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Multi-focal optical-resolution photoacoustic microscopy *in vivo*

Liang Song, Konstantin Maslov, and Lihong V. Wang*

Optical Imaging Laboratory, Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, Missouri 63130

Abstract

Although ultrasound arrays were exploited in photoacoustic imaging to improve imaging speed, ultrasound-array-based optical-resolution photoacoustic microscopy (OR-PAM) has never been achieved previously. Here we present our development of multi-focal OR-PAM using a microlens array for optical illumination and an ultrasound array for photoacoustic detection. Our system is capable of imaging hemoglobin concentration and oxygenation in individual microvessels *in vivo* at high speed. Compared with a single focus, multiple foci reduce the scanning load and increase the imaging speed significantly. The current multi-focal system can acquire $1000 \times 500 \times 200$ voxels at $\sim 10\text{-}\mu\text{m}$ lateral resolution within 4 min.

Due to the vital role of the microcirculation in the regulation of hemodynamics and metabolism, imaging of the microvasculature is crucial to both clinical practice and preclinical research [1]. Many high-resolution optical imaging modalities—including confocal microscopy, two-photon microscopy, and optical coherence tomography—have been adopted for microvascular imaging [2–4]. However, they either require exogenous fluorescent agents or have insufficient sensitivity to image a single capillary. In addition, none of them can accurately assess the oxygen saturation (sO_2) of hemoglobin—an important functional parameter in disease diagnosis.

In contrast, optical-resolution photoacoustic microscopy (OR-PAM) provides extremely high sensitivity to optical absorption, with optical diffraction limited lateral resolution [5]. It is capable of imaging both the total concentration and sO_2 of hemoglobin in microvessels—including capillaries—*in vivo*, making it a viable tool for microvascular imaging [6]. However, the imaging speed of OR-PAM using single-element ultrasonic detection is usually limited by mechanical scanning.

Previously, to improve the imaging speed of OR-PAM, a Galvo mirror was used to rapidly scan the illumination laser beam confined within the field of view of an unfocused ultrasonic transducer [7]. A hybrid optical-mechanical scanning OR-PAM using a cylindrically focused ultrasonic transducer was also developed [8]. However, since the ultrasonic transducer was either unfocused or focused in only one axis, the signal-to-noise (SNR) ratio of the system was inherently limited. In addition, because only a single laser beam and a single ultrasonic transducer were used, these systems demand a laser pulse repetition rate as high as 5 – 10 kHz to significantly improve the imaging speed. Presently, lasers with such high pulse repetition rates usually lack the wavelength tunability needed for measuring sO_2 . Furthermore, although ultrasound arrays were used to improve the speed of photoacoustic imaging systems [9–10], and although optical focusing was used to improve the elevational resolution of such systems [11], none of them achieved optical resolution in both the lateral and elevational directions.

*Corresponding author: lhwang@biomed.wustl.edu.

In this work, we developed multi-focal optical illumination in conjunction with ultrasonic array detection to achieve both optical resolution and high speed for photoacoustic imaging. Figure 1 illustrates the principles of the multi-focal optical-resolution photoacoustic microscopy (MFOR-PAM). For photoacoustic excitation, a tunable dye laser (Cobra, Sirah, Germany), pumped by a Q-switched Nd:YLF laser (INNOSLAB, Edgewave, Germany), was used. The laser system (with Rhodamine 6G laser dye) had a peak output at 570 nm with a ~20-nm tuning range, and was operated at a pulse repetition rate up to 1.3 kHz. To provide multi-focal optical illumination, a microlens array (11-1640-123-000, SUSS MicroOptics, Switzerland) was used. The microlens array consists of a 20×40 quadratic grid of spherical microlenses with 250- μm center-to-center spacing. Each microlens has a diameter a few micrometers smaller than 250 μm and an effective focal length ~1.2 mm—which is also its working distance. In theory, it can produce a diffraction-limited focus with a radius of ~4 μm at waist and a Rayleigh length of ~80 μm . In this study, a line of 20 microlenses from the microlens array was used, providing 20 focused optical illumination spots along x (Figure 1). The distance between two nearby spots, determined by the pitch of the microlens array, was 250 μm , greater than the ~80- μm acoustic lateral resolution of our ultrasound array.

For ultrasonic detection, a linear ultrasound array was used, simultaneously detecting photoacoustic emissions from all illumination beams. The ultrasound array, with a center frequency of 30 MHz and a receiving-only bandwidth of 70%, consists of 48 elements (each has a dimension of 82 $\mu\text{m} \times 2$ mm) spaced 100 μm along x . Further technical details about this array can be found in our previous publications [10, 12]. To acquire a volumetric image, the sample was scanned in the x - y plane. At each scanning position, the signals acquired from different illumination sites were reconstructed by using a photoacoustic delay-and-sum algorithm [10] followed by integrating the delay-and-summed signals into corresponding illumination lines. Of note, the use of the ultrasound array with photoacoustic reconstruction allows the spacing between adjacent optical illumination beams to be as small as the acoustic lateral resolution of the ultrasound array. Fundamentally different from simply combining multiple assemblies of a single optical focusing element and a single ultrasonic transducer, this approach enables us to position optical foci much closer to each other and potentially to scan optically at high speed.

Due to the simultaneous photoacoustic excitation from the 20 illumination beams, the number of scanning steps along x was reduced 20-fold compared with that in conventional single-focus OR-PAM. In theory, this reduction could result in a 20-fold increase in imaging speed. In our system, as a proof of concept, data from the 48-channel ultrasound array are multiplexed to an 8-channel data acquisition card (Octopus 8389, GaGe Applied Technologies, USA) [10, 12], reducing the possible speed increase by 6 times. However, even with the multiplexing, our MFOR-PAM system can acquire a data set of $1000 \times 500 \times 200$ voxels within 4 min, three to four times faster than existing mechanical scanning single-focus OR-PAM.

As measured previously, the axial resolution of our system, determined by the receiving ultrasonic bandwidth, is ~25 μm [12]. To quantify the lateral resolution, which is determined by the optical focus of the microlens array, we imaged two 6- μm diameter crossed carbon fibers, using a scanning step of 2.5 μm . Note that, after the implementation of optical focusing, the aforementioned lateral and elevational resolutions with respect to the ultrasound array have now become undistinguishable with respect to the imaging system. Hence, hereafter, both resolutions will be referred to as lateral resolution. Figure 2(a) shows a maximum amplitude projection (MAP) image of the two carbon fibers. In this report, all MAP images were formed by projecting the maximum photoacoustic amplitudes along the z axis to the x - y plane. Figure 2(b) shows the FWHM of the vertical fiber, demonstrating that

the lateral resolution of our system is at least as fine as 10 μm . The contrast-to-noise ratio (CNR), measured from Fig. 2(a), is as high as 38 dB.

To demonstrate the *in vivo* imaging capability of our system, we imaged the microvasculature in the right ear of an anesthetized nude mouse (Hsd, Athymic Nude-Foxn1NU, Harlan Co.) weighing ~20 g. In order to couple the photoacoustic waves to the ultrasound array, a thin layer of ultrasonic gel was applied to the mouse ear. The image was acquired completely noninvasively, without using any optical clearing agent. After imaging, the animal naturally recovered without observable laser damage. All experimental animal procedures were carried out in compliance with protocols approved by the Animal Studies Committee of Washington University.

Figure 3(a) shows an *in vivo* MAP image of the mouse ear microvasculature acquired by our MFOR-PAM system at 570 nm, sensing the intrinsic optical absorption contrast of hemoglobin. Microvessels in diameters down to 10 μm are clearly imaged. Figure 3(b) is a snapshot of a 3D animation (Video 1) showing the mouse ear microvasculature from various perspectives. In addition to 570 nm, laser light at 565 nm was also used to image the mouse ear, enabling sO_2 measurement, as demonstrated previously with OR-PAM [13]. While Fig. 3(c) shows the microvasculature of a selected area, Fig. 3(d) shows the corresponding vessel-by-vessel sO_2 mapping, where the arteries and veins are differentiated based on the measured sO_2 . On average, the sO_2 values in arteries and veins were estimated to be 0.96 ± 0.4 and 0.75 ± 0.5 , respectively, consistent with previous results from OR-PAM. In the experiment, the incident laser energy on each microlens was limited to ~100 nJ per pulse, resulting in a maximum fluence of ~8 mJ/cm^2 per pulse on the skin surface (assuming the optical focus was 200 μm below the skin), which is less than the 20- mJ/cm^2 ANSI laser safety standard [14].

In summary, using a microlens array and a high-frequency linear ultrasound array, we developed multi-focal optical-resolution photoacoustic microscopy. The system provided volumetric imaging of optical absorption contrast in scattering biological tissue *in vivo*, at depths up to the optical transport mean free path (~1 mm in the skin), and with optical diffraction limited lateral resolution at least as fine as 10 μm (the axial resolution was ~25 μm). With this high spatial resolution and multiple wavelengths, our system was capable of imaging both the concentration and oxygen saturation of hemoglobin in capillary-level microvessels *in vivo*. Moreover, in MFOR-PAM, the combination of multi-focal optical illumination and ultrasonic array detection enabled a significant improvement in imaging speed over existing mechanical scanning single-focal OR-PAM. In the future, by eliminating the 6:1 multiplexing in data acquisition, the imaging speed can be increased 6-fold. In addition, a more densely packed microlens array (e.g., with a spacing of 100 μm), together with optical scanning within the lens array pitch, can be used to further increase the imaging speed. To enable reflection-mode imaging, a beam combiner similar to that used in OR-PAM [5] but with smaller dimensions need to be developed. In addition, a microlens array of a longer working distance, or any other multi-focal optical device with a relatively long working distance, may be needed. In clinical practice, high imaging speed is critical to reduce motion artifacts, cost, and patient discomfort. This proof-of-principle study has demonstrated that MFOR-PAM represents a promising direction for translating photoacoustic microscopy technology to the clinic.

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Fig. 1.
(Color online) Schematic of the multi-focal optical-resolution photoacoustic microscopy system. The widths of the slit and the aperture along y are $50\ \mu\text{m}$ and $5\ \text{mm}$, respectively.

Fig. 2. (Color online) Lateral resolution of the multi-focal optical-resolution photoacoustic microscopy system. (a) Photoacoustic (PA) maximum amplitude projection (MAP) image of two crossed 6-μm diameter carbon fibers. (b) Distribution of the PA amplitude (dots) along the dashed line in (a); the solid line is a Gaussian fitted curve.

Fig. 3. (Color online) *In vivo* photoacoustic image of a mouse ear microvasculature. (a) MAP image acquired at 570 nm. (b) Snapshot of a 3D animation (Video 1) showing the microvasculature in (a). (c) Close-up of the vasculature in the dashed box in (a). (d) Oxygen saturation (sO_2) mapping for vessels in (c).