Three-dimensional hydrodynamic focusing with a single sheath flow in a single-layer microfluidic device†

Myung Gwon Lee,‡ Sungyoung Choi‡ and Je-Kyun Park*

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We report a contraction–expansion array (CEA) microchannel that allows three-dimensional hydrodynamic focusing with a single sheath flow in a single-layer device. The CEA microchannel exploits centrifugal forces acting on fluids travelling along the contraction and expansion regions of the microchannel. Around an entrance of the contraction region, the centrifugal forces induce a secondary flow field where two counter-rotating vortices enable to envelop a sample flow with a sheath flow in three dimensions. We herein describe an underlying principle and a design of the CEA microchannel and demonstrate complete sheathing of a sample fluid (water and human red blood cells) in three dimensions. The focusing characteristics of the CEA microchannel are investigated in terms of the number of the rectangular structures, flow rate, and flow ratio between sample and sheath flows. This microfluidic channel for three-dimensional hydrodynamic focusing is easy to fabricate in a single-layer fabrication process and simple to operate with a single sheath flow.

1. Introduction
Flow cytometry is an essential resource in a number of fields, including cell biology, molecular biology, and immunology. It offers high-throughput quantification of single particles (even single molecules) suspended in a fluid stream, continuously counting and sorting the particles (typically in a rate of several thousand particles every second). However, flow cytometry has been developed in large, complex, and expensive instruments that require a high volume of sample and a trained operator. There is much interest in the development of flow cytometry to reduce operational cost, complexity of the system, and consumption of sample volume by utilizing microfluidic and microfabrication techniques for a wide variety of microfluidic applications.

Replacing conventional flow chambers in flow cytometers with microfabricated devices is one of the most concerning issues in miniaturizing cytometers, because focusing the particles in the flow chamber is necessary for them to be detected by a tightly focused laser in a small region. Conventional methods for focusing single particles are compression of an inner fluid stream containing suspended particles with outer sheath streams: horizontal focusing (two-dimensional focusing). The major drawbacks of two-dimensional particle focusing are a broad detection volume in the vertical direction, and subsequent impairment of illumination and signal collection efficiency. If particles are allowed to flow through the broad detection volume, any measures to obtain quantitative information will be confounded by variations in their flow speed of a parabolic velocity profile and illumination. To overcome the above limitations, many efforts have been addressed for the realization of three-dimensional focusing methods in microfabricated devices. Sundararajan et al. surrounded a sample flow with a cylindrical sheath flow in both vertical and horizontal dimensions, but it needed a chamber comprised of five channel layers for sample and sheath flows and as many as six sheath inlets. Simonnet and Groisman used a main channel intersected with side channels to focus a sample flow with three sheath inlets. Chang et al. slightly modified the microchannels from Simonnet and Groisman in a two-layer device with three sheath inlets and focused sample flow in three dimensions. Yang et al. fabricated quite complex structures by tilted exposures by twice using three masks and focused the sample flow with two sheath flows. Kennedy et al. first focused the sample flow on top of the main channel and secondly focused it toward the bottom of the main channel using four sheath flows intersected with the main channel. Howell Jr et al. wrapped a sample flow with only one sheath flow in a chevron groove design but the design needed to be fabricated with two layers. Mao et al. achieved vertical focusing by Dean flow at a curved channel and horizontal focusing by two sheath flows in single-level microchannel but the device required a total of three sheath inlets. Most of the three-dimensional focusing methods used multi-layer or multi-level devices for injecting sheath flows in vertical and horizontal directions, thereby causing complexity of fabrication and, substantially, many needs for sheath inlets.

Accordingly, three-dimensional focusing methods by single-layer microchannel and fewer sheath inlets are highly desirable for simplicity of fabrication and operation. Here, we describe a single-layer microchannel with contraction–expansion array (CEA) structures needing just one sheath inlet for three-dimensional hydrodynamic focusing. This advance significantly reduces the number of sheath flow required, and complexity of

‡ These authors contributed equally to this work.

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fabrication due to simple one-step lithography. In this paper, we injected one sheath flow to wrap a sample flow by a secondary flow, Dean flow induced by the CEA structures. The sample flow wrapped by the injected sheath flow appears to be focused in horizontal and vertical directions. In order to observe the focused streams, we introduced fluorescein isothiocyanate (FITC) for a sheath flow and deionized water (DIW) for a sample flow in the CEA microchannel with various flow ratios between FITC and DIW and various total flow rates. We then demonstrated three-dimensional hydrodynamic focusing of human red blood cells (RBC) in the CEA microchannel. The cross-sectional images of the channel were obtained by confocal microscopy. For comparison with the experimental results, we performed numerical simulation using computational fluid dynamics (CFD) software.

2. Experimental

2.1. Design principle

Fig. 1 shows a schematic of the CEA microchannel for three-dimensional hydrodynamic focusing. Sample and sheath fluids flowing in the CEA microchannel experience a secondary flow, Dean flows at contraction regions. When the fluids flow from the expansion region to the contraction region, they are influenced by centrifugal forces which push them to side 1 of the microchannel, generating pressure gradients along a lateral direction. These centrifugal effects induce a secondary flow, transverse flow field characterized by two counter rotating vortices in the upper and lower plane of symmetry of the channel. The sheath fluid exposed to the Dean flow at the contraction region vertically wraps the sample fluid on the top and bottom close to side 2 of the microchannel. Simultaneously, the sample fluid wrapped by the sheath fluid begins to horizontally shift toward side 1 of the microchannel, resulting in the sample fluid being three-dimensionally focused in a cross-sectional plane of the microchannel. To achieve complete wrapping for three-dimensional focusing, a direction of rotation of the Dean flow needs to be sustained throughout the CEA microchannel. The proposed channel design utilizes the abrupt change in the cross-sectional area of the channel that curves the fluid streams, and accelerates or decelerates the flow velocity along the contraction and expansion region, respectively. When the fluid enters the contraction region, the streamlines from the wider part of the upstream microchannel accelerate and follow a curved path. In contrast, the fluid entering the expansion region decelerates due to the increase of cross-sectional area and any Dean-like flow effects are offset by the deceleration. Therefore, centrifugal effects are dominant at the contraction region, resulting in Dean vortices in fixed directions.

2.2. Channel design and fabrication

The CEA microchannel was 350 μm wide by 53 μm deep, having contraction regions of 50 μm wide and 300 μm long. The interval between contraction regions was 300 μm. The CEA microchannel was fabricated in poly(dimethylsiloxane) (PDMS) using soft lithography techniques. A mixture of PDMS prepolymer and curing agent (Sylgard 184; Dow Corning, MI) in the ratio of 9:1 was poured on SU-8 photore sist molds and cured for 3 h in a convection oven at 65 °C. To bond between a PDMS replica and glass slide, we treated both of them with oxygen plasma (200 mTorr, 200 W).

2.3. Computational fluid dynamics simulation

Computational fluid dynamic simulation for flow characteristics of the CEA microchannel was performed using a commercial CFD solver (CFD-ACE+; ESI-CFD Inc., Huntsville, AL). The physical properties of water were applied to fluids traveling through the CEA microchannel (density $\rho = 997$ $kg\ m^{-3}$ and dynamic viscosity $\mu = 8.55 \times 10^{-4}$ $kg\ m^{-1}\ s^{-1}$). Generally, the diffusion coefficient of fluorescein in water is $\approx 10^{-10}$ $m^2\ s^{-1}$ but to minimize diffusion effect we used the smaller coefficient value of $10^{-12}$ $m^2\ s^{-1}$. The applied total flow rates (the sum of the flow rates of the injected sample and sheath flow) were varied from 5.5 to 16.5 $mL\ h^{-1}$, while the outlet was set to a fixed-pressure boundary condition. The flow and user scalar modules were used to solve behaviors of two specified fluids in the CEA microchannel. The Algebraic MultiGrid (AMG) solver was used for pressure correction, and the Conjugates Gradient Squared (CGS) and Preconditioning (Pre) solvers were used for velocity and two species fluids. The convergence limit and iteration were set to $10^{-4}$ and $10^4$ time steps until flow reached the outlet.

2.4. Experimental setup

The two sample fluids: (1) DIW and (2) RBC of 4% hematocrit level diluted with phosphate buffered saline (PBS, Invitrogen Corporation, CA) before injection to the device were introduced with the corresponding two sheath fluids: (1) FITC (Sigma-Aldrich Co.) solution with a concentration of 50 μg mL$^{-1}$ and (2) PBS in the CEA microchannel using syringe pumps (KDS200; KD Scientific Inc., Holliston, MA) at specified flow rates: 3.3 to 16.5 $mL\ h^{-1}$, varying the flow ratio between the sample and sheath flow rate as 1:10, 1:2 and 1:1. The trajectories of the two fluids were visualized using a confocal microscope (LSM 510, Carl Zeiss Inc., Germany) for cross-sectional view using Z-stacked series of fluorescent images scanned at 2 μm intervals, and a fluorescence microscope (TS100; Nikon Co., Japan).

Fig. 1  Schematic of the contraction–expansion array (CEA) microchannel for three-dimensional hydrodynamic focusing (s1: side 1, s2: side 2).
equipped with a charge-coupled device (DS-2MBWc; Nikon Co., Japan) for top view. The RBC samples were obtained from the Republic of Korea National Red Cross Organization (Daejeon, Korea) in compliance with safety regulations.

2.5. Measurement

The acquired images with the fluorescence microscope were processed with ImageJ software (http://rsb.info.nih.gov/ij/). The mean lateral position ($Y_{\text{DIW}}$) of the focused sample flow (DIW) was calculated according to the following formulation:

$$Y_{\text{DIW}} = \frac{\sum_{i=1}^{n} y_i I_i}{\sum_{i=1}^{n} I_i}$$

where $y_i$ refers to pixels (between 0 and 1) measured relative to the lateral position in the contraction region, and $I_i$ refers to the grayscale values of each pixel. Each pixel from the acquired images represents 2 μm of the lateral position.

3. Results and discussion

3.1. Effect of the number of rectangular structures

Fig. 2 shows the experimental and simulation results as a function of the number of the contraction regions. The sample flow (DIW) was injected into side 2 of the channel at 0.5 mL h⁻¹, and the sheath flow (FITC) also was injected into side 1 of the channel at 5 mL h⁻¹ (at a flow ratio of 1:10). In Fig. 2a, the cross-sectional images were experimentally and numerically obtained at x-axial position of 35 μm from the beginning of the contraction region in the second, fourth and seventh contraction regions. From the numerical simulation results, two counter-rotating vortices representing Dean flow appear to be completely developed around x-axial position of 35 μm apart from the beginning of the contraction region (see ESI Fig. S1†). The left and right frames of the images represent confocal microscope and numerically simulated images, respectively. As shown in the figures, similar flow distributions were observed in the experimental and simulated images relative not only to the vertical and horizontal positions but also the shape of the focused sample stream. At the second contraction region (top images), under the influence of Dean flow in the contraction region, the injected sample flow begins to be wrapped by the injected sheath flow at the top and bottom close to side 2 of the channel, and simultaneously shifts toward side 1 of the channel. The wrapped sample flow becomes located at the center of the cross-section of the channel, finally forming a three-dimensionally focused stream at the fourth contraction region (middle images). After the fourth contraction region (bottom images), the focused sample stream still shifts toward side 1 of the channel, escaping from the center of the channel due to the influence of Dean flow being repeatedly induced at every contraction region.

Mean lateral positions of the sample flow were calculated from images captured with the fluorescent microscope using the formulation as described in the section 2.5. The resolution (2 μm/pixel) is sufficient to differentiate the change of lateral position by ≈10 μm per contraction region of sample flow. The calculated mean lateral positions of the sample flow were located at $Y_{\text{DIW}} = 43.1, 35.3, 27.3, 21.6, 15.9$ and 7.9 μm corresponding to the contraction regions from second to seventh, respectively. At the fourth contraction region, the sample flow was closely positioned in the center of the lateral position across the channel. The tendency of the simulated lateral positions of the focused sample flow is similar to the experimental ones. The lateral position of the sample flow gradually moves toward side 1 of the channel with the increase of the passing number of the contraction regions (Fig. 2b and c).

For complete three-dimensional focusing, it is important to maintain a stable flow throughout the CEA microchannel.

![Fig. 2](image)

**Fig. 2** Experimental and simulated results as a function of the number of contraction regions. (a) The cross-sectional images obtained from the confocal microscope (left frame) and CFD simulation (right frame) at the x-axial position of 35 μm apart from the beginning of the second, fourth and seventh contraction regions (images from top to bottom). The sheath flow (FITC) and sample flow (DIW) were injected on side 1 and side 2 of the channel, respectively. The blurring at the bottom boundaries of the confocal images might be due to scattering at the surface of the glass substrate. The dashed lines are drawn to clarify the bottom boundaries of the channel. (b) Plot of the mean lateral position of the sample flow across the channel. --- indicates the middle of the lateral position in the contraction region. (c) The top-view image obtained from the fluorescent microscope when the sample (DIW) and sheath (FITC) flows were injected in the CEA microchannel. The dashed arrows indicate positions where cross-sectional images were obtained for panel (a).
Above a certain flow rate, the flow separates at the edge of the contraction region and then reattaches in the expansion region. Thereby, a vortex is generated in the expansion region which enlarges as the flow rate increases. However, the vortex generated sustains a steady state even at high \( Re \) causing an asymmetric flow pattern in a symmetry expansion region.\(^{19} \) In the CEA microchannel, even though the vortex is observed by flow separation in the expansion region, above a total flow rate of 5.5 \( \text{mL h}^{-1} \) the vortex does not disturb the three-dimensional focusing process due to its stable flow pattern. The three-dimensional focusing might be further considered in not only the rectangular structure, but also the semicircular structure where vortex generation is suppressed; and the performance between the rectangular and semicircular structure could be discussed in further studies.

### 3.2. Effect of total flow rates

Fig. 3 shows the experimental and simulated results with the increase of total flow rate at the fourth contraction region. The total flow rate of the sample and sheath flow was specified at 3.3 to 16.5 \( \text{mL h}^{-1} \), maintaining the same flow ratio of 1:10 (sample : sheath). The trajectories of the focused sample flow in the \( xy \) plane were experimentally observed with a fluorescent microscope. As the total flow rate increased, the mean lateral position of the focused sample flow became positioned toward side 2 of the channel (see the arrows in Fig. 3a). In Fig. 3b, the cross-sectional images of the focused sample flow were numerically simulated at the fourth contraction region. The Dean number, \( De \), is a dimensionless number as a measure of the Dean flow, and is defined as \( De = Re(Dh/2R)^{1/2} \), where \( Re \) is the Reynolds number, \( Dh \) is the hydraulic diameter and \( R \) is the radius of curvature. From the above equation, if the other conditions except \( Re \) are constant, the Dean flow is proportional to the \( Re \) which is proportional to the \( x \)-axial flow velocity relative to the total flow rate in the CEA microchannel. In summary, the higher the total flow rate is, the larger Dean flow induced. However, there is a relatively large discrepancy between the intensities of the total flow rate and the transverse flow rate induced by the Dean flow. The nonlinear change of the difference between the two intensities as a function of the total flow rate implies that the critical flow rate for three-dimensional focusing exists at a given number of the contraction region. These phenomena might be attributed to the formation of vortex by flow separation at the entrance to the expansion region. Above the critical flow rate of 5.5 \( \text{mL h}^{-1} \), the vortex began to grow as \( Re \) increased. The expansion vortex as a dead volume can deform the curved path at the entrance of the contraction region, producing Dean vortices. The deformation of the curved path by the expansion vortex might have a negative effect on the formation of Dean vortices, thereby decreasing both the curvature of the curved path and the transverse velocity. In order to understand the above phenomena, we calculated and plotted the maximum \( x \)-axial and transverse velocities of the cross-sectional plane as the increase of the total flow rate. The \( x \)-axial velocity increases more steeply than the transverse velocity relative to the Dean flow (Fig. 3c). The sample flow initially injected in side 2 of the channel still remains in its initial position without shifting at the high flow rate of 16.5 \( \text{mL h}^{-1} \). This means that there is not enough time for shifting of the sample fluid toward the center of the channel at high flow rates. Mean lateral positions of sample flow also were calculated, and the calculated mean lateral positions of the focused sample flow were located at \( Y_{DIW} = 31.8, 25.6, 38.4, 39.6 \) and 44.1 \( \mu \text{m} \), corresponding to 3.3, 5.5, 7.7, 11.0 and 16.5 \( \text{mL h}^{-1} \), respectively. At 5.5 \( \text{mL h}^{-1} \), the sample flow was closely shifted to the center of the lateral position across the channel. The lateral position of the sample flow gradually moves toward side 2 of the channel as the total flow rate increases (Fig. 3d).

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**Fig. 3** Experimental and simulated results with an increase of the total flow rate in the fourth contraction region. (a) The top-view images were obtained by fluorescent microscopy. The arrows indicate the focused sample flow (DIW). (b) The cross-sectional images at the \( x \)-axial position of 35 \( \mu \text{m} \) apart from the beginning of the fourth contraction region. The images show the concentration distributions between FITC and DIW; and transverse velocity vectors. (c) Plot of the maximum \( x \)-axial and transverse velocities at the \( x \)-axial position mentioned in panel (b). (d) Plot of the lateral position of the sample flow across the channel as the total flow rate increases at the \( x \)-axial position mentioned in panel (b). --- indicates the middle of the lateral position in the contraction region.
3.3. Effect of flow ratio

Fig. 4 shows the experimental and simulated results at the various ratios between sample and sheath flow rate. The cross-sectional images of the second, fourth and seventh contraction regions were experimentally obtained with a confocal microscope at a flow ratio of 1:1 (sample : sheath). As shown in Fig. 4b, similar distributions were observed in the simulated images relative to not only the vertical and horizontal position but also the shape of the focused sample stream. The flow ratio between the sheath and sample flow rates determined a cross-sectional diameter of the focused sample flow in both horizontal and vertical directions. Because increasing the proportion of sheath flow suppresses that of sample flow, the diameter of the focused sample flow begins to shrink. Modulating the diameter of the focused sample flow by the flow ratio between the sample and sheath flow rate is possible, but altering the vertical position of the focused sample flow is not possible. However, to achieve successful three-dimensional hydrodynamic focusing, it is necessary for the focused sample flow to be vertically and horizontally sustained at the center position in the cross-section of the contraction region. Regardless of the modulating the diameter of the focused sample flow by the flow ratio, the center of the focused sample flow becomes located in the middle of the channel height due to Dean flow characterized by two counter rotating vortices in the upper and lower plane of symmetry of the channel. This means that the focused sample flow is vertically located in the middle of the channel height without any additional sheath flow, taking advantage of three-dimensional hydrodynamic focusing with just one sheath flow.

3.4. Three-dimensional hydrodynamic focusing with cells

At high \( Re \), inertial lift forces act on flowing particles of more than 7 \( \mu \)m in diameter. \(^{20,21} \) Therefore, the optimization of device geometry and operational condition such as flow rate and the diameter of the focused sample flow became large at the ratio of 1:1 (Fig. 4a). The cross-sectional images of the second, fourth and seventh contraction region were simulated and obtained at the flow ratio of 1:10, 1:2 and 1:1 (sample : sheath). As shown in Fig. 4b, similar distributions were observed in the simulated images relative to not only the vertical and horizontal position but also the shape of the focused sample stream. The flow ratio between the sheath and sample flow rates determined a cross-sectional diameter of the focused sample flow in both horizontal and vertical directions. Because increasing the proportion of sheath flow suppresses that of sample flow, the diameter of the focused sample flow begins to shrink. Modulating the diameter of the focused sample flow by the flow ratio between the sample and sheath flow rate is possible, but altering the vertical position of the focused sample flow is not possible. However, to achieve successful three-dimensional hydrodynamic focusing, it is necessary for the focused sample flow to be vertically and horizontally sustained at the center position in the cross-section of the contraction region. Regardless of the modulating the diameter of the focused sample flow by the flow ratio, the center of the focused sample flow becomes located in the middle of the channel height due to Dean flow characterized by two counter rotating vortices in the upper and lower plane of symmetry of the channel. This means that the focused sample flow is vertically located in the middle of the channel height without any additional sheath flow, taking advantage of three-dimensional hydrodynamic focusing with just one sheath flow.

Fig. 4  Experimental and simulated results at the various ratios between sample and sheath flow rate. (a) The cross-sectional images from the confocal microscope at the x-axial position of 35 \( \mu \)m apart from the beginning of the second, fourth and seventh contraction regions (images from left to right). The sheath flow (FITC) and sample flow (DIW) were injected on side 1 and side 2 of the channel, respectively. The dashed lines are drawn to clarify the bottom boundaries of the channel. (b) The cross-sectional images from CFD simulation at the various flow ratios of 1:10, 1:2 and 1:1 (sample : sheath) at the x-axial position mentioned in panel (a).

Fig. 5  Microscope images of red blood cells (RBC) focused by a phosphate buffered saline (PBS) flow in the \( xy \) plane. (a) The RBC flow as a sample flow and PBS flow as a sheath flow were introduced in the CEA microchannel at flow rates of 0.1 and 4.0 \( \text{mL h}^{-1} \), respectively. (b–d) Enlarged images at the sixth contraction region at a sample flow rate of 0.1, 0.4 and 0.6 \( \text{mL h}^{-1} \) (RBC), respectively; and a fixed sheath flow rate of 4.0 \( \text{mL h}^{-1} \) (PBS).
number of contraction regions should be considered for three-dimensional hydrodynamic focusing of these particles. In order to investigate the effectiveness of three-dimensional hydrodynamic focusing for cells and particles, the hydrodynamic focusing of RBC was implemented in the CEA microchannel.

Fig. 5 shows microscope images of RBC focused by PBS in the xy plane. A three-dimensional hydrodynamic focusing of RBC with PBS was demonstrated in the CEA microchannel where the depth of the channel was 38 μm. The RBC as a sample flow was injected into side 2 of the channel varying at 0.1, 0.4 and 0.6 mL h⁻¹, and the PBS as a sheath flow was also injected into side 1 of the channel at 4 mL h⁻¹; the corresponding flow ratio is 1:40, 1:10 and 1:7 (sample : sheath), respectively. The RBC flows are wrapped by PBS flows due to the influence of Dean flow in the contraction region and are shifted toward side 1 of the channel as the passing number of contraction regions increases (Fig. 5a). The wrapped RBC flows are located near to the center of the channel at the sixth contraction region. The widths of wrapped RBC flows in the xy plane enlarge as the proportion of the PBS flows decreases (Fig. 5b–d). This focusing tendency is similar to that of DIW and FITC as discussed in section 3.1 and 3.3. Even though the confocal microscope images representing the cross-sections of the contraction region were not investigated here, the similar focusing tendency suggests that the RBC flows were successfully wrapped by PBS flows and three-dimensionally focused in the CEA microchannel.

Whenever cells flow through a capillary with varying widths such as the CEA microchannel at high Re, hemolysis of the cells should be considered because the cells can be exposed to sudden changes of flow velocity and shear rate. Hemolysis of RBC occurs over a specific hemolytic threshold value in terms of Reynolds shear stresses (RSSs) and the exposure time of RBC to the flow fields. Of various hemolytic threshold values experimentally determined, the commonly used value is 400 Nm⁻², with an exposure time of 1 ms. ²² The maximum values for RSSs and the exposure time in the CEA microchannel were calculated to 6.5 Nm⁻² and 0.5 ms at total flow rate of 4.1 mL h⁻¹, which are lower than the hemolytic threshold values. From this calculation, we can conclude that hemolysis of RBC is negligible during the three-dimensional hydrodynamic focusing process in the CEA microchannel.

4. Conclusion

We have demonstrated a CEA microchannel that allows three-dimensional hydrodynamic focusing in a single-layer device with a single sheath flow. Sample and sheath flows flowing through the CEA microchannel are exposed to Dean flow in the contraction regions. The sheath flow wraps the sample flow by Dean flow, resulting in the sample flow to be horizontally and vertically focused in the cross-section of the contraction region. By modulating the flow rate or the number of the contraction region, we can easily control the focused position of the sample stream in three-dimensions. This advance significantly reduces the number of required sheath inlets and the complexity of fabrication for three-dimensional hydrodynamic focusing, and enables an easy integration with other microfluidic components.

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