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High resolution ultrasound and photoacoustic imaging of single cells

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ABSTRACT

High resolution ultrasound and photoacoustic images of stained neutrophils, lymphocytes and monocytes from a blood smear were acquired using a combined acoustic/photoacoustic microscope. Photoacoustic images were created using a pulsed 532 nm laser that was coupled to a single mode fiber to produce output wavelengths from 532 nm to 620 nm via stimulated Raman scattering. The excitation wavelength was selected using optical filters and focused onto the sample using a 20× objective. A 1000 MHz transducer was co-aligned with the laser spot and used for ultrasound and photoacoustic images, enabling micrometer resolution with both modalities. The different cell types could be easily identified due to variations in contrast within the acoustic and photoacoustic images. This technique provides a new way of probing leukocyte structure with potential applications towards detecting cellular abnormalities and diseased cells at the single cell level.

1. Introduction

Scanning acoustic microscopy (SAM) was first developed in the early 1970's at Stanford University. When using frequencies at 1000 MHz, the ultrasound imaging resolution approaches 1 μm; various biomedical applications including the imaging of single cells became readily apparent [1,2]. In SAM, the image contrast depends on the biomechanical properties of the cell assessed through the scattering and attenuation of the acoustic waves [3]. This enables high resolution images of single cells and cellular organelles with contrast not possible using optical microscopy [4–12].

Photoacoustic microscopy (PAM) uses similar hardware as acoustic microscopy, but with the addition of a pulsed laser to create the photoacoustic waves. In PAM, chromophores within the cell absorb the incident laser energy and emit an acoustic wave [13]. The acoustic wave, called a photoacoustic wave, is then detected by the same ultrasound transducer that is used in SAM. PAM is well suited to creating images of single cells using their natural endogenous chromophores, which include hemoglobin in erythrocytes [14–17], melanin in melanoma cells [14,18], DNA in cell nuclei [19] and cytochrome C in the mitochondria [17,20]. Photoacoustic images of single cells can also be created using exogenous chromophores such as dyes or nanoparticles [16,21].

Optical-resolution photoacoustic microscopy (OR-PAM) provides sub-micrometer lateral resolution, which is achieved by focusing the laser to a diffraction limited spot (approximately 200 nm using a 532 nm laser) [14,22]. The emitted photoacoustic waves are then detected using an ultrasound transducer with central frequencies up to about 100 MHz [17,23–25]. The diffraction limited laser spot provides excellent lateral resolution as photoacoustic waves are generated from within the focal spot only, however axial resolution suffers as it depends on the acoustic bandwidth of the transducer. Transducers used in these studies typically have lateral and axial resolutions in the 20–50 μm range [26], and thus are unsuitable for acoustical imaging of single cells.

We have pioneered the use of PAM using frequencies at 1000 MHz [27–29]. Combined with traditional pulse-echo acoustic microscopy, our system is capable of either acoustic or photoacoustic imaging with micrometer resolution. Images with excellent lateral and axial resolution are obtained; a quantitative analysis of the signals can also be performed to extract biomechanical or structural information about the cell [18,30–35]. To date, combined acoustic and photoacoustic imaging of the same structures at 1000 MHz has not been demonstrated. Here we demonstrate the combined imaging approach on single cells in a blood smear.

A blood smear is a monolayer of blood cells fixed and stained on top of a glass substrate [36]. The smear is composed of erythrocytes and leukocytes that are spatially separated, and as a result are ideal for both acoustic and photoacoustic microscopy. Blood smears are routinely examined by hematologists using optical microcopy to identify and diagnose blood disorders [37]. However leukocytes

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are transparent in the visible spectrum, and difficult to identify without the use of a colorimetric stain. Metachromatic Romanowksy type dyes such as the Wright stain differentially stain cellular organelles and are often used for visual identification [38]. However, even when stained it can be difficult to identify abnormalities present in the blood cells.

Using a photoacoustic microscope outfitted with a fiber-coupled pulsed laser and a 1000 MHz transducer, this study demonstrates combined ultrasound and photoacoustic images of stained leukocytes at optical wavelengths of 532 and 600 nm. This technique has the potential to identify blood-related abnormalities and reveal structural detail that can be difficult to assess using optical microscopy alone.

2. Method

2.1. Sample preparation

Blood smears were prepared from a fresh drop of blood extracted via fingerprick from a healthy volunteer in accordance with Ryerson University Ethics Review Board (REB #2012-210), then fixed in methanol. Staining was performed by adding 1 mL of Wright–Giemsa stain (Sigma Aldrich, USA) to the slide for one minute, then 1 mL deionized water for 2 min. The slide was then washed with deionized water to remove excess stain and left to dry before imaging.

2.2. Acoustic/photoacoustic microscope

The acoustic/photoacoustic microscope (Kibero GmBH, Germany) is an inverted optical microscope equipped with a pulsed 532 nm laser (Teem Photonics, France) and an ultrasound transducer positioned above the sample. The sample was placed on the microscope translation stage where the microscope optics were used to view the sample and focus the laser, and the ultrasound transducer positioned above the sample was used to record the ultrasound and photoacoustic signals (Fig. 1A). The focal spots of the ultrasound transducer and laser were co-aligned, and crosshairs on the optical view provided by a CCD camera enable targeting of specific cells (Fig. 1B). Images were acquired by raster scanning the stage and recording the acoustic and photoacoustic signals. A schematic demonstrating photoacoustic waves emitted from a single cell is shown in Fig. 1C.

A pulsed 532 nm laser was coupled to a 2 m long single mode fiber and the collimated output was directed into the optical path of the microscope. Through cascaded stimulated Raman scattering (SRS) within the fiber, additional output wavelengths between 532 and 620 nm were observed as shown in Fig. 2A [39–42]. Adding a fiber to an existing laser setup is an inexpensive method for increasing the range of wavelengths available for photoacoustic signal generation. Using this technique, fiber-coupled 532 nm lasers with wavelength output up to 800 nm have been demonstrated [41]. Alternative methods of multispectral photoacoustic imaging use multiple dye or diode lasers, or a tunable optical parametric oscillator (OPO) laser; however, these devices are expensive. The absorption spectrum of the Wright–Giemsa stain is shown in Fig. 2B, and was measured using a UV-3600 spectrophotometer (Himadzu, Japan). Two laser wavelengths were used to probe the samples, 532 and 600 nm; the desired wavelength was selected by using optical bandpass filters (Olympus, Japan). Output energies at the selected wavelengths ranged from 1 to 5 nJ/pulse, resulting in a laser fluence between 30 and 160 mJ/cm^2 using a 1 μm diameter focal spot. The fiber-coupled laser was focused by a 20× objective (0.45 numerical aperture) onto the sample, and was co-aligned with the 1000 MHz center frequency transducer. Using these frequencies and optical focusing components, the resolution was estimated to be about 1 μm for both ultrasound and photoacoustic imaging [24,43].

Acoustic images were performed first, followed by photoacoustic imaging at 532 nm, then at 600 nm. Raster scans approximately 20 × 20 μm were made using a 0.33 μm step size, and all signals were averaged 100 times to increase the signal–noise ratio (SNR). All signals were amplified by a 40 dB low noise amplifier (Miteq, USA) and digitized at 8 GS/s (Acqiris, USA). Images were created by normalizing the acoustic and photoacoustic signals to a 256 greyscale value, with white being the strongest signal, and black the lowest. Composite photoacoustic images combining the 532 and 600 nm scans were made by assigning the 532 nm image a green channel, the 600 nm a red channel and then merging the images. Further details on the acoustic and photoacoustic methods can be found in [18,30,35].

3. Results

The ultra-high frequency acoustic/photoacoustic microscope (UHF–APAM) is a hybrid of optical resolution (OR–PAM) and acoustic resolution (AR–PAM) photoacoustic microscopy. Using ultrasound and photoacoustic frequencies at 1000 MHz, this system is capable of micrometer-resolution imaging with high sensitivity. This enhanced photoacoustic sensitivity is achieved as the photoacoustic signal generated at the micrometer laser spot is amplified by the geometrical gain of the focused ultrasound

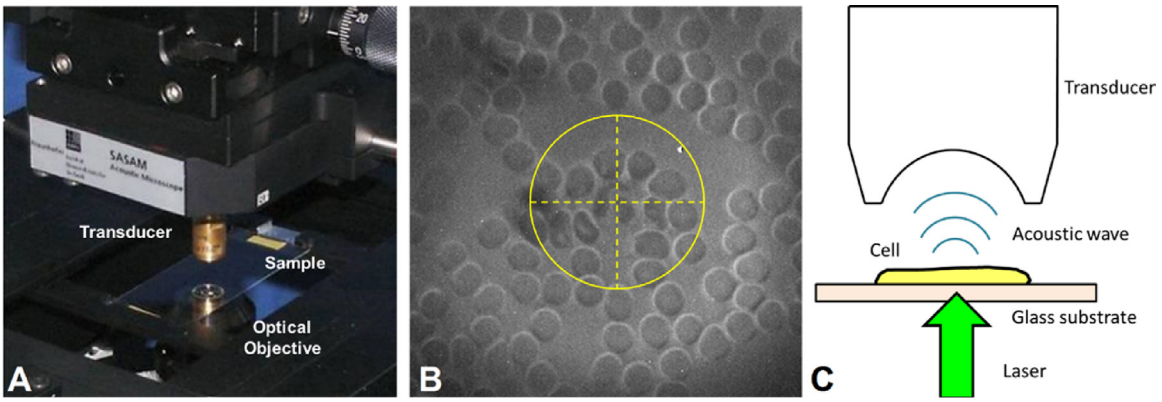

Fig. 1. The acoustic microscope: (A) the transducer is positioned above the sample slide, and the optics below are used to focus the laser and to view the sample. (B) An optical view of a stained blood smear with the transducer in place, the crosshairs are used to target each cell. A neutrophil is visible within the crosshairs. (C) A schematic showing photoacoustic waves generated from a cell on top of a glass slide travelling towards the transducer.