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Bond-selective imaging of deep tissue through the optical window between 1600 and 1850 nm

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Abstract

We report the employment of an optical window between 1600 nm and 1850 nm for bond-selective deep tissue imaging through harmonic vibrational excitation and acoustic detection of resultant pressure waves. In this window where a local minimum of water absorption resides, we found a 5 times enhancement of photoacoustic signal by first overtone excitation of the methylene group CH₂ at 1730 nm, compared to the second overtone excitation at 1210 nm. The enhancement allows 3D mapping of intramuscular fat with improved contrast and of lipid deposition inside an atherosclerotic artery wall in the presence of blood. Moreover, lipid and protein are differentiated based on the first overtone absorption profiles of CH₂ and methyl group CH₃ in this window.

VPA imaging of atherosclerotic plaque with the presence of blood.

Keywords

optical window; deep tissue imaging; photoacoustic imaging; atherosclerosis

1. Introduction

Optical imaging of deep tissue has been a formidable challenge due to tissue absorption and scattering of both incident photons and generated signals. Owing to reduced scattering and minimal tissue absorption, near infrared (NIR) excitation and emission represent an effective method to enhance the optical penetration depth. NIR contrast agents including the indocyanine green [1], fluorescent protein [2], quantum dots [3], and single-walled carbon nanotubes [4] were developed and applied to live animal imaging. NIR fluorescence using contrast agents has enabled in vivo and ex vivo imaging of atherosclerosis [5–7]. On the excitation side, non-linear optical microscopy increased the penetration depth by using near-infrared (NIR) excitation [8–12]. In particular, excitation between 1000 to 1300 nm has allowed multiphoton imaging of vasculature in mouse brain at a depth of 1.0 mm [13]. Furthermore, use of adaptive optics has allowed deep tissue imaging at enhanced spatial resolution and signal strength [14].

Beyond the transport mean free path of the NIR light, which is around 1.0 mm in a soft tissue, photon propagation changes from the ballistic regime to diffusive regime [15]. Photoacoustic imaging [16], which utilizes absorbed light to produce local heating and subsequent thermal expansion and detection of the transient pressure waves by an ultrasound transducer, has allowed visualization of vasculature at visible wavelengths [17, 18]. PA imaging at NIR wavelengths is demonstrated by using nanostructures as contrast agents [19–22]. Moreover, PA imaging at NIR wavelength region has also been applied to artery imaging and even towards the endoscopy [23–28]. Among those studies, 1200 nm excitation has been demonstrated to allow label-free detection of lipid inside an artery wall [23, 26–28]. In vibrational photoacoustic (VPA) microscopy, the contrast arises from harmonic vibration [23]. Compared to NIR spectroscopy [29], photoacoustic detection avoids scattering and allows depth-resolved signal collection.

Until now, the consensus is that the gold optical window lies between 650 and 1300 nm (grey shadow region). It is commonly believed that the window stops at 1300 nm due to significant water absorption at longer wavelengths. Nevertheless, we have realized that the water absorption between 1.0 and 3.0 μm is modulated by the vibration transition of H_2O , namely the fundamental symmetric vibration ν_1 and asymmetric vibration ν_3 at ~ 2900 nm, ν_2 (bending) + ν_3 at ~ 1938 nm, ν_1 + ν_3 at ~ 1453 nm, second combinational transition at ~ 1200 nm, and second overtone transition at ~ 979 nm [30–32] (Figure 1A). We note that a valley exists between 1600 and 1850 nm, where the absorption coefficient of pure water is at the same level as that of heme proteins in whole blood around 800 nm. Considering the reduced scattering and diminished phototoxicity at longer wavelength excitation, the wavelength region from 1600 to 1850 nm is appealing as a new optical window for deep tissue imaging (light red shadow region). Importantly, the first overtone of CH vibration, which has higher transition strength by one order of magnitude compared to the second overtone, is located at the same window of 1600 to 1850 nm. Such spectral features make it promising to perform label-free imaging by first overtone excitation and acoustic detection. In this paper we show photoacoustic imaging of arterial plaques by excitation of the first overtone of CH bond at 1730 nm from the lumen through a layer of whole blood. Furthermore, we show selective imaging of lipids and proteins by excitation of CH_2 and CH_3 in the same optical window.

2. Experimental

2.1 Vibrational photoacoustic spectroscopy and imaging

A Nd: YAG pumped optical parametric oscillator (OPO, Panther Ex Plus, Continuum) was utilized as the excitation source (Figure S1). The excitation module provides 10 Hz, 5 ns

pulses laser with the wavelength range from 400 nm up to 2500 nm. The near-infrared light, produced at the idler beam from the OPO, was directed to an inverted microscope (IX71, Olympus) for spectroscopy and imaging purposes. The laser irradiation was focused by an achromatic doublet lens (30 mm focal length, Thorlabs). A focused-type, 20 MHz ultrasound transducer with a 50% bandwidth (V317, Olympus NDT) was employed to detect the photoacoustic signal. A 30 dB low noise preamplifier (5682, Olympus NDT) and a receiver (5073PR-15-U, Olympus NDT) with adjustable gain were applied for receiving signal. The signal was then sent to a digitizer (USB-5133, National Instrument), recorded by PC via a LabVIEW (National Instrument) program. VPA spectra were taken by automatic laser wavelength scanning of the OPO system. For the 3 dimensional (3-D) VPA imaging, a 2 dimensional (2-D) scanning stage (ProScan H117, Prior) was employed for the raster scanning. All 2-D images were reconstructed using a MATLAB program, while 3-D images and movies were built via ImageJ and Voxx [33], respectively.

2.2 Intramuscular fat sample

The muscle samples were harvested from goat and then preserved in 10% neutered-buffered formalin. The muscle chunk was then cut into $\sim 10 \times 10 \times 4$ mm pieces. The small muscle piece which contains a considerable amount of intramuscular fat was then placed in a glass bottom dish and embedded in D₂O-agarose gel for imaging purposes.

2.3 Phantom construction

To measure the effect of water absorption, a PDMS wedged well was created in a cover glass bottom dish. Water was added into the well and covered by a polyethylene film which generated the VPA signal. The polyethylene film was then covered with 2.5% agarose-water gel. VPA amplitude as function of water layer thickness can be obtained by scanning the sample along the wedged direction. To measure the effect of the present of blood, we replaced the water with blood.

For lipid-protein differentiation, phantom was constructed by solid butter fat and tendon which extracted from the tail of a Sprague Dawley rat. Butter fat and rat tail tendon were embedded in agarose-D₂O gel, and then covered by D₂O as acoustic coupling medium.

2.4 Monte Carlo simulation

For evaluation of the effect of blood scattering and absorption to the VPA signal, a Monte Carlo simulation according to photon propagation in multilayered tissue method [34] was performed to calculate the excitation light attenuation by whole blood.

2.5 Artery samples from Ossabaw miniature swine model

Swine were fed high-fat/cholesterol/fructose diet, which was composed of 2% cholesterol, 20% kcal from fructose, and 43% kcal from hydrogenated soybean oil coconut oil, and lard [35]. Iliac arteries, including the bifurcation of the internal and external iliac arteries, were harvested and then preserved in 10% phosphate-buffered formalin. Before imaging was performed, arteries were washed by PBS and incised longitudinally for luminal imaging.

Detailed information on the materials and methods is included in the *Supporting Information*.

3. Results and discussion

To identify the valid contrast in the new window, we recorded the VPA spectra of major functional groups (see schematics of VPA imaging and spectroscopy system in Figure S1). Figure 1B shows the VPA spectra of CH₂-rich polyethylene, CH₃-rich trimethylpetane,

water, and deuterium oxide (D_2O). The new window is indicated by light red shadow. The spectrum of polyethylene provides the absorption profile of the methylene group (CH_2), for which the first overtone ($2\nu CH_2$) shows two primary peaks around 1730 nm (5800 cm^{-1}) and 1760 nm (5680 cm^{-1}). The stronger peak at 1730 nm is generally thought to be a combination band of asymmetric and symmetric stretching ($\nu_a + \nu_s$) [32, 36]. The 1760 nm peak is assigned to the first overtone of stretching [32]. The second combination band of CH_2 , located between 1350 and 1500 nm, is attributed to the combination of harmonic stretching and non-stretching, such as bending, twisting and rocking ($2\nu + \delta$) [32]. The second overtone of CH_2 is peaked around 1210 nm. Noticeably, the VPA amplitude at 1730 nm is around 6.3 times of that at 1210 nm.

The spectrum of trimethylpentane is mainly contributed by the absorption profile of methyl group (CH_3). The primary peak around 1700 nm (5880 cm^{-1}) is assigned to the first overtone of CH_3 stretching. It is a remarkable fact that the CH_2 and CH_3 groups have distinguishable profiles at the first overtone region. The second combination band of CH_3 starts from 1350 to 1500 nm with the main peak around 1380 nm, which is generally thought to be $2\nu + \delta$ [32]. The second overtone of CH_3 shows the primary peak around 1195 nm.

The H_2O band around 1450 nm, generally referred to as the first overtone of OH stretching, is a combination band of OH asymmetric and symmetric stretching ($\nu_1 + \nu_3$) [32]. The peak around 1940 nm is assigned to combination of bending and asymmetric stretching ($\nu_2 + \nu_3$) [32]. Excitingly, no water absorption peak is found in between the two combination bands, where the strong first overtones of CH_2 and CH_3 are located. Therefore, a potential optical window for imaging CH bond in deep tissue can be created at the water absorption 'valley' between 1600 and 1850 nm. For D_2O , no significant absorption peak is found below 1900 nm, which indicates that D_2O can be an ideal VPA coupling medium between excitation light and samples for VPA imaging and spectral measurements.

We used intramuscular fat to compare the amplitudes of VPA signals produced by first and second CH_2 overtone absorption (Figure 1C). With the same pulse energy ($45\text{ }\mu\text{J}$) for the 1730 and 1210 nm beams, the level of signal by first overtone absorption is 5 times of that by second overtone absorption. As the signal-to-noise ratio (SNR) in our setup is current limited by the electronic noise, which is constant when changing the laser intensity and wavelengths, using 1730 nm excitation also increased the SNR 5 times compared to the 1210 nm excitation. To confirm that the contrast indeed arose from the CH_2 vibration, a VPA spectrum was taken from a fat marked with a green cross in the top panel of Figure 1C. As seen in the bottom panel, two primary peaks around 1730 and 1760 nm were detected, consistent with the CH_2 absorption profile of polyethylene. A 3-D map of lipid network formed by intramuscular fat is shown in Movie S1. It was found that 3.5 mm penetration into the muscle tissue could be reached.

Because water absorption at 1730 nm is about 5 times larger than that at 1210 nm, we studied the effect of water absorption on the first and second CH_2 overtone excitation, using a phantom shown in Figure 2A. By moving the sample from right to left and scanning the excitation wavelengths, the VPA spectra of polyethylene at different water thickness were obtained (Figure 2B). By increasing the water layer thickness, a greater decrease of signal at 1730 nm was seen. Nevertheless, the VPA signal produced by the first overtone remained larger than that by the second overtone when a water layer of 2.9 mm was added to the beam path. To validate the data, we theoretically computed the local light flux using the Beer-Lambert law for water absorption and calculated the PA signal ratio between the first and second overtone excitation, as shown in Figure 2C (red solid line). The calculation agrees well with the data, showing that 1730 nm excitation is beneficial even through 3 mm water absorption. With average pulse energy of $50\text{ }\mu\text{J}$ in the focal volume, the temperature

increase due to water absorption at 1730 nm is calculated to be 1.0 Kelvin. Experimentally, no cell damage was found at the pulse energy as high as 150 μJ (Figure S2).

Next we examined the benefit of 1730 nm excitation when scattering is taken into account. This is especially the case for detection of arterial plaques through whole blood which has scattering coefficient ($\mu_s = 600 \text{ cm}^{-1}$ at 1200 nm) much larger than absorption coefficient ($\mu_a = 1.2 \text{ cm}^{-1}$ at 1200 nm). To estimate how blood absorption and scattering affect the VPA signal, we used the same phantom construction as shown in Figure 2A, except that water was changed to rat whole blood in the wedged well. The VPA signals from polyethylene by 1730 and 1210 nm excitation were measured as a function of blood layer thickness. The results are shown in Figure 2D. At 0.5 mm blood, the signal at 1210 nm is fairly weak, whereas the signal at 1730 nm is as large as the signal at 1210 nm generated with 0.1 mm blood in the beam path. To validate the data, we calculated the light delivery efficiency using Monte Carlo simulation (see methods). The calculated signal amplitude agrees well with the experimental data (Figure 2D). The VPA signal at 1730 nm is 3 times larger than that at 1210 nm in the entire range of blood layer thickness from 0 to 0.8 mm. Specifically, when 0.5 mm blood layer presents, 1730 nm excitation gains 5 to 6 times in signal level compared to 1210 nm excitation. Such enhancement is largely owing to larger absorption by first CH overtone excitation.

We then employed the optical window between 1600 to 1850 nm to VPA imaging of an atherosclerotic artery from the lumen through a 0.5 mm thick layer of whole blood (Figure 3). The atherosclerotic iliac artery was extracted from an Ossabaw pig atherogenic diet for 6 months [35]. The artery sample was cut open longitudinally and placed in a sample container (Figure 3A). A 0.5 mm thick layer of whole blood extracted from rat was inserted in the space between the sample and the coverslip. A focused ultrasound transducer was placed across the sample for forward receiving. The 3-D image by 1730 nm excitation is shown in Figure 3B and Movie S2. Lipid depositions as deep as 3.0 mm underneath the blood layer were detected. The blood layer gave a strong contrast, possibly because of the VPA signal from the blood components such as the heme proteins and water. Importantly, the artery sample and the blood layer can be well differentiated owing to the depth resolvability of photo-acoustic imaging. The advantage of using 1730 nm over 1210 nm excitation was shown by b-scan VPA imaging (Figure 3C, D). A signal reduction of 6 times was found when switching the laser to 1210 nm, resulting in disappearance of contrast from the lipid depositions (Figure 3D). The VPA spectrum (Figure 3E) of the lipid deposition matches the profile of first overtone of CH_2 .

We further demonstrated bond-selective VPA imaging of lipid and protein by making use of the distinctive spectral feature of CH_2 and CH_3 groups in the first overtone region. Phantoms composed of butter fat containing CH_2 -rich lipid and rat tail tendon containing type I collagen were used to demonstrate this concept. Figure 4A shows the VPA spectra of butter fat and rat tail tendon. The fat shows two peaks at 1730 and 1760 nm, corresponding to the first overtone transitions of CH_2 . The type I collagen shows a shoulder that appears below 1700 nm, contributed by the CH_3 groups. Note the much lower VPA signal from the tendon, which is more discernable in the spectrum because the display gain was 15-fold higher. VPA imaging of the phantom sample was performed (Figure 4B, C). The result shows that lipid and protein can be differentiated using 1730 and 1640 nm excitation. Such capability was applied to 3-D VPA imaging of the outer layer of an intact artery composed of perivascular fat and adventitia. The artery was placed in the glass bottom dish and stabilized by D_2O agarose gel (Figure S3A). Figures 4D–F show images at selected x - z plane and Figures 4G–I show images at selected x - y plane through the adventitia. The 3-D image is shown in Figure S3B and Movie S3. The contrast at 1640 nm illustrates the location of proteins whereas the contrast at 1730 shows the distribution of vascular fat. VPA

spectra taken at selected position (Figure S3C) confirmed that these contrasts were contributed by signals from CH₃ and CH₂, respectively. Moreover, bond-selective 3-D imaging of an atherosclerotic plaque by 1640 and 1730 nm excitation from the lumen side (Movie S4) showed different contrasts from protein content (cyan color) and lipid deposition (yellow color).

4. Conclusion

A variety of advanced techniques have been developed to characterize the atherosclerotic plaque [37, 38], including multidetector spiral computed tomography [39], magnetic resonance imaging [37, 40], intravascular ultrasound [41], optical coherent tomography [42] and intravascular near infrared spectroscopy [29]. As shown here, label-free imaging atherosclerotic plaques can be performed through optical excitation of first overtones of CH bonds and acoustic detection of the generated ultrasound waves in a previously underappreciated optical window (1.6 to 1.85 μm). Furthermore, selective VPA imaging of collagen and lipids heralds the potential in diagnosis of vulnerable plaques through detection of the thickness of the collagen cap and the location of the lipid-laden plaque inside the arterial wall without molecular labeling that could alter tissue composition. Collectively the current work opens new opportunities for label-free imaging of lipid-laden plaques in atherosclerosis and other medical conditions.

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optical imaging tools and nano-technologies for challenging applications in biomedicine such as early detection of tumor spread and early nerve repair after traumatic spinal cord injury.

Figure 1.

(online color at: www.biophotonics-journal.org) Spectroscopy and imaging in the new optical window. (A) Absorption spectra of whole blood and pure water adapted from Ref. [30, 43]. (B) PA spectra of polyethylene, trimethylpentane, water and deuterium oxide. (C) VPA imaging of muscular fat. The maximum amplitude projection images were acquired by CH₂ first (top panel) and second (middle panel) overtone excitation. The VPA spectrum of muscular fat is shown in the bottom panel. Scale bar: 1 mm.

Figure 2. (online color at: www.biophotonics-journal.org) Phantom study to evaluate the effect of water and blood on VPA imaging. **(A)** Schematic of the constructed phantom. PE: polyethylene. **(B)** VPA spectra of PE at different water layer thickness. **(C)** PA amplitude ratio between the first and second overtone excitation as a function of water layer thickness. **(D)** PA amplitude as a function of blood layer thickness.

Figure 3.

(online color at: www.biophotonics-journal.org) VPA imaging and spectroscopy of arterial plaque in the presence of blood. (A) Schematic for VPA imaging of an artery through whole blood. (B) 3-D imaging of arterial plaque by 1730 nm excitation. Lumen and blood are indicated with red arrows. To precisely control the thickness at 0.5 mm, the blood was sandwiched between two cover glasses. As the result, the ultrasound signal from the blood layer was reflected by the glasses for multiple times, which generated an interference pattern of the signal from blood. (C) VPA image at selected x - z plane under 1730 nm excitation. (D) VPA image at selected x - z plane under 1210 nm excitation. (E) VPA spectrum taken at the lipid deposition pointed by red arrow in (C). Scale bar: 1 mm.

Figure 4.

(online color at: www.biophotonics-journal.org) Bond-selective VPA imaging to differentiate lipid and protein. (A) VPA spectra of butter fat and rat tail tendon. (B–C) B-scan VPA imaging of butter fat – tendon phantom using 1640 nm (B) and 1730 nm (C) excitation. (D–E) VPA images at selected *x-z* plane under 1640 nm (D) and 1730 nm (E) excitation. (F) Merged image. (G–H) VPA images at selected *x-y* plane in adventitia under 1640 nm (G) and 1730 nm (H) excitation. (I) Merged image. Scale bars: 1 mm.