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Particle Focusing in Staged Inertial Microfluidic Devices for Flow Cytometry

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Abstract

Microfluidic inertial focusing has been demonstrated to be an effective method for passively positioning microparticles and cells without the assistance of sheath fluid. Because inertial focusing produces well-defined lateral equilibrium particle positions in addition to highly regulated interparticle spacing, its value in flow cytometry has been suggested. Particle focusing occurs in straight channels and can be manipulated through cross sectional channel geometry by the introduction of curvature. Here, we present a staged channel design consisting of both curved and straight sections that combine to order particles into a single streamline with longitudinal spacing. We have evaluated the performance of these staged inertial focusing channels using standard flow cytometry methods that make use of calibration microspheres. Our analysis has determined the measurement precision and resolution, as a function of flow velocity and particle concentration that is provided by these channels. These devices were found to operate with increasing effectiveness at higher flow rates and particle concentrations, within the examined ranges, which is ideal for high throughput analysis. Further, the prototype flow cytometer equipped with an inertial focusing microchannel matched the resolution provided by a commercial hydrodynamic focusing flow cytometer. Most notably, our analysis indicates that the inertial focusing channels virtually eliminated particle coincidence at the analysis point. These properties suggest a potentially significant role for inertial focusing in the development of inexpensive flow cytometry-based diagnostics and in applications requiring the analysis of high particle concentrations.

Flow cytometry is used for a host of research and clinical diagnostic applications ranging from CD4+ progression analysis to high-throughput fluorescence activated cell sorting (FACS). Typical flow cytometers hydrodynamically focus cells and microparticles into a narrow sample stream traveling at meter per second linear velocities that are interrogated by a tightly focused laser beam. Using this approach, flow cytometers provide precise optical

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measurements on a particle-by-particle basis at analysis rates approaching 50 000 sec^{-1}. However, the high linear velocities required to maintain high particle analysis rates necessitate the use of low noise laser sources, high-speed data acquisition systems, and highly sensitive optical detectors; all of which increase the cost and reduce the portability of traditional flow cytometers. Furthermore, this sample delivery approach results in a stochastic arrival of cells to the analysis point, which significantly limits the maximum analysis and sorting rates due to particle coincidence at the point of interrogation. Thus, while providing precise particle positioning for optical analysis, hydrodynamic focusing also limits the analysis rate, portability, and affordability of flow cytometry.

The recent development of microfluidic inertial focusing, which exploits inertial fluidic forces within microfluidic channels to precisely position particles,[1] has the potential to overcome many of the limitations imposed on flow cytometers by hydrodynamic focusing. First, it focuses particles without the use of liquid sheath, a critical consideration in the development of low-cost portable flow cytometers.[1] In this sense, inertial focusing provides a similar benefit as other efforts to focus particles in the absence of sheath fluid that include acoustic fields,[2] optical traps,[3] and dielectrophoretic focusing.[4] These methods use fluidic forces or externally applied fields to position particles to desired streamlines. Acoustic focusing, in which a standing acoustic wave positions particles to the center of a channel, without moving the fluid, based on compressibility differences between the particles and surrounding media, has been shown to be effective for flow cytometry.[5] Optical particle positioning, which creates forces based on the difference in the index of refraction between particles and surrounding media, has been used in microfluidics to sort and analyze particles.[3,6,7] Dielectrophoresis, which takes advantage of electric permittivity differences between particles and surrounding media, positions particles based on electrode positions.[4] In comparison, the potential use of inertial focusing has the additional advantage of being passive in that does not require additional power beyond what is required to pump the sample stream through a microfluidic channel. Furthermore, it is very simply implemented in a disposable microfluidic format. These features are conserved by alternative passive particle positioning techniques that exploit the properties of the microfluidics themselves[8] or via herringbone patterns that position particles via induced flow patterns.[9,10]

Beyond their potential utility in portable flow cytometry, each of these sheathless focusing technologies raises the possibility of dramatically increased sampling rates because they align particles without increasing their velocity or the overall volumetric flow rate of carrier fluid. Consequently, alternative focusing approaches, such as acoustic focusing, have been noted to provide focusing at high volumetric flow rates and lower linear velocities, an important consideration when handling suspensions of living cells. Achieving higher throughput without sheath flow will, therefore, be important to bioprocesses with living cells or applications in which cell or particle concentrations may be very low such as environmental process monitoring[11] or rare cell identification.[12] In these applications, particle concentration technologies simultaneously increase the particle analysis rates and the volumetric delivery rate. It has been anticipated that inertial focusing approaches, which concentrate particles to discrete streamlines, will likely retain these desirable properties.[13]

Spontaneous lateral focusing of random suspensions of particles and cells within straight channels occurs as a result of inertial lift forces and was first observed in cylindrical capillaries by Segre and Silberberg.[14] Microfabrication offers the ability to miniaturize and define the cross-sectional geometry of the fluidic conduits, thereby allowing the equilibrium behavior of focused particles to be directed to predictable, finite positions. For instance, Figure 1a shows that straight channels with square and rectangular cross sections focus particles to four and two discrete face-centered equilibrium positions, respectively. This focusing behavior and its dependence upon particle Reynolds Number (the Reynolds

number based on the shear rate at the particle length scale), particle concentration, and geometry have been recently described and documented for a range of channel geometries and particle sizes.[1,13,15] Equilibrium focusing positions may be further modified through the incorporation of channel curvature,[1,16] which introduces secondary flows, known as Dean flow.[17] Dean flow may be leveraged to shift the lateral position of centered focused particles, lending the appearance of a single focused stream of particles, actually consisting of two vertically overlapping streamlines.[13] As such, the benefits of passive ordering by inertial forces are complicated by the complexity of ordering behavior and the inability to generate less than two focused positions in a straight or curved channel. We overcome that limitation here by coupling curved and straight, high-aspect ratio rectangular channel sections in series to produce a single focused stream of particles with periodic longitudinal spacing.

Beads are focused laterally and vertically by competing lift forces[18] and spaced longitudinally by hydrodynamic repulsion, a particularly compelling feature of microfluidic inertial particle focusing for flow cytometry applications.[19] This effect has been shown to reduce coincidence during analysis in a flowing system,[15] which if successfully translated to a flow cytometry platform would dramatically increase the maximum achievable analysis rate, currently limited partly by random arrival of particles in the analysis region.[20] Notably, this feature is relatively unique among the sheathless focusing approaches and indicates that inertial focusing may have an impact on flow cytometry that is broader than just use in miniaturized or portable flow systems.

In this work, we have developed a staged microfluidic inertial focusing device for the passive ordering of particles into a single focused stream with lateral, vertical, and longitudinal definition. We have also evaluated the ability of microfluidic inertial focusing channels to support precise high-resolution flow cytometry using a series of fluorescence standardized calibration microspheres and a custom optical platform optimized for flow cytometry. Additionally, we have characterized device behavior by determining optimal flow rates and particle concentrations required for flow cytometry. Finally, we investigated the prediction that inertial focusing will reduce coincidence events and benchmarked the performance of our system against a commercially available flow cytometer. On the basis of this performance evaluation, we extrapolate the limits of performance capabilities for flow cytometers developed using staged inertial focusing.

METHODS AND MATERIALS

Microfluidic Inertial Focusing Channels

Microfluidic, inertial focusing chips were designed and fabricated as previously described by replicating photolithographically patterned silicon wafers in poly(dimethylsiloxane) (PDMS).[21] Inertial focusing channels used here were all 50 μm tall with curved regions and straight regions of varying width. The smallest lateral dimension of the curved channels, defining the channel segment responsible for focusing by Dean Flow, was 100 μm. Curved channels tapered smoothly into straight channels that were 30 μm in width. Fluidic connections between the microfluidic chip were made using 1/16 in. OD × 0.030 in. ID PEEK tubing (IDEX, Corp., IL). Sample was pumped into the channel using a syringe pump (#N3000 Chemyx, Inc., TX) to flow particles at desired volumetric flow rates. Long exposure streak images of 9.9 μm Dragon Green polystyrene beads (ThermoScientific) were captured with a CCD camera (SPOT). High speed video of focused particles was taken at a 2 μs exposure with a Phantom v4.2 high speed camera.

Flow Cytometry System

An epifluorescence flow cytometry system was built to evaluate the samples (Figure 1a). In addition to backscatter, this system was modified to incorporate off-angle scatter collection detection. Excitation was provided by a 5 mW (lasersale.com), 532 nm DPSS laser module, which has been demonstrated to support precise flow cytometry measurements.[22] The laser was focused into the sample using a 50×, 0.6 NA objective lens (Leitz, Germany), which also served to collect the epifluorescence. A photomultiplier (#H9656-02 Hamamatsu, Japan) with a band pass 585/42 filter was used to detect fluorescence, and a photodiode (#PDA36A ThorLabs, NJ) with a band pass 535/30 filter was used to collect scatter. Fluorescence data was acquired, and collection was triggered on scatter. Flow cytometry data acquisition was performed using a custom digital microcontroller data acquisition system, which provided data files in Flow Cytometry Standard (FCS) 3.0 format.[23,24] FCS files were analyzed using FlowJo 8.8 (TreeStar Software, Inc., OR).

Flow Cytometry Calibration Particles

Nile Red labeled alignment microspheres (10.2 μm; #FP 10056-2 Spherotech, IL) and custom manufactured 10.2 μm microspheres (Spherotech) that were dyed to contain varying levels of fluorescence were used. The Nile Red beads allowed us to perform basic testing to find the parameters, described below, at which the inertial focusing channels would effectively focus polystyrene beads. These microspheres are virtually identical, except for the different fluorescent dye, to the green polystyrene microspheres, which were used to visually characterize device behavior under varying flow conditions. As such, all flow cytometry data was taken with Spherotech beads while experiments to generate the corresponding micrographs were performed with the ThermoScientific beads, as described earlier.

RESULTS AND DISCUSSION

In previous work, we have reported the focusing of microspheres and cells[15] within straight microchannels with square and rectangular cross sections. These devices are effective at focusing particles into a finite number of equilibrium positions. Square channels focus particles to four equilibrium positions, each centered at a face of a channel wall. Rectangular channels focus particles to two positions, each centered upon the faces of the channel's long walls. However, there has been no straight channel configuration observed to inertially focus particles to a single streamline with high fidelity. We overcome this limitation by adding an asymmetrically curved channel before and in series with the straight channel section. As previously described, fluid drag upon particles results from the secondary flow generated as inertia carries fluid centrifugally toward the outer edge of the curved channel. This motion, known as Dean flow, produces a primary flow along the channel centerline as well as corresponding recirculating vortices along the top and bottom channel faces. In our devices, the recirculating flow modifies the two equilibrium focusing positions by dragging them toward the inner edge of the focusing curve. Thus, particles are biased toward one half of the channel prior to their introduction into the straight channel. As predicted, we find that these particles do not, in fact, migrate laterally to cross the midplane as they are conveyed the length of the straight channel. They are, however, focused vertically and laterally while establishing the longitudinal spacing that is characteristic of inertially focused particle trains.

Inertial focusing of particles was visually observed under a microscope with bright field imaging or fluorescence imaging, while the quality of focusing was measured using standard flow cytometry coefficient of variation (cv) measurements with a photomultiplier tube for single channel fluorescence, and a silicon photodiode for scatter. The tightness and, thus, quality of the particle focusing stream was found to be velocity and concentration

dependent. We found the optimal flow concentrations and velocities through a series of recursive optimizations: using the particle concentration that produced the lowest cv in initial trials, we established the velocity dependence. We then used the flow velocity that yielded the lowest cv to determine the concentration dependence of the channels.

Conditions under which particles focus within straight channels are described by the particle Reynolds Number (Re_p), which is related to the channel Reynolds Number (Re_c) but rescaled to account for the proportion of the channel cross section occupied by a particle. This yields $Re_p = Re_c(a^2/D_h^2)$ with $Re_c = U_m D_h/\upsilon$, where a is the particle diameter, D_h is the channel hydraulic diameter ($D_h = 2wh/(w + h)$), $\nu = \mu/\rho$ is the kinematic viscosity of the fluid, μ is the fluid viscosity, ρ is the fluid density, and U_m is the maximum fluid velocity. This quantity captures the relative inertial to viscous forces at the particle scale due to the shear rate imposed by the channel geometry. A description of focusing in curved channels requires an additional quantity, the Dean Number ($De = Re(D_h/2r)^{1/2}$), which estimates the magnitude of the secondary fluid flow induced by channel curvature. Previously, it was shown that asymmetrically curved channels begin to focus particles to single streamlines between De = 1 and 10^1. Figures 1 and 2 show focusing of particles within both curved and straight channel sections and the resulting shift from two focal positions to a single equilibrium position. The quality of particle focusing within staged inertial focusing microchannels was also found to be velocity dependent (Figure 3). Particles translating at low flow rates (≲10 μl/min) were not focused in either the curved channel or the straight channel. At intermediate velocities (20–100 μl/min), two streamlines were clearly observed in the straight channel with both fluorescence and scatter measurements. Here, focusing in the curved channel gradually improves with velocity, but two equilibrium streamlines still occur in the straight channel, as would be generated by focusing a randomly distributed suspension of particles. The corresponding streak intensity profiles show that, as the velocity increases, the distribution of particles is increasingly skewed toward the side to which particles are focused by the curved channel. Additionally, two distinct transit times were also observed in the flow cytometry data. As particles flow through the focused laser spot, particles in multiple, discrete, and thus independent streamlines, encounter different cross sections of the laser spot resulting in distinct transit times, even at a constant flow velocity (Figure 2). Fluid velocities approaching or greater than 100 μL/min focus particles to a single streamline. A minimum cv of 6% was found when flowing the 10.2 μm Nile Red particles at 100 μL/min. The optimal cv achieved is very close to the commercial system that achieved a 5% cv.

The ability to focus particles within the microfluidic inertial focusing samples was found to be concentration dependent (Figure 4). As the concentration increased, the width of the focused particle streamline decreased resulting in a lower measured cv. For these experiments, the velocity was held constant at 100 μL/min, which corresponds to the best measured cv as shown in Figure 3. Varying the particle concentration again resulted, at best, in a minimum cv of 6% at a concentration of 0.1% w/v (or about 2×10^6 particles/mL). This dependence reflects a concentration dependence that we also report elsewhere, which is presumably a result of repulsive interparticle, hydrodynamic interactions that assist in reaching focusing equilibrium positions faster.[25] This dependence is currently being investigated more closely. Notably, this concentration dependence will impose a lower limit on sample concentration, which will need to be addressed through design optimizations for maximal effectiveness in low particle concentration applications such as environmental monitoring. Nonetheless, in rare event applications, such as detecting rare cells in a high concentration of cells, this focusing approach should be adaptable and effective.

After establishing optimal operating windows for 10.2 μm polystyrene microparticle concentration and velocity within the staged inertial focusing channels, the quality of the

focusing technology was tested with custom 10.2 μm rainbow beads. This test allowed for the resolution of the inertial focusing samples to be gauged relative to the commercial cytometer with a well characterized reference standard. As shown in Figure 5, under consistent sample and flow conditions (100 μL/min, 0.1% w/v 10.2 μm polystyrene microparticles), our inertial focusing system rivals the performance of a commercial flow cytometer. Comparing the cv's of the rainbow beads, the inertial focusing chip system resolves each peak slightly better than the Accuri C6 system. This is likely largely attributable to the precise focusing of the inertial focusing system, particularly the longitudinal spacing of particles, but could also be a composite effect of the entirely digital data acquisition system that we built, the tightness of particle focus, laser spot, or any number of other system parameters. Nonetheless, we can confidently conclude that particle alignment in the inertial focusing system is comparable to what a commercial system can accomplish.

One advantage that our inertial focusing approach possesses over current hydrodynamic focusing is the lack of particle coincidence at the point of analysis. This has been predicted previously and further verified in our data,[15] in that we observe only single populations of particles and no doublets. This means that the particles are effectively spaced longitudinally in the sample streamline, allowing for high linear particle velocities (>1 m/s) and cleaner data collection. At our tested volumetric flow rates of 100 μL/min through a 30 μm × 50 μm rectangular channel, the average linear velocity is 1.1 m/s. From this velocity, assuming 10 μm particle diameters and a 5 μm wide tightly focused laser spot, we would expect to achieve pulse widths of about 13.5 μs as the particles enter and exit the interrogating laser. Given that our visual data (Figure 2B) clearly shows that we can analyze particles spaced by just two diameters, this would imply that at linear velocities of 1.1 m/s it is possible to analyze a single particle every 40 μs. At this frequency, we would achieve an analysis rate of 25 000 particles per second. On the basis of the critical fluid velocity above which the Dean vortex limit is reached in the curved channel sections, the maximum linear velocity supported by the reported channel geometry is 2.16 m/s. In this system, it should, therefore, be possible to achieve particle analysis rates of about 49 000 per second without coincidences. The primary limitation to higher analysis rates is the upper limit on the linear velocity imposed by the geometry of the curved inertial focusing channel sections. Regardless, we have shown that an experimental cytometer with extremely high analysis rates can be constructed around a microfluidic channel that utilizes inertial particle focusing.

We have created a staged microfluidic inertial focusing device capable of passively focusing particles to a single streamline with periodic longitudinal particle spacing. We have also developed complementary optics and data acquisition systems to evaluate the performance of these microfluidic inertial focusing devices using varying intensity fluorescent, 10.2 μm polystyrene microparticles. The major advantages that inertial focusing possesses over hydrodynamic focusing are passivity to cells, the elimination of sheath fluid and particle coincidence, low cost, and portability. Inexpensive, portable, point of care flow cytometers have been the goal for many groups hoping to bring the technology into every day clinics and resource poor areas of the world such as AIDS progressions testing in Africa.[26–28] This motivation has resulted in many commercial cytometers aimed at second and third world areas such as the Guava EasyCyte,[29] the Partex CyFlow,[30] and the Accuri C6 flow cytometer. The majority of these systems have directed efforts at miniaturizing light sources, data acquisition systems, and detectors, while only one has changed the particle alignment scheme from a hydrodynamically focused sample stream approach. Because a flow cytometer is a compilation of many smaller subsystems, in addition to alternate particle positioning schemes, lower power light sources[22] and data acquisition systems[23] are being investigated to create a truly compact flow cytometer. Inertial focusing offers a new avenue for passive particle alignment and, under the correct circumstances, matches or surpasses

that which can be achieved using a commercial flow cytometer. In this work, we have established optimal conditions under which these samples effectively focused particles resulting in cv's on the order of, and in some cases slightly better than, a commercial flow cytometer without any doublet events, allowing for the potential for very high analysis rates.

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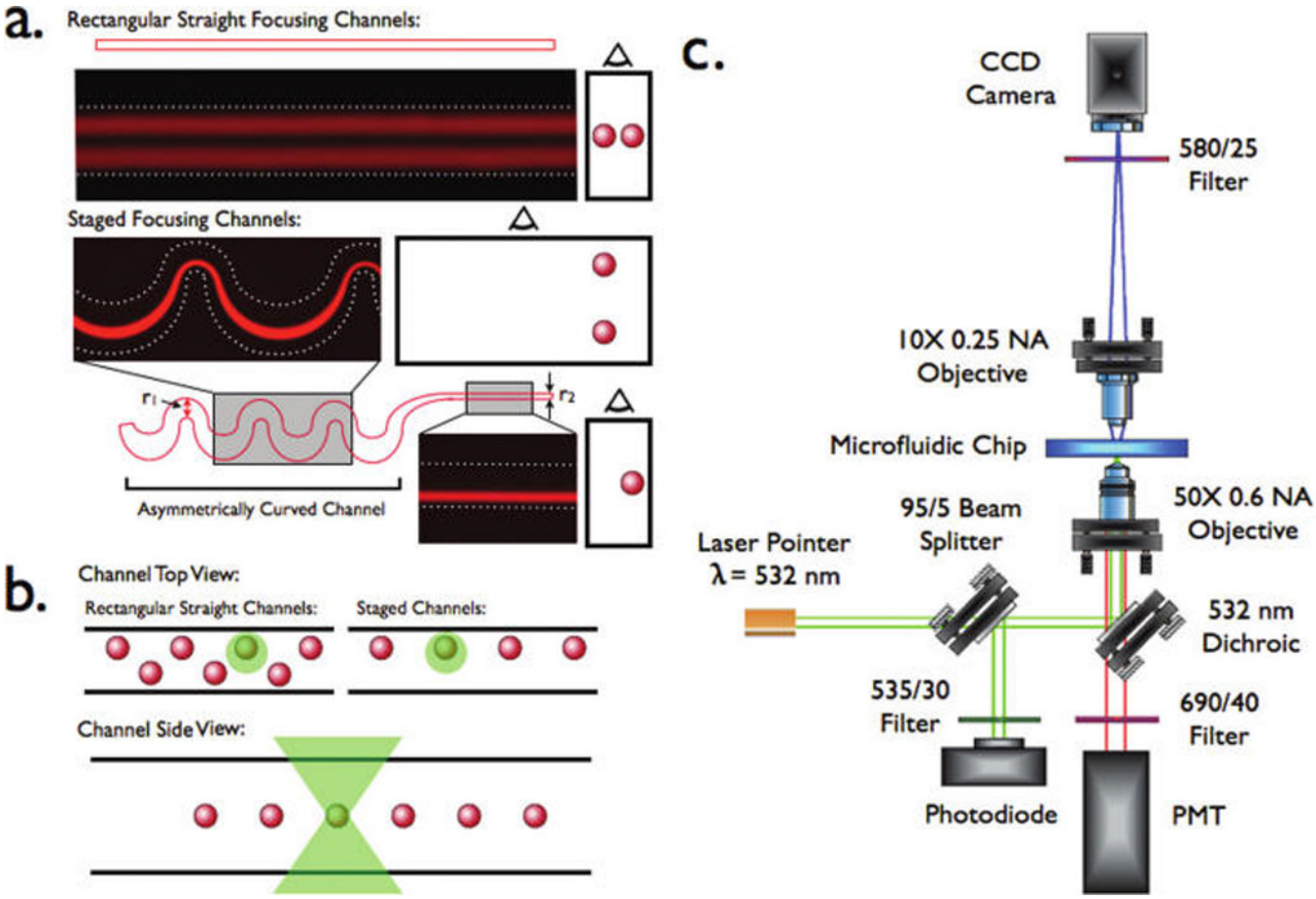

Figure 1.

(a) Overview of focusing behavior: inertial focusing in straight channels and curved channels with varying modes of symmetry are shown. All streak images are taken from the top of the channel, as indicated by the cross-sectional schematics that show particle equilibrium focusing positions. High aspect ratio channels focus to two lateral positions while asymmetrically curved channels focus to vertical streamlines. Combining these channels in series biases the entire particle population to one half of the channel where they are focused to a single vertical streamline within the straight channel. (b) Channel and particle orientation for various flow conditions (one and two equilibrium positions) relative to the focused laser beam in our cytometer apparatus. (c) Schematic of the flow cytometry setup. A green laser is focused upon the microchannel using a 50× 0.6 NA objective. Backscatter and fluorescence are collected using the same objective, passed through a 535/30 to a photodiode and a 690/40 photomultiplier tube, respectively.

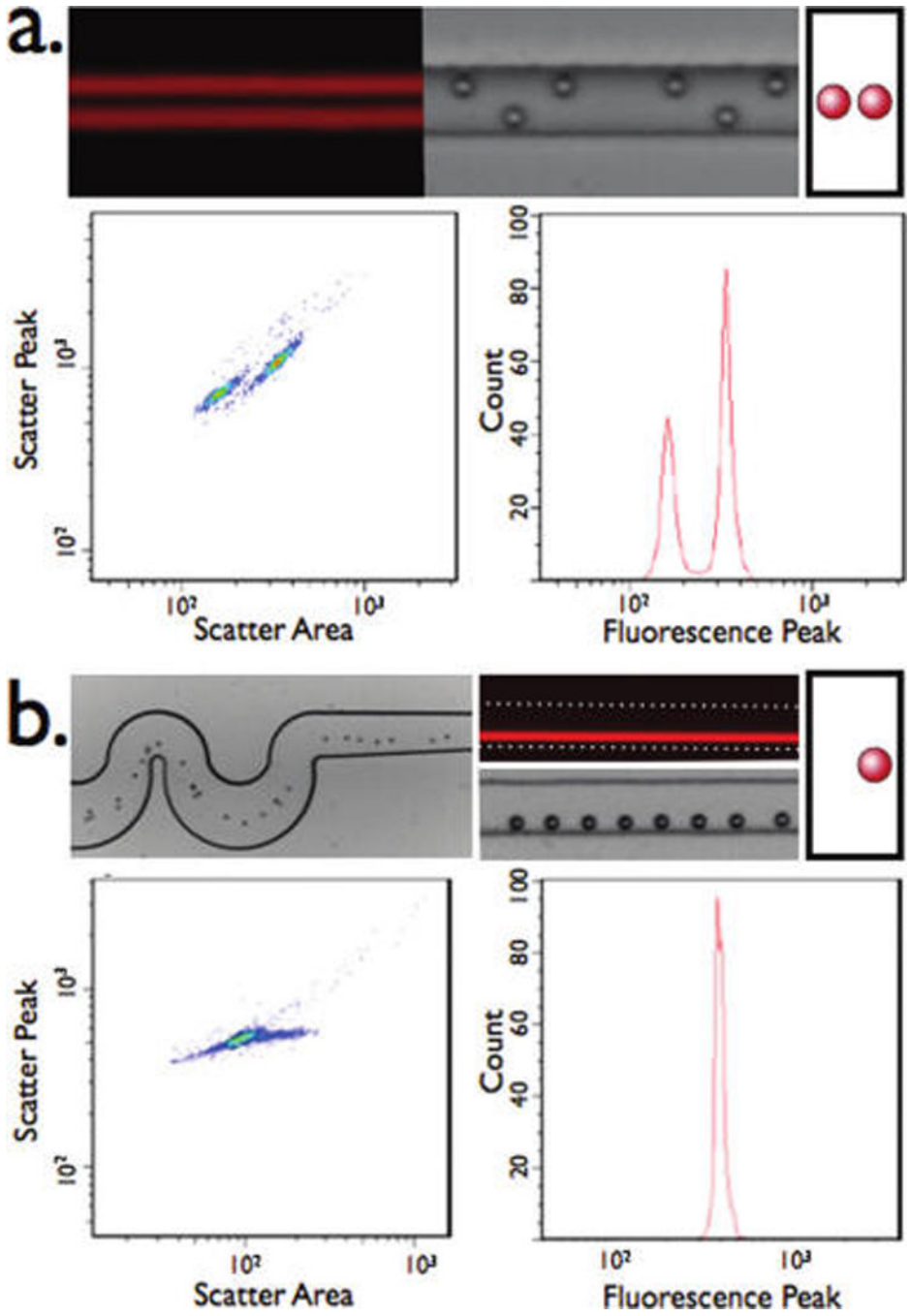

Figure 2.

Focusing behavior in staged inertial focusing channels as measured by flow cytometry under two distinctly different flow regimes. (a) At slower flow rates, no focusing occurs in the curved channel but does occur in the straight channel, and two discrete streamlines clearly emerge. (b) Once the flow rate is increased to the point at which focusing occurs in the curved channel, a single streamline with a minimum cv% of 6% is observed in the straight channel. The same sample measured with an Accuri C6 flow cytometer had a cv% of 5%. Shown in each panel are streak images from the straight channel section, high speed video frames from relevant channel positions, and corresponding measured particle peaks.

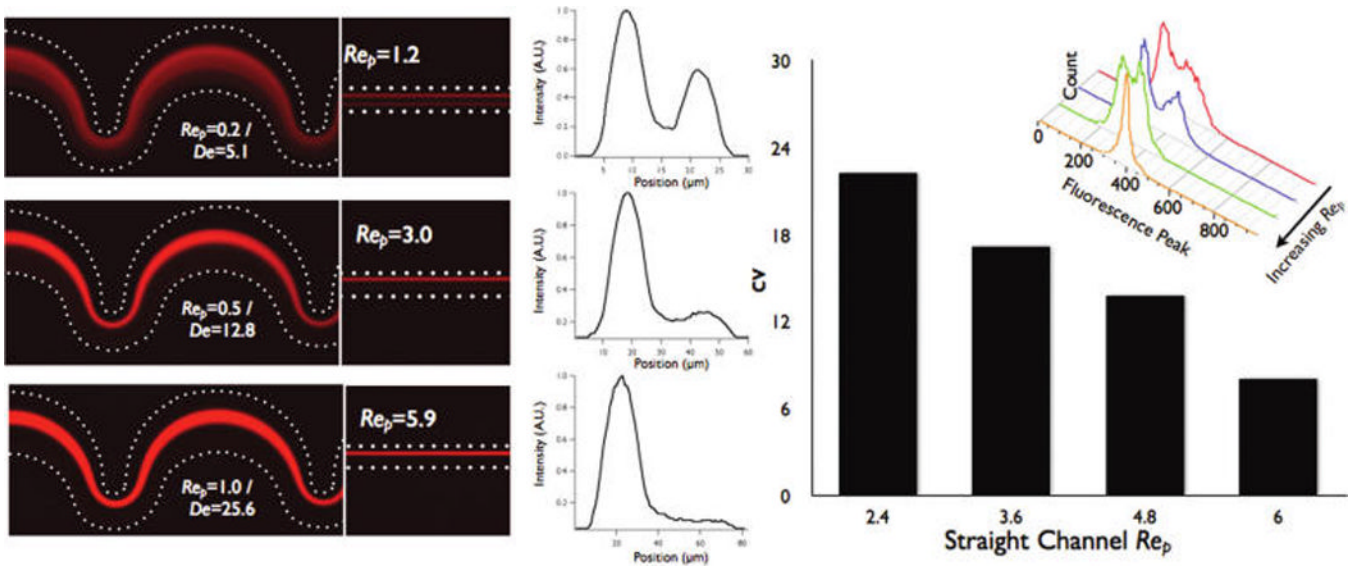

Figure 3.

Streamlines show focusing behavior in staged inertial focusing channel under increasing volumetric flow rate. As shown in the micrographs at left, when particles are unfocused in the curved channel, particles focus to two equilibrium positions in the straight channel; when focusing occurs in the curved channel, a single equilibrium focusing position results in the straight channel. Profiles of fluorescent intensity, measured across the straight channels at the same position at which flow cytometry measurements were performed, indicate that, as particle focusing improves in the curved channel at higher flow rate, the relative distribution of particles within the straight channel's primary focused streamline increases. The graph at right shows decreasing measured cv values at increasing Re_p, as measured at the end of the straight channel (inset: corresponding histogram for each flow rate). Re_p corresponds to volumetric flow rates as follows: Re_p = 2.4 (40 μL/min), Re_p = 3.6 (60 μL/min), Re_p = 4.8 (80 μL/min), and Re_p = 6.0 (100 μL/min).

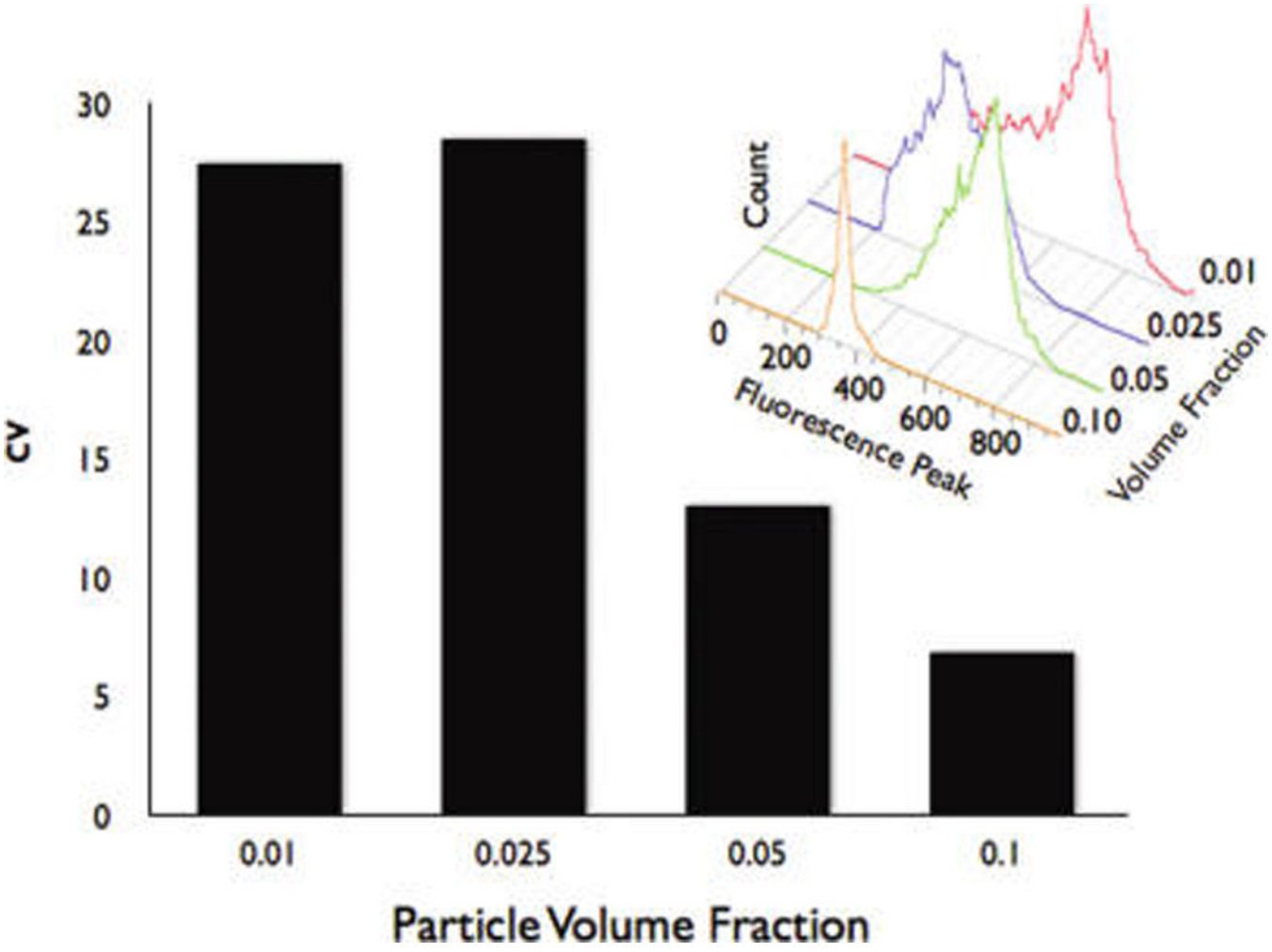

Figure 4.

Focusing performance (as measured with cv) dependence on particle concentration at Re_p = 6.0 (100 μL min^{-1} in the straight channel section). As the concentration is increased, a single streamline with a minimum cv of 6% results at a particle concentration of 0.1% (w/v).

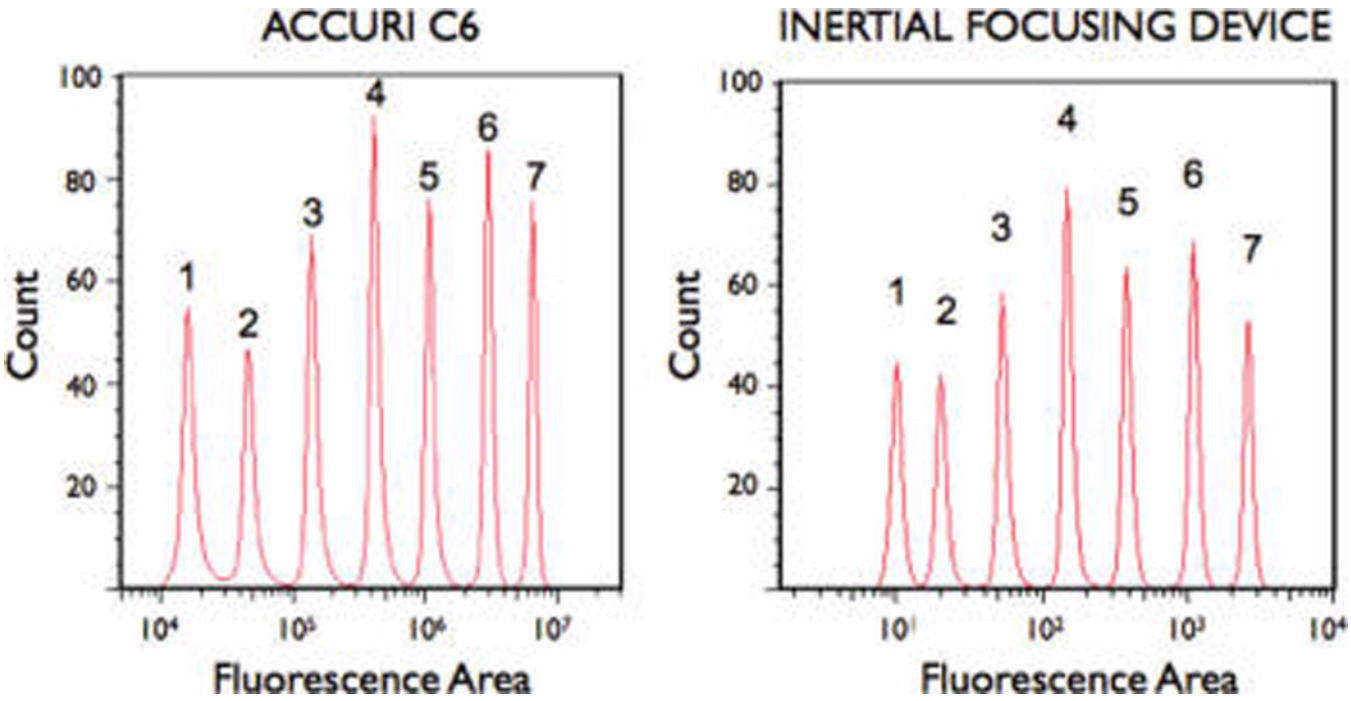

Figure 5.

7-peak, 10.2 μm rainbow beads (0.1%w/v) ran through the inertial focusing microfluidic sample at Re_p = 6.0 (100 μL/min) and the Accuri C6 flow cytometer. A slight improvement in cv is shown in the microfluidic system over the Accuri C6. Peaks 1, 2, 3, 4, 5, 6, and 7 in the Accuri are 11.00%, 9.63%, 9.38%, 8.79%, 8.24%, 6.29%, and 5.41% respectively, while peak 1, 2, 3, 4, 5, 6, and 7 in the inertial focusing sample are 8.7%, 8.34%, 8.62%, 7.94%, 7.18%, 6.23%, and 5.58%.