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Pulsed Laser Activated Cell Sorting with Three Dimensional Sheathless Inertial Focusing

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The fluorescence-activated cell sorter (FACS) is a powerful tool for high-throughput single cell analysis in both fundamental biomedical research and clinical applications.^[1–9] Conventional FACS focuses a cell suspension into a single stream with three-dimensional sheath flows through a small nozzle. Droplets leaving the nozzle are selectively and electrostatically charged and sorted based on the fluorescence of an encapsulated cell at a

typical throughput lower than 25000 cells s^{-1} .^[4,5,10] This throughput facilitates both research and clinical applications, such as isolating sub-populations of immune cells for molecular analysis and sorting of circulating tumor cells (CTCs) from blood samples in a reasonable time.^[11] However, current aerosol based FACS suffers several drawbacks. One shortcoming is the potential for sample contamination, which can generate a personal safety issue for FACS operators due to infectious aerosols.^[12] Perhaps more importantly, aerosol-based FACS does not provide a convenient interface for integration with upstream pre-sort or downstream post-sort sample preparation or analysis modules, especially with many of the novel microfluidic modules developed over the past decade.

Microfluidic FACS provides a fully enclosed, sterile environment for cell sorting^[13] and can easily interface with many upstream and downstream modules. For example, integration with an upstream label-free “rough” sorting module, such as one based on inertial or dielectrophoretic forces can increase the throughput for rare cell sorting.^[14–16] Similarly, upstream integration with lysis and solution exchange sample preparation^[17] is possible, creating a complete diagnostic solution—from body fluid to result.^[18] Also, integration with downstream modules facilitates single cell analysis by keeping small numbers of sorted cells on the same chip, which reduces the risk of losing rare cells.^[19] Furthermore, microfluidic FACS can handle a small number of cells while consuming less reagents and producing less chemical waste.^[11,20–22]

Rapid progress in microfluidic FACS systems over the past decade has quickly narrowed the sorting performance gap with conventional aerosol-based FACS devices. Most microfluidic FACS systems demonstrated so far still utilize conventional optics for detection and analysis.^[23] This feature gives all microfluidic FACS systems the same optical potential for multi-parameter analysis as that in aerosol-based FACS machines. The main innovation in microfluidic FACS systems is the sorting actuation mechanism, which includes electro-osmotic flows,^[24] pneumatic valve control,^[25] solenoid valve control,^[26] optical forces,^[27] piezoelectric actuators,^[28] acoustic waves,^[29] sol-gel deformation^[30–32] and a pulsed laser activated cell sorter (PLACS).^[33,34] Among these, PLACS recently achieved a sorting performance of 90% sort purity at 23000 cells s^{-1} and 45% sort purity at 45000 cells s^{-1} using 3D sheath flows provided by multilayer PDMS structures.^[34] However, the large amount of diluting sheath fluid needed for tight 3D focusing requires a high initial cell concentration in samples ($>10^7$ cells ml^{-1}), which can result in cell clogging in channels, and high pressure pumps to drive fluid through microchannels at high speeds. The need for 3D microfluidic channels also increases the complexity and the cost of device fabrication. In this context, sheathless 3D cell focusing provides a potential solution for this multi-faceted issue.

Several sheathless 3D cell-focusing solutions have been demonstrated so far. These include focusing using acoustic pressure,^[35,36] optical,^[37] magnetic,^[38] and dielectrophoretic forces,^[39,40] hydrophoresis^[41] and inertial focusing.^[42–45] Among these, inertial focusing is a promising sheathless mechanism for integration with PLACS since both can operate at high speed (>1 m s^{-1}). Other mechanisms, although providing a 3D cell focusing function, can only operate at relatively low throughput and are therefore not ideal for integration with high-throughput PLACS. Furthermore, particle-particle interaction phenomena^[46,47] in

inertial microfluidics can be utilized to regulate the inter-particle separation distance between two closely positioned particles, or cells, to improve purity in high-throughput sorting.

Here, we demonstrate an inertial focusing integrated sheathless PLACS for high-purity and high-throughput cell sorting in a single layer PDMS channel that can be easily and inexpensively fabricated. We also demonstrate an approach to enlarge the inter-particle distance of closely positioned particles, which may positively impact the sort purity at high-throughput, by utilizing particle-induced hydrodynamic interactions in an expansion chamber. As shown in Figure 1, the upstream channel design consists of a single low aspect ratio straight channel (80 μm in width and 40 μm in height) with a series of 30 vertical constrictions (20 μm in width, 18 μm in height, and 1 mm in periodicity), followed by a symmetric laterally expanded chamber structure (160 μm in width and 800 μm in length with an expanding slope of 2.86 degree). This main sample channel is geometrically engineered to enable 3D particle/cell focusing and inter-particle spacing control. The sample channel bifurcates into two outlets, collection and waste, after the switching junction. The train of focused particles/cells initially flows into the waste channel. Once fluorescence is detected by a photomultiplier tube, a cavitation bubble will be excited in a separate, parallel channel, which is 150 μm in width and expands to 300 μm at the bubble excitation location to allow enough space for bubble expansion, that is then connected with the main sample channel through a nozzle (20 μm in width and 50 μm in length) at the bifurcation. A rapidly expanding cavitation bubble triggered by the pulsed laser induced breakdown of water pushes high-speed fluid jets through the nozzle into the main channel to deflect the target particle or cell passing by into the collection channel.

Cavitation bubble dynamics are shown in the time-resolved images in Figure 2. A disk-shaped bubble expands to a maximum diameter of 160 μm within 3 μs (Figure 2c) and fully collapses by 20 μs (Figure 2d, see Supporting Information Movie S1 for details). Red dye (Allura, 67 mg ml^{-1} , Sigma-Aldrich) is added to reduce the threshold energy required to excite a cavitation bubble. With a liquid jet induced by a cavitation bubble through a nozzle, particles which are initially focused along a streamline that exits into the waste channel (Figure 2e) are pushed into the collection channel path (Figure 2f, see Supporting Information Movie S2 for details). The switching efficiency profile, which is defined as the probability of a successful switching event at various time delays, is experimentally constructed by counting successful switching events in 150 captured particle fluorescence images. The proper delay time between detection and trigger of a laser pulse is set at the time that has the highest switching efficiency. Here, a critical parameter to tune is the fluid flow rate in the bubble excitation channel. The peak switching efficiency is lower with a lower flow rate (for example 500 $\mu\text{L min}^{-1}$, as shown in Figure 3) in the bubble channel because the trace of the particles/cells is more biased away from the channel-separation tip, thus requiring a larger perturbation bubble to switch. By increasing the flow rate to 650 $\mu\text{L min}^{-1}$, the highest switching efficiency can be increased to 93% (Figure 3) while still keeping the focused particles/cells flowing into the waste channel. The optimized switching window for the sorter configuration here is 16 μs in time or 32 μm in distance (16 $\mu\text{s} \times 2 \text{ m s}^{-1}$).

Fluid inertia is used to focus particles without sheath flows, using three separate channel regions: (i) a straight focusing region, (ii) a stepped region to bias a single stream, and (iii) an expansion region to maximize inter-particle spacing uniformity. With a particle/cell speed of 2 m s^{-1} and Reynolds number ($Re = \rho v D_h / \mu$, the ratio of inertial to viscous forces, D_h is $53.33 \text{ }\mu\text{m}$ calculated based on the dimension of the channel) of 107, particles/cells are focused by fluid inertia and equilibrate into two equilibrium positions in the initial low aspect ratio straight channel segment. This occurs due to a balance of two inertial lift forces originating from the shear gradient and the presence of the wall.^[46] In the second region of the channel, vertical steps induce a pair of top-bottom asymmetric helical secondary flows, which cause the upper focusing position to become unstable and direct particles/cells to the other equilibrium position. This results in 3D focusing of cells and particles into a single stream in the channel (see Supporting Information Figure S1 for details). Figure 4a shows the focusing result of $10 \text{ }\mu\text{m}$ polystyrene microspheres at a concentration of 0.1% w/w, which corresponds to a throughput of $10000 \text{ particles s}^{-1}$ at a flow rate of $400 \text{ }\mu\text{L min}^{-1}$. A standard deviation plot from 8000 high-speed images (Figure 4a) shows that all particles are focused tightly into the waste channel. Previous studies found that the inertially focused particles/cells, due to the particle-particle interaction effects, tend to form trains with certain inter-particle separation distances,^[47] which for our channel dimensions and particle sizes is $22 \text{ }\mu\text{m}$ (Figure 4b), a value smaller than the PLACS switching window measured in our experiments (Figure 3). Figure 4b shows a high-speed image of particles focused at the bottom inertial equilibrium position after the stepped structure but before entering the expansion chamber. To increase this inter-particle spacing we have implemented a third region in the channel – an expansion chamber which acts to slow down the particles and bring them closer into a hydrodynamic repulsive interaction regime, in which the viscous disturbance flows act to repel neighboring particles and push them apart.^[47] After passing through the expansion chamber, inertial lift forces again act to focus particles and maintain them at enlarged separation distances, as shown in Figure 4c. This process is possible because the train of particles is segmented, which means there are still large intervening regions with no particles. After slight widening of inter-particle separation distances, the particles are spread more uniformly while still satisfying conservation of mass.

Using an expansion chamber increases the sorting purity at high sort rates by making the spacing between particles more uniform and less dependent on Poisson arrival statistics. Without the expansion region, we observed that 41% of the particles have inter-particle separation distances smaller than $32 \text{ }\mu\text{m}$, which is the switching window. This means that non-target neighboring particles have a high probability to be switched into the collection channel. As a result, the sort purity in the device without an expansion chamber for regulating inter-particle separation distances drops to as low as 15% at a throughput of $10\,000 \text{ particles s}^{-1}$. In contrast, by passing 3D focused particles through an expansion chamber, the percentage of particles with a separation distance smaller than the switching window is reduced to 13%.

Sorting performance is assessed by sorting $10 \text{ }\mu\text{m}$ (CV 2.4%) polystyrene microsphere mixtures at various concentrations corresponding to different throughputs. The sample flow rate is fixed at $400 \text{ }\mu\text{L min}^{-1}$ while the bubble channel flow rate is $650 \text{ }\mu\text{L min}^{-1}$. Both the

initial mixtures and the post-sort samples are analyzed by a commercial flow cytometer (BD FACSCanto II). The sorting results with throughputs ranging from 6000 particles s^{-1} to 30 000 particles s^{-1} are shown in Figure 5. The sorter can achieve > 90% sort purity at throughputs lower than 10000 particles s^{-1} . The obtained post-sort purities were $91.3 \pm 1.2\%$ (mean $\pm$ s.d.) and $92.3 \pm 1.4\%$ for throughputs of 6000 particles s^{-1} and 10000 particles s^{-1} , respectively. The post-sort purity drops at higher throughputs because of higher particle concentrations and smaller inter-particle separation distances. At throughputs of 20000 particles s^{-1} and 30000 particles s^{-1} , the obtained post-sort purities were $73.6 \pm 2.2\%$ and $45.9 \pm 5.4\%$, respectively.

Mammalian cell sorting is also demonstrated using Ramos human Burkitt lymphoma cells, which are $11.29 \mu m$ in diameter on average but have a larger size variation (CV 9.3%) than particles (CV 2.4%). Stained cells by Vybrant CFDA SE Cell Tracer (Invitrogen) are mixed with unstained cells at desired ratios. After tuning the density of the cell suspension, which is approximately 10^6 cells ml^{-1} , the mixture is introduced into the microchannel. After sorting, the collected cells are stained with propidium iodide (PI) dye to test viability and are analyzed by a commercial flow cytometer (Table 1). A sort purity of 80.1% is achieved at a throughput of 6000 cells s^{-1} . At higher throughputs, more unfocused cells stack at the bifurcation tip in a short time and cause the shifting of the bifurcation point. This not only changes the switching timing, decreases switching efficiency, but also causes device clogging. These unfocused cells partly come from the beginning of sample introduction before a stabilized fluid flow is established. After the fluid flow becomes stable, there is still a small percentage (less than 1%) of unfocused cells.^[45] Decreasing the number of unfocused cells or increasing focusing efficiency by adding more steps at the inertial focusing stepped region may help reduce clogging probability. Widening the channel can also prevent device clogging.^[48] The reasons for lower sort purity for cells than that for particles at the same throughput arise from both mis-focusing and mis-sorting. (1) The focusing efficiency for cells is lower compared to particles given the same experimental conditions because cells are more deformable and have larger variations in size. Non-target cells can flow into the collection channel due to mis-focusing of variably sized cells. (2) The chamber structure is less effective in enlarging the distance between cells than it is for particles, partly because of size differences which lead to variations in cell velocities and inter-particle distances. Thus, there are more cells, compared to particles, with inter-cell distances smaller than the switching window, causing more non-target cells to be sorted into the collection channel.

In conclusion, we presented a 3D inertial focusing integrated PLACS for high-throughput, sheathless, fluorescence activated cell sorting. Inertial focusing, which replaces 3D sheath flow focusing, uses a 10-fold lower initial concentration of cell samples than sheath-based PLACS in order to avoid severe dilution effects from high volume sheath flows at the same throughput. The unique particle-particle interactions in inertial flow also allow more uniformly distributed particles/cells to increase sort purity during high-throughput sorting. With an optimized switching window (peak switching efficiency of 93% and width of $16 \mu s$), sort purity >90% has been achieved at a throughput of 10000 particles s^{-1} . Sorting mammalian cells with >80% sort purity at a throughput of 6000 cells s^{-1} is also demonstrated. To further improve the sorting performance, throughput and purity, a superior

channel design is required to further reduce the chance of cell clogging and enlarge the inter-cell separation distances.

Experimental Section

Microfabrication

SU-8 2010 and SU-8 2025 were patterned by standard photolithography on bare wafers for the first and second layers, respectively. Then PDMS was poured on the mold and cured at 80 °C for 2 h. After being peeled off and cut into the desired size, PDMS with structures were bonded to a piranha-cleaned thin cover glass (150 µm thick). Finally, the whole device was placed in an oven at 80 °C for 4 h to enhance bonding strength.

Experimental setup

A Q-switched Nd:YVO₄ pulsed laser (EKSPLA, JAZZ 20, 532 nm wavelength, 8 nsec pulse width) was focused into the middle of the bubble channel through a 100×/N.A. 0.9 objective lens. A CW laser (Crytalaser, DL-488-010, 10 mW) at 488 nm was used for fluorescence excitation through a 25×/N.A. 0.4 objective lens. The emission fluorescence was collected by the same objective lens and detected by a photo-multiplier tube (PMT) (Sens-Tech, P30CWAD501) which connected to a DAQ card (National Instruments, PCI 7831R) for signal acquisition and processing. FPGA logic was programmed using Labview to perform real-time detection, threshold comparison, and timed triggering of the pulsed laser. To observe and characterize the bubble, a flash lamp (High-Speed Photo-Systems, NANOLITE, KL-M) was used to capture time-resolved images on a CCD camera (Zeiss, AxioCam MRm).

All fluid flows were driven by syringe pumps (Harvard Apparatus, MA, USA). A high-speed camera (Vision Research, NJ, USA) mounted on a Nikon inverted microscope (Nikon, Japan) was used to take series of high-speed images at 4000 fps with a 1 µs shutter speed. The recorded images were processed by ImageJ and then analyzed using R.

Mammalian Cell Preparation

Ramos human Burkitt lymphoma cells were cultured using a standard protocol. Cell staining was performed by adding 1 µM carboxyfluorescein diacetate, succinimidyl ester (CFDA SE) at 37 °C for 15 min, followed by centrifugation and 3 washes with 1x PBS, pH 7.4. The mixture was suspended in 1x PBS, pH 7.4, with 10% fetal bovine serum (FBS) and 1 mM ethylenediaminetetraacetic acid (EDTA) to prevent aggregation. Dust and large cell clumps were filtered using a 40 µm pore-size cell strainer (Thermo Fisher Scientific, MA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic of inertial PLACS. (i)–(ii) Randomly arriving particles/cells are inertially focused into two positions in a 1:2 aspect ratio microchannel. (iii) A stepped microchannel induces secondary flows that lead to lateral migration of upper particles/cells in the channel. (iv) After passing through these stepped microstructures, the particles/cells are focused into a single stream. The y–z and x–z planes depict the particle/cell migration process. The focused particles enter an expansion chamber structure whose function is to enlarge the separation distance between two closely positioned particles by particle-particle interaction in inertial flows to reduce the coincident events. Particles passing through the stepped structure and the expansion chamber are then interrogated in the fluorescence detection zone. Once a target particle is identified, a laser pulse is triggered to excite a cavitation bubble at a proper delay time to induce a liquid jet through the nozzle to deflect the target particle/cell into the collection channel.

Figure 2.

(a) Detection spot and pulsed laser spot location. (b) Flow rates of DI water in the main channel and fluid with red dye in the bubble channel were $400 \mu\text{L min}^{-1}$ and $650 \mu\text{L min}^{-1}$, respectively. The flow in the main channel was biased toward the bubble channel through the nozzle to minimize color dye following into the waste and collection channels. (c–d) Time-resolved images showing cavitation bubble dynamics. A laser pulse triggered, disk-shape bubble that was $160 \mu\text{m}$ in diameter expanded to its maximum size in $3 \mu\text{s}$ and fully collapsed by $20 \mu\text{s}$. (e) The fluorescence trace of a particle passing through the switching zone into the waste channel without triggering a bubble. (f) The fluorescence trace of a target particle that triggered a cavitation bubble and was deflected into the collection channel.

Figure 3.

The tested switching efficiency profile, defined as the probability of a successful switching event at various time delays by counting successful switching events in 150 captured particle fluorescence images with different dye flow rate settings. The proper delay time between detection and triggering of a laser pulse is set as the time that has the highest switching efficiency. By increasing the flow rate up to $650 \mu\text{L min}^{-1}$, the highest switching efficiency is increased to 93%. The optimized switching window is $16 \mu\text{s}$ in time or $32 \mu\text{m}$ in distance ($16 \mu\text{s} \times 2 \text{ m s}^{-1}$).

Figure 4.

Inertial focusing performance. (a) An image without flowing particles and an image showing the particle trace by stacking 8000 high-speed images of particles focused into a single stream and flowing into the waste channel. (b) Without an expansion chamber structure, interparticle separation distances have a distribution with a peak at 22 μm. There are 41% of particles whose separation distances with neighboring particles were smaller than the 32 μm switching window. (c) After passing through a chamber structure, the distribution of interparticle separation distance shows a two-peak pattern with the major peak at 80 μm and another peak at 22 μm. The percentage of the particles with interparticle distance smaller than the switching window was reduced to 13%.

Sorting Performance

Figure 5.

Particle sorting results at different throughputs. The initial mixture contains non-fluorescent 10 μm polystyrene particles with a low percentage of fluorescent particles. After sorting, greater than 90% sort purity of the fluorescent particles was achieved for throughputs below 10000 particles s^{-1} . The obtained post-sort purities were $91.3 \pm 1.2\%$ (mean $\pm$ s.d.) and $92.3 \pm 1.4\%$ for throughputs of 6000 particles s^{-1} and 10 000 particles s^{-1} , respectively. At throughputs of 20 000 particles s^{-1} and 30000 particles s^{-1} , the obtained post-sort purities were $73.6 \pm 2.2\%$ and $45.9 \pm 5.4\%$, respectively. All the purity data were confirmed by a conventional flow cytometer. For higher throughputs, the sort purity decreases because of decreased interparticle separation distances due to higher initial particle concentrations.

Table 1

Results of sorting mammalian cells at different throughputs.

Throughput (cells per s)	3000	6000
Cell sample density ($\times 10^6$ cells per mL)	0.45	0.9
Initial purity (viability)	6.4% (93.9%)	4.3% (81.1%)
Collection purity (viability)	85.6% (90.7%)	80.1% (78.5%)
Waste purity (viability)	1.5% (95.5%)	2.4% (71.9%)