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Research Article

High frequency label-free photoacoustic microscopy of single cells

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ABSTRACT

Photoacoustic measurements of melanoma cells and red blood cells (RBCs) using ultra-high frequency (UHF) wide-bandwidth transducers are reported. In this detection system, the resolution typically depends on the parameters of the receiving transducer, and not the focus of the laser. A single melanoma cell was imaged with 200, 375 and 1200 MHz transducers. As the frequency increased, the resolution increased, resulting in greater detail observed. A single RBC was imaged at 1200 MHz, showing the contours of the cell. While lateral and axial resolutions approaching 1 μm are possible with this microscope, the key advantage is the ability to perform a wide-bandwidth quantitative signal analysis of the photoacoustic signals. The power spectrum of the signals measured from RBCs showed distinct spectral minima around 800 and 1500 MHz which are directly related to the RBC geometry. This study reports on the high-resolution imaging capabilities and quantitative analyses using UHF photoacoustic microscopy.

1. Introduction

Photoacoustic microscopy uses focused lasers with ultrasound transducers as receivers to create sub-micron resolution images of cells and tissue [1]. Typically high frequency transducers (20–100 MHz) are used which have native ultrasound resolutions greater than 20 μm [2]. To achieve micron-resolution photoacoustic imaging, a laser is focused to a small spot to ensure only a small region of the sample is illuminated and subsequently generates a photoacoustic wave. Several different photoacoustic microscope designs exist, in which the laser is positioned opposite the transducer [3–6], integrated into the transducer [7–9], beside the transducer [10–12] or other configurations [13–16]. In most cases, the lateral resolution is limited by the ability to focus the laser to a small spot, which in turn is directly related to the numerical aperture (NA) of the optical lens.

Various contrast agents such as dyes and nanoparticles can be used to create photoacoustic images of cells and tissue in vitro and in vivo [17,18]. Endogenous optically absorbing components of cells provide unique opportunities to image and analyze single cells using label-free methods [19]. Hemoglobin, contained in red blood cells (RBCs), strongly absorbs visible and near-infrared light in comparison to other chromophores in tissue. Melanin, the pigment responsible for coloring skin and hair, is present in melanocyte cells and absorbs strongly throughout the visible spectrum. DNA/RNA in the cell nucleus absorb UV light, and can also be used for photoacoustic imaging [9,20]. Using endogenous optical absorbers enables imaging of live cells without fixation or staining, which can alter the cellular microstructure [21].

Most photoacoustic microscopes use transducers with frequencies under 100 MHz and achieve micrometer resolution using objectives to focus the laser with moderately high NAs (up to 1.0) [22]. Resolutions near the diffraction limit of 200 nm have been achieved using a high-NA (1.23) water-immersion lens [5]. Using transducers with frequencies less than 100 MHz has some disadvantages in photoacoustic imaging. First, the laser spot is tightly focused in the lateral direction, but less so in the axial direction. In this case, the axial resolution is limited by the ultrasound transducer, and can be many times larger than the lateral resolution [23]. As the laser power is focused to a very small spot, unwanted bioeffects may be introduced [24]. Second, the transducer bandwidth is relatively narrow, limiting the potential for quantitative analyses of the photoacoustic signals. Third, micron-sized objects typically generate photoacoustic waves where a significant portion of the photoacoustic wave energy is over 100 MHz. Typically, spectral amplitude peaks of the photoacoustic wave generated from micron sized objects are above 100 MHz. Therefore, when using transducers less than 100 MHz a portion of the photoacoustic signal energy is not recorded, reducing the measurement SNR.

Our system is capable of both a qualitative and quantitative photoacoustic analysis using a focused laser with ultra-high frequency (UHF) transducers up to 1200 MHz. Lateral and axial

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resolutions up to 1 μm can be achieved, based on the transducer parameters [2]. The overall image resolution is a combination of the transducer resolution and the size of the laser spot; when the transducer resolution is smaller than the laser focus area, then the resolution primarily depends on the transducer resolution, and not the laser spot. Due to the wide bandwidth of these transducers (typically hundreds of MHz or more), a quantitative analysis of the power spectrum of the photoacoustic signal from single cells can be performed, giving information about the cell not obtainable through imaging methods alone. Moreover, photoacoustic can be integrated with ultrasound pulse-echo imaging to perform simultaneous photoacoustic and ultrasonic imaging in one pass with similar resolutions in each modality. This is not possible with lower frequency transducers (<60 MHz) used in photoacoustic microscopy, which have pulse-echo ultrasound resolutions typically greater than 25 μm. The disadvantage of using frequencies over 100 MHz is the high signal attenuation; therefore these high frequency measurements cannot be used in vivo and are limited to thin samples and cell culture studies.

This paper describes a photoacoustic microscope that uses a focused laser and UHF transducers with frequencies over 200 MHz to achieve resolution in the micrometer range. A quantitative analysis of the signals can be performed using the wide-bandwidth photoacoustic wave power spectrum, enabling a new type of single cell analysis not available to traditional photoacoustic microscopes.

2. Method

The photoacoustic microscope was designed and built by Kibero GmbH (Saarbrucken, Germany). It combines an inverted optical microscope with an ultrasound transducer positioned above the optics (Fig. 1A). A 532 nm laser (Microchip STG-03E, Teem Photonics, France) is focused into the side port of the microscope and reflected onto the sample using the same optical objective that is used to view the sample. The laser and transducer can be aligned under optical guidance to within 1 μm, with the sample positioned within the target path (Fig. 1B). The laser has a pulse width of 330 ps and operates at a pulse repetition frequency of 4 kHz. The laser fluence could be adjusted up to 650 mJ/cm^2 when focused to a 5 μm spot size using a 10x objective (0.25 NA). The photoacoustic signals are recorded passively using three different ultrasound transducers (200, 375 and 1200 MHz supplied by Kibero GmbH) and amplified by a low noise 40 dB amplifier (Miteq, USA). Signal digitization is synchronized to the laser trigger, with a data acquisition rate up to 8 GHz using an Acqiris DC252 digitizer (Agilent, USA). Images were created by scanning the sample stage in a raster pattern through the target area while keeping the laser and transducer co-aligned. The entire microscope is contained in a temperature controlled chamber kept at 36 °C, which ensures live cell viability and reduces the ultrasound attenuation though the medium at the high frequencies used.

Two types of cells were measured, melanoma cells (B16-F1) and human red blood cells (RBCs). Melanoma cells were cultured with Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS) and passed every few days. Cells were dissociated with trypsin then transferred to a 35 mm diameter glass bottom dish (Mattek, USA) and allowed to grow for 24 h. The cells were then fixed with 10% formalin for 6 min and then placed in the microscope for imaging with PBS used as the coupling fluid. Hoechst 33342 was added to mark the nucleus using fluorescence. For the RBC measurements, a blood smear on a glass slide was prepared using freshly extracted blood from a human volunteer in accordance with the Ryerson University Ethics Board (REB #2012-210). The blood smear was fixed with methanol, and then placed in the microscope for imaging. For melanoma cells, 160 μm × 160 μm areas were scanned using a step size of 0.75–1.0 μm and a laser fluence of 350 mJ/cm^2. For RBCs, 20 μm × 20 μm areas were scanned with a 0.2 μm step size and a laser fluence of 125 mJ/cm^2. All signals were averaged 100 times to increase signal to noise (SNR). Acquisition times ranged from 10 to 15 min for each cell since signal averaging was used to increase the SNR. Images were generated by converting the maximum signal amplitude of each measured a-scan to grayscale values.

Three transducers with increasing frequency and bandwidth were used for photoacoustic imaging with center frequencies of 200, 375 and 1200 MHz. The transducer parameters such as the

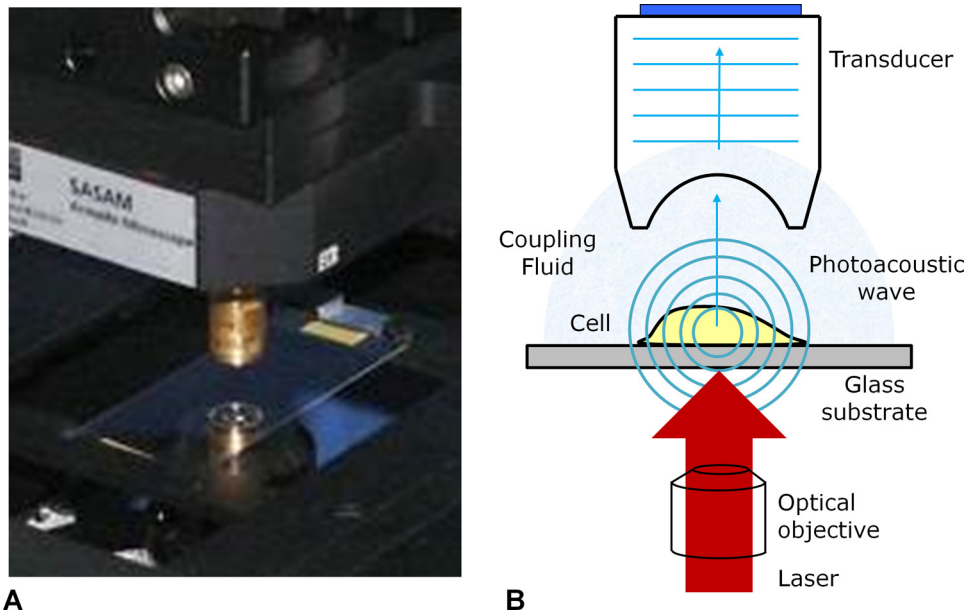

Fig. 1. The photoacoustic microscope. (A) A view of the sample holder, with the transducer positioned above the sample and microscope optics. (B) A schematic of the sample measurement area. The cells on top of the substrate are irradiated by the focused laser, with the transducer recording the resulting photoacoustic signals.