Featuring work from the group of Prof. Ian Papautsky at University of Cincinnati and collaborators at VTT Technical Research Centre of Finland, members of the NSF Center for Advanced Design and Manufacturing of Integrated Microfluidics (CADMIM).

A disposable, roll-to-roll hot-embossed inertial microfluidic device for size-based sorting of microbeads and cells

An inertial microfluidic device was developed for size-based sorting of cells and microparticles. The simplicity of the design allows high-throughput roll-to-roll hot embossing, leading to disposable devices for real-world biomedical and clinical applications.

As featured in:
A disposable, roll-to-roll hot-embossed inertial microfluidic device for size-based sorting of microbeads and cells

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Inertial microfluidics has been a highly active area of research in recent years for high-throughput focusing and sorting of synthetic and biological microparticles. However, existing inertial microfluidic devices always rely on microchannels with high-aspect-ratio geometries (channel width $w < $ channel height $h$) and small cross-sections ($w \times h < 50 \times 100 \mu m^2$). Such deep and small structures increase fabrication difficulty and can limit manufacturing by large-scale and high-throughput production approaches such as roll-to-roll (R2R) hot embossing. In this work, we present a novel inertial microfluidic device using only a simple and low-aspect-ratio (LAR) straight microchannel ($w > h$) to achieve size-based sorting of microparticles and cells. The simple LAR geometry of the device enables successful high-throughput fabrication using R2R hot embossing. With optimized flow conditions and channel dimensions, we demonstrate continuous sorting of a mixture of 15 $\mu m$ and 10 $\mu m$ diameter microbeads with >97% sorting efficiency using the low-cost and disposable R2R chip. We further demonstrate size-based sorting of bovine white blood cells, demonstrating the ability to process real cellular samples in our R2R chip. We envision that this R2R hot-embossed inertial microfluidic chip will serve as a powerful yet low-cost and disposable tool for size-based sorting of synthetic microparticles in industrial applications or cellular samples in cell biology research and clinical diagnostics.

Introduction

Inertial focusing in microchannels has attracted much interest in recent years for focusing and sorting of synthetic and biological microparticles. This technique uses inertia of fluid surrounding microparticles to drive them across flow streamlines into equilibrium positions in microfluidic channels. By manipulating the microchannel geometry, focused cells can be entrained into different cross-sectional positions depending on their size and deformability, and sorted further downstream into different outlets. A number of promising applications have been demonstrated including shearless flow cytometry, label-free cell separation, rare cell enrichment and volume reduction with orders of magnitude higher throughput and much simpler external instrumentation than active microfluidic systems.

To make inertial microfluidic devices commercially viable, the ability to manufacture disposable devices with low cost and high throughput is critical. Roll-to-roll (R2R) hot embossing is a high-volume thermoforming process for large-scale production of microfluidic devices. In the R2R process, a thermoplastic foil is continuously fed through a pressurized area between a heated embossing cylinder and a blank counter cylinder to generate microscale geometries with high throughput. Due to the continuous web feed and short cycle times compared to e.g. injection moulding, R2R hot embossing is a fast and cost-efficient production method. Compared to soft lithography which has been widely used in microfluidic prototyping, R2R hot embossing offers high throughput, high automation and decreased device cost. Although it has demonstrated promising advantages, R2R hot embossing has process-inherent limitations regarding feature width and depth due to the use of thin film substrates which are fed into the embosser unit as a sheet of roll. Due to the short contact time between an embossing roller and a substrate material, the entire micromoulding process (i.e. substrate heat-up, tool imprint, and de-moulding) takes place in a relatively short window of time, typically <1 s. Both the low substrate thickness (typically <500 $\mu m$ films are used in R2R hot embossing) and small time window of material flow set limits to the maximum depths that can be imprinted. As a general rule, tool structures with an aspect ratio (AR) of >1 ($w < h$) are difficult to create using R2R hot embossing.
processes. However, existing inertial microfluidic sorting devices always rely on microchannels with high-aspect-ratio geometries \((w < h)\) and small cross-sections \((w \times h < 50 \times 100 \mu m^2)\).\(^6,8,10,15\) As discussed above, such deep and narrow structures are challenging to fabricate using R2R hot embossing.

Herein, we present a novel inertial microfluidic device using only a simple low AR (LAR) \((w > h)\) straight microchannel for size-based sorting of microparticles.\(^14\) Our design consists of a straight microchannel for inertial focusing of microparticles which symmetrically splits into two straight microchannels downstream to reposition and displace cells based on their sizes. The device has been optimized to comply with the fabrication requirements of the R2R process to enable successful replication of the device in a PMMA film using R2R hot embossing with low cost and high throughput. With optimized flow conditions and channel dimensions, we demonstrate continuous sorting of a mixture of 15 μm and 10 μm diameter microbeads with >97% efficiency using the R2R-manufactured device. We further demonstrate size-based sorting of bovine white blood cells, indicating the capability of processing real cellular samples using this disposable chip. We envision that this R2R hot-embossed inertial microfluidic sorter will serve as a powerful yet low-cost and dispos-

**Results and discussion**

**Inertial focusing and sorting in our low-aspect-ratio microchannel**

Our microfluidic device consists of two segments of LAR straight microchannels. It combines inertial migration and flow bifurcation to achieve size-based sorting of microbeads or cells (Fig. 1a). In straight microchannels, inertial migration is caused by the balance of lift forces arising from the curvature of the velocity profile known as the shear-induced lift \(F_s\) and the interaction between microparticles and the channel wall known as the wall-induced lift \(F_w\).\(^2,3\) In a straight microchannel with a rectangular cross-section, the migration process can be described into two stages.\(^3\) As Fig. 1b shows, microparticles first equilibrate into bands along the top and bottom walls where these two dominant lift forces balance each other. Once this initial equilibrium is reached, the lateral motion of microparticles is dominated by the rotation-induced lift force \(F_\Omega\) which drives them towards

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Fig. 1  Schematic of the device principle. (a) Top-view schematic of the device principle. The device uses a low-aspect-ratio microchannel to focus microparticles into the centers of the top and bottom walls after two-stage inertial migration (1, 2). The flow is then symmetrically bifurcated into the two LAR channels downstream (3). Focused particles are repositioned into either of the channels close to the corners. Further downstream, the lateral positions of microparticle streams are differentiated due to size-based inertial migration (4) and sorted at the outlet (5). The grey dashed line represents the central streamline in the upstream channel which is bifurcated into downstream channels near the side walls. The red dashed lines represent flow splitting positions at the outlets. (b) Cross-sectional schematics of two-stage inertial migration in the upstream LAR channel and size-based inertial migration in the downstream LAR channel. The red dashed line indicates the flow splitting position. Due to the symmetry of the downstream channels, only one of the channel cross-sections is illustrated.
the centers of the top and bottom walls. As a result, particles equilibrate at the center of each sidewall.

Based on inertial migration, we first designed a LAR microchannel upstream to focus microparticles at the centers of the top and bottom walls (Fig. 1a). The minimum downstream length $L$ necessary for focusing microparticles with diameter $a$ in a channel with cross-sectional dimensions $w \times h$ is given by:

$$L = \frac{3\pi \mu D_{h}^{2}}{4 \rho U_{f} a^{3}} \left( \frac{h}{C_{L}^{+}} + \frac{w}{C_{L}^{-}} \right), h < w$$

(1)

where $\mu$ is the fluid viscosity, $\rho$ is the fluid density, $U_{f}$ is the average flow velocity, $D_{h} = 2wh(w + h)$ is the hydraulic diameter, $C_{L}^{-}$ is the negative lift coefficient related to the first stage of migration and $C_{L}^{+}$ is the positive lift coefficient related to the second stage of migration.\(^3\) Values for $C_{L}^{-}$ and $C_{L}^{+}$ can be obtained from our earlier work.\(^3\)

To further separate these pre-focused microparticles based on size, we designed two LAR microchannels with identical dimensions to symmetrically bifurcate the flow. Following the central streamline in the upstream microchannel, the center-focused microparticles are re-positioned to the inner corner in either of the downstream microchannels. As a consequence, the microparticles experience the wall-lift force $F_{w}$ and migrate away from the side walls. Further away from the side walls, the rotation-lift force $F_{\Omega}$ takes over and drives the microparticles towards the centers of the top and bottom walls. Since the magnitude of the inertial lift forces scales with the size of the microparticles as $F_{w} \sim a^{6}$ and $F_{\Omega} \sim a^{2}$,\(^1,3\) the larger microparticles experience larger forces, and thus migrate faster toward these equilibrium positions. This size-dependent migration leads to the differentiation of the lateral positions of the larger and smaller particles.

To demonstrate the device concept, we first fabricated the device in PDMS using soft lithography and showed sorting of a mixture of $18 \mu m$ and $15 \mu m$ diameter microbeads. Guided by eqn (1), the device has a $25 \text{ mm}$ long upstream microchannel with a LAR cross-section of $w \times h = 75 \mu m \times 50 \mu m$ and two $10 \text{ mm}$ long downstream microchannels with the same cross-sectional dimensions. The mixture of microparticles was introduced into the device at $Re = 40$. As the fluorescence images and bright-field images show (Fig. 2a), the FITC-labeled $18 \mu m$ and TRITC-labeled $15 \mu m$ diameter microbeads fully focus at the center of the top and bottom walls in the upstream channel shown as a single overlapped fluorescent streak. After the flow bifurcation, the green fluorescent streaks are gradually separated from the red streaks and eventually exit from different outlets, fulfilling size-based sorting. Line scans of the two fluorescent streaks at downstream positions from $0 \text{ mm}$ to $35 \text{ mm}$ indicate that the lateral positions of the $18 \mu m$ and $15 \mu m$ diameter particles first overlapped (inseparable), then separated into a $6 \mu m$ gap and sorted at the outlets (Fig. 2b and c). Our proof-of-concept experiment indicates that the device consisting of only a simple LAR straight microchannel can successfully sort microparticles based on size.

Characterization of the R2R inertial microfluidic sorter

Roll-to-roll hot embossing is a promising approach for mid-to large-scale manufacturing (Fig. 3a and b).\(^2,8\) By restricting the channel design to large-width, low-aspect-ratio microchannels ($150 \mu m \times 50 \mu m$, $w \times h$), we are able to manufacture the device using the R2R process on a PMMA foil (Fig. 3d). We note, however, that due to the increase in the cross-sectional dimensions, the length of the R2R channel is increased to $50 \text{ mm}$ to ensure focusing of microparticles or cells with diameters $>10 \mu m$.

Compared to the soft lithography device which has a rectangular cross-section, the R2R hot-embossed chip has a trapezoidal cross-section (Fig. 3c). This slanted side wall angle of the microchannel features on the embossing mold originates from the tooling process and facilitates mold release during de-moulding, thus causing differences between the channel top and bottom widths. The average widths of the top and
Fig. 3  Roll-to-roll hot embossing of inertial microfluidic chip. (a) The roll-to-roll pilot printing line used in this study. An embossing module with a oil-heated embossing cylinder and a counter pressure cylinder is circulated in the image. (b) Schematic of the principle of the R2R hot embossing process. A thermoplastic foil is fed by unwinders between the heated counter cylinder and embossing cylinder carrying the microfluidic patterns. By controlling the web speed, nip pressure and temperature, microfluidic channels are embossed to the polymer. (c) A SEM image of the solvent-bonded microfluidic chip cross-section. (d) A photograph of the R2R hot-embossed inertial microfluidic chip on the PMMA foil.

Fig. 4  Inertial focusing in the R2R chip. (a) Fluorescence images at different downstream positions from inlet to 50 mm showing gradual focusing of 15 μm diameter microparticles. The white dashed lines indicate the outlines of the channel top wall. The blue dashed lines indicate the outlines of the channel bottom wall. The scale bar is 100 μm. (b) Fluorescence intensity profiles at the corresponding downstream positions. (c) Full width of half maximum of the intensity peaks at different downstream positions. The inset images illustrate the cross-sectional view of focusing. (d) Comparison of focusing of 15 μm diameter microparticles in the PDMS device and in the R2R device. The scale bars are 75 μm. (e) Fluorescence intensity profiles of focused microparticle streaks in the PDMS and R2R devices indicating comparable focusing quality. The inset image shows the FWHMs of both intensity peaks to be ~15 μm.
bottom channel walls were \( w_t = 220 \pm 30 \, \mu m \) and \( w_b = 90 \pm 15 \, \mu m \), and the average height of the channel was \( h = 40 \pm 5 \, \mu m \). Although the R2R chip has minor geometric imperfections, it has a surface roughness of only 20 nm as well as good optical quality which allows clear observation of particle focusing and sorting via bright-field and fluorescence imaging. In the following sections, we will investigate the inertial focusing and sorting in the R2R chips and will show focusing and sorting of microparticles with high performance despite the trapezoidal cross-section and the dimensional variations.

### Inertial focusing in the R2R chip

We first investigated the inertial focusing in the R2R chip to evaluate the impact of non-vertical side walls. We pumped a solution of TRITC-labeled 15 μm diameter microbeads into the R2R device at \( Q = 200 \, \mu L \, \text{min}^{-1} \) (Re = 30) to investigate inertial focusing in the upstream microchannel. As the top-view fluorescence images in Fig. 4a and the corresponding fluorescence intensity profiles (Fig. 4b) show, the 15 μm diameter microbeads disperse in the channel at 0 mm shown as a wide fluorescence band. Further downstream after 10 mm, they focus at the center into a narrow fluorescent streak indicating tight focusing. The full width at half maximum (FWHM) of the fluorescence intensity profile decreases from \( \sim 80 \, \mu m \) to \( \sim 16 \, \mu m \) at the 10 mm downstream position and remains at \( \sim 15 \, \mu m \) from 20 mm to 50 mm downstream (Fig. 4c). The FWHM matches the diameter of the microbeads indicating the complete focusing of the microparticles after 20 mm downstream. As Fig. 4d and e show, the R2R device can focus microparticles into tight streams with the same FWHM indicating comparable focusing performance with the PDMS device. One may notice that the microbeads were initially focused as a band with FWHM = \( \sim 80 \, \mu m \) at 0 mm instead of spreading across the entire channel. This might be due to the limited space and larger wall-lift forces in the top \( \sim 45^\circ \) corners.

We investigated the inertial focusing of different sized microbeads to determine the feasible size range for the R2R device. To mimic the size range of cells, we prepared microbead suspensions with diameters of 25 μm, 15 μm, 10 μm and 7 μm and individually pumped each sample into the R2R device at \( Q = 200 \, \mu L \, \text{min}^{-1} \) (Re = 30). Both fluorescence and bright-field images were taken at 50 mm downstream. As Fig. 5a shows, microbeads with the diameter from 10 μm to 25 μm are focused into tight streaks indicating high quality of focusing. However, the 7 μm microbeads are focused as a band in the upstream LAR channel. We measured the FWHMs of the fluorescence intensity peaks and calculated the ratio of the FWHM and the corresponding particle diameter \( a \) to quantitatively evaluate the quality of focusing. The ratios for the 15 μm and 25 μm diameter microbeads are both \( \sim 1 \) indicating complete inertial focusing. For the 10 μm diameter microbeads, the ratio increases to \( \sim 1.2 \) indicating a slight decrease in focusing quality. It further increases to \( \sim 3 \) for the 7 μm diameter microbeads implying incomplete focusing. This is because the inertial lift forces decrease with the size of the particle, leading to much slower inertial migration towards the equilibrium positions. Further improvement of the focusing quality may be achieved using a longer microchannel or a microchannel with a smaller cross-section. Our experimental results suggest that even with a trapezoidal cross-section, the R2R chip can precisely focus microbeads within a feasible size range.

#### Size-based inertial sorting in the R2R chip

To optimize the size-based sorting in the R2R chip, we investigated the inertial migration of microbeads with diameters from 7 μm to 25 μm at flow rates of \( Q = 100–300 \, \mu L \, \text{min}^{-1} \) (Re = 15–45) in the R2R downstream segment after flow bifurcation (Fig. 6a). We measured the separation distance \( d \) between the lateral positions of the microbeads and the centerline of the microchannel (y = 0 μm) at downstream positions from 1 mm to 25 mm. As Fig. 6a and b show, as \( Q \) increases from 100 (Re = 15) to 300 μL min⁻¹ (Re = 45), microbeads migrate faster towards the equilibrium positions. For example, at \( Q = 100 \, \mu L \, \text{min}^{-1} \) (Re = 15), the 15 μm diameter microbeads are 28 μm away from the centerline at 10 mm downstream. As \( Q \) increases to 300 μL min⁻¹ (Re = 45), they migrate faster because the lateral migration velocity \( U_L \) scales with the average flow velocity \( U_T \) as \( U_L \sim U_T^2 \). Thus, they become closer to the centerline with \( d = 10 \, \mu m \) at the same 10 mm downstream.

We compared the lateral positions of different sized microbeads at the indicated range of flow rates to serve as the design guideline for downstream segments of the R2R device (Fig. 6c). By designing the appropriate downstream length and flow conditions, different sized microbeads can be separated into sufficient lateral space leading to efficient separation at the outlets. For example, using \( Q = 100 \, \mu L \)
To sort the 15 μm and 10 μm diameter microbeads, one can design 25 mm long downstream segments with $Q = 100 \mu L \text{ min}^{-1}$ ($Re = 15$). Note that although 10 μm and 7 μm diameter microbeads defocus into a band at the beginning of the downstream microchannels, their lateral positions can still be differentiated from those of the 25 μm and 15 μm diameter particles with appropriate device dimensions and flow conditions. The results indicate that the R2R device can achieve robust size-based differentiation of lateral positions of microparticles despite the low-aspect-ratio trapezoidal cross-section.

The robustness of the size-based sorting stems from the relatively simple components of the forces and flow patterns in the straight microchannel geometry. Inertial microfluidic devices with curved or spiral channels can also achieve size-based sorting within large cross-sections, thus they can
with overlapping lateral positions. As the microbeads travel downstream along the flow, 15 μm diameter microbeads migrate faster than the 10 μm diameter ones in the lateral direction leading to clear separation of particle streaks. Further downstream, the 15 μm diameter microbeads are sorted into outlet 1 (O1), while the 10 μm diameter microbeads are sorted into O2–O4 (Fig. 7b). Note that the 15 μm diameter microbeads only flow to the top downstream channel instead of symmetrically flowing to both channels. This is because the flow splitting is slightly asymmetric due to the fabrication inaccuracy. Thus, the tightly focused 15 μm diameter microbeads only flowed to one of the channels. We measured the purity of these microbeads at the inlet and outlets O1–O4 (Fig. 7c). The results indicate that the purity of the 15 μm diameter microbeads increases from 16% to 86% in O1 after sorting, and the purity of the 10 μm diameter microbeads increases from 84% to ~100% in O2–O4 after sorting. The separation efficiencies of the 15 μm and 10 μm microbeads were measured to be $\eta = 99\%$ and 97% correspondingly (Fig. 7d). These results suggest that the R2R device can be used to achieve continuous size-based sorting of microbeads with high efficiency and purity, and thus could potentially be used to sort cell samples with similar size ranges.

To demonstrate the potential of size-based sorting of cellular samples using the R2R device, we used a bovine WBC sample (Fig. 8). Compared with human blood, bovine blood has similar WBC subtypes with similar size distributions and concentrations.30 Furthermore, bovine blood samples are inexpensive and do not carry human pathogens, and thus are

### Continuous size-based sorting of beads and cells using the R2R chip

Following the study of inertial focusing and migration in the R2R chip, we designed a device with the 50 mm upstream segment and 25 mm downstream segment to sort 15 μm diameter microbeads from 10 μm diameter microbeads. A total volume of 3 mL sample solution was pumped through the device at $Q = 100 \, \mu \text{L min}^{-1}$ (Re = 15). As shown in Fig. 7a and b, in the upstream microchannel, both microbeads are focused along the centerline. At the channel bifurcation, microbeads are relocated to the corner of the downstream microchannel.

### Fig. 7 Size-based sorting of microbeads in the R2R device.

(a) Bright-field images showing inertial focusing and size-based inertial migration of 15 μm and 10 μm diameter microbeads in a R2R device with a total length of 75 mm at a flow rate of $Q = 100 \, \mu \text{L min}^{-1}$ (Re = 15). The red arrows indicate 15 μm diameter microbeads. The blue arrows indicate 10 μm diameter microbeads. The scale bar is 200 μm. (b) Bright-field images showing successful sorting at the outlets (see Fig. 3d). (c) Purity of 15 μm and 10 μm diameter microbeads at the inlet and four outlets ($n = 3$). (d) Separation efficiency of 15 μm diameter microbeads into outlet 1 and 10 μm diameter microbeads into outlets O2–O4.

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more convenient to use for device characterization. Before introducing into the R2R device, bovine RBCs were first lysed using lysing buffer (Fig. 8b). The remaining cells were resuspended in PBS and pumped through the R2R device at $Q = 100 \mu$L min$^{-1}$ (Re $= 15$). As Fig. 8a shows, the cells were first focused in the upstream channel and sorted in the outlets downstream. The representative images from the outlet samples (Fig. 8c and d) indicate that larger cells are more likely to be extracted from O1 & O3. The size distributions of cells from the inlet and outlets (Fig. 8f) further suggest the sorting cutoff to be $\sim 13 \mu$m. One should note that since the WBCs have a continuous size distribution, the sorting result is not as ideal as that for the microbead sample with distinct size differences. Further enhancement of the sorting performance may be achieved by improving the geometric consistency of the R2R device. These results suggest that the R2R device can sort WBCs based on their sizes. Ultimately, the device is generalizable beyond sorting of blood samples and can be applied to sorting of any cellular samples within this size range.

**Conclusions**

In summary, we present an inertial microfluidic sorter that is compatible with the R2R hot embossing process for size-based sorting of microbeads and cells. Our design only consists of a large low-aspect-ratio straight microchannel. Due to the geometric simplicity of this design, we successfully manufactured the device using R2R hot embossing and demonstrated robust size-based sorting of microbeads with high efficiency and purity. We further demonstrated sorting of cellular samples indicating the feasibility of the chip for cellular sample preparation. With the high-throughput R2R manufacture and robustness of the size-based sorting, we envision that this low-cost and disposable R2R inertial microfluidic sorter can move one step further to bring microfluidic sorting techniques from lab prototypes to real-world biomedical and clinical applications.

**Methods**

**Roll-to-roll hot embossing**

The low-cost microchannels were fabricated by roll-to-roll hot embossing (Fig. 3b) on a thermoplastic polymethyl methacrylate film ($375 \mu$m thick PMMA, Plexiglas 99524, Evonik, Germany) as described in ref. 28. In short, the large area embossing shim ($200 \text{mm} \times 400 \text{mm}$) was manufactured via wet etching, welded into a cylindrical sleeve, and mounted onto the oil-heated hot embossing cylinder of a custom built roll-to-roll pilot printing machine (Fig. 3a, Technical Research Centre of Finland Ltd., Oulu, Finland). Embossing parameters, $105 ^\circ \text{C}$ for the embossing cylinder surface, $85 ^\circ \text{C}$ for the counter pressure cylinder surface, 20 bar nip pressure and $0.5 \text{ m min}^{-1}$ web speed, were optimized for the inertial microfluidics design. For lidding, a $175 \mu$m thick PMMA foil with drilled $2 \text{ mm}$ diameter fluid access ports was solvent-bonded to the channel layer as described in ref. 28.

**Preparation of microbead suspensions**

To investigate inertial focusing in the R2R device, we diluted TRITC-labeled microbeads with a diameter of $15 \mu$m (Invitrogen, Inc.) in deionized water to form a suspension with a concentration of $4 \times 10^4$ beads per mL. To investigate the focusing of different sized microbeads, suspensions of microbeads with diameters of $7 \mu$m, $10 \mu$m, $15 \mu$m and $25 \mu$m (Bangs Laboratories, Inc.) were also prepared at a
concentration of 4 × 10⁴ beads per mL. We added Tween-20 at 0.1% v/v (Fisher Scientific, Inc.) to the particle suspensions as a stabilizer to avoid clogging issues. For demonstration of sorting of 15 μm and 10 μm diameter microbeads, we prepared the mixture of 15 μm and 10 μm diameter microbeads with concentrations of 4 × 10⁴ beads per mL and 2 × 10⁵ beads per mL correspondingly.

Preparation and imaging of white blood cells
1 mL of bovine whole blood with Na-heparin anti-coagulant (Lampire Biological Laboratories, Inc.) was lysed with RBC lysing buffer (eBioscience, Inc.) and re-suspended in 3 mL of PBS following the protocol provided by eBioscience, Inc. After sorting, the outlet cellular samples were collected and re-suspended in bovine plasma. A smear was created from each sample using the same method as that for the standard smear of whole blood. The slides were then stained with Giemsa-Wright solution (Sigma Aldrich) and readied for imaging analysis.

Device operation and microscopic imaging
We first loaded a syringe with particle suspension and connected it to the device by using 1/16" PEEK tubing (IDEX) with appropriate fittings (IDEX). We pumped the particle solution into devices with designed flow rates using a syringe pump (Legato 180, KD Scientific). To visualize the trajectory of particles in bright field, we used an inverted epifluorescence microscope (IX71, Olympus, Inc.) equipped with a 12 bit high-speed CCD camera (Retiga EXi, QImaging). We set the exposure time to a minimum value (10 μs) and sequentially took 100–200 images with a minimum time interval. By stacking images in ImageJ, we established a complete view of the particle motion. To capture fluorescence images of particle focusing, we used the same microscope while setting the exposure time to 100–300 ms depending on the brightness of the fluorescent particles and sequentially taking 20–50 images. The images were then stacked and pseudo-colored in ImageJ.

Data analysis
We measured the line scans of the fluorescence profiles using ImageJ. To calculate the purity before and after sorting, we first measured the concentration in the inlet and outlets. We injected inlet or outlet samples into a hemocytometer (Hauser Scientific) to form a monolayer of particles and then took bright-field images and used Image Pro Plus to automatically count and size the particles. The concentration and purity were then calculated from the counts. To calculate the separation efficiency, we combined the concentration and corresponding sample volume to estimate the count of each sized particles from each outlet and normalized the counts as separation efficiency.

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