Modulation of aspect ratio for complete separation in an inertial microfluidic channel†

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Inertial microfluidics has been attracting considerable interest in recent years due to immensely promising applications in cell separations and sorting. Despite the intense attention, the moderate efficiencies and low purity of the reported devices have hindered their widespread acceptance. In this work, we report on a simple inertial microfluidic system with high efficiency (>99%) and purity (>90%). Our system builds on the concept of two-stage inertial migration which permits precise prediction of particle or cell position within the microchannel. Our design manipulates the inertial equilibrium positions by modulating channel aspect ratio to achieve a complete separation. Here, we successfully demonstrate a complete separation of particles and isolation of rare cells in blood spiked with human prostate epithelial tumor (HPET) cells. Based on the planar structure, large separation spacing and predictable focusing, we envision promising applications and easy integration of our system with existing lab-on-a-chip systems for cell separations.

Introduction

Cell separation and sorting are a critical step in many diagnostic, therapeutic and cell biology applications. Clean and pure outcome is expected as it is critical in numerous applications, such as early detection of cancer. Traditionally, flow cytometry is used to perform these separations. While it offers high-throughput with high information density, its wide adoption in clinical settings is limited due to system complexity, need for user training, and high cost. In addition, the necessary cell labeling can affect cell properties. When selectivity is critical, as in detection of circulating tumor cells (CTCs) for example, immunoselection is the preferred approach. Nevertheless, high cost and low throughput limit the wide acceptance of such systems. Furthermore, recent evidence suggests that surface biomarkers recognized during the immunoselection may not be homogeneously expressed.

New technologies based on microfluidics have received considerable attention for cell separations and sorting due to a number of promising advantages, including high throughput, high efficiency, label-free, low cost and simplicity. Active microfluidic systems that rely on optical, magnetic, electro-kinetic, dielectrophoretic, acoustic and centrifugal forces have been reported. These active methods typically provide excellent separation efficiency, but often offer limited throughput and require complex sample preparation and sophisticated external control. Passive microfluidic systems based on hydrodynamic forces, size filtration, pinched flow fractionation, and sedimentation are generally simpler and easier to operate. These label-free methods can significantly reduce operational complexity, and thus are generally preferable.

Recently, manipulation of hydrodynamic forces for label-free and membrane-free sorting of cells has attracted intense interest. The interaction of these forces on particles/cells in flowing fluid leads to specific migration behavior depending on cell size and deformability and has been demonstrated in microchannels of various geometries, such as square and serpentine channels. These platforms generally offer excellent separation efficiency but with a much higher throughput. For example, demonstrated >80% efficiency at flow rates as high as 400 μL min⁻¹. Our previous demonstration in a spiral channel has shown even better performance in terms of throughput (~ 1 mL min⁻¹) and efficiency (~90%), subject to an appropriate outlet system. Much higher efficiency (~99%) can also be achieved as demonstrated recently. However, comparisons on the basis of purity of the separated sample are difficult since few investigators report it.

Achieving high purity of targeted cells in separation is challenging (due to sample heterogeneity) yet critical for downstream analysis, such as subpopulation counting or molecular assays. One of the most size-sensitive inertial microfluidic designs is based on microvortex extraction, but only offers ~40% sample purity which is far from the practical needs. A much higher (>99%) sample purity was
reported by Wu et al.\textsuperscript{21} but the low throughput of their system (maximum flow rate $< 18 \mu \text{L min}^{-1}$) and the complexity of the system make it less practical. The most promising results that offer high purity ($\sim 95\%$) coupled with high efficiency ($\sim 89\%$) were reported by Choi et al.\textsuperscript{52} However, the multi-layer device fabrication is complex and its throughput is low (flow rate $\sim 14 \mu \text{L min}^{-1}$ for single channel) subjected to P-selectin coating which potentially introduces contamination. In short, the few reports demonstrating high separation purity suggest tradeoffs with throughput, efficiency, or system/operational complexity.

In this work, we introduce a new microfluidic device concept that has the potential to address the aforementioned challenges. Our system offers high purity ($> 90\%$) and ultra-high separation efficiency ($> 99\%$) without compromising throughput (with operational flows $\geq 100 \mu \text{L min}^{-1}$) or operational complexity. The throughput may even be enhanced further by paralleling due to the straight, planar design. Our approach is based on manipulation of inertial forces acting on particles/cells according to our model of two-stage inertial migration.\textsuperscript{53} The design modulates the microchannel aspect ratio (AR) to achieve a complete sample separation. The design consists of a high-aspect-ratio segment that focuses particles/cells at the height-centered equilibrium positions. These fully-focused streams of particles/cells are then led into a downstream low AR microchannel to further differentiate them based on size. Such a simple platform capable of continuous sorting can be easily integrated with downstream detection or assays for automated on-chip sample preparation and processing. Ultimately, we envision that this elegant design can finally facilitate translation of the inertial microfluidic technique from laboratory benchtop to a clinical setting.

**Design principle**

Particles or cells flowing in a microchannel are subjected to two dominant forces: the viscous drag ($F_D$), which entrains particles along streamlines, and the inertial lift force ($F_L$) that leads to migration across the streamlines. Away from channel walls, a shear-induced lift force ($F_s$) due to the parabolic nature of the velocity profile drives particles toward the channel walls.\textsuperscript{16,42} As particles approach channel walls, a wall-induced lift force ($F_w$) acting up the velocity gradient repels them away from the walls.\textsuperscript{16,42} It is the net lift force, a balance of the shear-induced and wall-induced lift forces, that is responsible for particle equilibration near channel sidewalls. Segré and Silberberg\textsuperscript{54,55} were the first to observe these equilibrium positions in 1960s in the form of an annulus near walls for neutral buoyant particles flowing in a pipe. A number of investigators have confirmed this phenomenon in microfluidic channels in recent years.\textsuperscript{56,57}

The balance of the two lift forces has been widely used in inertial microfluidics to explain equilibration of particles and cells in microfluidic channels. While this force balance explains annulus distribution of particles in capillaries, it is incomplete for radially-asymmetric geometries of square and rectangular microchannels that are commonly used in microfluidic systems. Specific equilibrium positions of particles are observed both numerically and experimentally in square channels. Chun and Ladd\textsuperscript{58} have numerically shown that particles flowing in a square microchannel possess four or eight equilibrium positions depending on flow $Re$. While our previous work\textsuperscript{36,42} reported eight positions at low $Re$, others\textsuperscript{59,60} showed four face-centered positions contemporaneously. Recent work by us\textsuperscript{53} and others\textsuperscript{61} in rectangular microchannels has shown that the number of equilibrium positions can be further reduced to two under certain conditions. These results reveal additional cross-lateral migration that is not explained by a balance of the two lift forces, suggesting a supplementary lift force that influences particle motion near walls.

Indeed, our model of two-stage migration revealed a rotation-induced lift force ($F_{\text{r}}$) that acts up the velocity gradient and directs particle/cell migration to the face-centered equilibrium positions.\textsuperscript{53} In essence, migration in microchannels occurs in two stages. First, particles away from microchannel walls migrate toward the walls under the influence of the shear-induced lift force. In the second stage, near the channel walls, the shear-induced lift is balanced by the wall-induced lift force, and the rotation-induced lift leads to particle migration toward the walls’ centers. While the first stage is fast, the second stage is slow since $F_{\text{r}}$ is an order of magnitude smaller than $F_s$.\textsuperscript{52} Particles in a radially-symmetric channel (i.e., capillary) undergo only the first stage of migration due to absence of particle spinning in the tangent direction. On the contrary, particles in radially-asymmetric (e.g., rectangular) channels undergo both stages and equilibrate into distinct positions.

The MARCS (modulation of aspect ratio for complete separation) system we describe in this work builds on our model of two-stage migration in rectangular microchannels, and contains two segments as illustrated in Fig. 1. The first, upstream segment (segment I) consists of a high AR channel where randomly dispersed particles at the input complete both stages of inertial migration and order in two equilibrium positions at the centers of the channel sidewalls (or at $\frac{1}{2}H$). The second, downstream segment (segment II) reverses the channel aspect ratio, transitioning into a low AR microchannel, where equilibrium positions shift to centers of the top and bottom walls (or at $\frac{1}{2}W$). This change in equilibrium positions causes particles to regain migration velocity in order to move toward the new equilibrium locations. Since the migration velocity $U_m$ is strongly dependent on particle diameter $a_p$ scaling as $U_m \propto a_p^{3/2}$,\textsuperscript{42} larger particles complete their migration much faster than the smaller particles. Thus, larger particles require much shorter downstream channel length $L$, which scales inversely with the particle diameter as $L \propto a_p^{-2}$.\textsuperscript{53} Consequently, in the downstream segment of our system, the slowly-migrating smaller particles remain near channel sidewalls while the larger particles equilibrate near centers of the
The downstream particles are fully focused, the channel expands into a low AR channel (segment II), which modifies the inertial lift and shifts equilibrium positions to the middle of channel width (at \( \frac{1}{2}W \)). Here, particles regain migration velocity and move toward the new positions. Due to the strong dependence of migration velocity on particle size, larger particles complete the refocusing much faster than the smaller ones.

For example in the upstream segment of our system, \( d_m^a = \frac{1}{2}W_1 \) and \( d_m^a = \frac{1}{2}H \). Similarly, in the downstream segment, \( d_m^a = \frac{1}{2}H \) and \( d_m^a = \frac{1}{2}W_2 \). We designed the upstream segment to be 27 \( \mu \)m (\( W_1 \)) x 50 \( \mu \)m (\( H \)), and downstream segment to be 100 \( \mu \)m (\( W_2 \)) x 50 \( \mu \)m (\( H \)) to demonstrate complete separation of 10 \( \mu \)m and 20 \( \mu \)m diameter particles. Using lift coefficients we measured in our recent work,\(^53\) \( L_1 = 10.3 \) mm for the 10 \( \mu \)m diameter particles in a 27 \( \mu \)m x 50 \( \mu \)m channel (\( C_L^1 = 0.012 \)) and \( C_L^1 = 0.013 \) at \( Re = 40 \). Similarly, \( L_2 = 9.2 \) mm for the 20 \( \mu \)m diameter in a 100 \( \mu \)m x 50 \( \mu \)m channel (\( C_L^1 = 0.3 \) and \( C_L^1 = 0.05 \) at \( Re = 20 \)).

We confirmed these calculations experimentally by measuring the fluorescent intensity distribution progressively in the microchannel as various downstream positions (Fig. 2).

Since the focusing length scales with flow rate,\(^53\) we measured it under different flow conditions to determine an optimal \( Re \) where \( L \) is minimized. Linescans of fluorescent images at downstream positions disclose the progressive increase of intensity of the central peak, indicating particle migration to the center of channel width (Fig. 3a). Full width at tenth maximum (FWTM) was used to characterize the focusing length where FWTM becomes constant, as shown in Fig. 3b. We found focusing length to scale with \( a_p^{-5/7} \) (Fig. 3c), which is expected considering that \( C_L^2 \propto a_p^{-1} \) and \( d_m^a/C_L^2 \) in eqn (2).\(^31\) To find the optimal flow conditions, we plotted the focusing lengths (\( L_1 \) and \( L_2 \)) as a function of \( Re \) in Fig. 3d. Since \( Re \) in the downstream segment is half of the input \( Re \) in the upstream segment, we plotted the total focusing length as \( L_1(Re) = L_1(Re) + L_2(\frac{1}{2}Re) \). The data reveal the optimal flow conditions at \( Re = 40 \), with the total focusing length of \( L = 19 \) mm (\( L_1 = 10 \) mm and \( L_2 = 9 \) mm); any change in either direction leads to an increase in...
focusing length. While these calculated dimensions are sufficient for separation of 10 μm and 20 μm diameter particles, to ensure complete focusing of particles with diameter as small as 7 μm (approximate size of RBCs) in the upstream segment, we extended its length to 20 mm. In addition, we also elongated the downstream segment to 16 mm to accommodate channel expansion and minimize any transitional effects (as reported in previous work).

In summary, we designed the MARCS system using our model of two-stage inertial migration to completely separate particles based on size. The channel AR reversed from 1.86 to 0.5 as channel expands from the upstream segment to the downstream segment. The designed optimal performance is at input Re = 40. Next, we demonstrate the complete separation using this device and discuss its characterization and optimization.

Separation of a binary particle mixture

Successful demonstration of a complete separation is shown in Fig. 4 using a binary mixture of 20 μm and 9.94 μm diameter particles. Our results reveal that the complete separation occurs in three steps. First, randomly distributed particles at the input (Fig. 4a) gradually migrate to their equilibrium positions near sidewalls of the high-AR upstream segment, forming two streams for each particle. Traces of smaller red particles are indistinguishable due to proximity of the equilibrium positions of both particles, with the larger particles appearing as brighter green streaks (Fig. 4b). To show the positions of the particle streams, we measured the lateral distance dm of each stream from peak maxima in intensity linescans (Fig. 5a). The particle positions at the end of the upstream segment indicate that the smaller red particles are closer to channel sidewalls, which is expected since they experience less wall-induced lift force (Fw \( \propto a_p^{3/2} \delta \)), where \( \delta \) is the minimum distance between particle and wall. The green particles were observed at \( \delta = 1 \) μm (\( \delta = \frac{1}{2}W - d_m - \frac{1}{2}a_p, d_m = 5 \) μm), while the smaller red particles were at \( \delta = 2 \) μm (\( d_m = 9 \) μm) at L = 19.5 mm downstream.

In the second step, as the microchannel expands to reverse the aspect ratio, the increasing \( \delta \) leads to a substantial reduction in \( F_w \) and activates lateral motion under the influence of the shear-induced lift force \( F_s \). As a result of the strongly size-dependent migration velocity (eqn (1)), larger particles move laterally faster and cross the trajectory of smaller particles (red curve in Fig. 5a). In this transient state, the larger 20 μm diameter particles are closer to channel sidewalls at \( \delta = 2 \) μm (\( d_m = 38 \) μm), and the 9.94 μm diameter particles are at \( \delta = 12 \) μm (\( d_m = 33 \) μm) at L = 23 mm downstream. Such
distinct positions indicate that a separation has already occurred; nevertheless the relatively small gap between the streams ($\Delta \delta = 10 \mu m$) makes it difficult to design an outlet system to fractionate the sample. This gap however is substantially amplified further downstream, following the second trajectory crossing.

In the third and final step, once the large particles reach their maximum lateral distance, the migration velocity reverses as a result of rotation-induced lift force (Fig. 4c–g). The green particles begin to migrate in the direction opposite to that of the smaller particles at $L \geq 23 \text{ mm}$, making red streams of the smaller particles visible near sidewalls (Fig. 4c–d). Since migration velocity of red particles is smaller but their transient equilibrium position is closer to sidewalls, they continue migrating toward the sidewalls. For example, the red particles reach the maximum lateral distance of $D_{m} = 38.1 \mu m$ at $L = 28 \text{ mm}$ downstream and then slowly reverse the velocity direction. Even at $L = 36 \text{ mm}$, where green particles already arrive at their new equilibrium positions, red particles only migrate $\sim 1 \mu m$ toward channel center ($D_{m} = 36.8 \mu m$). Consequently, the second trajectory crossing occurs at $L \sim 24 \text{ mm}$ (Fig. 5a). Here, the large particles migrate rapidly to the channel centerline, dominated by the rotation-induced lift force, resulting in the tremendous distance between the two particle stream centers ($\Delta D_{m} = 37 \mu m$) which is ideal for separation. This distance is more than $7 \times$ that the spacing in the second step. Focusing length is strongly dependent on particle size ($L \propto a_p^{-2}$), and thus the smaller (9.94 $\mu m$) particles will not migrate to the width-centered equilibrium positions until $\sim 60 \text{ mm}$ downstream, offering a flexible option to fractionate the flow and separate particles. The particle-free spacing ($\sim 20 \mu m$) observed between the two particle streams (Fig. 4f–g) without any further channel expansion is remarkable, permitting an easy design of an outlet system for separations. Indeed, we demonstrated a complete separation using a simple three-channel outlet, with both fluorescent and brightfield images confirming that small particles exit through the two side outlets while the large particles elute from the central outlet.
Physics of particle migration in the expansion

The migration trajectory of 20 μm diameter particles appears linear with respect to downstream length after the second crossing (Fig. 5a). Indeed, \( d_m \) can be estimated as \( d_m = L \times U_m / U_f \). At given \( Re \), \( U_f \) is constant and the average lateral migration velocity \( U_m \) remains the same. In fact, the high linearity (\( R^2 > 0.997 \), from 23 to 32 mm) indicates little variation of the lift coefficient along the migration path and thus constant migration velocity. In other words, \( d_m \) can scale with \( L \). Based on our measurements, the average \( U_m \) of 20 μm diameter particles is calculated as \( U_m = U_f \times d_m / L = 1.27 \text{ mm s}^{-1} \), where \( L = 9 \text{ mm} \) and \( d_m = 38 \mu m \) (Fig. 5a). Using Stoke’s Law, the rotation-induced lift force is then calculated to be \( F = 3 \pi \mu a_U L \mu = 0.24 \text{ nN} \), which is small but important for particle focusing and cannot be neglected.

In addition to \( F_0 \), we are able to estimate the shear-induced lift force \( F_s \) from the measurements in Fig. 5a. While the focusing length in the downstream segment remains similar (~9 mm) to that in the simple low-AR channel (Fig. 2d-f), an additional length \( L_{ext} = 3 \text{ mm} \) is necessary for the 20 μm diameter particles following the channel expansion at \( L = 20 \text{ mm} \) downstream. This is illustrated in Fig. 5b. As channel expands, reduction in \( F_s \) causes particles to regain lateral migration velocity; this velocity has to reduce to zero before velocity reversal (the third step), which results in the additional \( L_{ext} = 3 \text{ mm} \) downstream length. Assuming that this velocity decreases linearly and the acceleration force is the averaged rotation-induced force, we can then estimate the lateral velocity \( U_e \) at the end of the expansion. Using the Newton’s second law \( F_{\Omega} = ma \), where \( m \) is the particle mass and \( a \) is the particle acceleration) and considering the time for reduction of velocity is \( t_e = L_{ext} / U_e \), the lateral velocity in the expansion is given by \( U_e = a_t t_e = F_{\Omega} L_{ext} / m U_f \). Further assuming that the particle is accelerated undergoing constant net force in the expansion \( (L_{exp} = 150 \mu m) \), we can obtain the other acceleration \( (a_2) \) in the opposite direction in the expansion as \( a_2 = U_e / t_2 \), where \( t_2 = L_{exp} / U_f \). Since the migration in the expansion is dominated by the shear-induced lift force, it can be estimated as \( F_s = ma_2 \). Our calculations for 20 μm diameter particle indicate \( F_s \approx 5 \text{ nN} \), which is ~20 × higher than the rotation-induced lift force. In short, similar to the calculation of \( F_{\Omega} \), the estimate of \( F_s \) is in agreement with values reported in literature based on numerical models³⁰ and our previous outcomes using Asmolov’s equation³¹ (we note, however, that this estimate neglects variation of \( Re \) in the expansion).

The additional channel length \( L_{ext} \) is necessary due to the non-zero lateral velocity \( (U_e) \) at the end of the expansion. Considering the net force acting on particles is primarily modulated by \( F_s \) and \( F_{\Omega} \), with the former inversely dependent on \( \delta \), \( L_{ext} \) can be modulated by the expansion angle. Early work⁶¹ has shown that rapid expansion shift particle equilibrium position to the centerline, while gradual expansion acts in the opposite way. Gradual expansion (small angle) keeps the side wall in proximity to particles, which leads to a small net force for outwardly lateral acceleration. As a result, \( U_e \) is small and thus the \( L_{ext} \) is short. Conversely, the sharp expansion (large angle) results in a larger \( \delta \), compromising \( F_s \) and increasing the net force. As a result, \( U_e \) is larger causing longer \( L_{ext} \). The angle of our channel is ~12.8°, leading to \( L_{ext} \approx 3 \text{ mm} \) for 20 μm diameter particles at \( Re = 40 \).

Using the known \( L_{ext} \) and considering scaling \( L \propto a_p^{-2} \), we can estimate the cutoff particle size \( a_{cutoff} \) for the complete separation. Since the central outlet channel \( (W = 30 \mu m) \) is larger than particle diameter, it is not necessary for particles to reach full focusing for separation. As illustrated in the electronic supplementary information (ESI† Fig. 1), a migration distance of 33 μm is theoretically sufficient for particle sorting in this device (Fig. 5a). As a result \( L_{20 \mu m} = 8 \text{ at } Re = 40 \text{ and the cutoff size is refined to 15.7 μm} \). To confirm this calculation, we used 15.5 μm diameter particles with a standard deviation of 1.5 μm. Following separation in our device, particles were counted by size at each outlet. The results show that our device is analogous to a high/low-pass electrical filter with a cut-off \( a_{cutoff} \approx 15.65 \mu m \). Hence, the central outlet rejects particles with \( a_p < 15.65 \mu m \) (high pass filter) and the side outlets contain particles with diameter \( a_p < 15.65 \mu m \) (low pass filter). These results are quite close to our estimates using eqn (2) and consistent with the implication of high sensitivity from \( \Delta L/\Delta a_p \approx a_p^{-3} \). Since the flow rate modifies the focusing length (Fig. 3d) and its inverse dependence on particle size, this cutoff size can be tuned either by flow rate (input \( Re \)) or by length of downstream segment. The scaling of \( L \propto a_p^{-2} \) also implies an easy separation of mixtures, since a small variation in particle size results in significant difference in focusing length.

Separation purity and efficiency

Our results show excellent separation performance of the MARCS system (Fig. 6). Samples from side outlets (#1 and #3) were mixed together due to symmetry and the volumes of all samples were adjusted to the same as the control for better comparison. Both of our fluorescent images and quantitative counts show at input \( Re = 40 \) (flow rate of 100 μL min⁻¹), almost every large particle is collected from outlet #2, with efficiency \( \eta = 99.3% \) and purity = 90.8% for 20 μm diameter particle. Similarly, \( \eta = 94.5% \) and purity = 99.6% for smaller 9.94 μm diameter particles in side outlets. An independent samples t-test shows statistical significance between 20 μm particles in outlet #2 and control \((t(5) = 5.29, p < 0.001)\), and 9.94 μm particles in outlets #1 & #3 and control \((t(5) = 10.26, p < 0.001)\). We should note that sedimentation can remarkably alter the initial particle concentration. For example, the initial concentration ratio of the mixture was prepared to be 1 : 1, but the concentration of the control sample at the input shows the actual species ratio of 1 : 3 at input. Our previous work reported up to 90% efficiency in spiral channel⁴⁶ and Mach et al.⁵¹ showed 40% purity in their trapping system. Thus, the
outcomes reported here are superior to the existing inertial systems.

As the device is designed to work at input Re = 40, we observed the optimal performance at this flow condition where maximum efficiency and purity were reached (Fig. 6b–c). Although we expected nearly 100% efficiency and purity at optimal Re due to the complete separation nature and Fig. 2, measurements reveal efficiencies η > 95% and the purities >90% for the two species. The decrease in efficiency and purity are primarily attributed to the altered AR of the upstream segment where full focusing of particles into two equilibrium positions is necessary. Measurements in the actual channel showed AR = 1.56 despite the designed AR = 1.86. Previous work42,59 revealed that particles in square channel (AR = 1) focus into four face-centered positions. Hur et al.15 showed that as channel AR increases from unity, particles focus predominantly into two positions (Fig. 2 in this work). More recently, we quantitatively showed channel AR effect on particle focusing: particles in a channel with AR closer to unity (square channel) employ smaller lift coefficient, which means longer channel length is required for full focus in upstream segment.53 In addition Lim et al.61 showed that smaller particles have higher possibility to stay in the positions centered short faces of rectangular channel than larger particles do. In summary, the altered AR in upstream segment leads to downgrade of focusing quality and an increase of focusing length, especially for smaller particles (9.94 μm). Consequently, a portion of smaller particles may remain not well focused at the end of the upstream segment whose length can be insufficient. Since the particle equilibrium positions shift to the middle of top and bottom faces as the AR change after the expansion, these smaller particles stop migrating to the side walls. These smaller particles are then collected from the central outlet together with larger 20 μm diameter particles. Consequently, they contaminate the target particles and reduce the purity as well as compromise the efficiency of smaller particles.

Deviations in input Re from the optimal conditions undermines the device performance. While the large particles show stable efficiency η > 98.6% for Re > 30, it varies with Re for 9.94 μm diameter particles due to the limited length of the upstream segment. As we already discussed, the small AR of upstream segment reduces the focusing performance, especially for smaller particles. Moreover, according to Fig. 3d and our recent work,53 focusing length increases after Re = 40. Hence higher Re requires longer upstream segment to achieve full focus for particles. At fixed upstream length, the focusing quality downgrades. Since full focus of all particles into equilibrium positions centered channel height is the prerequisite of downstream complete separation, the performance in terms of efficiency and purity decrease at higher Re.

Separation of blood

One target application of our system is to process a blood sample to extract cells of interest. Since the whole blood is rather viscous (female Ht = 40%), a dilution is necessary to achieve Newtonian fluid and reduce the cell–cell interaction before processing through the device. Typically 0.5% is used to perform cell separation in inertial microfluidic systems.47,63 Higher hematocrit (less dilute) blood is preferred to save processing time. To find the minimum dilution factor that offers acceptable performance, we introduced blood samples of diminishing hematocrit into the device. We took brightfield images near the outlet and measured the full width at half maximum (FWHM) of the intensity distribution across the channel width (Fig. 7). Since RBCs are the majority of the
blood constituents, the intensity distribution primarily represents focusing of the RBCs. As the hematocrit decreases with increasing dilution factor, FWHM first drops rapidly and then converges to $7\,\mu m$ (mean size of RBCs) when hematocrit $Ht < 1\%$. Thus we conclude that our device works for blood samples with hematocrit less than 1\%. For example 50\,6 dilution ($Ht = 0.8\%$) can offer good focusing of cells (Fig. 7b).

These results are in agreement with previous work by Bhagat et al.\textsuperscript{47} who used $Ht = 0.5–1\%$ in their devices and showed good focusing, while $Ht = 2\%$ led to unfavorable results. Thus, we chose 50\,6 dilution ($Ht = 0.8\%$) to demonstrate separation of spiked blood samples next.

We successfully demonstrated the separation of circulating tumor cells (CTCs) from blood as shown in Fig. 8. We first used particle spiked blood to show the feasibility of separation of tumor cells. Stacked brightfield image in Fig. 8a shows that RBCs mimic the behavior of small particles and entrained themselves at side outlets while larger particles flow through central outlet. Most of the blood cells here should be RBCs since it is difficult to identify WBCs in the brightfield images due to their low concentrations. Next we used human blood spiked with human prostate epithelial tumor (HPET) cells to show separation of CTCs. HPET cells are derived from a high grade (Gleason 9) prostate punch biopsy and recapitulate the histopathology of human prostate cancer \textit{in vivo}\textsuperscript{65–67} and it can be present in blood stream.\textsuperscript{68} The HPET cells are 18–22\,\mu m in diameter, and cannot be separated with conventional flow cytometry due to their fragile nature (relatively high shear rates in the flow cytometer tend to lyse these cells). The cells were stained with green live cell tracker dye (ER-Tracker\textsuperscript{TM} Green, Invitrogen) to permit visualization. The fluorescent streak in the middle (Fig. 8c) indicates HPET cells behave similar to 20\,\mu m particles and exit from central outlet still intact. The corresponding brightfield image (Fig. 8b) shows most of the RBCs flow out the channel from side exits, which is confirmed by sample collected from the three outlets. The sample from side outlets is red while the central is clear (Fig. 8d). Most of WBCs which are the major contaminants in discrimination of CTCs could also be depleted from the outlet #2 considering the cutoff size ($\sim 15.7\,\mu m$) of current system. Further demonstration of separating CTCs from WBCs will be necessary to validate the power of our platform for cell sorting. Nevertheless, our results indicate that this system offers promise to isolation of CTCs considering its high efficiency and purity.

### Challenges in cell sorting

Cells differ from rigid microparticles in many properties which can affect the inertial focusing and thus device performance. Cells may have distinct shapes which could behave different from spherical beads.\textsuperscript{4} For example, RBCs in peripheral blood are usually discoid, which may lead to a different response to inertial forces. Nonetheless, recent work\textsuperscript{69} reported that generally the maximum dimension of the object determined its lateral focusing position within microchannel, which is also shown in this work (Fig. 7 and 8) that RBCs mimic the behavior of microparticles and focus near the sidewalls. Deformability is the second cell property that impact cell equilibration within channel cross-section.\textsuperscript{34} Cells were found to equilibrate in positions closer to channel centerline than rigid particles.\textsuperscript{44} However, cancer cells focused into
positions closer to sidewalls than WBCs, which is actually good for cell sorting.\textsuperscript{44} In our device, the deformability should not have significant impact on the system performance since the equilibrium positions of different cells are remarkably distant at downstream segment. Unlike the deformability, the variation of cell size could pose great challenge to have high profile of separation performance in our device. Although the cutoff size of current device is approximately 15.7 μm, the diameters of WBCs range from 6 to 20 μm. Those WBCs larger than the cutoff size could still introduce contamination in the case of CTC isolation and compromise the separation purity, due to the potentially extreme rarity of CTCs (1–10 cells per mL). Hence a second step such as immunoselection may be required for downstream analysis.

High throughput is desired in isolation of the rare CTCs from bulk blood cells.\textsuperscript{70} Although our throughput (∼100 μL min\textsuperscript{−1}) is much better than other existing platforms,\textsuperscript{21,52} it is still insufficient to process relatively large volume (∼7.5 mL) of a blood draw. Moreover, as the general limitation of inertial devices, at least 40× dilution is required before sample processing (Fig. 7c). This dilution translates in the sample ballooning to even larger volume (∼300 mL) and the processing time of 50 h in a single channel. Multiplexing can tremendously reduce this processing time. For example, parallelization of 100 channels can reduce this time to 30 min. Due to the planar straight nature of our channel, it is easy to scale the throughput by multiplexing. Several groups\textsuperscript{47,52,63} have successfully demonstrated the massive parallelization of as many as 256 channels on the tradeoff of increased device footprint.

Conclusions

In this work, we have successfully demonstrated the complete separation in our straight MARCS system. We have shown efficiency η > 99% and purity > 90% depending on Re, which outperform the existing inertial microfluidic platforms including our spiral (efficiency η = 90%)\textsuperscript{46} and the trapping system (η = 20% and purity = 40% purity).\textsuperscript{51} Moreover, our device shows a wide range of flow conditions (30 < Re < 80) that offer excellent performance. In addition, we have also demonstrated the complete separation of rare cells using HPET cell spiked blood in our system. Such outcome suggests our approach is a promising alternative to isolation of CTCs, which remains a grand challenge facing the microfluidic community. Our results further show that Ht < 1% blood works well in the device. In short our device shows high promising performance that permits a wide applications including isolation of rare cells.

Furthermore, we have shown a predictable design of separation system using our model of two-stage migration.\textsuperscript{53} Using our model and the experimentally measured lift coefficients, we are able to precisely calculate the channel length for particle focusing and thus accurately predict rather than guess where to place the outlet system, which reduces a lot of experiment load. For instance, it is subtle and difficult to place the outlets at an appropriate downstream position of a spiral channel as there is no accurate model. Guided by the model, we are now able to anticipate the optimal flow condition and the best performance of the design system. The throughput of current system is ∼100 μL mm\textsuperscript{−1} (Re = 40). However this can be remarkably enhanced simply by parallelizing. And the continuous processing implies a theoretically unlimited processing volume. Due to the planar structure, large separation spacing and predictable focusing, we envision promising applications and easy integration of our platform with existing lab-on-a-chip systems for cell separations.

Experimental

The flows of the fluorescnetly-labeled microparticles in microchannels was visualized at successive downstream positions using an inverted epi-fluorescence microscope (Olympus IX71) equipped with a 12-bit CCD camera (Retiga EXi, QImaging). Analogous to microparticle streak velocimetry (μ-PSV), flowing particles generated streaks across each frame, and we analyzed fluorescent intensities and locations of these particle streaks. At least 100 frames were obtained and stacked using ImageJ\textsuperscript{8} at each downstream position. Fluorescence intensity linescans were used in quantitative analyses. Particle suspensions were injected into device with a syringe pump (NE-1000, New Era Pump Systems, Inc.) to sustain stable flow rate. The loaded syringe was connected to 1/16” Peek tubing (Upchurch Scientific) using proper fittings (Upchurch Scientific) and then secured to the device inlet.

Particle suspensions and cell sample were prepared at different concentrations. Fluoreocently-labeled, neutrally-buoyant polystyrene particles 9.94, 15.5 and 20 μm in diameter were used in this work. Particles were mixed at 0.025% volume fraction (VF) in deionized water to minimize the particle–particle interactions. Particles were purchased from a number of vendors, depending on size (Bangs Lab Inc., Polyscience Inc., and Life Technologies Inc.). A small drop (~1% volume fraction) of Tween-20 was added to avoid clogging channels. Female human blood (Hoxworth blood center) was diluted in Saline solution to achieve different VF. Basically we prepared 40%, 2%, 0.8%, 0.4%, 0.08% and 0.004% hematocrit corresponding to the dilution factor of 1 ×, 20 ×, 50 ×, 100 ×, 500 × and 1,000 ×. In terms of particle spiked blood, we directly added 20 μm particles into the diluted sample. Fluorescently labeled HPET cells were spiked into the diluted blood (Ht = 0.8%) at the concentration of 250 cells per mL.

Hemocytometry was used to count particles in the sample collected from each outlets and determine the concentrations. Each sample was counted at least 3 times to reduce the potential errors. Briefly, sample was first stirred for 2 min to ensure the uniform particle suspension. The particle counting was performed on the microscope stage with fluorescent excitation illuminating. 20 μm diameter particles were counted.
using FITC filter cube and TRITC filter cube was used to count 9.94 diameter particles.

Microchannels were fabricated using standard soft lithography. Briefly we used the dry resist film (PerMX 3050 series, DuPont Electronic Technologies) to pattern the microchannels on a 3” polished silicon wafer by conventional photolithography. Polydimethylsiloxane (PDMS) (Dow Corning) was casted on the wafer and peeled after 2 h curing on 80 °C hotplate. Replicated channels in PDMS were bonded to 1”× 3” Corning® glass slides (Fisher Scientific Inc.) using surface treater (Electro-Technic Products Inc.). The inlet and outlet ports were punched manually using stainless flat head needles.

Straight microchannels were fabricated in pairs to allow imaging from long and short edge of cross-section. For example, 50 μm × 27 μm (width × height) channel was used to obtain topview image and the sideview was imaging using 27 μm × 50 μm channel. Essentially the rectangular channel was rotated 90° for topview and sideview. Our MARCS system comprised two segments (Fig. 1). The upstream segment was designed to be a 20 mm long, 27 μm × 50 μm in cross-section but measured to be 32 μm × 50 μm. The downstream segment is a 16 mm long low AR channel with 100 μm in width and 50 μm in height.

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