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Accelerated Articles

Free Flow Acoustophoresis: Microfluidic-Based Mode of Particle and Cell Separation

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A novel method, free flow acoustophoresis (FFA), capable of continuous separation of mixed particle suspensions into multiple outlet fractions is presented. Acoustic forces are utilized to separate particles based on their size and density. The method is shown to be suitable for both biological and nonbiological suspended particles. The microfluidic separation chips were fabricated using conventional microfabrication methods. Particle separation was accomplished by combining laminar flow with the axial acoustic primary radiation force in an ultrasonic standing wave field. Dissimilar suspended particles flowing through the 350-μm-wide channel were thereby laterally translated to different regions of the laminar flow profile, which was split into multiple outlets for continuous fraction collection. Using four outlets, a mixture of 2-, 5-, 8-, and 10-μm polystyrene particles was separated with between 62 and 94% of each particle size ending up in separate fractions. Using three outlets and three particle sizes (3, 7, and 10 μm) the corresponding results ranged between 76 and 96%. It was also proven possible to separate normally acoustically inseparable particle types by manipulating the density of the suspending medium with cesium chloride. The medium manipulation, in combination with FFA, was further used to enable the fractionation of red cells, platelets, and leukocytes. The results show that free flow acoustophoresis can be used to perform complex separation tasks, thereby offering an alternative to expensive and time-consuming methods currently in use.

Although separation of suspended micrometer-sized or smaller particles is of fundamental importance in the biological and biomedical sciences, challenges remain. The most widespread methods for cell and particle separation are the centrifugal methods, which exploit differences in size and density.[1,2] Chromatography,[3,4] electrophoresis,[5] immunoisolation,[6,7] and application specific methods[2] are used as complements or alternatives to centrifugation. An emerging family of potentially useful methods is field-flow fractionation.[8] More recently, novel magnetic,[9] pinch flow,[10] hydrodynamic,[11] deterministic lateral displacement,[12] and optical lattice interaction[13] methods have also been reported. Unfortunately, separation methods often involve time-consuming steps, noncontinuous operation, very low throughput, or risks of damaging biological samples due to the substantial mechanical stress exerted or the high electrostatic fields employed. There is clearly still a need for a simple, low-cost, high-throughput, and gentle method for separation of microparticles.

Acoustic forces have been used extensively for separation of suspended micrometer-sized particles, from their medium or from

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other particles,[14−17] as well as in trapping of particles.[18,19] Recently, acoustic force-based size separation was reported by Kapishnikov et al.[20] In general, these methods utilize the axial acoustic primary radiation force (PRF) to move or trap particles in a continuous flow situation. Acoustic separation systems realized by silicon microfabrication technology have also been used in different applications.[21−23] Microfabrication provides the precision needed to fabricate the high-frequency acoustic resonators in which the acoustic standing wave forces are generated. The advantages of acoustic separation and trapping systems are that they offer a continuous mode of operation, are relatively easy to run, and handle biological samples gently.[19,24−27]

This paper presents a novel method, free flow acoustophoresis (FFA), which is capable of separating micrometer-sized or smaller particles into multiple fractions in a continuous flow mode. Separation is obtained by acoustic standing wave force-induced lateral displacement in the laminar flow of a silicon microchannel. Separation based on size and density is demonstrated on both blood cell suspensions and reference polymer bead suspensions. Pure compressibility-based separation is also possible but was not investigated in the current study.[28] The FFA process starts the separation when suspended particles enter a rectangular cross-section flow channel through two side inlets of a main channel. The same medium, but without the suspended particles, enters the main channel through the single center inlet, occupying most of the channel width. A half-wavelength acoustic standing wave, generated between the side walls of the channel using a piezo ceramic actuator, gives rise to an acoustic force field perpendicular to the direction of flow. As the particles advance along the flow path, they are translated toward the channel center by the acoustic force, at a rate determined by their size, density, compressibility, and acoustic pressure amplitude (Figure 1). If the flow rate, acoustic force, and particle mixture are correctly balanced, a particle gradient will have developed across the channel at its end (Figure 2). Because of the laminar flow properties, this gradient can be fractionated further down the channel, using several outlets (Figure 3). The method is capable of separating micrometer-sized particles in a continuous mode without the risk of harming sensitive species. In applications demanding high throughput, several separation channels can be operated in parallel, similar to the approach performed in microchip-based blood washing.[24] The presented results demonstrate the potential of free flow acoustophoresis as a novel method for preparation of particle-based biological and nonbiological samples.

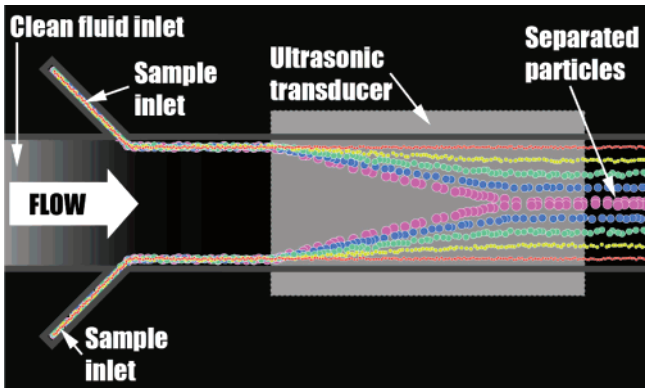

Figure 1. Illustration of a particle suspension passing over the transducer where the particles are moved toward the center of the separation channel at a rate determined by their acoustic properties. Because of the laminar flow almost no mixing takes place.

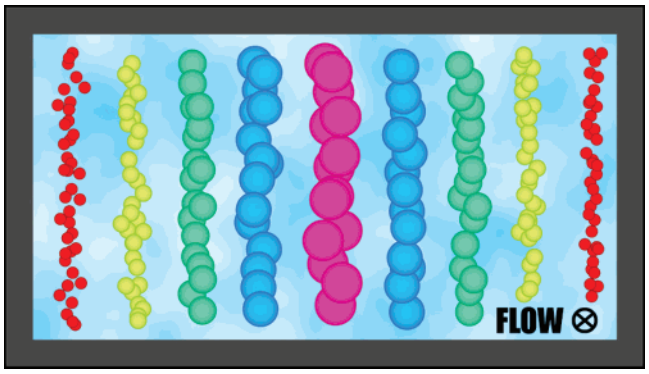

Figure 2. Illustration of the cross section of the separation channel directly after the transducer. The particles that are most affected by the axial PRF have reached the stable position in the center of the channel while the others are located somewhere between the walls and the center.

RESULTS

Device Design and Operation. The rectangular cross-section separation channel (370 μm wide, 125 μm deep) had three inlets: one particle-free medium inlet, located at the beginning of the separation channel, and two sample inlets, one at each side wall 1.5 mm down the channel (Figure 4). Eleven outlets, of which 10 were pairwise connected, were located at the end of the separation channel. This outlet configuration enabled the extraction of up to six particle fractions. The number of fractions in use was defined by blocking one or more outlets. The channel length was determined by the desired flow rate, the particle mixture used, and the employed acoustic input power. These parameters were tuned such that the particle size distribution occupied the full width of the channel when reaching the flow splitter outlets. The upper limit of the input power is set by the attenuation in the piezo ceramic actuator, causing overheating of the separation channel. A 30-mm-long channel was designed for separation of

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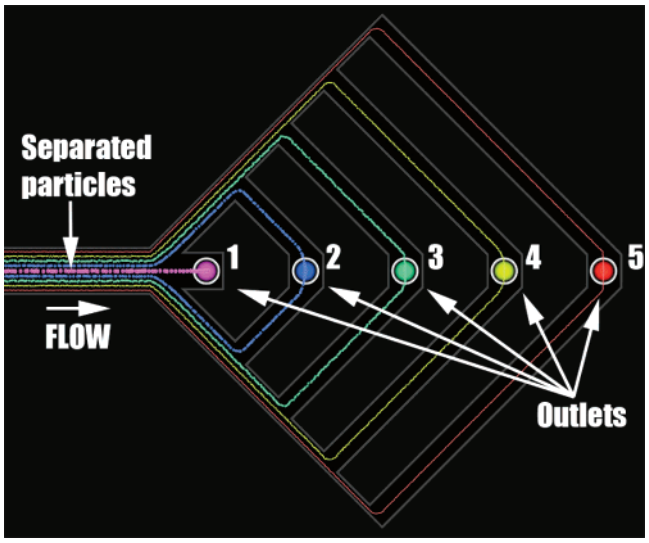

Figure 3. Illustration of the fractionation of the separated particles at the end of the separation channel, in this case, through five consecutive outlets/fractions. Since the separation is symmetrical along the center of the channel; eight of the fractionation outlet channels are parewise connected.

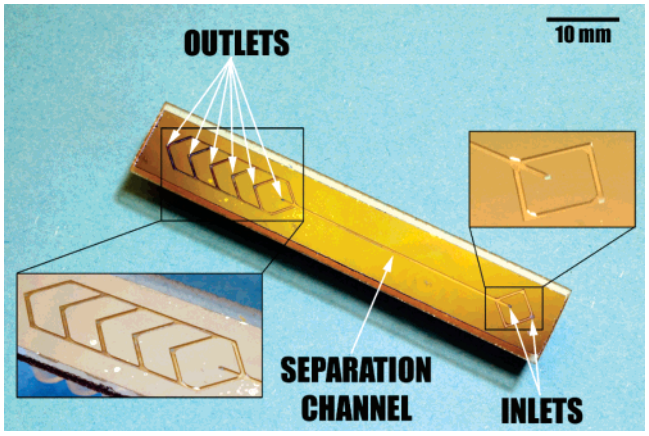

Figure 4. Photograph showing the basic chip design that consists of a silicon wafer with wet-etched channel structures. The channels have been sealed with an anodically bonded glass sheet, and the inlets and outlets have silicon tubing connections on the back side of the chip.

the suspensions used in this study. The net power applied to the piezoelectric element ranged from ~0.5 to ~2.0 W, depending on the size of the particles, the desired lateral displacement of a certain particle type, and the suspending medium. The spatial positioning of the actuator under the separation channel was uncritical.

The separation process was driven by the axial acoustic PRF,[29] in this context the dominant among several acoustic forces.[30,31] A particle in an acoustic standing wave field experiences this force if its acoustic contrast factor, ϕ (eq 1), is nonzero. The magnitude of the axial PRF acting on a particle is determined by the properties of the medium and the particles as well as the amplitude and wavelength of the standing wave (eq 2). Particles can only be separated from each other if they differ in volume, density, compressibility, or a combination of these.

$$\phi = \frac{5\rho_p - 2\rho_m}{2\rho_p + \rho_m} - \frac{\beta_p}{\beta_m} \quad (1)$$

$$F_r = -\left(\frac{\pi p_0^2 V_p \beta_m}{2\lambda}\right)\phi(\beta,\rho)\ \sin\ (2kx) \quad (2)$$

In eqs 1 and 2, the densities of the medium and the particles are denoted ρ_m and ρ_p, respectively and the corresponding compressibilities β_m and β_p, respectively, p_0 is the pressure amplitude, V_p is the volume of the particle, λ is the ultrasonic wavelength, k is defined by $2\pi/\lambda$, and x is the distance from a pressure node.

During operation, suspended particles were laminated along the side walls with the particle-free medium, via the side inlets (Figure 1). The ratio between the sample inlet flow rates and the clean medium inlet flow rate decided whether a narrow distribution of each particle type across the width of the channel or high throughput was the priority. Particles with positive ϕ-factors were continuously translated toward the center of the 370-μm-wide channel, when it was actuated at its fundamental resonance criterion, i.e., ~2 MHz in aqueous solution. The rate of particle movement perpendicular to the direction of flow was determined by the magnitude of the axial PRF and the opposing viscous drag force.[16,28,32] The power supplied to the piezoelectric ceramic was adjusted such that the particles most affected by the acoustic force arrived at the center of the channel just before reaching the end of the transducer and thus exited the system through the single center outlet, 1 (Figure 3). Since the particle gradient created by the axial PRF was symmetrical around the center of the channel, the successive fractions on each side exited through the pairwise connected outlets 2−5 (Figure 3). If needed, each outlet flow rate can be adjusted individually for optimal separation of a specific particle mixture.

Device Characterization. In the first step of the characterization of the FFA process, 7-μm polystyrene spheres (positive ϕ-factor, concentration: ~1.0% by volume) were addressed to a predetermined outlet by adjusting the power supplied to the piezoelectric ceramic. This was done in order to study the expected performance (lateral dispersion) of the system when processing more complex particle mixtures. The separation chip was operated in a four-fraction outlet mode. The two remaining outlets were sealed during the experiment. Samples collected from each outlet (1−4) were analyzed using a Coulter counter. The chart in Figure 5 shows that the first and fourth outlets could be addressed easily; in both cases, ~99% of the particles arrived at the intended outlets. This was expected as at zero power input the particles remain laminated along the side walls and exit via outlet 4, and at a high-power input, all particles are focused in the center of the channel, exiting via outlet 1. When addressing the two middle outlets, the power was tuned by visual feedback. Also, the impact of bubbles and flow speed fluctuations was stronger when collecting the particles in these two fractions. The second and third fractions comprised 60 and 64% of the particles,

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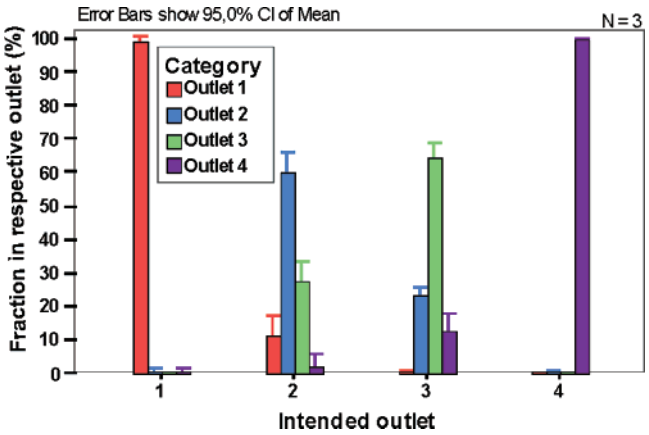

Figure 5. Bar plot showing the fraction of 7-μm polystyrene particles in each outlet fraction out of four when the acoustic power was set to direct particles to a specific outlet (outlets numbered according to Figure 3).

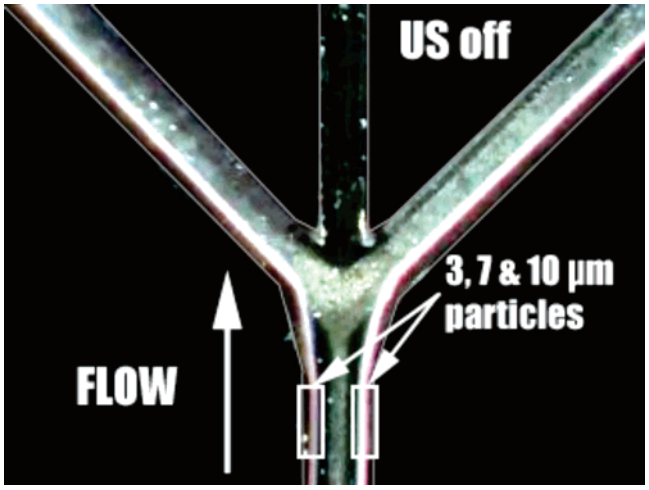

Figure 6. Microscope image showing the end of the separation channel. When the ultrasound is off, all three particle sizes remain close to the side wall because of the laminar flow properties.

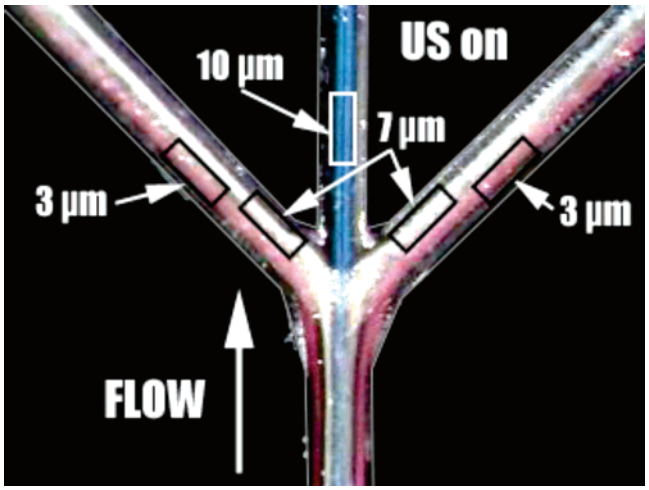

Figure 7. Particle size gradient formed in the separation channel when the ultrasound is turned on. Each particle fraction exits through one of the three active outlets. As seen in the image, the blue 10-μm particles exit through the center outlet.

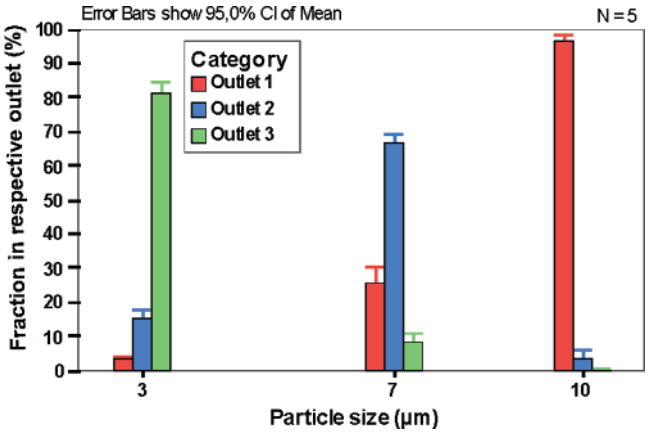

Figure 8. Distribution of each of the three particle sizes (3, 7, and 10 μm) over the three outlet fractions when polystyrene particles were separated (~50 000 particles/μL per size). Each size clearly dominates one fraction (outlets numbered according to Figure 3).

respectively. Thus, the dispersion of the particle band was clearly seen when addressing outlet 2 or 3. All outlet flow rates were set to 0.10 mL/min, and the sample suspension flow rate was 0.04 mL/min.

The second step in characterizing the FFA system comprised a simultaneous fractionation of three sizes of polystyrene spheres (red 3 μm, white 7 μm, and blue 10 μm). Initially, the sample suspension contained ~50 000 particles/μL of each size. During operation, the power was tuned such that virtually all blue 10-μm particles just reached the center outlet (Figures 6 & 7), while observing that the red 3-μm particles moved only slightly off the side walls. Thereby it was ensured that the axial PRF formed a particle size gradient across the full half-width of the channel. The chart in Figure 8 shows the resulting particle size distribution in each fraction, almost all 10-μm particles, 96%, exited through the center outlet while 67% of the 7-μm and 81% of the 3-μm particles exited through the second and third outlets, respectively. During the experiment, all outlet flow rates were set to 0.13 mL/min and the sample suspension flow rate was 0.04 mL/min. The experiment was repeated with the sample suspension containing the same volume of particles (~2.0% by volume of each particle size) instead of equal numbers. This resulted in an improvement of the overall separation results; 93% of the 10-μm particles exited through the center outlet while 76% of the 7 μm and 87% of the 3-μm particles exited through the second and third outlets, respectively (Figure 9). Separation of four polystyrene particle sizes (2, 5, 8, and 10 μm) was also investigated. The concentration of each of the three larger particle sizes in the sample suspension was ~1.0% by volume and ~0.5% for the 2-μm particles in order to make their number less dominant in the subsequent Coulter counter analysis. The total flow rate, 0.4 mL/min (Re < 30), was equally divided between the four outlets, and the sample suspension flow rate was 0.04 mL/min. As shown in Figure 10, 94% of the 10 μm, 62% of the 8 μm, 66% of the 5 μm, and 88% of the 2-μm particles were collected in separate fractions. Eight parallel bands of particles were observed during the separation, one for each inlet and particle size. The distances between the 2- and 5-μm bands and the 8- and 10-μm bands were significantly shorter than that between the 5- and 8-μm bands, which is in good agreement with what can be expected from the magnitude of the acoustic force and the viscous drag on the four particle sizes.

The final FFA characterization experiment separated particles of the same size but with different densities. It was found that it

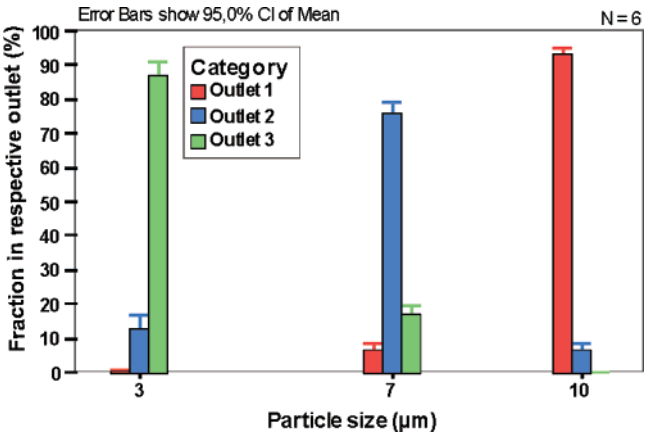

Figure 9. Distribution of each of the three particle sizes (3, 7, and 10 μm) over the three outlet fractions when polystyrene particles were separated (concentration ~2.0% by volume per size). The overall separation result was improved compared to the run with the same number of particles (outlets numbered according to Figure 3).

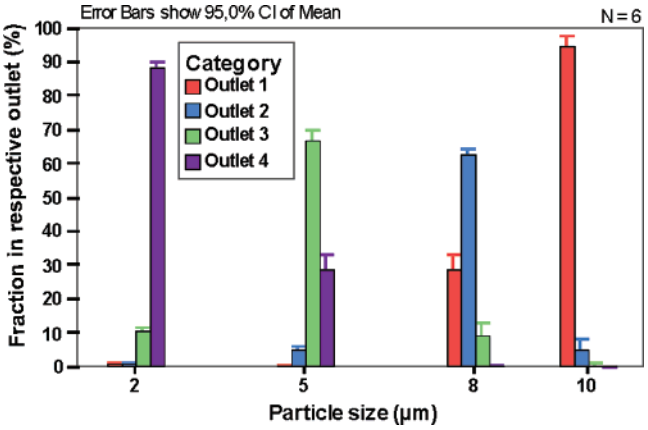

Figure 10. Distribution of each of the four particle sizes (2, 5, 8, and 10 μm) over the four outlet fractions when polystyrene particles were separated (concentration of 5, 8, and 10 μm particles ~1.0% by volume, 2-μm particles ~0.5% by volume). Each size clearly dominates one of the four fractions (outlets numbered according to Figure 3).

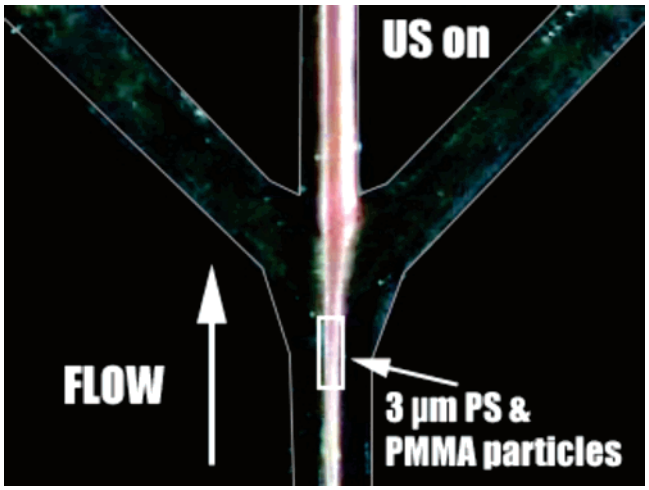

Figure 11. Microscope image showing the end of the separation channel. No separation of 3-μm red polystyrene (PS) and white poly(methacrylate) (PMMA) particles was observed when suspended in distilled water.

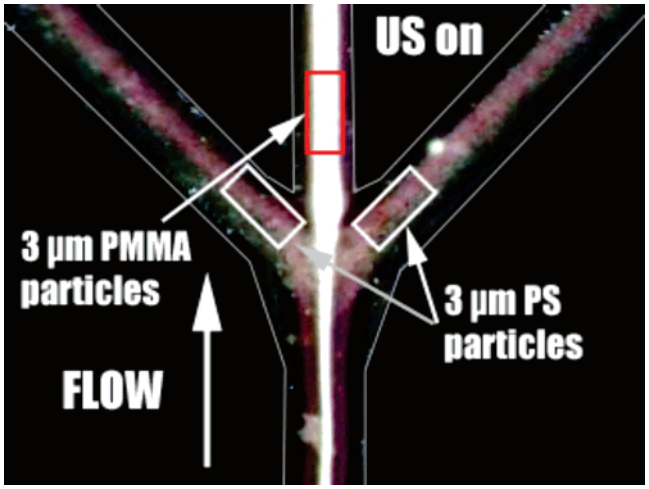

Figure 12. CsCl (0.22 g/mL) added to distilled water, in order to change the density. 3-μm red PS and white PMMA particles could then be separated.

was not possible to separate 3-μm polystyrene (density 1.05 g/cm^3, red, concentration ~2.5% by volume) and poly methyl methacrylate (density 1.22 g/cm^3, white, concentration ~2.5% by volume) particles suspended in distilled water since both particle types were affected almost identically by the axial PRF (Figure 11). As stated in eq 1, the ϕ-factor is determined by the density and compressibility of the particles and the medium. If two particle types behave similarly in an acoustic force field, this situation will change if the density of the medium is adjusted. Thus, 0.22 g/mL cesium chloride was added to the medium entering the channel through the center inlet. The medium density was thereby set between the densities of the two particle types (1.16 g/cm^3), which also was confirmed through centrifugation of the sample suspension. The medium-density manipulation resulted in a distinctive separation of the red and white particles (Figure 12). The total flow, 0.4 mL/min, was equally divided between the two outlets used and the sample flow rate was 0.04 mL/min. By counting red and white particles under a microscope, it was determined that 96% of the poly(methacrylate) particles exited through the first outlet while 88% of the polystyrene particles exited through the second outlet.

Separation of Red Cells and Platelets. The combination of FFA and medium-density manipulation was subsequently used in separation of red cells and platelets. While suspended in saline solution (0.9 mg/mL) with nutrient additives, the two blood components were affected similarly by the axial PRF and were thus difficult to separate. A sample suspension containing red cells (~7 μm in diameter, ~2 μm thick, density ~1.100 g/mL) and platelets (2−4 μm in diameter, density ~1.058 g/mL), ~1.0% by volume of each cell type, was processed using a chip with two active outlets (flow rate 0.10 mL/min per outlet).[33,34] When saline solution entered through the center inlet (flow rate 0.18 mL/min), no significant separation of the two cell types was achieved; virtually all cells ended up in the first fraction. However, when CsCl (0.22 g/mL) was added to the saline solution, it resulted in

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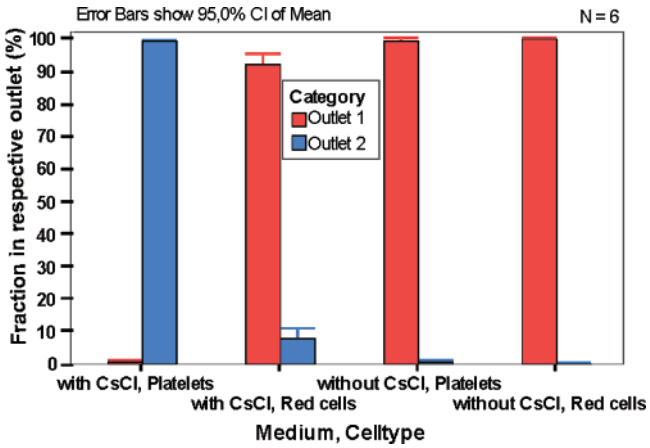

Figure 13. Separation of platelets and red cells with and without CsCl (0.22 g/mL) added to the suspending medium (outlets numbered according to Figure 3). The medium manipulation resulted in clear separation of the two cell types.

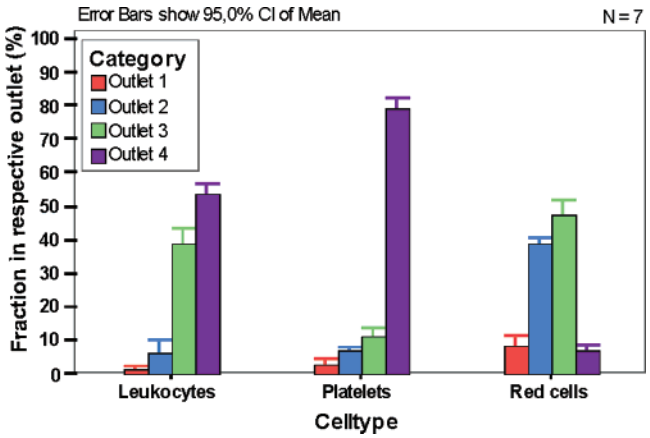

Figure 14. Separation of platelets, red cells, and leukocytes with CsCl (0.22 g/mL) added to the suspending medium (outlets numbered according to Figure 3). The red cells were intentionally directed to the second and third outlets.

an almost perfect separation of the two blood constituents; 92% of the red cells ended up in the first fraction while 99% of the platelets ended up in the second (Figure 13).

Separation of Leukocytes, Red Cells, and Platelets. Separation of buffy coat, an intermediate fraction from centrifuged whole blood units containing red cells, platelets, and leukocytes (5−20 μm in diameter, density 1.062−1.082 g/mL), was also investigated.[33,34] Buffy coat is used for platelet concentrate preparation and typically contains a high concentration of red cells ($\sim 50 \times 10^{11}$/L) and platelets ($\sim 15 \times 10^{11}$/L) and a lower concentration of leukocytes ($\sim 30 \times 10^{9}$/L). When saline solution with CsCl (0.22 g/mL) entered a chip with four active outlets (flow rate 0.10 mL/min per outlet) through the center inlet (flow rate 0.36 mL/min), 79% of the platelets exited through the fourth outlet while 86% of the red cells exited through the second and third outlets (Figure 14). Of the leukocytes larger than red cells, i.e., ~90% of the total number, 92% ended up in the third and fourth fractions.[33,34] The red cells were intentionally directed to the second and third outlets in order to find out if any optically nonobservable particles would exit through the first outlet. The distributions in Figure 14 show that leukocytes were more affected by the acoustic force field than platelets but less than red cells, with the CsCl addition to the saline.

DISCUSSION

System Performance. The characterization experiments showed that a particle size gradient was indeed formed by the axial PRF and that it was possible to choose the outlet or outlets through which a specific particle size would exit the FFA chip. The latter was accomplished by tuning the power supplied to the piezoelectric ceramic, and it was shown that a certain particle size fraction can be placed in virtually any outlet. The lateral distribution of the single particle sample across the different outlets gave a clear indication as to which separation resolution could be expected when separating more complex suspensions into multiple outlets, which also agreed well with subsequent experiments. A clearly influencing factor in this respect is the varying duration time of particles in the acoustic force field due to the parabolic flow profile in the vertical direction, between channel bottom and top. A more focused particle stream in the center of the vertical flow profile would thus be desired, reducing the lateral dispersion.

When several different particle sizes were present, the separation could be tuned such that the larger particles reached the stable pressure node position in the middle of the channel while the smallest particles were not significantly affected and remained close to the side walls. Furthermore, it was shown that the properties of the suspending medium can be altered in such a way that particle types that would be impossible to separate in their original medium could be separated. Since density gradient centrifugation is a routinely used method, there are several alternatives to CsCl as density manipulation additive for different applications.[35] Laminated streams of media with different acoustic properties are also a possibility that can be explored. The above system characteristics, in combination with the possibility to set all inlet and outlet flow rates individually, make free flow acoustophoresis a versatile method for continuous and parallel separation of a wide range of biological and nonbiological particle suspensions.

Blood component therapy for transfusion, i.e., the use of pure red cells, platelets, and plasma, is standard procedure in modern health care and offers several advantages compared to the use of whole blood. Pure leukocytes are also necessary for transplantation purposes. The preparation of, for example, platelet concentrates, is, however, a time-consuming and costly procedure. Platelets are easily activated, and gentle preparation methods are of importance. Modern transfusion therapy is based on leukocyte-depleted platelets and various techniques are in use, e.g., filtration. It is therefore of great interest to find cost-effective methods that meet the quality requirements with high purity and yield.[34,36] As an effort to address this need, platelets and red cells were separated using medium-density manipulation and acoustic standing wave forces. Furthermore, it was shown that leukocytes behave differently in an acoustic force field compared to the other two cell types. These results strongly indicate that it is possible to obtain pure fractions of the different blood cells simultaneously by combining FFA and medium-density manipulation. By using

(35) Rickwood, D.; Ford, T. C.; Steensgaard, J. *Centrifugation: essential data*; Wiley: New York, 1994.
(36) *Directive 2002/98/EC of the European Union Parliament and the Council of 27*, January 2003.

more parallel outlets, a balanced suspending medium, and a slightly stronger acoustic force field, good separation results should be feasible.

There were no indications of damage to the red cells during the blood component separation experiments. The hemolysis level in the original sample suspension was below 0.3 g/L, and the level in the outlet fractions was too low to be measured with standard equipment because of the dilution factor. These findings are in agreement with previous research showing that acoustic manipulation causes no perceptible harm to red cells and other biological particles.[19,24−27] The rise in temperature was below 10 °C, which is insignificant for most room-tempered biological samples.

MATERIALS AND METHODS

Device Fabrication. The particle separation chips were fabricated using conventional silicon microfabrication techniques.[21] The flow channels were anisotropically wet-etched in ~350-μm-thick, 3-in. <100>, silicon wafers. The wafers were diced and the chips sealed with 1.1-mm-thick boron silica glass lids through anodic bonding. The side walls of the separation channels were bounded by <100> planes defined by the anisotropic etching, thus ensuring good resonance cavities. Fluid inlets and outlets were etched through the wafers and ~5-mm-long pieces of silicon tubing were attached to each inlet and outlet using silicone glue.

Sample Collection. Twelve-port injectors (25.EPC12W, VICI Valco Instruments, Houston, TX) were used to acquire samples from the outlets. Teflon tubing (0.3-mm i.d.), inserted in the silicone tubing, connected the chip and the injectors. Each injector collected samples from two outlets in 100-μL Teflon tubing (0.8-mm i.d.) loops.

Flow Rate Control. Syringe pumps (WPI SP260P, World Precision Instruments Inc., Sarasota, FL) were used to control the flow rates in the chip. All outlets were individually connected to high-precision glass syringes (1005 TLL and 1010 TLL, Hamilton Bonaduz AG, Bonaduz, Switzerland) via the injectors using Teflon tubing, allowing independent control of the outlet flow rates. The clean fluid inlet was connected to a syringe pump and the particle suspension inlet to a 50-mm-long piece of Teflon tubing (0.3-mm i.d.) with its other end submerged in a beaker from which the sample suspension was aspirated at a rate defined by the difference between the net outlet flow and the clean fluid inlet flow.

Ultrasonic Actuation. The ultrasound used to induce the standing wave between the walls of the separation channel was generated using a 20 × 20 mm piezoelectric ceramic (Pz26, Ferroperm Piezoceramics AS, Kvistgard, Denmark) attached to the back side of the chip as presented earlier.[23] Ultrasonic gel (Aquasonic Clear, Parker Laboratories Inc., Fairfield, NJ) ensured a good acoustic coupling between the two. The piezoelectric ceramic was actuated via a power amplifier (model 75A250, Amplifier Research, Souderton, PA) connected to a function generator (HP 3325A, Hewlett-Packard Inc., Palo Alto, CA). Even though the acoustic waves entered the chip from the back side, a standing wave was induced between the side walls of the separation channel as a result of the coupling of the mechanical vibrations along the three axes of the crystal structure.

Process Control. The separation process was monitored using a standard microscope and a wattmeter (43 Thruline Wattmeter, Bird Electronic Corp., Ceveland, OH). The process could subsequently be controlled by tuning the signal frequency, the actuation power, and the flow rates.

Particle Size Measurement. The particle size distributions in the samples were analyzed using a Coulter counter (Multisizer 3, Beckman Coulter Inc., Fullerton, CA). Each sample was mixed with an electrolyte (Isoton II, Beckman Coulter Inc.) and analyzed using a 100-μm aperture. All particles used were polystyrene or poly(methacrylate) Fluka microparticles with a size standard deviation of ~0.2 μm (Sigma-Aldrich Corp.).

Hemolysis Measurement. The level of hemolysis, i.e., the concentration of free hemoglobin from damaged red cells, was measured using a photometer (Plasma/low HB Photometer, HemoCue AB, Ängelholm, Sweden).

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