Published in final edited form as:

Anal Chem. 2015 June 2; 87(11): 5596–5604. doi:10.1021/acs.analchem.5b00370.

Concurrent Isolation of Lymphocytes and Granulocytes Using Prefocused Free Flow Acoustophoresis

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Abstract

Microchip-based free flow acoustophoresis (FFA) in combination with two-dimensional cell prefocusing enables concurrent multiple target outlet fractionation of leukocytes into subpopulations (lymphocytes, monocytes and granulocytes); we report on this method here. We also observed significantly increased accuracy in size-based fractionation of microbeads as compared to previously presented FFA multiple outlet systems. Fluorescence microscopy illustrates the importance of two-dimensional prefocusing where a sample mixture of 3-, 7- and 10-micrometer beads are separated into well-confined particle streams and collected in their respective target outlets. Flow cytometry data for lymphocytes and granulocytes, respectively, in their corresponding outlets verify concurrent isolation of leukocyte subpopulations with high purity ($95.2 \pm 0.6\%$ and $98.5 \pm 0.7\%$) and high recovery ($86.5 \pm 10.9\%$ and $68.4 \pm 10.6\%$). A relatively low purity and high recovery of monocytes ($25.2\% \pm 5.4\%$ and $83.1 \pm 4.3\%$) was obtained in the third target outlet. No subpopulation bias was observed. These data demonstrate an unprecedented separation of leukocyte subpopulations at flow rates of $\sim 100 \mu\text{l}/\text{min}$ and $\sim 1\text{M}$ cells/ml sample concentrations, not previously reported in acoustofluidic systems. Two-dimensional prefocusing FFA with multiple target outlets is a viable alternative to current methods for particle fractionation and cell isolation, requiring a minimum of sample preparation, and lowering analysis time and cost.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s). Dr. Hans Lilja holds patents for free PSA, intact PSA, and hK2 assays. Cecilia Magnusson, Carl Grenvall, and Thomas Laurell are listed as inventors of a patent application based on the reported method: Title: System and method to separate cells and/or particles; Inventor: Per Augustsson, Cecilia Magnusson, Carl Grenvall, and Thomas Laurell; Publication info: P9716SE00.

INTRODUCTION

This paper presents a method to fractionate leukocytes or other complex particle- or biosuspensions into pure subpopulations by combining acoustophoresis with two-dimensional (2D) acoustic prefocusing in a chip with multiple target outlets. Recent years have demonstrated an increased activity in the Lab-on-a-Chip (LOC) field, aimed at integrating miniaturized analytical tools for biotechnical applications to decrease cost or to allow handheld devices in point-of-care diagnostics.^{1–4} The development of rapid and precise low-cost methods for fractionation and analysis of blood, a matter of vital interest for clinicians as well as researchers, is one of the areas benefitting from this technology. Among the possible applications for such improved methods is the purification of leukocytes into subpopulations that may then be used to analyze gene expressions, interleukin signaling between different subpopulations during immune responses, and help to distinguish between viral and bacterial infections, as well as detect leukemia.^{5–8}

Traditionally the preparation and fractionation of blood require several steps of pipetting, centrifugation, and labeling, which are time-consuming, expensive, and require trained personnel. The methods also risk introducing unwanted artifacts like fibers, hair, or dirt particles into the samples during the manual handling; may affect cell viability or phenotype profile.^{9–14} An automated and operator independent process for differentiation of blood cells into subpopulations is therefore highly sought-after and microfluidic technology clearly offers opportunities that challenge the performance of macroscale methods.

Several groups have presented novel cell handling methods based on preprocessed samples that are analyzed on chip or on chips that process samples for subsequent analysis off-chip.^{4,15–19} However, there is a significant unmet need for strategies to handle small volumes and to perform label-free or simultaneous fractionation into multiple subpopulations. We propose to address these challenges by using free flow acoustophoresis (FFA), which is based on the intrinsic acoustic properties of particles or cells and does not require any excessive pretreatment of the sample. Furthermore, FFA is a gentle method, able to handle a wide range of sample volumes with high throughput. FFA can also fractionate cells into several subpopulations simultaneously.^{20–23,25}

Free Flow Acoustophoresis

Petersson et al.²³ demonstrated continuous FFA size separation of particle mixtures in the range of 2–10 μm , as well as blood component fractionation. FFA can be considered a further developed subgroup of SPLITT field flow fractionation (FFF)²⁴, where the employed external force field is realized by the acoustic standing wave force field.

More recently size-dependent cell cycle separation using acoustic sorting was reported.²⁶ In FFA, the particle stream is normally laminated at the side wall of a flow channel that supports a $\lambda/2$ standing wave. The acoustic force then causes the particles to migrate to the standing wave pressure node in the channel center. The migration speed for individual particles is highly dependent on particle volume and will result in a lateral distribution of particles across the channel according to size. The laminar flow regime in the channel allows

fractionation of this distribution, each fraction containing particles with similar sizes, through different channel outlets.

A key limiting factor in conventional FFA separation systems, as well as in many other microchip separation or detection methods, is the initial spatial distribution of particles as they enter the separation channel.^{27–29} This limitation is mainly caused by the fact that the acoustic force field and the flow speed vary with the spatial location in the channel. The variation causes particles to experience a different acoustic force based on their initial position in the channel, which in turn induces dispersion. The acoustic profile directly affects the forces acting on the particles, while the inherent parabolic flow profile of the microchannel will affect particle retention time in the channel, thus indirectly affecting the exposure time to the acoustic force field. These phenomena lead to “field dispersion,” in which particles of the same size will end up in different outlets due to varying initial positions in the channel. Even though clever channel design can alleviate some of the particle alignment problems, there are often drawbacks in terms of complex and expensive designs, decreased flow speeds, brittle structures, and increased risk of clogging in small inlets.^{30,31}

In order to overcome the problems with sample dispersion and to facilitate an integration of FFA in LOC devices together with miniaturized analysis tools, this paper presents for the first time a multiple- (5-)target outlet FFA chip with an integrated 2D prefocusing zone. The method confines the particles to a distinct position in a prefocusing channel, yielding uniform flow velocities and acoustic exposure for the particles in the separation channel, without the drawbacks of having to design complex channel structures. Previously, 2D continuous flow focusing has been reported to improve performance in systems that are dependent on precise alignment of particles. These include single-target outlet systems for acoustically activated cell sorting and circulating tumor cell extraction from blood, as well as fluorescence- and impedance-based cytometers.^{32–36}

Our multi-outlet FFA system demonstrated an unprecedented performance in separating microparticles and leukocyte subpopulations with respect to purity, recovery, and throughput. Confocal z-scan images confirmed that the 2D prefocusing method confined particles laterally as well as vertically before separation. Size sorting was performed and visualized using fluorescent polystyrene beads. Sorted monodisperse polystyrene beads of different sizes were counted in a Coulter counter (the gold standard) to compare the system with earlier work. As final proof of concept, a bench-top flow cytometer was used to confirm successful isolation of sorted blood components (white blood cell [WBC] fractionation).

MATERIALS AND METHODS

The 150×150 µm prefocusing channel was operated in a laminar flow domain. Typical flow rates were 100 µL/min. Once laminar flow is established, it is also important to ensure that all particles flow in the same 2D location in the cross-section of the separation channel to minimize sample dispersion. Early FFA systems suffered from having the sample distributed across the entire channel height as it entered the separation zone, which resulted in sample

dispersion and modest separation performance. Because particles entered the sorting channel at different locations and thus experienced non-uniform acoustic field retention times, they ended up laterally spread across the channel even after “size sorting” and consequently left the channel through different outlets because of the laminar flow. By sheath flow laminating the sample into a narrow stream before entering the separation section, sorting performance can be improved. Recent research has eliminated the need for elaborate pre-alignment fluidics and has increased sample throughput by adding a 2D acoustic prefocusing zone prior to separation.^{32,36,37} The chip presented here expands this method by combining 2D prefocusing with a FFA channel that has multiple outlets to allow concurrent cell fractionation into several subpopulations (Fig. 1).

Theory – Acoustic Force

The use of acoustic standing wave forces in suspended particle manipulation has been well researched. In the past decade, significant efforts have been made to implement acoustic particle manipulation in microchip formats with integrated microfluidics.^{21,38,39} Acoustophoresis utilizes the primary acoustic radiation force F_{rad} (Eq. 1) to focus particles into the acoustic pressure nodes or antinodes, in microfluidic channels. Depending on their density and compressibility in comparison to the suspending media, particles will travel toward either the nodes or antinodes. The determining factor for this translation is the acoustic contrast factor Φ (Eq. 2). Denser particles such as cells tend to have a positive contrast factor in commonly used flow media including water, thus they focus into the acoustic pressure nodes. Less dense particles such as lipid vesicles have a negative contrast factor and focus into the pressure antinodes. The primary acoustic radiation force scales linearly with an increased contrast factor, Φ , and the particle volume, a^3 . Studies have shown that acoustophoresis, along with other acoustic force-based particle manipulation techniques in microfluidic systems, are gentle to cells and may consequently be used for biofluid preconditioning with maintained cell viability.^{22,40–42}

$$F_{\text{rad}} = 4\pi a^3 \Phi k_y E_{\text{ac}} \sin(2k_y y) \quad (1) \text{ Primary acoustic radiation force}$$

where

$$\Phi = \frac{\kappa_o - \kappa_p}{3\kappa_o} + \frac{\rho_p - \rho_o}{2\rho_p + \rho_o} \quad (2) \text{ Acoustic contrast factor}$$

and a ($\approx 10 \mu\text{m}$) is the particle radius, Φ is the acoustic contrast factor, $k_y = 2\pi/\lambda$ is the wave vector, E_{ac} ($\approx 100 \text{ J/m}^3$) is the acoustic energy density, y is the distance from the wall, κ_p ($\approx 2.5 \times 10^{-10} \text{ Pa}^{-1}$) is the isothermal compressibility of the particle, κ_o ($\approx 5 \times 10^{-10} \text{ Pa}^{-1}$) is the isothermal compressibility of the fluid, ρ_p ($\approx 1030 \text{ kg/m}^3$) is the density of the particle (cell) and ρ_o ($\approx 1000 \text{ kg/m}^3$) is the density of the fluid.

The FFA chip presented in this work translates particles towards the center node orthogonal to the flow. Since F_{rad} scales linearly with volume, even small differences in particle radius will result in large variations in the magnitude of the acoustic force. The size-dependent primary radiation force moves the particles toward the center node with a velocity that

scales with a^2 and results in a size-specific particle distribution across the different outlets (Fig. 1).

Chip Design and Fabrication

The microfluidic system was designed to allow acoustic prefocusing and FFA separation at 5 and 2 MHz actuation, respectively. The wet etching and glass bonding methods used to produce the microfluidic chip have previously been described.⁴² However, in this work [110] silicon was used to achieve the aspect ratio needed for the prefocusing channel (1:1 etching ratio compared to the normal 1:2 ratio when using [100] silicon to obtain rectangular channel cross-sections).

Acoustic Setup

The ultrasonic standing waves were induced using piezoceramic transducers. A 0.4-mm thick transducer (PZ26, Ferroperm, Kvistgaard, Denmark) was attached underneath the 150×150 μm channel section with glue (Super Glue, Loctite [Henkel], Dusseldorf, Germany) and actuated using a 4.82 MHz, ~5 V signal, for 2D prefocusing. A 1-mm thick transducer was attached underneath the 375×150 μm channel section and actuated using a 1.93 MHz, ~19 V signal, for 1D lateral separation (Fig. 2). Each transducer was actuated using sine wave signal generators (AFG 3022B, Tektronix, Bracknell, Berkshire, UK) that were amplified using in-house assembled amplifiers (LT1012, Linear Technology, Milpitas, California, USA). The chip temperature was controlled at ~33°C using a Peltier cooling system (Peltier-Controller TC0806, CoolTronic, Beinwil am See, Switzerland), attached to the backside of the piezoceramic transducers in order to maintain optimal acoustic actuation conditions regardless of ambient temperature and possible heating by thermal conduction from the transducers. Temperature readings for the cooling system were performed by a Pt1000 element attached to the 2 MHz transducer. A thermal compound (NT-H1, Noctua, Vienna, Austria) was used as thermal coupling between the transducers and the Peltier element.

Microfluidic Setup

Fluidic access to the chip was enabled using silicon tubing as previously described.²³ The fluidic network, Teflon tubing with 0.3 mm inner diameter (TFE 58697-U, Supelco [Sigma-Aldrich], St. Louis, Missouri, USA), connected the 1-ml sample syringe (Gastight 1001, Hamilton, Bonaduz, Switzerland) and the 5-ml sheath fluid syringe (Gastight 1005, Hamilton) to the channel inlets and the four first channel outlets to four separate 1-ml collection syringes (BD Plastipak, Becton Dickinson, Franklin Lakes, New Jersey, USA) while the fifth outlet flowed into an open container. Six microfluidic pumps (Nemesys, Cetoni GmbH, Korbussen, Germany), each individually operated by computer, were used to maintain stable flow. During cell experiments, sample inlet flow was set to 8 $\mu\text{L}/\text{min}$ while sheath inlet flow was set to 60 $\mu\text{L}/\text{min}$, resulting in a 1:10 ratio which supported lamination of the prefocused cells close to the channel wall, maximizing the separation distance between the original lateral particle position and the acoustic node in the middle of the channel. Sorting outlet flows were set to 12, 9, 5, and 7 $\mu\text{L}/\text{min}$ respectively in the first four outlets, leaving 35 $\mu\text{L}/\text{min}$ flowing into the last outlet. For polystyrene bead experiments, a seventh pump was connected to the last outlet. Inlet flows in these experiments were 10

μl/min for sample flow and 120 μl/min for sheath flow, while each of the first four target outlets was set to a flow of 15 μl/min and the fifth to 70 μl/min. These settings split the half of the channel containing the particles into five equally large fractions which can be analyzed individually.

Confocal Microscopy

In order to evaluate the 2D focusing, the acoustophoresis channel was perfused with a suspension of 4.8 μm green fluorescent polystyrene beads (Fluoro-Max G0500, Thermo Scientific, Waltham, Massachusetts, USA) in MQ-water and observed using a confocal microscope system (Fluoview 300 running on a BX51WI microscope, Olympus, Hamburg, Germany). A combination of two lasers (PS-Argon-Ion and PS-HeNe, Melles Griot, Rochester, New York, USA) was used to show bead positions and channel geometry simultaneously. Images were constructed from a 25-step 250 μm total scan in the z-direction and captured with the acoustic prefocusing turned off, operating at half amplitude (2 V) and at optimal amplitude (4 V) for subsequent size sorting. The same microscope, using a wide-spectrum mercury lamp instead of lasers, was used for capturing images of sorting performance with 2D prefocusing on and off (Fig. 3).

Polystyrene Bead Separation

A mixture of polystyrene beads (Fluka, Sigma-Aldrich) suspended in water was used as a model system for initial experiments to evaluate channel performance. The bead mix contained beads of three different sizes, 3 μm (colored red), 7 μm (white), and 10 μm (blue), chosen to allow for reasonably large variations in the resulting acoustic forces while staying close to the cell size regime that was expected to be analyzed in the chip. The concentration was 0.15% by volume for the 3-μm beads and 0.65% and 0.6% respectively for the 7- and 10-μm beads. MQ-water was used as suspending media with 0.01% detergent (Tween 20, Bio-Rad, Hercules, California, USA) added to the suspension. Samples from each of the five target outlets were then analyzed using a Coulter counter (Multisizer 3, Beckman Coulter, Brea, California, USA).

Blood Component Separation

To evaluate the system on a biological sample with clinical implications, a population of WBC was separated in the chip. The WBC subpopulations range from ~8 μm in diameter (small lymphocytes, T-cells) to ~11 μm (smaller granulocytes like neutrophils and eosinophils) and even ~14 μm and above for the largest subpopulations (large lymphocytes, monocytes, and larger granulocytes like basophils and macrophages). Our experiments were aimed at isolating pure populations of lymphocytes in outlet 2 and granulocytes in outlet 4 simultaneously, with maximum recovery, while also recovering most of the monocytes in outlet 3. Outlets 1 and 5 were used as waste/buffer outlets.

Cells were prepared as follows: Blood was acquired from five healthy donors at Skåne University Hospital, Lund, Sweden. The red blood cells were lysed by isotonic lysis (BD FACSTM Lysing Solution). WBC were fixed by 2% paraformaldehyde for 10 min on ice and stained with 4 different antibodies: CD45-APC, CD3-PE, CD14-PE-Cy7, and CD66b-FITC (BD Biosciences [Becton Dickinson]), for 20 min at room temperature. WBC were

diluted 1:5 in a FACS buffer (1xPBS, 1% fetal bovine serum, and 2 mM EDTA) before the acoustic separation. Outlet samples were collected in loops and analyzed using a FACS Canto II, (BD Biosciences). The use of fixed cells was preferred as the experiments and system operation was performed over full day experimental series, based on the fact that we have experienced that acoustofluidic studies on unfixed blood samples display a time dependent behavior when extending beyond several hours.

FACS analysis procedure: Sixty μ l of each outlet fraction was analyzed by flow cytometry. The different WBC were characterized as follows: lymphocytes (T-cells) CD45+ CD3+ CD14- and CD66b-; monocytes CD45+ CD3- CD14+ and CD66b-; and granulocytes CD45+ CD3- CD14+ and CD66b+.

RESULTS AND DISCUSSION

Chip Modeling and System Assembly

Simulations indicated that 4.98 MHz acoustic actuation of a 150 $\times$ 150 μ m cross-section channel would result in 2D prefocusing (Fig. 2a). Corresponding simulations in a 375 $\times$ 150 μ m cross-section showed that 1.99 MHz acoustic actuation would allow FFA sorting (Fig. 2c). Based on the modeling, the superposition of the two eigenmodes (vertical and lateral) at 4.98 MHz combined to focus particles in the center of the channel cross-section. The finished chip with glued transducers and fluidic access tubing was placed on an aluminum heat sink to allow cooling with a Peltier element that was placed next to the chip on the heat sink. Temperature readings for the Peltier system were acquired using a Pt1000 sensor attached to the 2 MHz transducer. Initial focusing experiments in the finished chip resulted in acoustic focusing at 4.82 in the prefocusing channel and 1.93 MHz in the sorting channel (Fig. 2b).

2D Prefocusing

Experiments with fluorescent polystyrene beads were performed to verify successful 2D prefocusing using confocal microscopy. With the acoustic focusing inactive, beads were evenly distributed across the entire channel cross-section (Fig. 3a). However, with the acoustic actuation set to 2 V, the beads started to focus into the center of the channel and at 4 V they were confined to a narrow center section < 15 μ m in diameter (microscope confocal depth limitations prevented more detailed measurements), confirming successful 2D prefocusing (Fig. 3b and 3c). Once it was established that particles could be prefocused, the channel was perfused with a mix of 3-, 7-, and 10- μ m beads to investigate lateral dispersion in the separation channel with prefocusing active and inactive. Top view images of the channel with 2D prefocusing active confirmed that particle positions varied less than 10 μ m laterally when entering the separation channel (Fig. 3d and 3e). When 2D focusing was active, three bands, corresponding to the three bead size populations, were clearly visible in the separation channel while when 2D was inactive, particles were more dispersed and not as precisely separated (Fig. 3f and 3g). It was possible to distinguish one bead population being separated into each target outlet with 2D active (Fig. 3h-j). With 2D inactive, no clear separation between subpopulations was seen in the different outlets (Fig. 3k-m).

Separation Efficiency – Beads

Results from separations of suspensions with 3-, 7-, and 10- μ m size polystyrene beads showed that size-based separation was possible with moderate performance when 2D prefocusing was inactive. In this case, the 3- μ m beads were targeted to outlet 2, resulting in this distribution over the five outlets: 13.7%, **55.9%**, 23.5%, 6.3%, and 0.7%, respectively. The 7- μ m beads were targeted to outlet 3 and had this distribution over outlets 1–5: 1.4%, 3.3%, **91.4%**, 3.7%, and 0.2%, respectively. The 10- μ m beads targeted to outlet 4 were distributed over outlets 1–5 as follows: 1.5%, 1.0%, 5.6%, **91.7%**, and 0.2%, respectively (Fig. 4a). These values correspond well with previous findings using similar non-prefocused FFA systems.²³

When prefocusing was active, there was a significant improvement in separation efficiency. 2D prefocused 3- μ m beads were again targeted to outlet 2, with a relative distribution of 3.8%, **87.5%**, 4.8%, 3.6%, and 0.3% in outlets 1–5 respectively. The 7- μ m beads were targeted to outlet 3, with a distribution over the five outlets of 0.6%, 1.0%, **96.2%**, 2.1%, and 0.1% respectively. Finally, the 10- μ m beads were targeted to outlet 4 and were recovered in outlets 1–5, respectively, as follows: 0.4%, 0.2%, 0.9%, **98.5%**, and 0.0% respectively (Fig. 4b).

These results show that separation performance of particles improves as size increases. A contributing factor is that the primary acoustic radiation force decreases rapidly with decreased particle size (volume), resulting in less efficient prefocusing on the 3- μ m beads compared to the 7- or 10- μ m beads, which leads to a loss in separation performance for the smaller particles. Separation data, as well as images for the larger beads, suggest that it is not the acoustics that limit the separation efficiency at this point but rather the small flow instabilities, the rare occurrence of gas bubbles in the channel, possible sample carryover and residues in the fluidic system. Any of these parameters will strongly impact system performance when separation efficiency is >95%, as is the case for 7- and 10- μ m beads.

Separation Efficiency – White Blood Cells

As in the polystyrene bead experiments, experiments on white blood cells also showed that separation efficiency for the prefocused system was much better than when prefocusing was inactive. White blood cell subpopulations overlap in size as well as density due to different nucleus composition; this compromises ideal separation using acoustic forces. Thus, we present the white blood cell separation data in terms of subpopulation purity and recovery rate as measured by fluorescence activated cell-sorting (FACS) analysis. It should be noted that the number of cells in the diluted fraction in outlet 5 during the 2D prefocused experiments was so low that the concentration fell below the FACS instrumentation analysis threshold (<72 gated events in 30 sec). Thus data were reported only for outlets 1–4. Sample concentrations throughout these runs were measured at ~1M cells/ml using the same flow cytometer that was used for sorted cell analysis. Sample and sheath inlet flows were set to 8 and 60 μ l/min, respectively. The outlet flows for target outlets 1–4 were set to 12, 9, 5, and 7 μ l/min respectively, leaving the 5th waste outlet to free flow at 35 μ l/min.

For the experiments without prefocusing (Fig. 5a), the lymphocytes were targeted to outlet 2 and the result was a fraction with 56.5% purity (standard deviation [SD] 15.0%) and 51.0% recovery (SD 7.3%); recovery represents number of lymphocytes detected in target outlet compared to total number of lymphocytes detected in outlets 1–4. The monocyte fraction was targeted to outlet 3 and had a 12.1% purity rate (SD 4.0%) and recovery rate of 40.9% (SD 15.9%). Granulocytes were targeted to outlet 4 and had 86.0% purity (SD 4.5%) and 47.5% recovery (SD 16.7%).

With prefocusing activated (Fig. 5b), lymphocytes (targeted to outlet 2) resulted in a clean fraction with 95.1% purity (SD 0.6%) and 86.5% recovery (SD 10.9%). The monocyte fraction, targeted to outlet 3, had a 25.2% purity (SD 5.4%) and recovery rate of 83.1% (SD 4.3%). Granulocytes were collected in outlet 4 with a purity of 98.5% (SD 0.7%) and a recovery rate of 68.4% (SD 10.6%). These data clearly demonstrate the importance of using prefocusing to accomplish an optimized separation performance. Notably, the lymphocyte/granulocyte ratio was 57:1 in outlet 2 and 1:158 in outlet 4. This huge difference in subpopulation ratios is only possible by introducing multiple outlets that allow interlacing outlets to act as buffers where the cells that overlap in acoustic properties are allowed to flow out. Also, 90% recovery with 25% purity for the monocytes would not be possible without multiple outlets combined with 2D prefocusing which allows extraction of this “center” fraction of the sample. All outlet fractions (1–4) were collected to compare subpopulation ratios in the original sample to the fractionated cells. The data show that the prefocused separation does not change the subpopulation ratios, while the non-prefocused samples have a higher ratio of lymphocytes, most probably due to lost monocytes and granulocytes in the fifth waste outlet (Fig. 5c). At the reported setting the system sorted ~150 cells/s (0.5M cells/h). Thus it offers rapid isolation of small samples compared to traditional centrifugation based isolation which require a set amount of time, regardless of sample volume. Currently the system throughput is limited by the short prefocusing channel. The throughput can be increased by designing longer prefocusing and separation channels and/or allowing a higher ultrasound input power (temperature control of the PZT actuator may then be needed). Systems with successful 2D prefocusing have been achieved at flow rates of up to 100 μ L/min at cell concentrations of 10^6 cells/mL have been achieved (unpublished).

CONCLUSION

This paper shows that the acoustofluidic FFA system with prefocusing is able to simultaneously fractionate white blood cells into subpopulations with high purity (95–99% for lymphocytes and granulocytes) and recovery (>90% for lymphocytes and monocytes). The system fractionates particles of different sizes into multiple outlets with higher accuracy than previously reported multiple outlet FFA systems.²³

Future efforts will be aimed at an increased sample throughput and the development of an ease of use standard scheme of leukocyte subpopulation separation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful for financial support from the Swedish governmental agency for innovation systems, VINNOVA, CellCARE, Grant No. 2009-00236, the Swedish Research Council, the Royal Physiographic Society, the Crafoord Foundation, the Carl Trygger Foundation, the SSF Strategic Research Centre (Create Health), Cancerfonden [Project No. 11-0624], Fundacion Federico SA, the National Cancer Institute [Grant Nos. R33 CA 127768-03, 1R01CA160816-01A1, and P50-CA92629], the Sidney Kimmel Center for Prostate and Urologic Cancers, and David H. Koch through the Prostate Cancer Foundation. A huge thank you is extended to Per Augustsson for his help in the design and lithography stages of chip development.

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Figure 1.

Microfluidic and acoustic schematic explaining 2D prefocused FFA. A laminar system with prefocused particles is needed to perform precise sorting since particle retention times in the acoustic field will otherwise vary (a). By inducing standing waves in both directions of a square channel, it is possible to prefocus particles prior to sorting (b, side view of the channel). The prefocused particles will enter the acoustic field at uniform positions and velocities and be translated towards the center pressure node according to their size and acoustic contrast factor (c). The separated particles then exit the sorting section of the chip (d, middle section) and are sorted into different target outlets according to their cross-sectional position in the laminar flow profile (d, rightmost section of chip).

Figure 2.

Schematic of the finished chip and acoustic simulations. Acoustic resonance in the prefocusing channel was indicated to be 4.98 MHz according to simulations, but the finished chip focused particles in two dimensions at 4.82 MHz actuation (a). These findings were consistent with results in the separation channel which focused particles at 1.93 MHz rather than the simulated 1.99 MHz (c). The finished chip had transducers and fluidic access ports glued to it and was placed on an aluminum heat sink which was actively cooled with a Peltier system in order to maintain optimal acoustic temperature conditions (b).

Figure 3.

Confocal (left inserts) and normal microscope images of 2D prefocusing and separation of polystyrene beads. With 2D prefocusing activated, particles could be positioned in a well-defined area of the channel cross-section using 4.82 MHz ultrasound (c vs. a and b). The particles were then laminated along the side of the separation channel (e and d). By actuating the separation channel at 1.93 MHz, the prefocused beads were separated into three well-defined streamlines in the separation channel while the non-prefocused beads were more dispersed (g vs. f). The particles were sorted into different target outlets according to their lateral positions in the flow profile, prefocused beads going into distinct outlets according to size while unfocused beads were spread out across the outlets (k–m vs. h–j).

Figure 4.

Separation efficiency of polystyrene beads with and without prefocusing. The non-prefocused beads were more dispersed as they entered the separation channel, which prevented precise separation (a). When prefocusing was activated, the beads could be sorted with high purity (b). Notably, the prefocusing also allowed very high sorting purities of 3- μm beads, not feasible in previously presented systems.

Figure 5.

Fractionation efficiency of white blood cells into subpopulations of lymphocytes, monocytes and granulocytes (error bars represent SD, $n=5$). (a) Due to overlap in size and density of lymphocytes, monocytes and granulocytes, it was not feasible to get high purity (or recovery) of any of these subpopulations when prefocusing was inactive - even with multiple outlets. (b) By contrast however, and when prefocusing was activate, it was possible to fractionate the white blood cells into subpopulations of either lymphocytes, monocytes or granulocytes with high purity. It should be noted that the monocyte purity is lowered by the overlapping acoustic mobilities of some lymphocytes and granulocytes. A comparison between ratios of subpopulations in sorted samples (1–4) and their respective original samples showed that the prefocused acoustophoretic sorting of white blood cells does not alter inter-subpopulations ratios, while unfocused samples are altered in ratio, most probably due to granulocytes spilling over into the “waste” outlet (c).