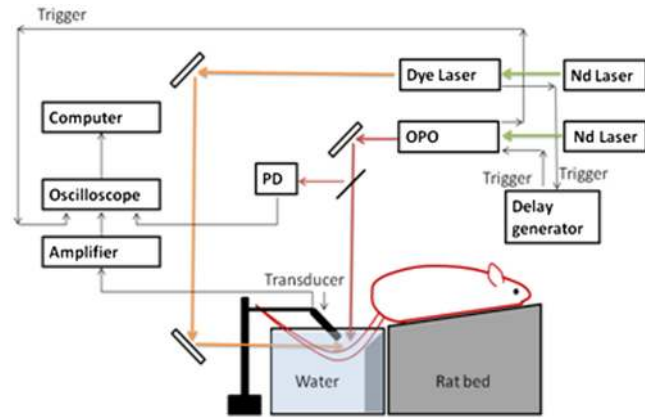


Fig. 1 The schematic of the experimental setup. PD: photodiode, OPO: optical parametric oscillator, Nd Laser: second harmonic of Nd:YAG laser. The dye laser is used to trigger the delay generator which, in turn, triggers the OPO after a set delay. The acoustic signal corresponding to different delay times is measured using the transducer.

probe beam and pump beam were operated at a 10 Hz repetition rate. During PALT measurement, the pump beam and the probe beam illuminate the same object, with incident light energy densities of about $18 \text{ mJ}/\text{cm}^2$ which is lower than the ANSI safety limit. A delay generator (DG535, Stanford Research Systems) was used to precisely control the delay between the pump (dye laser) and the probe (OPO) beams. The photoacoustic signals created by the two beams, respectively, were detected by a high-sensitivity, wide-bandwidth (133% at -6 dB with a center frequency of 10 MHz) ultrasonic transducer (V312, Panametrics) that was cylindrically focused with a focal length of 0.75 in. The signals received by the transducer were amplified and then digitized by a 500 MHz digital oscilloscope (TDS 540B, Tektronix) before being transferred and stored in a computer. The signal averaging was performed over 30 measurements for each data point. A beam splitter was used to direct a small fraction of the beam from the OPO to the photodiode (Model 2031, Newport Corporation), which was used to normalize the measured photoacoustic signal intensity so as to take into account any error due to fluctuations in the OPO output power.

3 Results and Discussion

We first checked the feasibility of the PALT for oxygen sensing using the G2 dye *in vitro*. We dissolved the dye in water containing 6% albumin. The G2 dye binds to albumin and the resultant absorbance and fluorescence gets significantly enhanced.^{12,13} During photoacoustic measurement, the G2 dye solution was injected, using a syringe pump, into a transparent soft tubing (0.58 mm- BD Intramedic, Sparks, MD, USA), mimicking a blood vessel. The photoacoustic signal from the dye, in response to the probe beam, was measured for a series of time delays, varying from 0 to $350 \mu\text{s}$. Examples of the photoacoustic signals for delays of 5, 60, and $100 \mu\text{s}$ are shown in Fig. 2(a), where a significant change in signal amplitude, as a function of the delay time can be seen. By reading the peak-to-peak signal amplitude for each delay time, a delay curve can then be drawn, as shown in Fig. 2(b). To demonstrate the feasibility of the G2 dye for oxygen sensing, the delay curves of the dye in two different media were measured, one in normal air-saturated water and the other in oxygen-saturated water prepared by purging with pure oxygen gas. The phosphorescence lifetime of the dye is

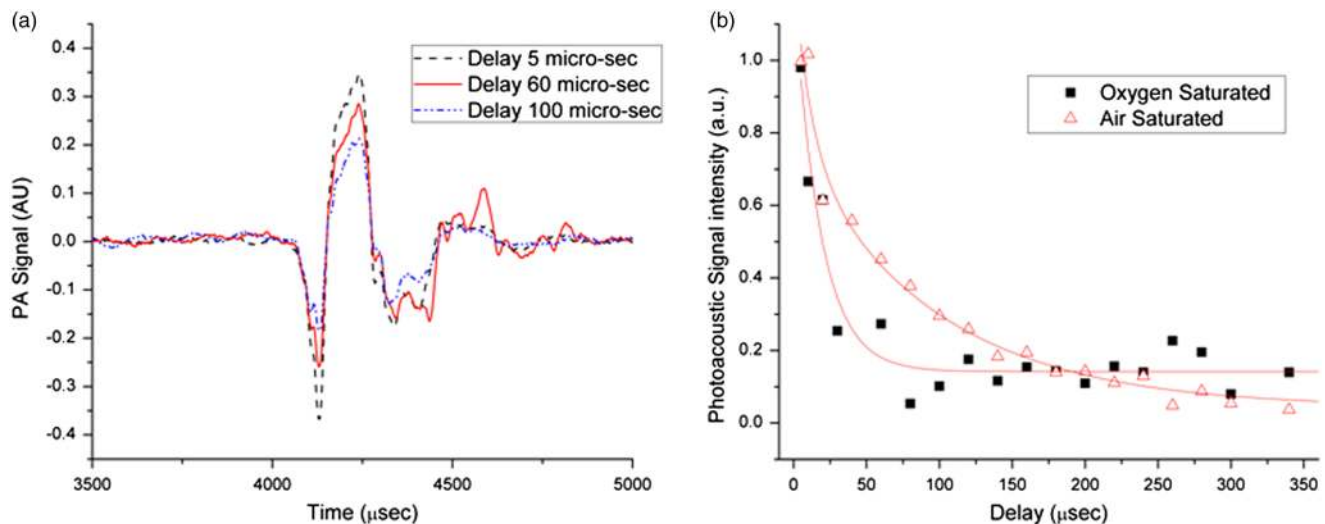


Fig. 2 (a) The photoacoustic (PA) signals from the G2 dye dissolved in water (containing 6% albumin), correspondent to different pump-probe delays. (b) The *in vitro* change in the photoacoustic amplitude with time. The exponential decay curves for oxygen saturated water and air saturated water, mixed with albumin, are shown for comparison.

about 250 μs in nitrogen-saturated water^{11,12} and decreases with increase in oxygen concentration.

The *in vivo* experiments were performed on live Sprague Dawley rats (100 g, Charles River Laboratory). Before measurement, general anesthesia was administered to the rat by an intramuscular injection of 87 mg/kg Ketamine plus 13 mg/kg Xylazine. During the experiment, the rat was placed on a heating pad and its tail was fixed firmly on a 45-deg angle inclined platform in a water tank to prevent motion. One lateral vein of the rat tail was identified, and a catheter (24 gauge, 19 mm long, BD Angiocath, Sandy, Utah) was placed for systemic administration of the dye solution. By illuminating the light beam at the ventral surface of the tail and pointing the ultrasonic transducer to the illuminated area, PALT measurement of blood oxygenation was conducted on the ventral caudal artery in the rat tail. At the same time, the arterial blood oxygenation level was also monitored by a tail sensor of a pulse-oximeter (NONIN, 8500A, Plymouth, MN) that was attached over the ventral caudal artery. The time-averaged readout of blood oxygen saturation from the pulse-oximeter provided a gold standard to validate the PALT results. The heart rate of the rat was also monitored continuously, using the pulse-oximeter, showing that the rat was in good condition during the course of the experiment.

The dye solution was prepared by dissolving the G2 dye in phosphate buffer saline (PBS) at a concentration of 5 mg/ml. We injected 0.9 ml of the dye solution into the circulatory system of the rat to have a final dose of 45 mg per kg body weight. During the experiment, the rat breathed through a breathing mask. By changing the content of the oxygen in the breathing gas, the global arterial oxygenation level of the rat was changed over different levels (77%, 83%, 88%, and 94%), with 94% corresponding to the normal blood oxygen level. No measurement was conducted below 77% since severe hypoxia could lead to the death of animals. At each arterial oxygenation level, the photoacoustic signal amplitude was measured, as a function of the delay time between the pump and the probe beams. The PALT decay curves correspondent to the two different blood oxygenation levels, 94% and 77%, are shown, as examples, in Fig. 3(a). We performed two sets of measurements for

each injection. We also monitored the photobleaching and the elimination of the dye from the animal for the duration of experiment by monitoring the photoacoustic signal of the dye from the tail vein. Over the time of measurement, these parameter changes were almost negligible but were still taken into account by incorporating the photobleaching/elimination time constant, during final analysis. The upper state lifetimes of the dye, for four different blood oxygen concentrations, as measured with PALT, are shown in Fig. 3(b). The lifetime measured with PALT shows a good linear relationship with the blood oxygenation level. The signal averaging was performed over 30 measurements, for each data point, while obtaining the exponential decay curve as shown in Figs. 2(b) and 3(a). Whereas each data point in Fig. 3(b) shows the average of the lifetime measurement calculated from two sets of data that were obtained for the same blood oxygen concentration. The horizontal error bars are from the pulse oxymeters. During our measurement, the blood oxygen level was fairly constant with occasional fluctuations, as shown by the error bar in Fig. 3(b). The observed lifetimes obtained using PALT are slightly longer than the reported phosphorescent lifetimes. This discrepancy can be attributed to the relatively low quantum yield of the dye ($\sim 10\%$). PALT is measuring the depletion of the excited state, rather than strictly the radiative transition, like phosphorescence. Thus, the lifetimes measured using the two different techniques should not be quantitatively compared. However, the important aspect is that the lifetime decreases with increase in oxygen concentration, which is quite consistent with the phosphorescence.

This system can further be modified for *in vivo* 3-D mapping of oxygen. For our current measurement, we used a single transducer, which essentially focuses on one spot and obtains the lifetime measurement in that region. 3-D imaging can be achieved through a 2-D raster scan of a single transducer, which, however, takes a long time for data acquisition. In order to improve the speed for 3-D PALT, a 2-D transducer array should be used, which could enable the acquisition of a 3-D image after a single laser pulse. In that case, 3-D PALT could be conducted in as short a time as taken in the present study that is only focused on one point.

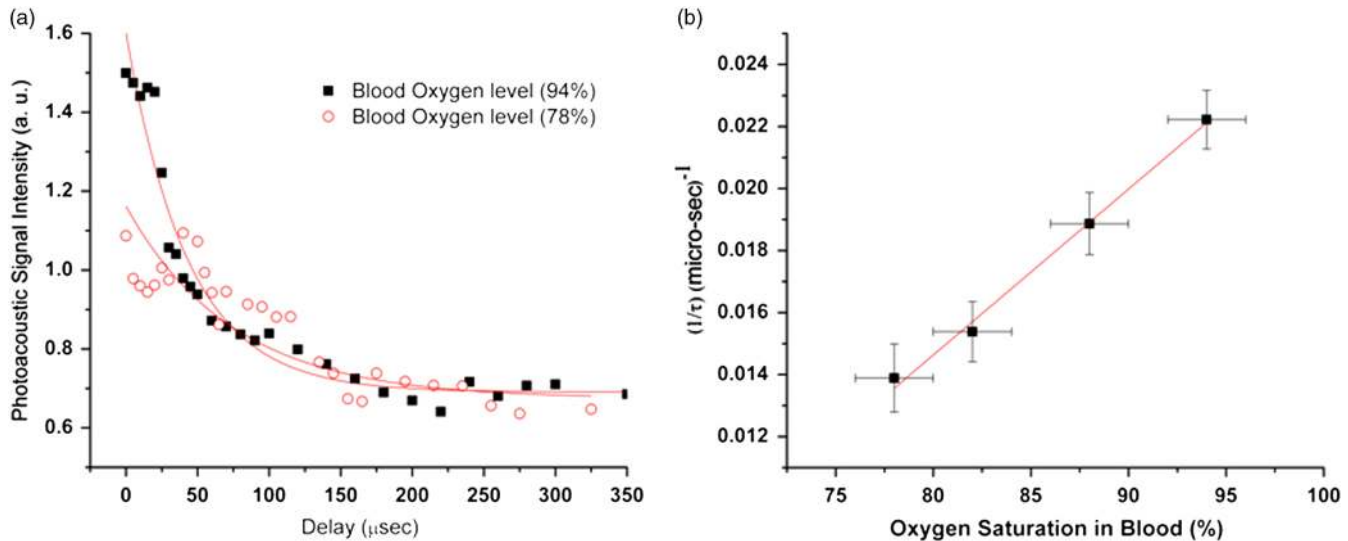


Fig. 3 (a) The *in vivo* change in the photoacoustic amplitude with time. The exponential decay curves for two different levels of oxygen saturation are shown. (b) The change in the upper-state lifetime of the dye measured with PALT for different blood oxygen saturations (R sq value: 0.99).

4 Conclusion

In conclusion, we demonstrated, for the first time, through experiments on live animals, the feasibility of quantifying blood oxygenation by using PALT measurements facilitated with an oxygen sensing dye. The measurements from the rat tail artery indicated that the lifetime of the dye quantified with the PALT technique showed a linear relationship with the blood oxygenation levels in the targeted artery. Unlike the spectroscopic PAI based on endogenous contrast, the PALT technique is not dependent on the optical absorption of hemoglobin for the oxygen level measurement. Therefore, this technique can be further extended to measure the oxygen concentration in any other media. This makes it a potentially powerful tool to evaluate oxygenation levels in tissues that are devoid of blood, such as the necrosis core of a solid tumor. With its high resolution and good imaging depth, PALT holds promise for evaluation of tumor hypoxia which is usually very heterogeneous in nature.

Acknowledgments

This work was supported by NIH Grants R33CA125297 (RK), R01AR055179 (XW) and R01AR060350 (XW), and NSFC Grant 11028408 (XW).

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