Lifetime-weighted photoacoustic imaging

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Abstract

Photoacoustic (PA) imaging has been utilized to quantify the lifetime profile of exogenous agents using a series of pump-probe pulses with a varying time delay; however, current techniques typically lead to long acquisition times which are sensitive to motion and cause absorption or photobleaching. We introduce a technique called lifetime-weighted imaging, which uses only three laser pulses to preferentially weight signals from chromophores with long lifetimes (including exogenous contrast agents with triplet excited states such as methylene blue and porphyrins) while nulling chromophores with short picosecond- to nanosecond-scale lifetimes (including hemoglobin). This technique detects the PA signal from a probe pulse either with or without a pump pulse. By subtracting the probe-only signal from the pump-present probe signal, we effectively eliminate signals from chromophores with short lifetimes while preserving PA signals from chromophores with long-lifetimes. We demonstrate the oxygen-dependent lifetime of both methylene blue and porphyrin-lipids and demonstrate both ground-state recovery and excited-state lifetime-weighted imaging. Lifetime-weighted PA imaging may have applications in many molecular imaging application including: photodynamic therapy dosimetry guidance and oxygen sensing.

Keywords: lifetime, lifetime-weighted, photoacoustic microscopy, porphyrins, methylene blue

(Some figures may appear in colour only in the online journal)

1. Introduction

Photoacoustic (PA) imaging has emerged as a powerful hybrid bio-imaging technology providing optical absorption contrast with ultrasonic or optical spatial resolution. There has been extensive research in PA imaging demonstrating utility for imaging angiogenesis and microvascular networks [1, 2], estimating oxygen saturation and oxygen flux [3, 4], imaging optical reporters such as chromoproteins and fluorescent proteins [5–8], the lacZ operon [9, 10] or an inducible tyrosinase gene [11]. In such applications, absorption spectra can be used to spectrally separate PA images into maps of dominant absorbing molecules. Excited-state lifetimes (ESLs) and ground-state recovery (GSR) times are additional molecular properties which can lead to new contrast mechanisms for PA imaging.

Although lifetime imaging techniques have been widely studied in other optical modalities, such as fluorescence imaging, and have the capabilities to indirectly study the local molecular environments (pH, analyte concentrate ion, etc [12, 13]), it has not been investigated greatly for PA imaging [14, 15]. Lifetime imaging typically involves a pump-probe experiment to first excite the molecules and then image the molecules in the transient state. Using this technique, Ashkenazi [14] demonstrated PA lifetime imaging of dissolved oxygen using methylene blue, a highly absorbing molecule that has long, oxygen-dependent lifetimes. By varying the time separation between the pump and probe pulses and fitting the resulting PA signal to an exponentially decaying curve, he was also able to determine the partial pressure of oxygen in a sample. Recently, due to their high optical absorption, stability, and biocompatibility, porphyrins have been investigated for use as a PA contrast agent in the form of biochemical constructs such as porphysomes, microbubbles,
and nanodroplets [16–18]. As with methylene blue, porphyrins have long lifetimes and can be used for lifetime-base PA imaging to probe the molecular environment surrounding a sample.

One issue with these lifetime imaging methods is that they can require many pump-probe pulse pairs at varying time separations. This makes imaging slow and subject to the accumulation of many errors such motion artifacts. Furthermore, the many pulses required for imaging may make the molecule undergo absorption bleaching or photobleaching. Finally, imaging over a long period of time may result in a slight change in the molecular environment around the particle.

Additionally, the many pulses required for imaging may make the accumulation of many errors such motion artifacts. Furthermore, imaging over a long period of time may result in a slight change in the molecular environment around the particle. Therefore, it is a slow process. Due to the slow process many interesting phenomena can be investigated more easily with phosphorescent molecules including ESL and GSR time of a molecule. Experiments investigating these phenomena typically involve a pump-probe approach where the molecule is first excited to a higher energy state and then the molecule is interrogated as it recovers back to the ground state.

For both ESL and GSR imaging, the wavelength of the pump pulse is often selected as the peak absorption of the molecule of interest. At time t after the pump pulse, the number of molecules that are in the triplet-excited state \( N_I(t) \) can be represent by an exponential decaying functions as given by equation (1), where \( I \) represents the intensity of the pump pulse, \( \tau_L \) represents the ESL of the molecule, \( N_0 \) represents the number of molecules, \( I_{sat} \) represents the saturation intensity, \( v_{pump} \) represents the frequency of the pump pulse, \( \sigma_{pump} \) represents the absorption cross-section at the wavelength of the pump, and \( h \) represents Planck’s constant.

For clarity, the intersystem crossing efficiency (50% for methylene blue [20]) is left out of the equation

\[
N_I(t) = \frac{N_0}{h\nu_I/\tau_L + 1} e^{-\frac{t}{\tau_L}}, \text{ where } I_{sat} = \frac{h\nu_{pump}}{\sigma_{pump} \cdot \eta}.
\]

For ESL imaging (figure 1(A)), the probe pulse has sub-bandgap energy and can only be absorbed by molecules in the triplet-excited state; therefore, the PA signal generated by the probe pulse is proportional to the number of molecules in the triplet-state. The lifetime-weighted signal, \( \text{LWI}(T) \), can be represented by the PA signal of the pump-present probe signal, \( \text{PA}_+(\lambda_{pump}, T) \approx P_e^{+} e^{-T/\tau} + P_{bkgd}^{+} \), subtracted by the pump-absent PA probe signal, \( \text{PA}_- \approx P_{bkgd}^{-} \), as given by equation (2). \( P_+ \) and \( P_{bkgd} \) are constants that are independent of the time interval, \( T \), between the pump and probe pulses and are related to \( P = \mu_A \Phi \Gamma \), where \( \mu_A \) represents the absorption coefficient, \( \Phi \) represents the fluence, and \( \Gamma \) represents the Grüneisen parameter. The ESL-weighted imaging signal computed for each PA image pixel is thus:

\[
\text{LWI}(T) = \text{PA}_+ - \text{PA}_- = P_e^{+} e^{-\frac{T}{\tau}}, \text{ where } P_e^{+} \approx \frac{\Gamma_{\text{hsl}} \sigma_{\text{pump}} \mu_{\text{pump}} N_0}{1 + h\nu_{\text{pump}} \sigma_{\text{pump}} / \Phi \Gamma}
\]

Equation (2) demonstrates that the lifetime-weighted image for ESL imaging exponentially weights the lifetime. Hence, this imaging technique is sensitive to molecules with long ESL, such as methylene blue and porphyrins, and rejects molecules with short lifetimes, such as blood.

For GSR imaging (figure 1(B)), the probe pulse has similar energy as the pump pulse and is only absorbed by the molecules that have returned to the ground-state. Therefore, the PA signal generated by the probe pulse is proportional to the number of molecules that have returned to the ground-state. A similar exponential decay can be found for GSL-weighted imaging. Equation (3) represents the ground-state population after a pump pulse, where \( \tau_{GSR} \) represents the

2. Theory

When a molecule absorbs energy it undergoes a transition from the ground state (\( N_0 \)) to a higher energy state (\( N_2 \)). The excited molecule returns back to ground state through several competing radiative and non-radiative relaxation pathways including fluorescence, resonant energy transfer, vibrational and rotational relaxation, and phosphorescence. Most of the relaxation pathways occur quickly (on the order of femtoseconds to nanoseconds); however, phosphorescence is relatively slow (on the order of microseconds). Phosphorescence typically involves an intersystem transition of an electron from a singlet-excited state (\( N_0 \)) to a triplet-excited state (\( N_1 \)). Since it requires an electron to flip its spin, the transition from the triplet-excited state back to the singlet-ground state is unfavoured and, consequently, it is a slow process. Due to the slow process many interesting phenomena can be investigated more easily with phosphorescent molecules including ESL and GSR time of a molecule. Experiments investigating these phenomena typically involve a pump-probe approach where the molecule is first excited to a higher energy state and then the molecule is interrogated as it recovers back to the ground state.
GSR lifetime

\[ N_0(t) \approx N_0 - \frac{N_0}{I_{\text{SAT}}} t + 1 e^{-\frac{t}{\tau_{\text{GSR}}}}. \quad (3) \]

The ground-state based lifetime-weighted signal, \( \text{LWI}_{\text{GSR}} \), can be represented by subtracting the pump-present probe signal, \( \text{PA}_{\text{GSR,}\lambda_{\text{probe}}}(T) \approx P_0 \left( 1 - \frac{1}{\zeta + 1} e^{-\frac{T}{\tau_{\text{GSR}}}} \right) \), by the pump-absent probe signal, \( \text{PA}_{\lambda_{\text{probe}}} \approx P_0 + P_{\text{bkgnd}} \), as given by equation (4).

\[ \text{LWI}_{\text{GSR}}(T) = \frac{P_0}{\zeta + 1} e^{-\frac{T}{\tau_{\text{GSR}}}} \quad \text{for } \tau_{\text{GSR}} > 0. \quad (4) \]

where \( P_0 = \Gamma_{\text{n}} h \sigma_{\text{probe}} \Phi_{\text{probe}} N_0 \) and \( \zeta = \frac{\tau_{\text{GSR,pump},\lambda_{\text{pump}}}}{\tau_{\text{GSR},\lambda_{\text{pump}}} \tau_{\text{pump}}} \).

Again, there is an exponential weighting of the GSR lifetime on the lifetime-weighted image. This imaging technique would be sensitive to molecules with long GSR times, such as methylene blue and porphyrins, and eliminate signal from molecules with short GSR times, such as blood. It should be emphasized that the theoretical work presented here is based on a 3 + 1 energy level model which may be an oversimplification for many molecular species with more complicated energy-level dynamics.

3. Materials and methods

Similar to [21], two Nd:YAG lasers (Surelite III, Continuum) were used in the lifetime-weighted PA system (figure 1(C)). The first Nd:YAG laser was used to pump a dye laser (Nd60000, Continuum) containing LGS 698 and generate a 5 ns, \( \sim 50 \text{ mJ} \) pulse at 680 nm. The second Nd:YAG laser was frequency-doubled and used to pump an OPO (optical parametric oscillator, Continuum) and generate either a 10 ns, \( \sim 40 \text{ mJ} \) pulse at 680 nm or a 10 ns, \( \sim 60 \text{ mJ} \) pulse at 810 nm. A pulse-delay generator was used as a master clock to trigger both the flashlamp and Q-switch triggers of the lasers and synchronize the pump and probe laser sources. The pump and probe light paths were combined and co-aligned through a beam splitter and a pair of objective lenses into a 600-micron multimode fiber. The output of the multimode fiber was collimated and then focused through a prism, which was designed to reflect light at 90° while allowing the transmission of ultrasound. A 50 MHz ultrasound transducer was used as a detector capable of 50 \( \mu \text{m} \) resolution. The output of the light was centered through an acoustic lens that was attached to the prism and co-aligned with the acoustic detector. The fluorescence at the surface of the sample was approximately 15 mJ cm\(^{-2}\) and 10 mJ cm\(^{-2}\) for the pump and probe pulses, respectively. A two-channel data acquisition card was used to collect (1) PA signals detected by the ultrasound transducer that were amplified with a 39 dB gain pulser-receiver and (2) a photodiode signal which was used to correct for pulse-to-pulse laser instability. A motorized stage was used to move the sample and acquire C-Scan images. A custom-designed user interface for data acquisition, motor control, and the pulse delay generator was used.

A 1 mm methylene blue sample and a phospholipid aqueous stock of 0.75 mg ml\(^{-1}\) 1-stearoyl-2-pyr-oephorphoride-sn-glycero-3-phosphocholine [20, 21] sample were prepared and deoxygenated by pumping nitrogen through the sample. A pO\(_2\) electrode (DO_166MT-1 Micro Dissolved Oxygen Electrode, Lazar Research Laboratories, Inc., USA) was used to estimate the partial pressure of oxygen in the samples. The samples were then drawn from the mixing chamber with a syringe pump into polyethylene tubing and placed under the imaging prism using ultrasound gel as a coupling medium. For ESL absorption imaging, the pump and probe wavelengths were set to the peak absorption wavelength of 680 and 700 nm for methylene blue and Pyro-18 lipids (porphyrin-lipids kindly provided by Prof Gang Zheng of the University of Toronto), respectively. For both samples,
810 nm was used as a probe wavelength (where transient absorption is significant but ground-state absorption is negligible). For GSR a pump and probe pulse of 680 nm was used. MATLAB was used to process the data.

4. Results and discussion

Experiments were performed to measure the ESL and GSR lifetimes of methylene blue and porphyrin-lipids. Figure 2(a) plots the GSR lifetime-weighted PA signal as a function of pump-probe delay in a sample of methylene blue. Curve-fitting produces estimates of the GSR lifetime for a measured pO2 level. Similar to the findings of [14], we found that there was a linear dependence between the decay rate (lifetime) of methylene blue and the oxygenation of the sample (figure 2(b)). Note that rates here are roughly an order of magnitude larger than the triplet excited state decay rates shown by Ashkenazi [14]. GSR rates may be faster than transient absorption decay rates as there may be multiple relaxation pathways contributing to ground state recovery in addition to the relaxation from the long-lifetime triplet excited state. Furthermore, we verified the dependency between the transient absorption decay rate of porphyrins and the oxygenation concentration in the sample (figures 2(c) and (d)).

GSR lifetime-weighted PA imaging (GSR-LWI) was first performed on samples containing either deoxygenated methylene blue, heparinized rat blood, or a 50–50 mixture of the two samples. The pump and probe pulses were at a wavelength of 680 nm. Figure 3(A) shows the PA signal of the probe signal with pump-probe delay of \( \sim 5 \) \( \mu \)s. Although, the PA signal from blood is slightly lower than the PA signal from the methylene blue sample it is still quite difficult to differentiate these tubes. Figure 3(B) shows the lifetime-weighted PA signal and demonstrates that the signal from blood is substantially reduced compared to the signal from methylene blue. For the PA image, the signal-to-noise ratio is 37.5, 32.5, and 37.2 dB for the tube containing methylene blue, blood, and the 50–50 mixture of methylene blue and blood, respectively. For the lifetime-weighted image, the signal-to-noise ratio is 33.8, 21.2, and 27.9 dB for the tube containing methylene blue, blood, and the 50–50 mixture of...
methylene blue and blood, respectively. For in vivo imaging, this would represent an enhanced contrast between the unwanted background signal (blood) and the methylene blue samples of approximately 2.5 fold.

A clear difference was seen between all three tubes. The deoxygenated methylene blue had the highest signal intensity of the three tubes, which is expected from previous work [14] since the lifetime is long. The signal from the blood tube was nullified in the lifetime-weighted imaging. This is due to the ps-scale ESL of hemoglobin or ns-scale GSR time. Finally, the 50–50 mixture of methylene blue and blood had a small signal intensity which implied that the lifetime was short. This is due to the blood being oxygenated and decreasing the lifetime-weighted methylene blue signal. These results were replicated using porphyrins.

Longitudinal images of the probe signal (at 810 nm) from tubes containing methylene blue and ICG (another short lifetime molecule) are presented in figures 3(C) and (E), respectively. The ESL-weighted images of the methylene blue and ICG are also presented in figures 3(D) and (F), respectively. The subtraction of the probe signal without the pump pulse from the probe signal with the pump pulse clearly emphasises the signal from the methylene blue tube while greatly reducing the signal from the ICG tube.

Our results show that background signals, such as blood, can be significantly muted using the proposed lifetime-weighted imaging technique. There are a number of competing factors, however, to consider when evaluating the lifetime-weighted image of a sample with PA imaging. It has been reported that PA signal changes depending on the temperature of the samples [21]. Since two pulses of light in quick succession are required to generate the images, it is possible that the pump laser generates heating which modulates the Gruneisen parameter causing PA intensity changes in the probe laser. We believe that the low laser fluence and the μs-scale time separation mitigated this effect. Furthermore, evidence that we are not just detecting this heating effect is our ability to estimate lifetimes dependent on partial oxygen pressure as shown in figure 2. It should be noted that the pulse-to-pulse stability of the pump laser may non-linearly effect the probe PA signal. Finally, another important factor to consider is that there are many different decay pathways (photobleaching, vibrational or electronic relaxation, resonance energy transfer, etc) which act simultaneously to restore the molecules to the ground state. Despite these other factors, we believe that the lifetime-weighted images presented here of methylene blue represent a mapping of the GSR lifetime and that further investigation is warranted for this imaging technique.

For in vivo imaging, the high concentration of methylene blue and porphyrins used in these phantom experiments may be difficult to achieve with intravenous injections. However, bolus subcutaneous injections can still prove useful and in vivo lifetime imaging have been demonstrated for subcutaneous injections with methylene blue concentrations as high as 5 mm [22]. Furthermore, figure 3(B) demonstrates that there is a large change in PA signal amplitude when both the pump and probe are used compared to just the probe pulse. The average signal change is approximately 2-fold greater than the background signal in the lifetime-weighted image. By averaging over more pulses and sacrificing the signal-to-noise, lower concentrations can be achieved.

We believe that the demonstrated lifetime-weighted imaging technique has many potential applications including the examination and guidance of photodynamic therapy oxygen depletion, the visualization of contrast agent distribution, the quantification of tumor partial pressure of oxygen, and other molecular imaging applications. Of course it is additionally possible to use multi-wavelength spectroscopic demixing to separate molecular species, however, such previous approaches provide no lifetime information useful, for example, for sensing partial oxygen pressure. In the future, a combination of lifetime and spectral separation techniques may provide maximal information content. Finally, since the GSR and ESL parameters are effect by multiple parameters including temperature, pH, and ion concentration, the
proposed lifetime-weighted imaging technique could be used to analyze these environmental conditions while minimizing the effects of photobleaching. This work should be examined in future research.

5. Conclusion

In summary, we demonstrate a technique to visualize the excited-state or GSR of a long-lifetime agent with a triplet-excited state in a lifetime-weighted PA images. While transient absorption PA imaging has been previously introduced as a function of scan-speed and laser-repetition-rate, we have introduced GSR PA imaging and show it is possible to obtain lifetime information even when pump and probe wavelengths are identical. The proposed GSR and transient absorption lifetime-weighted imaging techniques can be used to differentiate spectrally similar targets based on lifetime and can provide surrogate lifetime-related information such as pO2 which is not possible with existing spectral de-mixing techniques. We believe that the lifetime-weighted PA imaging technique has many potential applications in biomedical imaging including studying photodynamic therapy dosimetry guidance and oxygen sensing, and that it will open up new areas of research in molecular imaging.

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