Photothermal optical lock-in optical coherence tomography for \textit{in vivo} imaging

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Abstract: Photothermal OCT (PTOCT) provides high sensitivity to molecular targets in tissue, and occupies a spatial imaging regime that is attractive for small animal imaging. However, current implementations of PTOCT require extensive temporal sampling, resulting in slow frame rates and a large data burden that limit its \textit{in vivo} utility. To address these limitations, we have implemented optical lock-in techniques for photothermal optical lock-in OCT (poli-OCT), and demonstrated the \textit{in vivo} imaging capabilities of this approach. The poli-OCT signal was assessed in tissue-mimicking phantoms containing indocyanine green (ICG), an FDA approved small molecule that has not been previously imaged \textit{in vivo} with PTOCT. Then, the effects of \textit{in vivo} blood flow and motion artifact were assessed and attenuated, and \textit{in vivo} poli-OCT was demonstrated with both ICG and gold nanorods as contrast agents. Experiments revealed that poli-OCT signals agreed with optical lock-in theory and the bio-heat equation, and the system exhibited shot noise limited performance. In phantoms containing biologically relevant concentrations of ICG (1 µg/ml), the poli-OCT signal was significantly greater than control phantoms (p<0.05), demonstrating sensitivity to small molecules. Finally, \textit{in vivo} poli-OCT of ICG identified the lymphatic vessels in a mouse ear, and also identified low concentrations (200 pM) of gold nanorods in subcutaneous injections at frame rates ten times faster than previously reported. This work illustrates that future \textit{in vivo} molecular imaging studies could benefit from the improved acquisition and analysis times enabled by poli-OCT.

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References and links


1. Introduction

In vivo molecular imaging in animal models serves a vital role in medical research, providing fundamental insights into mechanisms of disease formation and progression [1], as well as drug discovery [2]. Unfortunately, current small animal molecular imaging tools supply either high resolution or a wide field of view, but not both. For example, fluorescence microscopy can image molecular contrast in vivo [3, 4] at subcellular (<1 µm) resolution, but only over a small, depth-limited field of view of a few hundred micrometers. Unlike microscopy, whole body imaging (e.g. bioluminescence imaging, positron emission tomography, magnetic resonance imaging) of contrast agents provide molecular images without practical limitations to imaging depth. However, whole body imaging approaches suffer from limited spatial resolution (nm-cm) [2]. Optical coherence tomography (OCT) fills the niche of high resolution (1-30 µm), wide field of view (5-10 mm), and deep (1-3 mm) three-dimensional optical imaging [5]. OCT is a cost-effective noncontact imaging tool, capable of MHz line rates [6]. Using low coherence interferometry of near infrared (NIR) photons, OCT provides depth-resolved images of tissue structure, vessel morphology [7], and blood flow [8]. Yet, OCT is not especially sensitive to molecular targets, since the index of refraction varies weakly amongst most molecular species in tissue.

Photothermal OCT (PTOCT), a functional extension of OCT, enhances the sensitivity and specificity of OCT to molecular sources of contrast [9, 10]. Similar to photoacoustic imaging, another emerging molecular imaging technique, PTOCT images the effects of photon absorption. Unlike photoacoustic imaging, PTOCT is a contact-free tool that images heat release after photon absorption, rather than the release of acoustic waves from thermally confined laser pulses. After photon absorption by a chromophore, heat released into the
microenvironment causes thermoelastic expansion and slight changes in local refractive index, which in turn alter the observed optical path length. PTOCT quantifies these photothermal-induced optical path length variations using phase-sensitive OCT measurements and post-acquisition frequency analysis. PTOCT has been used to image a range of exogenous and endogenous forms of contrast in vitro and ex vivo including gold nanospheres [9], gold nanorods [11, 12], gold nanoshells [10, 13, 14], carbon nanotubes [15], and blood [16]. Recently, PTOCT was expanded to in vivo applications, and used to track in vivo heterogeneities in intratumoral nanoparticle distributions [17]. Even with its recent success, PTOCT still suffers from impracticalities that limit its widespread use for in vivo molecular imaging. In the traditional PTOCT implementation for imaging trace concentrations of contrast agents, one A-scan position is temporally sampled hundreds to thousands of times, and heating dynamics are identified from the phase of the depth-resolved OCT signal using post-acquisition frequency analysis. This post-acquisition frequency analysis scheme requires time consuming data acquisition, excessive data collection, and lengthy offline signal analysis. These limitations make traditional PTOCT impractical and lengthy for multi-animal in vivo time-course studies, and have therefore restricted its use in research.

Optical lock-in detection, a real-time alternative to post-acquisition frequency analysis, has been previously implemented for photothermal microscopy of cell monolayers [18]. This approach was recently expanded to depth-resolved photothermal optical coherence microscopy [19] and two-dimensional wide field photothermal microscopy [20] of gold nanospheres in cell monolayers. Specifically, a photothermal optical lock-in optical coherence microscopy (poli-OCM) technique was developed, allowing for kHz line rate photothermal imaging with OCM systems [19]. By imposing a frequency shift on the reference arm light, this optical lock-in technique removes the temporal sampling constraints of photothermal OCM, thus reducing its data burden and effectively transforming the OCM signal to a real time image of photothermal heating. However, optical lock-in acquisition has yet to be demonstrated in optically thick samples or in vivo, where confounding factors such as motion and blood flow introduce artifacts into the image.

Here, optical lock-in techniques are implemented for real time imaging of NIR absorbing contrast agents in vivo using photothermal optical lock-in optical coherence tomography (poli-OCT). An in vivo poli-OCT system was built and tested using tissue-mimicking phantoms. In addition, motion artifact and blood flow, which are confounding factors unique to in vivo imaging, were characterized and attenuated. Finally, contrast agents were imaged in vivo using frame rates at least 10 fold faster than previous in vivo publications [11, 17]. Two contrast agents were imaged in vivo in order to demonstrate the versatility of poli-OCT to a range of contrast agents, including an FDA approved small molecule (indocyanine green: ICG) that has not been previously measured in vivo with PTOCT, as well as gold nanorods. Overall, the development of poli-OCT for in vivo use provides improved in vivo molecular imaging in the unique spatial niche of OCT.

2. Materials and methods
2.1. poli-OCT imaging
The theory for poli-OCT has previously been published [19]. In an interferometric imaging system, a carrier frequency is introduced by imposing a temporal frequency shift (ΩR) onto the reference arm. In the presence of photon-absorbing molecules and frequency matched photothermal-induced phase oscillations (Ωp), the resulting interference pattern is demodulated. Low pass filtering then distinguishes the demodulated signal from background. Temporal integration by the CCD serves as the low pass filter, attenuating the signal due to static scatterers to zero, and maintaining the demodulated signal due to absorption (Fig. 1(a)). To attenuate the signal due to scatterers, the integration time (τ) of the CCD must be set to a multiple (n) of the frequency shift period (T0, where τ = n/ΩR = nT0). This optical lock-in technique transforms the OCT signal as a function of depth (z), defined as the amplitude of the tomogram (|S(z)|) after Fourier transform of the interference signal, to a real time image of
photothermal heating (i.e. poli-OCT). The poli-OCT signal at a given depth ($|S(z)|$) where an absorber such as ICG is present is described by

$$|S(z)| \propto \alpha r(z) r_r \sqrt{P_s P_r}$$

(1)

where $\alpha$ describes the magnitude of the photothermal heating signal, $r(z)$ and $r_r$ are the reflectivities at depth $z$ in the sample and at the reference arm, respectively and $P_s$ and $P_r$ are the power to the sample and reference arm, respectively [19].

For in vivo imaging, a custom poli-OCT system was constructed with an 860 nm center wavelength, 40 nm bandwidth (Fig. 1(b), yellow, inset), superluminescent diode imaging laser (SLD, Fig. 1(b)). Light from the SLD was split between the sample and reference arms using a 50:50 fiber coupler (50:50, Fig. 1b). A programmable frequency shift ($\Omega_R$) was applied to the reference arm using the beat frequency of two acousto-optic modulators (AOMs) in serial configuration. The first AOM (AOM 1, Fig. 1(b)) performed a frequency downshift at the AOM central carrier frequency ($\omega_c$, where $\omega_c = 100$ MHz), while the second AOM (AOM 2, Fig. 1(b)) performed a slightly offset frequency upshift ($\omega_c + \Omega_R$). AOMs operate on the MHz time scale, therefore the beat frequency ($\Omega_R$) of AOM 1 and AOM 2 was used to shift the operational range to the Hz and kHz regime, where photothermal effects are large enough to image with our focused spot size (24 µm). A custom phase-locked AOM driver (Brimrose) controlled the frequency shifts and power throughput of both AOMs, with an operational range of 80-120 MHz and 1 Hz resolution. Knife edges removed zeroth-order output beams from the AOMs, and the reference arm light was focused into a fiber coupler for interferometry. In the sample arm, polarization optics were used to control power to the sample and minimize autocorrelation artifacts. Light output from the 50:50 fiber coupler was collimated and the polarization angle was tuned using a half wave plate (HWP, Fig. 1(b)). A linear polarizer (LP, Fig. 1(b)) then rejected any light off axis from the reflective mode of the polarization beam splitter (PBS, Fig. 1(b)). After reflection towards the sample by the PBS, a quarter wave plate (QWP, Fig. 1(b)) controlled the polarization shape of the sample arm light, from circular (45 degrees) to linear (0 degrees). A 3X scan lens (Thorlabs) then focused the light onto the sample (depth of field = 1.15 mm, 1/$e^2$ spot size = 24 µm). Any light backreflected from the sample that matched the transmission state of the PBS was collected into a fiber coupler for interferometry. The output interference signal from the fiber coupler was dispersed by a spectrometer and captured by a CCD with tunable integration time from 50 to 10,000 µs. To achieve optical lock-in detection, the integration time ($\tau$) of the CCD was set to a multiple (n) of the frequency shift period ($T_0$, where $\tau = n T_0$). The spectrometer, galvo drive electronics, and software from a commercial OCT system (Bioptigen) were used for the poli-OCT system.

Photothermal heating was achieved with a wavelength tunable Titanium:Sapphire (Fig. 1(b)) photothermal laser (PT laser). The wavelength of the PT laser (Fig. 1(b), green, inset) was tuned to match the absorption of the selected contrast agent, as shown for ICG (Fig. 1(b), inset). To induce photothermal phase oscillations, the PT laser was intensity modulated at the same frequency as the reference arm ($\Omega_R$, where $\Omega_R = \Omega_P$) using a third AOM (AOM 3, Fig. 1(b)), and then fiber coupled into the imaging system. Commercial software (Brimrose) controlled the frequency shifts in all three AOMs, while analog inputs (0-1 V) drove the intensity of the output RF signal. The RF signals sent to AOM 1 and AOM 2 were attenuated, mixed, and then low pass filtered using inline RF electronics to isolate the beat waveform. The beat waveform from AOM 1 and AOM 2 was digitally acquired, conditioned in LabVIEW to a square wave frequency matched to the beat of AOMs 1 and 2 ($\Omega_P = \Omega_R$), and output as an analog waveform to intensity modulate AOM 3.
2.2. Image processing

Unlike traditional PTOCT which requires Fourier analysis of the digitally sampled temporal phase data [11], poli-OCT images require the same image processing as standard OCT images. If the instrumentation and signal requirements are met, either an OCT ($\Omega_R = 0$ Hz) or poli-OCT ($\Omega_R \neq 0$ Hz and $\Omega_R = n/\tau$) image are acquired. To process images, data was first resampled from linearity in wavelength to linearity in wavenumber. The interference spectrum was then numerically dispersion corrected [21], followed by a Fourier transform along the wavenumber dimension to create the depth-resolved image. Fixed pattern noise removal was performed on the complex signal [22], and the poli-OCT signal was calculated as the non-log compressed magnitude of the depth-resolved data.

2.3. Imaging of phantoms

To quantify system performance and optimize the system for in vivo imaging, scattering phantoms were imaged with poli-OCT under varying parameters. Tissue mimicking phantoms containing ICG for photothermal absorption were created from clear silicone (Quantum Silicons) consisting of two parts (A and B) mixed at a 10:1 weight ratio. Rutile titanium dioxide (TiO$_2$, Sigma Aldrich) was added to a final concentration of 4.1 mg/g to mimic human skin scattering [23]. The TiO$_2$ was added to silicone component A, mixed for two minutes, and then degassed for two minutes using a planetary centrifugal mixer (Thinky USA). ICG diluted into a small volume of 70% ethanol was added to component B of the silicone mixture to a final concentration of 8 µg/ml, then mixed and degassed. Components A and B were then mixed and degassed. The final mixture was placed in a petri dish under vacuum at 29 inches of Hg for five minutes with brief returns to standard pressure every minute, and left to cure for 12 hours at 70 degree Celsius. Phantoms were imaged using a 2 mm B-scan (400 A-scans/B-scan) and data was averaged across each B-scan including the first 400 µm in depth in the phantom. Baseline acquisition parameters for phantom studies were 500 µW of SLD sample arm power, 25 mW average PT laser power to the sample, 6 ms CCD integration time, and 500 Hz frequency shift ($\Omega_P = \Omega_R$). In addition, reference arm power was adjusted to fill approximately 80% of the CCD dynamic range. Measurements
were acquired with the PT laser on (true poli-OCT signal), PT laser off (background poli-OCT signal), and with the sample arm blocked (noise floor). The poli-OCT signal was calculated as the non-log compressed OCT magnitude signal. Similar to previous work [19, 24], the signal to noise ratio (SNR) was defined as the squared magnitude of the poli-OCT signal with the PT laser on divided by the variance of the image signal with the sample arm blocked. Means and standard deviations were assessed over 10 repeated scans, and statistical significance was calculated using a non-parametric Wilcoxon Rank Sum test (p<0.05).

The integration time of the CCD and the sample arm power were altered individually, and the poli-OCT signal and SNR were calculated and compared to photothermal optical lock-in theory [19] to assess for shot-noise limited performance at millisecond integration times. First, while maintaining the system frequency shift at 500 Hz, the integration time of the CCD (τ = nT0, where T0 = 2 ms) was increased by integer multiples of the frequency shift period (T0) between 2 ms and 8 ms. For each integration time, the reference arm power was adjusted to fill 80% of the CCD dynamic range. In addition, while maintaining a constant integration time and frequency shift, the SNR was assessed using a non-parametric Wilcoxon Rank Sum test (p<0.05).

Finally, theoretical assumptions of poli-OCT were experimentally validated. According to theory [19], the presence of the photothermal signal in the poli-OCT image requires that the intensity modulation of the PT laser (Ωp) occurs at the same frequency as the reference arm frequency shift (Ωr). Therefore, Ωp was varied between 250 Hz and 750 Hz in 50 Hz increments, while maintaining Ωr at 500 Hz. In addition, in order to reject background scattering, the integration time of the CCD (τ) must be set to a multiple of the frequency shift period (T0). To experimentally validate this assumption, while maintaining a constant frequency shift of 500 Hz (T0 = 2 ms), the integration time was altered from 4 ms (τ = 2T0) to 8 ms (τ = 4T0) in 500 μs (T0/4) increments.

2.4. Effect of motion and blood flow

All animal studies were approved by the Vanderbilt University Animal Care and Use Committee and meet the NIH guidelines for animal welfare. Transitioning the imaging system to in vivo applications requires an understanding of the effects of motion and blood flow on the poli-OCT signal, specifically on the rejection of the background scattering signal (i.e. PT laser off). Therefore, a 4 by 4 mm (400 A-scans/B-scan, 400 B-scans/C-scan) rectangular volume was acquired of a mouse ear (The Jackson Laboratory, Foxn1nu/Foxn1nu) using 10 ms CCD integration time, 500 Hz frequency shift, and without the PT laser. A 1 by 1 mm (100 A-scans/B-scan, 100 B-scans/C-scan) rectangular volume was then acquired while varying the reference arm frequency shift (Ωr) from 100 to 2500 Hz. During imaging, the mouse was anesthetized using inhalant isoflurane (1.5-1.75%) while a closed-loop heating blanket maintained body temperature at 37 degrees Celsius.

2.5. In vivo imaging with poli-OCT

Photothermal imaging was performed in vivo in female nude mice (The Jackson Laboratory, Foxn1nu/Foxn1nu) using poli-OCT. Mice were anesthetized during imaging using inhalant isoflurane (1.5-1.75%), and a closed-loop heating blanket maintained body temperature at 37 degrees Celsius. Contrast agents were imaged from within intradermal locations of the mouse ear, an optically accessible biological site for preclinical studies of cancer [26], immune response [27, 28], and lymphatics [29–31]. The mouse ear was firmly attached to a 10 ml scintillation vial using double sided tape which was in turn taped to the imaging stage. En face
projections were created by taking the average signal across the depth dimension (~1 mm depth).

In the first demonstration of in vivo poli-OCT imaging to date, the lymphatic vessels of a healthy mouse ear were imaged with injected ICG in real time. Using a micromanipulator under OCT image guidance, a 31 gauge needle (Hamilton) was placed intradermally in the outer rim of the mouse ear. Approximately 5 µl of 500 µg/ml ICG [31, 32] was injected, and removal of ICG from the interstitial space via lymphatic vessel drainage was imaged with a 3 by 3 mm (300 A-scans/B-scan, 300 B-scans/C-scan) rectangular volume medial to the injection site. An integration time of 6 ms was used, with 500 Hz frequency shift ($\Omega_p = \Omega_b$), and 30 mW of average PT laser power (770 nm) on the sample. Image volumes were acquired with the PT laser on, the PT laser off, and the frequency shift ($\Omega_b$) set to 0 Hz (i.e. OCT). An accompanying speckle variance scan was acquired to identify native blood vessels over the 3 mm by 3 mm scan window. Speckle variance was calculated as the variance of the OCT magnitude signal over 10 repeated A-scans, acquired at a 10 kHz line rate [17].

Last, similar to previous publications [11], subcutaneous injections of gold nanorods were imaged with poli-OCT. Gold nanorods were manufactured and coated in poly(ethylene glycol) (PEG) according to previous methods [17], diluted to 400 pM concentration in water, then mixed 1:1 with Matrigel (Corning) to a final concentration of 200 pM. The Matrigel-gold nanorod solution was manually injected into the base of a mouse ear using a 28 gauge needle and allowed to solidify for 10 minutes. A 2.5 by 2.5 mm rectangular volume (250 A-scans/B-scan, 250 B-scans/C-scan) was imaged at the periphery of the injection site using 10 ms integration time, 500 Hz frequency shift ($\Omega_p = \Omega_b$), and 35 mW average PT laser power (740 nm) at the sample. Image volumes were captured with the PT laser on and off, and then again with a frequency shift ($\Omega_b$) of 0 Hz. In a separate mouse, a control injection using Matrigel diluted 1:1 in water was imaged using identical image parameters. In addition, an overlapping speckle variance scan was acquired to identify native blood vessels. For in vivo imaging, output poli-OCT image volumes were median filtered in the en face dimension to reduce the effects of motion artifact and then average filtered. The poli-OCT image contrast was also adjusted to exclude signals near the noise floor, and contrast adjustments were kept consistent across each set of images.

3. Results

3.1. Imaging scattering phantoms

Tissue-mimicking phantoms containing ICG (8 µg/ml) and TiO$_2$ were imaged under variable system parameters to quantify poli-OCT performance and to optimize the system for in vivo imaging. ICG phantoms did not exhibit significant photobleaching after repeated measurements in the same phantom location, and the phantoms maintained their integrity over multiple months. Results were acquired for the poli-OCT signal with the PT laser on (PT laser (+)), the background signal with the PT laser off (PT laser (-)), and the noise floor with the sample arm blocked. Both the poli-OCT signal (black, $r^2 = 0.97$, Fig. 2(a)) and SNR ($r^2 = 0.97$, Fig. 2(b)) scaled linearly with the integration time of the CCD while the integration time was set to a multiple of the frequency shift period. The poli-OCT signal scaled with the square root of the sample arm power (black, $r^2 = 0.98$, Fig. 2(c)), while the SNR of the poli-OCT signal increased linearly with the sample arm power ($r^2 = 0.98$, Fig. 2(d)). The trends observed in Fig. 2 agree with the theory established for photothermal optical lock-in [19], while the trends in SNR agree with the theory for shot noise limited performance, the optimal operation mode for OCT systems [19, 24].
Fig. 2. Poli-OCT signal characterization in phantoms containing ICG (absorber) and TiO₂ (scatterer) while altering image system parameters. Solid phantoms were imaged with the PT laser on (black, top), the PT laser off (blue, top), and the sample arm blocked (green, top). (a) The poli-OCT signal (black) increased linearly with integration time (τ) set to multiples (n) of the frequency shift period (T₀). (b) The SNR of the poli-OCT signal increased linearly as well. (c) The poli-OCT signal (black) increased with the square root of the sample arm power, while (d) the SNR increased linearly. The poli-OCT signal agrees with theory [19], and SNR results are consistent with shot noise limited performance [19, 24].

Parameters that affect the magnitude of the photothermal oscillations were also examined. Increasing the PT laser frequency (Ωₚ = Ω₀) from 200 to 800 Hz caused a nonlinear decay in the poli-OCT signal (Fig. 3(a)), while increasing the PT laser power resulted in a linear increase in poli-OCT signal (Fig. 3(b), r² = 0.99). Scattering phantoms with varying ICG concentrations from 0 to 17 µg/ml displayed a linear increase in poli-OCT signal (Fig. 3(c), r² = 0.99). Results in Fig. 3(c) include the removal of background signal (PT laser (-)) from the poli-OCT signal (PT laser (+)). Concentrations of ICG as low as 1 µg/ml displayed significantly increased (p<0.05, Fig. 3(c) inset) poli-OCT signal compared with scattering controls (red). ICG concentrations greater than 1 µg/ml can be reached at a tumor site when ICG is tagged to a systemically injected antibody, indicating that ICG and poli-OCT could potentially be used for in vivo molecular imaging studies in cancer, or to track delivery of antibody therapies (e.g. trastuzumab in breast cancer) [33]. The effects seen from increased PT laser frequency, PT laser power, and absorber concentration all agree with previous traditional PTOCT data and a model of the bio-heat conduction equation [11, 25].
Fig. 3. Poli-OCT signal characterization in phantoms containing ICG (absorber) and TiO₂ (scatterer) while altering photothermal signal magnitudes. (a) As the PT laser frequency (Ωₚ, where Ωₚ = Ωᵣ) is increased, the poli-OCT signal (black) decreases nonlinearly. (b) Increasing PT laser power caused a linear increase in poli-OCT signal. (c) Increasing the concentration of the ICG resulted in a linear increase in the poli-OCT signal. The average signal from n = 10 repeated scans showed a significant increase (*p<0.05, figure inset) with 1 µg/ml concentration of ICG compared to the control (red).

Theoretical assumptions for photothermal optical lock-in were experimentally validated as well. Mismatch between the PT laser modulation frequency (Ωₚ, Fig. 4(a) x-axis) and the reference arm frequency shift (Ωᵣ, Fig. 4(a) red vertical line) caused a large fall off in poli-OCT signal (Fig. 4(a)). There is however, a broad range of frequencies over which the poli-OCT signal remains high (FWHM ~150 Hz), indicating that Ωₚ and Ωᵣ do not need to be precisely frequency locked to measure photothermal absorption. It should be noted that mismatching Ωₚ and Ωᵣ resulted in temporal oscillations in the image signal (data not shown). Additionally, altering the integration time from 4 ms to 8 ms while maintaining a frequency shift of 500 Hz (T₀ = 2 ms) altered both the poli-OCT signal (PT laser (+), black, Fig. 4(b)) and background scattering signal (PT laser (-), blue, Fig. 4(b)). The poli-OCT background signal (PT laser (-), blue, Fig. 4(b)) approached the noise floor (Noise Floor, green, Fig. 4(b)) only when CCD integration times (τ) were integer multiples (n) of the frequency shift period (T₀) (Fig. 4(b), red vertical lines). Therefore, any source of alteration to the programmed frequency shift (Ωᵣ) in the signal could result in incomplete removal of static background scatterers from the poli-OCT image. Slight differences between background and noise floor were due to the autocorrelation signal.

Fig. 4. Poli-OCT signal characterization in phantoms containing ICG (absorber) and TiO₂ (scatterer), validating theoretical assumptions. (a) When the PT laser modulation frequency (Ωₚ, x-axis) is mismatched from the reference arm frequency shift (Ωᵣ, red vertical line), the poli-OCT signal (black) is attenuated. (b) The poli-OCT signal with the PT laser on (black) and off (blue) are both minimized at CCD integration times (τ) that are integer multiples (n) of the frequency shift period (T₀) (Fig. 4(b), red vertical lines). The scattering signal (blue) is effectively removed only when the integration time is equal to a multiple of the frequency shift period.
3.2. Effect of motion and blood flow

As demonstrated by phantom experiments (blue, Fig. 4(b)), a false positive background signal emerges when the CCD integration time is not divisible by the frequency shift period. Motion artifact and blood flow expected during in vivo imaging impose their own frequency shifts on the image signal, causing the interference pattern to carry a frequency shift different from the programmed one ($\Omega_R$). Therefore, it was hypothesized that motion artifact and blood flow would cause incomplete rejection of a non-absorbing sample. As predicted, motion artifacts due to breathing and cardiac cycles of the mouse as well as blood flow within vessels result in incomplete rejection of the background scattering signal (Fig. 5(a)). Although the background signal due to blood flow and motion seem indistinguishable in one B-scan (Fig. 5(a)), the en face average intensity projection from the complete image volume (Fig. 5(b)) reveals the transient and streaking features of the motion artifact (red arrows, Fig. 5(a)-5(b)), and the more structured nature of the flow signal (green arrows, Fig. 5(a)-5(b)). Motion artifact-induced streaking was reduced with a median filter in the en face dimension over a 4X4 pixel neighborhood. The filter maintains the structure of the flow found in the vessels, while attenuating streaking artifacts in the image (Fig. 5(c)). The overall background signal (i.e. average image signal with the PT laser off) including movement and blood flow from in vivo imaging can be further attenuated by increasing the frequency shift in the reference arm ($\Omega_R$, Fig. 5(d)). Increased photothermal frequency ($\Omega_R$) results in both reduced background signal and reduced photothermal signal strength (Fig. 3(a)). Therefore, the photothermal frequency must be carefully selected for each in vivo application.

![Fig. 5. Motion and blood flow affects in vivo poli-OCT signal. A 3D image volume of a mouse ear with the PT laser off shows motion artifact and flow manifested as poli-OCT background signal. (a) A representative B-scan shows false positive background signal due to blood flow in vessels (green arrow) and motion artifact (red arrow). (b) The same image volume represented as an en face projection shows the signals due to motion artifact and flow. (c) Median filtering the 3D image volume attenuates artifacts due to motion. (d) The average background signal in the image volume is attenuated with increasing frequency shifts in the reference arm ($\Omega_R$, x-axis). Red boxes show 2X zoomed image regions. Scale bar = 1 mm.](image-url)
3.3. **In vivo imaging with poli-OCT**

Recently, ICG has been used as a lymphatic imaging contrast agent due to its bright NIR fluorescence and efficient lymphatic removal [31, 32]. Therefore, as a demonstration of *in vivo* poli-OCT, ICG was imaged from within the lymphatics during its removal from the mouse ear. B-scans through the mouse ear include OCT (gray) and poli-OCT (green) data (Fig. 6(a), 6(b)). Morphological features are resolved through the full thickness of the ear in the OCT magnitude data (Fig. 6(a), 6(b)), and the lymphatics that drain the interstitial ICG are resolved in the poli-OCT data with the PT laser on (Fig. 6(b)). The high concentration of injected ICG (500 µg/ml) is similar to previous fluorescence based studies [31, 32] making the poli-OCT signal extremely bright. The network structure of the lymphatics is more evident when visualizing the *en face* average intensity projection of the image volume (Fig. 6(c), 6(d)). With the PT laser off, the background signal (green, Fig. 6(c)) overlaps only with the location of blood vessels identified by speckle variance OCT (red, Fig. 6(c)), as predicted by results found in Fig. 5. With the PT laser on, the poli-OCT signal identifies the lymphatic vascular networks removing ICG from the intradermal injection site towards the base of the ear (green, Fig. 6(d)). The green circular feature at the bottom of Fig. 6(d) corresponds to a surface leak of the ICG near the injection site.

![Fig. 6. In vivo poli-OCT of mouse ear lymphatics using ICG as a contrast agent. 3D image volumes were acquired of a mouse ear after intradermal injection of ICG. Representative B-scans containing tissue morphology (gray) and poli-OCT signal (green) with the PT laser (a) off and (b) on. (c) *En face* projections of the volume including the vasculature (red, speckle variance OCT) shows minimal extravascular poli-OCT signal (green) with the PT laser off. (d) With the PT laser on, the poli-OCT signal (green) increases in locations where the lymphatic vessels are draining ICG from the injection site. Scale bar = 1 mm.]

To demonstrate the breadth of potential imaging contrast agents for poli-OCT, gold nanorods were also imaged *in vivo* at 10 times faster frame rates than previously reported for *in vivo* imaging of similar concentrations of gold nanorods using traditional PTOCT [11]. Injections of either Matrigel alone or Matrigel with 200 pM of gold nanorods were imaged over 2.5 mm by 2.5 mm volumes with poli-OCT (green, Fig. 7). Overlapping speckle variance OCT scans identified the locations of blood vessels (red, Fig. 7). After injection of Matrigel alone into the mouse ear, *en face* average intensity projections revealed no significant extravascular poli-OCT signal with either the PT Laser off (Fig. 7(a)) or the PT Laser on (Fig. 7(b)). Additionally, after gold nanorod injections into the mouse ear, there were no extravascular regions of poli-OCT signal with the PT Laser off (Fig. 7(c)). However, a
significantly enhanced poli-OCT signal was present in the region surrounding the site of gold nanorod-loaded Matrigel injection (Fig. 7(d)). Unlike the ICG lymphatic imaging (Fig. 6), the gold nanorods remained localized near the injection site due to the solidification of the Matrigel at body temperature.

Fig. 7. In vivo poli-OCT imaging of subcutaneous injections of gold nanorods in two mice. 3D image volumes were acquired of a mouse ear after injection of Matrigel alone (top) or Matrigel plus 200 pM gold nanorods (bottom). En face average intensity projections contain the poli-OCT signal (green) and speckle variance OCT signal (red). With Matrigel alone, no poli-OCT signal is observed (a) without or (b) with the PT laser. (c) In the presence of gold nanorods, no extra-vascular poli-OCT signal is present without the PT laser. (d) Only with gold nanorods and the PT laser on, is there an observable poli-OCT signal. Scale bar = 1 mm.

4. Discussion

We have incorporated optical lock-in methods into traditional PTOCT, and for the first time demonstrated in vivo poli-OCT. The poli-OCT system and signal was characterized while imaging tissue-mimicking phantoms containing an FDA-approved small molecule fluorophore (ICG). We also assessed how motion artifact and blood flow, confounding factors unique to in vivo imaging, affect the background signal in poli-OCT. Finally, two contrast agents were imaged in vivo, demonstrating the versatility of poli-OCT for use with small molecule and nanoparticle contrast agents. The ability to image fluorophores common to molecular imaging [34] as well as gold nanoparticles under development for molecular imaging and drug delivery [35] demonstrates the breadth of potential poli-OCT applications. Given the improvements enabled by poli-OCT, the unique spatial imaging niche of OCT, and the range of potential contrast agents available for imaging, the optical lock-in techniques demonstrated in these studies could enable more widespread adoption of photothermal OCT for in vivo imaging.

In this study, poli-OCT was used to identify gold nanorods in real time at ten times faster frame rates and with 1000 times less digital data acquisition than a previous PTOCT study (Fig. 7 and [11]). The previous PTOCT study from our group imaged 400 pM gold nanorods subcutaneously injected into the mouse ear (2X higher concentration than the current study)
using 200 Hz photothermal laser modulation frequency, 3.2 kW/cm² average irradiance, and 100 ms observation time at each A-scan position. In the current study, a photothermal modulation frequency of 500 Hz was used to avoid background signal contribution (Fig. 5(d)), and the average irradiance was increased to 15.5 kW/cm² to account for the signal decrease at faster modulation frequencies (Fig. 3(a)). Even with increased irradiance, the tenfold increase in poli-OCT imaging speed reduced the total radiant exposure at each A-scan position by a factor of two, from 320 J/cm² in the previous PTOCT study [11] to 155 J/cm² in the current poli-OCT study. To better understand the impact of these advances for in vivo imaging studies, scan length times and data burden were calculated and compared for poli-OCT and traditional PTOCT [11], assuming 1024 pixels per A-scan saved as 16 bit unsigned integers. For each A-scan position, poli-OCT acquired one A-scan over 10 ms, while a previously reported traditional PTOCT system acquired 1000 A-scans over 100 ms [11]. For a study including 10 mice imaged over a 300 by 300 rectangular scan volume repeated at five time points, the previously reported PTOCT system would require 7500 minutes (125 hours) and 9.2 TB of data acquisition, while poli-OCT would require only 750 total minutes (12.5 hours) and 9.2 GB of data acquisition. Thus, poli-OCT provides more streamlined in vivo time-course studies than previously reported PTOCT systems. GPU processing [36] could also be used to mitigate the data acquisition and offline signal analysis burden of traditional PTOCT, and offers reduced optical complexity compared to poli-OCT. However, previous publications that implement the optical lock-in detection scheme used in poli-OCT have suggested significant SNR improvements over digital sampling of phase modulations [20], an advantage that GPU processing of traditional PTOCT data does not provide. Nevertheless, this paper demonstrates the first real time in vivo PTOCT images, at increased frame rates compared to previous publications that use traditional PTOCT to image similar samples [11, 17].

While this work demonstrates improvements of poli-OCT with respect to traditional PTOCT, the current poli-OCT design includes a few addressable shortcomings. The technical advancement from traditional PTOCT to poli-OCT has increased the complexity of the optical instrumentation. For example, the use of three AOMs to control the reference arm frequency shift (Ω_R) and photothermal laser intensity modulation (Ω_P) increases the cost and complexity over traditional PTOCT. AOMs are attractive for this application because they provide both frequency shifting and amplitude modulation, can be phase and frequency locked to each other, and supply a wide range of frequencies. However, as demonstrated in Fig. 4(a), precise frequency matching of Ω_P and Ω_R may not be required for poli-OCT, so approaches that are less precise but more cost effective than AOMs could be implemented (e.g. moving reference arm [37], offset scanning reference mirror [38]). An additional source of poli-OCT imaging system complexity is the one way optical paths in both the reference and sample arms, which are used to reduce unwanted back-reflections acquired during long CCD acquisition times. Long CCD acquisition times are governed by the photothermal laser modulation frequency (Ω_P), which is slow due to the inverse relationship between photothermal signal magnitude and photothermal laser modulation frequency (Fig. 3(b) and [11]). Furthermore, for in vivo poli-OCT, focusing optics with a larger depth of focus and correspondingly larger spot sizes than microscopy are desirable. These larger spot sizes require slower photothermal modulation frequencies (e.g. hundreds of Hz) than photothermal microscopy for optimal performance [18, 39]. Photothermal modulation frequencies (Ω_P) in the hundreds of Hz regime require millisecond CCD integration times (e.g. if Ω_R = Ω_P = 500 Hz, τ ≥ 2 ms). One-way optical paths help to reduce unwanted back-reflections that can consume a large portion of the CCD dynamic range at these long integration times.

In comparison to traditional PTOCT and OCT, poli-OCT requires additional unique considerations. First, the contribution of certain image signals is greatly enhanced in poli-OCT images compared to OCT. The poli-OCT cross-correlation signal due to photothermal heating is an attenuated version of the original OCT cross-correlation signal. However, the poli-OCT autocorrelation signal is not frequency shifted and therefore not attenuated compared to the original OCT image. Consequently, the intensity of the autocorrelation signal...
relative to the cross-correlation signal is large for poli-OCT compared to traditional PTOCT or OCT. In this work, the poli-OCT autocorrelation signal was attenuated using cross-polarized sample arm light that limited surface specular reflections [40–42]. Furthermore, the autocorrelation image remains largely unchanged with or without the PT laser, and therefore subtracting an image with the PT laser off from an image with the PT laser on can help minimize the autocorrelation signal and generate images with minimal artifact. Note that this was not required for these *in vivo* imaging applications (Fig. 6 and Fig. 7). In addition to the autocorrelation signal effects, the poli-OCT signal depends on the reflectivity of the sample and reference arms (Eq. (1)) [19]. Therefore, variations in image parameters or tissue optical properties can affect images taken on different days or between samples. Dividing the poli-OCT image by the OCT image ($\Omega = 0$) can account for day-to-day inter-sample and intra-sample variations in reflectivity, and aid in maximizing contrast in the poli-OCT signal. If OCT images are desired in addition to poli-OCT images, an OCT scan must be acquired separately from the poli-OCT scan. However, the OCT data can be captured at significantly faster integration times than poli-OCT, thus minimally affecting overall acquisition time. A remaining consideration is phase accumulation, or the integration of the PTOCT signal with depth [43]. Unlike traditional PTOCT, the poli-OCT signal is also a function of the OCT signal magnitude (Eq. (1)). Therefore, phase accumulation does occur, but the signal first increases with depth due to phase accumulation then decreases with depth due to OCT signal falloff (data not shown). Algorithms are currently under development to address this issue.

In conclusion, for the first time, we have demonstrated *in vivo* poli-OCT for real time imaging of both small molecules and gold nanoparticles. Using poli-OCT, PTOCT data can be acquired faster, with orders of magnitude less data burden, and real time signal analysis. Mitigating the slow acquisition and high data burden of traditional PTOCT gives poli-OCT the potential to significantly impact preclinical *in vivo* molecular imaging. Higher volume acquisition rates and real time display of photothermal images could enable *in vivo* PTOCT applications that are not feasible with traditional PTOCT, such as time-course tomograms of small molecule drug delivery [4]. In addition, poli-OCT is attractive for other applications where the high resolution, wide field of view, and accompanying label-free imaging of tissue and vessel morphology provide significant advantages over traditional imaging techniques. Potential applications are numerous: molecular imaging in mammary, dorsal, abdominal, and cranial window models of cancer [34]; noninvasive imaging of drug delivery kinetics to assess the impact of drug vector size [44, 45], surface chemistry [46], and shape [47]; imaging drug clearance [48]; and imaging local drug diffusion and release [49, 50]. Overall, by leveraging the existing high resolution and wide field of view of OCT as well as its abilities to image tissue and vessel morphology, poli-OCT holds significant promise in studies of drug discovery and drug delivery, molecular imaging, and biological studies of pathogenesis.

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