Photoacoustic probing of fluorophore excited state lifetime with application to oxygen sensing

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Abstract. A new method is developed to perform local measurements of fluorophore excited state lifetimes in turbid media without collecting the fluorescence emission. The method is based on a pump-probe approach where a first laser pulse excites the dye and then a second laser pulse is used for photoacoustic probing of the transient absorption. The photoacoustic response generated by the probe pulse is recorded by an ultrasound receiver. Repeating the measurement for increasing pump-probe time delays yields a series of photoacoustic signals that are used to extract the lifetime of the excited state. The method is validated by measuring the lifetime of an oxygen sensitive dye solution at different concentrations of dissolved oxygen. The dye is pumped with a 532-nm pulsed laser and the transient absorption at 740 nm is probed using a second pulsed laser system. The photoacoustic-based results are in close agreement with those obtained from time-dependent fluorescent measurements. The method can be extended to photoacoustic lifetime imaging by using a receiver array instead of a single receiver. Potential applications of this method include tissue oxygen imaging for cancer diagnostics and mapping molecular events such as resonant energy transfer and ion collisions in a biological environment. [DOI: 10.1117/1.2927466]

Keywords: fluorescence lifetime imaging; photoacoustic imaging; transient absorption; oxygen sensing; molecular imaging.

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The lifetime of fluorophore's excited state is typically an intrinsic property of the fluorophore, determined by the quantum transition amplitude between the initial and final states. In many cases, interactions with the environment can shorten the unperturbed lifetime. This mechanism forms the basis for a wide range of fluorescent sensors applied to biological systems. A typical example is oxygen sensitive dyes.[1,2] These dyes can be excited into a triplet state, which has a relatively long lifetime due to the "forbidden" (spin-flip) nature of the relaxation to the ground state. However, intermolecular collisions with oxygen (in its triplet ground state) can quench the excited state. Measuring the excited state lifetime provides a quantitative measure of oxygen concentration in the environment. This enables tissue oxygenation imaging,[3] an extremely valuable tool for cancer diagnostics, assessment of tumor aggressiveness, and treatment monitoring. Lifetime measurement was also utilized to measure intermolecular distances based on energy transfer between acceptor and donor molecules[4] [fluorescence resonance energy transfer (FRET) pair]. Shorter donor excited state lifetimes are observed at short intermolecular distances, due to efficient quenching by the acceptor molecule. This "spectral ruler" technique also enables monitoring protein dynamics in living cells. In these applications, the lifetime is evaluated by measuring the decay of the fluorescent signal. Compared to fluorescent intensity measurements, lifetime measurements are much more robust and stable due to insensitivity to dye concentration, excitation intensity, and light absorption in tissue.

One of the major difficulties in applying these methods for clinical imaging is the loss of spatial information due to strong light scattering. We present here an alternative technique for local measurement of a dye's excited state lifetime, one that does not require collection of the fluorescent emission but rather relies on photoacoustic probing.[5,6] The technique is based on a double-pulse illumination. A first pulse is used to excite the dye, and a second pulse is used to generate photoacoustic waves that provide information on the dye's optical absorption. Since the optical absorption of the excited state is different than that of the ground state, the amplitude of the photoacoustic signal does reflect the relative population of the excited and ground states. By changing the time delay between the pulses, the decay of the excited state population can be monitored and the excited state lifetime can be extracted. The wavelengths of the two pulses are independently optimized to maximize contrast. A schematic illustration of the principle of operation is given in Fig. 1. The method is well suited for tissue imaging because both the excitation and photoacoustic probing pulses are insensitive to light scatter-

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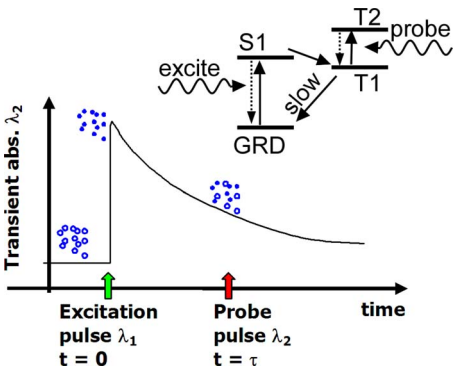

Fig. 1 Basic principle of photoacoustic lifetime measurement of a phosphorescent oxygen sensitive dye. Dye molecules at ground state (open circles) are excited at $t=0$ (closed circles) by a short laser pulse of wavelength λ_1 (532 nm in this paper). The dye molecule is excited to its first singlet state (S1) and then to a triplet state (T1) by internal system crossing. This process is relatively efficient (quantum yield of 50% for PtOEP dye) and occurs in a time scale of picoseconds. The population of the T1 excited state then decays back to ground state via phosphorescence and quenching by collisions with oxygen molecules. The decay rate is relatively slow (~50 μs for PtOEP) and depends on oxygen concentration in the environment. A probe pulse (at 740 nm in this paper) fired at $t=\tau$ generates a photoacoustic response due to optical absorption corresponding to the T1 to T2 transition. The absorption is transient because of the decay of the population of the T1 state. The amplitude of the photoacoustic signal generated at different delay times τ is used to measure the decay rate of the T1 excited state population.

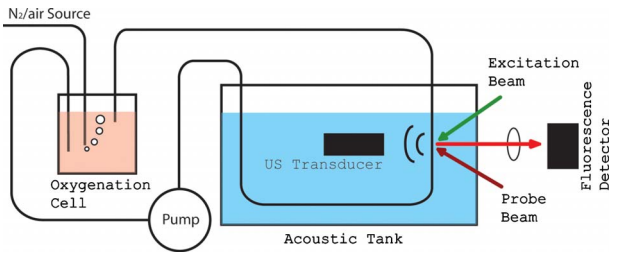

Fig. 2 Experimental setup including a closed flow circulating dye, in plastic tubing, a peristaltic pump, and an oxygenation cell. The excitation and probe beams illuminate a section of the tubing. Photoacoustic signals are detected by a focused ultrasound transducer. Water tank is used for ultrasound coupling between the tubing and the transducer. Time-dependent fluorescent signal is detected by a photodetector and recorder using a digital oscilloscope.

ing, while the generated acoustic waves propagate with minimal scattering and provide useful information for image reconstruction.

To demonstrate the new method we have used a Pt(II) octaethylporphine (PtOEP: Frontier Scientific Inc.) oxygen sensitive dye. Its excited state has an intrinsically long lifetime (91 μs in polystyrene medium[7]), however, it decreases in the presence of oxygen due to intermolecular collisions resulting in energy transfer to the oxygen molecule. Measuring the lifetime, therefore, yields a measure for the concentration of oxygen. The experimental system is described in Fig. 2. It consists of a closed flow system for circulating the dye and controlling the concentration of dissolved oxygen, and two pulsed lasers for excitation and photoacoustic probing. The airtight closed flow circulation system includes 0.8-mm-ID (3.8-mm-OD) transparent plastic tubing (Tygon Lab R-3603, L/S13, Cole-Parmer), a compatible peristaltic pump (MasterFlex 7524-00, Cole-Parmer), and an air-sealed gas exchange cavity for controlling the level of dissolved oxygen in the dye by either bubbling N_2 or air, for low and high oxygen concentration, respectively. A doubled YAG laser (QuantaRay, $\lambda=532$ nm, pulse energy=11 mJ, pulse width =12 ns, beam width=9 mm) was used for excitation. A doubled YAG laser pumped OPO system [Surelite I-20, Surelite OPOplus, Continuum, $\lambda=740$ nm, pulse energy with neutral density (ND) filters=10 mJ, pulse width=5 ns, beam width=4 mm) was used for photoacoustic probing. The two laser beams were directed to overlap on a section of the circulating dye tubing. A focused ultrasound (US) transducer (Panametrics V311, 10 MHz, f#1.5), focused at the beam overlap position, was used to detect the photoacoustic signal. The signal was amplified by a +40-dB amplifier (Panametrics 5072PR) and recorded by a digital oscilloscope (WaveSurfer 432, LeCroy Inc., bandwidth limited to 20 MHz, 20 averaging). The timing of the excitation and probing pulses was controlled by a programmable logic array that enables an accurate time delay in the range of −100 to +400 μs between the excitation and the probe pulses. The dye was first dissolved in toluene at a 1-mM concentration of stock solution which was then diluted with ethanol to prepare a 100-μM dye solution. An optical system for collecting the fluorescence light was added to enable simultaneous measurement of fluorescence and photoacoustic signals. The optical system includes a collecting lens ($f=50$ mm, diameter=25 mm), bandpass filter (615/60 nm, Chroma,Technology Corp.), and a photodiode (FDS010 Si Photodiode, Rload=2.5 Kohm, Thorlabs Inc.).

We measured the photoacoustic response for a set of excitation-probe time delays in the range of 0 to 100 μs. This set of measurements was repeated after 120 minutes of N_2 bubbling (low oxygen state) and after 10 min of air bubbling (high oxygen state). A reference signal with no excitation pulse, which corresponds to ground state constant absorption at the probe wavelength (740 nm), was also recorded for each oxygen state. The reference signal was subtracted from the measured photoacoustic signals at different delays to extract the contribution of transient absorption to the photoacoustic signals. An example of transient absorption phtoacoustic signals at three different time delays and a low oxygen state is shown in Fig. 3. To measure the amplitude of the signals we calculated the square root of the signal energy in a time window containing the first peak (see Fig. 3). The photoacoustic signal amplitudes, of both low and high oxygen states, as a function of the excitation-probe delay time are shown in Fig. 4. To compare the excited state lifetime based on the photoacoustic measurements with fluorescence lifetime, the fluorescence emission was collected. The photoacoustic and fluorescence lifetimes were measured by fitting an exponential decay function to data in the range of 4 to 30 μs. A close agreement was found between lifetimes measured in both methods. The results are summarized in Table 1. The results for the high oxygen state show significant deviation from a simple exponential decay. This deviation is primarily due to lack of dynamic range in the PA measurement.

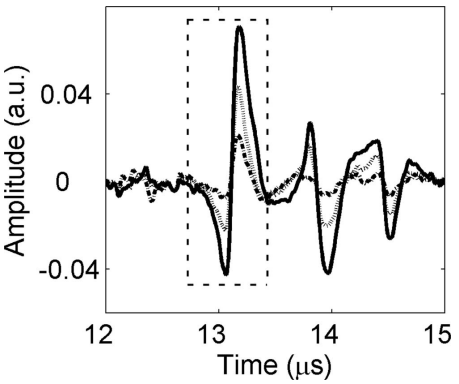

Fig. 3 Photoacoustic signals at three different time delays (6, 34, and 100 μs), after a reference signal that corresponds to infinite time delay (no excitation) was subtracted. The signals were measured at a low oxygen level. The rectangular frame (dashed line) indicates the time window used for signal processing. The first and second peaks in the signals correspond to the front and back dye-tubing interfaces.

To conclude, we presented a new technique for quantifying the excited state lifetime of a dye, based on PA probing. The technique was demonstrated by measuring different lifetimes of an oxygen sensitive dye that was subjected to different oxygen concentrations. The results obtained by the PA method were verified by an independent measurement of the lifetimes by conventional time-dependent FL.

The single position measurement technique can be extended to lifetime imaging by replacing the single US transducer with an ultrasonic array receiver and acquisition system.[8] PA lifetime imaging has a number of significant advantages over FL lifetime imaging:

1. Penetration is deeper in tissue since the excitation pulse and the PA probe pulse only traverse a single path to the target, while FL measurement requires two way light propagation. The typical penetration depth for PA imaging,[9] at a wavelength of 740 nm, exceeds 5 cm. However, effective dye excitation imposes an additional limitation on the penetration depth. In the near IR (NIR) region this would correspond to a depth of 2 to 3 cm.
2. Submillimeter resolution is maintained up to the full penetration depth.
3. 3-D imaging (with 2-D receiver arrays) is possible.

Table 1 Lifetimes measured by fitting an exponential decay function to photoacoustic (PA) and fluorescence (FL) decay data.

	Method	Lifetime (μs)
Low O_2	PA	33.1±2
	FL	33.2±0.2
High O_2	PA	11.3±0.7
	FL	13.9±0.2

A constant term was first subtracted from the data before fitting. For the photoacoustic data, the constant was determined from photoacoustic signals measured with no excitation pulse. For the fluorescence data, the constant was extracted from the photodetector signal at long delay (100 ms) after excitation.

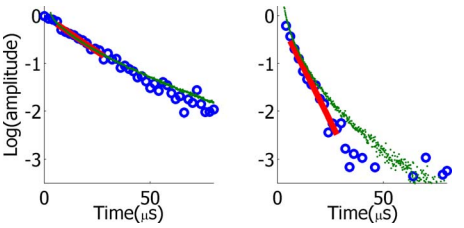

Fig. 4 Amplitude of photoacoustic response (open circles) and fluorescence emission (dots) as a function of the time delay following an excitation pulse, for low (left) and high (right) oxygen levels. An exponential decay fit to the photoacoustic data, calculated in the range of 4 to 30 μs, is shown (solid line).

In the work reported here we used green light (532 nm) to excite the dye, which has very shallow tissue penetration (less than 2 mm). To translate the method to medical applications it is essential to replace the dye with a red or NIR excitable dye. A potential candidate is Pd-tetra-(4-carboxyphenyl) tetrabenzoporphyrin dendrimer (PdTBP) (Oxygen Enterprises Ltd., Philadelphia, Pennsylvania), which can be excited[10] at 630 nm. To estimate the effective penetration depth in tissue we first calculate the optical fluence required for saturated absorption as $I_{sat}=h\nu/\sigma$, where h is Planck's constant, ν is the optical frequency, and σ is the single-molecule cross section for absorption. For PdTBP dye, this requirement yields a value of 1.5 mJ/cm^2. Considering tissue-effective attenuation at a wavelength of 630 nm, and assuming surface illumination of 20 mJ/cm^2 (complying with ANSI Z136.1 laser safety standard), efficient excitation would be achieved up to a depth of 2 cm.

Another improvement of the technique would be to use a long excitation pulse, but one that is still shorter than the resolution required for lifetime measurement. In our example, a 1-μs excitation pulse could be used to measure the long lifetime (10 to 90 μs) of PtOEP dye. Using a relatively long excitation pulse would enable efficient rejection of the unwanted photoacoustic signals generated by the pump pulse, by utilizing a high-pass filter, thus leaving the PA signals of the probe pulse free of interferences.

Developing the technique for clinical tissue pO_2 imaging could provide a valuable tool for cancer diagnostics, staging, and treatment monitoring. Note that over the past decade, studies have demonstrated high correlations of hypoxia with tumor aggressiveness and tumor resistance to therapy.[11] Delivering the dye to tumor *in vivo* is a challenging task. One of the promising ways to accomplish it, is using[12,13] dye nanoencapsulation technology such as PEBBLE (Probing Explorers for Bioanalysis with Biologically Localized Embedding). This enables specific tissue targeting by biocompatible nanoparticles embedded with the sensor dye.

Our specific implementation of the PA lifetime measurement relied on the fact that the lifetime is in the order of 10 s of microseconds, much longer than the laser pulse width. However, most fluorophores in biological systems (or those used to probe biological systems) have lifetimes shorter than the 5 ns laser pulse width.[14,15] In principle, the method can be scaled to measure shorter lifetimes by using laser pulse widths that are shorter than the required lifetime measurement resolution (e.g., picosecond lasers). In this case, the resulting PA

signal would combine contributions from the excitation pulse and the delayed probe pulse (transient absorption). The decay of the PA signal amplitude as a function of the excitation-probe delay time would still reflect the transient absorption. However, penetration depth would be compromised due to lower pulse energy in picosecond lasers, lower efficiency for PA generation, and reduced contrast due to large constant absorption term. In spite of these difficulties, we believe that PA probing of excited state decay in the nanoseconds range of endogenous species (e.g., hemoglobin) and functional exogenous molecular probes would open up a range of new applications for medical molecular imaging.

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