**In vivo** photothermal optical coherence tomography of gold nanorod contrast agents

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**Abstract:** Photothermal optical coherence tomography (PT-OCT) is a potentially powerful tool for molecular imaging. Here, we characterize PT-OCT imaging of gold nanorod (GNR) contrast agents in phantoms, and we apply these techniques for *in vivo* GNR imaging. The PT-OCT signal was compared to the bio-heat equation in phantoms, and *in vivo* PT-OCT images were acquired from subcutaneous 400 pM GNR Matrigel injections into mice. Experiments revealed that PT-OCT signals varied as predicted by the bio-heat equation, with significant PT-OCT signal increases at 7.5 pM GNR compared to a scattering control (p < 0.01) while imaging in common path configuration. *In vivo* PT-OCT images demonstrated an appreciable increase in signal in the presence of GNRs compared to controls. Additionally, *in vivo* PT-OCT GNR signals were spatially distinct from blood vessels imaged with Doppler OCT. We anticipate that the demonstrated *in vivo* PT-OCT sensitivity to GNR contrast agents is sufficient to image molecular expression *in vivo*. Therefore, this work demonstrates the translation of PT-OCT to *in vivo* imaging and represents the next step towards its use as an *in vivo* molecular imaging tool.

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**OCIS Codes:** (110.4500) Optical coherence tomography; (160.4236) Nanomaterials; (350.5340) Photothermal effects.

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1. Introduction

In vivo molecular imaging is widely used in pre-clinical studies of the diseases that cause the greatest burdens of morbidity and mortality in the developed world (e.g., cancer, cardiovascular disease, diabetes, etc.) [1]. When performed at high resolution, in vivo molecular imaging can provide insight into the mechanisms of disease progression and drug resistance on a cellular level, thereby unraveling heterogeneities in pathological expression and drug response that are critical for understanding and combatting these diseases [2]. Microscopy, including confocal and multiphoton microscopy, has been the standard for high resolution molecular imaging in live cells and tissues. However, these microscopy techniques suffer from relatively shallow imaging depths. Magnetic resonance imaging (MRI) and positron emission tomography (PET) have been the standard for functional imaging deep within the body. However, these methods lack cellular-level resolution.

Optical coherence tomography (OCT) fills a niche between high resolution microscopy and whole body imaging techniques with cellular-level resolution and penetration depths in tissue that exceed the imaging depths of microscopy. This three-dimensional, non-invasive imaging technique provides an especially attractive scale for monitoring mouse models of disease. However, contrast in standard OCT images is based largely on differences in scattering cross section, which can be minimal amongst certain molecular species. Thus, augmenting standard OCT images with sensitive and specific molecular contrast represents an area of significant interest.

Functional extensions of OCT, including magneto-motive (MMOCT) [3,4], spectroscopic [5–7], pump-probe [8,9], and photothermal OCT (PT-OCT), have demonstrated molecular contrast. Specifically, PT-OCT has recently received much attention [10–19] for a number of reasons. First, PT-OCT is able to identify and separate absorbing targets from the scattering background through active detection of photothermal heating [20] (which is also independent of tissue mechanical properties, unlike MMOCT). Second, PT-OCT is highly sensitive to absorbing targets in the sample due to lock-in detection and low background. Finally, PT-OCT can exploit the rapid recent advancements in nanotechnology to develop efficient, molecularly-targeted contrast agents. For example, gold nanoparticles with near infrared (NIR) plasmon resonance peaks have been investigated for imaging and photothermal therapy [21–24] and are particularly attractive contrast agents for PT-OCT.
PT-OCT leverages the photothermal heating phenomenon, where photon absorption by an imaging target of interest (e.g., an absorbing nanoparticle) leads to a temperature change in the environment surrounding the target [20]. These local temperature changes cause thermoelastic expansion of the sample and shifts in the local index of refraction [25]. The photothermal-induced shifts in the local index of refraction and geometric path length alter the local optical path length (OPL, the product of index of refraction and geometric path length). OPL changes due to photothermal heating can be directly imaged via the phase information in an OCT interferogram. The PT-OCT signal has been shown to increase with increasing pump beam power and absorber concentration, and the PT-OCT signal to noise ratio increases with the number of repeated photothermal cycles [10,11].

PT-OCT has previously been characterized and demonstrated in vitro and ex vivo with targeted gold nanospheres [10], non-targeted gold nanoshells [11,12], gold nanorods [14], gold nanorose [13], and carbon nanotubes [18]. However, to date, no contrast agents have been imaged with PT-OCT in vivo. Two studies have demonstrated in vivo PT-OCT of hemoglobin (for quantifying blood oxygenation) [15,16], although only point scans (not images) were collected over multiple second long acquisition times. The primary goal of this study is to demonstrate the ability of PT-OCT to image contrast agents in vivo.

Gold nanorods (GNRs) are especially appealing contrast agents for in vivo PT-OCT because of their resonance in the NIR tissue optical window (~650-900 nm), tunable optical absorption properties (based on their physical dimensions) [26], and efficient absorption (compared to gold nanoshells and nanospheres) [27]. GNRs are also on a more advantageous size scale for in vivo molecular imaging (tens of nanometers in size) compared to nanoshells (hundreds of nanometers in size). Finally, the full width half max (FWHM) of the GNR absorption peak is much smaller than that of nanoshells [28,29], which is desirable to avoid attenuation of the imaging beam by the contrast agent.

The idealized pairing of PT-OCT with GNR contrast agents could allow for three-dimensional in vivo molecular imaging in a currently unexploited regime of resolution and penetration depth. However, prior to in vivo molecular imaging, the PT-OCT signal must be characterized, optimized, and tested in simpler in vivo systems without the added complexity of targeted molecular imaging. In this paper, GNRs are demonstrated as robust PT-OCT contrast agents, achieving pM-scale concentration sensitivity. The PT-OCT signal is also characterized with respect to imaging speed, photothermal beam power, and OCT magnitude signal. In addition, experimental PT-OCT signals are directly compared to photothermal heating models. Finally, we demonstrate PT-OCT imaging of GNRs in phantoms as well as the first documented in vivo images of contrast agents using PT-OCT.

2. Methods

2.1. GNR Synthesis

GNRs were synthesized using a common seed-mediated growth method employing the surfactant hexadecyltrimethyl ammonium bromide (CTAB) [30]. Briefly, a gold nanoparticle seed solution was prepared by adding 600 μl of 10 mM ice cold sodium borohydride to a 10 ml 250 μM gold chloride solution in 100 mM CTAB, under vigorous stirring. The growth solution was prepared by adding 800 μl of 10 mM silver nitrate and 550 μl of 100 mM to 100 ml of a 500 μM gold chloride solution in 100 mM CTAB. Following complete reduction of the gold ions (change in color from yellow to clear), 120 μl of the seed solution was added, and the GNRs were left undisturbed overnight. Synthesized GNRs were purified by two rounds of centrifugation at 10,000 xg for 20 minutes, followed by re-suspension in fresh deionized (DI) water. CTAB surface molecules were then exchanged for 5000 dalton polyethylene glycol (PEG) chains to improve biocompatibility, stability in salt solutions and serum, and extend circulation times [27,31]. Briefly, 10 μl of 1 mM mPEG-SH and 100 μl of 2 mM potassium carbonate were added to 1 ml of 4 mM CTAB-coated GNRs and stirred.
overnight. PEG-GNRs were then purified by two rounds of centrifugation/resuspension. The peak absorption wavelength of the GNRs was approximately 725 nm both before and after PEG exchange (Fig. 1(a)), with minor blue shifts (<10 nm) in peak wavelength a month after fabrication (data not shown). The average size of the GNRs was determined to be 45.2 ± 5.7 nm long by 13.2 ± 1.8 nm wide (n = 20) by transmission electron microscopy (TEM), shown in Figs. 1(b) and 1(c). The presence of the PEG coating on the surface of the GNRs was confirmed by a small increase in hydrodynamic diameter measured with dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Ltd.) (Fig. 1(d)). The stability of the PEG coating was confirmed by unaltered spectrophotometry spectra after suspension in 10X PBS, a high salt solution, and 15% FBS, both of which cause aggregation (and spectral shifts) in CTAB coated GNRs. The GNR concentration was calculated using spectrophotometry curves [32].

2.2. Imaging instrumentation

A commercial spectral domain OCT system (Bioptigen, Inc.) was altered for photothermal imaging (Fig. 1(e)). The OCT system contains an 860 nm center wavelength 51 nm FWHM super luminescent diode (SLD) with 6.4 μm axial resolution in air, and 900 μW of power on the sample (95.6 dB signal to noise ratio at 176 μm in depth). The SLD light is fiber coupled and split between a reference mirror and sample arm using a 50/50 fiber coupler while X-Y galvos in the sample arm perform lateral scanning. Returning interference light is sent through an 860 nm center wavelength circulator to a spectrometer with a 2048 pixel CCD. A -line integration time for all experiments was 100 μs (10 kHz line sampling rate). A femtosecond pulse (<140 fs), 80 MHz repetition rate, titanium:sapphire laser (Coherent, Inc.) tuned to 725 nm serves as a quasi-CW photothermal laser source, which is fiber coupled with the OCT system via the 50/50 fiber coupler. A standard continuous wave laser can be used in its place, and the PT-OCT signal dynamics over the temporal and spatial scales used here remain unchanged between the two laser options (data not shown). For all experiments with the exception of the in vivo studies, a higher resolution lens (16.5 μm 1/e² spot at the focus for the OCT imaging beam) was used to focus both the OCT and photothermal light onto the sample, with 10 mW average photothermal laser power on the sample. For in vivo experiments, a lower resolution lens (40 μm 1/e² spot at the focus for the OCT imaging beam) was used for
an increased depth of focus and less divergent photothermal beam. The increased spot size of
the low resolution lens was accounted for by increasing the average photothermal power on
the sample to 40 mW, maintaining similar irradiance for all experiments. Prior to fiber-
coupling, the pump beam is attenuated with a half wave plate and polarizer, while a
mechanical chopper (Newport, Inc.) modulates the amplitude of the free space beam at a
predetermined frequency (200 Hz 50% duty cycle square wave unless otherwise noted). For
each A-scan, 1000 repeated axial depth scans (M-mode scans) are performed at the same
lateral point while the pump beam is amplitude modulated.

2.3. Signal processing

Temporal oscillations in phase have been previously quantified using OCT [33]. Following
resampling to wavenumber, dispersion correction [34], and DC reference spectrum and fixed
pattern noise removal [35], the OCT interference spectrum \( I \) as a function of wavenumber
\( k \), and depth \( z \), at a given scan time \( t_0 \), can be described by

\[
I(k, t_0) = 2|E_R E_S| \cos(2kz + \varphi)
\]

where \( E_R \) is the electric field from the reference arm, \( E_S \) is the electric field from the sample,
and \( \varphi \) is a random phase noise term. The magnitude and phase information as a function
of depth at time \( t_0 \) is calculated using a Chirp Z Transform (CZT) along the dimension of \( k \) [12].
The CZT operation allows for control over the frequency resolution as well as the locations of
magnitude and phase analysis in image space [36]:

\[
I(z, t_0) = CZT[I(k, t_0)]_k = |M(z, t_0)| \exp[i\Phi(z, t_0)]
\]

In Eq. (2), \( |M(z, t_0)| \) is the magnitude spectrum, and \( \Phi(z, t_0) \) is the phase spectrum as a
function of depth at time \( t_0 \). Using the photothermal laser and mechanical chopper, cyclical
amplitude modulations in OPL are achieved over time in the M-mode scan. Equation (2) is
repeated for all temporal lines in the M-mode scan to create a 2-D magnitude \( (M(z, t)) \) Fig.
2(a)) and phase spectrum \( (\Phi(z, t)) \) Fig. 2(b)). Photothermal oscillations approximated as a
sinusoid at the chopping frequency \( f_0 \) can be extracted from the phase signal of the OCT
image over time at a specific depth, \( z_0 \) (Fig. 2(c)):

\[
\Phi(z_0, t) = \frac{4\pi}{\lambda} A(z_0) \sin(2\pi f_0 t) + \varphi
\]

In Eq. (3), \( \lambda \) is the center wavelength of the imaging beam (860 nm) and \( A(z_0) \) is the
magnitude of the induced OPL changes due to photothermal heating at depth \( z_0 \). In order to
remove the random phase term \( (\varphi) \), center the signal around zero, and avoid phase wrapping,
the derivative of the phase signal is computed similar to Doppler OCT algorithms [33,37],

\[
\Delta\Phi(z_0, t) = \frac{8\pi^2}{\lambda} A(z_0) f_0 \cos(2\pi f_0 t) \Delta t + \xi(t) = \tan^{-1}\left[ \frac{\text{Im}[I(z_0, t)I^*(z_0, t - 1)]}{\text{Re}[I(z_0, t)I^*(z_0, t - 1)]} \right]
\]

where \( \Delta t \) is the sampling period (100 μs) and \( \xi(t) \) is a phase noise term due to the photon,
thermal, and electronic noise of the system [33]. A subsequent Fourier transform in the
temporal direction allows for the magnitude of OPL changes at the laser amplitude
modulation frequency \( f_0 = 200 \text{ Hz} \) to be calculated with high SNR (Fig. 2(d)):

\[
FT[\Delta\Phi(z_0, t)]_0 = |p(z_0, f)| \exp[i\theta(z_0, f)] = \frac{8\pi^2}{\lambda} A(z_0) f_0 \Delta f \frac{1}{2} \left[ \delta(f - f_0) + \delta(f + f_0) \right]
\]
This operation is repeated at all depths to find the photothermal-induced optical path length changes as a function of depth (z):

\[
OPL(z) = A(z) = \frac{|p(z, f_0)| \lambda}{4\pi^2 f_0 \Delta t}
\]  

In Eqs. (5) and (6), \(FT\) is the Fourier transform operation in the temporal dimension, \(|p(z, f)|\) is the magnitude of the Fourier transform of the temporal phase signal at line \(z\) as a function of temporal frequency \((f)\), \(\theta(z, f)\) is the phase spectrum of the same signal, and \(\delta\) is a delta function. As shown by Eq. (6), the PT-OCT signal \((OPL(z))\) is calculated from the magnitude of the Fourier transform of the processed phase data at the chopping frequency \((|p(z, f_0)|)\). The 1000 repeated temporal scans were then separated to 20 overlapping 512 point scans, and Fourier transforms over these shorter temporal windows were averaged. Frequency averaging over small, overlapping windows within a discretely sampled signal allows for less variability in the estimate of the true spectrum, and more accurate estimation of signal and noise [38,39]. The final PT-OCT signal included the subtraction of photothermal noise, defined as the mean magnitude of nearby frequencies (270-370 Hz) in the FT of the phase signal (Eq. (5)). This noise subtraction accounted for the baseline spectral intensity of the FT. The final PT-OCT signal was filtered in the axial direction using a trimmed inner mean filter to reduce noise, thresholded using the intensity image to remove noise dominated PT-OCT pixels, and median filtered with a 4 pixel square kernel to reduce speckle noise. PT-OCT images were processed offline after image acquisition using a custom Matlab script.

Fig. 2. PT-OCT signal processing basics. (a) M-mode OCT magnitude scan (a.u.) as a function of time and depth. (b) Accompanying M-mode OCT phase (in radians) scan as a function of depth and time. (c) Representative temporal phase information at one point in depth (red arrow in 1b), showing amplitude modulated fluctuations of phase due to photothermal heating. (d) Fourier transform of temporal phase data, showing distinct peak at the photothermal modulation frequency (200 Hz) in units of nm displacement in OPL. Data taken in common path configuration.

2.4. Modeling the photothermal signal

To understand the effects of parameter changes on the PT-OCT signal, experiments were performed on GNR samples in 1X PBS and compared to previously tested theoretical models of photothermal heating dynamics [11,40]. The photothermal-induced temperature dynamics
(and thus the PT-OCT signal) in space and time due to the absorption of the photothermal beam can be modeled using the bio-heat conduction equation with a heat source term,

\[
\frac{\partial T}{\partial t} = \frac{\phi \mu_a}{\rho c} + \alpha \nabla^2 T
\]  

(7)

where \( T \) is temperature, \( t \) is time in seconds, \( \rho \) is the density of the medium (1000 kg/m\(^3\) for water), \( c \) is the specific heat of the medium (4186 J/kg K for water), \( \phi \) is the photothermal laser fluence rate at the sample, \( \mu_a \) is the absorption coefficient of the GNR sample (~1200 m\(^{-1}\) for a homogenous 800 pM nanorod sample), and \( \alpha \) is the thermal diffusivity of the medium, defined as \( \alpha = k / \rho c \), where \( k \) is thermal conductivity of the medium (0.6 W/K for water). For small spot sizes relative to the absorption depth, the heat conduction equation is dominated by radial heat transfer, and can be represented by a closed form solution in cylindrical coordinates. Over one modulation period of the chopper (\( 2t_L \)), the change in temperature at the heat source over time can be modeled according to previous work [11,40]:

\[
\Delta T(t, r = 0) = \frac{P \mu_a}{4 \alpha \pi \rho c} \ln\left(1 + \frac{t \alpha}{\omega^2/8}\right), \quad \omega \ll \frac{1}{\mu_a}, t < t_L
\]  

(8)

\[
\Delta T(t-t_L, r = 0) = \frac{P \mu_a}{4 \alpha \pi \rho c} \ln\left(1 + \frac{t_L \alpha}{\omega^2/8 + \alpha(t-t_L)}\right), \quad \omega \ll \frac{1}{\mu_a}, t \geq t_L
\]  

(9)

In Eqs. (8) and (9), \( P \) is photothermal laser power, \( \omega \) is the 1/e\(^2\) beam radius of the photothermal beam, and \( t_L \) is the dwell time of the laser on the sample before mechanical interference from the chopper transitions photothermal power to zero. Note that this model is applied to a continuous wave (CW) heating beam that is amplitude-modulated by the optical chopper at the frequency of interest. Although we use a femtosecond laser in our experiments, as stated above, we assume its behavior as quasi-CW due to the high repetition rate (80 MHz) among other considerations. The photothermal heating dynamics of the femtosecond source have been experimentally validated to be equivalent to a CW source over the spatial and temporal scales of these experiments (data not shown). Solutions over time for the model in Eqs. (8) and (9) were compiled with a custom Matlab file.

2.5. Parameter characterization and PT-OCT sensitivity

It is important to note that, although this model has been used to understand the photothermal phenomena as imaged with PT-OCT [11], here we directly compared it’s outputs to PT-OCT signals during parameter perturbations. To do so, PT-OCT signal from a GNR solution was assessed with respect to pump beam laser power, chopping frequency, and OCT magnitude signal. To assess the effect of these parameters on the PT-OCT signal, and to confirm that experimental PT-OCT data mimics the physical photothermal heating model (Section 2.4), the following experiments were conducted. PT-OCT imaging was performed on the GNR sample while altering the parameter of interest, leaving all remaining instrumentation and sample properties unchanged (\( n = 10 \)). A 5 μl 800 pM concentration sample of GNRs in 1X PBS was placed on a microscope slide. The PT-OCT signal at the bottom of the sample (~150 μm) was assessed. For chopping frequency and irradiance experiments, the sample was covered with a coverslip, positioned with the coverslip at the focal point of the OCT beam, and the reference arm was fully attenuated. The top coverslip reflection thus served as the reference reflection (i.e., common path imaging), in order to increase phase stability (common path imaging phase stability = 2.1 mrad, reference arm imaging phase stability = 119.9 mrad). Phase stability was measured as the standard deviation of the derivative phase signal (Eq. (4)) at the bottom of a coverslip over 1000 repeated A-scans. The rate at which the photothermal beam was amplitude modulated [the chopping frequency \( f_0 \), where \( f_0 = 1/(2t_L) \)] was tested at a number...
of frequencies from 50 Hz to 700 Hz. The same sample was then imaged with varying levels of photothermal laser power from 3.5 mW to 15 mW peak power on the sample. The effect of OCT magnitude signal on the PT-OCT signal was also determined. For OCT magnitude signal experiments, the reference arm was incorporated and repeatedly attenuated to alter the reflectivity of the OCT magnitude image between experiments. OCT magnitude signal was calculated as 20 times the common logarithm of the peak reflectivity signal in the image divided by the standard deviation of the background signal. OCT magnitude signal over a 25 dB range was tested.

Linearity and sensitivity of the PT-OCT system to GNR contrasts was determined by imaging varying concentrations of GNRs using the imaging methods above with common path imaging geometry. Sensitivity was determined as the smallest concentration sample with a significantly different signal from a control scattering phantom (1% Intralipid sample). Linearity was determined using least squares regression and statistical significance was assessed with unpaired t-tests (p<0.01).

2.6. Phantom imaging

Phantoms were created to assess spatial specificity of the PT-OCT signal. A 4% low gelling temperature agarose solution was prepared with 1X PBS. Agarose was heated above 85 degrees Celsius to dissolve it into solution. GNRs were then added to the heated agarose to create a 2% agarose, 400 pM GNR experimental sample. A control phantom was also made by diluting down the 4% agarose to 2% with 1X PBS. Samples were mixed well with a pipette, and then loaded into a capillary tube via capillary action to allow for solidification for 10 minutes at room temperature. After solidification, capillary tubes were imaged with the PT-OCT system, incorporating the reference arm. Cross sectional images of one control and experimental capillary tube were each imaged at 5 different locations, and an approximately 1 mm by 0.75 mm square region of interest (ROI) within the capillary tube was chosen for each image for further analysis. The mean and standard deviation across OCT magnitude and PT-OCT ROIs were compared between agarose/GNR- (control) and agarose/GNR+ (experimental) capillary tube images.

2.7. In vivo imaging

PT-OCT imaging of contrast agents in vivo was performed in a nude mouse using a protocol approved by the Vanderbilt University IACUC. While the mice were maintained under 2% isoflurane anesthesia, Matrigel was directly injected into the subcutaneous tissue of the left ear, and a 400 pM GNR Matrigel solution was injected into the right ear. The surface of the tissue was rinsed with PBS to remove any contrast agent that leaked onto the skin, and imaging was performed under isoflurane anesthesia 20 minutes after Matrigel injections. OCT, Doppler OCT, and PT-OCT scans were performed on each mouse ear to compare the measured PT-OCT signal with and without GNRs in vivo. Doppler OCT was assessed using the same repeated scans as the PT-OCT data (Doppler number = 999), and processed using the mean derivative phase signal over the repeated Doppler scans [37].

3. Results

3.1. Photothermal model and parametric results

As shown in Fig. 3(a), experimental PT-OCT data (solid line, taken from an 800 pM solution of GNRs) over one chopping period (f₀ = 200 Hz) along with the local heating and cooling dynamics predicted by the photothermal model in Eqs. (8) and (9) (dashed line) are in agreement. Over one chopping period, the temperature at the photon absorption point increases logarithmically when the photothermal pump beam is on. Once the photothermal pump beam is blocked, the temperature at the photon absorption point decreases logarithmically.
Changes in the PT-OCT signal as a function of pump beam laser power and chopping frequency \( f_0 \) also agree with theory (Figs. 3(b)–3(c)). The modeled photothermal signal is defined as the peak temperature increase observed over one heating cycle, as shown in Eq. (10) below. The peak PT-OCT signal occurs at \( t = t_L \), the point at which the irradiance goes from maximum to zero due to the chopper’s mechanical interference. At that point in time, the maximum photothermal modeled signal is given by

\[
\Delta T(t = t_L, r = 0) = \Delta T_{\text{max}} = \frac{P \mu_a}{4 \alpha \pi \rho c} \ln \left( 1 + \frac{t_L \alpha}{\omega^2 / 8} \right)
\]

(10)

For the purposes of comparison, the experimental PT-OCT signal and modeled photothermal signal (Eq. (10)) are normalized to the signal observed at either the highest laser power in the plot (15 mW, Fig. 3(b)), or the minimum chopping frequency in the plot (50 Hz, Fig. 3(c)). As predicted in Eqs. (8) and (9), both the experimental PT-OCT and modeled photothermal signal linearly increase \( (r^2 = 0.998) \) with photothermal pump laser power \( P \), Eq. (10) on the sample (Fig. 3(b)). However, increasing the chopping frequency causes a more complex change in the PT-OCT signal. The chopping frequency \( f_0 \) is directly related to the chopping period \( 2t_L \) by \( f_0 = 1 / (2t_L) \). Therefore, the effect of the chopping frequency on the maximum photothermal temperature increase in one chopping period can be calculated from Eq. (10) and the relationship between \( t_L \) and \( f_0 \):

\[
\Delta T_{\text{max}} = \frac{P \mu_a}{4 \alpha \pi \rho c} \ln \left( 1 + \frac{\alpha / (2f_0)}{\omega^2 / 8} \right)
\]

(11)

Equation (11) shows that the maximum photothermal temperature change logarithmically decays with increased chopping frequency \( f_0 \), which is evident in both the modeled and
experimental results (Fig. 3(c)). Therefore, faster chopping frequencies degrades PT-OCT signals. However, faster chopping frequencies are desirable for practical in vivo studies, because they allow for faster image acquisition speeds (note that the PT-OCT SNR increases by capturing a greater number of photothermal cycles per A-scan [11], which can be more quickly captured with a faster chop frequency). Consequently, the practical choice of chopping frequency is a trade-off between irradiance on the sample, imaging speed, and PT-OCT SNR.

The OCT magnitude signal should not affect the PT-OCT signal, since the OCT magnitude signal (the intensity of the OCT image) does not affect the thermodynamics which control photothermal heating (Eq. (8) and 9). Experimental results confirm that altering the OCT magnitude signal does not have an effect on the mean PT-OCT signal (Fig. 4(a)). Conversely, decreasing the OCT magnitude signal does increase the noise in the PT-OCT signal (Fig. 4(b)), which accounts for the increased variance in the PT-OCT signal as OCT magnitude signal decreases (error bars, Fig. 4(a)). The increase in PT-OCT noise due to decreased OCT magnitude signal is predicted by the power-law relationship between OCT phase stability and OCT SNR [41].

Fig. 4. The effect of OCT magnitude signal on the photothermal signal. (a) Decreases in the OCT magnitude signal cause no change in the mean PT-OCT signal, as demonstrated by the low correlation coefficient and horizontal linear fit. (b) Decreasing the OCT magnitude signal increases the noise in the PT-OCT signal, due to decreased phase stability of low SNR image points as previously predicted [41,43]. All experimental data collected with a reference arm. Values are plotted as signal ± s.d.

3.2. Linearity and sensitivity of photothermal signal

According to the theoretical model in Eqs. (8) and (9), increasing the concentration of the absorber (GNRs) in the sample (and thus the absorption coefficient) linearly increases the photothermal signal. Accordingly, the PT-OCT signal linearly increases with GNR concentration ($r^2 = 0.997$, Fig. 5).

Notably, Fig. 5 demonstrates that the magnitude of the heating increase as measured by PT-OCT scales linearly with the concentration of contrast agent, even though the heating process itself is dynamic and nonlinear (Fig. 3(a)). The PT-OCT system described above (common-path mode) exhibits 7.5 pM (~0.54 mg/L) sensitivity to GNRs when compared to a scattering control (1% Intralipid, red in Fig. 5, p<0.01).
3.3. Phantom imaging

Capillary tube imaging was performed using the reference arm in order to demonstrate the ability of PT-OCT to spatially distinguish the presence of GNRs at concentrations anticipated to be achievable in vivo and in an imaging environment with reduced phase stability. GNR positive and negative capillary tubes display similar OCT magnitude signals, but significantly different PT-OCT signals (Fig. 6). The OCT magnitude images measured from n = 5 capillary tube images are similar between GNR positive and negative regions (GNR positive region = 4.07 ± 0.003 a.u., GNR negative region = 4.02 ± 0.004 a.u.), while the GNR positive capillary tube exhibits a 15 fold enhanced PT-OCT signal with respect to the agarose control (GNR positive region = 3.81 ± 0.24 nm, GNR negative region = 0.24 ± 0.05 nm). Small phase accumulations cause an apparent increase in PT-OCT signal in the GNR + tube as depth increases.

Fig. 6. PT-OCT images of capillary tube phantoms. (a) Example OCT magnitude images of GNR + (left) and GNR- (right) solid agarose capillary tube phantoms (a.u.). (b) Mean ± standard deviation OCT signal (a.u.) from a series of n = 5 capillary tube images. (c) Example PT-OCT images of capillary tubes from Fig. 5(a), displaying the ability of PT-OCT to distinguish GNR + from GNR- sample (units of nm optical path length displacement). (d) Mean ± standard deviation of PT-OCT signal (units of nm optical path length displacement) from a series of n = 5 capillary tube images. Data collected with the reference arm intact.
3.4. In vivo PT-OCT Imaging

Representative PT-OCT images in the experimental and control ears of a mouse are shown in Fig. 7. Doppler OCT overlays in Fig. 7 (red and blue channels) illustrate the presence of small blood vessels crossing the B-scan. There is a dramatic increase in PT-OCT signal in the experimental ear where GNRs are present compared to control ears with Matrigel only. Signal increases occur in discrete locations spatially separate from the vessels, indicating that there is PT-OCT signal not due to blood absorption. The PT-OCT signal gains in the GNR + mouse ear are similar to that from ex vivo GNR injections into chicken breast using the same 400 pM GNR injection (data not shown). Additional B-scan images (not shown) demonstrate the same representative increases in PT-OCT signal in GNR + mouse ears compared to control injections.

![Fig. 7. In vivo PT-OCT of GNRs. (a) PT-OCT signal in the control mouse ear injected with only Matrigel. (b) The OCT image (grayscale channel) of the control ear, with Doppler (red and blue channels) and PT-OCT (green channel) overlaid. (c) PT-OCT signal in the experimental mouse ear injected with 400 pM GNR in Matrigel. (d) OCT image (grayscale channel) of experimental ear with Doppler (red and blue channels) and PT-OCT (green channel) overlaid. Data collected with the reference arm intact.](image)

4. Discussion

We have demonstrated PT-OCT of highly absorbing GNR contrast agents in the near infrared wavelength region, including validation in phantoms and feasibility studies in vivo. We characterized the photothermal signal with the bio-heat equation, and directly compared modeled and experimental PT-OCT data. The PT-OCT system in common path configuration demonstrated high (pM-level) sensitivity to GNR contrast agents (Fig. 5), with detection levels appropriate for in vivo imaging of GNR uptake in mouse tumors [42]. The linear relationship between contrast agent concentration and PT-OCT signal leaves open the possibility of quantitative molecular imaging with PT-OCT. In addition, GNR contrast agents were successfully imaged in vivo, demonstrating a first step towards in vivo molecular imaging with PT-OCT.

Before pursuing imaging applications involving complex in vivo systems with PT-OCT, it is important to thoroughly characterize and optimize the imaging system in more controlled environments. Therefore, we performed experiments to assess the photothermal signal as a...
function of chopping frequency, laser power (and thus irradiance), and OCT magnitude signal. These experimental PT-OCT results were compared to the results of a quantitative photothermal heating model. The physical basis of the PT-OCT signal is a change in OPL. Since the transition from temperature change to OPL is essentially linear, but difficult to accurately solve [11], the modeled (maximum temperature change, Eq. (10)) and experimental (maximum OPL change, Eq. (6)) results were compared after normalization (Fig. 3). From Eqs. (8) and (9), it is evident that the only controllable imaging parameters governing the photothermal temperature change are the chopping frequency (i.e., how rapidly the photothermal laser is amplitude modulated) and the laser irradiance (a function of spot size and laser power). Therefore, we tested the effects of these two variables on the photothermal signal, in comparison to the predictions of a well-established closed form solution for the heat conduction equation [11,40]. Our results indicate that the heat conduction model accurately represents experimental PT-OCT data. As expected, an increase in irradiance linearly increases the PT-OCT signal (Fig. 3(b)), while increased chopping frequency logarithmically degrades the PT-OCT signal (Fig. 3(c)).

The photothermal heating of a sample (Eqs. (8) and 9)) is not dependent upon a traditional OCT image, only on the sample and heating parameters. Therefore, the PT-OCT peak signal should be independent of the OCT magnitude signal. It is essential to demonstrate independence between OCT magnitude signal and PT-OCT signals because of the speckle and heterogeneities in OCT magnitude images. As predicted, the OCT magnitude signal did not affect the mean PT-OCT signal (Fig. 4(a)). However, the OCT magnitude signal did impact the noise in the PT-OCT signal (Fig. 4(b)), in agreement with the predicted power-law degradation of phase stability with decreased OCT SNR [41,43].

This work demonstrates the first reported in vivo imaging of contrast agents with PT-OCT and is an important step towards the use of PT-OCT in molecular imaging studies in mice. However, there are some remaining considerations to address before robustly applying PT-OCT toward in vivo molecular imaging. First, motion artifacts must be limited during PT-OCT imaging. Motion artifact can be minimized by physically restraining the tissue, and by gating images between breathing cycles. In addition, the derivative operation in Eq. (4) and subsequent Fourier transform (Eq. (5)) help to separate oscillations due to motion artifact from the OPL oscillations that form the PT-OCT signal. The results shown here indicate the effects of motion artifacts can be minimized if the PT-OCT chopping frequency does not overlap with frequencies due to motion artifacts. Common path imaging using a secured coverslip above the sample or by surface coating with a diffuse scattering compound [44] could also assist with motion artifact compensation. Algorithms and image registration methods for removing motion artifacts in OCT phase and magnitude signals also currently exist to help reduce the affects from sample motion [45–47]. Second, the divergence and scattering of the photothermal beam through tissue complicates calculations of irradiance as a function of depth. Phantom measurements of the photothermal beam size as a function of depth along with Monte Carlo simulations could address this problem, or ratiometric measurements could be acquired at different pump beam wavelengths [48]. Another obstacle to account for is integration of the photothermal signal with depth. Since phase images measure changes in OPL, and OPL is an integrated signal, photothermal signals accumulate in depth, as is evident in Fig. 6(c). Previous work has accounted for this integration by taking the local slope of the photothermal signal [17]. However, using local linear curve, highly concentrated samples cannot be accurately characterized with linear models. Finally, the imaging speed for photothermal optical coherence microscopy (OCM) has recently been improved with the use of optical lock-in methods [49]. It is expected that these methods can also be applied to PT-OCT to improve imaging speeds, reduce motion artifacts, and further translate these methods toward in vivo use. Regardless of these confounding factors, PT-OCT has been proven to be a sensitive method to image both endogenous and exogenous absorptive targets at depths greater than any microscopy method, making it a molecular
imaging tool with great potential in biomedical science applications. It is expected that further development of this relatively new technology will overcome these remaining obstacles.

In conclusion, PT-OCT is a promising technology, with the potential to enable molecular imaging with an impressive combination of spatial resolution and imaging depth. We have characterized and translated this technology to in vivo use, illustrating the potential of PT-OCT to address critical needs in pre-clinical molecular imaging. We have demonstrated that PT-OCT can image a contrast agent at physiologically relevant concentrations [42] at depths approaching 1 mm in vivo, exceeding the imaging depths of traditional microscopy (including two-photon) techniques. An additional strength of this method is that it provides images of the spatial distribution of contrast agent along with the complementary existing features of OCT (structural imaging and Doppler OCT of blood flow).

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