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Photoacoustic Elastography and Next-generation Photoacoustic Tomography Techniques Towards Clinical Translation

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Photoacoustic Elastography and Next-generation Photoacoustic Tomography Techniques Towards Clinical Translation

by

Pengfei Hai

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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Pengfei Hai

Washington University in St. Louis

May 2018
Dedicated to my family.
ABSTRACT OF THE DISSERTATION

Photoacoustic Elastography and Next-generation Photoacoustic Tomography

Techniques towards Clinical Translation

by

Pengfei Hai

Doctor of Philosophy in Biomedical Engineering

Washington University in St. Louis, 2018

Professor Lihong V. Wang, Co-Chair

Professor Mark A. Anastasio, Co-Chair

Ultrasonically probing optical absorption, photoacoustic tomography (PAT) combines rich optical contrast with high ultrasonic resolution at depths beyond the optical diffusion limit. With consistent optical absorption contrast at different scales and highly scalable spatial resolution and penetration depth, PAT holds great promise as an important tool for both fundamental research and clinical application. Despite tremendous progress, PAT still encounters certain limitations that prevent it from becoming readily adopted in the clinical settings. This dissertation aims to advance both the technical development and application of PAT towards its clinical translation.

The first part of this dissertation describes the development of photoacoustic elastography techniques, which complement PAT with the capability to image the elastic properties of biological tissue and detect pathological conditions associated with its alterations. First, I demonstrated vascular-elastic PAT (VE-PAT), capable of quantifying blood vessel compliance changes due to thrombosis and occlusions. Then, I developed photoacoustic elastography to noninvasively map the elasticity distribution in biological tissue. Third, I further enhanced its
performance by combing conventional photoacoustic elastography with a stress sensor having known stress–strain behavior to achieve quantitative photoacoustic elastography (QPAE). QPAE can quantify the Young’s modulus of biological tissues on an absolute scale.

The second part of this dissertation introduces technical improvements of photoacoustic microscopy (PAM). First, by employing near-infrared (NIR) light for illumination, a greater imaging depth and finer lateral resolution were achieved by near-infrared optical-resolution PAM (NIR-OR-PAM). In addition, NIR-OR-PAM was capable of imaging other tissue components, including lipid and melanin. Second, I upgraded a high-speed functional OR-PAM (HF-OR-PAM) system and applied it to image neurovascular coupling during epileptic seizure propagation in mouse brains in vivo with high spatio-temporal resolution. Last, I developed a single-cell metabolic PAM (SCM-PAM) system, which improves the current single-cell oxygen consumption rate (OCR) measurement throughput from ~30 cells over 15 minutes to ~3000 cells over 15 minutes. This throughput enhancement of two orders of magnitude achieves modeling of single-cell OCR distribution with a statistically meaningful cell count. SCM-PAM enables imaging of intratumoral metabolic heterogeneity with single-cell resolution.

The third part of this dissertation introduces the application of linear-array-based PAT (LA-PAT) in label-free high-throughput imaging of melanoma circulating tumor cells (CTCs) in patients in vivo. Taking advantage of the strong optical absorption of melanin and the unique capability of PAT to image optical absorption, with 100% relative sensitivity, at depths with high ultrasonic spatial resolution, LA-PAT is inherently suitable for melanoma CTC imaging. First, with a center ultrasonic frequency of 21 MHz, the LA-PAT system was able to detect melanoma CTCs clusters and quantify their sizes based on the contrast-to-noise ratio (CNR). Second, I developed an LA-
PAT system with a center ultrasonic frequency of 40 MHz and imaged melanoma CTCs in patients *in vivo* with a CNR greater than 12. We successfully imaged 16 melanoma patients and detected melanoma CTCs in 3 of them. Among the CTC-positive patients, 67% had disease progression despite systemic therapy. In contrast, only 23% of the CTC-negative patients showed disease progression. This study lays a solid foundation for translating CTC detection to bedside for clinical care and decision-making.
Chapter 1 Introduction

1.1 Photoacoustic Tomography

By converting interior structures (anatomy) and functions (physiology) to comprehensive visual illustrations, biomedical imaging has revolutionized fundamental life sciences research and modern healthcare. Among various biomedical imaging modalities, optical imaging techniques are uniquely positioned to probe biomolecules with high resolution and rich contrast because light occupies a specific range of the electromagnetic spectrum. However, due to the strong light scattering, high-resolution optical imaging is usually restricted to the ~1 mm optical diffusion limit in biological tissue\(^1\).

First discovered by A. G. Bell back in 1880, the photoacoustic effect describes the phenomenon of converting light absorption by an object to a pressure rise that propagates as acoustic waves\(^2\). Based on this effect, photoacoustic tomography (PAT, also called optoacoustic tomography) forms high-resolution images of optical absorption in biological tissue beyond the optical diffusion limit by detecting the acoustic waves\(^3\). There are five essential steps in PAT\(^4\). (1) The object is irradiated by a short-pulsed or an intensity-modulated laser beam. (2) Light is absorbed by the object and partially or fully converted into heat. (3) The heat causes a thermos-elastic expansion that gives a pressure rise. (4) The pressure rise propagates as an ultrasonic wave, referred to as photoacoustic wave. (5) The photoacoustic wave is detected by one or more ultrasonic transducers and an image is formed based on the detected signals.

As a hybrid imaging modality, PAT has four unique advantages by combing optical excitation with ultrasonic detection. (1) PAT has a 100% relative sensitivity to optical absorption (i.e., a given
percentage change in the optical absorption coefficient yields the same percentage change in the photoacoustic amplitude). (2) Exploiting the rich optical contrast, PAT achieves structural, functional, metabolic, molecular, and genetic imaging of biological systems. (3) Taking advantage of the ultrasonic transparency of biological tissue, PAT breaks the optical diffusion limit and enables high-resolution imaging of optical contrasts at depths. (4) PAT provides multiscale imaging capability with highly scalable penetration depth and spatial resolution based on a consistent contrast mechanism of optical absorption.

With these unique advantages, PAT can be implemented in many forms according to the desired imaging parameters and specific biological applications. Based on the image-formation method, there are two major types of implementation of PAT, photoacoustic computed tomography (PACT) and photoacoustic microscopy (PAM). PACT uses an inverse reconstruction imaging formation method by wide-field illumination and multi-position acoustic detection with an array of transducers. PACT can be configured with different transducer array geometries, including linear, half ring, full ring, and hemisphere. In PACT, the axial (along the acoustic axis) and lateral (perpendicular to the acoustic axis within the imaging plane) resolutions are derived from image reconstruction. The elevation (orthogonal to the imaging plane) resolution is determined by the cylindrical acoustic focusing. PAM utilizes a focused-scanning imaging formation method by focusing both the optical excitation and acoustic detection and scanning either the imaging focus or the object imaged. For each laser pulse, a one dimensional (1D) photoacoustic signal (referred as to A-line) along the axial direction is acquired. To form a three dimensional (3D) photoacoustic image, lateral raster scanning is usually performed. Based on the relative size of the optical and acoustic focus, PAM can be further classified into optical-resolution PAM (OR-PAM) and acoustic-resolution PAM (AR-PAM). In OR-PAM, the optical focus is tighter than the acoustic
focus. The excitation laser beam is usually tightly focused to achieve optical diffraction-limited lateral resolution\textsuperscript{20}. In AR-PAM, the acoustic focus, smaller than the diffused optical beam, determines the lateral resolution. For both OR-PAM and AR-PAM, the axial resolution is determined by the acoustic time of arrival\textsuperscript{21}. In addition to PAM and PACT, PAT can also be implemented for endoscopy\textsuperscript{22}. Based on the desired imaging parameters regarding penetration depth, spatial resolution, temporal resolution, and imaging contrast, a particular PAT system can be selected to solve the specific biological and medical problems.

1.2 Motivation

This dissertation aims to push the frontier of PAT in the following three aspects.

First, this dissertation aims to develop photoacoustic elastography (Chapter 2). Although PAT can provide multi-dimensional information of biological tissue, one critical dimension missing is the elastic property. Alterations of elastic properties are often associated with pathological states in biological tissue\textsuperscript{23}. Physicians have long used manual palpation to detect such alterations. Inspired by such manual palpation, elastography, an imaging technique that is typically implemented using existing medical imaging techniques, was developed to map the elasticity distribution in biological tissue\textsuperscript{24}. So far, elastography has not been successfully implemented using PAT. So, I aim to develop photoacoustic elastography to complement PAT with the capability to image elastic properties of biological tissue.

Second, this dissertation aims to further enhance the performance of OR-PAM (Chapter 3). I want to achieve greater penetration depth and finer lateral resolution in the deeper regions with OR-PAM, which can be realized by employing near-infrared (NIR) light for illumination.
target to improve OR-PAM to image fast dynamic biological processes such epilepsy propagation in mouse brain in vivo with high spatio-temporal resolution. Additionally, we want to further boost the functionality of OR-PAM by developing the capability of performing label-free high-throughput single-cell oxygen consumption rate measurement and characterizing intratumoral metabolic heterogeneity with single-cell resolution.

Third, this dissertation aims to develop linear-array-based PAT systems for label-free high-throughput imaging of circulating melanoma tumor cells in vivo (Chapter 4). Metastasis accounts for more than 90% of cancer deaths\textsuperscript{25}. In metastasis, the rare circulating tumor cells (CTCs) are the key determinants of metastatic propensity\textsuperscript{26}. Effective detection and characterization of the rare CTCs will greatly contribute to understanding tumor metastasis and improving cancer therapy. Current CTC detection techniques all encounter certain limits that impedes their clinical translation. We aim to overcome these challenges by developing linear-array-based PAT systems capable of label-free high-throughput imaging of circulating melanoma tumor cells in patients in vivo.
Chapter 2 Photoacoustic Elastography

In this chapter, I introduce the development of photoacoustic elastography techniques. Elastography is a medical imaging technique can noninvasively map the elastic properties of biological tissue. Photoacoustic elastography combines the elasticity imaging capability with the unique advantages of PAT. In the first section, I developed the vascular elastic PAT (VE-PAT) system that can measure the blood vessel compliance. Then, in the second section, I demonstrated photoacoustic elastography using a linear-array-based PACT system. Last, I report quantitative photoacoustic elastography (QPAE) capable of measuring Young’s modulus of biological tissue \textit{in vivo} in humans.

2.1 Vascular Elastic Photoacoustic Tomography

2.1.1 Background

Mechanical properties of biological tissue are directly related to its underlying structure and can be altered by pathological states such as tumors and arteriosclerosis. Particularly, the elastic properties of blood vessels are strongly affected by hemodynamic changes in the circulation system and may indicate the presence of thrombosis and vessel occlusion, which can lead to severe conditions such as stroke, acute heart attack, and pulmonary embolism\textsuperscript{27}. Partially or fully clogged blood vessels under thrombosis usually are harder to compress\textsuperscript{28}. Thus, characterizing the elastic properties of blood vessels under different hemodynamic states can potentially contribute to the detection of thrombosis, and help prevent it from developing into acute life-threatening conditions.

Inspired by manual palpation, medical imaging technologies have been applied to image the elastic properties of biological tissue\textsuperscript{24}. By measuring the tissue’s deformation under loading, elasticity
imaging techniques usually map the mechanical properties of biological tissue onto an image called an elastogram. An elastogram conveys the local variations of stiffness in the region of interest and adds a new dimension of information that may help clinical diagnosis and therapy. Besides its primary application in breast cancer diagnosis, elasticity imaging techniques also find applications in blood vessel stiffness characterization. Vascular elasticity imaging was first implemented invasively by using endovascular imaging catheters. Based on various imaging modalities, noninvasive vascular elasticity imaging was later achieved, including ultrasound elastography (USE), magnetic resonance elastography (MRE), and optical coherence elastography (OCE). USE can image blood vessels in deep regions, but the poor ultrasonic scattering contrast between blood and extravascular tissue makes it insensitive to the deformations of small blood vessels. MRE can potentially penetrate the whole human body, but its spatial resolution (~1–3 mm) is sufficient for assessing the stiffness of large blood vessels only. Benefiting from the high spatial resolution of optical coherence tomography, OCE has a typical spatial resolution of 1–10 μm and can detect displacement at the sub-micrometer level. Thus, it is capable of mapping small blood vessel elasticity. However, OCE suffers from strong optical scattering in biological tissue, limiting its penetration depth to ~1 mm, which is insufficient for assessing elastic properties of blood vessels in the optical diffusive regime in tissues.

With strong optical absorption contrast provided by hemoglobin in red blood cells, PAT has achieved structural and functional imaging of vasculature in both animals and humans. PAT has also been applied to measure the elastic properties of biological tissue ex vivo. However, by measuring the phase delay between the dominant frequency photoacoustic wave and the reference signal, the current photoacoustic technique is able to detect only the viscosity-elasticity ratio
instead of the elasticity, which is not applicable to assessing the vascular elastic properties in humans \textit{in vivo}\cite{42}.

In this section, I report my work on developing VE-PAT, which is capable of measuring blood vessel compliance changes at depths in humans. Because PAT is highly sensitive to blood, the term “blood vessel” in this section specifically refers to blood perfused vessels. By measuring the compliance changes of blood vessel phantoms with and without simulated thrombosis, we demonstrated the feasibility of VE-PAT in characterizing blood vessel elasticity. We further applied this technique to humans and detected a decrease of blood vessel compliance after downstream vessel occlusion, demonstrating the potential of VE-PAT in clinical thrombosis detection.

\section*{2.1.2 Methods}

\textbf{Experimental system}

In VE-PAT, the major device is a linear-array-based photoacoustic imaging probe\cite{43,44}. The probe consisted of a linear array ultrasonic transducer (Visualsonics Inc. LZ250, 21 MHz center frequency, 78\% one-way bandwidth, 256 elements, 23 mm $\times$ 3 mm array size) with two optical fiber bundle strips (20 mm $\times$ 1.25 mm) mounted on each side (Fig. 2.1a). The laser beams coming out of the two optical fiber bundle strips had an angle of incidence of 30$^\circ$ with respect to the imaging plane. The two optical fiber bundles were bifurcated from a single optical bundle that was incorporated into the probe together with an ultrasound signal cable. A tunable optical parametric oscillator laser (680 nm to 970 nm, 20 Hz pulse repetition rate) was coupled into the single fiber optical bundle for photoacoustic excitation. The wavelength was set to 850 nm to achieve deep penetration for vascular elasticity imaging. In our experiments, the fluence on the sample surface
was 10 mJ/cm², well within the safety limit set by the American National Standards Institute (20 mJ/cm²). Each element of the transducer array was cylindrically focused with a focal length of 15 mm. For each of four laser pulses, photoacoustic signals were captured sequentially on one quarter (i.e., 64 elements) of the transducer array elements. After all data was acquired from the four segments, we reconstructed a two-dimensional photoacoustic image using the filtered back-projection algorithm⁴⁵. The reconstructed two-dimensional photoacoustic image was referred to hereafter as the B-scan photoacoustic image. An imaging station (Vevo LAZR, Visualsonics Inc.) displayed the reconstructed B-scan photoacoustic images at 5 frames/second, as determined by the 20 Hz laser repetition rate and the four-to-one multiplexing in the image acquisition system. The VE-PAT system has spatial resolutions of 119 μm in the lateral direction, 86 μm in the axial direction, and 1237 μm in the elevational direction⁴⁴. The minimum displacement that can be resolved is 18.3 μm, which is determined by the data acquisition sampling rate of 84 MHz and the average speed of sound in biological tissue of 1540 m/s. The typical signal-to-noise ratios (SNRs) of the VE-PAT system were around 25 dB in the following phantom experiments and 20 dB in the \textit{in vivo} experiments in humans.

To implement VE-PAT, a customized compression stage was developed and incorporated into the probe. The compression stage had an aluminum plate controlled by a three-dimensional translation stage, which could position the plate in the x-y plane and induce precise displacements along the z-axis for accurate sample compression (Fig. 2.1b). Above the compression plate, a water tank held water for acoustic coupling. An imaging window slightly larger than the probe was machined in the center of the compression plate. To ensure the compression force applied to the sample was normal and uniaxial, a piece of fully stretched polymethylpentene (TPX) plastic membrane was attached to the bottom of the compression plate, which allowed the transmission of both the optical
excitation light and generated photoacoustic wave. An aluminum block on an optical table held the sample against compression.

**Figure 2.1.** Schematic of VE-PAT. (a) Photoacoustic imaging probe. (b) VE-PAT setup for a blood vessel phantom imaging. The imaging probe is incorporated with a customized compression stage.

**Phantom preparation**

Silicone microtubes with 0.3 mm (60985-700, VWR) and 3.4 mm inner diameters (ID) (60985-720, VWR) perfused with bovine blood (905, Quad-Five) mimicked small and large blood vessels, respectively. Blood was pumped into the microtubes through a syringe, and the flow speed was controlled by a syringe pump (BSP-99M, Braintree Scientific). The thrombosis was simulated by injecting a small drop of glue into the microtube downstream from the measurement point, which hardened on the tube wall and partially blocked the flow. The microtube was then embedded in tissue-mimicking gelatin phantoms 3 mm deep (Fig. 2.2a). To achieve stiffness similar to that of soft tissue, the gelatin concentration in the phantoms was 100 g/L. To achieve similar optical scattering as in biological tissue, 1% intralipid was added to the gelatin phantoms.
Sample compression and photoacoustic image acquisition

Elastic property changes induced by simulated thrombosis were investigated in both the large and small blood vessel phantoms. As shown in Figure 2.1b, the axis of the phantom blood vessel was perpendicular to the imaging plane. A cross section of the phantom was first imaged right after the compression plate contacted the sample with a minimum load. For the big vessel (ID = 3.4 mm), we applied ten small steps of compression with a step size of 50 μm and two large steps of compression with a step size of 500 μm. For the small vessel phantom (ID = 0.3 mm), only one 50 μm step compression was applied instead of twelve steps. For all the compression steps, corresponding photoacoustic images were acquired when the phantom had stabilized. The same compression and imaging procedure was performed in both conditions with and without simulated thrombosis.

*In vivo* blood vessel elasticity was assessed on the middle finger of the right hand of a 29-year-old male volunteer. All of the experiments were conducted in accordance with the human studies protocols approved by the Institutional Review Board at Washington University in St. Louis. The right hand was placed on the object holder, and the middle finger was fixed in position and imaged by the VE-PAT system. Similar to the procedures in our phantom experiments, a cross section of the finger was first imaged just after the compression plate contacted the finger with a minimum load. Then, we applied five small steps of compression with a step size of 50 μm and two large steps of compression with a step size of 300 μm. Photoacoustic images were acquired after each step of compression. To simulate thrombosis, a vessel occlusion was created on the near end of the middle finger. The same compression and imaging procedure was repeated to obtain the elastic
property changes of a finger vessel due to the occlusion. The occlusion time was short enough (60 seconds) to avoid any potential harm.

**Strain calculation**

Strain values of the blood vessel phantoms and the finger blood vessels under compression were obtained via analyzing the reconstructed B-scan photoacoustic images before and after compression based on the following method. First, the photoacoustic images at preload were segmented based on a threshold of 6 dB with respect to the noise level, and the original distances \( L \) between the top and bottom boundaries of the blood vessels were calculated at each reconstructed A-line. Then, corresponding reconstructed A-lines in the photoacoustic images before and after compression were analyzed with a sliding-window cross-correlation method\(^{24}\). The sliding window was set slightly larger than the visible vessel boundaries in the photoacoustic images. By doing cross-correlation between the corresponding signals within the window before and after compression, axial displacements of the top and bottom boundaries, denoted as \( d_t \) and \( d_b \), were estimated. The strain \( \varepsilon \) was then calculated by

\[
\varepsilon = \frac{d_t - d_b}{L}. \tag{2.1}
\]

As the compliance \( C \) is proportional to the strain \( \varepsilon \), the ratio of the compliances \( C_1 \) and \( C_2 \) of two samples under the same stress equals the ratio of their strains \( \varepsilon_1 \) and \( \varepsilon_2 \):

\[
\frac{C_1}{C_2} = \frac{\varepsilon_1}{\varepsilon_2}. \tag{2.2}
\]
2.1.3 Results

Standard compression test of the large blood vessel phantom

![Diagram of the large blood vessel phantom](image)

**Figure 2.2.** Characterization of a large blood vessel phantom. (a) Diagram of the large blood vessel phantom embedded 3 mm deep in gelatin. (b) Stress–strain response measured for the large blood vessel phantom with and without simulated thrombosis. Stress–strain curves with strain smaller than 10% were fitted to linear functions to calculate the compliances as shown by the dashed lines.

Before the microtubes were embedded into tissue-mimicking gelatin phantoms, the compliances of the perfused large blood vessel phantoms with and without simulated thrombosis were measured with a standard compression test. In the standard compression test, the large blood vessel phantom was placed on a high-precision digital weighing scale (S200, Ohaus) and compressed using the customized compression stage. The compression stress was calculated based on the difference in the scale readings before and after compression. The axial displacement of the top boundary was read from the translation stage that moved the compression plate along the z-axis for precise sample compression. Because the bottom boundary did not move, the strain was calculated as the
deformation over the original diameter of blood vessel phantom (Eq. 2.1). Consistent with the previous elasticity imaging studies\textsuperscript{47}, the stress–strain relationship appeared linear with the coefficient of determination ($R^2$) of 0.993 and 0.987 when the strain was smaller than 10% (Fig. 2.2b). The ratio of the compliances between with and without simulated thrombosis was 0.64.

**VE-PAT of large blood vessel phantom**

Photoacoustic images of a cross section of the large blood vessel phantom before and after ten small steps of compression with a step size of 50 μm are shown in Figure 2.3. The diameter of the large blood vessel phantom before compression was measured to be 3513.6 μm. The axial displacements of the top and bottom boundaries and the strain values of the large blood vessel phantom are summarized in Table 2.1. Under the same stress, the blood vessel phantom without simulated thrombosis (Fig. 2.3a and b) underwent larger deformation than that with simulated thrombosis (Fig. 2.3c and d). The strain values of the large blood vessel phantom with and without simulated thrombosis were plotted at each compression step (Fig. 2.3i). During the ten small steps of compression, the stress–strain response still appeared linear, as shown in Figure 2.3i. We quantified the relative compliance by fitting the two data sets to linear functions. Compliance ratio between the two states was estimated to be 0.67, which is close to the ratio 0.64 measured in the standard compression test.

Figure 2.3e–h show B-scan photoacoustic images of a cross section of the large blood vessel phantom before and after two large steps of compression with a step size 500 μm, during which the strain reached the apparent nonlinear stress–strain response regime. The blood vessel phantom became harder to compress in the presence of simulated thrombosis (Fig. 2.3g and h), akin to the linear stress–strain response result. The strain values at the first compression state for the blood
vessel phantom with and without simulated thrombosis were 12.9 ± 1.3% and 16.3 ± 1.1%, respectively. At the second compression state, they were 15.8 ± 1.4% and 22.4 ± 1.7%, respectively (Fig. 2.3i). Since the same compression forces were applied to the blood vessel phantom with and without simulated thrombosis at each state, the smaller strains indicated that a decrease in compliance of the blood vessel phantom was induced by the simulated thrombosis.

**Figure 2.3.** VE-PAT of a large blood vessel phantom. Photoacoustic images of a cross section in the large blood vessel phantom, without thrombosis before (a), after ten small steps of compression (b), and after two large steps of compression (e-f). Photoacoustic images of the cross section in the large blood vessel phantom with simulated thrombosis before (c), after ten small steps of compression (d), and after two large steps of compression (g-h). (i) Strain curves under twelve steps of compression for the large blood vessel phantom with and without simulated thrombosis. Dashed lines: linear fits with the coefficient of determination ($R^2$) of 0.998 and 0.995 for $\leq 13\%$ strain. Error bars: standard deviation.
Table 2.1. VE-PAT of axial displacements and strains of the large blood vessel phantom. The linear compression is the result from 10 total compression steps.

<table>
<thead>
<tr>
<th></th>
<th>Linear compression</th>
<th>Nonlinear compression step 1</th>
<th>Nonlinear compression step 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without thrombosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top boundary displacement (μm)</td>
<td>475.8</td>
<td>732.0</td>
<td>1006.5</td>
</tr>
<tr>
<td>Bottom boundary displacement (μm)</td>
<td>43.9</td>
<td>161.0</td>
<td>219.6</td>
</tr>
<tr>
<td>Deformation (μm)</td>
<td>431.9</td>
<td>571.0</td>
<td>786.9</td>
</tr>
<tr>
<td>Strain (%)</td>
<td>12.3</td>
<td>16.3</td>
<td>22.4</td>
</tr>
<tr>
<td>Standard deviation of strain (%)</td>
<td>0.9</td>
<td>1.1</td>
<td>1.7</td>
</tr>
<tr>
<td>With thrombosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top boundary displacement (μm)</td>
<td>320.3</td>
<td>567.3</td>
<td>695.4</td>
</tr>
<tr>
<td>Bottom boundary displacement (μm)</td>
<td>34.8</td>
<td>113.4</td>
<td>139</td>
</tr>
<tr>
<td>Deformation (μm)</td>
<td>285.5</td>
<td>453.9</td>
<td>556.4</td>
</tr>
<tr>
<td>Strain (%)</td>
<td>8.1</td>
<td>12.9</td>
<td>15.8</td>
</tr>
<tr>
<td>Standard deviation of strain (%)</td>
<td>0.6</td>
<td>1.3</td>
<td>1.4</td>
</tr>
</tbody>
</table>

VE-PAT of small blood vessel phantom

Photoacoustic images of a cross section of the small blood vessel were acquired before and after compression, with and without simulated thrombosis (Fig. 2.4a–d). The diameter of the small blood vessel phantom before compression was measured to be 329.4 μm. The axial displacements of the top and bottom boundaries and the strain values of the small blood vessel phantom are summarized in Table 2.2. Similar to the large blood vessel phantom, the small blood vessel phantom was harder to compress with the simulated thrombosis. The strain for the small blood
vessel phantom without simulated thrombosis was 13.3 ± 0.9% while the strain with simulated thrombosis was only 6.7 ± 0.4% (Fig. 2.4e). Under the same compression force, the decrease in strain indicated a decrease in compliance due to the simulated thrombosis.

Figure 2.4. VE-PAT of a small blood vessel phantom. Photoacoustic images of a cross section of the small blood vessel phantom, without simulated thrombosis before (a) and after compression (b), and with simulated thrombosis before (c) and after compression (d). (e) Strain values for the small blood vessel phantom with and without thrombosis. Statistics: paired student’s t-test. P values: **<0.05.

Table 2.2. VE-PAT of axial displacements and strains of the small blood vessel phantom.

<table>
<thead>
<tr>
<th></th>
<th>Without thrombosis</th>
<th>With thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top boundary displacement (μm)</td>
<td>71.4</td>
<td>45.8</td>
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<tr>
<td>Bottom boundary displacement (μm)</td>
<td>27.5</td>
<td>23.8</td>
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<tr>
<td>Deformation (μm)</td>
<td>43.9</td>
<td>22.0</td>
</tr>
<tr>
<td>Strain (%)</td>
<td>13.3</td>
<td>6.7</td>
</tr>
<tr>
<td>Standard deviation of strain (%)</td>
<td>0.9</td>
<td>0.4</td>
</tr>
</tbody>
</table>
VE-PAT of blood vessel in a human

Figure 2.5 shows the VE-PAT results of the middle finger blood vessels of the human subject with and without vessel occlusion. The diameter of the blood vessel in the human finger before compression was measured to be 786.9 μm. The axial displacements of the top and bottom boundaries and the strain values of the finger blood vessel are summarized in Table 2.3. During five small steps of compression with a step size of 50 μm, the blood vessel in the finger underwent smaller deformation with vessel occlusion downstream (Fig. 2.5a–d). Strain values for the blood vessels at each step of compression were calculated for both normal and vessel occlusion conditions (Fig. 2.5e). Since the largest deformation was still within the linear stress–strain response regime, we estimated a relative decrease of compliance as 30.0% when the vessel was occluded.

**Figure 2.5.** VE-PAT of a human finger in the linear stress–strain response regime. Photoacoustic images of a cross section of the human finger, without vessel occlusion before (a) and after compression (b), and with vessel occlusion before (c) and after compression (d). (e) Strain curves under five steps of compression for the human finger with and without vessel occlusion. Dashed lines: linear fits with the coefficient of determination ($R^2$) of 0.993 and 0.987. Error bars: standard deviation.
Table 2.3. VE-PAT of axial displacements and strains of the finger blood vessel *in vivo*. The linear compression is the result from 5 total compression steps.

<table>
<thead>
<tr>
<th></th>
<th>Linear compression</th>
<th>Nonlinear compression step 1</th>
<th>Nonlinear compression step 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Without vessel occlusion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top boundary displacement (μm)</td>
<td>234.2</td>
<td>456.1</td>
<td>585.6</td>
</tr>
<tr>
<td>Bottom boundary displacement (μm)</td>
<td>155.5</td>
<td>161.0</td>
<td>208.7</td>
</tr>
<tr>
<td>Deformation (μm)</td>
<td>78.7</td>
<td>295.1</td>
<td>376.9</td>
</tr>
<tr>
<td>Strain (%)</td>
<td>10.0</td>
<td>37.5</td>
<td>47.9</td>
</tr>
<tr>
<td>Standard deviation of strain (%)</td>
<td>2.8</td>
<td>5.8</td>
<td>6.1</td>
</tr>
<tr>
<td><strong>With vessel occlusion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top boundary displacement (μm)</td>
<td>165.6</td>
<td>259.9</td>
<td>475.7</td>
</tr>
<tr>
<td>Bottom boundary displacement (μm)</td>
<td>113.4</td>
<td>128.1</td>
<td>295.5</td>
</tr>
<tr>
<td>Deformation (μm)</td>
<td>52.2</td>
<td>131.8</td>
<td>180.2</td>
</tr>
<tr>
<td>Strain (%)</td>
<td>6.6</td>
<td>16.7</td>
<td>22.9</td>
</tr>
<tr>
<td>Standard deviation of strain (%)</td>
<td>0.8</td>
<td>3.4</td>
<td>4.5</td>
</tr>
</tbody>
</table>

The finger was then compressed with two 300 μm steps, during which the blood vessels underwent larger deformation that the stress–strain response became nonlinear (Fig. 2.6a–f). Again, the blood vessels with occlusion underwent smaller deformation than that without occlusion under the same load. Strain values for blood vessels without occlusion at the two steps were 37.5 ± 5.8% and 47.9 ± 6.1%, while they were 16.7 ± 3.4% and 22.9 ± 4.5% with vessel occlusion (Fig. 2.6g). These results demonstrated that VE-PAT can detect the decrease of vessel compliance in the human finger due to vessel occlusion in both the linear and nonlinear stress–strain response regimes.
Figure 2.6. VE-PAT of a human finger in the nonlinear stress–strain response regime. Photoacoustic images of a cross section of the human finger without vessel occlusion before compression (a), after compression step 1 (b), and after compression step 2 (c). Photoacoustic images of the same cross section of the human finger with vessel occlusion before compression (d), after compression step 1 (e), and after compression step 2 (f). (g) Strain values for the blood vessels with and without thrombosis in two compression steps. Statistics: paired student’s t-test. P values: **<0.05.

2.1.4 Discussion

VE-PAT detected vascular compliance changes due to simulated thrombosis in large and small blood vessel phantoms at depths and in blood vessels in a finger of a human subject in vivo. In the large blood vessel phantom, the compliance ratio between the two states with and without simulated thrombosis measured by VE-PAT agreed with the value measured in the standard compression test. By imaging the small blood vessel phantom embedded 3 mm deep in the gelatin phantom, VE-PAT demonstrated its capability to detect elasticity changes of small blood vessels at depths that are difficult for either USE or OCE to image. In addition, VE-PAT was applied to assess blood vessel elasticity changes due to vessel occlusion in the human finger, demonstrating its potential for clinical vessel elasticity characterization.
The detection sensitivity of the VE-PAT system is limited by the minimum detectable displacement, i.e., 18.3 μm. First, the minimum detectable displacement limits the minimum diameter of blood vessels whose elasticity can be assessed by VE-PAT in the linear stress–strain response regime, which is usually considered to be less than 10% strain in biological tissue. To stay within the linear stress–strain response regime, the blood vessel diameter should be at least 183 μm, i.e., 10 times (reciprocal of 10%) the minimum detectable displacement. For blood vessels with diameters smaller than 183 μm and larger than the minimum detectable displacement of 18.3 μm, their elasticity can still be assessed, but only in the nonlinear stress–strain response regime. For blood vessels with diameters smaller than 18.3 μm, their deformation cannot be resolved, which is the ultimate detection limit of the current VE-PAT system.

To the best of our knowledge, this is the first time that PAT has been applied for quantitative assessment of blood vessel elastic properties in humans in vivo. In addition to the information including structure, flow velocity, molecule concentration, and oxygen saturation level of hemoglobin, that photoacoustic imaging can provide\textsuperscript{21,48–50}, VE-PAT adds important elasticity information.

2.2 Quasi-static Photoacoustic Elastography

2.2.1 Background

Elastography was first performed using ultrasound imaging in 1991\textsuperscript{51}. Later, elastography was implemented using magnetic resonance imaging, namely magnetic resonance elastography, at the whole-body level with a spatial resolution of \(\sim 1–3\) mm\textsuperscript{52}. Elastography was also implemented in optical coherence tomography (OCT), which has greatly improved the spatial resolution to \(\sim 1–10\)
μm, but OCT has limited imaging depth (~1 mm in the skin) due to the strong optical scattering in biological tissue.

PAT is a hybrid imaging technique that combines rich optical absorption contrast and high ultrasonic spatial resolution. PAT has proven capable of imaging anatomical, functional, molecular, and metabolic information of biological tissue. There have been several photoacoustic studies on measuring the elastic properties of biological tissue. In one study, the viscoelasticity of biological tissues was imaged by photoacoustic technique, but the elasticity could not be measured because the detected photoacoustic signal phase delay was related to the viscosity-elasticity ratio instead of the elasticity alone. In a second study, the volume-averaged Young’s modulus of soft tissue was measured by a photoacoustic sensing technique, which, however, possessed no spatial resolution. Yet another study introduced speckles in photoacoustic images for sample displacement estimation but demonstrated the concept only with simulation data. So far, elastography has not been successfully implemented using photoacoustic imaging. Here, we demonstrate photoacoustic elastography, capable of high-resolution strain imaging of biological tissue based on the contrast of Young’s modulus. Implemented using PACT, photoacoustic elastography can map the mechanical contrast in biological tissue while maintaining high spatial resolution and excellent penetration depth.

2.2.2 Methods

The photoacoustic elastography was developed based on a linear-array PACT system, which is capable of high-resolution imaging of the elasticity distribution in tissue in vivo. A 10 ns pulsed laser beam at 680 nm was used for photoacoustic excitation with a 20 Hz pulse repetition rate. Light was first coupled into a fiber bundle. The fiber bundle was then split into two rectangular
light bars mounted on each side of a linear ultrasonic transducer array (LZ250, VisualSonics Inc., 21 MHz center frequency, 256 elements), which detected the generated photoacoustic waves. Photoacoustic signals were sampled at 84 MHz. For each laser pulse, one quarter of the 256 ultrasonic array elements were used for detecting photoacoustic signals. Acquired with four laser pulses, the full data set was used to reconstruct a cross-sectional photoacoustic image, yielding a frame rate of 5 Hz. The spatial resolutions of the PACT system were 119 μm in the lateral direction, 86 μm in the axial direction, and 1.2 mm in the elevational direction.

In our photoacoustic elastography system, an aluminum compression plate larger than the object exerted a small axial compressive force on the object (Fig. 2.7a). An imaging window slightly larger than the ultrasonic transducer probe was opened at the center of the compression plate (Fig. 2.7b). A piece of fully stretched polymethylpentene (TPX) plastic membrane was attached to the bottom of the compression plate to provide uniform and uniaxial force to the object while passing the illumination laser beam. Ultrasound gel was used for acoustic coupling between the compression plate and the object, without changing the elasticity of the gelatin phantoms. Ultrasound gel is also convenient for in vivo animal imaging and potential clinical applications. For acoustic coupling between the probe and the compression plate, water was chosen as the medium. The compression plate was adjusted by a manual translation stage to provide precise compression to the object against a rigid object holder. The total displacement of the object surface was read from the translation stage. The object and the object holder were placed on a high-precision digital weighing scale (S200, Ohaus). The compression stress applied to the object was calculated from the difference in the scale readings before and after compression:
\[ \sigma = \frac{g(m_a - m_b)}{A}. \] (2.3)

Here, \( \sigma \) is the compression stress, \( g \) is the acceleration of gravity, \( m_a \) and \( m_b \) are the scale readings before and after compression, and \( A \) is the area on which the compression force is applied.

**Figure 2.7.** Schematic of the photoacoustic elastography system. (a) Side view of the photoacoustic elastography system. (b) Top view of the compression plate with the imaging window at the center.

### 2.2.3 Results

**Photoacoustic elastography of homogeneous gelatin phantoms**

To demonstrate quantitative elasticity measurement, photoacoustic elastography was first used to image four homogeneous gelatin phantoms with respective gelatin concentration of 40, 60, 80, and
100 g/L. To provide absorption contrast for photoacoustic imaging, 50 μm microspheres were mixed in the gelatin phantoms at a concentration of ~5 microspheres per mm$^3$. Each gelatin phantom was imaged with the photoacoustic elastography system before and after compression with an external stress of 53 Pa (Fig. 2.8a and b). The maximum surface displacement in the experiments was 120 μm, and the maximum strain was 4%, which was considered to be within the linear strain response regime of the phantom$^{46}$. Time-resolved A-line signals before and after compression were cross-correlated to calculate the axial displacement due to compression$^{59}$, generating a cross-sectional map of displacements after compression (Fig. 2.8c). Here, short-window cross-correlation between corresponding A-lines was computed. We slid a 90-μm-wide window along the A-lines acquired before and after compression and computed the cross-correlations to find the displacement between the A-lines at each window position. Displacements were then averaged among the microspheres at each depth (Fig. 2.8d). The slope of the linear fitting of the displacements versus depths—i.e., the magnitude of the average gradient of the displacement—quantified the average strain of each gelatin phantom. The same data processing was performed on 20 measurements, 5 each for four gelatin phantoms (Fig. 2.8e). The measured strains were then fitted by the model below$^{46}$:

$$\varepsilon = \frac{\sigma}{K} \frac{1}{C^2}. \quad (2.4)$$

Here, $\varepsilon$ is the strain of the gelatin phantom, $\sigma$ is the stress applied to the phantom, $K$ is a constant factor, and $C$ is the gelatin concentration. Note $K$ is affected by the equilibrium temperature, the temperature and duration of the gelatin mixing process, and the molecular weight of gelatin.
**Figure 2.8.** Photoacoustic elastography of a single-lay gelatin phantom. (a-b) Cross-sectional PA images of a gelatin phantom (40 g/L gelatin concentration) mixed with 50 μm microspheres acquired (a) before and (b) after compression. (c) Displacement image obtained from (a) and (b). (d) Average displacement versus the depth. The strain of the phantom was estimated as the slope of the linear fitting. (e) Measured strains of gelatin phantoms with 4%, 6%, 8%, and 10% concentration in weight. The data was fitted to a quadratic model that describes the relationship between the strain and the gelatin concentration (Equation 2.4).

**Photoacoustic elastography of a bilayer gelatin phantom**

Photoacoustic elastography was then used to image a bilayer gelatin phantom with different gelatin concentrations in each layer. The top layer had a gelatin concentration of 50 g/L and a thickness of 2.5 mm. The bottom layer had a gelatin concentration of 100 g/L and a thickness of 2.0 mm. Again, 50 μm microspheres were mixed in the gelatin phantom at the concentration of 5 microspheres per mm$^3$. The bilayer phantom was imaged by the photoacoustic elastography system before and after compression with a stress of 98 Pa (Fig. 2.9a and b). A displacement image was
generated using the same method as above (Fig. 2.9c). Displacements of the microspheres were averaged at each depth and fitted by a linear function for each layer (Fig. 2.9 d). The slopes of the two linear fittings reflect the strains in the two layers. The strain ratio between the two layers is $4.0 \pm 0.2$, which agrees with the theoretical value of 4.

![Figure 2.9](image)

**Figure 2.9.** Photoacoustic elastography of a bilayer gelatin phantom. (a-b) PA images of a bilayer gelatin phantom mixed with 50 μm microspheres acquired (a) before and (b) after compression. (c) Displacement image obtained from (a) and (b). (d) Average displacement versus depth. The data was fitted by a linear function for each layer.

**Photoacoustic elastography of a mouse leg in vivo**

A mouse leg was then imaged *in vivo* by photoacoustic elastography. All experimental animal procedures were carried out in conformity with laboratory animal protocols approved by the Animal Studies Committee at Washington University in St. Louis. The mouse leg was imaged
before and after applying an external compression force of 12 mN (Fig. 2.10a and b). A displacement image was obtained by cross-correlating the photoacoustic images before and after compression, using the image pixels with photoacoustic signal amplitudes above the noise level. A raw strain image was then obtained by numerically differentiating the axial displacements, assuming that the applied stress was uniaxial (Fig. 2.10c). The raw strain image was then superimposed on the structural photoacoustic image (Fig. 2.10e). The regions of tissue with larger strains were softer than regions with smaller strains, and thus were thought to have more fat. The photoacoustic elastography was validated by USE using the same linear-array imaging probe, which showed a similar distribution of strains (Fig. 2.10d and f). In USE, structural ultrasound images were acquired simultaneously with structural photoacoustic images before and after the compression. The displacement and strain images in the USE were computed using the same data processing method as in the photoacoustic elastography. The average strains over the entire cross-sectional image were 0.84 ± 0.49% in photoacoustic elastography and 0.82 ± 0.29% in USE. Here it is worth noting that there were differences between the strain distribution measured by photoacoustic elastography and USE. Although both methods measured the elastic property of the tissue, they were based on different contrast mechanisms. Within each resolution voxel (~119 μm by 86 μm by 1.2 mm) of the strain image, optical-absorption-based photoacoustic elastography measured the integrated elasticity of the tissue only through the displacement of the vasculature, while acoustic-scattering-based USE mapped the integrated elasticity through the displacement of more tissue components, including vasculature, muscle fibers, and fat. Under compression, there may be more relative displacements between vasculature and other tissue components in the soft regions, resulting in higher strain values than those in USE. Another possible reason for the discrepancy between the measured strain images is the different number of useful pixels in
photoacoustic and ultrasound elastography images. Lacking speckles and imaging only the blood vasculature, photoacoustic elastography has fewer useful pixels than USE, resulting in fewer effective pixels for interpolation, especially in the tissue region with less blood.

Figure 2.10. Photoacoustic elastography of a mouse leg in vivo. (a-b) PA images of a mouse leg in vivo before and after compression. (c) A strain image of the mouse leg obtained by photoacoustic elastography in vivo. (d) A strain image of the mouse leg obtained by USE in vivo. (e) The strain image of the mouse leg obtained by photoacoustic elastography superimposed on the structural PA image. (d) The strain image of the mouse leg obtained by USE on the structural ultrasound image.

2.2.4 Discussion

Compared to previous studies, our photoacoustic elastography technique based on a linear-array PACT has the following distinctive features. First, photoacoustic elastography maps the Young’s modulus of biological tissue, but previous photoacoustic viscoelasticity studies are based on the contrast of the viscosity-elasticity ratio. Second, photoacoustic elastography is able to provide
cross-sectional strain images of biological tissue with high axial resolution, while viscosity-elasticity ratio measurement has no axial resolution. Third, photoacoustic viscoelasticity imaging is based on an intensity-modulated continuous-wave laser excitation, yielding a much lower signal to noise ratio than that in photoacoustic elastography using a pulsed laser excitation\textsuperscript{60}. Fourth, photoacoustic elastography is implemented on a commercial linear-array-based PACT system, which can measure other biological parameters including vasculature density, tumor volume, oxygen saturation of hemoglobin, and blood flow velocity\textsuperscript{61}.

We would like to point out that the motivation of this work is not to prove that photoacoustic elastography is superior to USE. Instead, the major motivation is to demonstrate the feasibility of elasticity measurement by using PAT as an independent device: Not all the photoacoustic imaging systems have the capability of ultrasound transmission, and thus USE is not always available. Photoacoustic elastography can be implemented on existing photoacoustic imaging systems, as an additional function, to provide more comprehensive information about the tissue’s mechanical and functional information.

Further, PAT can potentially measure elasticity concurrently with other functional parameters, including the oxygen saturation of hemoglobin, which may provide more comprehensive information for disease diagnosis and treatment evaluation\textsuperscript{9}. Noninvasive imaging of elasticity distribution expands the functionality of PAT and is expected to find potential applications in clinical practice, such as cancer detection and arterial plaque assessment. To advance the current photoacoustic elastography technique for clinical applications, several challenges remain to be solved. One challenge is to apply normal uniform compression force \textit{in vivo}. A special compression mechanism needs to be developed for curved tissue surface. Another challenge is to
achieve 3D volumetric strain imaging. To achieve this, the current linear-array photoacoustic probe can be linearly scanned orthogonally.

2.3 Quantitative Photoacoustic Elastography

2.3.1 Background

To quantify the elastic properties of biological tissue, elastography has been developed in various modalities including USE\(^{51}\), MRE\(^{37}\), OCE\(^{38}\), and photoacoustic elastography\(^{62}\). In elastography, tissue deformation is induced by a static or dynamic load and imaged. If the stress distribution is known, the deformation can be converted to an image of elasticity called elastogram. However, unless the stress is known in absolute values, elastography techniques can image elasticity only in relative values, which are not sufficient for longitudinal monitoring.

Various methods have been developed to achieve absolute elastography. By measuring shear wave propagation, USE and MRE can quantify the shear modulus of biological tissue\(^{52,63}\). Although the Young’s modulus \(E\) is directly related to the shear modulus \(G\) in soft tissue by

\[
E = 2G(1 + \nu).
\]

(2.5)

It is also affected by the Poisson's ratio \(\nu\) of soft tissue, which can vary from 0.46 to 0.49\(^{64}\). Thus, the absolute Young’s modulus is still unknown without the knowledge of both the shear modulus and Poisson’s ratio. OCE has achieved quantitative measurement of absolute Young’s modulus. In one study, compression OCE was combined with a stress sensor to measure both strain and stress, from which the absolute Young’s modulus of biological tissue was calculated\(^{65}\). In another study, the absolute Young’s modulus was obtained from the phase velocity of the surface acoustic
wave, which was measured by phase-sensitive optical coherence tomography. However, both methods suffer from limited imaging depth (~1 mm) due to strong optical scattering in biological tissue.

By acoustically detecting optical absorption, PAT achieves high sensitivity, multicontrast imaging of biological tissue with highly scalable spatial resolution and penetration depth. PAT has successfully measured the elastic properties of biological tissue, including strain, the viscosity-elasticity ratio, and vascular compliance. Yet, all the aforementioned photoacoustic elastic imaging techniques measure only relative elastic properties. Here, we report QPAE capable of measuring the absolute Young’s modulus in vivo in humans. By introducing a stress sensor into photoacoustic elastography, QPAE measures the local stress and strain simultaneously and quantifies the absolute Young’s modulus.

2.3.2 Methods

To implement QPAE, a linear-array-transducer based photoacoustic imaging system (Vevo LAZR Imaging System, VisualSonics Inc., Toronto, Canada) was modified to be combined with a customized compression system (Fig. 2.11). A Nd:YAG laser pumped a tunable optical parametric oscillator laser to provide illumination with wavelengths from 680 nm to 970 nm at a repetition rate of 20 Hz. An excitation wavelength of 850 nm was chosen to achieve deep penetration for QPAE. The laser beam was then coupled into an optical fiber bundle that was incorporated into the photoacoustic imaging probe. The optical fiber bundle bifurcated into two rectangular fiber bundles (20 mm × 1.25 mm). Laser beams emerging from the two rectangular fiber bundle strips illuminated the object to be imaged at an angle of incidence of 30° with respect to the imaging plane. The fluence on the tissue surface was about 10 mJ/cm², below the 20 mJ/cm²
safety limit set by the American National Standards Institute. The generated photoacoustic waves were detected by a linear array ultrasonic transducer (23 mm × 3 mm), which was placed coaxially and confocally with the illuminating fiber bundles to maximize the system’s sensitivity. The linear array ultrasonic transducer had 256 elements, a central frequency of 21 MHz, and a one-way bandwidth of 78%. For each laser pulse, ultrasonic signals from 64 out of the 256 elements in the linear array were acquired by the data acquisition system. Thus, to obtain a two-dimensional image with full width, four laser pulses were needed, which reduced the two-dimensional imaging frame rate to 5 Hz, corresponding to one fourth of the laser pulse repetition rate of 20 Hz. The full data set from all the elements in the linear array ultrasonic transducer was then used to reconstruct a two-dimensional photoacoustic image, referred as a B-scan photoacoustic image, by using the filtered back-projection algorithm.

Figure 2.11. Schematic of QPAE system. (a) Photoacoustic imaging probe at lateral and elevational view. FB, fiber bundle; IP, imaging probe; LB, laser beam; SC, signal cable; TA, transducer array. (b) QPAE system setup. CP, compression plate; O, object to be imaged; OH, object holder; SS, stress sensor; TM, TPX membrane; WT, water tank.
The compression system consisted of an aluminum compression plate with an open imaging window at the center. A translation stage moved the compression stage along the z-axis to exert a small axial compression force on the object to be imaged. To ensure the compression force was normal, a piece of fully stretched polymethylpentene (TPX) plastic membrane was attached to the bottom of the compression plate. A stress sensor made of translucent silicone rubber was placed between the TPX plastic membrane and the object to be imaged to measure the local stress. The stress sensor had a Young’s modulus of 30 kPa. An object holder held the object to be imaged against compression. To provide acoustic coupling, the photoacoustic imaging probe head was submerged in a water tank above the compression plate. Ultrasound gel maintained good acoustic contact between the compression plate and the sensor, and between the sensor and the object.

To obtain the Young’s modulus of the object, both the local stress and strain were measured in each experiment. After the compression plate contacted the stress sensor with a minimum load, a B-scan photoacoustic image of both the sensor and the object was first obtained, from which the baseline thickness of the stress sensor $l_b(x)$ was measured. Then we exerted a small axial compression by moving the compression plate along the z-axis. After the object had stabilized, we obtained another B-scan photoacoustic image of the same cross section of the sensor and the object, from which the compressed thickness of the stress sensor $l_c(x)$ was measured. The strain of the stress sensor $\varepsilon_{ss}(x)$ at each lateral location was then calculated by

$$\varepsilon_{ss}(x) = \frac{l_b(x) - l_c(x)}{l_b(x)}.$$  \hspace{1cm} (2.6)

The local stress $\sigma(x)$ at each lateral position was obtained from the stress–strain curve of the sensor material (Fig. 2.12a), which was generated by an independent compression test. A two-
dimensional short-window cross-correlation between the two B-scan images was calculated to obtain a map of displacement. By numerically differentiating the displacement map, we obtained a strain image of the object \(\varepsilon_{sa}(x, z)\). The Young’s modulus value at each location \(E_{sa}(x, z)\) was then calculated by

\[
E_{sa}(x, z) = \frac{\sigma(x)}{\varepsilon_{sa}(x, z)}.
\]  
(2.7)

**Figure 2.12.** Characterization of the stress sensor. (a) Stress–strain curve of the stress sensor material. (b) Validation of the stress measurement by the stress sensor. The measured stress (black dots) agreed well with the applied stress.

To ensure the accuracy of the Young’s modulus measurement, we first validated the stress measurement by the stress sensor. Placed on a high-precision digital weighing scale (S200, Ohaus), the stress sensor was imaged by QPAE before and after compression. The applied stress was calculated by

\[
\sigma_A(x) = \frac{g(m_a - m_b)}{A}.
\]  
(2.8)
Here, \( \sigma_A(x) \) is the compression stress, \( g \) is the acceleration of gravity, \( m_a \) and \( m_b \) are the scale readings before and after compression, and \( A \) is the area on which the compression force is applied. The local compression stress was also obtained by analyzing the photoacoustic images before and after compression with the method described above and averaged over the entire cross section. The stress measured by the stress sensor agreed well with the applied stress (Fig. 2.12b).

### 2.3.3 Results

**QPAE of agar phantoms**

We first demonstrated the feasibility of QPAE by imaging agar phantoms with different concentrations. A small portion of black ink was mixed with the agar to provide optical absorption. Agar phantoms with concentrations of 20 g/L, 25 g/L, 30 g/L, 35 g/L, and 40 g/L were embedded in gelatin at a concentration of 100 g/L. To mimic optical scattering in biological tissue, 1% intralipid was added to the agar phantoms and the gelatin background. The five phantoms were imaged by QPAE, and maps of Young’s modulus were obtained using the method described above (Fig. 2.13a-e). The entire cross-sections of the five agar phantoms, with depths between 2.5 mm and 3.0 mm, were all clearly resolved by QPAE. Then the Young’s modulus at each agar concentration was calculated by averaging over all the pixels with signal to noise ratios (SNRs) above 6 dB in the entire Young’s modulus map. Two methods were adopted to validate the Young’s modulus measurement in the phantoms. First, the average Young’s modulus values were fitted to the following empirical relationship based on the agar concentrations\(^{46}\) (Fig. 2.13f)

\[
E = kC^{1.87}.
\]  

(2.9)
Here, $E$ is the Young’s modulus at a given concentration $C$, and $k$ is a factor related to several parameters, including the molecular weight of agar used in the experiments and the agar mixing duration and temperature. The fitting results show a good agreement between the Young’s modulus measurement by QPAE and the empirical relationship based on the agar concentrations, with an $R^2$ value of 0.99. Second, the Young’s modulus measurements of the agar phantoms were validated by an independent standard compression test (SCT). In the SCT, stress–strain curves of the agar phantoms fabricated with the same procedure as above were generated. Young’s modulus values were calculated based on stress–strain curves with strain $< 0.1$ \cite{69}. The Young’s modulus values of agar phantoms measured by QPAE agree well with those measured by SCT, further demonstrating the accuracy of QPAE in quantifying the absolute elasticity (Fig. 2.13g).

**Figure 2.13.** QPAE of agar phantoms. (a–e) QPAE images of agar phantoms at agar concentrations of 20 g/L, 25 g/L, 30 g/L, 35 g/L, and 40 g/L, respectively. (f) Young’s modulus measured by QPAE as a function of agar concentration. The results were fitted by Equation 2.8. (g) Validation of Young’s modulus measurement by SCT. The Young’s modulus values of the agar phantoms measured by the two methods agreed well.
QPAE of a human arm *in vivo*

To demonstrate quantitative measurement of Young’s modulus *in vivo*, we imaged the right arm of a healthy human volunteer. All of the experiments were conducted in accordance with the human study protocols approved by the Institutional Review Board at Washington University in St. Louis. The biceps muscle was chosen because the volunteer would have sufficient control of the arm to avoid motion artifacts and could maintain the same arm position and same elbow angle of 90° throughout the experiment. The right arm was chosen to reduce the possible motion artifacts induced by the movement of the chest wall and the heart. During the experiments, the volunteer was asked to place his arm as flat as possible on the object holder. Then he held a hand grip attached to a cable with different loadings pulling his arm straight but was tasked with keeping his elbow at a 90° angle during imaging. The stress sensor was placed on the biceps with ultrasound gel in between to keep good acoustic contact. The compression system and the photoacoustic imaging probe were on top of the stress sensor. Different loadings of 0.0 kg, 2.5 kg, 5.0 kg, 7.5 kg, and 10.0 kg were applied. At each loading, a B-scan photoacoustic image of a cross section of the stress sensor and arm was obtained first. In the B-scan photoacoustic image, three layers of structures were resolved, including the skin layer, the blood vessels, and a muscle layer (Fig. 2.14a). Then an axial compression force was exerted by moving the compression plate down along the z-axis. Another B-scan photoacoustic image of the same cross section of the stress sensor and arm was obtained. At each loading, a map of Young’s modulus was calculated based on the method described above (Fig. 2.14 a–e). With QPAE, we were able to obtain the Young’s modulus values of the bicep up to 6 mm deep, within which the SNR was sufficiently high to calculate the displacements. We also calculated the average Young’s modulus values for each layer (Fig. 2.14f). The skin had an average Young’s modulus value of 15.9 kPa, and we found that it stayed invariant
with increasing loadings of the arm. The Young’s modulus of the muscle layer increased linearly with the loading applied. The result indicated that the elastic modulus of the biceps muscle has a linear relationship with the loading applied, which agrees with previous shear modulus measurements by MRE\textsuperscript{70}. A slight increase of the Young’s modulus was also observed in the cephalic vein, which possibly resulted from the increased blood supply to the arm due to repeated loading applied during the experiments. The \textit{in vivo} results in human arm further demonstrated the capability of QPAE in measuring the Young’s modulus quantitatively.

![Figure 2.14. QPAE of a human biceps muscle \textit{in vivo}. QPAE images of the human biceps muscle \textit{in vivo} at different loadings: 0.0 kg (a), 2.5 kg (b), 5.0 kg (c), 7.5 kg (d), and 10.0 kg (e). The skin layer, blood vessel boundaries, and skeletal muscle can be observed. (f) Young’s modulus value averaged in each layer as a function of loading.]

\subsection*{2.3.4 Discussion}

Note that in the phantom and \textit{in vivo} experiments above, the maximum deformation of the phantom and the biological tissue was controlled to be smaller than 0.1. This ensures that the stress–strain
response stayed in the linear range, so the Young’s modulus calculation was valid. However, this requirement was not necessary for the stress sensor because we had characterized its stress–strain behavior with strain up to 0.5. The Young’s modulus was calculated only on the pixels with SNRs above 6 dB because the displacement calculation based on the cross-correlation was only valid for pixels with SNRs above 6 dB.

The spatial resolutions of QPAE are 86 μm in the axial direction, 119 μm in the lateral direction, and 1237 μm in the elevational direction, determined by the linear-array-based photoacoustic imaging probe. The minimum detectable displacement is 18.3 μm, which is determined by the data acquisition sampling rate of 84 MHz and the average speed of sound in biological tissue of 1540 m/s. The range of Young’s modulus measurement by QPAE depends on two factors. One is the range of strain measurement by PAE. The other is the ratio of the elasticity of the stress sensor to that of the object to be imaged. The maximum measurable Young’s modulus is determined by the maximum stress measured by the stress sensor and the minimum strain of the object to be imaged, and vice versa for the minimum measurable Young’s modulus. For a given stress sensor and PAE system, the maximum measurable Young’s modulus $E_{\text{max}}$ is

$$E_{\text{max}} = \frac{\sigma_{\text{max}}}{\varepsilon_{\text{min}}} = \frac{\sigma_{\text{max}}}{d_{\text{min}}/l}$$  \hspace{1cm} (2.10)

Here, $d_{\text{min}}$ represents the minimum detectable displacement in PAE, which is 18.3 μm in our system. The original object thickness $l$, is around 6 mm in the above experiments. The maximum measurable stress $\sigma_{\text{max}}$ is theoretically limited to the stress at which the sensor breaks down. In practice, if the stress sensor works in the linear stress–strain response range (strain < 0.1), the maximum measurable stress for the sensor would be 3 kPa, resulting in a maximum measurable
Young’s modulus of 983 kPa. If we reach a strain of 0.4 for the stress sensor, the maximum measurable Young’s modulus would be 28.3 MPa. The minimum measurable Young’s modulus $E_{\text{min}}$ can be calculated by

$$E_{\text{min}} = \frac{\sigma_{\text{min}}}{\varepsilon_{\text{max}}} = \frac{d_{\text{min}}/l_{\text{ss}}}{\varepsilon_{\text{max}}}.$$  \hspace{1cm} (2.11)

Here, $l_{\text{ss}}$ represents the thickness of stress sensor and $\varepsilon_{\text{max}}$ is the maximum strain of the object. For valid Young’s modulus calculation, the stress–strain response of the object needs to stay in the linear range, thus the maximum strain of the object should be 0.1. For a 2-mm-thick stress sensor in our QPAE system, the minimum measurable Young’s modulus would be 3.2 kPa.

To the best our knowledge, this is the first quantitative imaging of absolute elasticity in biological tissue by PAT. QPAE achieves mapping of the absolute Young’s modulus in vivo up to 6 mm deep, which is in the optical diffusive regime, and thus enables longitudinal imaging of tissue elasticity. QPAE can be exploited for potential clinical applications, especially for long term measurement of tissue elasticity such as monitoring softening of the cervix during pregnancy.
Chapter 3 Advanced Photoacoustic Microscopy Techniques

In this chapter, I introduce the development of advanced PAM techniques. In the first section, I report NIR-OR-PAM. By utilizing NIR light for photoacoustic excitation, we have achieved greater penetration depth and finer lateral resolution in the deeper regions. Then, in the second section, I demonstrated a high-speed functional OR-PAM (HF-OR-PAM) system for epilepsy imaging in mouse brain in vivo with high spatial-temporal resolution. Last, I developed a single-cell metabolic PAM (SCM-PAM) capable of label-free high-throughput single-cell oxygen consumption rate measurements. By improving the current single-cell metabolism measurement throughput limit by two orders of magnitude, from ~30 cells over 15 minutes to ~3000 cells over 15 minutes, SCM-PAM enables imaging and quantification of intratumoral metabolic heterogeneity with single-cell resolution.

3.1 Near-infrared Optical-resolution Photoacoustic Microscopy

3.1.1 Background

With 100% sensitivity to optical absorption, OR-PAM can provide both anatomical and functional information with high spatial resolution\textsuperscript{71–73}. In OR-PAM, the high lateral resolution is achieved by tightly focusing the laser beam into a diffraction-limited spot\textsuperscript{20}. In a highly scattering medium such as biological tissue, the focusing capability of OR-PAM degrades with the imaging depth\textsuperscript{74}. In previous OR-PAM studies, ultraviolet (UV) or visible (VIS) light was used for illumination, mainly because of the strong absorption of DNA/RNA, cytochromes, hemoglobin, and melanin in
these wavelength regions\textsuperscript{75,76}. However, the strong optical scattering limits the penetration depth of OR-PAM in biological tissue.

To improve the imaging depth, we propose NIR-OR-PAM. Compared with visible illumination, NIR illumination yields several benefits. First, NIR-OR-PAM can penetrate deeper in tissue for a given SNR, primarily due to weaker absorption of NIR light by blood\textsuperscript{77}. Second, with weaker scattering of NIR light, NIR-OR-PAM can maintain its lateral resolution at greater depths. Third, NIR-OR-PAM can image other tissue components, such as water and lipid\textsuperscript{78,79}. The optical absorption spectra of four tissue components from 250 nm to 1150 nm are shown in Figure 3.1a\textsuperscript{80,81}. Fourth, the American National Standard Institute (ANSI) permits stronger NIR light intensity on the tissue surface\textsuperscript{82}. Here, we applied NIR light in OR-PAM and demonstrated these advantages.

\section*{3.1.2 Methods}

In the NIR-OR-PAM system (Fig. 3.1b), an Nd:YLF laser (INNOSAB, Edgewave, GmbH) generates laser pulses at its fundamental wavelength of 1046 nm. The laser pulses then pass through a second harmonic generator to generate 523 nm laser pulses. After the second harmonic generator, 523 nm and residual 1046 nm laser beams are split by a dichroic mirror. A dye laser (CBR-D, Sirah, GmbH) is pumped by the 523 nm beam and emits laser pulses with tunable wavelengths. We selected 570 nm for photoacoustic imaging, referred as VIS-OR-PAM, to compare with NIR-OR-PAM. A flip mirror is used to select either the 570 nm or the 1046 nm laser beam. After this, the laser beam is reshaped by an iris (ID25SS, Thorlabs) and then attenuated by a neutral density filter (NDC-50C-2M, Thorlabs). A condenser lens (LA1131, Thorlabs) and a 50 \textmu m diameter pinhole (P50C, Thorlabs) spatially filter the beam further. The laser beam is then
focused into the sample by an objective (AC127-050-A, Thorlabs; numerical aperture: 0.1 in air). A high-frequency ultrasonic transducer is placed confocally with the objective to detect the generated photoacoustic waves. The acoustic-optical coaxial alignment is achieved by using a beam combiner composed of a thin layer of silicone oil sandwiched between a right-angle prism (NT32-545, Edmund Optics) and a rhomboid prism (NT49-419, Edmund Optics). Each laser pulse generates a one-dimensional photoacoustic image (A-line) by recording the time course of the photoacoustic signal. Volumetric imaging is provided by two-dimensional motor scanning.

3.1.3 Results

Penetration depth of NIR-OR-PAM

To measure the penetration depth of the NIR-OR-PAM, we imaged a 50 μm diameter black human hair obliquely inserted into a piece of fresh chicken breast tissue. To conform to the ANSI safety limit, the light fluences on the tissue surface at 570 nm and 1046 nm were adjusted to 20 mJ/cm² and 100 mJ/cm², respectively. NIR-OR-PAM was able to image the black hair 3.2 mm beneath the skin surface with an SNR of 6 dB compared to only 2.3 mm by VIS-OR-PAM with the same SNR (Fig. 3.2a and b). NIR-OR-PAM can penetrate deeper than VIS-OR-PAM: First, NIR light undergoes weaker optical attenuation than VIS light, which allows it to penetrate deeper; Second, ANSI permits stronger NIR light fluence on tissue surface, which also gives us larger penetration depth. The same time-gain compensation (TGC, 0.75 dB/MHz/cm) for acoustic attenuation was applied to improve the visibility in both cases.
Figure 3.1. Absorption spectra and the schematic of NIR-OR-PAM. (a) Absorption spectra of oxy-hemoglobin (HbO2) (150 g/L or 2.3 mM in blood), deoxy-hemoglobin (HbR) (150 g/L or 2.3 mM in blood), water (80% by volume in tissue), and lipid (20% by volume in tissue) from 250 nm to 1150 nm. (b) Schematic of the NIR-OR-PAM system. ConL, condenser lens; CorL, correction lens; DC, dye cell; DM, dichroic mirror; FM, flip mirror; M1, M2, and M3, mirrors; ND, neutral density filter; OL, objective lens; PH, pinhole; PL, pump laser; RAP, right-angle prism; RhP, rhomboid prism; SHG, second harmonic generator; SOL, silicone oil layer; UT, ultrasonic transducer; WT, water tank.
Figure 3.2. Penetration depths of the two OR-PAM systems. VIS-OR-PAM (a) and NIR-OR-PAM (b) images of a black human hair inserted obliquely into a piece of fresh chicken breast tissue. The hair was imaged with an SNR of $\geq 6$ dB up to 3.2 mm deep in the tissue by NIR-OR-PAM and 2.3 mm deep in the tissue by VIS-OR-PAM.

Figure 3.3. Lateral resolutions of the two OR-PAM systems. (a-b) Line spread functions (black dotted lines) extracted from the fitted edge spread functions (solid red lines) of VIS-OR-PAM (a) and NIR-OR-PAM (b) acquired in water. Here, the lateral resolution was quantified as the FWHM of the line spread functions. The lateral resolution of NIR-OR-PAM in water is 6.2 $\mu$m, while the lateral resolution of VIS-OR-PAM is 2.9 $\mu$m. (c) Lateral resolution of NIR-OR-PAM and VIS-OR-PAM as a function of imaging depth in scattering media. Here, the lateral resolution was quantified as the FWHM of the numerical first-derivative of the measured edge spread function.
Lateral resolution of NIR-OR-PAM

To measure the lateral resolution of the NIR-OR-PAM system in clear media, a sharp metal edge was imaged with a step size of 0.625 μm and a scanning range of 100 μm along the x-axis. By fitting the measured data to an edge spread function, the full width at half maximum (FWHM) of the corresponding line spread function was estimated as the lateral resolution. The lateral resolution of NIR-OR-PAM in a clear medium was estimated to be 6.2 μm (Fig. 3.3b), and that of VIS-OR-PAM was estimated to be 2.9 μm (Fig. 3.3a), both of which are close to the theoretical lateral resolutions of OR-PAM, which are determined by the focal diameters of the laser beams. The axial resolution of NIR-OR-PAM was estimated to be ~27 μm, based on the transducer bandwidth and the speed of sound in tissue. In scattering media such as biological tissue, both the lateral and axial resolutions decrease with imaging depth due to optical scattering and frequency-dependent acoustic attenuation in tissue.

The lateral resolutions of NIR-OR-PAM and VIS-OR-PAM at different imaging depths were measured in scattering media. A sharp metal edge obliquely inserted into a piece of fresh chicken breast tissue was imaged at different depths while the optical focus was maintained on the metal edge. As optical focusing deteriorated in tissue, the line spread functions were no longer Gaussian functions, thus the lateral resolution could no longer be estimated by fitting the edge spread functions using error functions, as we did for the clear medium. Therefore, we estimated the lateral resolution as the FWHM of the numerical first-derivative of the measured edge spread function. Figure 3.3c shows the lateral resolutions of both NIR-OR-PAM and VIS-OR-PAM as functions of imaging depth. Due to optical scattering in the chicken tissue, the optical focusing degrades with depth, deteriorating the lateral resolution of OR-PAM. At 1046 nm, scattering in chicken

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breast tissue is weaker than that at 570 nm. With weaker optical scattering, NIR light can focus better than VIS light after traveling through a certain depth, which results in finer lateral resolution in NIR-OR-PAM over VIS-OR-PAM. For VIS-OR-PAM, the lateral resolution is finer than 40 μm up to ~1.6 mm depth, while for NIR-OR-PAM the lateral resolution is finer than 40 μm up to ~2 mm depth. Close to the tissue surface (<0.6 mm), NIR-OR-PAM has a worse lateral resolution than VIS-OR-PAM due to the longer wavelength; at ~0.6 mm depth, NIR-OR-PAM and VIS-OR-PAM show similar resolutions. As the imaging depth goes beyond 0.6 mm, NIR-OR-PAM has finer lateral resolutions than VIS-OR-PAM, until the optical focusing becomes so ineffective that OR-PAM turns into acoustic-resolution PAM, where the acoustic focusing of the 50 MHz ultrasonic transducer (~43 μm) determines the lateral resolution.

**NIR-OR-PAM of a mouse ear in vivo**

A mouse ear was imaged in vivo with NIR-OR-PAM. All experimental animal procedures were carried out in conformity with the laboratory animal protocol approved by the Animal Studies Committee at Washington University in St. Louis. A 2 × 2 mm² area of the mouse ear was imaged by both VIS-OR-PAM and NIR-OR-PAM (Fig. 3.4a and b). VIS-OR-PAM is able to image only the blood vessels (Fig. 3.4a). NIR-OR-PAM, however, is able to image more contrasts (Fig. 3.4b). In addition to blood vessels, the circular structures imaged by NIR-OR-PAM are suspected to be fat cells surrounding sebaceous glands. Close-up images and B-scan images (Fig. 3.4c and d) further demonstrate the improvement of NIR-OR-PAM in lipid contrast and imaging depth in vivo.

**NIR-OR-PAM of a mouse brain in vivo**
We also imaged a mouse brain \textit{in vivo} with NIR-OR-PAM. The same area of the mouse brain was imaged by VIS-OR-PAM and NIR-OR-PAM. The scalp was removed but the skull was intact. Due to the strong blood absorption and brain tissue scattering, few blood vessels deeper than 0.5 mm can be imaged by VIS-OR-PAM (Fig. 3.5a and b). However, NIR-OR-PAM can detect signals from blood vessels deeper than 0.5 mm (Fig. 3.5c and d).

![Figure 3.4](image)

**Figure 3.4.** Comparison of mouse ear images by the two OR-PAM systems. (a) A maximum amplitude projection (MAP) image of a mouse ear acquired by VIS-OR-PAM \textit{in vivo} within depths up to 300 μm. Two close-ups show the PA signals from blood vessels, as indicated by the dashed boxes. (b) An MAP image of the same mouse ear acquired by NIR-OR-PAM \textit{in vivo} within depths up to 300 μm. (c-d) B-scan images along the dashed lines in (a) and (b), respectively, on a logarithmic scale after acoustic attenuation compensation with an acoustic attenuation coefficient of 1 dB/MHz/cm.

NIR-OR-PAM can not only penetrate deeper than VIS-OR-PAM but also image structures in deep regions with finer resolution. As shown in Figure 3.6a and b, at both illumination wavelengths, OR-PAM was able to clearly image the cortical vascular network. However, close-ups of the vessels more than 200 μm deep in figure 3.6a and b show that the vessels are less blurred in the NIR-OR-PAM images than in the VIS-OR-PAM images, as shown in figure 3.6c. The lateral resolution improvement for \textit{in vivo} imaging by NIR-OR-PAM was estimated by comparing the
apparent diameters of vessels at the same depths. The apparent diameters of two representative vessels were 120 μm and 160 μm in VIS-OR-PAM, while they were 50 μm and 80 μm in NIR-OR-PAM, respectively (Fig. 3.6d). The apparent diameters of blood vessels were ~2 times smaller in NIR-OR-PAM.

**Figure 3.5.** Comparison of *in vivo* imaging depths in a mouse brain. (a) x-z projection of a VIS-OR-PAM image of the mouse brain acquired *in vivo*. (b) x-y projections of the mouse brain at different depths. No structure can be detected at depths beyond 500 μm. (c) x-z projection of a NIR-OR-PAM image of the same mouse brain acquired *in vivo*. (d) x-y projections of the mouse brain at different depths. Structures can be detected at depths up to 750 μm. The dashed lines in the x-z projections mark the approximate location of the skull.

Due to strong absorption, an overlying blood vessel may shadow vessels underneath in VIS-OR-PAM. At 1046 nm, the optical absorption of hemoglobin is about 40 times less than that at 570 nm (Fig. 3.1a). Therefore, 1046 nm NIR light has a 1/e penetration depth of 1.2 mm in blood, and is
able to image multiple layers of blood vessels. This advantage was demonstrated by imaging a bleeding area in a mouse brain. The bleeding layer appears weaker in NIR-OR-PAM than in VIS-OR-PAM (Fig. 3.7a and b). By peeling off the bleeding layer in data processing, we can clearly see that the optical shadowing from the bleeding layer is weaker in NIR-OR-PAM, and the vascular network underneath can thus be better resolved with greater SNR. However, due to the strong absorption, nearly all of the VIS light is absorbed by the blood pool and generated strong photoacoustic signals that were reverberated by other underlying brain structures, showing as a ‘ghost’ image of the blood pool.

Figure 3.6. Comparison of in vivo lateral resolutions in the mouse brain. (a-b) In vivo mouse brain MAP images acquired by VIS-OR-PAM (a) and NIR-OR-PAM (b), respectively. (c) Close-ups of the dashed boxes in (a) and (b) show that more blood vessels can be imaged by NIR-OR-PAM. (d) Normalized PA amplitudes across two selected blood vessels at 225 μm (solid lines) and 330 μm (dashed lines) depths in (c), respectively. Both vessels have smaller apparent diameters in the NIR-OR-PAM image than in the VIS-OR-PAM image, because they are less blurred.
Figure 3.7. Comparison of penetrations in blood. (a) Top: a VIS-OR-PAM MAP image of a mouse brain acquired in vivo within depths up to 300 μm, in which blood vessels were covered by a bleeding layer. Bottom: a VIS-OR-PAM MAP image of the same area in the mouse brain acquired in vivo within depths from 100 μm to 300 μm after digitally peeling off the bleeding layer. The underneath blood vessels can barely be resolved. (b) Top: an NIR-OR-PAM MAP image of the same region as (a) within depths up to 300 μm. The signal strength from the bleeding layer is weaker than that from the VIS-OR-PAM counterpart. Bottom: an NIR-OR-PAM MAP image of the same area in the mouse brain acquired in vivo within depths from 100 μm to 300 μm after digitally peeling off the bleeding layer. The underneath blood vessels can be clearly resolved.

3.1.4 Discussion

It is worth pointing out that lower optical absorption in the NIR region allows deeper penetration but results in weaker photoacoustic signals. However, with nanosecond pulse excitation, the tissue
damage mechanism is mainly photothermal\textsuperscript{86}. Therefore, lower absorption allows for stronger excitation fluence, which can partially compensate for the reduced signal strength.

### 3.2 High-speed Functional Photoacoustic Microscopy

#### 3.2.1 Background

Epilepsy is a neurological disorder characterized by recurring seizures that affects more than 50 million people worldwide\textsuperscript{87}. Imaging and characterizing the neurovascular coupling during epileptic seizure can help understand the disease\textsuperscript{88,89}. To study the highly localized correlation between the metabolic demands of neural activities and the cerebral hemodynamics, capillary-level spatial resolution is needed\textsuperscript{90}. To investigate the propagation of epileptic seizures through the cerebral hemodynamic responses, high temporal resolution is also needed.

Modern functional brain imaging has enabled better understanding of epilepsy and contributed to its diagnosis and treatment\textsuperscript{91–95}. However, functional magnetic resonance imaging (fMRI), the major clinical functional brain imaging method, lacks the required spatial and temporal resolutions for documenting complex paroxysmal brain activities during epileptic seizures\textsuperscript{96}. Functional ultrasound (fUS) imaging has imaged the cross-sectional propagation of epileptic seizure with greatly improved temporal resolution at adequate depths, but still lacks the required spatial resolution for studying capillary-level hemodynamics\textsuperscript{97}. Optical imaging methods have also played a major role in understanding epilepsy. Voltage-sensitive dye (VSD) imaging provides information of neural behaviors during epileptic seizure with sufficient temporal resolution but suffers from tissue toxicity\textsuperscript{98,99}. Optical coherence tomography (OCT) has reported a significant decrease in the backscattering of light in the mouse brain during seizure progression but is insensitive to changes in blood perfusion\textsuperscript{100}. 

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PAT has gained great attention for biomedical imaging by providing structural, functional, molecular, mechanical, and metabolic information\textsuperscript{8,9,101,102}. PACT was previously applied for imaging epileptic seizure in the mouse brain, but the spatial resolution was insufficient to resolve capillary-level hemodynamic changes\textsuperscript{103,104}. OR-PAM, a major microscopic implementation of PAT, is well suited for cerebral hemodynamic imaging because of its excellent contrast between blood vessels and background tissues\textsuperscript{105}. Given the deficiencies of the current brain imaging modalities, we report an HF-OR-PAM system capable of imaging epileptic seizure propagation in the mouse brain with sufficiently high spatial and temporal resolutions. This HF-OR-PAM system, with high speed (~0.5 Hz volumetric frame rate) and high spatial resolution (~3 μm lateral resolution), was able to image the spread of microvascular hemodynamic responses to the propagation of epileptic seizure. We observed increased blood perfusion along the propagation path of the seizure, reflected by increases in total hemoglobin concentration and blood vessel diameters.

3.2.2 Methods

Animal preparation

Female ND4 Swiss Webster mice (n = 3) between 16 to 20 g and 4 to 6 weeks old (Harlan Laboratory, Inc) were used for this study. All experimental animal procedures were carried out in conformity with the laboratory animal protocols approved by the Animal Studies Committee at Washington University in St. Louis. Before the experiment, each mouse was first treated with 1.5% vaporized isoflurane for scalp removal and 4-AP injection. Anesthetic depth was assessed by periodically checking the toe pinch withdrawal reflex. The mouse was mounted on a custom-built animal holder for HF-OR-PAM. The scalp of each mouse was removed. For epilepsy
induction, a 500 μm skull opening with the dura intact was created by using a dental drill (Fine Science Tools) about 2 mm posterior and 1.5 mm lateral to the Bregma point. A dosage of 10 μL 4-aminopyridine (4-AP; Sigma-Aldrich) solution at a concentration of 1.5 mM was injected through a 150-μm-diameter cannula, which was inserted 0.2 to 0.3 mm below the dura mater surface. Note that the 4-AP solution used in our study had a ten times lower concentration but a 10 times larger volume than that used in the previous study. After the 4-AP injection, each mouse was carefully treated with an intraperitoneal dose of 400 mg/kg chloral hydrate for a smooth transition from isoflurane to chloral hydrate, an anesthetic selected for its relatively mild effect on cerebral hemodynamics. The animal’s temperature was kept at 37 ºC by a heating pad during the entire experiment.

**Electroencephalogram recording**

To monitor the neural activity that induced the hemodynamic changes during epileptic seizures, we applied a single-channel electroencephalogram (EEG) recording through an electrode attached on top of the skull. A reference electrode was mounted on the tooth bar that was in direct contact with the nasal bone. The EEG signals were amplified by an AC/DC differential amplifier (A-M Systems, Model 3000), lowpass filtered with a cut-off frequency of 200 Hz, and concurrently sampled with the photoacoustic signals.

**Propagation map construction**

X-y projected photoacoustic images were formed by taking the MAP along the depth direction. Photoacoustic images were then analyzed by comparing the images with the baseline image and taking the fractional change. The propagation map at the $i^{th}$ frame ($i \geq 2$) was calculated by
\[
\Delta A_i = \frac{\left(\frac{A_{i-1} + A_i + A_{i+1}}{3} - \frac{A_1 + A_2 + A_3}{3}\right)}{\left(\frac{A_1 + A_2 + A_3}{3}\right)}.
\]

(3.1)

Here \(A_i\) is the \(i^{th}\) frame. The propagation map was then superimposed onto the corresponding structural image to show the spatio–temporal propagation of epileptic seizure. Here, because three consecutive frames were used in computing the activation map, the effective temporal sampling rate of the activation map was about 0.17 Hz, which was still sufficiently high to monitor the seizure propagation.

**Blood vessel segmentation and vessel diameter quantification**

To distinguish blood vessels from the background, photoacoustic amplitudes were first extracted by taking the Hilbert transform of the raw photoacoustic signals. The threshold for distinguishing blood vessels from background was set to be 3 dB above the noise level. The photoacoustic images were then converted into a binary image based on the threshold, and the blood vessels were segmented automatically\(^3\). The cross sections of the blood vessels were then identified, and the transverse pathlengths across the vessels were calculated at different angles. The shortest transverse pathlength was used as the vessel diameter.

**3.2.3 Results**

**HF-OR-PAM of mouse brain vasculature**

To image mouse brain vasculature, we updated an OR-PAM system enhanced by a water-immersible microelectromechanical systems (MEMS) scanning mirror\(^1\). HF-OR-PAM is capable of simultaneously providing high spatial and temporal resolutions. The schematic of HF-OR-PAM is shown in Figure 3.8a. The system employs a 3-ps pulsed laser (APL-4000-1064, RPMC Lasers,
Inc.) emitting at 532 nm wavelength with a 200 kHz pulse repetition rate. The laser beam is first attenuated by a neutral density filter. After being spatially filtered by a condenser lens (LA1131, Thorlabs, Inc.) and a 50-μm-diameter pinhole (P50C, Thorlabs, Inc.), the laser beam is focused by an optical objective lens (AC127-050-A, Thorlabs, Inc.; NA: 0.1 in air). The laser beam is then directed to the sample by a beam combiner and a MEMS scanning mirror. The fluence on the tissue surface was 5 mJ/cm², well within the safety limit (20 mJ/cm²) set by the American National Standards Institute (ANSI). The beam combiner is composed of an aluminum-coated prism (NT32-331, Edmund, Inc.) and an uncoated prism (NT32-330, Edmund, Inc.), and provides acoustic-optical coaxial alignment. The two prims are glued together. Here, the thin aluminum coating reflects light but transmits sound. The loss of the acoustic signal crossing the interface of the two glass prisms is approximately 25%, which depends on the acoustic impedance mismatch at the interface and the thickness of the glue layer. An optical correction lens is attached to the top surface of the combiner to correct aberration due to the prism. An acoustic lens with an NA of 0.25 (NT45-010, Edmund, Inc.) is attached to the right surface of the combiner. The generated photoacoustic signal is reflected by the MEMS scanning mirror and detected by an ultrasonic transducer (V214-BB-RM, Olympus-NDT, Inc.; 50 MHz central frequency; 100% one-way bandwidth), which is placed confocally with the objective lens. Both the excitation laser beam and generated photoacoustic beam are scanned confocally by the MEMS scanning mirror, providing high detection sensitivity within a large field of view (FoV).
Figure 3.8. HF-OR-PAM of mouse brain vasculature. (a) Schematic of HF-OR-PAM with a fast-scanning water-immersible MEMS mirror. AC, aluminum coating; AL, acoustic lens; CorL, correction lens; ConL, condenser lens; FSM, fast scanning MEMS mirror; ND, neutral density filter; OL, objective lens; UT, ultrasonic transducer; WT, water tank. (b) Scanning scheme of HF-OR-PAM. 3D imaging is achieved by fast MEMS mirror scanning along the x axis and slow motor-stage scanning along the y axis. (c) A representative x-y projected brain vasculature image acquired by MEM-OR-PAM through an intact skull. (d) The depth-enhanced x-z projected brain vasculature image of the red dashed box in (c). The location of the skull is indicted by the yellow dashed lines.

The scanning scheme of HF-OR-PAM for 3D imaging is shown in Figure 3.9b. The MEMS mirror is driven by electromagnetic force and submerged in water for ultrasound coupling. A one-dimensional photoacoustic image (A-line) is formed by recording the time course of the photoacoustic signal generated by one single laser pulse. A two-dimensional photoacoustic image (B-scan) is formed by scanning the laser pulses along the x-axis with the MEMS mirror at 400 Hz. A three-dimensional photoacoustic image (C-scan) is formed by stacking the B-scans acquired...
with slow linear step-motor scanning of the object along the y-axis. Bi-hemispheric and uni-
hemispheric imaging were performed to provide comprehensive epileptic seizure propagation patterns (Fig. 3.9).

Figure 3.9. Photographs of mouse brains with skull intact showing the scanning protocols for bi-hemispheric (black-dashed box) (a) and uni-hemispheric imaging (red-dashed box) (b) of epileptic seizure in the mouse brain, where the fast MEMS mirror scanning directions are marked by the arrows. (c) The position of the EEG recording electrode and the imaging FoV (shown by the blue dashed box).

A 4 × 3 mm² area in the mouse cortex was imaged by HF-OR-PAM with a volumetric frame rate of 0.5 Hz (Fig. 3.8c and d). The axial resolution of HF-OR-PAM was ∼27 μm, estimated from the transducer bandwidth (50 MHz) and the speed of sound in tissue (1540 m/s). Photoacoustic signals acquired at 532 nm, which is close to an isosbestic point of oxyhemoglobin and deoxyhemoglobin, reflected the relative total hemoglobin concentration.

**HF-OR-PAM of epileptic seizure propagation in mouse brain**

We performed photoacoustic imaging in a mouse epilepsy model (n = 3). We induced acute ictogenesis by focal injection of a potassium channel blocker (4-aminopyridine, 4-AP) at 0.2 mm depth in the mouse cortex, and then performed PAM imaging and electroencephalography (EEG)
recordings simultaneously (Fig. 3.10a). We imaged the dynamics of total hemoglobin concentration in response to epileptic seizures (Figs. 3.11 and 3.12). The total hemoglobin concentration increased along the propagation path of epileptic seizure. The increase in the total hemoglobin concentration started first at the injection site and then propagated bilaterally to adjacent areas.

**Figure 3.10.** HF-OR-PAM of epileptic seizure in a mouse brain. (a) Schematic setup for epilepsy imaging by HF-OR-PAM. Red-dashed box shows the imaging area in the mouse cortex. 4-AP was injected locally in the cortex, and an electrode was attached to the skull surface for EEG recording. (b) Comparison of the EEG signals (blue line) and the PA signals (red line) recorded simultaneously, showing the correlation between neural activities and hemodynamic changes. (c) Baseline structural PA image showing the brain vasculature. (d) Fractional changes in PA amplitude during epileptic seizure. (e) Superposition of the fractional change image (d) on the structural PA image (c) mapping the wave propagation.

We observed different propagation patterns, including waves of photoacoustic signal changes along one or both daughter branches of blood vessels, local activations, and waves spreading bilaterally in both hemispheres (Figs. 3.11 and 3.12). In one representative experiment, during 12
minutes after 4-AP injection, five groups of waves were observed, with an average duration of 69.6 ± 34.5 seconds. The duration was defined as the time interval during which PA signal increase was 3 dB above the noise level. Two groups of waves propagated along both daughter vessels of a mother vessel until they exited the FoV. Two other waves propagated along only one daughter vessel of the mother vessel. Another wave quickly diminished after entering the FoV. The propagation delay maps were computed using the same method by Macé et al. for representative ictal events to show the propagation path and time course (Figs. 3.11d, 3.11h, and 3.12f).

Figure 3.11. HF-OR-PAM of epileptic seizure propagation in one hemisphere. (a-c) Snapshots of representative wave of PA signal changes propagating along both daughter branches of a blood vessel. (d) Propagation delay map of the waves shown in (a-c), where the propagation delay in seconds is color encoded and the propagation direction is indicated by arrows. (e-g) Snapshots of representative wave of PA signal changes propagating along only one daughter branch of the same blood vessel. (h) Propagation delay map of the wave shown in (e-g). The timestamps are correlated with the EEG signal shown in Figure 3.10b.

To detect the neural activities that induced the changes in total hemoglobin concentration observed by HF-OR-PAM, we performed simultaneous EEG signal recording. The PA signal increase was highly correlated with the EEG signal showing the occurrences of epileptic seizures (Fig. 3.10b). The correlation coefficient was 0.95 between the PA signal change and the envelope of the EEG
signal. We also found that the seizure durations measured by PAM and EEG were highly correlated, with a correlation coefficient of 0.89.

**Figure 3.12.** HF-OR-PAM of epileptic seizure propagation in bi-hemispheres. (a-f) One representative wave of high fractional PA signal changes propagated bilaterally in both hemispheres. The timestamps are correlated with the EEG signal shown in (g). (h) Propagation delay map of the wave in (a-f). Propagation delay in seconds is color encoded. Arrows represent the wave propagation direction.

We then investigated the propagation speed of epileptic seizures by measuring the arrival time of the wave of PA signal changes traveling from a reference location (Fig. 3.13a-c). By using a set of 30 spatial points along the blood vessel, we found the epileptic seizure propagation speed was 3.42 mm/min on average (n = 3 mice, 21 blood vessels, 90 total seizures) (Fig. 3.13d), similar to that reported in previous studies.
Figure 3.13. HF-OR-PAM of epileptic seizure propagation speed. (a) Fractional changes in PA amplitudes are plotted for two different pixels chosen along a blood vessel in the representative ictal seizure event shown in Figure 3.11. (b) Cross-correlation function between the two signals. The propagation delay is given by the peak position of the cross-correlation function. (c) Measurement of epileptic seizure propagation speed. A set of 30 points are selected along the blood vessel. The delay time is linear with the distance from a reference point, and the data is fitted by a linear function. The slope of the linear function provides the epileptic seizure propagation speed. (d) Histogram of 90 measurements of epileptic seizure propagation speed.

**HF-OR-PAM of blood vessel diameter change in response to epileptic seizure**

Vessel diameter changes play a key role in regulating cerebral blood flow for neurovascular coupling during transient brain activities like epileptic seizure. HF-OR-PAM was applied to measure the blood vessel diameter change in response to epileptic seizure (n = 3). After the injection of 4-AP, HF-OR-PAM was used to image the mouse cortex vasculature with a frame rate of 0.5 Hz (Fig. 3.14a). We applied a customized vessel segmentation algorithm to distinguish the blood vessels from the background. Because the transverse resolution of HF-OR-PAM (~3 μm) is
finer than the axial resolution (~27 μm), the vessel diameter was calculated along the transverse direction.

Figure 3.14. HF-OR-PAM of blood vessel dilation in response to epileptic seizures. (a) A representative HF-OR-PAM image of the mouse brain vasculature. (b) The diameter changes of a representative vessel highlighted by the red-dashed box in (a). Green dashed lines indicate the resting-state blood vessel boundaries at 10 s. The timestamps are correlated with the EEG signal shown in (c). (c) Upper panel: time course of the vessel diameters indicated by the white box in (b) during epileptic seizures. Lower panel: EEG signals acquired simultaneously. (d) Fractional change in vessel diameter as a function of distance from the injection site. (n = 3 mice, 35 blood vessels, 117 total seizures). Blue line: running average over 3 sampling points.

HF-OR-PAM monitored the blood vessel diameter changes in response to epileptic seizures (Fig. 3.14b). The change in blood vessel diameters correlated well with the envelope of the EEG signals (Fig. 3.14c), with a correlation coefficient of 0.91. The distances of the responding vessels relative to the injection site were measured from the structural image. We found that the magnitudes of blood vessel diameter changes decreased with increasing distance from the 4-AP injection site (n
= 3 mice, 35 blood vessels, 117 total seizures) (Figs. 3.14d and 3.15). Within a 1 mm radius of the injection site, blood vessel diameters increased by an average of $67.1 \pm 15.8\%$ during epileptic seizures.

![Fractional changes of vessel diameter in response to epileptic seizures](image)

**Figure 3.15.** Fractional changes of vessel diameter in response to epileptic seizures. Histogram of 117 measurements of fractional changes of vessel diameter.

### 3.2.4 Discussion

In this study, a 4-AP induced epilepsy model was used to demonstrate the feasibility of HF-OR-PAM of hemodynamic responses to epileptic seizure propagation with high spatial and temporal resolution. The 4-AP model is commonly used as an acute model of epileptic ictal seizure\textsuperscript{109}. We believe HF-OR-PAM of 4-AP induced epilepsy will be of great interest to the epilepsy research community.
Although the hemodynamic response imaged by HF-OR-PAM was highly correlated and localized with the neural activities, it only provided information about the metabolic need during epileptic seizures. Further development of PAM based on direct sensing of neural activity using membrane potential or calcium sensitive contrast agents would provide new insights into the disease. In addition, by applying multi-wavelength measurements in HF-OR-PAM, we can quantify oxygen saturation of hemoglobin (sO$_2$) and target specific biomarkers$^{19,21}$. HF-OR-PAM can measure blood flow by using photoacoustic Doppler effect or cross-correlation methods$^{49,110}$. With sO$_2$ and blood flow information, HF-OR-PAM can further quantify the cerebral metabolic rate of oxygen (CMRO$_2$) during brain activities$^{111}$. HF-OR-PAM can also be used to study vascular compliance and the functional connectivity in the mouse brain during epileptic seizures$^{53,112}$. With high detection sensitivity, a large FoV, high spatio–temporal resolution, and functional imaging capabilities, HF-OR-PAM has the potential to become a powerful tool for epilepsy studies.

### 3.3 Single-cell Metabolic Photoacoustic Microscopy

#### 3.3.1 Background

Tumors consist of heterogeneous populations of cancer cells that have distinct genetic and phenotypic profiles. The heterogeneity within a tumor, namely intratumoral heterogeneity, has become the greatest challenge to effective cancer therapy$^{113}$. It exists among almost all the hallmarks of cancer, including acquired cell motility that leads to metastasis, elevated angiogenic potential that leads to angiogenesis, and altered cellular metabolism$^{114}$. Tumor cells are well known to have significantly altered metabolic profiles compared to normal cells, and many treatment methodologies target the cellular metabolism of tumor$^{115}$. Assessing intratumoral metabolic heterogeneity would greatly contribute to the understanding of tumor growth, invasion, and drug
resistance. It will also help design effective and personalized treatment strategies\textsuperscript{116}. While advances in genome sequencing have revealed intratumoral metabolic heterogeneity on the genetic level\textsuperscript{117}, little is known about its phenotypic landscape.

With greatly improved biomedical imaging tools, the phenotypic landscape of intratumoral metabolic heterogeneity now can be studied directly on bulk populations of cells\textsuperscript{118}. However, the limitations of the current methods usually prevent us from understanding intratumoral metabolic heterogeneity on a single-cell level. Metabolic heterogeneity has been directly visualized by positron emission tomography (PET) \textit{in vivo} in humans\textsuperscript{119}. However, PET lacks the spatial resolution to study metabolic heterogeneity at the single-cell level. Fluorescence-based methods have also been used, but the required labelling may perturb the original microenvironments of tumor cells\textsuperscript{120}. Optical imaging techniques based on endogenous contrasts, such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), can assess metabolic states without labeling\textsuperscript{121}. However, these techniques can only quantify heterogeneous cell populations, they cannot provide metabolic information for single cells\textsuperscript{122}.

The oxygen consumption rate (OCR) of a cell is directly related to its metabolism\textsuperscript{123}. The distribution of the single-cell OCRs within a tumor would be an important indicator of its metabolic heterogeneity. To date, most OCR measurements have been performed on bulk populations of cells, and they provide little information on cell-to-cell metabolic heterogeneity. To measure single-cell OCR accurately, each cell must be sealed into a small oxygen-diffusion-limited environment, where the temporal change of oxygen content can be monitored. To create such an environment, microwell arrays are usually designed to trap a single cell in each microwell for OCR measurement\textsuperscript{124}. Previously, single-cell OCR measurements have been performed by
electrical and fluorescent methods, which require microscale oxygen sensors to monitor the oxygen content change\textsuperscript{125}. To perform single-cell OCR measurements on a large population of cells with these methods, a massive array of microscale oxygen sensors has to be embedded into the microwell array, making it extremely difficult to fabricate and use. Moreover, the embedded microscale oxygen sensors may adversely affect the normal metabolism of the cells, rendering the OCR measurement inaccurate. Limited by these issues, single-cell OCR measurement has been demonstrated with only ~30 cell capability\textsuperscript{126}. However, to obtain statistically sound data for intratumoral metabolic heterogeneity studies, single-cell OCRs of a large number of cells need to be measured without altering the normal metabolism of the cells. To better understand intratumoral metabolic heterogeneity and improve cancer therapy, new high-throughput methods are needed to provide label-free single-cell OCR measurements of a large population of cells.

With 100\% relative sensitivity to optical absorption (i.e., a given percentage change in the optical absorption coefficient yields the same percentage change in the photoacoustic amplitude), photoacoustic imaging can provide anatomical, functional, molecular, mechanical, and metabolic information about biological tissues\textsuperscript{8,13,21}. OR-PAM, the major microscopic implementation of photoacoustic imaging, achieves diffraction-limited lateral resolution, which is sufficient for single cell imaging\textsuperscript{20}. By employing two excitation wavelengths, OR-PAM can measure the oxygen saturation of hemoglobin (sO\textsubscript{2}) in blood\textsuperscript{127}. Further, by temporally monitoring sO\textsubscript{2}, photoacoustic imaging can detect changes in blood oxygen content and quantify oxygen metabolism without labeling\textsuperscript{111}. Here, we developed SCM-PAM by combining a high-density microwell array with wide-field fast-scanning functional OR-PAM. SCM-PAM provides label-free high-throughput single-cell OCR measurements for a large population of cells. Each target cell is trapped in a microwell that constitutes a small oxygen-diffusion-limited environment. By using hemoglobin as
both an oxygen supplier and sensor, SCM-PAM continuously monitors the sO$_2$ in each microwell to detect oxygen content changes and quantify the OCR of each target cell. With the capability of fast-scanning over a wide FoV, SCM-PAM can monitor thousands of microwells, thus achieving label-free high-throughput single-cell OCR measurements for a large population of cells.

3.3.2 Methods

SCM-PAM system

To achieve label-free high-throughput single-cell metabolic imaging, we combined a functional OR-PAM system with a high-density microwell array (Fig. 3.16). For sO$_2$ measurement, the system employs a solid-state laser at 532 nm (SPOT, Elforlight) and a dye laser (CBR-D, Sirah) at 559 nm. The combined laser beam is reshaped by a 2 mm aperture iris (ID25SS, Thorlabs) and attenuated by a neutral density filter (NDC-50C-2M, Thorlabs). A pair of condenser lenses (LA1131, Thorlabs) and a pinhole (P50C, Thorlabs; diameter: 50 μm) are used to spatially filter the laser beam. A beam sampler (BSF10-A, Thorlabs) and a photodiode are used to monitor the laser intensity fluctuation. The filtered laser beam is then coupled into a single-mode photonic crystal fiber (LMA-10, NKT Photonics). The output of the single-mode fiber is collimated by an objective lens (RMS4×, Thorlabs), reflected by a mirror, and focused on the object by another identical objective lens. A beam combiner, composed of a thin layer of silicone oil sandwiched by a rhomboid prism (NT49-419, Edmund Optics) and a right-angle prism (NT32-545, Edmund Optics), provides acoustic-optical coaxial alignment. The generated photoacoustic waves are detected by an ultrasonic transducer with a central frequency of 50 MHz (V214-BB-RM, Olympus-NDT) placed confocally with the objective lens.
Figure 3.16. System schematic and working modes of SCM-PAM. a, System schematic of SCM-PAM. b, High-resolution mode of SCM-PAM, with optical diffraction limited lateral resolution. c, High-throughput mode of SCM-PAM, with single-cell metabolism measurement throughput of ~3000 cells over 15 minutes.

Lateral resolution of SCM-PAM

The lateral resolution of the SCM-PAM system was measured by imaging a sharp metal edge with a scanning step size of 0.625 µm and a scanning range of 100 µm along the x axis (Fig. 3.17). The measured data were fitted to an edge spread function (ESF), and the line spread function (LSF) was calculated based on the fitted ESF. The full width at half maximum (FWHM) of the LSF was quantified as the lateral resolution. The experimentally measured lateral resolution of SCM-PAM was 2.71 µm, close to the theoretical value of 2.66 µm.

High-density microwell array

To achieve label-free high-throughput single-cell metabolic imaging, we designed and fabricated a high-density microwell array capable of trapping a single cell in each microwell. To ensure
trapping at most one cell, each microwell was designed to be 40 μm in diameter and 50 μm in depth, and the microwells were set 80 μm apart. SU-8 50 (NANO™) resist was used for fabrication of the microwell array. To enable effective loading of cells and blood without trapping air in the microwells, the array was fabricated on an anodisc inorganic filter membrane (Whatman®). To improve adhesion between the SU-8 substrate and the anodisc inorganic filter membrane, a 400 nm thick layer of aluminum was deposited between them.

![Figure 3.17. Lateral resolution of SCM-PAM. The measured data (blue circles) were fitted to deduce an edge spread function (ESF, red solid line). The line spread function (LSF, black dotted line) was calculated by differentiating the fitted ESF. The lateral resolution was quantified as the full width at half maximum (FWHM) of the LSF.](image)

The detailed fabrication process was as follows (Fig. 3.18). First, a 400 nm thick layer of aluminum was deposited on one side of a 60 μm thick anodisc inorganic filter membrane by an e-beam evaporator (Kurt J. Lesker®). Second, SU-8 50 was spin coated on the aluminum layer at 500 rpm for 10 seconds in the spread cycle and at 4000 rpm for 30 seconds in the spin cycle to generate a 50 μm thick layer of SU-8 substrate. The entire substrate was then soft baked in an oven at 90°C for 3 hours, after which the SU-8 was exposed to near ultraviolet light at 200 mJ/cm². After post-
exposure baking, microwells were developed in an SU-8 developer. The entire microwell array was then immersed in aluminum etchant, during which the aluminum layer beneath the microwells was etched away, while the remaining part of the aluminum layer was kept.

Figure 3.18. Fabrication and operation of the high-density microwell array. a-d, Fabrication process of the high-density microwell array. a, A 400 nm thick layer of aluminum (gray) was deposited on one side of a 60 μm thick Anodisc inorganic filter membrane (black and white). b, A 50 μm layer of SU-8 (blue) was coated on top of the aluminum layer. c, Microwells were developed in the SU-8 substrate. d, The aluminum layer beneath the microwells was etched away, while the remaining part of the aluminum layer was kept. e-h, Operation of the high-density microwell array. e, Single-cell suspension mixed with blood was spread on the surface of the microwell array. f, Blood-cell mixture settled in the microwells. The blood was able to enter the Anodisc while the target cells could only enter the microwells because of the size. g, The cells and blood outside the microwells were removed by flushing the microwell array with blood and fresh cell culture medium and drawing a small rubber squeegee across the flat surface. h, The microwell array was sealed by immersing it into silicone oil (yellow).
Cell culture

Three types of cells, the B16 mouse melanoma cell line, the RAW264.7 murine macrophage cell line, and the A549 human epithelial lung cancer cell line, were obtained from the Tissue Culture and Support Center at the Washington University School of Medicine. The B16 mouse melanoma cells and the RAW264.7 murine macrophage cells were cultured in Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO₂. The A549 human epithelial lung cancer cells were cultured in F-12K Medium at 37°C in 5% CO₂. At 75% to 80% confluence, cells were harvested with 0.25% trypsin-EDTA solution (Invitrogen) to generate a single-cell suspension at a concentration of 1×10⁶ cells/mL.

Cells from patient specimens

After informed consent was obtained, breast cancer and normal tissues were collected from women with newly diagnosed clinical stage I/II breast cancer undergoing breast-conserving surgery. The protocol was approved by the Institutional Review Board at Washington University in St. Louis. After excision, the breast tissue was transported in saline immediately from the operating room to the lab and dissociated to a single-cell suspension with gentleMACS Dissociator (Miltenyi Biotec) with minimal ischemia time.

Label-free high-throughput single-cell OCR measurement by SCM-PAM

To achieve label-free high-throughput single-cell OCR measurement, 0.1 mL of the single-cell suspension containing ~10,000 single cells was mixed with the same volume of fully oxygenated blood and loaded into the high-density microwell array. After waiting for the cells to settle into the microwells, the high-density microwell array was gently flushed with fully oxygenated blood
and fresh cell culture medium to remove cells outside the microwells and subsequently a small rubber squeegee was drawn across the flat surface. After gently immersing the microwell array in silicone oil for oxygen sealing, the entire assembly was transferred to SCM-PAM for OCR measurement. During the OCR measurement, a lab-made heating pad maintained the temperature.

Because the amount of oxygen dissolved in blood is negligible compared to its counterpart bound to hemoglobin, the sO$_2$ change in a microwell can be considered reflective of the oxygen content change in the microwell. The OCR of a single cell in a microwell can be calculated by:

$$\text{OCR} = \frac{\Delta O_2}{\Delta t}. \quad (3.2)$$

Here $\Delta O_2$ represents the amount of oxygen change in the microwell over a certain time period $\Delta t$.

The oxygen change in a microwell can be calculated by:

$$\Delta n = V_b \times \Delta C_{bO_2}. \quad (3.3)$$

Here $V_b$ is the volume of blood in the microwell, which is $4.9 \times 10^4$ fL, and $\Delta C_{bO_2}$ is the change of oxygen concentration in the blood, which is proportional to the sO$_2$ change $\Delta$SO$_2$:

$$\Delta C_{bO_2} = 4 \times \Delta sO_2 \times C_{\text{Hb}}. \quad (3.4)$$

Here $C_{\text{Hb}}$ is the concentration of hemoglobin in blood, and the factor 4 is the bonding ratio between oxygen and hemoglobin. Combining the above three equations, the OCR can be calculated by:

$$\text{OCR} = \frac{4 \times V_b \times \Delta sO_2 \times C_{\text{Hb}}}{\Delta t}. \quad (3.5)$$
**Coefficient of variation and chi-squared goodness-of-fit**

The coefficient of variation, $C_v$, is defined as the ratio of the standard deviation $\sigma$ to the mean $\mu$ of a distribution:

$$C_v = \frac{\sigma}{\mu}. \quad (3.6)$$

It is a normalized measure of the dispersion of the single-cell OCR distribution and thus a parameter to quantify and compare intratumoral metabolic heterogeneity.

The chi-squared goodness-of-fit is used to assess how close the single-cell OCR distribution was to a normal distribution. The data is first grouped into bins, and the observed and expected counts for the bins are then calculated. The chi-squared value $\chi^2$ is then calculated by

$$\chi^2 = \sum_{i=1}^{N} \frac{(O_i - E_i)^2}{E_i}. \quad (3.7)$$

Here $O_i$ denotes the observed counts, and $E_i$ denotes the expected counts based on the normal distribution. A smaller chi-squared value indicates a better fit to the normal distribution and a lower level of intratumoral cellular metabolic heterogeneity.

**3.3.3 Results**

**SCM-PAM of single-cell trapping and oxygen sealing in a high-density microwell array**

The system schematic of SCM-PAM is shown in Figure 3.16a. In brief, the laser beams are focused on the high-density microwell array, and the generated photoacoustic signals are detected by an ultrasonic transducer. By recording the time course of the photoacoustic signal from each laser
pulse, a one-dimensional photoacoustic image (A-line) is acquired. Cross-sectional images (B-scans) or volumetric images (C-scans) can be obtained by linear or raster motor scanning. To provide comprehensive single-cell metabolic information, we operate SCM-PAM in two modes, high-resolution mode and high-throughput mode. In the high-resolution mode (Fig. 3.16b), the microwell array is placed at the optical and acoustic focal plane of the SCM-PAM. With a scanning step size of 1.25 μm, a photoacoustic image with a FoV of 1 mm × 1 mm (corresponding to 100 microwells) is acquired by SCM-PAM in 400 seconds. In the high-resolution mode, we achieve optical diffraction limited lateral resolution, which is 2.71 μm for the current system (Fig. 3.17). Multiple parameters, including the cell size and the well filling ratio, can be quantified in the high-resolution mode. In the high-throughput mode (Fig. 3.16c), the laser beam is slightly defocused so that the laser spot size on the microwell array is 10 μm. A photoacoustic image with a FoV of 7.2 mm × 7.2 mm (corresponding to 3600 microwells) is then acquired by SCM-PAM with a scanning step size of 20 μm. Each image in the high-throughput mode takes 720 seconds to acquire. In the high-throughput mode, four spots in each microwell are sampled to provide accurate measurement of sO₂ within the microwell. The throughput can be further improved by sampling only one spot in each microwell, at the expense of sO₂ measurement accuracy within the microwell.

We first imaged the high-density microwell array with the SCM-PAM in high-resolution mode. Because the aluminum layer generates strong photoacoustic signals, each microwell can be clearly identified in the photoacoustic images as a negative contrast (Fig. 3.19a). The microwell diameters estimated from the photoacoustic images were ~40 μm, which agreed well with the values set during fabrication. To test the single cell trapping efficiency of the microwell array, we imaged it loaded with B16 melanoma cells. Individual B16 melanoma cells were clearly identified in the microwells (Fig. 3.19b). By optimizing the cell trapping procedure, we achieved a cell trapping
efficiency of 73%. We then imaged the microwell array loaded with fully oxygenated blood (sO$_2$ = 100%) and fully deoxygenated blood (sO$_2$ = 0%). The sO$_2$ maps of the high-density microwell arrays were obtained by employing two wavelengths (532 and 559 nm) for photoacoustic excitation (Fig. 3.19c and d). The sO$_2$ values were measured accurately, and the microwells were clearly distinguished from the background based on the sO$_2$ values.

To ensure that the OCR for each single cell was measured accurately, we verified the oxygen sealing of the microwell array. We first studied the oxygen diffusion between the blood in the microwells and the outside air by monitoring the sO$_2$ in the microwells. The array was loaded with blood with sO$_2$ values of 0%, 25%, 50%, 75%, and 100%, and the sO$_2$ values in the microwells were monitored for 300 minutes, which is 20 times as long as the 15 minute time interval between two sO$_2$ measurements in later experiments. The sO$_2$ remained unchanged for 300 minutes during the imaging (Fig. 3.19g), showing that the microwells were fully sealed.

To avoid crosstalk between single-cell OCR measurements in adjacent microwells, we then tested the oxygen diffusion between microwells. First, we induced differences in adjacent microwells by selectively heating the microwells, because a temperature change can shift the oxygen–hemoglobin dissociation curve and thus change the sO$_2$ in the microwells. The baseline sO$_2$ of the blood loaded in the array was ~60%, at which value a small shift of the oxygen–hemoglobin dissociation curve can yield a relatively large change in sO$_2$. Then, every other column of microwells was heated by scanning a continuous-wave (CW) laser (532 nm wavelength; MLL-III-532, General Optoelectronic) along the column. During heating, the maximum photoacoustic amplitude increase at 532 nm was ~8.5%, corresponding to a temperature rise of 1.9°C. To ensure that only the blood in the microwells was heated, the CW laser spot size was 15 μm and the
scanning step size was 80 μm. The laser spot size was smaller than the microwell diameter of 40 μm, and the scanning step size matches the spacing between microwells of 80 μm. After heating, the array was sealed immediately with silicone oil, and the sO₂ in the microwells was monitored for 300 minutes (Fig. 3.19e and f). At the beginning of the monitoring period, an average decrease of 12% in sO₂ was observed, which was due to heating. During the 300 minute monitoring, the sO₂ in microwells with and without heating stayed unchanged, demonstrating that there was no oxygen diffusion between the microwells in the high-density microwell array (Fig. 3.19h).

Figure 3.19. Single-cell trapping and oxygen sealing of the high-density microwell array. a, SCM-PAM of a microwell array without loading. Each well can be clearly identified. b, SCM-PAM of a microwell array loaded with a single
B16 melanoma cell per well. c, SCM-PAM of a microwell array loaded with fully oxygenated blood ($sO_2 = 100\%$). d, SCM-PAM of a microwell array loaded with fully deoxygenated blood ($sO_2 = 0\%$). e, SCM-PAM of a microwell array loaded with blood immediately after heating the selected columns. f, SCM-PAM of the microwell array loaded with blood 300 minutes after heating the selected columns. The columns with heating are labelled with “H”, and the columns without heating are labelled with “N”. g, Oxygen sealing of an array from the outside air with various initial $sO_2$ values. The $sO_2$ of the blood in the microwells remained unchanged during 300 minute monitoring, showing that the microwell array was fully sealed and there was no oxygen diffusion between the microwells and the outside air. h, Oxygen sealing of an array between microwells. The $sO_2$ of the blood in the microwells remained unchanged during 300 minute monitoring after heating the selected columns, showing that the array was fully sealed and there was no oxygen diffusion between the microwells.

**SCM-PAM of metabolic cellular heterogeneity in cultured cells**

To validate the basic capability to image cellular metabolic heterogeneity, we first applied SCM-PAM to image cultured normal and cancer cells. Two cell lines, RAW264.7 murine macrophage (a normal cell line) and A539 human epithelial lung cancer (a cancer cell line) were used. The single-cell OCRs of RAW264.7 cells were first measured in high-resolution mode (Fig. 3.20a). Of 100 microwells, 92 were filled with a single RAW264.7 cell each well. The $sO_2$ in each microwell was monitored for 45 minutes to measure the oxygen consumed by the RAW264.7 cell (Fig. 3.20b). Based on the oxygen consumed in 45 minutes, the OCR of each cell was calculated. The average OCR of the 92 RAW264.7 cells was $0.84 \pm 0.08$ fmol/min. Then we switched to the high-throughput mode and monitored the $sO_2$ in 3600 microwells. Among the 3600 microwells, 2746 showed significant changes in $sO_2$ within 45 minutes of monitoring (Fig. 3.20c), indicating that each of these microwells was loaded with a single living RAW264.7 cell. For the remaining microwells, it could be that RAW264.7 cells were either not loaded or not alive. The oxygen consumed in each microwell was calculated based on the $sO_2$ change (Fig. 3.20d), and the OCRs
of the 2746 RAW264.7 cells were calculated accordingly. The average OCR of the 2746 RAW264.7 cells was $0.81 \pm 0.11$ fmol/min.

**Figure 3.20.** SCM-PAM of cellular metabolic heterogeneity in cultured cells. a-d, SCM-PAM of single-cell oxygen consumption rates (OCRs) of cultured RAW264.7 murine macrophage cells. a, SCM-PAM of sO$_2$ changes in the microwells in high-resolution mode. b, Oxygen consumption curves of 92 RAW264.7 cells measured by SCM-PAM in high-resolution mode. c, SCM-PAM of sO$_2$ changes in the microwells in high-throughput mode. d, Oxygen consumption curves of 2746 RAW264.7 cells measured by SCM-PAM in high-throughput mode. e-h, SCM-PAM of single-cell OCRs of cultured A539 human epithelial lung cancer cells. e, SCM-PAM of sO$_2$ changes in the microwells measured in high-resolution mode. f, Oxygen consumption curves of 86 A539 cells measured by SCM-PAM in high-resolution mode. g, SCM-PAM of sO$_2$ changes in the microwells in high-throughput mode. h, Oxygen consumption curves of 2761 A539 cells measured by SCM-PAM in high-throughput mode. i, Single-cell OCR distributions of the above two cell lines measured by SCM-PAM in high-throughput mode.
Similarly, the single-cell OCRs of A539 cells were first measured in high-resolution mode (Fig. 3.20e). Of 100 microwells, 86 were filled with a single A539 cell each. As before, the sO\textsubscript{2} in each microwell was monitored for 45 minutes, and the oxygen consumed were calculated (Fig. 3.20f). The OCR of each cell was then calculated based on the oxygen consumed, and the average OCR of the 86 A539 cells was 1.86 ± 0.43 fmol/min. In the high-throughput mode, among the 3600 microwells, 2761 showed significant changes in sO\textsubscript{2} within 45 minutes of monitoring (Fig. 3.20g), indicating that a single A539 cell was loaded in each of these wells. The oxygen consumed in each microwell was calculated based on the sO\textsubscript{2} change (Fig. 3.20h), and the OCRs of the 2761 A539 cells were calculated accordingly. The average OCR was 1.82 ± 0.47 fmol/min. To show the cellular metabolic heterogeneity, the distributions of the single-cell OCRs of the above two cell lines are shown in a histogram (Fig. 3.20i). The cancer cell line (A539 human epithelial lung cancer) showed a broader and more irregular distribution of single-cell OCRs, indicating a higher level of cellular metabolic heterogeneity compared to the normal cell line (RAW264.7 murine macrophage). The initial results in cultured cells demonstrate SCM-PAM’s ability to perform label-free high-throughput single-cell OCR measurements and show its potential for imaging intratumoral metabolic heterogeneity.

**SCM-PAM of intratumoral metabolic heterogeneity in breast cancer patients**

To fully demonstrate its potential in imaging intratumoral metabolic heterogeneity, we applied SCM-PAM to measure single-cell OCR distributions of normal and cancerous breast tissues from 3 breast cancer patients. After surgical excision, the normal and cancerous breast tissues were dissociated into single-cell suspensions and placed into the device. The single-cell OCRs of normal breast tissue cells from patients were first measured in high-resolution mode (Fig. 3.21a). Of 100
microwells, 87 were each filled with a single cell. We monitored the sO₂ in each microwell for 45 minutes to quantify the oxygen consumed and calculate the OCR of the cell in the microwell (Fig. 3.21b). The average OCR of the 87 normal cells was 1.40 ± 0.22 fmol/min. Then we switched to the high-throughput mode. Among the 3600 microwells imaged, 2438 showed significant changes in sO₂ within 45 minutes of monitoring (Fig. 3.21c), indicating that a single cell was loaded in each of these wells. The oxygen consumed in each microwell was calculated based on the sO₂ change (Fig. 3.21d), and the OCR of each single cell was calculated accordingly. The average single-cell OCR of the 2438 cells from the normal breast tissues was 1.41 ± 0.23 fmol/min.

Similarly, we measured the single-cell OCRs of the breast cancer cells from patients (Fig. 3.21e-h). In high-resolution mode, the measured average OCR of 93 single cancer cells was 2.27 ± 0.42 fmol/min. In high-throughput mode, the measured average OCR of 2463 single cancer cells was 2.21 ± 0.45 fmol/min. To illustrate the cellular metabolic heterogeneity in breast cancer patients, the distributions of single-cell OCRs of the normal and cancer cells were plotted (Figs. 3.21i, 3.22, and 3.23). Even though tumors contain multiple populations of cells beside the cancer cells, the cancer specimen had a significantly higher average OCR, due to its higher rate of metabolism and cell proliferation than those of the normal specimen. Paralleling the results in cultured cells, the cells from the cancer tissue also showed a broader and more irregular distribution of single-cell OCRs, indicating a higher degree of cellular metabolic heterogeneity than the normal cells. The results from breast cancer patients fully prove the capability of SCM-CAM in imaging intratumoral metabolic heterogeneity and show its potential for clinical translation.
Figure 3.21. SCM-PAM of intratumoral metabolic heterogeneity in a breast cancer patient. a-d, Single-cell OCRs of normal breast tissue cells measured by SCM-PAM. a, SCM-PAM of sO$_2$ changes in the microwells in high-resolution mode. b, Oxygen consumption curves of 87 normal cells measured by SCM-PAM in high-resolution mode. c, SCM-PAM of sO$_2$ changes in the microwells in high-throughput mode. d, Oxygen consumption curves of 2438 normal cells measured by SCM-PAM in high-throughput mode. e-h, Single-cell OCRs of cancerous breast tissue cells measured by SCM-PAM. e, SCM-PAM of sO$_2$ changes in the microwells in high-resolution mode. f, Oxygen consumption curves of 93 cancer cells measured by SCM-PAM in high-resolution mode. g, SCM-PAM of sO$_2$ changes in the microwells in high-throughput mode. h, Oxygen consumption curves of 2463 cancer cells measured by SCM-PAM in high-throughput mode. i, SCM-PAM of single-cell OCR distributions of normal and cancer cells from the patient in high-throughput mode.
To show the full capability of SCM-PAM in quantitatively characterizing intratumoral metabolic heterogeneity, we calculated and compared several key parameters of single-cell OCR distributions of both cultured cells and cells from three breast cancer patients. Using cultured cells, we first calculated the coefficients of variation (CoVs) of the single-cell OCR distributions in}

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normal (RAW264.7 murine macrophage) and cancer (A539 human epithelial lung cancer) cells. The cancer cells showed a significantly higher CoV than the normal cells \( n = 5 \) groups; 14,055 normal cells; 13,257 cancer cells; \(*p < 0.05\), indicating an elevated level of cellular metabolic heterogeneity (Fig. 3.24a). To quantify how close the single-cell OCR distributions are to normal distributions, we calculated the chi-squared goodness-of-fit to normal distributions for the cultured cells (Methods). The cancer cells had a significantly higher chi-squared value than the normal cells \( n = 5 \) groups; 14,055 normal cells; 13,257 cancer cells; \(*p < 0.05\), indicating a lower similarity to normal distributions (Fig. 3.24b). In other words, there was a higher level of chaotic cellular metabolic heterogeneity in the cancer cells than in the normal cells.

In subsequent tests using cells from three breast cancer patients, the cancer cells had a higher average single-cell OCR, showing an increase in oxygen consumption compared to normal cells (Fig. 3.24c). We also measured the CoVs and chi-squared values of the single-cell OCR distributions of the normal and cancer cells. The cancer cells showed a significantly higher CoV than the normal cells \( n = 3 \) patients; 7,549 normal cells; 6,807 cancer cells; \(*p < 0.05\), indicating an elevated level of cellular metabolic heterogeneity (Fig. 3.24d). In addition, the cancer cells also had a significantly higher chi-squared value than the normal cells \( n = 3 \) patients; 7,549 normal cells; 6,807 cancer cells; \(*p < 0.05\), indicating a lower similarity to normal distributions, thus a higher level of chaotic cellular metabolic heterogeneity (Fig. 3.24e). Quantitative characterization of the intratumoral metabolic heterogeneity further shows the capability of SCM-PAM and its potential as a tool for both preclinical cancer research and clinical cancer therapy.
Figure 3.24. Elevated and chaotic cellular metabolic heterogeneity in cancer by SCM-PAM. a-b, SCM-PAM of elevated cellular metabolic heterogeneity in cultured cells. a, Coefficients of variation (CoVs) of single-cell OCR distributions in cultured normal (RAW264.7 murine macrophage) and cancer (A539 human epithelial lung cancer) cells (n = 5 groups; 14,055 normal cells; 13,257 cancer cells; *p < 0.05). b, Chi-squared goodness-of-fit to normal distributions of single-cell OCR distributions in cultured normal and cancer cells (n = 5 groups; 14,055 normal cells; 13,257 cancer cells; *p < 0.05). c, Average single-cell OCRs of normal and cancerous breast tissue cells from three breast cancer patients. In all three patients, the cancer cells consumed oxygen faster than the normal cell on average (Patient 1: *p < 0.05; Patient 2: *p < 0.05; Patient 3: *p < 0.05.). d-e, SCM-PAM of elevated cellular metabolic heterogeneity in breast cancer patients. d, CoVs of single-cell OCR distributions in normal and cancer cells from breast cancer patients (n = 3 patients; 7,549 normal cells; 6,807 cancer cells; *p < 0.05). e, Chi-squared goodness-of-fit to normal distributions of single-cell OCR distributions in normal and cancer cells from breast cancer patients (n = 3 patients; 7,549 normal cells; 6,807 cancer cells; *p < 0.05).

SCM-PAM of oxygen consumption of cancer and normal cells in hypoxia

To show the versatility of SCM-PAM as a research tool, we applied it to study the oxygen consumption of single cells in hypoxia, an important hallmark of cancer. By changing the sO_2 of the blood (i.e., the oxygen supplier and sensor) in the microwells, we measured how the OCRs of normal and cancer cells depend on the environmental oxygen levels. In cultured cells, both the cancer and normal cells showed decreases in average OCRs in hypoxia, due to the lower oxygen supply (Fig. 3.25a). The cancer cells, however, showed a smaller relative decrease than normal cells, indicating better adaptation to hypoxia. We also studied how cellular metabolic...
heterogeneity changes in hypoxia by measuring the CoVs and chi-squared values of single-cell OCR distributions. The CoVs of both normal and cancer cells increased in hypoxia, indicating more diverse metabolism of single cells under a lower oxygen supply. The cancer cells had an even higher relative change in CoVs, showing a greater increase in cellular metabolic heterogeneity (Fig. 3.25b). The chi-squared values of both normal and cancer cells also became larger in hypoxia, showing that the chaotic levels of cellular metabolic heterogeneity increased under a lower oxygen supply (Fig. 3.25c).

Figure 3.25. Oxygen consumption of cancer and normal cells in hypoxia by SCM-PAM. a-c, Oxygen consumption of cultured cells in hypoxia measured by SCM-PAM. a, Normalized OCRs of cultured normal (RAW264.7 murine macrophage) and cancer (A539 human epithelial lung cancer) cells at different sO2 levels. b, Normalized CoVs of single-cell OCR distributions in cultured normal and cancer cells at different sO2 levels. c, Normalized chi-squared values of single-cell OCR distributions in cultured normal and cancer cells at different sO2 levels. d-f, Oxygen consumptions of normal and cancer cells from breast cancer patients in hypoxia measured by SCM-PAM. d, Normalized OCRs of normal and cancer cells from breast cancer patients at different sO2 levels. e, Normalized CoVs of single-cell OCR distributions in normal and cancer cells from breast cancer patients at different sO2 levels. f, Normalized chi-squared values of single-cell OCR distributions in normal and cancer cells from breast cancer patients at different sO2 levels.
An interesting observation is that the normal cells had a higher relative change in chi-squared values, showing a greater relative increase in the chaotic levels of cellular metabolic heterogeneity. This could be due to two reasons. First, the cancer cells started with a higher baseline level of chaos in cellular metabolic heterogeneity. Second, the cancer cells better adapted to hypoxia than the normal cells, leading to smaller further increases in the chaotic levels of cellular metabolic heterogeneity (Fig. 3.26). Similar results were obtained in cells from breast cancer patients (Fig. 3.25d-f), further validating the results and showing the additional potential of SCM-PAM as an important research tool.

Figure 3.26. Absolute changes of oxygen consumption in hypoxia by SCM-PAM. a-c, Absolute changes in oxygen consumption of cultured cells in hypoxia measured by SCM-PAM. a, Average single-cell OCRs of cultured normal (RAW264.7 murine macrophage) and cancer (A539 human epithelial lung cancer) cells at different sO₂ levels. b, CoVs of single-cell OCR distributions in cultured normal and cancer cells at different sO₂ levels. c, Chi-squared values of single-cell OCR distributions in cultured normal and cancer cells at different sO₂ levels. d-f, Absolute changes in oxygen consumption of normal and cancer cells from breast cancer patients in hypoxia measured by SCM-PAM. d, Average single-cell OCRs of normal and cancer cells from breast cancer patients at different sO₂ levels. e, CoVs of single-cell OCR distributions in normal and cancer cells from breast cancer patients at different sO₂ levels. f, Chi-squared values of single-cell OCR distributions in normal and cancer cells from breast cancer patients at different sO₂ levels.
3.3.4 Discussion

By ultrasonically probing the optical absorption of hemoglobin, which acts as both an oxygen supplier and sensor for single cells, photoacoustic imaging achieves label-free OCR measurements. Without the need for exogenous oxygen sensors or labelling, a large population of cells can be screened for single-cell OCR measurement by creating an oxygen-diffusion limited microenvironment for each cell, using a high-density microwell array. Combining photoacoustic imaging with the high-density microwell array, SCM-PAM successfully overcomes all the major challenges in high-throughput single-cell OCR measurements and enables metabolic imaging of cellular heterogeneity in cancer. In addition, by mixing cancer cells with blood, SCM-PAM avoids possible adverse effects on normal cell functioning that might be caused by embedded oxygen sensors or labelling, such as oxygen consumption by electrode sensors or photo-toxicity by fluorescent sensors. We initially proved the capability of SCM-PAM in label-free high-throughput single-cell OCR measurement by imaging cultured normal and cancer cells. We further showed the full potential of SCM-PAM for clinical translation by imaging the intratumoral metabolic heterogeneity in breast cancer patients. By measuring and comparing the key parameters of single-cell OCR distributions of normal and cancer cells, we demonstrated the capability of SCM-PAM to quantify intratumoral metabolic heterogeneity. In addition, we showed other potential applications of SCM-PAM as a powerful research tool to study oxygen consumptions of single cells in hypoxia. More importantly, we characterized the chaotic feature of intratumoral metabolic heterogeneity for the first time with and without hypoxia.

Both elevated and chaotic metabolic heterogeneity was observed in cancer cells by SCM-PAM in our results. The elevated cellular metabolic heterogeneity can be attributed to the genomic instability in cell cloning and propagation, the high potency in cell differentiation—
characteristic of cancer stem cells\textsuperscript{131}, and the presence of mixed cell populations in the patient specimens. First, cancer cell propagation results in constant mutation acquisitions from unstable genome replications, resulting in genomic diversity within a single tumor\textsuperscript{132}. Second, cancer stem cells produce a variety of cell types in a tumor through a differentiation hierarchy\textsuperscript{133}. Third, tumors are comprised of multiple populations of cells beside the cancer cells themselves, such as inflammatory cells, which may contribute to the metabolic heterogeneity. The genomic diversity, distinct cancer cell types, and mixed cell populations within a single tumor result in elevated intratumoral metabolic heterogeneity. Further, the gene expression unpredictability within one cancer cell type leads to chaotic cellular metabolic heterogeneity. The high unpredictability of gene expression yields different mitochondrial contents at different times, creating a chaotic metabolic system even within a single cancer cell\textsuperscript{134}. Together, these factors contribute to the elevated and chaotic intratumoral metabolic heterogeneity observed by SCM-PAM.

The OCR of an individual cell is the sum of the OCRs of all the subcellular mitochondria, which are responsible for aerobic glycolysis, i.e., oxygen consumption. Mathematically, we have

\[
S_n = \sum_{i=1}^{n} M_i .
\]  

(3.8)

Here, \(S_n\) denotes the single-cell OCR, \(M_i\) represents the \(i\)th mitochondrion OCR, and \(n\) denotes the virtual number of mitochondria in the cell. Within one cell type, because \(n\) is highly consistent\textsuperscript{135}, it can be treated as a constant. According to the central limit theorem, \(S_n\) follows a normal distribution if \(M_i\) follows the same distribution regardless of its shape. By sampling a massive number of single cells simultaneously, we can accurately model the distributions of \(S_n\) and compare them to a normal distribution using the chi-squared test. We found that cancer cells, both
in the cultured cell lines as well as the patient tumors, deviated farther from normal distributions, i.e., with greater chi-squared values, than normal cells, probably due to the aforementioned factors. First, both the genomic diversity and the multiple types of cancer cells within a tumor generate distinct $n$ values and $M_i$ distributions. Second, even within one cancer cell type, the unpredictability of gene expression produces incongruent distributions of $M_i$. This observation characterized the chaotic feature of intratumoral metabolic heterogeneity for the first time at the mitochondrial level.

In an effort to further understand the contributions of distinct cancer cell subclones to intratumoral metabolic heterogeneity, we tried to calculate the number of distinct cell subclones within the cancer cells sampled. By assuming that the single-cell OCR of each cell type follows a normal distribution, we fitted the single-cell OCR distributions of the cancer cells to the sum of multiple normal distributions (Fig. 3.27). The number and weight of the normal distributions were obtained during fitting. The fitting results showed that the single-cell OCR distributions of the cancer cells were the sum of multiple normal distributions, indicating that there may be several cell subclones or types within the cancer cell population examined.

The functionality of SCM-PAM can be further enhanced in two aspects. First, the imaging throughput can be further improved by using an array with more microwells and increasing the imaging speed. To make an array with more microwells, more robust fabrication materials and processes are required. To increase the imaging speed, new scanning mechanisms, such as microelectromechanical system mirror based scanning, can be used. Second, additional parameters can be measured to provide more information on single-cell metabolism. Cell sizes can
be more accurately quantified in three dimensions by using the photobleaching effect\textsuperscript{71}, and pH values can be measured with a pH-sensitive fluorescent dye\textsuperscript{136}.  

**Figure 3.27.** Fitting of single-cell OCR distributions. a, The single-cell OCR distribution of cancer cells in Patient 1 was fitted to the sum of two normal distribution with an $R^2$ value of 0.92, indicating that there may be two major types of cancer cells (indicated by the cyan dashed line and blue dotted line) within the cell population examined. b, The single-cell OCR distribution of cancer cells in Patient 2 was fitted to the sum of two normal distribution with an $R^2$ value of 0.84, indicating that there may be two major types of cancer cells (indicated by the cyan dashed line and blue dotted line) within the cell population examined. c, The single-cell OCR distribution of cancer cells in Patient 3 was fitted to the sum of two normal distribution with an $R^2$ value of 0.88, indicating that there may be two major types of cancer cells (indicated by the cyan dashed line and blue dotted line) within the cell population examined.
SCM-PAM holds great potential for clinical translation, especially in the emerging area of targeting hypoxia, understanding metabolic diversity within and between tumors, and investigating the effect this heterogeneity on tumor aggressiveness. By utilizing excised tumor tissue and quickly dissociating it into single-cell suspension for high-throughput single-cell OCR measurements, SCM-PAM can be conveniently adopted for clinical use. Although in this paper we have focused on breast cancer, SCM-PAM can be applied to image intratumoral metabolic heterogeneity of virtually all solid tumors that can be sampled. The microwell size can be adjusted to better accommodate different cancer cells of varying size. The obtained single-cell metabolism information and intratumoral metabolic heterogeneity will be particularly valuable to cancer therapy. Single-cell metabolism information about cancer cells can be used for therapy response monitoring and evaluation, which can help screen drug combinations and develop personalized cancer therapy strategy. Apart from cancer therapy, by measuring the single-cell OCR distributions of different tumors in one patient, SCM-PAM can also study intertumoral heterogeneity and response to hypoxia. Moreover, SCM-PAM is particularly suitable for studying the metabolic states of CTCs, the key determinants of cancer metastatic propensity, because blood is the original biological environment for CTCs.

In summary, we have developed and optimized SCM-PAM, which combines a wide-field fast-scanning functional OR-PAM with a high-density microwell array, for label-free high-throughput single-cell imaging of intratumoral metabolic heterogeneity. We have demonstrated its capability by measuring the single-cell OCR distributions of cultured cells and showed its potential for clinical translation by imaging intratumoral metabolic heterogeneity in breast cancer patient specimens. In addition, with rich optical absorption contrast, the wide-field fast-scanning functional OR-PAM subsystem of SCM-PAM can provide multiple dimensions of information.
about tumors, including angiogenesis, metastasis, and drug responses, along with intratumoral heterogeneity\textsuperscript{141–143}. With its unique capability for label-free high-throughput single-cell OCR measurement and the potential of providing multidimensional information about tumors, SCM-PAM is a promising tool for both fundamental cancer research and clinical personalized cancer therapy.
Chapter 4 Photoacoustic Tomography of Circulating Melanoma Tumor Cells

In this chapter, I introduce the development of linear-array-based PAT systems for label-free high-throughput circulating melanoma tumor cell detection. In the first section, I report a linear-array-based PAT (LA-PAT) system to detect and quantify circulating melanoma tumor cell clusters, which greatly elevate the metastatic potential of cancer compared to single circulating melanoma tumor cells. Then, in the second section, I demonstrated label-free high-throughput PAT of circulating melanoma tumor cells in patients \textit{in vivo}. Taking advantage of the strong optical absorption of melanin, we maximized the contrast between circulating melanoma tumor cells and blood by picking the optimal excitation wavelength and successfully imaged circulating melanoma tumor cells in patients \textit{in vivo}.

4.1 Label-free High-throughput Detection and Quantification of Circulating Melanoma Tumor Cell Clusters by Linear-array-based Photoacoustic Tomography

4.1.1 Background

Metastasis, the spread of cancer cells from a primary site to distant organs leading to the growth of new tumors there, accounts for the majority of cancer deaths\textsuperscript{144}. Blood-borne metastasis comprises multiple steps, including intravasation, survival in the blood circulation, extravasation, and secondary growth in the distant organs, in which the rare CTCs are the key determinants of metastatic propensity\textsuperscript{145}. While most CTCs survive in the blood circulation in the form of single cells, CTC clusters also exist and elevate the metastatic potential by 23 to 50 folds\textsuperscript{146}. Detection and quantification of CTC clusters will provide valuable insights into metastasis as well as cancer
therapy. Because the presence of CTCs is positively correlated with metastatic propensity\textsuperscript{147}, the detection and enumeration of rare CTCs will help to better evaluate the melanoma stage and predict metastatic potential during the diagnosis. In addition, tracking the concentration of CTCs in the circulatory system during treatment will likely be valuable indicators of both tumor responses to therapy and melanoma progression.

Taking advantage of tumor-specific physical and biological properties, such as sizes and surface biomarkers, multiple technologies have been developed to capture and isolate single CTCs and CTC clusters\textsuperscript{124,148}. However, most of these existing technologies require blood to be drawn to isolate and capture CTCs \textit{ex vivo}, reducing the effective CTC detection sensitivity. Optical imaging technologies, such as confocal microscopy, \textit{in vivo} flow cytometry, and optical coherence tomography, have been applied to detect CTCs \textit{in vivo}\textsuperscript{149–151}. However, suffering from strong optical scattering in biological tissue, these techniques have shallow penetration, limiting them to imaging only small blood vessels, which results in low throughput for CTC detection.

By integrating optical excitation with acoustic detection, PAT combines rich optical absorption contrasts with high ultrasonic spatial resolution at depths\textsuperscript{3}. With 100\% relative sensitivity to optical absorption, i.e., a given percentage change in the optical absorption yields the same percentage change in the photoacoustic signal, PAT\textsuperscript{8,40,62,111} achieves structural, functional, metabolic, and mechanical imaging of biological tissue. Taking advantage of the strong optical absorption of melanin, photoacoustic techniques have been successfully used for imaging and sensing melanoma CTCs. Photoacoustic flowmetry successfully detected circulating melanoma cells in human blood\textsuperscript{152,153}. Photoacoustic flow cytometry enables long term monitoring of melanoma CTCs \textit{in vivo}. However, these sensing techniques provide inadequate spatial resolution to further
characterize melanoma CTCs. The microscopic implementation of PAT, OR-PAM, images single melanoma CTCs in the blood flows with high spatial-temporal resolution. While providing valuable insights into CTCs on a single cell level, OR-PAM suffers from low throughput.

Here, we apply a LA-PAT system for label-free high-throughput melanoma CTC cluster detection and quantification in vivo. Exploiting the strong optical absorption of melanin in the melanoma tumor cells, LA-PAT can achieve label-free detection of melanoma CTC clusters in vivo. By analyzing the contrast-to-noise ratios (CNRs) of the photoacoustic signals, LA-PAT can quantify the number of cells in the CTC clusters and study their circulating kinetics in the bloodstream.

4.1.2 Methods

Linear-array-based photoacoustic tomography system

To image melanoma CTC clusters, we applied an LA-PAT system based on a handheld probe and improved the image reconstruction. A tunable optical parametric oscillator laser (680 to 970 nm, 20-Hz pulse repetition rate) was used for illumination. An excitation wavelength of 680 nm was chosen to achieve the optimal contrast between melanoma tumor cells and blood (Fig. 4.1a). The laser beam was coupled into a fiber optical bundle that bifurcated into two optical fiber bundles. Laser beams coming out of the two optical fiber bundle strips (20 mm × 1.25 mm) excited the object at an angle of incidence of 30 degrees relative to the imaging plane (Fig. 4.1b and c). During the experiments, the fluence on the tissue surface was about 10 mJ/cm², well within the 20 mJ/cm² safety limit set by the American National Standards Institute. The generated photoacoustic waves were detected by a linear array ultrasonic transducer (Visualsonics, Inc., LZ250, 21 MHz center frequency, 78% one-way bandwidth, 256 elements, 23 mm × 3 mm array size). The spatial
resolutions of the system were 119 μm in the lateral direction, 86 μm in the axial direction, and 1237 μm in the elevational direction. Because there were only 64 channels in the data acquisition unit, four-to-one multiplexing was applied during image acquisition. For each laser pulse, the generated photoacoustic signals were captured sequentially by a quarter segment of the linear array (i.e., elements 1–64, 65–128, 129–192, and 193–256). Once the data were acquired from all four quarter segments, a two-dimensional photoacoustic image was reconstructed with the universal back-projection algorithm developed by our group. The reconstructed photoacoustic images were displayed in an imaging station (Vevo LAZR, Visualsonics, Inc.). Determined by the 20 Hz laser repetition rate and the four-to-one multiplexing in image acquisition, the frame rate was 5 frames/second.

**Tumor cell culture and cluster preparation**

B16F10 melanoma tumor cells obtained from ATCC® were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C in air with 5% CO₂. Upon reaching 80% confluence, cells growing in a monolayer were incubated with 0.25% trypsin-EDTA solution (Invitrogen) to generate floating cell clusters of various sizes. After adding serum-containing medium to neutralize trypsin, the floating cell clusters were handled in two ways. To mimic melanoma CTC clusters of various sizes, floating cell clusters totaling 1×10⁶ cells were directly suspended in 1 mL of bovine blood for *ex vivo* experiments or in 1 mL of collected rat blood for tail vein injection. To generate melanoma cell clusters of a certain size for CNR analysis, instead of directly suspending the floating cell clusters in blood, the clusters were mechanically dissociated by pipetting to generate a single-cell suspension. The single cells were then suspended in a blood-agar mixture at a desired concentration and then cooled to become
solid. The concentration was controlled so that within each resolution voxel of LA-PAT there was a certain number of melanoma cells, i.e., 5, 10, 20, 30, 40, or 50. The solid mixture was then mechanically dissociated into particles smaller than the resolution voxel of LA-PAT to generate cell clusters of a certain size. The particles were then suspended in 1 mL of bovine blood for CNR analysis.

Figure 4.1. Absorption spectra and views of the hand-held probe for LA-PAT. (a) Absorption spectra of melanin (black dashed line), oxyhemoglobin (red solid line), and deoxyhemoglobin (blue dotted line). (b) Lateral view of the hand-held probe. (c) Elevational view of the hand-held probe with a zoomed-in view.

**Phantom preparation**
Silicone microtubes with 0.76 mm inner diameter (11-189-15C, Fisher Scientific) were perfused with bovine blood (905, Quad-Five) to mimic blood vessels. The microtubes were embedded 3 mm deep in tissue-mimicking gelatin phantoms. Optical scattering similar to that in biological tissue was achieved by adding 1% intralipid to the gelatin phantoms. Melanoma tumor cell clusters suspended in bovine blood were pumped through the microtubes with a syringe. A syringe pump (BSP-99M, Braintree Scientific) controlled the blood flow speed.

**Experimental animals and CTC cluster injection**

All experimental animal procedures were carried out in conformity with the laboratory animal protocol approved by the Animal Studies Committee at Washington University in St. Louis. Male nude rats (Hsd:RH-Foxn1nu/Foxn1+, Harlan Co.; 12–13 weeks old; ~330–350 g body weight) were used for all *in vivo* experiments. Throughout the experiments, the rats were kept under anesthesia with 2% vaporized isoflurane. A home-made animal holder immobilized the rats during the experiments.

**4.1.3 Results**

**CNR analysis of melanoma tumor cell clusters**

To detect and quantify CTC clusters, we first used LA-PAT to image melanoma tumor cell clusters of known sizes and analyzed their CNRs. Clusters with an average of 5, 10, 20, 30, 40, and 50 cells were suspended in blood and imaged by LA-PAT. The CNR for each cluster was calculated (Fig. 4.2a). The average CNRs were plotted as a function of the number of cells in the clusters, as measured with an optical microscope (Eclipse TS100, Nikon) (Fig. 4.2b). The experimental data were fitted to a linear function:
\[ CNR = 0.46 \times N. \]  

Figure 4.2. CNR analysis of melanoma tumor cell clusters. (a) CNR distributions measured with LA-PAT for melanoma tumor cell clusters of different sizes. (b) Average CNRs in LA-PAT as a function of the numbers of cells in melanoma tumor cell clusters. (c) The numbers of cells in melanoma tumor cell clusters measured by LA-PAT agreed well with those measured by an optical microscope (OM).

Here \( N \) represents the number of cells in a melanoma tumor cell cluster. Based on a 6 dB CNR threshold, a minimum of four cells can be resolved although this minimum was not directly observed. To verify the relationship between the CNR and the number of cells in the clusters, another independent experiment was performed. Melanoma tumor cell clusters consisting of a uniform but unknown number of cells were equally divided into six groups. Three randomly chosen groups were suspended in blood and imaged by LA-PAT. The numbers of cells in the clusters were calculated based on the CNRs and the relationship above. The other three groups were examined with an optical microscope to determine the numbers of cells in the clusters. The
number of cells in the melanoma tumor cell clusters measured by LA-PAT and optical microscopy agreed well with each other (Fig. 4.2c), confirming the relationship between the numbers of cells in the clusters and the CNRs in LA-PAT.

**LA-PAT of CTC clusters *ex vivo***

We then demonstrated the ability of LA-PAT to detect and quantify CTC clusters *ex vivo*. A mixture of melanoma tumor cell clusters of various sizes was equally divided into six groups. Three randomly chosen groups were suspended in blood, pumped through microtubes to mimic flowing melanoma CTC clusters, and imaged by LA-PAT. Each melanoma tumor cell cluster was captured by LA-PAT multiple times, and the average CNR was used to calculate the number of cells in the cluster. Based on the calculated number of cells in the clusters, the melanoma tumor cell clusters were divided into three categories: \( \leq 10 \), 11–20, and \( \geq 21 \). Typical photoacoustic images of the three categories are shown in Figure. 3a–c. The other three groups were measured with an optical microscope to determine the numbers of cells in the clusters and were divided into the same three categories. The size distributions of the clusters measured by LA-PAT and optical microscopy agreed well with each other with a correlation coefficient of 0.96 (Fig. 4.3d), and the results further demonstrated the ability of LA-PAT to detect and quantify melanoma CTC clusters.

**LA-PAT of CTC clusters *in vivo***

To demonstrate the feasibility of LA-PAT *in vivo*, we imaged melanoma CTC clusters injected into rats. Melanoma tumor cell clusters suspended in 1 mL of rat blood at a concentration of \( 1 \times 10^6 \) cells/mL were injected into the same rat from which the blood was collected a few minutes earlier. The rat tail vein was then monitored by LA-PAT for 45 minutes to study the circulation kinetics.
and the clearance rates of CTC clusters. CTC clusters were detected by LA-PAT immediately after injection and during circulating in the rat bloodstreams (Fig. 4.4a and b). The numbers of cells in the CTC clusters were calculated based on the CNRs. The CTC clusters were categorized into three groups based on the calculated number of cells and the clearance curves of the three groups are shown in Figure 4.4c. Larger CTC clusters showed faster clearance rates from the bloodstream than smaller CTC clusters.

Figure 4.3. LA-PAT of CTC clusters ex vivo. LA-PAT of a flowing melanoma tumor cell cluster with (a) 7 cells, (b) 14 cells, and (c) 29 cells. The cell numbers are calculated based on the CNR curve in Figure 4.2. (d) Distributions of the numbers of cells in melanoma tumor cell clusters, as measured by LA-PAT and OM.
4.1.4 Discussion

Compared to single CTCs, CTC clusters are rarer, but have a higher metastatic potential. CTC clusters have a faster clearance rate, i.e., a shorter lifetime, in the bloodstream than single CTCs, making them even more difficult to detect. The melanoma CTC detection sensitivity of LA-PAT is determined by the CNR. Because the noise is dominated by the fluctuating counts of red blood cells in each resolution voxel, the detection sensitivity is largely dependent on the spatial resolutions of LA-PAT. On one hand, to achieve single cell sensitivity in CTC detection, a higher frequency ultrasonic transducer could be used, at the expense of penetration depth. On the other hand, to achieve deeper imaging, a lower frequency ultrasonic transducer can be employed, at the expense of CTC detection sensitivity. The CTC detection sensitivity can be further improved by performing spectral unmixing using multi-wavelength illumination. To achieve multi-wavelength imaging of flowing CTCs, a second laser with a different wavelength needs to be incorporated into the system.
It is worth pointing out that currently LA-PAT detects only CTCs originating from primary tumors in which cells express melanin. To detect CTCs originating from amelanotic melanoma tumors, other tumor-specific physiological properties, such as sizes and surface biomarkers, can be utilized. The quantification of CTC cluster sizes is indeed based on the assumption that the melanin content is relatively uniform in the tumor cells. This assumption often holds true for tumor cells originating from the same primary tumor. However, if there are several primary tumors, it may affect the accuracy of quantifying CTC cluster sizes.

LA-PAT has the potential to be a powerful tool for both preclinical tumor metastasis study and clinical cancer diagnosis and therapy. As a useful technique for researchers and scientists to better understand the relationship between CTCs and tumor metastasis, LA-PAT can employ ultrasonic transducers of different frequencies for target vessels at various depths and different animal models. Other physiological parameters, including vessel diameter and blood flow speed, can be measured concurrently for further understanding of CTC flow dynamics. LA-PAT can also be used by clinicians to monitor the changes of CTC concentrations in patients’ circulatory systems and to evaluate the outcome of cancer therapy. To further improve LA-PAT for such clinical applications, an automatic CTC detection and counting algorithm should be developed.

Metastasis is a complex biological process that involves multiple steps and many parameters. Photoacoustic imaging, with the ability to quantify numerous parameters at multiple scales based on the same optical absorption contrast, can provide comprehensive information about tumor metastasis. OR-PAM can study CTCs on a single cell level. LA-PAT has been demonstrated to detect and quantify CTC clusters. In addition, photoacoustic imaging can measure other important biological parameters, including the oxygen saturation of hemoglobin, metabolic rate, and tumor
stiffness\textsuperscript{21,102,156}, all of which are closely related to metastasis\textsuperscript{142,157,158}. With the capability to detect and quantify CTC clusters and the potential to provide multidimensional information on tumor metastasis, LA-PAT is a promising tool for both preclinical cancer metastasis study and clinical tumor therapy.

4.2 Label-free High-throughput Photoacoustic Tomography of Circulating Melanoma Tumor Cells in Patients \textit{in Vivo}

4.2.1 Background

More than 90% of cancer-associated mortality are caused by metastasis\textsuperscript{25}, which refers to the process of cancer cells spreading from primary tumor site to surrounding tissues and distant organs to form new tumors. During the entire metastatic process, a cancer cell needs to execute multiple steps, including acquiring the invasive phenotype at the primary tumor, invading the surrounding tissue, enter the local blood or lymphatic vessel (intravasation), surviving and circulating in the blood stream, exiting through microvessels to distant tissues (extravasation), adapting to the new microenvironment, and growing into metastases\textsuperscript{26}. In this metastatic cascade, the rare CTCs are the key determinants to the metastatic propensity\textsuperscript{159,160}. Considered as the “seeds” to metastases, the presence and concentration of CTCs are closely correlated with the tumor progression, metastases, and survival rates in patients\textsuperscript{145}. Thus, efficient detection and characterization of CTCs will make critical contributions to cancer diagnosis and staging, therapy response assessment, and residual disease evaluation after surgery.

Recent advances in biomedical techniques have enabled intensive study on the presence of CTCs in the bloodstream to gain further insights of metastasis\textsuperscript{161}. These techniques usually utilize tumor-
specific physical and biological properties to detect, capture, or isolate CTCs from the bloodstream. However, they all encounter certain limits that prevent effective detection of CTCs in patients. Most ex vivo CTC detection assays, including the CTC microchip and the FDA-approved CellSearch (CELLSEARCH®), rely on epithelial markers as the tumor biomarker. A critical subpopulation of CTCs could be missed in such assays because some CTCs can shield their epithelial markers or express fewer markers after transitions. A new generation of microchip was developed to isolate CTC clusters based on size. However, it still suffers from very limited blood sample volume in the ex vivo assays relative to the patient’s entire blood volume, which significantly reduces the effective sensitivity of CTC detection, and thus leads to inaccurate diagnosis. To improve the detection sensitivity, in vivo optical CTC imaging techniques have also been developed, including in vivo flow cytometry, multiphoton microscopy, and inverse spectroscopic optical coherence tomography. However, most of these techniques require CTC labelling using targeted nanoparticles or fluorescent probes, the toxicity of which impedes their in vivo clinical translation. More importantly, strong optical scattering in biological tissue severely limits the imaging depth of these optical imaging techniques, restricting the imaging targets to only small vessels, resulting in low throughput for CTC detection.

By ultrasonically imaging optical absorption contrast, PAT achieves label-free imaging of biological tissue in vivo beyond the optical diffusion limit and has significantly advanced cancer imaging. The unique combination of optical excitation and acoustic detection allows PAT to break all the aforementioned limits for effective CTC detection. First, the weak ultrasonic scattering in soft tissue provides PAT the access to bigger vessels with greater flow rates at depths, thus achieving much higher throughput for CTC detection than pure optical techniques. Second, the rich endogenous optical absorption contrast enables high-sensitivity imaging of CTCs in vivo.
by proper excitation wavelength selection and/or robust spectral unmixing, without the need of exogenous labelling agents. In this work, we focus on CTC detection in melanoma, the most fatal type of skin cancer with more than 87,000 new diagnoses and 10,000 deaths each year\textsuperscript{168}. Melanoma has a high tendency of metastasis, after which the 5-year survival rate decreases from 98.5% to less than 20%. Effective detection of melanoma CTC is critical for understanding melanoma metastasis and improving its therapy. PAT is particularly suitable for melanoma CTC detection owing to the strong optical absorption of the highly expressed melanin, which serves as a perfect intrinsic contrast\textsuperscript{169}. Photoacoustic sensing techniques, including photoacoustic flowmetry and flow cytometry, has successfully infer the appearances of melanoma tumor cells by identifying the peaks in the 1D signal, but they do not provide the spatial resolution to visualize and characterize them\textsuperscript{152,154}.

Here, we developed a PAT system based on a linear ultrasonic transducer array (LA-PAT) and achieved label-free high-throughput imaging of melanoma CTCs in patient \textit{in vivo} for the first time. We first imaged flowing melanoma tumor cells in microtubes to initially demonstrate the capability of LA-PAT. Then, 16 Stage III and IV melanoma patients were imaged by LA-PAT and we successfully detected melanoma CTCs in 3 of them. The CTC imaging results were validated with CNR analysis compared to healthy volunteers and CTC-negative patients and the clinical relevance of CTC detection was studied by follow-up monitoring of the patients imaged.

\textbf{4.2.2 Methods}

\textbf{Optimal excitation wavelength for photoacoustic imaging of circulating melanoma tumor cells}
Figure 4.5. Optimal excitation wavelength for photoacoustic imaging of melanoma CTCs. a, Optical absorption spectra of melanin (major absorber in melanoma CTCs) and hemoglobin (major absorber in blood). HbO$_2$, oxyhemoglobin; HbR, deoxyhemoglobin. b, Absorption coefficient ratio of melanin and venous blood with an oxygen saturation level of 85%. An excitation wavelength of 680 nm was chosen to maximize the melanoma CTC detection sensitivity.

To achieve the highest detection sensitivity of melanoma CTCs in blood, an excitation wavelength that maximizes the contrast between melanoma CTCs (target) and blood (background) should be chosen. We compared the optical absorption spectra of melanin, the major optical absorber in melanoma CTCs, and hemoglobin, the major optical absorber in blood (Fig. 4.5a). Based on the absorption spectra, we calculated the optical absorption coefficient ratio of melanin and venous blood (oxygen saturation level of 85%) to select the wavelength that maximizes the contrast. Venous blood was used as the background here because veins, with shallow depth in tissue and
large blood volume, are ideal imaging targets in patients for melanoma CTC detection in patients. Based on the optical absorption coefficient ratio, an excitation wavelength of 680 nm was chosen to maximize the contrast between melanoma CTCs and blood and achieve the highest detection sensitivity (Fig. 4.5b).

**Linear-array-based photoacoustic tomography system for circulating melanoma tumor cell imaging**

To achieve label-free high-throughput imaging of melanoma CTCs, we developed a PAT system based on a linear array ultrasonic transducer (LA-PAT) (Fig. 4.6). After careful analysis, an excitation wavelength of 680 nm was chosen to maximize the contrast between melanoma CTCs and blood and another excitation wavelength of 850 nm was chosen to locate blood vessels in melanoma patients. To provide excitation wavelengths of 680 nm and 850 nm, a tunable optical parametric oscillator laser (680 to 970 nm, 20-Hz pulse repetition rate) was used and the excitation wavelength can be manually switched. To ensure the safety of patients, the laser beam was coupled into a fiber optical bundle. The optical fiber bundle bifurcated into two and laser beams coming out of the optical fiber bundles illuminate the imaging area at an angle of incidence of 30 degree relative to the imaging plane. The fluence on the tissue surface during the experiments was \( \sim 11 \text{ mJ/cm}^2 \), well within the 20 mJ/cm\(^2\) safety limit set by the American National Standards Institute. The generated photoacoustic waves were detected by a linear array ultrasonic transducer (Visualsonics, Inc., MS550D, 40 MHz center frequency, 33 MHz bandwidth, 256 elements). A four-to-one multiplexing was used in image acquisition because there were 256 elements in the linear array transducer and 64 channels in the data acquisition unit. In detail, for each laser pulse, the generated photoacoustic signals were captured sequentially by a quarter segment of the linear
array (i.e., elements 1 to 64, 65 to 128, 129 to 192, and 193 to 256). A 2D photoacoustic image was reconstructed with the universal back-projection algorithm after the data were acquired from all four quarter segments. The linear array ultrasonic transducer was connected to an imaging platform (Visualsonics Inc., Vevo LAZR), where the reconstructed photoacoustic images were displayed. The imaging frame rate was 5 frames/second, determined together by the 20 Hz laser repetition rate and four-to-one multiplexing in image acquisition. The frame rate can be adjusted by utilizing fewer elements in the linear array transducer, at the expense of smaller imaging FoV.

Figure 4.6. System schematic of LA-PAT for melanoma CTC imaging in patients.

Spatial resolutions of LA-PAT system

The spatial resolutions of the LA-PAT system were quantified by imaging a carbon fiber with a diameter of 6 µm. First, the carbon fiber was placed perpendicular to the imaging plane and a cross section image of the carbon fiber was acquired by LA-PAT (Fig. 4.7a). The photoacoustic amplitude profile of the carbon fiber along the axial direction was fitted to a Gaussian function and the full width half maximum (FWHM) of 43 µm was quantified as the axial resolution of the
LA-PAT system (Fig. 4.7b). The LA-PAT system was then scanned along the elevational direction with a step size of 20 μm. To quantify the lateral resolution, the MAP image along the axial direction of the carbon fiber was obtained (Fig. 4.7c). Similarly, the photoacoustic amplitude profile of the carbon fiber along the lateral direction was fitted to a Gaussian function and the FWHM of 94 μm was quantified as the lateral resolution of the LA-PAT system (Fig. 4.7d). To quantify the elevation resolution, the carbon fiber was placed along the lateral direction of the LA-PAT system. The LA-PAT system was then scanned along the elevational direction with a step size of 20 μm and an MAP image along the axial direction of the carbon fiber was obtained (Fig. 4.7e). The photoacoustic amplitude profile of the carbon fiber along the elevational direction was fitted to a Gaussian function and the FWHM of 633 μm was quantified as the elevational resolution of the LA-PAT system (Fig. 4.7f).

**Phantom preparation**

To mimic blood vessels of different diameters, silicone microtubes (Fisher Scientific) with various inner diameters of 0.3 mm, 0.5 mm, 0.64 mm, 0.76 mm, 1 mm, 1.5 mm, 2 mm, 2.64 mm, and 3.35 mm were perfused with bovine blood (905, Quad-Five). The microtubes were embedded at different depths in tissue-mimicking gelatin phantoms. To achieve optical scattering similar to that in biological tissue, 1% intralipid was added to the gelatin phantoms. Melanoma tumor cells suspended in bovine blood were pumped through the microtubes with a syringe. A syringe pump (BSP-99M, Braintree Scientific) controlled the blood flow speed.
Figure 4.7. Quantification of spatial resolutions of LA-PAT system. a, A cross section image of a carbon fiber by LA-PAT. b, The photoacoustic amplitude profile along the green dashed line in (a). The profile was fitted to a Gaussian function and the full width half maximum (FWHM) of 43 µm was quantified as the axial resolution of the LA-PAT system. c, An MAP image along the axial direction of the carbon fiber by LA-PAT. d, The photoacoustic amplitude profile along the green dashed line in (c). The profile was fitted to a Gaussian function and the FWHM of 94 µm was quantified as the lateral resolution of the LA-PAT system. e, An MAP image along the axial direction of the carbon fiber by LA-PAT. f, The photoacoustic amplitude profile along the green dashed line in (e). The profile was fitted to a Gaussian function and the FWHM of 633 µm was quantified as the elevational resolution of the LA-PAT system.
**Catching efficiency calculation**

To quantify the melanoma tumor cell catching efficiency of LA-PAT, ~1000 single melanoma tumor cells were suspended in 10 mL bovine blood and pumped through a microtube with a syringe. The melanoma tumor cells and blood were collected at the other end of the microtube. After ~50 melanoma tumor cells were captured by LA-PAT, the collected melanoma tumor cells and blood mixture were examined with an optical microscope to count the number of melanoma tumor cells pumped through the microtube. The catching efficiency $E$ is defined as

$$E = \frac{n}{N}.$$  \hspace{1cm} (4.2)

Here, $n$ stands for the number of melanoma tumor cells capture by LA-PAT and $N$ stands for the number of melanoma tumor cells pumped through the microtube.

**Imaging procedure for melanoma patients and healthy volunteers**

After informed consent was obtained, the patients and volunteers were asked to sit down or lie down in a comfortable position on the patient chair or bed. The protocol was approved by the Institutional Review Board at Washington University in St. Louis. Depending on the location of primary melanomas, either the cephalic veins or the small saphenous veins were imaged. First, an excitation wavelength of 850 nm was used to locate the target vein and proper imaging segment. Then, we switched to 680 nm to detect the melanoma CTCs and each patient was usually imaged for 20 minutes. During the entire imaging session, laser safety glasses with optical density greater than 5 at 680 nm and 850 nm were required to ensure the safety of patients.

**Flow speed of melanoma tumor cells**
Figure 4.8. Estimation of the melanoma CTC flow speed. a, A representative photoacoustic image of a single melanoma CTC in a patient. b, A representative photoacoustic image of a melanoma CTC cluster in the patient. c, The time trace plot of each pixel along the yellow dashed line in (a) in the space-time domain. The slope of the red solid line, computed by linear fitting as 10.3 mm/s, represents the flow speed of the single melanoma CTC. d, The time trace plot of each pixel along the yellow dashed line in (b) in the space-time domain. The slope of the red solid line, computed by linear fitting as 5.4 mm/s, represents the flow speed of the melanoma CTC cluster.

To make robust measurement of the flow speed of melanoma tumor cells, we converted their motions to the space-time domain. The time traces of each pixel along the yellow dashed line in Figure 4.8a and b were extracted and the signals were shown as an image in the space-time domain (Fig. 4.8a and b). The data were fitted to a linear function and the slope of the linear function in the space-time domain was used to calculate the speed of the melanoma CTC.

Detection sensitivity of melanoma CTC by LA-PAT
The ability to visualize a melanoma tumor cell in blood depends on the CNR, defined as

$$\text{CNR} = \frac{\Delta PA}{\sigma_{PA}}.$$  \hspace{1cm} (4.3)

Here, $\Delta PA$ stands for the photoacoustic amplitude variation due to the strong optical absorption by the single melanoma CTC, and $\sigma_{PA}$ stands for the standard deviation of the varying background photoacoustic amplitude due to the fluctuation of RBC numbers.

The photoacoustic amplitude variation $\Delta PA$ is defined as the photoacoustic amplitude difference between two adjacent voxels with and without a melanoma CTC

$$\Delta PA = PA_w - PA_{wo}. \hspace{1cm} (4.4)$$

And it can be further expressed as

$$\Delta PA = PA_{CTC} + PA_{RBC} \ast [RBC]_w - PA_{RBC} \ast [RBC]_{wo}. \hspace{1cm} (4.5)$$

Here $PA_{CTC}$ stands for the photoacoustic signal from one melanoma CTC and $PA_{RBC}$ stands for the photoacoustic signal from one RBC. $[RBC]_w$ and $[RBC]_{wo}$ stand for the number of RBCs in a resolution voxel with and without a melanoma CTC.

Because the fluctuation of RBC number in each resolution voxel follows Passion distribution, the noise $\sigma_{PA}$ can be expressed as

$$\sigma_{PA} = PA_{RBC} \ast \sqrt{[RBC]_{wo}}. \hspace{1cm} (4.6)$$

Combining equations (3) and (4), the CNR can be expressed as
\[
\text{CNR} = \frac{PA_{CTC} + PA_{RBC} \cdot [RBC]_w - PA_{RBC} \cdot [RBC]_{wo}}{PA_{RBC} \cdot \sqrt{[RBC]_{wo}}}. \quad (4.7)
\]

It can be further simplified by canceling \(PA_{RBC}\)

\[
\text{CNR} = \frac{PA_{CTC}/PA_{RBC} + [RBC]_w - [RBC]_{wo}}{\sqrt{[RBC]_{wo}}}. \quad (4.8)
\]

The photoacoustic amplitude ratio between one melanoma CTC and one RBC is determined by two factors, the absorption volume and absorption coefficient at the excitation wavelength. The volume ratio between a melanoma CTC and a RBC is typically \(\sim 10\). By careful analysis of the absorption spectra of melanin (major optical absorber in melanoma CTCs) and hemoglobin (major optical absorber in RBCs), the maximum absorption coefficient ratio is \(\sim 150\) (Fig. 4.5). So the typical value for \(PA_{CTC}/PA_{RBC}\) is \(\sim 1500\). Because a melanoma CTC only takes up the space for \(\sim 10\) RBCs, the difference between \([RBC]_w\) and \([RBC]_{wo}\) is \(\sim 10\), less than 1\% of \(PA_{CTC}/PA_{RBC}\), and can be neglected. So the CNR can be further expressed as

\[
\text{CNR} = \frac{PA_{CTC}/PA_{RBC}}{\sqrt{[RBC]_{wo}}}. \quad (4.9)
\]

The \([RBC]_{wo}\) is determined by the density of RBCs in blood and the resolution voxel size. The CNR, i.e. melanoma CTC detection sensitivity, can be enhanced by reducing the resolution voxel size using an ultrasonic transducer with higher frequency, at the expense of penetration depth. In this study, the spatial resolutions of LA-PAT system are 43 µm in axial direction, 94 µm in lateral direction and 633 µm in elevational direction. The resolution voxel size is \(\sim 2 \times 10^{-3} \mu \text{L}\). The typical RBC density is \(\sim 5 \times 10^6 \text{ cells/µL}\), so the \([RBC]_{wo}\) is \(\sim 1 \times 10^4 \text{ cells}\). Plugging in the values
of $PA_{CTC}/PA_{RBC}$ and $[RBC]_{wo}$, the typical CNR of a single melanoma CTC is $\sim 15$, which is much higher than the threshold of 2 and ensures the high detection sensitivity of melanoma CTCs by LA-PAT.

It is worth noting that in the model above, noises from resources other than the fluctuation of RBCs are not considered because they are usually much smaller than the photoacoustic amplitude variation due to the fluctuation of RBCs. However, as the imaging depth increases, the photoacoustic signals from RBCs attenuate and the noise $\sigma_{PA}$ can be expressed as:

$$\sigma_{PA} = PA_{RBC} \sqrt{[RBC]_{wo}} + \sigma_o. \quad (4.10)$$

Here $\sigma_o$ stands for noises coming from resources other than the fluctuation of RBCs, and it mainly comes from three resources: thermal acoustic noise from the medium, thermal noise from the ultrasonic transducer, and electronic noise from the amplifier. The CNR is then expressed as:

$$\text{CNR} = \frac{PA_{CTC}}{PA_{RBC} \sqrt{[RBC]_{wo}} + \sigma_o}. \quad (4.11)$$

The CNR decreases as the imaging depth increases.

**Estimation of total number of melanoma CTCs in patients**

The total number of melanoma CTCs $N_t$ in a patient can be estimated by the following equation assuming a random distribution of melanoma CTCs in blood.

$$N_t = N_d \frac{V_t}{V_f(t)}. \quad (4.12)$$
Here $N_d$ stands for the number of melanoma CTCs detected by LA-PAT, $V_t$ stands for the total blood volume in the patient, and $V_f(t)$ stands for the fresh (i.e. unrepeated) blood volume examined by LA-PAT in a given imaging time $t$.

To determine the fresh blood volume $V_f(t)$ we can sample during a given observation time $t$, we tackle the problem by assuming the followings: (1) After each complete cycle of the blood stream we randomly sample a $\gamma$ fraction of the entire blood volume; and (2) The blood is completely mixed at the end of each cycle. Each cycle is defined as the time it takes to pump the entire blood volume through the body

$$T = \frac{V_t}{Q_{CO}}. \quad (4.13)$$

Here $Q_{CO}$ stands for the average cardiac output (as a volumetric flow rate, or volume of blood per unit time) defined as

$$Q_{CO} = r_{HR} \times V_{SV}. \quad (4.14)$$

Here $r_{HR}$ stands for the heart rate of the patient and $V_{SV}$ represents for the stroke volume. So the cycle duration becomes

$$T = \frac{V_t}{r_{HR} \times V_{SV}}. \quad (4.15)$$

The sampling ratio $\gamma$ of the entire blood stream by the observed vein is defined as

$$\gamma = \frac{Q_0}{Q_{CO}} = \frac{Q_0 T}{V_t}. \quad (4.16)$$
Here $Q_O$ stands for the volumetric flow rate of blood observed by LA-PAT (the subscript “O” stands for “observed”). Because we want to capture the flowing melanoma CTC multiple times, $Q_O$ is always limited by the volumetric flow rate of the vein monitored and can be expressed as

$$Q_O = \pi \times \frac{D^2}{4} \times v. \quad (4.17)$$

Here $D$ stands for the diameter of the vein and $v$ stands for the average blood flow speed in the traverse direction.

During a given observation time $t$, if $t \leq T$, in which case there is no repeated blood sampled by LA-PAT, the fresh blood volume can be calculated by the following equation

$$V_f(t) = Q_O \times t. \quad (4.18)$$

If $t > T$, in which case multiple cycles occur during the observation time, at cycle $i$, the fresh blood volume we can observe is

$$V_i = Q_O T (1 - \gamma)^{i-1}. \quad (4.19)$$

Therefore, the total fresh blood volume we can observe during the observation time $t$ is

$$V_f(t) = \sum_{i=1}^{\left\lfloor \frac{t}{T} \right\rfloor} Q_O T (1 - \gamma)^{i-1} + Q_O \left( t - \left\lfloor \frac{t}{T} \right\rfloor T \right) (1 - \gamma)^{\left\lfloor \frac{t}{T} \right\rfloor}$$

$$= Q_O T \frac{1 - (1 - \gamma)^{\left\lfloor \frac{t}{T} \right\rfloor}}{\gamma} + Q_O \left( t - \left\lfloor \frac{t}{T} \right\rfloor T \right) (1 - \gamma)^{\left\lfloor \frac{t}{T} \right\rfloor} \quad (4.20)$$
\[ = V_t [1 - \left(1 - \frac{Q_o T}{V_t}\right)^\lfloor \frac{t}{T} \rfloor] + Q_o \left(t - \left\lfloor \frac{t}{T} \right\rfloor T\right) \left(1 - \frac{Q_o T}{V_t}\right)^\lfloor \frac{t}{T} \rfloor]. \]

Here \( [a] \) is the largest integer that is less than or equal to the real number \( a \). Briefly, the expression consists of two parts: the sum of a geometric series representing a decreasingly efficient sampling of the blood stream at each full cycle and a residual term at the last incomplete cycle. If we had infinite time, mathematically, we can ignore the residual term and take the infinite limit of the sum

\[ \lim_{t \to +\infty} V_f(t) = \lim_{N \to +\infty} V_t \left[1 - \left(1 - \frac{Q_o T}{V_t}\right)^N\right] = V_t. \]  

(4.21)

Therefore, the entire blood volume can be sampled. In summary, the total number of melanoma CTCs \( N_t \) can be estimated by

\[ N_t = N_d \frac{V_t}{V_f(t)} \]

(4.22)

\[ = \begin{cases} 
N_d \frac{V_t}{Q_o t} & \text{if } t < T \\
N_d \frac{V_t}{V_t(1 - \left(1 - \frac{Q_o T}{V_t}\right)^\lfloor \frac{t}{T} \rfloor)} + Q_o \left(t - \left\lfloor \frac{t}{T} \right\rfloor T\right) \left(1 - \frac{Q_o T}{V_t}\right)^\lfloor \frac{t}{T} \rfloor & \text{if } t > T
\end{cases} \]

If \( t < T \), in which case there is no repeated blood sampled by LA-PAT, the fresh blood volume can be calculated by the following equation:

\[ V_f(t) = Q_o \times t. \]  

(4.23)

If \( t > T \), in which case multiple cycles occur during the observation time, the fresh blood volume can be calculated by the following equation:
\[ V_f(t) = V_t (1 - (1 - \frac{Q_O T}{V_t})^t) + Q_O \left( t - \left\lfloor \frac{t}{T} \right\rfloor T \right) \left( 1 - \frac{Q_O T}{V_t} \right)^t. \] (4.24)

Here \( \lfloor a \rfloor \) is the largest integer that is less than or equal to the real number \( a \), \( Q_O \) stands for the volumetric flow rate of blood observed by LA-PAT (the subscript “O” stands for “observed”), and \( Q_O T / V_t \) stands for the sampling ratio of the entire blood stream by the observed vein. Because we want to capture the flowing melanoma CTC multiple times, \( Q_O \) is always limited by the volumetric flow rate of the vein monitored and can be expressed as

\[ Q_O = \pi \times \frac{D^2}{4} \times v. \] (4.25)

### 4.2.3 Results

**LA-PAT of melanoma tumor cells in phantoms**

To initially demonstrate the capability of LA-PAT, we first applied LA-PAT to image melanoma tumor cells in phantoms. To mimic melanoma CTCs in blood, cultured B16 melanoma tumor cells were suspended into bovine blood and pumped through microtubes. They were then imaged by LA-PAT with an excitation wavelength of 680 nm, which maximizes the contrast between melanoma tumor cells and blood (Fig. 4.5). Due to the strong optical absorption of melanin at 680 nm, the flowing melanoma tumor cells showed much stronger photoacoustic signals than the blood in the background and were detected by LA-PAT (Fig. 4.9a).

The ability to detect and visualize the melanoma tumor cells in the bloodstream depends on the CNR. To determine the maximum depth that melanoma tumor cells can be detected by LA-PAT, we studied how the imaging depth affected the CNR. The microtubes mimicking blood vessels
were embedded into scattering gelatin phantoms at different depths and melanoma tumor cells suspended in blood were pumped through the microtubes. We used LA-PAT to image the flowing melanoma tumor cells at different depths (Fig. 4.9a–d) and calculated the CNR at each depth (Fig. 4.9e). The CNR decreases with the increasing imaging depth because the acoustic signal attenuation. With a CNR threshold of 2, the maximum depth that a melanoma tumor cell can be detected by LA-PAT was ~3.5 mm. The depth is adequate for imaging most of the superficial veins in humans.

We also studied the melanoma tumor cell detection efficiency of LA-PAT. With the high detection sensitivity, there are two situations when a melanoma tumor cell can be missed by LA-PAT. In one situation, when the diameter of the blood vessel is larger than the slice thickness of the ultrasonic detection, i.e. elevational resolution, chances are that the melanoma tumor cells may flow outside of the FoV and will not be detected by LA-PAT. In the other situation, when the blood flow speed is so fast that melanoma tumor cells may flow through the imaging FoV before they are detected by LA-PAT. To quantify the melanoma tumor cell detection efficiency, we imaged melanoma tumor cells with different flow speeds in microtubes with different diameters by LA-PAT and calculated the detection efficiencies at different parameter combinations (Fig. 4.9f). The results show that the detecting efficiency decreases with increasing blood flow speed and vessel diameter. The detection efficiency curves may serve as a reference to estimate the total number of melanoma CTCs based on the number detected by LA-PAT.
Figure 4.9. LA-PAT of single melanoma tumor cells in phantoms. a, Photoacoustic snapshots of a single melanoma tumor cell 0 mm deep in phantom with a CNR of 16.4. The yellow arrows show the microtube boundaries. The red arrows highlight the flowing single melanoma tumor cell. b, Photoacoustic snapshots of a single melanoma tumor cell 1.5 mm deep in phantom with a CNR of 14.2. The yellow arrows show the microtube boundaries. The red arrows highlight the flowing single melanoma tumor cell. c, Photoacoustic snapshots of a single melanoma tumor cell 3 mm deep in phantom with a CNR of 4.3. The yellow arrows show the microtube boundaries. The red arrows highlight the flowing single melanoma tumor cell. d, Photoacoustic snapshots of the phantom at depth 4 mm. No melanoma tumor cells could be detected. e, CNRs of the melanoma tumor cells in photoacoustic images degrade with increasing imaging depth. With a CNR threshold of 2, the maximum depth that a melanoma tumor cell can be detected was ~3.5 mm. f, The melanoma tumor cell detection efficiency of LA-PAT decreases with increasing blood flow speed and vessel diameter.
LA-PAT of a single melanoma CTC and a melanoma CTC cluster in Patient 1

In the first case, we imaged a patient who had recurrent metastatic melanoma in her right lower extremity and went through multiple resections for in-transit metastases (Table 4.1). We imaged the small saphenous vein in the right leg of the patient. The vein had a diameter of ~1.7 mm and was ~3.1 mm deep from skin surface to lower vessel boundary. In the photoacoustic images, structures including skin, vessel boundaries, and subcutaneous fat layer were clearly resolved (Fig. 4.10a).

**Figure 4.10.** LA-PAT of a single melanoma CTC in patient 1. a, Photoacoustic snapshots of the single melanoma CTC in the patient. The yellow arrows show the structures including skin, vessel boundaries, and subcutaneous fat layer. The red arrows highlight the flowing single melanoma CTC. b, Differential photoacoustic images showing only the flowing single melanoma CTC. c, Differential photoacoustic images (b) superimposed on structural photoacoustic images (a), highlighting the flowing single melanoma CTC.
In one imaging session, a single melanoma CTC was detected in this patient by LA-PAT. The CNR of the melanoma CTC was ~12.6. The single melanoma CTC was captured 5 times while it flew through the entire imaging FoV (Fig. 4.10a). To highlight the flowing melanoma CTC, differential photoacoustic images were obtained (Fig. 4.10b). The differential photoacoustic images were then superimposed on the structural images to better illustrate how the single melanoma CTC flew through the entire imaging FoV (Fig. 4.10c). By tracking the melanoma CTC and analyzing the movement in the space-time domain, the flow speed of the melanoma CTC was computed (Fig. 4.8). The flow speed of this single melanoma CTC was 10.3 mm/s. Although the flow speed of the melanoma CTC does not necessarily match the flow speed of blood, it is a close approximation given the low concentration of melanoma CTC and the large vessel diameter.

In another imaging session, a melanoma CTC cluster was detected in this patient by LA-PAT. The CNR of the melanoma CTC cluster was ~11.6. The melanoma CTC cluster was captured 9 times when it flew through the entire imaging FoV (Fig. 4.11a). It was captured more times than the single melanoma CTC because of its smaller flow speed. Similarly, to highlight the flowing melanoma CTC cluster, differential photoacoustic images were obtained (Fig. 4.11b). The differential photoacoustic images were then superimposed on the structural images to better illustrate how the melanoma CTC cluster flew through the entire imaging FoV (Fig. 4.11c). Applying the same method as in the single melanoma CTC, the flow speed of the melanoma CTC cluster was computed to be 5.4 mm/s. There are two possible factors contributing to the smaller flow speed of the CTC cluster compared to the single melanoma CTC. First is the location in the blood vessel. The melanoma CTC cluster was closer to the vessel boundary while the single melanoma CTC was closer to the vessel central line. Assuming a parabolic flow model, the flow speed near the vessel central line may be larger compared to that near the vessel boundary. Second
is the CTC size. Because the melanoma CTCs were driven by the blood flow, a smaller single melanoma CTC could be driven to a higher flow speed than a CTC cluster.

**Figure 4.11.** LA-PAT of a melanoma CTC cluster in patient 1. a, Photoacoustic snapshots of the melanoma CTC cluster in the patient. The yellow arrows show the structures including skin, vessel boundaries, and subcutaneous fat layer. The red arrows highlight the flowing melanoma CTC cluster. b, Differential photoacoustic images showing only the flowing melanoma CTC cluster. c, Differential photoacoustic images (b) superimposed on structural photoacoustic images (a), highlighting the flowing melanoma CTC cluster.

**LA-PAT of a single melanoma CTC in Patient 2**

In the second case, we imaged a patient who had metastatic melanoma in his brain and heart while there was no known primary melanoma (Table 4.1). We imaged the cephalic vein in the right arm of the patient. The vein had a diameter of ~1.0 mm and was ~3.2 mm deep from skin surface to
lower vessel boundary. In the photoacoustic images, structures including skin, vessel boundaries, and subcutaneous fat layer were clearly resolved (Fig. 4.12a).

![Figure 4.12. LA-PAT of a single melanoma CTC in patient 2. a, Photoacoustic snapshots of the single melanoma CTC in the patient. The yellow arrows show the structures including skin, vessel boundaries, and subcutaneous fat layer. The red arrows highlight the flowing single melanoma CTC. b, Differential photoacoustic images showing only the flowing single melanoma CTC. c, Differential photoacoustic images (b) superimposed on structural photoacoustic images (a), highlighting the flowing single melanoma CTC.]

In one imaging session, a single melanoma CTC was detected in this patient by LA-PAT. The CNR of the melanoma CTC was ~9.4. The single melanoma CTC was captured 5 times while it flew through the entire imaging FoV (Fig. 4.12a). Differential photoacoustic images were obtained to highlight the flowing melanoma CTC (Fig. 4.12b). To better illustrate how the melanoma CTC flew through the entire imaging FoV, the differential photoacoustic images were then
superimposed on the structural images (Fig. 4.12c). Using the same method of converting the movement of the single melanoma CTC to the space-time domain, the flow speed of the melanoma CTC was estimated to be 9.6 mm/s.

**LA-PAT of a melanoma CTC cluster in Patient 3**

![Image](image.png)

**Figure 4.13.** LA-PAT of a melanoma CTC cluster in patient 3. a, Photoacoustic snapshots of the flowing melanoma CTC cluster in the patient. The yellow arrows show the structures including skin and vessel boundaries. The red arrows highlight the flowing melanoma CTC cluster. b, Differential photoacoustic images showing only the flowing melanoma CTC cluster. c, Differential photoacoustic images (b) superimposed on structural photoacoustic images (a), highlighting the flowing melanoma CTC cluster.

In a third case, we imaged a patient who had recurrent metastatic melanoma in her scalp (Table 4.1). We imaged the cephalic vein in the right arm of the patient. The vein had a diameter of ~1.0 mm and was ~2.2 mm deep from skin surface to lower vessel boundary. In the photoacoustic images, structures including skin and vessel boundaries were clearly resolved (Fig. 4.13a). In one imaging session, a melanoma CTC cluster was detected in this patient by LA-PAT. The CNR of the melanoma CTC cluster was ~12.5. The melanoma CTC cluster was captured 3 times while it
flew through the entire imaging FoV (Fig. 4.13a). Again, to highlight the flowing melanoma CTC, differential photoacoustic images were obtained (Fig. 4.13b). The differential photoacoustic images were then superimposed on the structural images to better illustrate how the melanoma CTC cluster flew through the entire imaging FoV (Fig. 4.13c). Using the same method as above the flow speed of the melanoma CTC cluster was estimated to be 8.6 mm/s.

**CNR analysis of melanoma CTCs**

![CNR analysis of melanoma CTCs](image)

**Figure 4.14.** CNRs comparison between melanoma CTCs and phantoms. a, A representative photoacoustic image of the phantom without melanoma tumor cells. b, A representative photoacoustic image of the phantom with a melanoma tumor cell. The flowing object with the highest CNR was identified as a melanoma tumor cell. c, CNRs of melanoma tumor cells were significantly higher than peak CNRs in the microtube in phantom ($n = 105$ melanoma tumor cell events, $***p < 0.001$).

To fully validate that the flowing objects detected by LA-PAT were melanoma tumor cells, we did a thorough CNR analysis of the flowing objects in phantoms and in patients. In phantoms, blood without melanoma tumor cells were first pumped through the microtube and imaged by LA-PAT.
No flowing objects were detected in the photoacoustic images (Fig. 4.14a). We searched the entire area within the microtube to find the pixel with the highest CNR, referred as the peak CNR in the microtube. Then, melanoma tumor cells suspended in blood were pumped through the microtube and imaged by LA-PAT. Flowing objects were detected in the photoacoustic images (Fig. 4.14b). For each flowing object, we calculated the average CNR of all its appearances. The CNRs of the flowing objects were significantly higher than the peak CNRs in the microtube (Fig. 4.14c). Because only melanoma tumor cells were added to the blood compared to the control experiment, the flowing objects with the high CNRs must be the melanoma tumor cells. At an excitation wavelength of 680 nm, only melanin, with much stronger optical absorption than blood, can generate such high photoacoustic signals. In addition, the average CNR of the flowing objects was ~14.7, close the theoretically estimated value of 15.

In patients, to further validate the flowing objects in blood vessels detected by LA-PAT were melanoma CTCs, we did a similar CNR analysis as in phantom, with more types of control groups. First, we calculated the average CNR of each flowing object in the photoacoustic images (Fig. 4.15a). For the first type of control group, we compared the CNRs of the flowing objects with the peak CNRs in the blood vessels in patients in whom flowing objects were detected, referred as CTC-positive patients. In the frames without flowing objects, we searched the entire area within the blood vessel and found the pixel with the highest CNR (Fig. 4.15b). The CNRs of the flowing objects were significantly higher than the peak CNRs in the blood vessels in CTC-positive patients (Fig. 4.15e). For the second type of control group, we compared the CNRs of the flowing objects with the peak CNRs in the blood vessels in patients in whom no flowing objects were detected, referred as CTC-negative patients. Similarly, we searched the entire area within the blood vessel and found the pixel with the highest CNR (Fig. 4.15c). The CNRs of the flowing objects were also
significantly higher than the peak CNRs in the blood vessels in CTC-negative patients (Fig. 4.15f). For the third type of control group, we compared the CNRs of the flowing objects with the peak CNRs in the blood vessels in healthy volunteers (Fig. 4.15d). A similar result that the CNRs of the flowing objects were significantly higher than the peak CNRs in the blood vessels in healthy volunteers further showed that only melanoma CTCs can generate such high photoacoustic signals in the blood vessels.

![Figure 4.15. CNR comparison between melanoma CTCs and blood vessels in vivo.](image)

- a, A representative photoacoustic image of a melanoma CTC in a blood vessel in a patient, referred as a positive patient. The flowing object with the highest CNR was identified as a melanoma CTC.
- b, A representative photoacoustic image without melanoma CTCs in the positive patient.
- c, A representative photoacoustic image in a patient in whom no melanoma CTCs were detected, referred as a negative patient.
- d, A representative photoacoustic image in a healthy volunteer.
- e, CNRs of melanoma CTCs were significantly higher than peak CNRs in blood vessels in positive patients ($n = 23$ melanoma CTC events, $***p < 0.001$).
- f, CNRs of melanoma CTCs in positive patients were significantly higher than peak CNRs in blood vessels in negative patients ($n = 23$ melanoma CTC events, $***p < 0.001$).
- g, CNRs of melanoma CTCs were significantly higher than peak CNRs in blood vessels in health volunteers ($n = 23$ melanoma CTC events, $**p < 0.01$).
Estimation of the total number of CTCs and the clinical relevance of CTC imaging in patients

To demonstrate the clinical translational potential of melanoma CTC imaging by LA-PAT, we estimated the total number of CTC in patients based on the imaging results and tracked the statuses of the patients after photoacoustic imaging (Table 4.1). Based on the number of melanoma CTC detected and the effective blood volume screened by LA-PAT, the total number of melanoma CTCs in the three patients were estimated to be 214, 333, and 370, in the same range as reported previously using ex vivo CTC detection methods\(^\text{170,171}\). We then monitored the statuses of patients imaged by LA-PAT for up to one year to see whether the detection of CTCs is predictive of patient response to treatment and disease progression\(^\text{172}\). Because the clinical meaning of the melanoma CTC imaging results is presently unclear, the data was strictly protected from patients and their treating physicians and did not affect any clinical decisions. The follow-up results showed that 2 out of 3 CTC-positive patients had disease progression despite on systemic therapy. In contrast, only 3 out of 13 CTC-negative patients had disease progression (Table 4.2). The higher disease progression percentage of the CTC-positive patients over the CTC-negative may indicate that the number of CTCs detected by LA-PAT is possibly predictive of treatment response and prognostic outcome.
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Clinical Stage at Time of Scan</th>
<th>CTC Events Imaged by LA-PAT</th>
<th>Effective Blood Volume Examined</th>
<th>Estimated Total CTC Count</th>
<th>Clinical Status at Time of Scan</th>
<th>Follow-up Clinical Status</th>
<th>Time of Follow Up Post LA-PAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3</td>
<td>IIIb</td>
<td>2</td>
<td>46.8 mL</td>
<td>214</td>
<td>• Had recurrent metastatic melanoma in right lower extremity • Went through multiple surgical resections of in-transit cutaneous metastases • Received systemic immunotherapy • Showed no evidence of systemic disease at time of imaging</td>
<td>Disease Progression on Therapy</td>
<td>12 months</td>
</tr>
<tr>
<td>M11</td>
<td>IV</td>
<td>1</td>
<td>15.0 mL</td>
<td>333</td>
<td>• Had no known primary melanoma • Received targeted and systemic immunotherapy • Had metastatic melanoma in brain and heart at time of imaging</td>
<td>Disease Progression on Therapy</td>
<td>8 months</td>
</tr>
<tr>
<td>M14</td>
<td>IIIc</td>
<td>1</td>
<td>13.5 mL</td>
<td>370</td>
<td>• Had no known primary melanoma • Received course of local immunotherapy and started systemic immunotherapy • Showed interval progression disease at time of imaging</td>
<td>No disease Progression on Therapy</td>
<td>7 months</td>
</tr>
</tbody>
</table>
Table 4.2: List of melanoma patients imaged by LA-PAT

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Gender</th>
<th>Photoacoustic Imaging Result</th>
<th>Clinical Stage at Time of Scan</th>
<th>Follow-up Clinical Status</th>
<th>Time of Follow Up Post LA-PAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>Male</td>
<td>Negative</td>
<td>IV</td>
<td>Disease Progression on Therapy</td>
<td>11 months</td>
</tr>
<tr>
<td>M2</td>
<td>Male</td>
<td>Negative</td>
<td>IIIc</td>
<td>No Disease Progression off Therapy</td>
<td>4 months</td>
</tr>
<tr>
<td>M3</td>
<td>Female</td>
<td>Positive</td>
<td>IIIb</td>
<td>Disease Progression on Therapy</td>
<td>12 months</td>
</tr>
<tr>
<td>M4</td>
<td>Female</td>
<td>Negative</td>
<td>IIIa</td>
<td>No Disease Progression off Therapy</td>
<td>11 months</td>
</tr>
<tr>
<td>M5</td>
<td>Female</td>
<td>Negative</td>
<td>IV</td>
<td>No Disease Progression on Therapy</td>
<td>9 months</td>
</tr>
<tr>
<td>M6</td>
<td>Female</td>
<td>Negative</td>
<td>III</td>
<td>Disease Progression on Therapy</td>
<td>10 months</td>
</tr>
<tr>
<td>M7</td>
<td>Female</td>
<td>Negative</td>
<td>IV</td>
<td>No Disease Progression off Therapy</td>
<td>5 months</td>
</tr>
<tr>
<td>ID</td>
<td>Gender</td>
<td>Status</td>
<td>Stage</td>
<td>Disease Progression</td>
<td>Time</td>
</tr>
<tr>
<td>-----</td>
<td>--------</td>
<td>--------</td>
<td>-------</td>
<td>---------------------</td>
<td>--------</td>
</tr>
<tr>
<td>M8</td>
<td>Male</td>
<td>Negative</td>
<td>IV</td>
<td>Disease Progression on Therapy/Death</td>
<td>8 months</td>
</tr>
<tr>
<td>M9</td>
<td>Female</td>
<td>Negative</td>
<td>III</td>
<td>No Disease Progression on Therapy</td>
<td>8 months</td>
</tr>
<tr>
<td>M10</td>
<td>Female</td>
<td>Negative</td>
<td>IV</td>
<td>No Disease Progression off Therapy</td>
<td>7 months</td>
</tr>
<tr>
<td>M11</td>
<td>Male</td>
<td>Positive</td>
<td>IV</td>
<td>Disease Progression on Therapy</td>
<td>8 months</td>
</tr>
<tr>
<td>M12</td>
<td>Female</td>
<td>Negative</td>
<td>IV</td>
<td>No Disease Progression off Therapy</td>
<td>7 months</td>
</tr>
<tr>
<td>M13</td>
<td>Female</td>
<td>Negative</td>
<td>III</td>
<td>No Disease Progression on Therapy</td>
<td>8 months</td>
</tr>
<tr>
<td>M14</td>
<td>Female</td>
<td>Positive</td>
<td>IIIC</td>
<td>No Disease Progression on Therapy</td>
<td>7 months</td>
</tr>
<tr>
<td>M15</td>
<td>Female</td>
<td>Negative</td>
<td>IV</td>
<td>No Disease Progression off Therapy</td>
<td>6 months</td>
</tr>
<tr>
<td>M16</td>
<td>Male</td>
<td>Negative</td>
<td>IV</td>
<td>Disease Progression off Therapy</td>
<td>7 months</td>
</tr>
</tbody>
</table>
4.2.4 Discussion

Ultrasonically imaging optical absorption, PAT is inherently suitable for melanoma imaging and offers four unique advantages. (1) PAT has a 100% relative sensitivity to optical absorption (i.e., a given percentage change in the optical absorption coefficient yields the same percentage change in the photoacoustic amplitude) thus allowing high-sensitivity detection of strongly absorbing targets\(^40\); (2) Melanoma tumor cells express high-concentration melanin, which has much stronger optical absorption than hemoglobin in the red to NIR spectrum and serves as a perfect contrast for high-sensitivity photoacoustic detection of melanoma CTCs in the bloodstream\(^{169}\); (3) Taking advantage of the ultrasonic transparency of biological tissue, PAT achieves high resolution at depths, thus providing access to screen large blood vessels for high-throughput melanoma CTC detection; (4) LA-PAT, the linear-array-based implementation of PAT, well aligns the imaging FoV with the blood vessel, which maximizes the blood imaging efficiency (i.e. the ratio between the volume of blood examined and the entire volume imaged by LA-PAT) and the chance to detect melanoma CTCs in patients \textit{in vivo}. Owing to these advantages of LA-PAT, we successfully achieved label-free high-throughput imaging of melanoma CTCs in patients \textit{in vivo} for the first time. After initially demonstrating the capability of LA-PAT to detect and characterize melanoma CTCs in phantoms, we successfully imaged 16 Stage III and IV melanoma patients and detected CTCs in 3 of them. By optimizing the excitation wavelength, we maximized the CNR of melanoma CTC in LA-PAT, thus achieving the highest melanoma CTC detection sensitivity. By carefully selecting the imaging sites and target blood vessels, we tried to maximize our chances to detect melanoma CTCs in patients.

To successfully detect the rare melanoma CTCs in patients \textit{in vivo}, the key is to differentiate the single melanoma CTCs from the blood background, which mainly consists of red blood cells
(RBCs). The melanoma CTC detection sensitivity of LA-PAT ultimately depends on the CNR, which characterizes the ability to visualize a melanoma CTC from the huge number of RBCs in the background. The CNR can be expressed as following when ignoring noises other than the fluctuation of RBC number in each resolution voxel

\[
\text{CNR} = \frac{PA_{CTC}/PA_{RBC}}{\sqrt{[RBC]_{wo}}}. \tag{4.26}
\]

The photoacoustic signal ratio of a melanoma CTC and a RBC is determined by the optical fluence, the absorption coefficient ratio, and the absorption volume ratio, among which the absorption coefficient ratio can be maximized by optimizing the illumination wavelength. The number of RBCs in each resolution voxel is determined by the RBC density and the resolution voxel size, which is affected by the ultrasonic transducer frequency. A higher ultrasonic frequency gains the melanoma CTC detection sensitivity by reducing the resolution voxel size, at the expense of shallower penetration depth. In our study, we not only optimized the illumination wavelength by analyzing the optical absorption spectra of melanin and venous blood, but also considered the tradeoff between imaging depth and resolution voxel size. An excitation wavelength of 680 nm not only maximizes the photoacoustic signal ratio of a melanoma CTC and a RBC, but also allows sufficient light penetration in biological tissue to access large blood vessels at depths. However, there is a minor drawback that locating the blood vessels in patients may be difficult given the weak optical absorption of hemoglobin at 680 nm. This was overcome by applying an excitation wavelength of 850 nm to locate the blood vessels in patients before switching to 680 nm for melanoma CTC imaging. A 40-MHz center frequency of the ultrasonic transducer array was chosen to have sufficient sensitivity to detect single melanoma CTCs while the imaging depth is
adequate to screen large blood vessels for high-throughput melanoma CTC imaging. On one hand, a lower ultrasonic frequency, such as 30 MHz, can achieve deeper penetration in biological tissue and the required sensitivity to detect single melanoma CTCs at tissue surface. However, as the depth of the target blood vessel increases, the melanoma CTC detection sensitivity drops quickly, and it fails to detect single melanoma CTCs in deeper blood vessels. On the other hand, a higher ultrasonic frequency, such as 50 MHz, can achieve an even higher melanoma CTC detection sensitivity, but suffers from stronger acoustic attenuation and cannot reach the deep target vessels for high-throughput melanoma CTC imaging. So, an illumination wavelength of 680 nm and a detection center frequency of 40 MHz is the perfect combination to enable label-free high-throughput melanoma CTC imaging in patients in vivo.

It is worth noting that there is a type of skin cancer in which the cells do not highly express melanin. This type of skin cancer, namely amelanotic melanoma, only makes up ~5% of the total melanoma cases. Since some amelanotic melanoma cells also produce a small amount of melanin, the high detection sensitivity of LA-PAT may still help to detect these cells. As an alternative, other tumor biomarkers can be used detect CTCs originating from amelanotic melanoma.

The functionality of LA-PAT can be further boosted. The melanoma CTC detection sensitivity can be improved by spectrally unmixing melanoma CTCs and RBCs. To achieve spectral unmixing, two or more illumination wavelengths should be employed. The blood screening throughout can be enhanced by optimizing the blood imaging efficiency. To optimize the blood imaging efficiency, a larger blood vessel should be targeted, and the imaging frame rate can be adjusted to better match the blood flow speed, thus minimizing the blood examined repeatedly. Moreover, by applying a second laser pulse with higher energy immediately after identifying a melanoma CTC,
we can achieve selective laser fragmentation of melanoma CTCs and induce immunoresponse with the released antigens to further treat the primary tumor, CTCs and metastases\textsuperscript{155,175}. In addition to CTC detection, LA-PAT can also measure other parameters, including depth, pH, oxygen saturation, stiffness, glucose metabolism, angiogenesis, and therapy response, to provide comprehensive information on tumor\textsuperscript{41,58,62,176–179}.

Label-free high-throughput imaging of melanoma CTCs by LA-PAT in patients holds great promise for clinical translation. First, the label-free noninvasive nature of LA-PAT provides an inherent advantage for clinical translation. Second, the high blood screening throughout and melanoma CTC detection sensitivity give it the potential as a reliable routine clinical tool for melanoma progression monitoring and therapy evaluation. Third, although we have focused on melanoma in this work, LA-PAT can image CTCs of other types of cancer by studying their absorption spectra and selecting proper illumination wavelengths. Fourth, it is not limited to CTCs in the bloodstream. LA-PAT can be applied to image CTCs in the lymphatic system\textsuperscript{180}, in which the detection sensitivity can be even higher given the low optical absorption of lymph. Last, our results showed that the CTC-positive patients imaged by LA-PAT had greater tendency for disease progression than the CTC-negative patients, further indicating its clinical translational potential.

In summary, we have developed and optimized LA-PAT and achieved melanoma CTCs imaging in patients \textit{in vivo} for the first time. We demonstrated its abilities in phantoms and clinical feasibility in melanoma patients. Follow-up monitoring of the melanoma patients further revealed the clinical translational potential of LA-PAT. Our initial success in melanoma patients not only showed its feasibility detect and characterize melanoma CTCs \textit{in vivo}, but also established the full
potential of LA-PAT as an important clinical tool to incorporate CTC detection for cancer diagnosis and therapy.
Chapter 5 Summary

In this dissertation, I have further advanced PAT by developing photoacoustic elastography to enable elasticity imaging with PAT, improving the performance of PAM for various applications, and accomplishing label-free high-throughput photoacoustic imaging of melanoma CTCs in patients in vivo.

In Chapter 2, I have developed three novel photoacoustic elasticity imaging techniques. First, I have developed VE-PAT capable of detecting blood vessel elasticity changes due to abnormal hemodynamic states by incorporating a linear-array-based photoacoustic imaging probe with a customized compression stage. We demonstrated the capability of VE-PAT by detecting vascular compliance changes caused by simulated thrombosis in phantoms and in a human subject in vivo. VE-PAT has potential for use in many clinical applications, including detection of deep venous thrombosis, characterization of vulnerable plaque, and evaluation of atherosclerotic tissues. Second, I have developed and demonstrated photoacoustic elastography on gelatin phantoms and in vivo. Lacking speckles, photoacoustic elastography can still measure tissue displacements using optical absorption contrast provided by abundant endogenous biomolecules, especially hemoglobin in red blood cells. Photoacoustic elastography has a 100% relative sensitivity to optical absorption contrast, which means a given percentage change in the optical absorption coefficient yields the same percentage change in the photoacoustic signal amplitude. Photoacoustic elastography is well suited for mapping the elastic properties of diseased tissues with highly vascularized structures, such as carcinoma and glioblastoma. At longer wavelengths where water and lipids have relatively strong absorption, photoacoustic elastography can potentially map the elastic properties of tissues by using water and lipids as the contrast. Third, I have further
enhanced the capability of photoacoustic elastography by developing QPAE. Surpassing conventional photoacoustic elastography, QPAE achieves quantification of absolute Young’s modulus instead of relative values by utilizing a piece of silicone rubber with known stress–strain behavior as a reference stress sensor. An important underlying assumption in QPAE is that the compression stress is uniform along each A-line (the depth). During the phantom and in vivo experiments, to maintain the validity of the assumption by eliminating boundary effects, the compression plate was made much larger than the object to be imaged. Although internal structures of the object can also affect the assumption, the method should remain sufficient for tissues with laminar structures, such as the skin and muscles\textsuperscript{183}. To obtain more accurate results without the assumption of uniform stress along depth, an inverse problem for the three-dimensional distribution of stress within the object needs to be solved\textsuperscript{184}.

In Chapter 3, I have further boosted the performance of PAM systems. First, we have developed and demonstrated NIR-OR-PAM that offers new imaging contrast and better resolution at greater imaging depths than VIS-OR-PAM. By employing NIR light with our fast-scanning OR-PAM\textsuperscript{107} and incorporating multiple wavelengths\textsuperscript{21}, we should be able to measure hemodynamic parameters, including blood flow speed, the oxygen saturation of hemoglobin, and the metabolic rate of oxygen\textsuperscript{111}. Then, we have upgraded an HF-OR-PAM to image cerebral microvascular responses during epileptic seizure propagation, including changes in total hemoglobin concentration and vessel diameters, in mouse brains in vivo. Hemodynamic responses to epileptic seizure propagation were imaged with high spatial (~3 μm lateral resolution) and temporal (~0.5 Hz volumetric frame rate) resolutions simultaneously for the first time. With HF-OR-PAM, we were able to capture the waves of photoacoustic signal changes in response to epileptic seizure propagation. Different propagation patterns of the waves were observed. Since the waves of photoacoustic signal changes
observed were induced by the epileptic waves of neuron cluster hyper-excitation, the observed hemodynamic changes reflected the elevated neural activities. Possible reasons for the various behaviors of the waves are the different strengths of neural activities and randomness in neuron cluster triggering. Blood vessels dilated in response to epileptic seizures. The magnitude of vessel diameter changes decreased with the increasing distance from the 4-AP injection site. All the changes detected by HF-OR-PAM correlated well with EEG signals recorded simultaneously.

Last, we have developed and optimized SCM-PAM, which combines a wide-field fast-scanning functional OR-PAM with a high-density microwell array, for label-free high-throughput single-cell imaging of intratumoral metabolic heterogeneity. We have demonstrated its capability by measuring the single-cell OCR distributions of cultured cells and showed its potential for clinical translation by imaging intratumoral metabolic heterogeneity in breast cancer patient specimens. In addition, with rich optical absorption contrast, the wide-field fast-scanning functional OR-PAM subsystem of SCM-PAM can provide multiple dimensions of information about tumors, including angiogenesis, metastasis, and drug responses, along with intratumoral heterogeneity. With its unique capability for label-free high-throughput single-cell OCR measurement and the potential of providing multidimensional information about tumors, SCM-PAM is a promising tool for both fundamental cancer research and clinical personalized cancer therapy.

In Chapter 4, we have accomplished label-free high-throughput imaging of melanoma CTCs in patients in vivo with LA-PAT systems. First, we applied an LA-PAT system with a 21 MHz center frequency to detect and quantify melanoma CTC clusters in rats in vivo. Since the spatial resolutions of the 21MHz LA-PAT were not enough to resolve single melanoma tumor cells, the numbers of cells in the CTC clusters were quantified based on the CNRs. A linear relationship between the CNRs and the numbers of cells in the CTC clusters was found and verified. In ex vivo
experiments, LA-PAT quantified the numbers of cells in the CTC clusters, and the results were validated by an optical microscope. In experiments in vivo, LA-PAT detected and quantified melanoma CTC clusters immediately after injection, as well as when they were circulating in the rat bloodstreams. The results by LA-PAT also showed that larger CTC clusters have faster clearance rates. Then, we have developed a LA-PAT system with a 40 MHz center frequency. We initially demonstrated its capability by imaging CTC-mimicking melanoma tumor cells in phantoms, and then successfully achieved melanoma CTC imaging in patients in vivo. Out of the 16 melanoma patients imaged, we detected melanoma CTCs in 3 of them. Follow-up monitoring of their statuses further showed the clinical translational potential of label-free high-throughput imaging of melanoma CTCs in patients in vivo.
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