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Second-generation optical-resolution photoacoustic microscopy

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ABSTRACT

We developed a second-generation optical-resolution photoacoustic microscope (OR-PAM) with a novel acoustic detection scheme, which improved upon the sensitivity of our first-generation system by 18 dB. Moreover, translating the imaging head instead of the living object improved the scanning speed by a factor of 5, widening the field of view within the same acquisition time. The anatomy and hemoglobin oxygen saturation of an entire mouse ear was imaged *in vivo*.

Keywords: Photoacoustic imaging, photoacoustic microscopy, high sensitivity, wide field of view.

1. INTRODUCTION

In many potential clinical applications it is necessary to image anatomic structures with high resolution, to identify structures according to their optical spectral properties, or to detect very small biomarkers such as conjugated nanoparticles. There is also a significant amount of research performed *in vivo* on animal models, such as studies of angiogenesis or cancer metastatic activity by monitoring of circulated cancer cells. In such research a minimally invasive imaging technique suitable for longitudinal studies with at least the cellular resolution is required.

As an emerging high-resolution imaging modality, photoacoustic microscopy (PAM) images optically absorbing microstructures by detecting transient acoustic waves generated from laser-induced thermal-elastic expansion. PAM is directly sensitive to optical absorption, which provides quantitative measurements of physiologically important endogenous molecules (e.g., hemoglobin and melanin) as well as a variety of widely used optical contrast agents (e.g., methylene blue and indocyanine green). In acoustic-resolution PAM systems, both the transverse and axial resolutions are defined by ultrasonic detection. However, because of the overwhelming attenuation of high-frequency ultrasound beyond 100 MHz in biological tissue, it is difficult to achieve a transverse resolution below 10 μm with ultrasonic focusing without severely compromising the imaging depth. To overcome this obstacle, we recently proposed optical-resolution PAM (OR-PAM), whose transverse resolution is defined by a tight optical focus. Using an optical microscope objective with an NA of 0.1 we experimentally obtained 5- μm transverse resolution, and *in vivo* microvasculature was imaged down to the single-capillary level [1–6]. However, in our first-generation (G1) OR-PAM, the optical-acoustic combiner suffers significant acoustic loss [1]. Moreover, the G1 system could cover only a small field of view (FOV) within a reasonable acquisition time because translating the living object for scanning must be slow enough to avoid possible disturbance to the object. Here, we introduce our second-generation (G2) OR-PAM with improved detection sensitivity and imaging speed. In G2 OR-PAM, we have designed a novel optical-acoustic combiner, which improves the detection sensitivity by 18 dB over the G1 system. Moreover, the fiber-based light delivery allows scanning the imaging head rather than the living object, thereby enabling fast *in vivo* imaging over a large FOV.

2. METHODS AND MATERIALS

Our G2 OR-PAM [7], consists of both optical and ultrasonic components. The optical irradiation source is a dye laser pumped by a Nd:YLF solid-state pulsed laser. The output laser beam is attenuated by a neutral-density filter and coupled

into a single-mode optical fiber to allow mechanically scanning the microscope imaging head rather than the living object. To create a tight optical focus, the optical field of the single-mode fiber output is mapped into the imaging object by two identical microscope objectives. A beam sampler and a photodiode are inserted between the collimation objective and a stationary mirror to monitor the fluctuation in laser intensity. A CCD camera is added to view the imaging region through the reverse path of the optical illumination. The ultrasonic detection involves a home-made acoustic-optical beam combiner connected to 50-MHz central frequency ultrasonic transducer. The combiner aligns the optical irradiation and the acoustic detection coaxially. Different from the G1 OR-PAM system, where two right-angle prisms sandwich a thin layer of silicone oil for acoustic-optical coaxial alignment [1], G2 OR-PAM replaces the lower right-angle prism with a rhomboid prism to enhance the detection sensitivity. In the G1 system, the obliquely incident (45°) acoustic signal is reflected by a single solid-liquid interface (Fig. 1), where the energy of the longitudinal acoustic wave is coupled into both shear and longitudinal waves. According to the boundary conditions, 85% of the incident acoustic energy is transformed from longitudinal mode to shear mode at the interface. The second inclined surface provided by the rhomboid prism in the G2 system is able to transform 97% of the shear-wave energy back into longitudinal waves, to which the ultrasonic transducer is sensitive. In comparison to the combiner in the G1 system, the new design can theoretically result in a 20-dB increase in the detected longitudinal-wave energy. Our experimental results show that, at the same optical irradiation level, the signal-to-noise ratio (SNR) of the G2 system was 18 dB higher than that of the G1 system. The actual improvement in SNR is in good agreement with the theoretical estimation.

Into the bottom of the combiner a spherical cavity is ground to produce an acoustic lens with a numerical aperture of 0.5 in water, which provides an acoustic focal diameter of $43\ \mu\text{m}$ at the 50-MHz central frequency. To maximize the detection sensitivity, the optical and acoustic foci are aligned confocally. The OR-PAM system also includes electronic amplifiers, an analog-digital converter, a mechanical scanner, and a scanner controller. The digitized signal is transferred to a computer for image formation. The software converts the raw data into a sequence of cross-sectional B-scan images as the microscope head raster scans the object. The recorded images can then be displayed as individual cross-sectional images, 2D maximum amplitude projection images, or 3D renderings. A water tank is used to house the microscope imaging head. We cut an imaging window into a Petri dish and seal it with an ultrasonically and optically transparent polyethylene membrane. The photoacoustic wave generated from the object is acoustically coupled to the ultrasonic detector, first by ultrasonic gel between the object and the polyethylene membrane and then by deionized water in the Petri dish.

Figure 1. Experimental setup of the second-generation optical-resolution photoacoustic microscope.

The much improved imaging speed enables G2 OR-PAM to image a large FOV with single capillary resolution and sensitivity. Figure 2 shows the distributions of total concentration (Fig. 2A) and oxygen saturation (Fig. 2B) of

hemoglobin in a living nude mouse ear. Densely packed capillary beds are clearly resolved (Fig. 2C). The typical acquisition time of such a dual-wavelength, bidirectionally scanned image (image size: 10 mm × 10 mm; step size: 2.5 μm × 5 μm) is ~80 min, which is 5 times as fast as that for the G1 system.

Figure 2. *In vivo* functional optical-resolution photoacoustic microscopy of a nude mouse ear. MAP images of (A) the total hemoglobin concentration (acquired at 570 nm) and (B) the hemoglobin oxygen saturation (acquired at 561 nm and 570 nm). (C) Close-up of the boxed region in panel (A). The scale bar in panel (A) applies to both (A) and (B).

The lateral resolution of the G2 system was experimentally quantified to be 2.6 μm by imaging an Air Force resolution test target at 570 nm. Although capable of resolving single capillaries, the high lateral resolution of the G2 OR-PAM also raises a practical challenge: the small source of the photoacoustic wave generates a small amount of acoustic power, which must exceed the noise level to be effectively detected.

Let us consider a small optically absorbing volume, V , occupying the focal area of the objective. Due to its small dimension in comparison to the wavelength of the detected acoustic wave, the photoacoustic source can be approximated by a monopole (point) source of ultrasound. The peak power of the generated acoustic wave can be easily calculated [8]. At a distance r , the photoacoustic pressure generated by a small optically absorptive object illuminated by a laser pulse

of a Gaussian shape $I = \frac{1}{\sqrt{2\pi}} \frac{F_0}{\tau} \exp(-t^2 / 2\tau^2)$ can be expressed as [8]:

$$p = \frac{\sigma \beta F_0}{4\pi \tau^2 C_p r} \left[\frac{-2t}{\tau \sqrt{2}} \exp(-t^2 / 2\tau^2) \right] \quad (1)$$

where F_0 is the optical energy fluence, β is the isobaric volume expansion coefficient, σ is the optical absorption cross section of the absorbing volume, and C_p is the specific heat. For an absorption coefficient μ_a , whose reciprocal is small in comparison to the size of the absorbing volume, $\sigma \approx \mu_a V$. Equation (1) can be used to calculate the peak power of the photoacoustic wave on an ultrasonic transducer with a numerical aperture $NA = 0.5$:

$$W_{\max} \approx \frac{1}{\pi e \rho c} \left(\frac{\beta \mu_a F V}{C_p \tau^2} \right)^2 \cdot \frac{N A^2}{2} \approx 4 \cdot 10^{-7} W, \quad (2)$$

where ρ is mass density and c is the speed of sound. Here we insert in (2) realistic parameters: the optical absorption coefficient of blood $\mu_a \sim 300 \text{ cm}^{-1}$, focal volume $V \sim 100 \mu\text{m}^3$, which corresponds to an optical resolution of approximately $2.5 \mu\text{m}$, and $F_0 = 20 \text{ mJ/cm}^2$ which is the ANSI safety limit for unintended exposure of the human skin [9]. The full width at half maximum (FWHM) pulse width of the laser pulse was 4.5 ns which gives $\tau = FWHM / 2\sqrt{2 \ln 2} \sim 2 \text{ ns}$.

As one can see, the calculated peak power of the photoacoustic signal is well above the input thermal noise level of ideally matched electrical amplifier:

$$W_n = 2kT\Delta f = 4 \cdot 10^{-13} W, \quad (3)$$

where k is the Boltzmann constant, $T = 300 \text{ K}$ is the ambient temperature, and $\Delta f = 10^8 \text{ Hz}$ is the signal frequency band. That suggests for $2.5 \mu\text{m}$ resolution PAM signal to noise ratio (SNR) about 60dB. However, at near gigahertz frequencies ultrasonic absorption in the tissue and immersion liquid must be taken into account. To reach transducer surface photoacoustic wave propagate distance approximately equal to imaging depth in tissue and much large distance approximately equal to transducer focal length in immersion liquid (water). Ultrasonic attenuation in water is given by $\exp(-\alpha f^2 L)$, where $\alpha = 25 \cdot 10^{-15} \text{ Hz}^{-2} / \text{m}$ is ultrasound absorption coefficient, f ultrasound frequency, and $L = 6 \text{ mm}$ is ultrasonic transducer focal length. Taken ultrasonic absorption into account in (1), acoustic pressure at transducer surface is expressed as

$$p = \frac{\sigma \beta F_0}{4\pi C_p L} \left[\frac{-t\sqrt{2}}{(\tau^2 + \alpha L / 2\pi^2)^{3/2}} \exp(-t^2 / 2(\tau^2 + \alpha L / 2\pi^2)) \right]. \quad (4)$$

Corresponding peak power for $\tau^2 \ll \alpha L / 2\pi^2$ reduces to:

$$W_{\max} \approx \frac{\pi^3}{2e} \frac{1}{\rho c} \left(\frac{\beta \mu_a F_0 V}{C_p \alpha L} \right)^2 \cdot \frac{N A^2}{2} \approx 8 \cdot 10^{-8} W, \quad (5)$$

which is still much higher than noise level.

Additionally measurable signal peak power must include transducer losses, which for broad-band transducers used in experiment was 31 dB. Correspondingly, a reasonable SNR at ANSI safety limit at light focus can be as high as 22dB without signal averaging. ANSI does not regulate fluence level in the focal area of a tightly focused beam, it sets the safety limit for skin exposure for a collimated laser beam at 20 mJ/cm^2 . The ANSI limit notwithstanding, previously published works [10, 11] have put the safe exposure limit for RBCs to 0.5 J/cm^2 and even higher for other types of cells. That level of fluence should be enough to achieve an near 50 dB SNR.

Experimentally measured SNR in the acoustic-optical dual foci was $150 \mu\text{m}$ beneath the ear surface measured to be equal to 42.7 dB (136.5) at laser pulse energy before entering the ear of 80 nJ and operation wavelength 570 nm . Assume that the thicknesses of epidermis and dermis are $60 \mu\text{m}$ and $90 \mu\text{m}$, respectively. According to [http://omlc.ogi.edu/news/jan98/skinoptics.html], the net epidermal absorption coefficient $\mu_{a_{\text{epi}}} = 44 \text{ cm}^{-1}$ and the net dermal absorption coefficient is $\mu_{a_{\text{derm}}} = 12.5 \text{ cm}^{-1}$. Thus, the laser fluence at $150 \mu\text{m}$ beneath the ear surface is $0.6 \cdot F_0$, where F_0 is optical fluence on skin surface. Assuming $2.6 \mu\text{m}$ lateral resolution at $150 \mu\text{m}$ beneath the ear surface, the laser fluence in the dual foci is 0.75 J/cm^2 . Assuming that blood hemoglobin concentration is 150 g/liter , and blood has 75% HbO_2 and 25% HbR [http://omlc.ogi.edu/spectra/hemoglobin/] the estimated blood absorption coefficient at 570 nm by is 240 cm^{-1} . Comparing (5) and (3) and including transducer and waveguide losses theoretical estimate gives SNR of 52 dB which is slightly better than experimentally measured value. Slight discrepancy between theory and experiment can be explained by additional acoustic losses in tissue, light reflection from tissue surface and influence of

limited transducer bandwidth which were not taken into account in calculations. Thus, the noise-equivalent sensitivities in absorption coefficient is $\mu_{a_{NES}} = 1.8 \text{ cm}^{-1}$ which is equivalent to molar concentration of the hemoglobin $HbT_{NES} = 16.8 \mu\text{M}$ or $4 \cdot 10^{-3}$ of red blood cell volume.

CONCLUSION

We have developed G2 OR-PAM with high-sensitivity acoustic detection and instrument-scanning. Compared with G1 OR-PAM, the detection sensitivity and the scanning speed are improved by 18 dB and 5-fold, respectively. Large-FOV, volumetric microscopy of vascular anatomy and sO_2 are demonstrated *in vivo*.

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