Classification of biological cells using a sound wave based flow cytometer

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ABSTRACT

A flow cytometer that uses sound waves to determine the size of biological cells is presented. In this system, a microfluidic device made of polydimethylsiloxane (PDMS) was developed to hydrodynamically flow focus cells in a single file through a target area. Integrated into the microfluidic device was an ultrasound transducer with a 375 MHz center frequency, aligned opposite the transducer was a pulsed 532 nm laser focused into the device by a 10x objective. Each passing cell was insonfied with a high frequency ultrasound pulse, and irradiated with the laser. The resulting ultrasound and photoacoustic waves from each cell were analyzed using signal processing methods, where features in the power spectra were compared to theoretical models to calculate the cell size. Two cell lines with different size distributions were used to test the system: acute myeloid leukemia cells (AML) and melanoma cells. Over 200 cells were measured using this system. The average calculated diameter of the AML cells was 10.4 ± 2.5 μm using ultrasound, and 11.4 ± 2.3 μm using photoacoustics. The average diameter of the melanoma cells was 16.2 ± 2.9 μm using ultrasound, and 18.9 ± 3.5 μm using photoacoustics. The cell sizes calculated using ultrasound and photoacoustic methods agreed with measurements using a Coulter Counter, where the AML cells were 9.8 ± 1.8 μm and the melanoma cells were 16.0 ± 2.5 μm. These results demonstrate a high speed method of assessing cell size using sound waves, which is an alternative method to traditional flow cytometry techniques.

Keywords: Flow cytometry, microfluidics, ultrasound, photoacoustics

1. INTRODUCTION

Cytometry is a technique that measures the physical and/or chemical properties of biological cells. Flow cytometry streams cells through a target area or an orifice, where a large population of cells can be identified and counted in a short amount of time. The most common techniques use light scattering, fluorescence and/or electrical impedance[1]. Light scattering uses the amplitude and direction of scattered laser light from each cell to determine the cell size and internal structure. Fluorescence uses the presence or absence of fluorescent markers on specific cells to count them. Electrical impedance techniques, commonly used in Coulter Counters, detects changes in the impedance of cells passing through an orifice which is related to the volume of each cell.

An new type of flow cytometer that uses sound waves was developed to identify, count and size cells. Ultra-high frequency ultrasound in the 100-500 MHz range has a wavelength that is comparable to the size of a single cell. In this regime, the sound waves are highly sensitive to the physical and biochemical properties of the cell such as the cell size, shape, structure and composition. The scattered ultrasound wave from a cell contains information about the cell size and morphology, and the photoacoustic wave contains information about the cell structures that absorb the laser. These two independent but complementary measurements enable a multi-parameter analysis where the unique acoustic signatures from each modality enable identification and sizing of single cells[2–4]. Current acoustic/photoacoustic microscopy

Photons Plus Ultrasound: Imaging and Sensing 2016, edited by Alexander A. Oraevsky, Lihong V. Wang
Proc. of SPIE Vol. 9708, 97081A · © 2016 SPIE · CCC code: 1605-7422/16/$18 · doi: 10.1117/12.2211740

techniques can measure the ultrasound and photoacoustic signals from single cells[5,6], however this method is slow and laborious, limiting the measurements to a small number of cells only.

This study describes the development of an acoustic flow cytometer that can rapidly measure the high frequency ultrasound and photoacoustic signals from flowing cells. Recent studies have demonstrated flow systems that use ultrasound to measure signals from particles[7,8], however cells are significantly less echogenic than solid particles, and thus are particularly difficult to detect and analyze using ultrasound. In the acoustic flow cytometer system, a microfluidic device steams cells single file through a target area where each cell is insonified with an ultrasound pulse and irradiated with a laser pulse as shown in figure 1A. A sample of cells can be rapidly measured, and the cell size calculated by comparing the measured signals to theoretical models. In this proof of concept study, we demonstrate that the ultrasound and photoacoustic signals can be used to determine the size of cells using two cell lines with different average cell diameters, acute myeloid leukemia cells and melanoma cells. Biological applications of a sound-wave based flow cytometer include counting, sizing and identifying cells in a sample, assessing morphology of red blood cells, and detecting specific cells using targeted colorimetric stains, fluorescence dyes and nanoparticles.

2. METHOD

2.1 Flow device

A microfluidic device was developed to flow cells into a narrow stream through a target area where they were probed with ultrasound and a laser. The device was made of polydimethylsiloxane (PDMS) using a standard soft lithography technique[9]. The device mold was made using SU8 on a silicon wafer using photolithography[10], and then the PDMS was cured for 3 hours at 70°C. After curing, the device was plasma bonded onto a glass substrate and then a 200 μm needle was inserted into cell inlet. The resulting PDMS device consisted of three inlets, one for cell flow and the other two for sheath flow. All channels were 300 μm wide and 300 μm in height. The two sheath flows operating at 100 μL/min bisected the protruding cell inlet operating at 5 μL/min, hydrodynamically focusing the cells to the middle of the stream laterally and axially. The PDMS flow device is shown in figure 1B, and an optical view of the device flow focusing 6 μm beads is shown in figure 1C.

2.2 Ultrasound and laser hardware

A custom developed hardware system controlled through a computer via software written in Matlab (Mathworks, USA) was used to control all hardware components and acquire the data. A picture of the system is shown in figure 2. The computer consisted of a PCI card trigger source (Spincore, USA) and an 8-bit Cobramax digitizer card (Gage Applied, USA). The trigger source was used to trigger the ultrasound and laser pulses, and acquire signals through the digitizer. The ultrasound source consisted of a 300 MHz pulser (Geozondas, Lithuania) that sent monocycle pulses to an ultrasound transducer with a 375 MHz center frequency (Kibero GmbH, Germany). Signals detected from the transducer were amplified by 30 dB (Miteq, USA) then digitized at 4 GS/s. The laser was directed through a dichroic mirror, and then focused by a 10x optical objective onto the microfluidic device opposite the transducer (figure 2). A xiQ CCD camera (Ximea GmbH, Germany) was positioned behind the dichroic mirror to view the sample and align the transducer and laser spot. Translation stages were used to position the transducer and optics system. Syringe pumps (Harvard Apparatus, USA) were computer controlled and used to push fluid through the microfluidic device.

2.3 Signal acquisition and analysis

Two cell lines were examined in this system: acute myeloid leukemia cells (OCI-AML5) and melanoma cells (A375) obtained from ATTC, USA. These cell lines have different size distributions, and were used to test the diameter-fitting algorithm. Each cell line was measured separately in the flow system. For photoacoustic measurements, the cells were dyed with neutral red, a membrane permeable dye. Ultrasound and laser pulses were triggered at a pulse repetition rate of 4 kHz, and signals were acquired constantly during the measurement period. As a cell passed through the target region, an ultrasound or photoacoustic signal was detected. The noise floor of the system was 2 mV; any signal that was greater than 5 mV was considered a positive detection event and saved to file. Once acquired, all signals were filtered (100-600 MHz bandpass filter), windowed using a Hamming window and the power spectrum calculated. Signal averaging was not used. An algorithm was used to determine the best fit between the measured spectra and theoretical predictions. The ultrasound spectra were compared to the Anderson scattering model[11], and the photoacoustic spectra were compared to the Diebold thermoelastic expansion model[12]. In these models, the cell size, density and sound speed are parameters used to calculated the theoretical signal spectra. The sound speed and density of the cells were known

from previous acoustic microscopy studies[13],[14], and were kept constant in the model calculations. A best fit between the model and measured signal was performed by minimizing the error between the spectral width and location of spectral minima. Specific details in the algorithm fitting method can be found in reference [4].

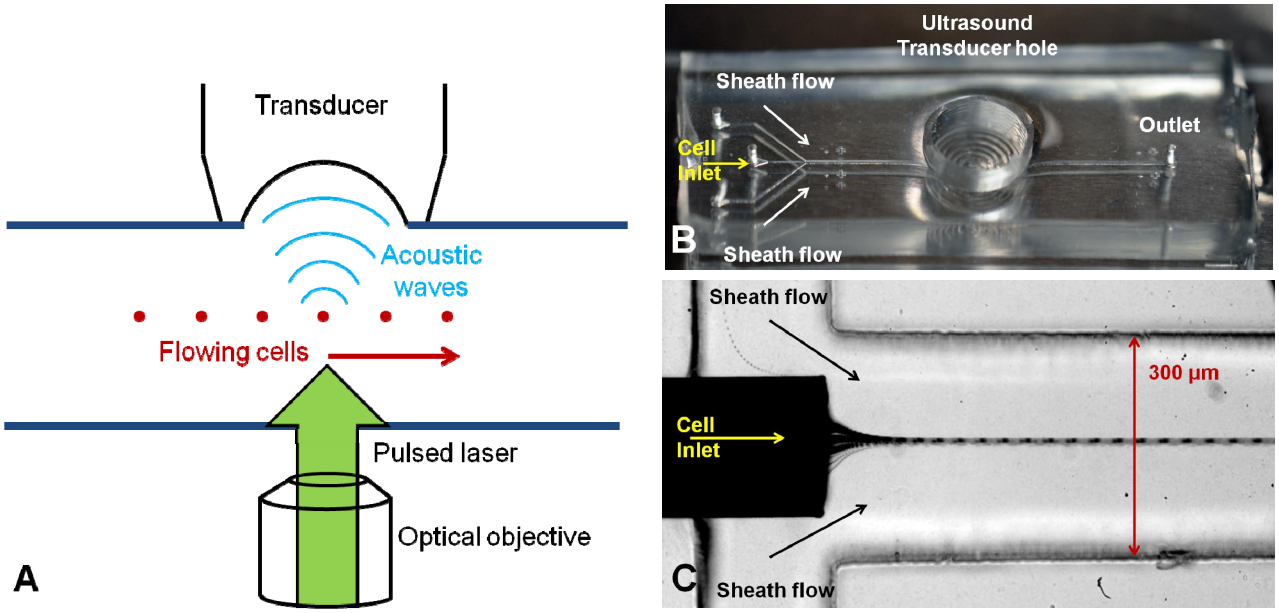

Figure 1: Design of the microfluidic flow device. A) System concept showing cells flowing through the ultrasound and laser target area, B) The PDMS microfluidic device containing the channels and hole for the transducer, C) Optical view inside the flow system demonstrating flow focusing of 6 µm beads through the cell inlet.

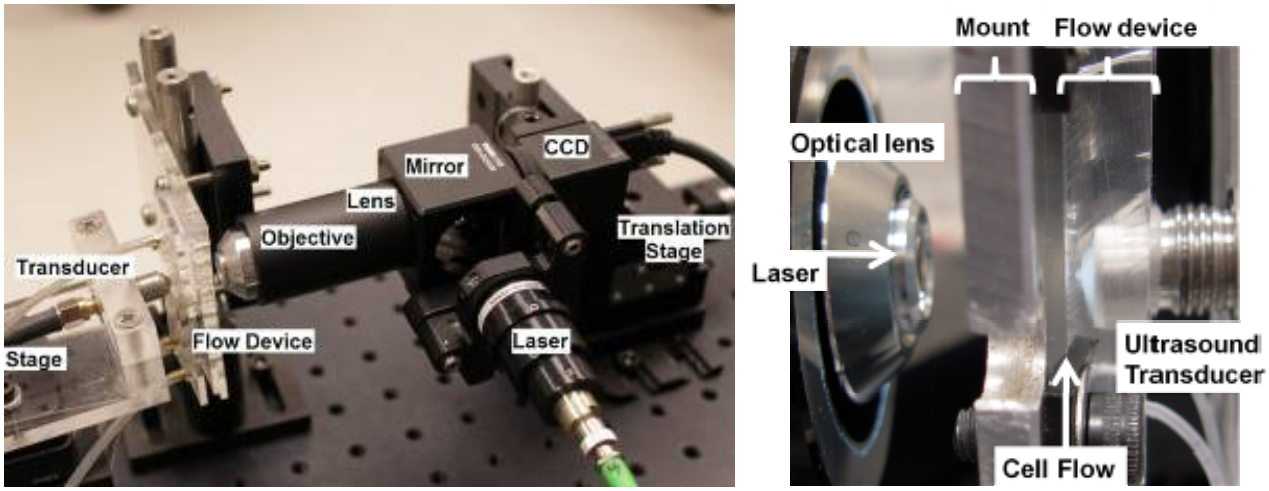

Figure 2: (Left) The ultrasound, optical hardware and flow device, (Right) A close-up view of the objective lens for focusing the laser into the microfluidic device with the transducer positioned inside the microfluidic device.

3. RESULTS AND DISCUSSION

The microfluidic flow device focused the cells into a narrow stream, flowing through the co-aligned ultrasound and laser beams. A representative ultrasound signal and the calculated power spectrum are shown in figure 3. A periodic spectral pattern consisting of sharp minima was observed, where the spectral width between minima is related to the cell size and sound speed; as the cell diameter increases, the spectral width decreases. Our assumption is that the variations in the sound speed between cells is small compared to the variations in the cell diameter, and thus the changes in the spectral width are predominantly due to variations in the cell size. The average spectral width and standard deviation for each cell was calculated and compared to theoretical predictions using the Anderson scattering model[11] to determine the cell size. The best fit theoretical result is shown as a dashed line overlaid on the measured spectrum as shown in figure 3. A representative photoacoustic signal and the calculated power spectrum from a cell are shown in figure 4. The photoacoustic spectra exhibit the same periodic pattern as observed in the ultrasound signals, however the photoacoustic spectral width is approximately double what would be expected in the ultrasound spectra for the same sized cell. This is expected due to the two-way propagation of the ultrasound wave within the cell, compared to one-way propagation of the photoacoustic wave. The photoacoustic spectral width was compared to the Diebold thermoelastic expansion model[12] to determine the cell size in a similar way as with the ultrasound model.

The cell diameter calculated for the AML and melanoma cells using both ultrasound and photoacoustic techniques are shown in table 1. Over 200 cells were analyzed for each cell type and measurement method. The diameter of the AML cells was calculated to be 10.4 ± 2.5 μm using ultrasound, and 11.4 ± 2.3 μm using photoacoustics. The diameter of the melanoma cells was calculated to be 16.2 ± 2.9 μm using ultrasound, and 18.9 ± 3.5 μm using photoacoustics. These numbers agree with the cell measurements using a Multisizer 4 Coulter Counter, where the average AML cell diameter was 9.8 ± 1.8 μm, and the average melanoma cell diameter was 16.0 ± 2.5 μm. In both cell types, the diameter found using the photoacoustic signals were higher than when using the ultrasound signals. The cells were dyed with neutral red for photoacoustic measurements, but not dyed for ultrasound measurements. We have previously observed an increase in ultrasound attenuation with some dyes[15]; it is possible that the dye alters the biomechanical properties of the cells, which may change the spectral width, altering the size estimation. Further work is required to investigate the root cause of the increase in diameter estimation using photoacoustics.

These results demonstrate that ultrasound and photoacoustic measurements from flowing cells can be used to determine the cell size. Future work will focus on using stains and targeted nanoparticles to target specific cells. The nuclear:cytoplasmic ratio can be calculated from the ultrasound signal (from the cell) and the photoacoustic signal (from a stained nucleus) to independently probe the nucleus and cell[16]. This calculation can be used to identify different cell types that may be present in a blood sample.

Table 1: The diameter of the AML and melanoma cells measured using ultrasound and photoacoustics, compared to expected cell diameter measured using a Coulter Counter Multisizer 4. Over 200 cells were measured for each event.

Cell type	Diameter using ultrasound	Diameter using photoacoustics	Expected cell diameter
AML cells	10.4 ± 2.5 μm	11.4 ± 2.3 μm	9.8 ± 1.8 μm
Melanoma cells	16.2 ± 2.9 μm	18.9 ± 3.5 μm	16.0 ± 2.5 μm

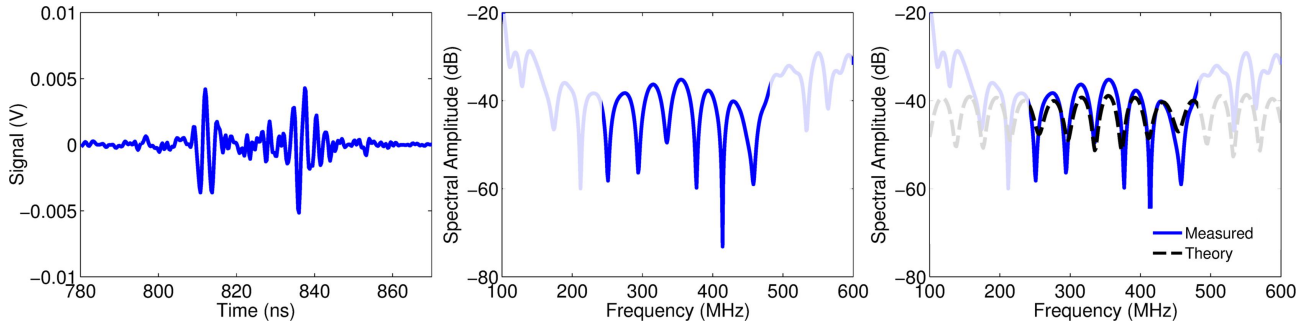

Figure 3: A representative measured ultrasound signal (left) and calculated spectrum (middle) from a single melanoma cell. Areas outside of the transducer bandwidth have been shaded. (Right) The spectrum (blue) with the best-fit theoretical spectrum (dashed black) overlaid on top. Good agreement between the measured and theoretical spectrum were observed. From this fit, the cell diameter was calculated to be 20.2 μm.

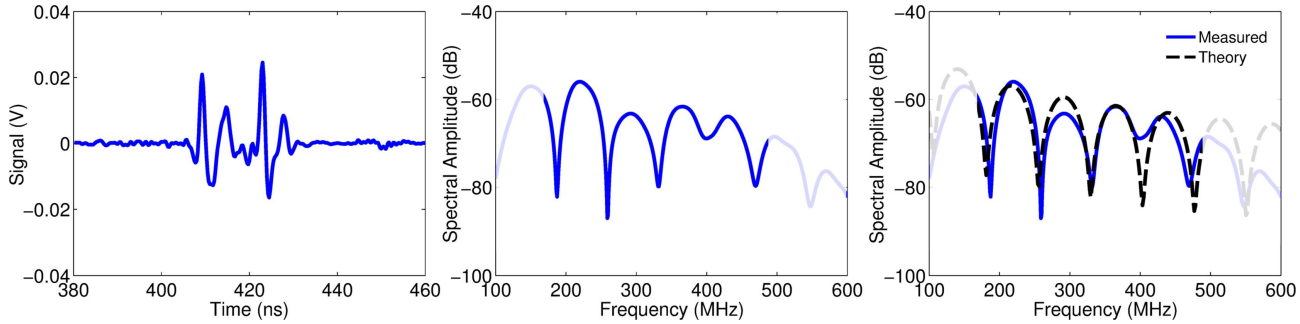

Figure 4: A representative measured photoacoustic signal (left) and calculated spectrum (middle) from a single melanoma cell. Areas outside of the transducer bandwidth have been shaded. (Right) The spectrum (blue) with the best-fit theoretical spectrum (dashed black) overlaid on top. Good agreement between the measured and theoretical spectrum were observed. From this fit, the cell diameter was calculated to be 21.3 μm.

4. CONCLUSIONS

An acoustic flow cytometer that measures the ultrasound and photoacoustic signals from flowing cells has been demonstrated. Using a microfluidic system, cells flowing single file through the ultrasound and laser target area were probed and the ultrasound and photoacoustic signals from each cell were used to determine the cell size of two different cell types: AML and melanoma cells. The diameter of the AML cells was 10.4 ± 2.5 μm using ultrasound, and 11.4 ± 2.3 μm using photoacoustics. The diameter of the melanoma cells was 16.2 ± 2.9 μm using ultrasound, and 18.9 ± 3.5 μm using photoacoustics. These measurements were within expected ranges. Future work will focus on using the ultrasound and photoacoustic signals to count, size and identify specific cells in a population.

5. ACKNOWLEDGEMENTS

The authors thank Arthur Worthington (Ryerson University) for technical support, and Michael Moore (Ryerson University) for helpful discussions. This research was undertaken, in part, thanks to funding from The Canadian Cancer Society generously supported by the Lotte & John Hecht Memorial Foundation, the Natural Sciences and Engineering Research Council of Canada, and the Canada Research Chairs Program. Funding to purchase the equipment was provided by the Canada Foundation for Innovation, the Ontario Ministry of Research and Innovation, and Ryerson University.

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