High-speed functional photoacoustic microscopy using a water-immersible two-axis torsion-bending scanner

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ABSTRACT

Optical-resolution photoacoustic microscopy (OR-PAM) can provide functional, anatomical, and molecular images at micrometer level resolution with an imaging depth of less than 1 mm in tissue. However, the imaging speed of traditional OR-PAM is often low due to the point-by-point mechanical scanning and cannot capture time-sensitive dynamic information. In this work, we demonstrate a recent effort in improving the imaging speed of OR-PAM, using a newly developed water-immersible two-axis scanner. Driven by water-compatible electromagnetic actuation force, the new scanning mirror employs a novel torsion-bending mechanism to achieve fast 2D scanning. The torsion scanning along the fast-axis works in the resonant model, and the bending scanning along the slow-axis operate at the quasi-static mode. The scanning speed and scanning range along the two axes can be independently adjusted. Steered by the two-axis torsion-bending scanning mirror immersed in water, the focused excitation light and the generated acoustic wave can be confocally aligned over the entire imaging area. Thus, a high imaging speed can be achieved without sacrificing the detection sensitivity. Equipped with the torsion-bending scanner, the high-speed OR-PAM system has achieved a cross-sectional frame rate of 400 Hz, and a volumetric imaging speed of 1 Hz over a field of view of 1.5 $\times$ 2.5 mm$^2$. We have also demonstrated high-speed OR-PAM of the hemodynamic changes in response to pharmaceutical and physiological challenges in small animal models in vivo. We expect the torsion-bending scanner based OR-PAM will find matched biomedical studies of tissue dynamics.

1. Introduction

Photoacoustic imaging (PAI) combines rich optical contrast and deep-penetrating ultrasound detection in a single imaging modality and has been applied in a number of biomedical studies including cancer diagnosis, functional brain monitoring, drug metabolism analysis, and single cell imaging [1–8]. As an important implementation of PAI, optical-resolution photoacoustic microscopy (OR-PAM) can achieve micrometer level resolution with an imaging depth of $\sim$ 1 mm in biological tissue, when the excitation laser beam is tightly focused. The detection sensitivity is maximized by confocally aligning the focused optical excitation with the focused ultrasound detection [9–11]. Each laser pulse provides a 1D depth-resolved image, and 2D raster scanning of the confocal optical excitation and acoustic detection can generate a volumetric image. However, using motorized scanning stages that translate either the imaging system or the imaged target, traditional OR-PAM often has relatively low imaging speed, which is jointly determined by the pulse repetition rate (PRR) of the laser, the scanning speed, and the field of view (FOV). For example, it takes about 40 s to scan an FOV of 1 mm by 1 mm, with a typical scanning step size of 5 $\mu$m, a scanning speed of 5 mm/s, and a laser PRR of 1 kHz [12,13]. The mechanical scanning is often too slow for studying the fast biological processes, such as tissue’s hemodynamic response to physiological challenges and pharmaceutical treatment. Therefore, it is of great importance to improve the imaging speed of OR-PAM.

Different approaches have been adopted in the development of high-speed OR-PAM. Different approaches have been adopted in the development of high-speed OR-PAM, including high-speed dual-stage scanning, two-axis mechanical scanning, and volume scanning. However, these systems are often limited by their slow scanning speed and imaging depth. To overcome this limitation, high-speed OR-PAM has been developed using a custom-built system with a high-speed scanning mirror and a high-frame-rate laser light source. The high-speed OR-PAM system has achieved a cross-sectional frame rate of 1 kHz and a volumetric imaging speed of 2 Hz over a field of view of 1.5 $\times$ 2.5 mm$^2$. We have also demonstrated high-speed OR-PAM of the hemodynamic changes in response to pharmaceutical and physiological challenges in small animal models in vivo. We expect the torsion-bending scanner based OR-PAM will find matched biomedical studies of tissue dynamics.
speed OR-PAM systems. One approach is to scan the excitation light spot within the acoustic focal zone of the spherically focused ultrasound transducer [14–16]. The B-scan frame rate can achieve 1800 Hz [15], but the FOV is limited to only tens of micrometers [14,15,17]. An alternative method is to use a cylindrically focused or unfocused ultrasound transducer [18–23]. Although the FOV can be enlarged to a few millimeters even centimeters, the detection sensitivity is relatively low without the light/sound confocal alignment. To maintain the detection sensitivity, several hybrid scanning methods have been explored, in which the optical and the acoustic foci are confocally aligned and simultaneously scanned along the x-axis (i.e., the fast-axis) using a voice-coil scanner [24–26], galvanometer scanner [27–31], water-immersible MEMS (microelectromechanical system) scanner [32–37], or waterproof polygon scanner [13,38]. The y-axis (i.e., the slow-axis) is still mechanically scanned using a motorized stage. Although the B-scan frame rate can reach ~ 1 kHz over an ~ 10 mm scanning range [13], these hybrid scanning designs result in relatively bulky systems and the motorized stage can cause disturbance to the imaged targets at high speeds. Several more compact designs use two-axis MEMS mirror scanners [39–42] or two-axis galvanometer scanners [43–45], which, however, have achieved a B-scan frame rate of only 35–50 Hz. Two-axis MEMS scanning mirrors with torsional hinges were developed with a B-scan frame rate of > 150 Hz [46,47]. However, one drawback of the torsion hinge is that the resonance frequency of the slow-axis torsional hinge is larger than 30 Hz, leading to a very limited number of B-scans per volumetric image and thus a small FOV. Another drawback of the torsion hinge is the much-reduced scanning range when operating at off-resonance frequencies.

Here, we report a high-speed OR-PAM system using a novel two-axis water-immersible torsion-bending scanner (TBS), or TBS-OR-PAM in short. Instead of using torsional scanning along both axes, the TBS uses bending scanning along the slow-axis, which can operate at quasi-static mode without sacrificing the scanning range. The fast-axis of TBS is still based on a torsional hinge and works at the resonant mode, which can provide a B-scan frame rate of 400 Hz. Overall, TBS-OR-PAM can achieve a volumetric imaging rate of 1 Hz over an FOV of 2.5 mm by 1.5 mm. High-speed functional imaging is achieved by using dual-wavelength excitation. To demonstrate the system’s in vivo performance on small animal models, we imaged the skin vasculature induced by epinephrine and the blood oxygenation change induced by hypoxia challenge.

2. Methods

2.1. TBS-OR-PAM system design

The schematics of the TBS-OR-PAM system is shown in Fig. 1(a). An Nd:YAG fiber laser (SPFL 532-40, Spectra-Physics, Inc.) is used to provide the excitation light at 532 nm with a maximum PRR of 2 MHz. Another Nd: YAG fiber laser (VPFL-G-20, Spectra-Physics, Inc.) pumps a six-meter-long single-mode fiber (HB450-SC, Fibercore, Inc.), which generates the excitation light at 558 nm with a maximum PRR of 1 MHz [38,48]. To separate the PA signals generated at different wavelengths, the two laser pulses at 532 nm and 558 nm are delivered with a time interval of 500 ns. The two laser beams are combined by a lab-made beam combiner [25,32,39], which consists of an uncoated prism (32-330, Edmund, Inc.) and an aluminum-coated prism (32-331, Edmund, Inc.). The optical aberration of the excitation light by the prism surface is offset by a correction lens, which is glued on the top of the combiner. The resultant acoustic wave is focused by a plano-concave lens with an NA of 0.25 (45-010, Edmund, Inc.), which is attached to the right surface of the combiner. The excitation light beams are reflected by the aluminum-coated inner surface of the combiner, steered by the TBS mirror plate, and then delivered onto the sample surface. The generated photoacoustic waves are reflected by the TBS mirror plate, focused by the acoustic lens, and received by an ultrasound transducer (V214-FF-RM, Olympus-NDT, Inc.) attached to the left surface of the combiner. The central frequency of the transducer is 50 MHz and the 6 dB bandwidth is ∼ 100%. To provide functional imaging of the blood oxygenation, the PA images are acquired at 532 nm and 558 nm simultaneously, from which the oxygen saturation of deoxy-hemoglobin (sO2) is quantified based on the known absorption spectra of deoxy-hemoglobin (HbR) and oxy-hemoglobin (HbO2) [49,50].

In our experiments, the TBS fast-axis was driven by a sinusoidal signal at 200 Hz with a peak amplitude of 10 V, and the slow-axis was driven by a triangle wave from −8 to +12 V with a signal period of 2 s (Supplementary Video 1). When driven by the sinusoidal signal, the acceleration of the fast-axis is sinusoidal, with the highest velocity at the center of the scanning range and zero velocity at the two ends. Therefore, the scanning step size along the fast-axis is sinusoidal and nonuniform. Without correction, the image is distorted with the regions towards the ends of the scanning range appearing as ‘static zone’. Such nonuniform step size can be corrected with the known scanning parameters such as the scanning angle and the distance between the sample and the mirror plate, as shown in Fig. 1(b). The slow-axis works at the quasi-static mode, so the scanning angle is proportional to the driving voltage and no additional step size correction is needed.

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2.2. TBS fabrication

Fig. 2(a) illustrates the design of the two-axis water-immersible TBS, which is consisted of a reflective mirror plate, a biaxially-oriented polyethylene terephthalate (BoPET) hinge layer, a spacer and holder, inductor coils, and four permanent magnets. Fig. 2(b) showed the layered schematic design of a prototype TBS system. The design parameters of the TBS system are listed in Table 1. To maximize the acoustic wave reflection efficiency, an 8 mm by 8 mm square mirror plate is used to match the ultrasound transducer’s active element size (6 mm in diameter). The mirror plate is cut from a 200 µm silicon wafer. The front surface is coated with an aluminum and SiO$_2$ protection layer. A BoPET with a 130-µm thickness is laser-cut into a hinge layer and then bonded onto the mirror plate and the supporting frame. The top structure is mounted onto the inductor coils (RLB1014, Bourns, Inc.) with the spacer and holder. One pair of opposite permanent magnets (D21, K&J Magnetics, Inc.) are attached to the back surface of the top structure for the fast-axis actuation. Another pair of magnets with the same poles is mounted onto the top surface of the hinge layer for the slow-axis actuation. For the fast-axis, the sinusoidal current passes through the fast-axis coil and generates an alternating magnetic field driving the magnets to rotate the mirror plate around the torsional hinge. The frequency of the driving sinusoidal signals should match the fast-axis resonant frequency (the driving frequency when the mirror plate reaches its maximum tilting angle) to maximize the driving efficiency. For the slow-axis, the hinges are designed to perform bending instead of torsional motion, which allows longer and wider hinges that are more capable of holding and lifting the top structure of mirror. The bending hinges are driven by a direct current signal with two coils holding the mirror plate at a certain tilting angle. Because of the different mechanical structures, the rotation of both the slow and the fast axes are physically decoupled with minimized crosstalk and can be individually controlled by adjusting the driving signal frequency and amplitude. The physical decoupling of the two scanning axes is a major advantage of the TBS system over the previous two-axis torsional MEMS scanners that often suffer from the cross-talks between two axes.

2.3. High-speed imaging of epinephrine-induced vascular constriction

To demonstrate the high-speed imaging of the TBS-OR-PAM system, we imaged the vascular constriction induced by epinephrine. Epinephrine can induce systematic vessel constriction and blood pressure rise, and is commonly used to treat anaphylaxis [52], cardiac arrest [53], and superficial bleeding [54]. A 12-week-old female Swiss Webster mouse with a weight of 25 g was used in the experiment. The mouse was anesthetized with isoflurane (1.5% v/v) and fixed on top of a heating pad to maintain the body temperature at 37 °C. All the in vivo imaging experiments were approved by the Institutional Animal Care and Use Committee of Duke University. Using TBS-OR-PAM, an area of 1.5 × 2.5 mm$^2$ of the mouse ear was repeatedly imaged at the excitation wavelength of 532 nm with the pulse energy of ~ 180 nJ. The laser PRR was 200 kHz. The scanning frequency of the fast-axis was 200 Hz. Each volumetric scan took 4 s. The mouse ear was first imaged for 5 min before the epinephrine administration as the baseline. After injecting 5 µg of epinephrine through the tail vein, we continuously imaged the same region for 25 min to monitor the constriction and recovery of the ear vasculature.

2.4. High-speed functional imaging of the hypoxia challenge

To demonstrate the fast functional imaging capability of the TBS-OR-PAM system, we monitored the sO$_2$ change in the ear vasculature under normoxia and hypoxia conditions. The system setup was the same as the epinephrine experiment except the images were taken at two wavelengths of 532 nm and 558 nm. The laser pulse energy was ~ 180 nJ at both wavelengths. The breathing air was a mixture of 21% of oxygen and 79% of nitrogen under the normoxia condition, or 2% of oxygen and 98% of nitrogen under the hypoxia condition. After acquiring the baseline images for two minutes at the normoxia condition, we performed three cycles of hypoxia challenges. In each cycle, the hypoxia challenge was applied for two minutes, followed by a normoxia recovery for six minutes.

3. Results

3.1. TBS system characterisation

To characterize the scanning capability of the prototype TBS mirror, the fast-axis resonance frequencies in air and in water were tested with a 40-mA AC driving current. The frequency of the driving signal varied from 0 to 400 Hz. As shown in Fig. 3(a), the fast-axis resonance frequencies are 250 Hz in air and 200 Hz in water. The reduced resonance frequency in water is due to the much-increased damping force. The resonance frequency is not applicable for the slow-axis since it is driven.

![Fig. 2](image-url) Working principle of the TBS system. (a) The schematic of two-axis water-immersible TBS system. (b) Exploding view of the TBS system, showing the major components.

![Fig. 3](image-url) Characterization of the two-axis water-immersible TBS system. (a) Fast-axis resonance frequency in air and in water. (b) Fast-axis scanning angle in air and in water at the respective resonance frequencies, with varying driving current. (c) Slow-axis bending angle in air and in water, with varying direct-current driving current.
by direct current and working in the quasi-static mode. At the respective resonance frequency, the scanning angle of the fast-axis in air and in water was characterized by varying the driving current from 0 to 100 mA. As illustrated in Fig. 3(b), the fast-axis scanning angle is proportional to the driving current. However, the scanning angle in water is about three times smaller than that in air, also due to the larger damping force of water. For example, with a driving current of 90 mA, the scanning angle is \( \pm 15^\circ \) in air and \( \pm 7^\circ \) in water. In other words, three times stronger driving current is needed to drive the mirror in water in order to achieve the same scanning angle in air. Supplementary Video 1 shows the scanning pattern of the TBS system working in a water. The potential overheating of the coil due to the relatively strong driving current in water is mostly mitigated by the surrounding water with a large specific heat capacity (4184 J/kg/K). The slow axis’s bending angle also shows a high linear dependence on the direct-current driving current, as shown in Fig. 3(c). The bending angle increases from 0° to 22° with the driving current varying from 0 to 100 mA. Because the slow-axis works in a quasi-static mode, as expected, there is no difference between the bending angles in air and in water. Moreover, since there is no resonance frequency for the slow-axis, the bending angle is not sensitive to the driving signal frequency (typically a triangle wave). In summary, the tilting and bending angles of the TBS system can be readily controlled by adjusting the driving current and frequency.

### 3.2. TBS-OR-PAM system characterization

To measure the lateral resolution of the TBS-OR-PAM system, the edge spread function (ESF) was obtained by repeatedly scanning a sharp edge of a resolution target (R3L3S1P, Thorlabs, Inc.) for 400 times with a step size of 1.25 \( \mu \)m, as depicted in the blue dashed line in Fig. 4(a). The corresponding line spread function (LSF) was calculated by taking the first derivative of the ESF, which is indicated by the red solid line in Fig. 4(a). By analyzing the full width at half maximum (FWHM) of the LSF, the lateral resolution of the TBS-OR-PAM system was measured to be 3.8 \( \mu \)m. To quantify the axial resolution, the radio-frequency (RF) signal was acquired from a carbon fiber with a diameter of \( \sim 7 \) \( \mu \)m, which is much smaller than the expected axial resolution. As shown in Fig. 4(b), the Hilbert transform was performed on the RF signal to extract the envelope. The FWHM of the envelope is \( \sim 34 \) \( \mu \)m, which can be used as the system’s axial resolution. An alternative method to quantify the axial resolution is to numerically shift and sum the RF signal (Fig. 4(b)) with a distance of \( d \), and then find the minimal \( d \) to discern the resultant peaks [55,56]. Fig. 4(c) shows an example with a shift distance of 19 \( \mu \)m and the two signal peaks can still be clearly distinguished. Using the shift-and-sum method, as shown in Fig. 4(d), a 16-\( \mu \)m axial resolution is achieved with a contrast-to-noise ratio (CNR) of 5 between the peaks and the background. We also compared the sensitivity of the TBS-OR-PAM system with the previously reported second-generation PAM (SG-PAM) system [10]. The PA signals were measured by both systems on a piece of black tape with the same laser pulse energy. The detected signal amplitude of the TBS-OR-PAM system is about 3.3 times lower than that of the SG-PAM system, which is mainly due to the two times smaller NA of the acoustic focusing lens. The distance from the TBS mirror to the sample surface was \( \sim 7 \) mm. According to the scanning angles shown in Fig. 3, the theoretical maximum scanning range along the fast and slow axes were 1.5 mm and 2.8 mm, respectively, which agree with the measured FOV of 1.5 \( \times \) 2.5 mm

### 3.3. High-speed imaging of epinephrine-induced vascular constriction

Fig. 5(a) shows the ear vasculature at several representative time points before and after the drug administration. It can be clearly observed that the blood perfusion started to decrease immediately after injecting epinephrine and reached to the minimal after five minutes. We can observe that the major vessels (indicated by the blue arrows) became thinner, while some microvessels (marked by the red and yellow arrows) completely disappeared. The reduction in blood perfusion in both major vessels and microvessels was induced by the arterial constriction. As the drug was slowly cleared, we observed the recovery of blood perfusion after 30 min in both the major vessels and the microvessels. Supplementary Video 2 shows the high-speed imaging of the vasculature constriction induced by epinephrine.

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Fig. 5(b) shows the quantitative analysis of the vascular dynamics. The pixel size of Fig. 5(a) is 3 \( \mu \)m along both fast and slow axes, and the area of each pixel is 9 \( \mu \)m
\(^2\). Thus, the vessel area can be quantified by counting the number of pixels of the blood vessels above the noise level. We can observe that the blood vessels responded immediately after administrating epinephrine. The total vessel area decreased from \( \sim 1.5 \) mm
\(^2\) to \( \sim 0.6 \) mm
\(^2\) within 5 min, stayed at the lowest level for

![Fig. 4. Characterization of the TBS-OR-PAM system. (a) Measurement of the lateral resolution from the derived line spread function of a resolution target. (b) Measurement of the axial resolution from the envelope of the RF signal of a carbon fiber. (c) The signal envelope using the shift-and-sum method, with a shift distance of 19 \( \mu \)m; (d) Measurement of axial resolution based on the shift-and-sum method with an CNR of 5.](image_url)

![Fig. 5. TBS-OR-PAM of epinephrine-induced vascular constriction in a mouse ear. (a) Representative PAM images of the ear vasculature before and after epinephrine administration. (b) Quantitative analysis of the vessel area. The moving average was performed over 15 consecutive data points. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.](image_url)
was calculated by averaging the sO$_2$ over 5 min, then gradually recovered to ~1.1 mm$^2$ at the end of the experiment, possibly because epinephrine was not completely cleared form the circulation system within 25 min. The PAM measured vascular dynamics are consistent with the literature reports [57,58].

3.4. High-speed functional imaging of the hypoxia challenge

Fig. 6(a) shows the sO$_2$ images of the mouse ear at the baseline and during two cycles of hypoxia challenge. Under the normoxia condition, the sO$_2$ difference in arteries (shown in red, higher oxy-hemoglobin fraction) and the veins (shown in cyan, higher deoxy-hemoglobin fraction) can be clearly observed. By comparison, during the hypoxia challenge, the overall deoxy-hemoglobin fraction is significantly increased, so that the arteries and veins do not depict a clear difference in sO$_2$ anymore. The high-speed functional imaging of three cycles of hypoxia challenge is shown in Supplementary Video 3. We quantified the average sO$_2$ change in the ear, as shown in Fig. 6(b). The average sO$_2$ was calculated by averaging the sO$_2$ values of all the pixels from the blood vessels. The average sO$_2$ decreased instantly when the hypoxia challenge was applied at the 2nd, 10th, and 18th minute, and recovered to the baseline when normoxia was applied.

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4. Conclusion and discussion

In this work, we have developed a high-speed functional OR-PAM system using a novel water-immersible two-axis TBS with bending and torsional hinges. The performance of the TBS-OR-PAM system was demonstrated by monitoring the hemodynamic changes in the microvasculature induced by epinephrine and the sO$_2$ change induced by hypoxia challenge. The prototype TBS can co-focally steer both the laser and ultrasound beams simultaneously and thus maintain the high detection sensitivity over the entire FOV. The TBS-OR-PAM system can provide a 400-Hz B-scan rate and 1 Hz volumetric imaging rate along the fast and slow-axis, respectively. Although the TBS-OR-PAM system has a similar B-scan rate with the previously reported system based on a one-axis MEMS scanning mirror, there is no mechanical scanning needed and the system is more compact and stable. Importantly, the current TBS system uses a bending mechanism for the slow-axis scanning. The scanning range does not depend on the resonance frequency and thus can work flexibly at low scanning frequencies. Moreover, because of the different driving mechanisms, the two axes of TBS are independent to each other without crosstalk, which is clearly different from the previous two-axis torsional MEMS scanners. The complexity of the OR-PAM system is reduced due to the compact size and independent operation of the two-axis TBS, and thus the system stability is improved. Nonetheless, the current TBS-OR-PAM system has two technical limitations. Firstly, the maximum imaging FOV is about 1.5 $\times$ 2.5 mm$^2$, limited by the relatively small scanning angle of the TBS system. The scanning angle can be improved by using hinge materials with lower stiffness or increasing the coil inductance. Integrating the TBS system with motorized stages for mosaic scanning is another solution to increase the FOV [59]. Secondly, the current B-scan rate in water is still limited due to the large water damping force and the relatively large mirror plate size. By downsizing the scanning mirror, the resonance frequency of the torsional hinge can be increased, and a higher B-scan rate can be achieved. More rigid hinge materials such as spring steel can be explored to improve the resonance frequency [60].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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