Multispectral photoacoustic bioimaging using low power continuous wave lasers

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ABSTRACT

We have developed a low-cost, non-contact, multispectral photoacoustic microscope system to study the functional parameters of cellular choromophores. The system uses low power continuous wave lasers and a photoacoustic sensor made of a kHz microphone coupled to a resonant chamber. Methemoglobin has relatively high optical absorption at 500 nm and 630 nm. Moreover, it has an almost the same optical absorption as hemoglobin at the isosbestic point of 525 nm. Photoacoustic data collected from methemoglobin using our system at wavelengths of 473 nm, 533 nm, and 633 nm show the similar trends as the methemoglobin optical absorption spectrum. The PA amplitude at 473 nm is about 1.03 times greater than at 533 nm and about 2.4 times greater than at 633 nm. Similarly, it possesses optical absorption of about 1.08 greater than at 533 nm and 1.34 times greater than at 633 nm. The developed system can be used as a differential photoacoustic microscope.

Keywords: Microscope, photoacoustic, multispectral, hemoglobin, methemoglobin

1. INTRODUCTION

The principle of photoacoustic (PA) imaging is based on the generation of sound by light[1]. It has a potential application in the field of biomedical imaging as it provides functional information in deep tissues. Traditionally, PA systems use an ultrafast laser for excitation, high-frequency transducer for imaging, and acoustic coupling medium between the transducer and the sample.[2–4] The system is expensive and bulky and these shortcomings hinder wide clinical applications. Recently we developed a low-cost Photoacoustic microscope (PAM) system using a low power continuous wave (CW) laser, a mechanical chopper, lock-in amplifier and a specialized PA sensor.[5] The PA sensor is developed to amplify a low amplitude PA signal produced by a low power CW laser by resonance enhancement. The main component of the PA sensor is a low-frequency kHz microphone coupled to an acoustic resonant chamber. The measurement is non-contact through the air which would enable the study of materials where water may affect the physical, chemical, and biological properties of the sample. The principle of low power PA spectroscopy was adapted to develop this PA sensor.[6–9] The PA signal is due to non-radiative excitation (via heat) of the absorbed light by the sample. The PA signal is a function of thermal diffusion of the sample which is proportional to the modulation frequency. The thermal diffusion length μ is given by[6]

$$\mu = \frac{k}{\pi \rho f c}$$

where k is the thermal conductivity, ρ the density, c the specific heat and f is the chopping frequency. Depth profiling of the sample is possible by varying the chopping frequency. Low power PA spectroscopy has been used to study the spectroscopic properties of solid, liquids and gases, and widely applied to trace gas detection, with concentration sensitivity in parts per billion.[10–12]

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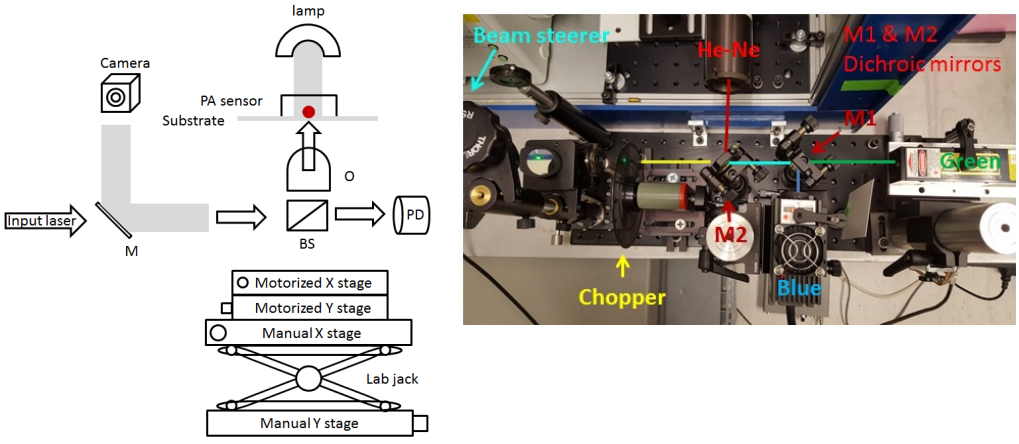
The developed PA system was used to study methemoglobin. Methemoglobin is a hemoglobin product in which the iron in the heme group is in the Fe3+ (ferric) state, which inhibits binding with free oxygen. People with an abnormal amount of methemoglobin suffer from the disorder called methemoglobinemia. A high level of methemoglobin concentration would also be observed in bleeding and tissue burning. The prosthetic group of hemoglobin is the other type of hemoglobin found in erythrocytes. This is formed due to intrinsic instability of hemoglobin. The heme's are released from host and accumulate in the erythrocytes.[13] The released heme is also in the Fe3+ state but in the form of hematin (Fe3+ protoporphyrin IX). Methemoglobin exhibits relatively high optical absorption at 500 nm and 630 nm compared to normal hemoglobin which has peak absorption at 420 nm and 570 nm. At the isosbestic point, 525 nm, both chromophores exhibit almost identical optical absorption. Analyses of the PA signals from blood using a single wavelength could not discriminate easily between the hemoglobin species. Multispectral studies enable the measurement of the ratios of the different chromophores and functional parameters that are based on these ratios. The information provided by multispectral imaging can allow us to deduce much more information about structural and functional properties of blood. The PA system allows multispectral PA data collection using three wavelengths: 473 nm (red), 533 nm (blue) and 632.8 nm (green) each operating at 3-4 mW. The present aim is to study the PA properties of methemoglobin at these wavelengths.

2. MATERIALS AND METHODS

2.1 Photoacoustic microscope

A dual modal optical/acoustic microscope system was developed as shown in figure 1. The optical system was built as an inverted microscope configuration consisting of a halogen lamp, an infinity-corrected 40 X and 0.60 NA Olympus objective (O), a beam splitter (BS) a mirror (M) and a Ximea xiQ camera (Ximea, USA). The system consists of motorized linear X-Y scanning stages (Zaber Technologies, USA) for raster scanning, and manual X-Y-Z stages (Thorlabs, USA) for manual adjustment. The multispectral PA system used three laser systems operating at wavelengths of 473 nm, 533 nm, and 633 nm. Figure 1 is a photograph of the three laser system. Three lasers were collimated using dichroic mirrors and directed into the PA microscope using a beam steerer. A mechanical optical chopper modulated the beam intensity. The laser path is indicated by the solid arrow in figure 1(a) and solid line in figure 1(b). The input laser light beam is split into two beams by the beam splitter (BS), one for the sample and the other for the reference. The reference beam is recorded by a photodiode (PD) and is used to normalize the recorded PA spectrum to correct for laser associated PA fluctuations.



(a) (b)

Figure 1. (a) A schematic of the multispectral photoacoustic microscope. (b) A multispectral laser system containing three lasers at wavelength 473 nm, 533 nm, and 633 nm respectively. M dichroic mirror, BS beam splitter, O objective, and PD photodiode.

The lock-in amplifier is operated with a single RC filter setting (6 dB RC roll off) with a time constant 300 ms. The PA sensor contains two chambers, one for the sample (sample chamber, SC) and another for the microphone.[5] The SC is connected to the microphone through the resonator of length 100 mm. The SC size is 8 mm in diameter and 3 mm height. The sensor has a maximum PA signal at a resonance frequency of approximately 390 Hz.

2.2 Procurement of blood

Blood was collected by netCAD (Vancouver, Canada), the research division of Canadian Blood Services, under protocol 2013-001 which involves standard Canadian Blood Services collection and testing procedures of whole blood, and delivery overnight at 4°C, with continuous monitoring during shipment to ensure no temperature deviations occur. This procedure has been approved by the research ethics boards of both Ryerson University and Canadian Blood Services.

2.3 Blood sample preparation

The guidelines on handling the blood were followed in accordance with the recommendations of the International Society for Clinical Hemorheology and the European Society for Clinical Hemorheology and Microcirculation.[14] Red blood cells (RBC) containing methemoglobin was prepared by treating normal oxy-hemoglobin with sodium nitrate ($NANO_3$, Sigma-Aldrich). $NaNO_3$ is a strong oxidizer. According to material data safety sheet (MSDS), it is harmful if swallowed and may cause irritation of the digestive tract, methemoglobinemia, cyanosis (bluish discoloration of skin due to deficient oxygenation of the blood), convulsions, and death. Two mg of $NANO_3$ was mixed with 1 ml of blood and shaken well. The optical absorption properties of the samples are studied using a USB 4000 Ocean optics fiber optics spectrometer. The glass slide was ultrasonically cleaned using isopropyl alcohol followed by water to remove any trace of oil or dirt. Then the blood was smeared on glass substrates for PA studies.

3. RESULT AND DISCUSSION

Whole blood exhibits optical absorption peaks in the visible region at 542 nm, and 579 nm. These peaks 542 nm and 579 nm are called Q band peaks due to singlet state transition from S_0 to S_1. The transitions are due to electron transfer from π to π^* porphyrin ring orbitals within the porphyrin ligand.[15,16] This can be explained by Gouterman Four-Orbital Model.[15] According to this model, the optical absorption in porphyrins is due to the electronic transition between two HOMOs and two LUMOs orbitals. Any structural change would split these two states of energy into a higher energy state with greater oscillator strength, giving rise to the Soret band, and a lower energy state with less oscillator strength, giving rise to the Q-bands. The optical absorption increase below 470 nm is due to strong Soret band transition.

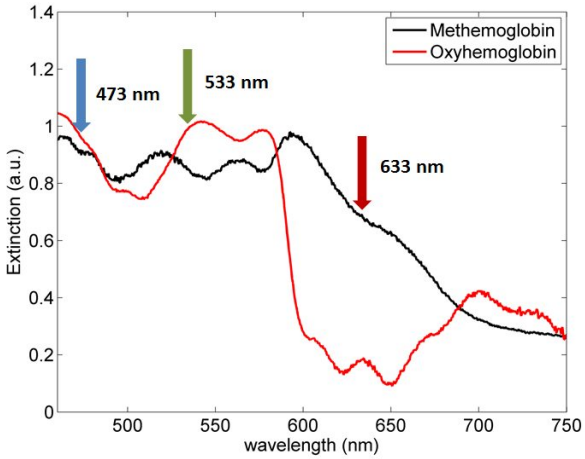


Figure 2. Measured optical extinction spectra of Oxyhemoglobin and methemoglobin.

Healthy human possesses methemoglobin in concentrations < 1% of total hemoglobin that is formed by spontaneous slow auto-oxidation of hemoglobin. Methemoglobin subsequently reduced to hemoglobin to maintain a steady-state of methemoglobin concentration in whole blood. People that suffer from methemoglobinemia possess an abnormal amount of methemoglobin. Methemoglobinemia can be either inherited or acquired. The inherited methemoglobinemia is genetically transferred whereas acquired methemoglobinemia is produced by the action of oxidants. When an individual is exposed to an exogenous oxidizing agent, the rate of methemoglobin formation can overwhelm the protective reduction mechanisms resulting to a condition called acquired methemoglobinemia.[17]

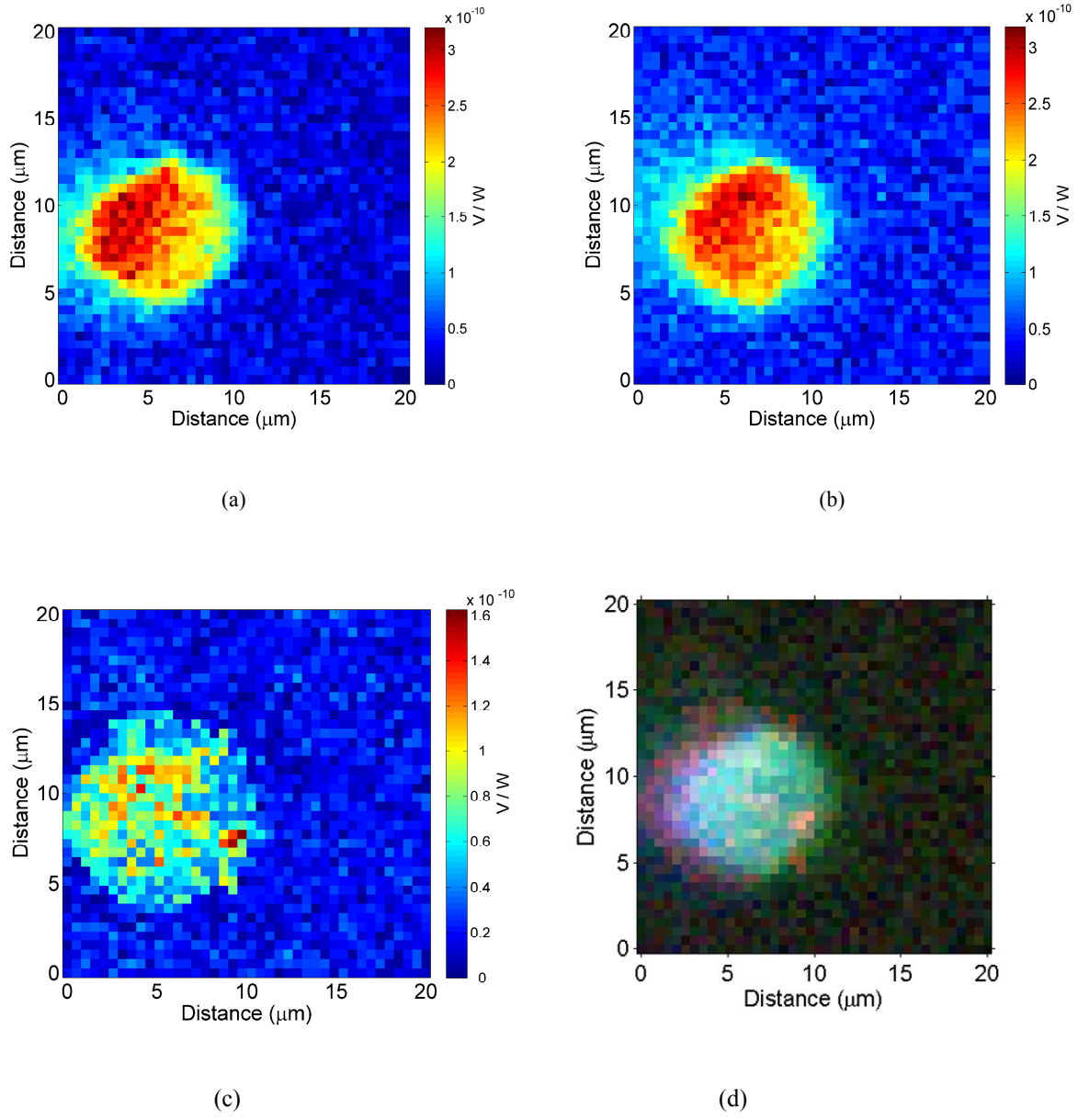


Figure 3. PA images of methemoglobin at the various optical wavelengths (a) 473 nm, (b) 533 nm, (c) 633 nm, and (d) RGB image.

The solid black line in absorption spectrum of Figure 2 represents RBC containing methemoglobin (RBC(Meth)). In these experiments, methemoglobin is obtained by oxidizing hemoglobin using $NaNO_3$ which can be reduced back to hemoglobin using methylene blue.[19] The iron Fe2+ (ferrous) in the heme group is oxidized to Fe3+ (ferric) state. This conformational change would inhibit bonding new free oxygen. This change inhibits the unloading of the bonded oxygen in tissues and predisposes the tissues to hypoxia due to an increased affinity towards its own bounded oxygen. This change would be observed as a leftward shift of the oxygen dissociation curve. The optical absorption spectrum of methemoglobin exhibits absorption peaks at 519 nm, 562 nm and 594 nm. The broad absorption peak from the measurements that is observed around 640 nm confirms the formation of methemoglobin.

Figure 3 is the PA images of a single RBC(Meth) at wavelengths of 473 nm, 533 nm, and 633 nm. The image was obtained by raster scanning the sample in step of 0.5 μm. The image represented by figure 3 took about 20 minutes to complete. The PA amplitude (V) was normalized with respect to input irradiance (W/cm^2). The legend on the right hand side represents the normalized PA signal. The PA image obtained at 473 nm exhibits the strongest PA signal compared to the other wavelengths. It exhibits PA amplitude about 1.03 times greater than green (533 nm) wavelength and about 2.4 times greater than red (633) wavelength. A single RBC contains about 270 million hemoglobin molecules. All the hemoglobin molecules present in a single RBC may not be the same type. The type of hemoglobin present in the RBC depends on its oxygen saturation state, and hereditary and acquired blood disorders.[13,20,21] The PA study at 633 nm shows the non-uniform distribution of methemoglobin in a single RBC. The PA studies can also be used to study the distribution of various types of hemoglobins in a single cell. Figure 3(d) is the RGB image obtained by fusing PA images (figure 3 (a), 3(b) and 3(c)) obtained at wavelength 473 nm, 533 nm and 633 nm. All the three images are converted to uint8 grayscale images and then concatenate along a third dimension for RGB image.

The similar trend is observed in the measured optical absorption spectrum. At 473 nm methemoglobin has a relatively large absorption of 1.08 times greater than at 533 nm, and about 1.34 times greater than at 633 nm wavelength. The developed system can also be used as a derivative PA spectroscopy.[22]

The developed technique can be used to study the hemoglobin concentration of blood which is usually determined by conventional techniques such as CO-oximeter, pulse oximeter, and blood gas analyzer. It also exhibits additional advantages of studying optically opaque samples such as highly concentrated methemoglobin and deep tissue properties.

4. CONCLUSION

The multispectral multimodal PA system operating at wavelengths 473 nm (red), 533 nm (blue) and 632.8 (green) nm was developed to study methemoglobin optical and PA properties. A demonstrated application was measuring the PA signal at the three different wavelengths to determine a spatial map of the hemoglobin and methemoglobin in a single red blood cell. Traditionally a CO-oximeter, pulse oximeter and blood gas analyzer are used to study methemoglobin. The developed PA imaging approach can be used to study these properties further, and enable the study of opaque samples such as highly concentrated methemoglobin.

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