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Multinode acoustic focusing for parallel flow cytometry

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Abstract

Flow cytometry can simultaneously measure and analyze multiple properties of single cells or particles with high sensitivity and precision. Yet, conventional flow cytometers have fundamental limitations with regards to analyzing particles larger than about 70 microns, analyzing at flow rates greater than a few hundred microliters per minute, and providing analysis rates greater than 50,000 per second. To overcome these limits, we have developed multi-node acoustic focusing flow cells that can position particles (as small as a red blood cell and as large as 107 microns in diameter) into as many as 37 parallel flow streams. We demonstrate the potential of such flow cells for the development of high throughput, parallel flow cytometers by precision focusing of flow cytometry alignment microspheres, red blood cells, and the analysis of CD4⁺ cellular immunophenotyping assay. This approach will have significant impact towards the creation of high throughput flow cytometers for rare cell detection applications (*e.g.* circulating tumor cells), applications requiring large particle analysis, and high volume flow cytometry.

INTRODUCTION

Flow cytometry is a powerful analytical technique used to measure many properties of cells, engineered microspheres, microscopic organisms, and particles in solution for applications that include biomedical diagnostics, monitoring of oceanic environmental states, and detection of biowarfare agents.¹ In conventional flow cytometry, sheath fluid hydrodynamically focuses cell or particle suspensions into a narrow sample stream. In combination with a tightly focused laser, this narrow sample stream creates a small interrogation volume that is analyzed via high numerical aperture optics. The tight positioning ensures highly precise analysis and the small interrogation volume enables largely homogeneous assays (wash-free), both of which are unique strengths of flow cytometry.¹ The collected light is typically distributed via conventional optics to several photodetectors to provide multiple parameters of fluorescence and scatter for each cell or particle. Conventional flow cytometers can analyze cells at rates as high as 50,000 cells/sec,¹ but for many applications that have clinical relevance (*e.g.* detection of circulating tumor cells present in blood at levels as low as a 100 cells per milliliter), the current analysis

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speed of conventional flow cytometers is inadequate.² As accurate and simple detection of circulating tumor cells in blood samples is becoming a highly sought after diagnostic for cancer detection and treatment monitoring applications,³ other technologies (*e.g.* MEMS, magnetic cell separation, wide-field imaging) have been developed to attempt to address rare-cell detection needs.⁴⁻⁶ However, these lack many of the strengths (populational analysis, homogeneous assays, single molecule sensitivity, etc.) of flow cytometers. Therefore, development of flow cytometers with higher analysis rates would clearly be of benefit. Additional rare-cell clinical applications, such as detection of fetal cells in maternal blood for prenatal diagnosis⁷ and endothelial progenitor cells that have roles in cancer and cardiovascular disease,^{8,9} which require analysis of billions of cells in regular basis, could also benefit from improved flow cytometer analysis rates.

While the need for high analysis rates to support rare event detection applications has been recognized, higher analysis rates are limited in conventional flow cytometry by several parameters that include: detector sensitivity, data acquisition electronics, and coincidence rates of particles within the analysis point of the flow cytometer. Detector sensitivity limits the rate of analysis since increasing particle analysis rates typically results in shorter interrogation times, which has led to the use of highly sensitive and fast detectors such as photomultiplier tubes or avalanche photodiodes. Extremely short transit times also pose a challenge for data acquisition where digitization of signals from multiple detectors with 100 MHz 14-bit analog-to-digital converters greatly increase the cost and complexity of the system. Finally, the maximum analysis rate is also determined by the stochastic nature of cellular arrival at the interrogation volume, which limits the concentrations of cells that can be used without causing an intolerable number of coincidences following the Poisson distribution of particle arrival times. Due to the above limitations, a conventional single stream flow cytometer is roughly limited to an analysis rate of 50,000 cells per second.¹

To achieve higher analysis rates it has become necessary to explore the use of parallel analysis streams. To a modest extent this approach has been successfully achieved via the use of four separate hydrodynamically focused streams analyzed by separate optical modules running in parallel. This approach enables analysis and sorting at rates reported at approximately 280,000 cells per second.¹⁰ However, the use of hydrodynamic focusing imparts acceleration to particles as they are focused and results in typical transit-times of one to ten microseconds that necessitate the use of expensive and power-hungry lasers, detectors, and data systems, all of which significantly drive up cost, size, and complexity of parallel hydrodynamically focused flow cytometers.

In part to address the expense, cost, and complexity of traditional flow cytometers, alternative approaches to particle focusing such as acoustic, inertial, and dielectrophoretic positioning have been developed and show promise in single stream flow cytometry applications.¹¹⁻¹³ Such sheathless focusing technologies have the advantage of concentrating particles to precise positions without the concurrent acceleration imparted by hydrodynamic focusing, which allows for high particle analysis rates at reduced linear velocities. Therefore, these approaches offer the potential to create many parallel streams with modest linear velocities, which might greatly simplify the creation of highly parallel flow cytometers with even higher analysis rates and greatly reduced system cost and complexity. Furthermore, these techniques do not require a sheath flow, thus fluid consumption and hazardous waste output is minimized.

The first attempt at highly parallel flow streams used highly parallel inertial focusing channels that were analyzed using wide-field imaging.¹⁴ This approach is promising, but does have some limitations as the channel widths must be designed specifically for the size of particles under analysis.¹⁵ As such, this approach may not be able to easily handle wide

range of cell or particle sizes in a single device. Nonetheless, the ability of this approach to precisely position cells and particles in a highly parallel array is clearly promising for high-throughput flow cytometry.

However, there may be distinct advantages to the use of acoustic focusing for parallel analysis. Acoustic focusing positions biological cells and other particles over a wide range of sizes, can utilize wide channels, and supports high volumetric flow rates for flow cytometry.^{8,16,17} To accomplish this, acoustic focusing employs an ultrasonic standing wave to position particles suspended in a fluid-filled cavity, via a time-averaged drift force that transports them to a nodal or anti-nodal position.¹⁷ Acoustic force on a particle is given by the equation (1).¹⁷

$$F = - \frac{(\pi p_0^2 V_p \beta_m)}{2\lambda} \Phi(\beta, \rho) \sin 2kx \quad (1)$$

$$\Phi(\beta, \rho) = \frac{5\rho_p - 2\rho_m}{2\rho_p + \rho_m} - \frac{\beta_p}{\beta_m} \quad (2)$$

From equation (1) we can obtain that acoustic force (F) is proportional to the particle volume (V_p), the applied frequency ($1/\lambda$), and the acoustic contrast factor (Φ). The acoustic contrast factor depends on the density of the particle (ρ_p), the density of the suspended medium (ρ_m), the compressibility of the particle (β_p), and the compressibility of the medium (β_m), as presented in equation (2).¹⁷ As particle volume is proportional to the cubed radius of the particle, the acoustic force is strongly dependent on the particle size. Except in the rare case where the density component equals the compressibility component in equation 2, a particle will experience an acoustic force as shown in equation 1. However, the sign of the acoustic contrast factor will determine the direction of particle movement. In general, particles that are denser and less compressible are driven to the pressure node of a standing wave (positive contrast) and those that are less dense and more compressible than the surrounding fluid (negative contrast) are driven to the pressure antinodes. Of course, it is possible for particles to exhibit a combination of these properties (less dense and less compressible or denser and more compressible), and in these cases the relative magnitude of the density and compressibility terms (in equation 2) will determine the contrast type of the particle. More detailed descriptions of acoustic forces on particles can be found in Laurell et al.¹⁷ and Wiklund et al.¹⁸

Acoustic focusing cells in a variety of forms have been developed for many applications.¹⁷ In planar standing waves, particles are typically regularly spaced at half-wavelength intervals parallel to the direction of acoustic wave propagation.¹⁷ Alternatively, the use of cylindrical transducing elements can drive standing waves with more complex structures that focus particles in two-dimensions to an axially positioned focusing node in the center of the capillary, which is analogous to how a traditional hydrodynamic focusing flow cell functions.^{11,16} As the optimal resonant frequency of all acoustic cells varies based on the viscosity, density, and temperature of the sample, this approach requires active control to maintain optimal focusing.^{11,16}

Here we explore the use of a simple planar acoustic standing wave to focus multiple parallel particle streams for flow cytometry. This approach is desirable as the number of flow streams can be varied easily by changing the frequency of a simple planar flow cell. We demonstrate the potential of these flow cells by implementing them within prototype flow cytometers that support precise measurements of flowing particles. We also demonstrate parallel analysis capabilities by employing multinode acoustic standing waves to

successfully focus particles and cells into multiple parallel streams in rectangular glass capillaries and larger machined flow cells that support up to 37 distinct particle flow streams simultaneously. Furthermore, we demonstrate acoustic focusing of particles with a range of sizes (from red blood cells to 107 μm diameter microspheres) at linear velocities and sufficient number of parallel streams to support analytical rates of a few thousand particles per second. Finally, we discuss these flow cell designs, which, with further optimization, will have great implications for high throughput cellular analysis via highly parallel flow cytometry and also impact less conventional applications, which include the analysis of larger particles including tumor microspheroids, one-bead-one compound particle libraries, and the analysis of small multicellular organisms.

METHODS AND MATERIALS

Flow Cell Construction

We fabricated acoustic flow cells either using commercially available glass capillaries or machined aluminum frames. Capillary based devices were used to create 1-3 parallel streams while machined devices were used to create 17 to 37 parallel streams. In either type, an acoustic transducer element was attached to one of the sides of the flow cell to drive the resonance frequency and in some cases one on the opposing side to pick up feedback signals.

Flow Cells from Glass Capillaries—Two lead zirconate titanate (PZT) ceramics (5 mm $\times$ 30 mm, APC International, Mackeyville, PA) were epoxy glued to two short walls of a rectangular fused silica capillary (Vitrocom, Mountain Lakes, NJ) with known dimensions (provided in the results). The frequency of the two PZTs were matched to the width of the glass capillary. Two silicone tubes with inner diameter matching to largest outer dimension of the glass capillary were attached to both ends of the capillary to serve as inlet and outlet for liquid connection. The assembled device was suspended on two blocks of poly(dimethylene siloxane) (1 cm $\times$ 1 cm $\times$ 0.5 cm) glued on to a standard microscopic glass slide, thus isolating two PZTs.

Flow Cells from Machined Aluminum Frames—A machined aluminum frame was sandwiched between two microscope glass slides (25 mm $\times$ 75 mm) and sealed with epoxy glue. The space between two glass slides was 730 μm . The metal frame had two through holes at the two vertical edges for liquid connection. Two silicone tubes were attached at the two through holes as the sample inlet and outlet. A circular piezoelectric transducer (2.5 cm diameter) was ultrasonic gel coupled to one side of the metal frame and used as the driving element. The acoustic coupling efficiency was monitored via a second circular piezoelectric element (1.25 cm diameter) gel coupled to the other side of the metal frame.

Sample Focusing

Devices were rinsed with skim milk, 1% Sodium dodecylsulfate (SDS) solution, and PBS buffer (pH 7.5) respectively prior to sample introduction to minimize non-specific adherence of particles onto the device walls. Samples (particles or blood) were flowed upward at a rate of 50 $\mu\text{L}/\text{min}$ or above through the device using a syringe pump (Nexus 3000, Chemyx Inc. Stafford, TX) while the focusing field was applied through the driving transducer element perpendicular to the direction of flow. The acoustic transducer was operated via a high power RF (radio frequency) amplifier (Empower RF Systems, Inglewood, CA) and the frequency was set by a waveform generator (33250A, Agilent, Santa Clara, CA). The frequency was fine tuned to generate the expected number of focused streams.

Focused Stream Analysis

The focusing efficiency of samples in capillary based devices was analyzed via an epi-fluorescence microscope and/or custom made flow cytometer. Highly parallel focusing in metal frame device was analyzed via a digital camera.

Imaging—In capillary based devices, we used a conventional epi-fluorescence microscope (Axio Imager –2, Carl Zeiss Inc.) equipped with a 10X objective (NA of 0.3), metal halide lamp, an EMCCD camera (Andor Luca S, Belfast, Ireland) and image analysis software (Andor Solis, Andor technology, Belfast, Ireland) to capture digital images. A filter cube set containing 545/25 nm band pass filter for excitation, a beam splitter with fluorescence transmission at 570 nm and 605/70 nm band pass filter for emission was used for image capturing. Image analysis was performed by line-scanning the image across the capillary. Screen shot captured images that were obtained from digital video recordings were used in the analysis of highly parallel focusing.

Flow Cytometry Analysis—The custom-made epi-fluorescence flow cytometer system used here was similar to the one that we reported before.¹³ The only modifications were that the backscatter PMT was replaced by a photodiode and instead of the CCD camera a simple video camera was used for initial sample alignment. The acoustic cell was angled at approximately 25 degrees to assure that we did not get direct reflection of laser light back down the light collection path. In this way, our scatter parameter actually collected low angle ‘backscatter’ and our fluorescence parameters collected low angle fluorescence rather than true epi-fluorescence. Data acquisition was performed using a custom digital microcontroller data acquisition system, which provided data files in Flow Cytometry Standard (FCS) 3.0 format.^{19,20} Data was collected using an inexpensive netbook (Samsung NC10), further decreasing size and cost since very little computing power is needed. FCS files were analyzed using FlowJo 7.6 and 9.0 (TreeStar Software, Inc., Ashland, OR) or FCSEXPRESS v.3 (DeNovo Software, Los Angeles, CA).

Preparation of Blood Samples

Preparation of White Blood Cells for CD4 Analysis—For CD4 analysis, a standard CD4⁺ panleukogating preparation was performed per published protocols.²¹ We used the anti-human CD4 antibody conjugated to Phycoerythrin (PE) and the anti-human CD45 antibody conjugated to PE.Cy5 (Biolegend, San Diego, CA) to label white blood cells. 100 μ L of IMMUNO-TROL, (Beckman Coulter, Brea, CA) positive process control whole blood samples were incubated with 10 μ L of each of the labeled antibodies listed above for 20 minutes in the dark. IMMUNO-TROL sample was then incubated in 1.5 mL of red blood cell lysis buffer (RBC Lysis buffer, 10X, Biolegend San Diego, CA) containing NH_4Cl , KCO_3 , and EDTA for 10 minutes to lyse red blood cells. This sample was then washed in cell staining buffer (Biolegend, San Diego, CA) and suspended in 1 mL of PBS buffer before analysis.

Preparation of Red Blood Cells—For focusing of red blood cells in the highly parallel acoustic flow cell, IMMUNO-TROL positive process control blood was diluted 10 times with PBS buffer prior to use.

RESULTS

Multinode Acoustic Focusing of Flowing Particles

The piezoelectric element converts electrical pulses into mechanical vibrations and vice versa. Using the PZT attached to the side-wall of the capillary, which vibrates at a resonant frequency of the rectangular flow cell, we are able to generate a variety of acoustic standing

waves with varying numbers of pressure nodes (Figure 1a). These pressure nodes are used to align flowing particles in multiple, discrete streamlines as they flow through the chamber (Figure 1c, d and Movie 1 and Movie 2 in the Supporting Information). The additional PZT was utilized to monitor the signal passing through the capillary, and is used to tune the resonance frequency (Figure 1a). These devices take advantage of a linear, standing, acoustic wave that can be tuned to various wavelengths, creating single or multiple harmonics.¹⁷ As predicted by the simple linear standing wave model, which has been shown to be accurate for rectangular channels, this results in single (Figure 1b, top) or multiple nodes (Figure 1b, middle and center) within the flow chamber. Notably, this model predicts that the dominant acoustic force drives particles in across the width of the channel into nodal positions and does not result in secondary positioning effects at the walls or elsewhere. This is consistent with the data shown in Figure 1c and 1d, as well as that seen in acoustic separation devices.¹⁷

Image and Flow Cytometry Analysis of Flowing Microspheres in a Multinode Flow Cell

To evaluate whether these flow cells support effective flow cytometry, calibration microspheres (10 μm Nile Red labeled polystyrene microspheres (NR-ps), Spherotech Inc., Lake Forest, IL) were focused into three streams at a resonance frequency of 1.49 MHz and a flow rate of 100 $\mu\text{L}/\text{min}$ in 0.1 mm (height) $\times$ 1 mm (width) $\times$ 7 cm (length) capillary device. First, the focusing was analyzed microscopically using an epi-fluorescence microscope. The field of view of the microscope objective (10X, NA of 0.3) was large enough to record across the entire width of the focusing cell, thus capturing all three streams simultaneously (See Movie 3 in the Supporting Information). Fluorescence images of unfocused (Figure 2a) and focused (Figure 2b) microspheres were captured and analyzed (Figure 2c, d). Further, to demonstrate the precision of our focusing system for quantitative flow cytometry analysis, the three streams were analyzed individually using a custom-made epi-fluorescence flow cytometer optimized for planar flow cells. The custom flow cytometer was aligned to create a flow cytometry analytical volume about 1.5 cm above the edge of two PZTs (Figure 1a) and analysis proceeded in the same fashion that has been described for microchip flow cytometers.¹³ The same calibration microspheres used for image analysis were interrogated with a 532 nm laser while backscattered light was used to trigger data collection for both backscatter and fluorescence. Fluorescence histograms obtained from flow cytometry analysis shows particle distributions and their intensity distribution at non-focused (Figure 2e) and focused (Figure 2f) conditions. The focused stream has a coefficient of variation (CV) of 15% and the unfocused stream has a CV of 51%. According to the manufacturer's specifications, the beads have an intrinsic CV in their fluorescence of 6%.

To demonstrate the capability of analyzing biological samples in our multinode focusing cell, we performed a standard flow cytometric test for CD4⁺ analysis. White blood cells (WBC's) from a pathogen free, normal human blood sample were prepared using standard methods and analyzed at a flow rate of 50 $\mu\text{L}/\text{min}$. We used CD45 antibodies conjugated with phycoerythrin-Cy5 to specifically label leukocytes with a fluorescent marker and CD4 conjugated with phycoerythrin to label CD4⁺ cells. Red blood cells were specifically lysed during the preparation using a commercial lysing solution that has been shown to not lyse white blood cells. Fluorescent image of focused WBC (Figure S-1) demonstrates that lysis process has little or no effect on focusing of WBC acoustically. Using a contour plot of backscatter parameter vs. CD45 fluorescence, we were able to create a 3 part white blood cell differential (Figure 3a). The differentiation of WBC populations is consistent with the expected populations. It shows a clear separation of the lymphocyte population from the granulocytes, the predicted position of the monocytes, and the cellular debris (Figure 3a). The lymphocyte population was gated (blue line) and displayed as a one-dimensional histogram, which clearly shows the CD4⁺ population (Figure 3b). Moreover, when the

positive population is analyzed, it gives the correct percentage of lymphocytes for the control blood sample (45 $\pm$ 5%).

Multinode Focusing and Analysis of Large Particles

To evaluate the ability of our parallel acoustic flow cell to focus large particles for analysis, we evaluated its performance using 107 μ m diameter red fluorescent polystyrene particles (Thermo Scientific, Fremont, CA). We created a larger flow cell (0.2 mm height $\times$ 2 mm width $\times$ 7 cm length) driven at 733 kHz, which resulted in three focusing nodes (Figure 1d). Particles (10^7 particles/mL) were flowed upward at a rate of 1.6 mL/min. We again used our custom flow cytometer to perform quantitative analysis of each stream individually. Again backscatter was used to trigger data collection and both backscatter and fluorescence were collected for each event. Each streamline of focused particles revealed outstanding precision (5 to 8% coefficient of variation) for each stream, when analyzed independently (Figure 4).

Highly Parallel Acoustic Focusing Flow Cells

Theoretically, it should be possible to predictably achieve extremely high numbers of parallel flow streams via acoustic focusing. To explore this possibility, we designed a very wide (1.6 cm across) flow cell with a volume of about 800 μ L, that was driven with varying frequencies to generate a high number of focused streams (Figure 5a). A sample containing 10 μ m NR-ps particles (1.36×10^5 beads/mL) was flowed upward at a flow rate of 250 μ L/min and a flow rate of up to 1000 μ L/min is possible. Once the frequency is fine tuned, particles were focused into highly parallel multiple streams at various resonant frequencies (Figure 5). Optical images in Figure 5 show two situations where 24 (Figure 5b) and 33 (Figure 5c and Movie 4 in the Supporting Information) streams of focused particles are generated. Two close range images (Figure 5d, e) indicate that these highly parallel streams are well resolved from their adjacent streams. We were able to generate similar data from drive frequencies that provided between 17 and 37 streams (Data not shown). Furthermore, when we focused whole blood (diluted 1:10 in PBS), we were able to obtain 33 well-focused streams of red blood cells at the predicted frequency (Figure 5f, g and Movie 5 in the Supporting Information).

DISCUSSION

Here we have demonstrated multinode planar acoustic standing waves to create parallel flow streams for flow cytometry analysis. Similar multinode positioning techniques have been demonstrated for moderately parallel one-dimensional trapping of particles (up to 4 nodes) in non-flowing systems and in particle separation systems using simple standing waves and surface acoustic waves.^{17, 22-25} Surface acoustic waves have also been used to create static arrays of cells in non-flowing systems.²³ While flow based systems for particle separation have been explored, the primary purpose of these works has been to explore how efficiently particles can be separated from either the carrier medium and/or other particles of different size and/or compositions.¹⁷ Here, we have effectively used flowing multinode acoustic focusing systems that enable effective and simultaneous analysis of particles in as many flowing streamlines as can fit in a single flow cytometry flow cell. As such, the methods used to evaluate the utility of multinode acoustic focusing have differed from those used to evaluate similar approaches for particle separation, which examine parameters such as collection efficiency and separation ratios that are not only related to the efficiency of acoustic focusing, but also to the fluid mechanical details of the collection channels created to collect particles.¹⁷ In this work, we have demonstrated the utility of these approaches for flow cytometry by directly evaluating several designs in conjunction with a custom flow cytometry platform.

Specifically, we have demonstrated effective moderately parallel acoustic focusing in rectangular glass capillaries over a wide range of particle sizes ranging from 10 μm to 107 μm by image analysis (Figure 1). These images demonstrated single file acoustic focusing of particles that was expected to support precision flow cytometry analysis. Fluorescence streak images of a three node parallel focusing glass capillary (Figure 2b) further confirmed that we could obtain highly precise focusing using 10 μm polystyrene microparticles. Interestingly, in this system the observed resonance frequency was 1.49 MHz for a three-node system, but calculation predicted a value of 2.22 MHz. The outer sidewalls of these glass capillaries where PZT is attached are not flat and a thin layer of epoxy-glue was used to attach the PZT to the capillary. Therefore, as first suggested by Evander et al., who observed similar discrepancies for acoustic focusing in etched glass devices,²⁴ disagreement in calculated and measured resonant frequencies may be due to such structural and material effects. Despite this discrepancy in resonance frequency, the three focused streams are placed in equal distance from each other (Figure 2d) and this distance is equal to half wavelength of corresponding resonance frequency. The presence of three distinct peaks found in a time based histogram of intensity (Figure 2d) further suggest that there is no particle crossings between focused streams, which is important as our flow cytometry analysis (see below) is only sensitive to particles that intersect the tightly focused laser.

The quantitative data obtained from our custom flow cytometer further indicate the precise particle focusing in these devices and demonstrate the effectiveness of this approach for flow cytometry analysis. The dramatic reduction of the CV for the focused sample in comparison to un-focused sample (Figure 2e, f) demonstrates that we are achieving effective particle focusing. However, as we expect that we are only focusing the particles into 'ribbons' (roughly a 1D focus where little to no focusing occurs in the short dimension of the flow cell) and not a tight core, it is likely that we have not achieved the tight focusing that has been achieved for more refined single stream acoustic flow cytometers based on structural excitation.²⁶ This ribbon focusing could result in particles moving at different flow rates at differing points in the parabolic flow profile and also result in differing laser intensities on the particles as they pass through different planes of the expected hourglass profile of the focused laser. Either of these effects could result in the good (5 to 15%) but not ideal CVs (typical flow cytometers can achieve 2%) measured in our flow cells. However, as has been seen in the development of other cytometers, we expect that after this proof-of-principle demonstration, potential improvements (e.g., the inclusion of small amounts of sheath or orthogonal PZT elements for top-to-bottom focusing, reducing channel height to restrict top-to-bottom position of particles, etc.) are likely to result in improved focusing precision of the system.

Analysis of white blood cells for CD4+ content in multiple streams demonstrates the potential of our flow cells to support high throughput analysis of clinically relevant samples via parallel acoustic focusing. Analysis of the histogram (Figure 3b) gave a CD4 positive fraction of 40%, which is within the expected range of the control values provided by the manufacturer (Beckman Coulter, Brea, CA) and the calibration test performed via Accuri C6 (Accuri Cytometers Inc., Ann Arbor, MI) flow cytometer. The poorly resolved monocyte population (Figure 3a), is likely due to non-optimized sample alignment and the 1D focusing provided by the flow cell, which will be refined in future systems.

One area where traditional hydrodynamically focused flow cytometry has had difficulty is the analysis and sorting of particles larger than approximately 70 μm .¹ These difficulties primarily occur due to the increasing likelihood of turbulent flow as channel dimensions increase to accommodate larger particle sizes – yet linear velocity is kept high to maintain high particle analysis rates that are important for most applications. As the onset of turbulent flow eliminates effective hydrodynamic focusing, its use is particularly difficult for

applications that include analysis of small multicellular organisms, multicellular spheroids, and molecular interactions at the surface of the large microspheres (> 100 μm particles) used in 'one-bead one-compound' combinatorial libraries.²⁷ In the analysis of large particle focusing (Figure 4), the low CV values in each stream imply that our focusing method tightly positioned particles for analysis.²⁸ The improved focusing for larger particles was likely due to the increasing acoustic force, which increases as particle size increases.²⁹

While traditional hydrodynamic focusing has difficulty in positioning larger particles, it can position particles as small as a single molecule for analysis.^{1,30} As the acoustic force on a particle is proportional to particle volume, it is unlikely that acoustic focusing will be effective for single molecule flow cytometry. However, planar standing waves in particle separation devices have effectively positioned particles as small as 3 microns in diameter¹⁷, structural acoustic focusing devices have effectively focused particles as small as 3 microns in diameter,²⁶ and here we have demonstrated effective focusing for 10 micron diameter particles and a white blood cell population that contains blood cells as small as 3 microns. As such, it is clear that acoustic focusing is effective for particles and cells that are 3 microns in diameter and larger. Furthermore, as increased particle residence time in the acoustic field (achieved by reducing flow rates and/or extending the focusing regions of our channels) and increased applied force to particles (achieved by improved acoustic coupling, and increased drive voltages) will improve the positioning of smaller particles, it is likely acoustic focusing will be effective for particles as small as a micron in diameter.^{17,26} Thus, in future work we will create highly optimized flow cytometry systems via the use of as long a focusing region as feasible, optimal PZT coupling, and explore the limits of applied drive power to achieve effective focusing for flow cytometry.

With regards to highly parallel flow cytometry, image analysis of our highly parallel flow cell clearly demonstrates that we can horizontally position particles to tightly focused streams that are tens of microns wide, using flow cells that provide up to 37 parallel flow streams. Interestingly, in the highly parallel acoustic focusing system the number of streams generated at a given frequency was identical to the theoretical half-wavelength value (Figure 6), which is unique for this planar flow cell as compared to the capillary based flow cells which significantly deviate from predicted frequencies (see above). The theoretical values for number of focused streams (nodes) can be calculated by the following equation.

$$n=2L \quad v/C \quad (3)$$

Where n is the number of nodes, L is the width of the channel, v is the applied resonance frequency and C is speed of sound in water (1480 m/s) at the operating temperature. The predictable number of nodes generated by this flow cell enable us to easily create focused streamlines ranging from 17 to 37 streams across the flow cell and we show images of a subset of these frequencies that generate 24 and 33 streams of 10 μm polystyrene particles at 1.17 and 1.54 MHz respectively (Figure 5b, d). Captured images at close range (Figure 5c, e) and captured video clip at long range (Movie 4 in the Supporting Information) indicate that focused streams are well resolved and have very good focusing without any sample cross-over. Overall, non-specific sample adherence to device walls occurs during extensive re-use of the device and inadequate rinsing of the device after each use. Captured real time video clips (Movies 1-5 in the Supporting Information) of both particle and blood focusing clearly show that sample loss due to non-specific adherence and sample cross-talk is minimal during sample runs using all devices.

Additionally, we have demonstrated analysis at volumetric delivery rates as high as 1.6 mL/min. This rate is an order of magnitude greater than traditional hydrodynamic systems¹ and almost two fold greater than other acoustic focusing systems reported to date.²⁵ This high

volumetric analysis rate could be of great value for analysis of samples that have very dilute cell or particle concentrations, which may be expected in some clinical and environmental samples.¹

Finally, while volumetric analysis rate is a useful metric, maximum particle analysis rate is also very important. When using traditional flow cytometry optical geometries, particle analysis rate is a complex function of concentration, interrogation volume, stochastic particle arrival rate, and linear velocity of the flow stream (as described above).¹ While the acoustic concentration effect has the distinct advantage of allowing simple online concentration of cells, the maximal particle analysis rates of our flow cells will be governed by the same factors as traditional flow cytometry cells. Assuming a 100 μm particle size, a particle concentration that provides average of 25% to 50% interrogation volume occupancy, and a 100 μm laser spot size, each stream of our capillary based flow systems (~ 7 cm/s linear velocity for large particles) could deliver 175 to 350 particles per second through the interrogation volume. As our capillary based flow cell currently supports 3 streams, this flow cell would be expected to support about 1000 events per second. While this seems modest, this already equals the best that highly refined conventional cytometers can achieve when analyzing large particles.¹

For cell sized particles (~ 10 μm diameter) our capillary flow cell has a linear velocity of ~ 2 cm/s, following similar logic as above (assuming 10 μm particle diameters and 10 μm laser spot height) this linear velocity would be expected to support an analysis rate of 500 to 1000 particles per second per stream or about 1500 to 3000 particles per second for a 3 stream capillary device. For similar particle size our machined device has a linear velocity of ~ 0.14 cm/s and thus it can deliver 1320 – 2640 particles per second for a 37-stream device. While it is interesting to note that use of higher frequencies to achieve higher numbers of focusing nodes also is predicted to provide increased force on particles within the standing wave (see equation 1), this simple prediction does neglect the effect of scattering from particles within the standing wave and attenuation of the wave as it propagates through the aqueous medium. While the data presented here did not show a strong relationship to either particle concentration or the number of nodes when observed in a single device type, the larger machined device was notably less efficient in focusing particles, which may be due to the attenuated acoustic forces experienced by the flow stream, the particle concentrations that scatter acoustic waves across the width of the channel, or poor coupling of the acoustic transducer. While poor coupling could be easily overcome by utilizing improved fabrication techniques that enable better integration of components and minimize material effects, the effects of wave attenuation across the liquid medium and the effects of particle concentration may eventually limit the number of nodes that can be used in single channel. Future work, will rigorously explore the limits of multinode acoustic focusing in flow-based systems based on detailed evaluations of these parameters.

Regardless, our estimates of particle analysis rate make it clear that parallel acoustic flow cells that support increased linear velocities will be required to achieve high analytical rates of cell sized particles. Considering that analytical rates approaching 10^4 per second (per stream) will be required to create flow cytometers that analyze 10^6 particles per second (in aggregate), flow cells that effectively focus cell sized particles at linear velocities 10 to 20 fold greater than our current cells will need to be developed. Improved flow cells could be developed in several ways: 1. By increasing the length of the focusing region, which has been successful for single node flow systems.²⁶ 2. Increasing the drive force applied. 3. Increasing the frequency of the standing wave, which is predicted to increase the relative acoustic force applied to particles.²⁹ Future work will pursue all of these approaches to create flow cells that support very high cellular analytical rates via parallel application of low cost flow cytometry optics, detectors, and data acquisition systems.

CONCLUSION

Here we have successfully implemented multi-node acoustic focusing flow cells and demonstrated their potential in developing high throughput, parallel flow cytometry. This approach has the capability to: 1. Align particles and blood cells into as many as 37 parallel streams. 2. Focus particle sizes ranging from that of a red blood cell to particles 107 μm in diameter. 3. Precisely position human white blood cells for a CD4+ cell analysis. 4. Provide volumetric throughputs greater than 1 mL/min. We have also demonstrated that our machined flow cells provide a predictable path to increasing the number of parallel streams. Conventional flow cytometry has most likely reached its throughput maximum due to fundamental limits on analyzing cells faster in hydrodynamically focused sample streams, which make it difficult to use current technology to develop systems with analysis rates sufficiently high for use in clinical and diagnostic applications that require rare event detections, such as the detection of circulating tumor cells. Furthermore, current technology hinders analysis of larger particles including tumor microspheroids, one-bead-one compound library particles, and the analysis of small multicellular organisms. Additionally, the very high volumetric sample flow rates supported by the large flow cells used here in combination with their concentration effects may make them useful for looking for rare particles in dilute samples, which would be of value in environmental monitoring for biothreat agents and naturally occurring organisms.^{30,31} Finally, as parallel flow cells support precise measurements, a wide range of particle sizes, and are easily manufactured, they offer a potential approach, in combination with low-cost lasers and data systems for flow cytometry,^{19,20} to the creation of affordable high-throughput flow cytometers that can both rapidly process samples containing large particles and analyze samples for very rare events. The acoustic based, parallel focusing techniques and multi-stream analysis methods we have demonstrated, will have significant impact towards designing high throughput flow cytometers using parallel analysis approaches. The ability to analyze large particles and handle large volumetric throughput will add additional significance to such an approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Multinode acoustic focusing in glass devices. (a) A schematic drawing of acoustic flow cell made with a rectangular glass capillary. (b) The location of first three pressure nodes for a fixed width capillary (Top – width = $\lambda/2$, center – width = λ , bottom – width = $3/2 \lambda$). Optical micrographs showing the focusing of two sizes of polystyrene particles into 3 nodes: (c) 10 μm in a 0.1×1 mm capillary and (d) 107 μm in a 0.2×2 mm capillary.

Figure 2.

Analysis of multinode acoustic focusing in $0.1 \text{ (height)} \times 1 \text{ (width)}$ mm glass capillaries. Fluorescent micrographs of (a) non-focused and (b) focused streams of $10 \mu\text{m}$ NR-ps particles. (c) Fluorescent image analysis of non-focused and (d) focused streams. Flow cytometric analysis of (e) non-focused and (f) focused streams.

Figure 3. Analysis of WBC in multinode acoustic flow cell. (a) Contour plot of stained WBC (b) Histogram of gated lymphocytes population with 40 % of CD4+ cells.

Figure 4.

Flow cytometric analysis of acoustic focusing of 107 μm red fluorescent polystyrene particles flowing at 1.6 mL/min. Each of the 3 focused streamlines was analyzed individually yielding CVs for the left (a) 5%, center (b) 6%, and right (c) 8% streams respectively.

Figure 5.

Formation of highly parallel focused streams in the machined device with different resonance frequencies. (a) A schematic of the machined flow cell for high numbers of parallel streams. The left hand photo panels show focusing captured at long range, while the right hand panels show the streams at close range. (b, d) 1.17 MHz, 24 streams of 10 μm polystyrene particles, (c, e) 1.54 MHz, 33 streams of 10 μm polystyrene particles. (f, g) 1.54 MHz, 33 streams of red blood cells.

Figure 6. Observed and theoretically expected number of focused streams of 10 μm NR-ps particles generated at varying applied resonance frequencies (circles – observed, line – expected)