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Doctoral Thesis

미세입자 및 세포 분리를 위한
유체영동기술
Hydrophoresis for Particle and Cell Separation

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Hydrophoresis for Particle and Cell Separation
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______________________________
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최 성용

위 논문은 한국과학기술원 박사학위논문으로 학위논문심사위원회에서 심사 통과하였음.

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Abstract

This thesis presents ‘hydrophoresis’, a novel flow-assisted separation principle for size separation of microparticles and cells. Hydrophoresis refers to the movement of suspended particles under the influence of a microstructure-induced pressure field. Particles subjected to lateral pressure gradients or flows induced by anisotropic microfluidic obstacles dynamically move from the one sidewall to the other sidewall without any active component. Therefore, hydrophoresis exhibits both advantages of field-based and flow-assisted methods that are dynamic particle manipulation and biocompatibility, respectively.

To demonstrate the hydrophoretic self-ordering of particles, I have designed and fabricated microfluidic obstacles slanted with respect to a fluid flow in a polymer device made of poly(dimethylsiloxane). Experiments were performed with micron, submicron beads, and DNA molecules to verify the strong nature of hydrophoresis such as dynamic particle manipulation and biocompatibility. Micron-sized particles ranged from 10 to 15 μm were discriminated with less than 6% resolution. DNA molecules of 49 and 115 kb were separated for 0.12 s over the channel length of 5 mm with the corresponding separation throughput of 1.7×10⁶ molecules/s.

For exact characterization of the hydrophoresis, I conducted three-dimensional (3D) measurement of particle positions in a hydrophoretic microchannel by using a mirror-embedded microchannel. The mirror ideally at 45 degrees reflects the side view of the channel and enables obtaining 3D positional information from two different orthogonal-axis images. With this method, I clearly revealed that hydrophoresis is governed by convective vortices and steric hindrance. I also observed that the hydrophoresis enables 3D particle focusing without sheath flows and an accurate flow-rate control.
I next developed a new class of a hydrophoretic device composed of slanted obstacles and filtration obstacles for effective separation of blood cells. Red blood cells (RBCs) are similar in diameter to that of white blood cells (WBCs), which makes difficult to separate two cell types based on their sizes. In the hydrophoretic filtration device, RBCs are aligned parallel to the filtration obstacles of 4.0 μm-height due to their small thickness and deformability, and thus pass through the obstacles, separating from WBCs. In the presented device, I separated WBCs from RBCs with an enrichment ratio of ~210-fold at a throughput of $4 \times 10^3$ s$^{-1}$.

The final section of the thesis deals with the use of hydrophoretic size separation to sort cells in target phases of the cell cycle entirely based on a hydrodynamic principle. With this method, I found that there is a linear relationship between a cell’s size and its position distribution in a hydrophoretic device. I also demonstrate the robustness of the hydrophoretic method for practical applications by sorting cells in G$_0$/G$_1$ and G$_2$/M phases out of original, asynchronous cells with a high level of synchrony of 95.5% and 85.2%, respectively.
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**NOMENCLATURE**

**Alphabetic Letters**

- $Re_p$: Particle Reynolds number
- $W_{ch}$: Width of the channel
- $H_{ch}$: Height of the channel
- $H_{ob}$: Height of the obstacle
- $L_{ob}$ or $C_{ob}$: Thickness of the obstacle
- $D_{ob}$ or $P_{ob}$: Pitch distance between the obstacles
- $H_g$: Height of the obstacle gap
- $D$: Diameter of the particle
- $x$: Position of the particle
- $R_s$: Separation resolution
- $U$: Maximum velocity of the fluid
- $D_h$: Hydraulic diameter
- $R_g$: Radius of gyration of the DNA molecule
- $H_{fo}$: Height of the filtration obstacle
- $Q$: Thickness of the filtration obstacle
\( S \)  
Pitch distance between the filtration obstacles

\( d \)  
Distance between a channel and mirror

\( n \)  
Refractive index of the medium

\( NA \)  
Numerical aperture of the objective lens

\( M \)  
Magnification of the system

\( e \)  
Pixel spacing of a CCD sensor

**Greek Letters**

\( \theta \)  
Slanting angle of the obstacle

\( \sigma_x \)  
Standard deviation for the particle position

\( \alpha \)  
Size selectivity of the hydrophoretic device

\( \rho \)  
Density of the fluid

\( \mu \)  
Dynamic viscosity of the fluid

\( \lambda \)  
Wavelength of an illuminating light
**Abbreviations**

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<th>Definition</th>
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<tr>
<td>AMG</td>
<td>Algebraic multigrid</td>
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<tr>
<td>CFD</td>
<td>Computational fluid dynamics</td>
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<tr>
<td>CGS</td>
<td>Conjugates gradient squared</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
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<tr>
<td>DEP</td>
<td>Dielectrophoresis</td>
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<tr>
<td>DOF</td>
<td>Depth of field</td>
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<tr>
<td>DPM</td>
<td>Discrete phase model</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>FFF</td>
<td>Field flow fractionation</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GrFFF</td>
<td>Gravitational FFF</td>
</tr>
<tr>
<td>LOC</td>
<td>Lab-on-a-chip</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
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<tr>
<td>PIV</td>
<td>Particle image velocimetry</td>
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<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Pre</td>
<td>Preconditioning</td>
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<td>PR</td>
<td>Photoresist</td>
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<td>PSV</td>
<td>Particle streak velocimetry</td>
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<td>PTV</td>
<td>Particle tracking velocimetry</td>
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<tr>
<td>3D</td>
<td>Three-dimensions</td>
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<td>2D</td>
<td>Two-dimensions</td>
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<tr>
<td>WBCs</td>
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1.1 Size Matters

The size of biological particles varies greatly with their environment and biological function (Fig. 1.1). Keeping a cell’s organelles or itself the right size is an important matter of life or death. The relationship between cell size and cell division has been extensively studied in yeast and is currently being investigated in mammalian cells [1,2]. Although there are many arguable points on a size-sensing mechanism of animal cells, it is obvious that a normal cell maintains its right size during its cell-cycle progression, called homeostasis and alteration of cell size can manifest as disease [3]. Identifying a minute change of cell size is an important matter for biology researches.

Blood is another size matter. One microliter of blood contains several millions of blood cells, proteins, glucose, ions and hormones suspended in blood plasma. For transfusion, each blood component such as red blood cells, platelets and plasma should be separated completely removing white blood cells (WBCs), which can cause febrile transfusion reactions and infections [4]. WBCs, cells of the immune system, last only a few hours outside a body and are sensitive to mechanical stresses and temperature changes. Purifying WBCs or their subsets in rapid and noninvasive ways is essential for inflammation studies and immunological evaluation. For these needs, the difference in blood
cell size is an important criterion for identifying and separating blood cells into individual component.

Separation by size at molecular level is a fundamental, analytical and preparative technique in forensics, molecular biology, genetics, and microbiology. Nucleic acid, ribonucleic acid, or protein molecules are routinely separated through a gel matrix in which an electric current is applied. Separated molecules in the gel matrix can be used for further characterization such as mass spectrometry, polymerase chain reaction (PCR), cloning and sequencing.

Identifying biological particles by size separation is a promising technique to cover abovementioned size issues. Recent technical advances of microfluidic devices for size separation provide new capabilities for integration of sampling, sample preparation, serial separation and detection, although the development of fully-integrated devices is yet to be achieved.

![Diagram of blood cell components and size comparison](image)

**Fig. 1.1.** Size matters in biology.
1.2 Field-based Separation

Cells and macromolecules are typically found in a heterogeneous mixture that requires precise identification of target cells or molecules. Many physical fields have been used for size separation of microparticles such as cells and bacteria, including dielectrophoretic (DEP), optical, and acoustic fields (Table 1.1).

DEP forces act on dielectric particles under a non-uniform electric field. Dielectric particles exhibit different DEP mobilities due to their size, shape, and lipid membrane characteristics (in the case of biological cells). The DEP forces acting on the particles also depend on the electrical properties of the particles and the medium as well as the frequency of the applied electric field. Consequently, a non-uniform electric field of a particular frequency allows the separation of microparticles such as cells and bacteria with high selectivity. DC-DEP has been demonstrated for size separation using a pinched channel under a DC bias between inlets and outlets [5-7]. The field gradient at the pinched region allows the separation of microbeads, blood cells and human breast adenocarcinoma cancer cell line (MCF-7). Slanted, interdigitated array electrodes fabricated in a microchannel have been used for sorting microbeads and human breast ductal carcinoma cell line (MDA-MB-231) by size [8-10]. The electric field gradients of the slanted electrodes generate a transverse force
component on the dielectric particles that make them differentially distribute across the microchannel. A trapezoidal electrode array has been developed for the differential lateral deflection of microparticles adopting the similar separation strategy with the slanted electrodes [11]. Although only the size separation by DEP is reviewed in this thesis, the different dielectric properties of cells with the same size have been exploited to purify target cells. Yang et al. revealed that the four subsets of WBCs (B- and T-lymphocytes, monocytes, and granulocytes) had different dielectric characteristics [12]. DEP field-flow fractionation (FFF) was used to discriminate these subsets of WBCs and human breast cancer MDA-435 cells through balancing between DEP and sedimentation forces [13]. Using the similar DEP approach, Becker et al. reported the separation of the human metastatic breast cancer cell line MDA231 from dilute peripheral blood [14].

Optical separation uses a scattering force of a laser light that pushes particles in the direction of laser beam propagation and differentially deflects them according to their size or optical property such as reflective index. Particles also can be separated by using an optical gradient force of a focused laser beam on the same principle with an optical tweezer. The optical force is proportional to the square of a particle diameter [15,16]. When balancing the
optical force with a drag force, the drift velocity induced by the optical force is proportional to the diameter of the particle. Consequently, a laser light of a particular wavelength allows the size separation of microparticles such as cells and bacteria. Optical chromatography has been demonstrated for size separation of microbeads and sperm cells using a radiation pressure induced by a laser [17-19]. In optical chromatography, the optical force acts in the opposite direction of a particle flow. Therefore, particles can be separated, having different retention times according to their size. For continuous-flow separation, Kim et al. have developed a microfluidic device for lateral optical separation that a laser beam propagates perpendicular to a particle flow in order to differentially distribute microbeads by size across the microchannel [20]. The optical forces can be also used for the separation of same-sized particles with different optical properties as well as for the size separation. MacDonald et al. reported the separation of same-sized silica and polymer beads using a holographic optical tweezer [21]. Hart et al. revealed that two closely related Bacillus spores exhibited different optical mobilities using an optical chromatography [22].

Acoustic forces have been employed into microfabricated devices for separation or manipulation of suspended micron-sized particles. Acoustic waves generated from resonating transducers induce pressure gradients in a liquid that
make particles migrate toward either pressure nodes or antinodes, depending on the density and compressibility of the particles and the medium [23,24]. The acoustic radiation force also depends on the volume of the particles. This acoustic principle was used for both size-based separation and density-based separation of polystyrene and poly(methyl methacrylate) (PMMA) beads, and blood cells in a microchannel with a piezo ceramic actuator [25]. Using the same acoustic approach, Petersson et al. reported the separation of lipid particle contaminants from erythrocytes [26].

In these field-based methods, the force acting on particles is proportional to their volume. The level of the force acting on macromolecules is several femto-Newton or less, which is insufficient to manipulate nucleic acids or proteins in flows. Dielectrophoretic trapping of DNA toward the high-field region has been proposed using dielectric constrictions and nanotubes, but these methods are limited by difficult sample recovery and low sample throughput [27,28]. One common approach for purifying macromolecules is to use electric fields in gels or microfabricated sieve structures [29-32]. Molecular separation in the nanoporous structures is achieved by repeated interaction between charged molecules and nanopores in Ogston, entropic, and electrostatic sieving modes. In other approaches, DNA molecules are continuously separated by
diffusion in asymmetric obstacle arrays [33].

Table 1.1 Previous researches on field-based separators.

<table>
<thead>
<tr>
<th>Dielectrophoresis</th>
<th>Optical separation</th>
<th>Acoustic separation</th>
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1.3 Flow-assisted Separation

Size-based separation is widely used in biotechnology, from cell separation to multianalyte, flow-assisted immunoassays [34-36]. For these purposes, conventional flow-assisted separation techniques and their microfabricated counterparts have been developed, such as field flow fractionation (FFF) and hydrodynamic chromatography [37-39]. Both techniques use a parabolic flow profile in which analytes are distributed differentially according to their size,
mass, and other physical properties. In this way, particles have different retention times along different velocity paths. Among the various FFF methods, sedimentation FFF, gravitational FFF (GrFFF), and their combination method with dielectrophoresis (DEP-FFF) have been used for applications of micron-sized particles, providing biocompatible and sterilized protocols. However, particle separation in GrFFF and DEP-FFF is not conducted in a continuous manner, which gives some disadvantages of both restricted amounts of samples within separation chamber volumes and relatively long separation times.

Flowing particles suspended in fluids are subjected to inertial lift forces such as shear-gradient and wall-induced lift forces [40,41]. These forces acting on the particles are negligible at relatively low particle Reynolds number ($Re_p$). However, at higher $Re_p$ of order 1, the inertial lift forces dominate particle behaviors even in micro-scaled channels. When balancing the inertial forces with a drag force, the drift velocity induced by the inertial forces is proportional to the volume of the particle. Carlo et al. have realized the inertial size-separation of microbeads and blood cells in curving channels (Table 1.2) [40,41]. By the interaction of both forces, micro-sized particles begin to migrate toward a particular equilibrium position across the channels. Inertial forces acting on relatively smaller particles are not enough to make them accurately
positioned to the equilibrium position. This size dependence of the inertial particle ordering causes the size separation. The inertial separation allows for the high-throughput separation of micro-sized particles. However, the inertial forces acting on particles are proportional to the fourth power of their diameter. The forces acting on macromolecules are insufficient to manipulate nucleic acids or proteins in flows. The inertial sorting is more suitable for cell separation than for molecular separation.

Microfabrication allows for engineering fluidic devices with accurate and well-defined channel dimensions at micro- and nano-scale. In microfabricated devices, a detailed understanding of fluid- and particle-transport has been contributed to the development of new techniques for sorting bioparticles without physical or external fields. The steric hindrance mechanism between a particle and a channel wall allows the size separation of micron and submicron particles (Table 1.2). Seki and coworkers developed ‘pinched flow fractionation’ that particles are pushed and aligned by a sheath flow toward a pinched channel wall [42-44]. The smaller the particles are, the closer their distance from the wall is. This size-dependent alignment allows differently-sized particles to flow into different positions across the channel. Austin and colleagues demonstrated size separation through deterministic lateral
displacement that particles interact with a large array of pillars, repeating the similar hindrance processes with pinched flow fractionation in many times [45-47]. By the repeated sieving processes, Austin and coworkers demonstrated continuous, high-resolution size separation of microbeads, DNA, and blood cells. These methods require the precise sheath control of a sample flow to prevent particles from flowing on multiple paths. The separation of large particles (negligible for Brownian motion) does not suffer from random motion and dispersion of the particles. However, most biomolecules such as nucleic acids and protein complexes are rather flexible and more diffusible that would be difficult to be separated. The purely hydrodynamic separation of macromolecules such as nucleic acids or proteins as well as cellular particles still remains a challenge.

Table 1.2 Previous researches on flow-assisted separators.

<table>
<thead>
<tr>
<th>Inertial separation</th>
<th>Pinched flow fractionation</th>
<th>Deterministic lateral displacement</th>
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1.4 Research Objective

Separation technologies in microfluidic environments can bring several more advantages over macroscale systems. These advantages include more accurate particle control without turbulent disturbance due to laminar flow at low $Re$ and higher process efficiency even with small numbers of particles. In the context of sample preparation methods for lab-on-a-chip (LOC) devices, recent technical advances of microfluidic separators will provide new capabilities for integration with other microfluidic components such as lysis, mixing, and detection units. (LOC refers to integrating several laboratory functions into a single chip of only a few square millimeters to centimeters in area.) Although the existing separators showed impressive results, the following issues should be resolved for fully-integrated devices.

Biocompatibility is a key point whenever making a choice for separation methods to be used in LOC devices. The biocompatibility of a separation device refers to the ability of the device to perform its intended function without undesirable physiological effects on biological samples. Although there are many arguable points for negative effects of physical fields on cells, field-based methods such as DEP (compared to flow-assisted methods) can cause more damages to their physiological characteristics. Archer et al.
revealed that DEP manipulation upregulated the gene transcription of fibroblast-like BHK 21 C13 cells [48]. Yang et al. reported that the immuno-reactivity of *Listeria monocytogenes* cells (especially for KPL anti-*Listeria* antibodies and to C11E9 monoclonal antibodies) was enhanced after DEP treatment [49].

Simplicity is another important issue to make the integration easier. Compared to flow-assisted methods, field-based methods require relatively complex fabrication processes such as patterning of piezoceramic plates and metal-electrodes. Optical methods depend on the accurate beam alignment between separation targets and complex optical instruments. Because flow-assisted methods utilize inherent hydrodynamic phenomena, they can disregard aforementioned problems. However, their dependence on laminar streams causes the complexity of fluidic control. Flow-assisted methods require confining particles in a certain position of a fluid stream with sheath flows. The separation methods based on sheath flows require an accurate flow control between the sample and sheath flows. Therefore, it can be difficult to apply the methods to the development of integrated microfluidic devices that require an operation on mobile and generate irregular flows.

In this study, ‘*hydrophoresis*’, a novel flow-assisted separation
principle was developed for size separation of biological particles, satisfying the aforementioned objectives. Hydrophoresis refers to the movement of suspended particles under the influence of a microstructure-induced pressure field. Particles subjected to lateral pressure gradients or flows induced by anisotropic microfluidic obstacles dynamically move from the one sidewall of a channel to the other sidewall without any active component. Therefore, hydrophoresis exhibits both advantages of the field-based and flow-assisted methods that are dynamic particle manipulation and biocompatibility, respectively. In addition, the size selectivity or sorting resolution of hydrophoresis is high enough to cover the abovementioned size matters. These features can facilitate the complete integration of LOC devices.

1.5 Chapter Outlines

This thesis is articulated into the following sections:

Chapter II first describes the principle concerning hydrophoresis, gives a computational description of fluidic phenomena in a hydrophoretic device and experimental characterization of the device with micron and submicron beads as a model particle. The demonstration of the high-resolution separation of the beads and DNA molecules was performed.
Chapter III deals with three-dimensional measurement of hydrophoretic particle ordering for exact characterization of hydrophoresis by using a mirror-embedded microchannel. This method was clearly revealed that hydrophoresis is governed by convective vortices and steric hindrance.

Chapter IV describes a brief review for blood cell separation in microfluidic devices and the principle concerning hydrophoretic filtration, an effective method to separate cells with a minute difference in size, thus allowing the isolation of WBCs from whole blood cells with high purity. The demonstration of the high-resolution separation of microbeads for device characterization and blood cell separation was performed.

Chapter V describes a practical application of hydrophoresis for sorting cells in target phases of the cell cycle entirely based on hydrophoretic size separation that uses convective, rotational flows induced from regularly patterned anisotropic microfluidic obstacles. With this method, I have demonstrated a linear relationship between a cell’s size and its position distribution in the hydrophoretic device and its use for sorting cells by size that were in different phases of the cell cycle, achieving a high level of cell synchrony.
Chapter VI summarizes the overall results of this thesis and discusses what remains to be determined by future researches.

Each chapter has been written, enabling to read independently. Thus, to facilitate the independent reading, the experimental and fabrication techniques used are sometimes described more than once.
References


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CHAPTER II: *Hydrophoresis for Size Separation*

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2.1 Basic Principle

The design of the hydrophoretic device consists of regular microscale obstacles with an anisotropic resistance with respect to the fluid flow as shown in Fig. 2.1 and 2.2. Upon application of a fluid flow along the \( y \)-axis, the anisotropic fluidic resistance of the obstacles generates rotational fluid streams as shown in Fig. 2.1(b) and (c) [1-4]. The channels are 50 \( \mu \text{m} \) wide (\( W_{\text{ch}} \)) and 38 \( \mu \text{m} \) deep (\( H_{\text{ch}} \)) with \( H_{\text{ob}} = 18 \ \mu \text{m} \), \( \theta = 10^\circ \), \( L_{\text{ob}} = 12 \ \mu \text{m} \), and \( D_{\text{ob}} = 21 \ \mu \text{m} \). The streamlines starting at the center of the \( z \)-axis deflect along the \( x \)-axis in (b). Following these streams, particles cross the channel and reach the right sidewall. The streamlines starting at the right sidewall move upward along the \( z \)-axis, i.e., Stream 1 in (c), and suddenly traverse the channel as they reach around the bottom of the obstacles, i.e., Stream 2 in (c). The overall flow streams rotate in a counterclockwise direction to the \( y \)-axis. Particles suspended in the fluid flow migrate toward the right sidewall following the rotational streams. At that time, the particle motion is determined by a kind of steric hindrance mechanism. Steric hindrance occurs when the obstacles prevent rotational flows of large particles that are observed in relatively smaller particles as illustrated in Fig. 2.1(d). In short, the particle-obstacle interaction deflects the large particles from their streamline and leads to equivalent flow paths for differently-sized
particles; this is called hydrophoretic ordering. A particle with a diameter that is
similar to the obstacle gap \(H_g = H_{ch} - H_{ob}\) will steer its position toward the
center of the \(z\)-axis due to the particle-wall interaction. The particle thus follows
Stream 1 and stays near the right sidewall without deviation. In contrast, the
smaller particle following streamlines will traverse the channel following
Stream 2, separate from the large particles as shown in Fig. 2.1(d).
Fig. 2.1. Hydrophoretic separation. (a) Schematic showing micron or submicron particles assuming hydrophoretic ordering under the influence of a microstructure-induced pressure field. (b) and (c) Simulated pressure fields and streamlines. (d) A particle with a diameter that is similar to the obstacle gap ($H_o = H_{ch} - H_{ob}$) will steer its position toward the center of the z-axis due to the particle-wall interaction. The particle thus follows Stream 1 and stays at the right sidewall without deviation. In contrast, the smaller particle following streamlines will traverse the channel following Stream 2.
Fig. 2.2. Microfluidic device incorporating anisotropic microfluidic obstacles. The device consists of a prefilter to remove particles with a diameter greater than 20 μm, the obstacles, and an expanded outlet region. The width ($W_{ch}$) of the anisotropic region is 50 μm; the other dimensions varied according to applications. (Scale bars = 100 μm)
2.2 Experimental

2.2.1 Device design and fabrication

The hydrophoretic device incorporating the anisotropic microfluidic obstacles was fabricated in poly(dimethylsiloxane) (PDMS) using soft lithographic methods. The mold for the obstacles and microchannels was defined on a Si wafer using two-step photolithography (see Appendix A for details). For microparticle applications, the channels were 50 μm wide ($W_{ob}$), and the obstacles had a gap height of 20 μm ($H_{ob}$), a thickness of 12 μm ($L_{ob}$), and a pitch distance of 21 μm ($D_{ob}$). The other dimensions such as the slant angle ($\theta$) of the obstacle ranged from 10° to 50° depending on applications. For applications involving submicron particles and DNA, the channels were 50 μm wide and 5.1 μm deep, with $L_{ob} = 21$ μm, $D_{ob} = 21$ μm, and the obstacles at a fixed angle of 30°. The deviations of all dimensions were less than 7.5%.

2.2.2 Sample preparation

Fluorescent polystyrene beads with nominal diameters of 0.52, 0.75, 1.1, 6 (actually 6.1), and 15 (15.4) μm were purchased from Polysciences (Warrington, PA) and Molecular Probes (Eugene, OR). The beads were prepared in 2%
pluronic F68 solution (Sigma-Aldrich, St. Louis, MO) in concentrations of 7.8×10^4, 4.1×10^4, 3.0×10^4, 5.4×10^2, and 1.7×10^2/μL, respectively. Non-dyed polystyrene beads with diameters of 8, 10 (10.1), 12 (11.9), and 15 (14.8) μm were obtained from Sigma-Aldrich. These beads were prepared in 2% pluronic solution in concentrations of approximately 3.7×10^2, 6.5×10^2, 2.8×10^2, and 2.5×10^2/μL, respectively, and had a coefficient of variation (CV) of less than 5% for particle size. For DNA separation, λ-phage (Takara Bio Inc., Tokyo, Japan) and micrococcus (Sigma-Aldrich) DNA were labeled with fluorescence dye (YOYO-1; Molecular Probes) in TBE 0.5× buffer. The dye to DNA base pair ratio was about 1:10 in the final concentration of approximately 20 ng/μL.

2.2.3 Position measurement

The beads and DNA molecules were introduced into the hydrophoretic devices using a syringe pump (Pump 11 Pico Plus; Harvard Apparatus, Inc., Holliston, MA). The mean flow speed at the obstacle constriction was calculated from the applied volumetric flow rate. The trajectories of the fluorescence beads and DNA were imaged using a fluorescence microscopy (DS-2MBWc; Nikon, Tokyo, Japan) with a long exposure time of 1 s or less. For the accurate measurement of non-dyed beads, their positions were recorded at the 1-mm-
wide expansion outlet of the device. The measured positions were then converted to corresponding positions in the 50-μm-wide anisotropic region under the assumption of linear amplification of fluid streamlines.

2.2.4 Computational fluid dynamics simulation

Simulations of the pressure fields and the gradients induced by the anisotropic obstacles were performed with commercial software (CFD-ACE+; ESI, Huntsville, AL).

2.2.5 Resolution calculation

The CV for particle position as a measure of resolution was calculated by CV = \( \sigma_x \times |dD/dx|/D \times 100\% \), where \( D \) is the particle diameter, \( x \) is the measured position, and \( \sigma_x \) is the standard deviation of \( x \) [5]. The size selectivity \( \alpha \) of the hydrophoretic device is defined as \( \alpha = CV \times D/100 \). The separation resolution \( R_s \) between two DNA streams is defined as \( R_s = 0.5 \times \Delta x/(\sigma_1 + \sigma_2) \), where \( \Delta x \) is the distance between the peaks of two streams and \( \sigma \) is the standard deviation of each stream width. A Gaussian function fit was used to determine the means and widths of DNA streams in their fluorescence profiles.
2.3 Results and Discussion

2.3.1 Hydrophoretic self-ordering of microparticles

To demonstrate the steric hindrance mechanism of microparticles, 15- and 6-μm beads were injected into the hydrophoretic device, which was 38 μm deep \( (H_{ch}) \) with obstacles 18 μm high \( (H_{ch}) \). Fig. 2.3(a) shows the fluorescence trajectories of the beads upon application of a fluid flow along the \( y \)-axis (approximately 6.7 and 3.3 mm/s for the 15-and 6-μm beads, respectively). The corresponding volumetric flow rates were 0.4 and 0.2 μL/min, respectively. The rotational flows induced by the anisotropic obstacles deflected both types of beads along the \( x \)-axis. When the beads reached the right sidewall, two different patterns of particle flow were observed: the 15-μm beads in hydrophoretic ordering and the 6-μm beads in free flow. The 15-μm beads in hydrophoretic ordering reached and stayed at the right sidewall without deviation. The 6-μm beads following the streamlines traveled back and forth between one sidewall and the other, following the rotational flows. The equivalent position of the 15-μm beads is about 9 μm from the right sidewall after passing through more than 40 obstacles (Fig. 2.4). To ensure the complete ordering of microparticles, the hydrophoretic device with 80 anisotropic obstacles was used at a flow speed of 33.3 mm/s (2
μL/min). On a microscale level, the diameter of particles assuming hydrophoretic ordering affects their equivalent flow paths. Particles in the diameter range 10–15 μm were clearly resolved into their own equivalent positions as shown in Fig. 2.3(b). The distribution of particle streams shifted upward as the obstacle angle increased from 10° to 50°. The maximum transverse flow in the staggered herringbone mixer was achieved for θ = 45° [2]. In a similar manner, the transverse flow in a hydrophoretic device with θ = 50° can be greater than in a device with θ = 10°, which results in the upward shift of the position distribution of differently-sized particles as the obstacle angle increases. The respective CVs for 10–15-μm beads at 10° are 5.6%, 2.6%, and 0.9%. The respective CVs for 10–15-μm beads at 50° are 3.6%, 2.0%, and 2.2%. The size selectivity values for 10–15 μm at 10° are 0.6, 0.3, and 0.1 μm, respectively. The size selectivity values for 10–15 μm at 50° are 0.4, 0.2, and 0.3 μm, respectively.
Fig. 2.3. Size-dependent ordering of microparticles. (a) Trajectories of microbeads passing through the slanted obstacles. (Scale bar = 100 μm) (b) Measured position distributions for bead diameters ranging from 8–15 μm at two different obstacle angles for a flow speed of 33.3 mm/s (2 μL/min).
Fig. 2.4. Measured position distributions of 15 μm-sized beads as a function of the number of the anisotropic obstacle. The beads were evenly distributed at the inlet. The respective bead positions after passing through 40, 80, and 120 obstacles are $9.7 \pm 0.6$, $8.5 \pm 0.6$ and, $9.0 \pm 0.6$ μm.
2.3.2 Effect of flow speed on hydrophoretic ordering

The flow speed affects the equivalent flow path of each particle in hydrophoretic ordering. The particle Reynolds number $Re_p$ is defined as the ratio of the particle inertia to the viscous force, $Re_p = (\rho D^2 U)/(\mu D_h)$, where $\rho$ is the fluid density, $D$ is the particle diameter, $U$ is the maximum fluid velocity, and $\mu$ is the dynamic fluid viscosity [6,7]. $D_h$ is the hydraulic diameter, defined as $D_h = 2wh/(w + h)$, where $w$ and $h$ are the width and height of the channel, respectively. For $Re_p >> 1$, the particle inertia is dominant. At such a high value of $Re_p$, inertial lift forces can affect the equilibrium position of particles under hydrophoretic ordering. As $Re_p$ increased from 0.1 to more than 0.7, the inertial lift away from the anisotropic obstacle resulted in the shift of the equivalent position of the 15-μm beads toward the channel center, i.e., inertial shifting as shown in Fig. 2.5. By using the repeated symmetric patterns of the anisotropic obstacles, this hydrophoretic device can easily augment the focusing throughput for high-throughput analysis without inertial shifting (Fig. 2.6). In the parallelized channel, the focusing throughput of 15-μm beads ($Re_p = 0.07$) was 57 particles/s (20 μL/min).
Fig. 2.5. Measured position distributions of 15-μm beads as a function of flow speed. The channel was 50 μm wide ($W_{ch}$) and 38 μm deep ($H_{ch}$), with obstacles 18 μm high ($H_{ob}$) and an angle of 10° ($\theta$). For $Re_p \gg 1$, the particle inertia is dominant. At such high particle Reynolds numbers, inertial lift forces can affect the equilibrium position of particles under hydrophoretic ordering. Because of the effect of inertia, the ordering position shifts toward the channel center along the anisotropic obstacle. The width of the fluorescence peaks corresponds closely to the diameter of the bead.
Fig. 2.6. Hydrophoretic ordering in the parallelized microchannel. (a) Microfluidic device incorporating the repeated symmetric patterns of the anisotropic obstacles. The channels are 1000 µm wide ($W_{ch}$) and 38 µm deep ($H_{ch}$) with obstacles 18 µm high ($H_{ob}$). The obstacle structure was constructed with ten-time repeat of the symmetric pattern of the anisotropic obstacle at an angle ($\theta$) of 10°. (b) Trajectories of 15 µm beads ($Re_p = 0.07$) in the parallel channel. The fluorescent trajectories (10 white-colored lines) were superimposed on the bright field images. By using the repeated symmetric patterns of the anisotropic obstacles, this hydrophoretic device can easily augment the focusing throughput for high-throughput analysis without inertial shifting. In the parallelized channel, the focusing throughput of 15-µm beads ($Re_p = 0.07$) was 57 particles/s (20 µL/min).
2.3.3 Effect of obstacle height on hydrophoretic ordering

The degree of the particle deflection along the $x$-axis is determined by $H_{ob}$ and thereby by the induced pressure gradients. As $H_{ob}$ was changed from 18 to 2 $\mu$m for a fixed obstacle gap ($H_g = 20$ $\mu$m), the 15-$\mu$m beads followed the applied flow along the $y$-axis without crossing as shown in Fig. 2.7. In the simulation, the magnitude of the pressure gradient at $H_{ob} = 18$ $\mu$m was three times higher than at $H_{ob} = 2$ $\mu$m (Fig. 2.8). The rotation of the fluid at $H_{ob} = 2$ $\mu$m was not sufficient to drive particle crossing or hydrophoretic ordering. At $H_{ob} \gtrsim H_g$, the magnitude of the pressure gradient is saturated and assures hydrophoretic ordering. The equivalent positions of particles at $H_{ob} = 43$ $\mu$m were similar to ones at $H_{ob} = 18$ $\mu$m (Fig. 2.3). The respective CVs for 10–15-$\mu$m beads at $H_{ob} = 43$ $\mu$m are 1.0%, 4.3%, and 1.3%. The size selectivity values for 10–15 $\mu$m at $H_{ob} = 43$ $\mu$m are 0.1, 0.5, and 0.2 $\mu$m, respectively.
Fig. 2.7. Effect of the height of the anisotropic obstacle on hydrophoretic ordering. (a) Trajectories of 15-μm beads for two different obstacle heights ($H_{ob}$): 43 μm (top) and 2 μm (bottom). (Scale bar = 100 μm) (b) Measured position distributions for bead diameters ranging from 8 to 15 μm in the device with $H_{ob} = 43$ μm. The equivalent positions of particles at $H_{ob} = 43$ μm were similar to ones at $H_{ob} = 18$ μm in Fig. 2.3.
Fig. 2.8. Simulated pressure fields and gradients in the microchannels with different obstacle heights ranging from 2 to 43 μm. (a) Simulated pressure fields and streamlines. A fluid flow was applied along the y-axis at the maximum flow speed of 33.3 mm/s. The channel was 50 μm wide ($W_{ch}$) and 38 μm deep ($H_{ch}$) with $H_{ob} = 18$ μm, $\theta = 10^\circ$, $L_{ob} = 12$ μm, and $D_{ob} = 21$ μm. The pressure gradient is generated in a way of counterclockwise direction to the y-axis. (b) Plots of pressure gradient along the line AB in panel a for three different obstacle heights at the fixed height of the obstacle gap ($H_g = 20$ μm). (c) Plot of the peak amplitudes of the simulated pressure gradients along the line AB in panel a for different obstacle heights ranging from 2 to 43 μm at fixed $H_g = 20$ μm.
2.3.4 Effect of obstacle-gap height on hydrophoretic ordering

Hydrophoretic ordering of submicron particles was demonstrated with micron and submicron beads with diameters of 1.1, 0.75, and 0.52 μm. A hydrophoretic device with 120 anisotropic obstacles was used to ensure complete ordering of the submicron particles. The beads were injected into the hydrophoretic device with a flow speed of 2.4 mm/s (0.01 μL/min); the device was 5.1 μm deep with \( H_g = 1.4 \) μm. The 1.1- and 0.75-μm beads remained near the right sidewall due to the steric hindrance mechanism as shown in Fig. 2.9(a). Their equivalent positions were 8.2 ± 2.0 μm and 12.2 ± 5.1 μm from the right sidewall for the 1.1- and 0.75-μm beads, respectively. The streams of these beads were not resolved, and they overlapped. This phenomenon can be presumed due to the low size selectivity of the device that is insufficient to resolve the micron and submicron beads. The hydrophoretic device can separate large particles in hydrophoretic ordering from relatively smaller particles in free flow. A mixture of 1.1- and 0.52-μm beads was introduced into the hydrophoretic device at a flow speed of 2.4 mm/s (0.01 μL/min). Fig. 2.9(b) shows the separation of the 1.1-μm beads in hydrophoretic ordering and 0.52-μm beads in free flow. The equivalent position of the 1.1-μm beads was 10.1 ± 2.3 μm from the right sidewall. The sorting efficiency was quantified by collecting the separated
beads into two reservoirs (Fig. 2.10). The ratio of the volume collected was 2:1 for the outlets of the 1.1- and 0.52-μm beads, respectively. The hydrophoretic device with 300 anisotropic obstacles was used at a flow speed of 23.8 mm/s (0.1 μL/min) for sorting submicron particles. The concentration ratio of the beads at the inlet was 2.6:1 for 0.52- to 1.1-μm beads. After separation, the concentration ratio collected at the outlet for the 0.52-μm beads was 261:1 for 0.52- to 1.1-μm beads. The purity of the 0.52 μm beads after separation was increased to 99.6% from the initial value of 72.2%. After hydrophoretic separation, the concentration ratio collected at the outlet for the 1.1-μm beads was 1.4:1 for 0.52- to 1.1-μm beads. The purity of the 1.1-μm beads was enhanced from 27.8 to 41.7%. The sorting throughput was approximately 90 particles/s.

The height of the obstacle constriction (Hₖ) determines whether particles assume hydrophoretic ordering as shown in Figure 2.9(c). As Hₖ was reduced from 1.4 μm to 0.65 μm keeping Hₐh constant at 5.1 μm, the beads with a diameter (D) of 0.52 μm became focused in their equivalent position of 12.9 ± 6.9 μm from the right sidewall as shown in Fig. 2.9(d), where the flow speed was 5.1 mm/s. For D/Hₖ ≥ 0.5, the obstacle constriction Hₖ begins to hinder the rotational flow of particles induced by the anisotropic obstacles and leads to
hydrophoretic ordering. As the obstacle constriction is reduced in nanoscale, this device could perhaps be used even to separate biomolecules such as protein complexes.

Fig. 2.9. Hydrophoretic ordering and sorting of submicron particles. (a) and (b) Fluorescence images and profiles of micron and submicron beads in the microchannel. (c) The ratio of particle diameter ($D$) assuming hydrophoretic ordering to $H_g$ as a function of $H_g$ for values of 1.4, 9.3, and 20.0 μm. Each data point represents the upper or lower limit of the particle diameter assuming hydrophoretic ordering for a given $H_g$. (d) Hydrophoretic ordering of 0.52-μm beads in the channel for $H_g = 0.65$ μm. (Scale bars = 100 μm)
Fig. 2.10. Separation procedures of a mixture of 1.1- and 0.52-μm beads in the hydrophoretic device. The device was 50 μm wide (W_{ch}) and 5.1 μm deep (H_{ch}) with H_{g} = 1.4 μm and θ = 30°. The bead mixture was introduced into the hydrophoretic device at the flow speed of 23.8 mm/s. The purity of the 0.52 μm beads after separation was increased to 99.6% from the initial value of 72.2%. After hydrophoretic separation, the concentration ratio collected at the outlet for the 1.1-μm beads was 1.4:1 for 0.52- to 1.1-μm beads. The purity of the 1.1-μm beads was enhanced from 27.8% to 41.7%. The sorting throughput was approximately 90 particles/s (0.1 μL/min). (Scale bar = 50 μm)
2.3.5 Separation of large DNA

The hydrophoretic device allows another separation mechanism based on size and deformability to enable continuous separation of biological samples (Fig. 2.11). When the obstacle gap ($H_g$) is smaller than the radius of gyration ($R_g$) of large DNA molecules, the molecules do not immediately pass through the gap and move toward the left sidewall. In this region, the DNA molecules temporarily change their shape due to their deformability and pass through the gap. DNA molecules with $R_g < H_g$ follow the hydrophoretic ordering and flow along their own equivalent path. The binary separation of DNA molecules by size was achieved using these mechanisms.

Fig. 2.11. Hydrophoretic separation of macromolecules. A large molecule with a radius of gyration that is greater than the obstacle gap does not pass through the gap, but moves toward the left sidewall. Smaller molecules, however, follow the hydrophoretic ordering, separated from the large molecule.
Long DNA molecules of 49 and 115 kb (λ-phage and micrococccus DNA, respectively) were prepared in TBE 0.5× buffer. These supercoiled molecules were condensed in the presence of YOYO-1 dye and can be considered to be spherical particles. The corresponding radii of gyration $R_g$ were 0.863 and 1.45 μm for the λ-phage and micrococccus DNA molecules, respectively [8]. These $R_g$ values are useful measures for defining the critical separation diameter or the gap height ($H_g$) of the anisotropic obstacle. Therefore, a microchannel was designed and fabricated with $H_g = 1.2$ μm between the $R_g$ values of λ-phage and micrococccus DNA molecules.

Fig. 2.12 shows differential molecular ordering of the λ-phage and the micrococccus DNA for a fluid flow of 111 mm/s (0.4 μL/min). When driven into the microchannel with 120 anisotropic obstacles, the λ-phage DNA molecules passed freely through the obstacles and followed the hydrophoretic ordering. In contrast, the micrococccus DNA molecules were blocked since their $R_g$ was greater than $H_g$, and they moved along the anisotropic obstacle. Fig. 2.12(c) shows a separation experiment for the mixture of the two DNA molecules in which the concentration of each molecule was 10 ng/μL. The DNA mixture was separated into two streams for the separation time of 0.12 s over the channel length of 5 mm. The separation resolution for the two DNA streams was 0.44.
The separation throughput was $1.7 \times 10^6$ molecules/s (0.4 μL/min). This rate is over 100 times faster than that of the DNA prism [9], one of the high-throughput DNA separators. The sample throughput of the hydrophoretic device can be further enhanced with parallel channels with repeated patterns of anisotropic obstacles.

Fig. 2.12. Differential molecular ordering of DNA. (a) Micrococcus (115 kb, top) and λ-phage (49 kb, bottom) alone and (b) a mixture of micrococcus and λ-phage. The channel was defined with an obstacle gap of 1.2 μm and an angle of 30°. The bars under the separation profile label the stream widths (± the standard deviation) centered at the means. (Scale bars = 100 μm)
2.4 Conclusion

The use of rotational flows induced by the anisotropic obstacles eliminates the precise sheath-control of a sample flow, allowing the self-ordering and -sorting of micron and submicron particles. Through two different hindrance mechanisms, different sizes of micron and submicron beads as well as DNA molecules were tracked into their distinct trajectories. I have successfully demonstrated the differential ordering and sorting of micron and submicron beads (0.5–15 μm in diameter), and DNA molecules of 49 and 115 kb.

This chapter clearly describes that there exists a critical particle diameter, the half of the obstacle gap, which ensures the hydrophoretic ordering of particles. Once above this criterion, the obstacle begins to hinder the rotational flow of the particles induced by the obstacle and leads to hydrophoretic ordering. This points to the importance of the height of the obstacle gap when engineering hydrophoretic devices for biological purposes. As the obstacle gap is reduced in nanoscale, this device could perhaps be used even to separate biomolecules such as protein complexes.
References


CHAPTER III: *Three-dimensional measurement of hydrophoretic particle ordering*

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3.1 Introduction

Grooved microchannels have been used to continuously separate particles of different sizes using a hydrodynamic principle known as "hydrophoresis" as shown in Chapter 2. This technique separates particles by forcing them to align to the groove surface and to follow different streamlines according to their size as a result of steric hindrance. Previous works have proved that this technique can separate biological molecules such as DNA as well as beads of 0.5 to 15 μm in diameter. The use of convective flows induced by groove patterns eliminates the precise sheath-control of a sample flow that is frequently required from existing separation methods, thereby allowing the self-sorting of particles. The convective flows show spatially varying characteristics in three-dimensions (3D). Therefore, it is inevitable to measure particle motions in 3D for exact characterization of hydrophoresis, although its separation results exhibit two-dimensional (2D) characteristics.

Particle image velocimetry (PIV) is an optical method that can be used to address the above needs by instantaneously obtaining velocity measurements and related properties in fluid [1-3]. The fluid is seeded with tracer particles that are assumed to have no influence on fluid characteristics. The velocity information of the fluid is calculated by processing the motion of the seeding
particles. In the strict sense, particle tracking velocimetry (PTV) or particle streak velocimetry (PSV) is more suitable to address the above needs because these techniques find the velocity information of individual tracer particle in the Lagrangian frame of reference and our main interest is to rather trace the particle position than find the velocity information in microchannels [4]. Holographic PIV/PTV uses an object and reference laser beam, thereby forming an interference pattern that contains the 3D information of seeding particles [5-7]. Stereoscopic PIV/PTV records two simultaneous but distinct off-axis views of the same object and reconstructs 3D images from the two different off-axis images [8, 9]. Defocusing PIV/PTV uses an optical aberration that the sharpness and contrast of an image reduce as the imaging plane translates away from the in-focus plane [10, 11]. In the presence of a pinhole mask, the aberration makes distinct, depth-dependent patterns. These methods of velocimetry enable the accurate, 3D measurement of velocity profiles even in microchannels. However, they typically require relatively complex and expensive equipments such as high-speed cameras, a high-power laser, a precise alignment between optical components, and specialized image processing algorithms. These disadvantages prevent easy implementation of 3D measurement of particle position and fluid stream in microchannels.
To overcome these limitations, I present a proof-of-principle mirror-embedded channel and its use for 3D measurement of hydrophoretic particle ordering for exact characterization of hydrophoresis by directly obtaining 3D positional information from two different orthogonal-axis images. A mirror ideally at 45 degrees reflects the side view of the channel and enables simultaneous imaging of the top and side views of the channel with a single lens. To enhance the reflectance from the mirror surface, an optical coating was conducted over a silicon wafer known as silvering that metals such as aluminum are deposited on a glass substrate to make a mirror. The aluminum coating can yield a reflectivity of around 80 to 90% over the visible spectrum [12]. I will describe here the characterization of an aluminum-coated silicon substrate for a mirror-embedded microchannel, comparing it with a bare, uncoated silicon substrate. Next, I will describe the 3D positional measurement of different particle orderings by size in a hydrophoretic microchannel and provide details about their characteristics. I will finally verify the hydrophoresis mechanism by conducting the computational modeling of hydrophoretic particle self-ordering.
3.2 Experimental

3.2.1 Design and fabrication of mirror-embedded channels

The photoresist (PR) mold for the mirror-embedded microchannels was defined on a silicon wafer using multi-step photolithography (Fig. 3.1). The first spin-coating of an UV-curable PR (SU8-3025; Microchem Corp., MA) defines a groove (31.7 μm depth) to place a mirror a certain distance away from the microchannel. The grooves also ensure that the mirror fully covers the sidewall of the channel. The second coating of the PR (SU8-2010; Microchem Corp.) defines the linear microchannel for imaging. For patterning grooved surfaces on the linear microchannel, an additional PR coating (SU8-3025) was conducted on the Si mold with linear channel structures. The grooved channel for hydrophoresis is 50 μm wide ($W_{ch}$) and 37 μm deep ($H_{ch}$) with $H_g = 19$ μm, $\theta = 60^\circ$, $C_{ob} = 46$ μm, and $P_{ob} = 63$ μm (Fig. 3.7) with 70 grooves. A poly(dimethylsiloxane) (PDMS) block was then placed at the same distance with the thickness of the PDMS block away from the groove. The PDMS mixture of PDMS and its curing agent (the ratio of 10:1) was poured on the PR mold and cured for 3 h in a convection oven at 65 °C for complete cross-linking. To seal the microchannel, two PDMS layers were manually aligned and bonded
(one for the mirror-embedded channel and the other for a stepped PDMS substrate) after brief exposure of oxygen plasma for 30 s. The gap between two PDMS layers was filled with liquid PDMS to prevent diffraction around the edges of the PDMS layers. For complete curing, the bonded PDMS layers were stored in a convection oven for 1 h. The mirrors were made by dicing a silicon wafer that an aluminum film (1000 Å in thickness) was deposited into each piece of 10 mm in width and 10 mm in length. The mirror was embedded in the PDMS device at an angle (θ) of $-41^\circ$.

### 3.2.2 Material preparation

Fluorescent polystyrene beads with diameters of 4 and 15 μm were purchased from Molecular Probes (Eugene, OR). Fluorescent melamine beads with a diameter of 5 μm were purchased from Sigma-Aldrich (St. Louis, MO). The beads were prepared in 2% pluronic F68 solution (Sigma-Aldrich) supplemented with a fluorescein isothiocyanate (FITC) dye (20 μg/mL) to visualize fluorescent streams of the microchannel.

### 3.2.3 Computational modeling

The liquid flow in the hydrophoretic microchannel was solved without particles
by using commercial computational fluid dynamics software (CFD-ACE+; ESI, Huntsville, AL). The channel for simulation is identical with the grooved channel geometry (Fig. 3.7). The total number of cells in the three-dimensional structured grid for the channel was 175959. Upwind scheme is used in the conjugates gradient squared (CGS) and preconditioning (Pre) solvers for velocity field, while algebraic multigrid (AMG) solver is used for pressure correction. The applied flow rate was 1 µL/min along the flow direction. The software treats particles as a point mass using a discrete phase model (DPM) implemented in most commercial CFD codes. In this method, the liquid flow is solved without particles and their trajectories are predicted by integrating the force balance acting on the particles in a Lagrangian reference frame. Since a point mass possesses its diameter just as a property, physical interactions such as steric hindrance can be ignored that is not appropriate for the modeling of hydrophoretic ordering. Therefore, the modeling of hydrophoresis was conducted by adding user-defined functions that describe steric hindrance into the simulated flow fields. Velocity components were extracted from CFD-ACE+ and transformed suitable for Matlab (MathWorks, Natick, MA). I then forced particles just to follow fluid streams as a point mass and corrected their positions whenever they collided with the obstacles. As a result, the particles
could exist at a distance from the channel walls that is further than their radius.

3.2.4 Data acquisition

The microchannels and beads were imaged through an inverted optical microscope (TS100; Nikon Co., Japan) with an objective lens (CFI Plan UW2×; Nikon Co.) of a magnification of 2× and a numerical aperture of 0.06, an objective lens (CFI Plan 4×; Nikon Co.) of a magnification of 4× and a numerical aperture of 0.10, and an objective lens (CFI ADL 10×; Nikon Co.) of a magnification of 10× and a numerical aperture of 0.25. A syringe pump (Pump 11 Pico Plus; Harvard Apparatus, MA) was used to produce 0.01 to 2 μL/min flows through the microchannels. A commercial image analyzing program, i-Solution (IMT i-solution Inc., Korea) was used to measure the positions of particles inside the microchannel. The program measures the lines drawn by users, and converts their pixel information into metric information.
3.3 Results and Discussion

3.3.1 Characterization of mirror-embedded microchannel

Figure 3.1 and 3.2 show a mirror-embedded microchannel consisting of a mirror and PDMS microchannel made by aligning and bonding two PDMS layers. The mirror ideally at 45 degrees reflects the side view of the channel and enables simultaneous imaging of the top and side views of the channel with a single lens. At the same time, the distance \(d\) between the mirror and channel is within the depth of field (DOF) that is determined by the distance from the nearest object plane to the farthest plane in focus (Fig. 3.1). Therefore, 3D locations of particles can be measured without calibration by directly observing the in-focus side and top views of the mirror-embedded channel. The proper functioning of the mirror-embedded microchannel was demonstrated by imaging the linear channel filled with FITC (Fig. 3.3). Figure 3.3A shows a fluorescence micrograph of the linear channel taken with 2× objective lens. The mirror even reflects the side view of the channel that is far from the mirror due to the high depth of focus of the lens. The DOF is given by Inoué and Spring as follows [13]:

\[
DOF = \frac{n_l}{NA^2} + \frac{ne}{NA \cdot M}
\]  

(1)
where, $n$ is the refractive index of the medium, $\lambda$ is the wavelength of illuminating light, $NA$ is the numerical aperture of the objective lens, $M$ is the magnification of the system, and $e$ is the pixel spacing of a CCD sensor. The calculated DOFs for the 2× and 4× lens are 394 and 130 μm for $\lambda = 700$ nm and $e = 24$ μm, respectively. These values are larger than the mirror-to-channel distance ($d$) that assures the in-focus imaging of both top and side views.

Figure 3.3B and 3.3C show the top (upper) and side views (lower) of the channel with the side view width of ~25.6 μm that well corresponds to the mold thickness of the linear channel. The images were taken with a bare silicon and aluminum-coated mirror, respectively. The fluorescence-intensity measurements (Fig. 3.3D) show that the maximum intensity of the side-view image reflected from an aluminum-coated mirror is approximately two times higher than that of the image reflected from a bare silicon mirror. As mentioned, this intensity enhancement is attributed to the increase of mirror reflectance by deposition of an aluminum film. A thin aluminum film is used to enhance the reflectance of a silicon mirror up to more than 80% that enables to image low light-level objects. The typical reflectance of a silicon mirror at wavelengths of 500–800 nm is approximately 35% [12].
Fig. 3.1. Schematic fabrication procedures of the mirror-embedded microchannel. (A) A mirror is placed on the micro-groove (31.7 μm depth) to fix it at a certain distance from the channel. (B) The PDMS mixture of PDMS and its curing agent was poured on the PR mold and cured for complete cross-linking. (C and D) For complete sealing of the microchannel, two PDMS layers are aligned and bonded (one for the mirror-embedded channel and the other for a stepped PDMS substrate). The gap between two PDMS layers is filled with liquid PDMS to prevent diffraction around the edges of the PDMS layers.
Fig. 3.2. Schematic view of a mirror-embedded microchannel for 3D measurement of hydrophoretic particle ordering for exact characterization of hydrophoresis. The mirror ideally at 45 degrees reflects the side view of the channel and enables the direct observation of 3D particle position from two different orthogonal-axis images with a single lens. The distance between the mirror and channel is within the depth of field that is determined by the distance from the nearest object plane to the farthest plane in focus.
Fig. 3.3. Mirror-embedded microchannel filled with FITC. (A) Fluorescence micrograph of the linear channel with an inlet, outlet, and mirror labeled that was taken with 2× objective lens. The aluminum-coated mirror reflects the side view of the channel. The inlet filter is used to remove dusts over 20 μm in diameter. (B and C) Fluorescence micrographs of the linear channel reflected from a bare silicon and aluminum-coated mirrors, respectively. The images were taken with 4× objective lens. The upper and lower stripes show the top and side views of the channel, respectively. (D) Fluorescence intensity profiles of the channel filled with FITC. The red and black profiles indicate the profiles obtained with a bare silicon and aluminum-coated mirror, respectively.
3.3.2 3D measurement of hydrophoretic particle ordering

Figure 3.4 shows two orthogonal-axis images of a grooved microchannel for hydrophoretic particle ordering. The DOF value of 130 µm for the 4× lens covers the mirror-to-channel distance (d) and assures the in-focus images of both top and side views (Fig. 3.4A and 3.4B). In contrast, the lower DOF value of 25 µm for the 10× lens results in blurring either of two images (Fig. 3.4C and 3.4D). To obtain sharply focused, orthogonal-axis images, the 4× lens was used in the following experiments.

The hydrophoretic microchannel (H_{ch} = 37 µm and H_g = 19 µm) is designed so that particles whose diameters range between 9 and 19 µm assume hydrophoretic self-ordering. The height of the groove gap H_g determines whether particles (D in diameter) assume hydrophoretic self-ordering by the steric hindrance mechanism. As shown in Chapter 2, I have experimentally found that the grooves of H_g ≤ 2D hinder the rotational flows of particles and lead to hydrophoretic ordering. To characterize hydrophoretic particle ordering in 3D, 4 and 15 µm-sized beads were injected into the mirror-embedded channel. Figure 3.5 shows the fluorescence trajectories of the beads upon application of a fluid flow along the x-axis (0.2 and 2.0 µL/min for the 4 and 15 µm beads, respectively). Hydrophoresis is governed by two kinds of
hydrodynamic phenomena: 1) convective vortices induced from the anisotropic fluidic resistance of the slant grooves and 2) steric hindrance resulted from the particle-groove interaction. In the presence of slant grooves, the microchannel generates rotational flows in a counterclockwise direction to the x-axis (Fig. 3.5). Such convective vortices make particles move along the transverse direction, following the rotational flows. Steric hindrance occurs when the particles reach near the sideway 1. At that position, the beads are exposed to the upward flows of the rotational flows. As shown in Fig. 3.5B, the 4 μm bead goes upward and approaches near the top surface of the channel, following the upward flows. In contrast, the large size of the 15 μm beads prevents them from approaching near the top surface of the channel that are observed in the 4 μm beads (Fig. 3.5C). This steric effect makes the 15 μm beads diffused out of the rotational streams, thereby staying near the sideway 1. After reaching the channel surface, the 4 μm bead crosses the channel from the sideway 1 to the sideway 2, following the transverse flows of the rotational flows. After crossing the channel, the bead goes downward and crosses the channel again in the opposite direction.

Figure 3.6A is a representative plot measured after 4 μm beads passed through all the grooves. As shown in the figure, there exists the constant, certain
rotational track of the 4 μm beads, even through the small beads travel back and forth between two sidewalls without converging. The steric effect also enables the self-ordering of the 15 μm beads along the z-axis as well as the y-axis (Fig. 3.6B). Their focusing position is (y, z) = (17.2 ± 3.1 μm, 22.8 ± 0.8 μm). The z-axis position of the 15 μm bead can be 11.5 μm at the groove gap (Hg = 19 μm), assuming that the bead is tightly aligned to the top surface of the groove. The z-axis position of the bead at the channel (Hch = 37 μm) can be determined by solving a propositional problem in terms of the bead diameter and both groove and channel depths. The calculated value for the z-axis position is 22.4 μm that is similar with the measured value of 22.8 μm. This result well supports that the steric effect confines the large particles in a certain position of the z-axis. This confinement allows 3D, sheathless focusing of particles assuming hydrophoresis.
Fig. 3.4. Two orthogonal-axis imaging of a grooved microchannel for hydrophoretic particle ordering. (A and B) Bright-field and fluorescence images of the channel taken with 4× objective lens. (C and D) Fluorescence micrographs of the channel taken with 10× objective lens. The images are in focus only at the top and side view images, respectively.
Fig. 3.5. 3D measurement of hydrophoretic particle ordering. (A) Fluorescence micrograph of 4 μm-sized beads passing through the slant grooves. The image was taken with a long exposure time to reveal several-particle trajectories. (B) Trajectory of a 4 μm-sized bead in the grooved channel. The bead approaches near the groove surfaces and then crosses the channel following rotational streams. (C) Fluorescence micrograph of 15 μm-sized beads assuming hydrophoretic self-ordering.
Fig. 3.6. Position measurement of particles after passing through all the grooves. (A) 4 μm-sized beads follow a certain rotational track without converging. (B) 15 μm-sized beads are focused at the averaged position of \((y, z) = (17.2 \ \mu m, 22.8 \ \mu m)\).
3.3.3 Computational modeling of hydrophoresis

To verify the hydrophoresis mechanism, a computational modeling of hydrophoretic particle ordering was conducted. Figure 3.7 shows the simulated velocity fields that were solved without particles. The channel for simulation is identical with the experimental one. The secondary flow patterns form due to the anisotropic fluidic resistance of the groove. As mentioned, the overall velocity fields rotate in a counterclockwise direction to the x-axis. In commercial CFD codes, particle trajectories are predicted without considering the steric effect in the grooved microchannel [14]. Therefore, they are not appropriate for modeling of hydrophoretic particle ordering. In my user-defined functions (see Appendix B for details), I still treat particles as a point mass, assuming that they follow the velocity vector at the particle center, but continuously check whether the particles collide with the grooves. The particle position is corrected whenever they exist at a distance from the channel wall and groove that is shorter than their radius. Especially for the 15 µm bead, the collision occurs around the front edge of the groove. At that time, I shifted its position along the slant groove to the distance that is larger than its radius. This correction forces the bead to be aligned to the top surface of the groove that makes it diffused out of its rotational streams (Fig. 3.8A). The z-axis position of
the 15 µm bead at the groove gap \((H_g = 19 \, \mu m)\) is 10.3 µm at the distance that is 1.2 µm from the groove surface. The \(z\)-axis position at the channel \((H_{ch} = 37 \, \mu m)\) is 19.5 µm that is smaller than both the estimated value of 22.4 µm and the measured value of 22.8 µm. Regardless of the inlet position, the 15 µm bead is focused at \((y, z) = (21.3 \, \mu m, 19.2 \, \mu m)\) after passing through 40 grooves in the simulation that is slightly different from the measured position of \((y, z) = (17.2 \pm 3.1 \, \mu m, 22.8 \pm 0.8 \, \mu m)\). In the simulation, the 4 µm bead before the channel crossing approaches near the groove surface at the distance that is 2.4 µm from the surface (Fig. 3.8B). The corresponding \(z\)-axis position is 14.6 µm that is higher than that of the 15 µm bead. After passing the groove, the 4 µm bead goes up to the \(z\)-axis position of 33.9 µm. At that position, the bead is exposed to the transverse flows along the \(y\)-axis and begins to cross the channel (see the \(y\)-axis velocity component in Fig. 3.7). Although these simulation results well verify the hydrophoresis mechanism that is greatly influenced by the particle size and its steric effect, some additional modifications are required for more accurate prediction of hydrophoretic particle ordering. For example, the particle motion at a given position can be determined by the change of linear and angular momentums around the particle surface rather than the velocity vector at the particle center.
Fig. 3.7. Simulated velocity fields. The channel for simulation is identical with the experimental one of $W_{ch} = 50 \, \mu m$, $H_{ch} = 37 \, \mu m$, $H_g = 19 \, \mu m$, $\theta' = 60^\circ$, $C_{ob} = 46 \, \mu m$, and $P_{ob} = 63 \, \mu m$. The overall velocity fields rotate in a counterclockwise direction to the $x$-axis. $U$, $V$, and $W$ indicate the velocity components along the $x$-, $y$-, and $z$-axis, respectively.
Fig. 3.8. Simulated particle trajectories. (A) The 15 μm-sized bead does not approach near the groove surfaces and is diffused out of the rotational streams, thereby assuming hydrophoretic self-ordering. (B) The 4 μm-sized bead goes upward near the groove surfaces and then crosses the channel without converging.
3.3.4 Effect of gravitational field on hydrophoresis

As mentioned before, the aligned position of a particle along the $z$-axis determines whether particles assume hydrophoretic ordering. For the hydrophoretic self-ordering, the distance between the particle center and the groove surface should be larger than a quarter of the groove gap. The distance is typically determined by particle size without the influence of external forces. Conversely speaking, the particle position can be modulated by applying external forces as in field-flow fractionation, and then hydrophoresis can turn the vertical particle motion into the horizontal motion. In order to evaluate the effect of the gravitational field as an external force, we injected melamine beads with a relatively larger density of 1.51 g/cm$^3$ and a diameter of 5 $\mu$m into the microchannel at a flow rate of 10 nL/min. The diameter range is out of the critical diameter ($D \geq \sim 10 \, \mu$m) for hydrophoretic ordering, but the beads will settle down under the gravitational force of $\sim 7.7$ pN and steer their position into the bottom wall. As shown in Fig. 3.9, the gravitational force makes the beads settle down into the bottom wall. The lateral flows along the $\gamma$-axis of the convective vortices (Fig. 3.7) finally enables the focusing of the 5 $\mu$m melamine beads near the sidewall 1 as in the 15 $\mu$m beads. The focusing position of the melamine beads is $(\gamma, z) = (2.5 \, \mu$m, 2.5 $\mu$m). At that position, the maximum
flow velocity along the $z$-axis by the convective vortices is \(~2.9\ \mu\text{m/s}\) from our CFD simulation. By Stokes’ law, the melamine bead in water at 20 °C has a settling velocity of \(~6.9\ \mu\text{m/s}\). By balancing two competing fields, the bead can go down to the bottom, and be finally focused to the sidewall 1. However, when we increase the applied flow rate to 0.2 μL/min, the maximum flow velocity along the $z$-axis also increase to \(~60\ \mu\text{m/s}\) and the gravitational effect is negligible. These results imply that the use of external fields allows hydrophoresis to separate particles by other physical properties such as particle density as well as size.

Fig. 3.9. 3D measurement of a melamine bead under an influence of a gravitational field. The time interval between each particle image is 1/3 s. The upper and lower stripes show the top and side views of the channel, respectively.
2.4 Conclusion

In summary, 3D measurement of hydrophoretic particle ordering have been presented using an optically coated mirror-embedded microchannel that enables easy implementation of 3D position measurement. The investigation clearly reveals the hydrophoresis mechanism that is governed by two kinds of hydrodynamic phenomena: 1) convective vortices induced from the anisotropic fluidic resistance of the slant grooves and 2) steric hindrance resulted from the particle-groove interaction. I also observed that the hydrophoresis enables the particle focusing along the vertical direction as well as the lateral direction. The ability of hydrophoresis for 3D particle focusing will be helpful for the development of microflow cytometry and other integrated microfluidic systems with advantages of a sheathless method, passive operation, and single channel. I finally verified the hydrophoresis mechanism by conducting the computational modeling of hydrophoretic particle ordering, and comparing the simulation results with the experimental measurements. The mirror-embedded microchannel is also particularly attractive because it can be easily fabricated with a single cast of PDMS and allows to easily obtain 3D positional information from two different orthogonal-axis images without relatively complex and expensive equipments and the modification of microscopes.
References


CHAPTER IV: Blood cell separation by

hydrophoretic filtration

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4.1 Introduction

Blood is one of the major measures to determine physiological and pathological states such as disease and organ function. Blood as a transport tissue flows throughout the entire body, provides oxygen and nutrients, and retrieves waste products. In the circulation, blood cells can be affected by many medical conditions such as hematological disorders.

Blood separation plays an important role when performing blood analysis for diagnosis. Since blood is a very complex mixture containing red blood cells (RBCs), white blood cells (WBCs), and platelets, blood samples need to be separated prior to analysis. One common approach to separate blood cells is by differentially settling blood samples in a tube using a centrifuge. However, it is difficult to draw a thin layer of WBCs (called buffy coat) on a layer of RBCs with high purity. This method is also inappropriate for applications that require purified blood cells immediately.

For blood cell separation, many of physical fields have been employed into microfluidic devices in which heterogeneous cells show different mobilities. Each subset of blood cells exhibits different dielectrophoretic (DEP) mobilities due to their different shape, size, and lipid membrane characteristics [1]. DEP-field-flow fractionation (FFF) isolates WBCs with a purity of 5% through
balancing between DEP and sedimentation forces. Magnetophoresis utilizes the different magnetic natures of blood cells for separation [3,4]. The relative magnetic susceptibilities of deoxyhemoglobin RBCs and WBCs are about $3.9 \times 10^{-6}$ and $-0.13 \times 10^{-6}$ in water, respectively. In the presence of high magnetic field gradients, such susceptibility difference used to separate WBCs from RBCs. An acoustic force was employed into a microfabricated device for separation of blood cells [5]. Under an acoustic standing wave field, an acoustic force is differentially exerted on blood cells according to their size, density, and compressibility. Such physical differences were used for separation of WBCs, RBCs, and platelets in a microchannel with a piezo ceramic actuator. Although these field-based methods showed impressive results, their efficacy should be demonstrated without sample treatment such as media exchange and the adjustment of the ratio of WBCs to RBCs. In addition, their enrichment ratios of WBCs were not enough high for further analysis.

Difference in cell size is one of the criteria for blood cell separation. The deterministic bump array separated WBCs with an enrichment ratio of 110-fold without clogging [6]. This device uses asymmetric bifurcation of laminar flow under which RBCs follow streamlines, and WBCs are bumped the array and laterally moved by hydrodynamic lateral drag. A microfluidic network with
splitting channels isolated WBCs in a continuous manner with an enrichment ratio of 29-fold [7]. At a branch point in the network, dividing volumes into each channel determine the critical separation diameter called Zweifach-Fung effect [8]. However, their dependence on a laminar stream makes it difficult to induce the dynamic movement of blood cells across the channel along field gradients as in the active methods. Therefore, it is necessary for cell separation to confine cells in a certain position of the fluid stream with sheath flows or to divide and re-distribute the fluid stream with complex channel networks. Thus, it is essential to develop simple and efficient methods to isolate WBCs for easy integration with downstream analysis.

This chapter presents an effective method for blood cell separation using a hydrophoretic device composed of slanted obstacles and filtration obstacles. This device exploits the size and deformability difference between RBCs and WBCs. The filtration obstacles are designed with the same shape as the slanted obstacles, only shorter to create a filtration pore. The microfluidic device was characterized with polystyrene beads with a minimum diameter difference of 7.3%. In the isolation of WBCs from RBCs, the microfluidic device isolated WBCs with 210-fold enrichment within a short filtration time of ~0.3 s.
4.2 Principle of Hydrophoretic Filtration

Fig. 4.1 illustrates overall focusing and filtering processes with schematic trajectories of cells passing the slanted obstacles and filtration obstacles. The slanted obstacles are formed on the bottom and top of the channel (Fig. 4.1a). Its height is defined as half of the channel height. Upon application of a fluid flow along the \( y \)-axis, the anisotropic fluidic resistance of the obstacles generates rotational fluid streams. In short, the top or bottom area of the obstacles has higher resistance than the side areas. When blood cells pass through the slanted obstacles, these cells migrate toward the right sidewall following the rotational streams. At that time, the cell motion is determined by a kind of steric hindrance mechanism. Steric hindrance occurs when the obstacles prevent rotational flows of large particles that are observed in relatively smaller particles. The cell-wall interaction deflects the blood cells from their streamline and leads to an equivalent flow path, the right sidewall. RBCs have a biconcave disk shape, and their diameters are 6–9 \( \mu \)m, a similar size to that of WBCs. Therefore, it can be difficult to separate two cell types based on their sizes. However, RBCs are easily deformed due to their low shear modulus of 2.5 \( \mu \)N/m and thereby pass through small capillaries even with a diameter of approximately 3 \( \mu \)m. To isolate WBCs from RBCs, the height of the filtration
obstacles set between the thickness of RBCs (2–3 μm) and the diameter of WBCs (6–10 μm) (Fig. 4.1b). The filtration obstacle has a filtration pore with the same geometry as the slanted obstacle. WBCs larger than the obstacle gap collides against the filtration obstacles and move into the filtration pore. In contrast, RBCs having smaller thickness than the obstacle gap freely pass through the obstacle gap and keeps their focused position. The height of the filtration obstacles or their gap in the filtering process defines the critical separation diameter.
Fig. 4.1. Hydrophoretic filtration. The obstacles are alternately formed on the bottom and top of a channel. A flow direction is along the $y$-axis. (a) The slanted obstacles at the bottom and the top wall drive helical recirculation. Along the transverse flows, blood cells are focused to a sidewall. (b) In the filtering process, the gap between the filtration obstacle and the top or the bottom wall is set between the diameters of the small and the large cells. Therefore, the larger cells than the gap are blocked by the filtration obstacles and move through the filtration pore. The smaller cells freely pass the gaps in the filtration obstacle areas and stay in their focused position.
4.3 Experimental

4.3.1 Device design and fabrication

The device consists of the slanted obstacles and filtration obstacles at an angle of $55^\circ$ respect to the applied fluid flow in the channel of 100 μm width (Fig. 4.2). The thickness and pitch distance of the obstacles are 70 and 210 μm, respectively. The channel area (1) for focusing has 20 slanted obstacles alternately formed on the bottom and top of the channel after assembly of two PDMS layers. In the channel area (2), 20 filtration obstacles are formed alternately on the bottom and top of the channel with filtration pores. All blood cells pass through the slanted obstacle in the area (1), while WBCs are blocked by the filtration obstacle in the area (2). Therefore, the height of the slanted obstacle and its gap should be higher than that of the filtration obstacle and its gap. For blood cell separation, the height of the filtration obstacles was ~3.9 μm. In the device, the cross-sectional area of the filtration pore was 20 μm width by 7.8 μm height. The widths of the initial filtration pores in the area (2) were designed with gradual decrease to prevent the trapping of WBCs at the obstacle gap. Blood can form clots by coagulation during blood draw. In-channel prefilters with a pore size of 20 μm can be designed to prevent channel clogging.
by the blood clots. For cell collection, the channel of the device was equally divided into two outlets; one for WBCs and another for RBCs.

Fig. 4.2. Hydrophoretic device for blood cell separation. (1) and (2) denote the channel areas containing the slanted obstacles and filtration obstacles, respectively. (a) Layouts of PDMS layers with upper and lower obstacles. (b) Side view of the device after assembly of two PDMS layers. These figures are not scaled. (c) A photography of the assembled device filled with a red dye solution. (D) Optical micrographs of the channel region (1) and (2) after bonding. (Scale bar = 100 μm)
The device for hydrophoretic filtration was fabricated by two-step photolithography. The first spin-coating of PR (photoresist, SU8-2002 or 2010) defines filtration obstacles and the second coating determines slanted obstacles. Accordingly, the height of the slanted obstacles is higher than the filtration obstacles. For particle separation, the exact heights of filtration obstacles with 8.6 and 11.6 μm nominal heights were 8.55 ± 0.07 and 11.58 ± 0.02 μm, respectively. The filtration obstacles of 4.0 μm nominal height for blood cell separation were 3.92 ± 0.04 μm in exact height. The mixture of PDMS prepolymer and curing agent (Sylgard 184; Dow Corning, MI) in the ratio of 5:1 was poured on the PR mold and cured for ~3 h in a convection oven of 65 °C. To align and bond the PDMS replicas (one with upper obstacles and another with lower obstacles), after their brief treatment with oxygen plasma (200 mTorr, 200 W), I dipped them into ethyl alcohol solution. Since the oxidized surface of PDMS is preserved under liquid solutions, the PDMS replicas could be aligned and bonded with each other.

4.3.2 Sample preparation
Plain polystyrene beads of 8, 9, 10, 11, and 12 μm nominal diameter (Sigma-Aldrich Co., MO) were used for the demonstration of hydrophoretic filtration.
Their exact sizes were 8.02, 9.26, 10.09, 10.98, and 11.85 μm, respectively. They had coefficient of variation (CV) less than 2% for particle size. The beads were prepared in 0.2% Tween20 aqueous buffer with concentrations of ~380, 260, 240, 250, and 150 μL⁻¹ for 8 to 12 μm beads, respectively.

For blood cell separation, Sprague-Dawley rat blood was drawn with EDTA or a mixture of EDTA and citrate as anticoagulant. Blood sample was used within 12 h of collection. I measured the size of blood cells through microscopic images. WBCs in suspension had a diameter in the range 4.1–7.9 μm (n = 20). RBCs in suspension had a diameter in the range 6.2–7.9 μm (n = 11) and a thickness in the range 1.7–2.6 μm (n = 6). When the WBCs are fixed to a glass slide to prepare a blood film, they can spread and become larger. To quantify separation efficiency, WBCs were stained with Hoechst (Invitrogen Co., CA), a nucleic acid-specific fluorescence dye. WBCs were identified from RBCs by fluorescence.

4.3.3 Experimental setup

Bead mixtures or cells were introduced into the microfluidic device using a syringe pump (Pump 11 Pico Plus; Harvard Apparatus, Inc., MA). Particle images and videos were taken with a CCD camera (DS-2MBWc; Nikon Co.,
Japan) attached to an inverted optical microscope (TS100; Nikon Co.). The lateral position of particles was measured from images captured in the expanded outlet region of 1 mm. A commercial image analyzing program, i-Solution (iMTecho Technology Co., Korea) was used to measure particle positions, cell sizes, and the number of unstained RBCs and Hoechst stained WBCs. The images for measuring particle position were acquired at a resolution of $1024 \times 768$ pixels. In each experiment, more than 100 particles were measured. The pressure distributions in the microchannel were simulated using a commercial CFD solver (CFD-ACE+; CFD Research Co., Huntsville, AL).

4.3.4 Differential count of white blood cells

The differential counts of WBC subpopulations in initial and separated samples were performed using a hematology analyzer (Cell-dyn 3500; Abbott Laboratories, IL). The separated sample was mixed with phosphate buffered saline in a minimum volume of $\sim 120 \mu$L for the hematology analyzer.
4.4 Results and Discussion

4.4.1 Hydrophoretic filtration of microparticles

To demonstrate the hydrophoretic filtration, I tested whether particles larger than the gaps of filtration obstacles can bump against the obstacles and change their flow direction. For this test, microspheres with diameters of 11 and 12 μm were individually injected into the microfluidic device, in which the heights of the slanted obstacles and filtration obstacles were 18.6 and 11.6 μm, respectively. In the device, the cross-sectional area of the filtration pore was 20 μm in width by 23.2 μm in height. The applied flow rate was 0.1 μL min⁻¹. Fig. 4.3(a) shows overlaid images of two different modes of separation. The time interval between particles in each image was 1/15 s. In the device with a critical separation diameter of 11.6 μm, the 11 μm bead stayed in its focused position (focusing mode), but the 12 μm bead collided against the 11.6 μm-height filtration obstacles and passed through the filtration pore (filtration mode).

The transition of the separation modes was then characterized with several beads ranging from 8 to 12 μm in diameter. The microfluidic devices were designed for two different types; one had 16.2 μm-height slanted obstacles and 8.6 μm-height filtration obstacles, and the other had 18.6 μm-height slanted
obstacles and 11.6 µm-height filtration obstacles. In the device with 8.6 µm-
height filtration obstacles, the cross-sectional area of the filtration pore was 20
µm in width by 17.1 µm in height. The particles were individually injected into
the microfluidic devices at 2 µL min⁻¹. On a given critical separation diameter,
particles with smaller diameters than the criterion value took the focusing mode,
and particles with larger diameters took the filtration mode (Fig. 4.3b). In the
device with 8.6 µm-height filtration obstacles, only 8 µm beads flowed in the
lateral range of 44.2 ± 26.4 µm (focusing mode). In contrast, only 12 µm beads
flowed in the range of 744.2 ± 7.8 µm in the device with 11.6 µm-height
filtration obstacles (filtration mode). Particles having a comparable size to the
filtration obstacles were aligned to the center of the channel height and exposed
to an identical pressure field. Therefore, they flowed to the same lateral region.
In the same way, 8 to 11 µm-sized particles were completely focused to a
sidewall within the range from 31.7 to 56.0 µm in the device with 11.6 µm-
height filtration obstacles. The advantage of the filtration obstacles over
conventional filters is that they play not only the role of filtering large particles
but also the role of focusing small particles able to pass through the gap in the
obstacle area. Therefore, through hydrophoretic filtration, the desired target can
be completely separated by size from unnecessary elements.
Fig. 4.3. (a) Optical micrographs showing trajectories of 11 and 12 μm beads passing the 11.6 μm filtration obstacles at 0.1 μL min⁻¹. (b) Lateral position of particles as a function of microsphere diameters, measured in the expanded outlet region of 1 mm.
4.4.2 Simulation of pressure fields around filtration obstacles

By applying a pressure drop across the simulation geometry with the filtration obstacles and filtration pores, I obtained pressure fields and velocity vectors, which lead to transverse motions of a microparticle. The geometric conditions were identical with the experimental ones in Fig. 4.3(a); $H_{f_0} = 12 \, \mu m$, $W = 100 \, \mu m$, $Q = 70 \, \mu m$, and $S = 210 \, \mu m$. The applied flow rate was 0.1 $\mu L \cdot min^{-1}$ along $y$-axis. The values of the field intensity in each cross-section were normalized for clear illustration. Fig. 4.4 shows the variation of the pressure field intensity generated by the filtration obstacles. The insets are the enlarged views of the projected velocity vectors around the lower filtration obstacle. The pressure field intensity is higher at the filtration pore and it becomes lower going to the right sidewall. There are no significant variations of the field intensity in the filtration pore. The pressure field gradient from the left to the right sidewall drives the focusing flows (see the insets of Fig. 4.4). The lateral flow induced by the transverse pressure gradient focuses small particles, which can pass through the gaps of the obstacles, to the right sidewall. On the other hand, large particles, which bump against the obstacles, move to the filtration pore. As shown in Fig. 4.3(a), the $11 \, \mu m$ particle smaller than the gap of the filtration obstacles was completely focused to the sidewall.
Fig. 4.4. Simulated pressure fields. The simulation geometry composed of lower and upper filtration obstacles and the cross-sectional plots of pressure distributions. The enlarged plots describe the projected velocity vectors to the cross-sectional surfaces.
4.4.3 Separation of blood cells

Applying the hydrophoretic filtration method, WBCs were isolated from RBCs. The microfluidic device was designed with slanted obstacles of 13.0 μm-height and filtration obstacles of 4.0 μm-height. In the device, the cross-sectional area of the filtration pore was 20 μm in width by 7.8 μm in height. The channel of the device was equally divided into two outlets; one for WBCs and another for RBCs (Fig. 4.5a). The applied flow rate for blood cell separation was 1 μL min⁻¹. The concentration of blood cells was ~5 × 10⁹ cells mL⁻¹ and WBCs occupied 0.28% of the total cells. The blood sample was diluted 1:20 in PBS buffer for this separation experiment. Erythrocytes have a biconcave disk shape, and their diameters are 6.2~7.9 μm, a similar size with that of leukocytes. Therefore, it can be difficult to separate two cell types based on their sizes. However, RBCs are easily deformed due to their large surface area to volume, thereby passing small capillaries even with diameter of ~ 3 μm [9]. In our device, RBCs were aligned parallel to the filtration obstacles of 4.0 μm-height due to their thin thickness of 1.7~2.6 μm and deformability. The majority of RBCs chose the focusing mode. Decrease of RBC deformability can make RBCs difficult to pass through the gap of the filtration obstacles as other conventional filtration methods used for quantifying RBC deformability [10,11]. In that case, impaired
RBCs would take the filtration mode. WBCs larger than the filtration obstacles chose the filtration mode. By the filtration process, WBCs were isolated with a purity of ~ 58% (710 WBCs in 1221 total cells) at a throughput of $4 \times 10^3$ cells s$^{-1}$ (Fig. 4.5b and 4.5c). Up to 85% of WBCs were recovered compared with their initial concentration. The hydrophoretic filtration device enriched WBCs to ~ 210-fold from RBCs in just a single round of separation. The deformability of WBCs can have a negative effect on their recovery. Since WBCs with diameters between 4 and 5 $\mu$m can deform within the gap of 4-$\mu$m height, they would pass through the gaps of the filtration obstacles in the focusing mode and decrease the recovered number of WBCs at the outlet for WBCs. The simplest way to increase the recovery ratio of WBCs is to reduce the gap size between the filtration obstacles and the top or the bottom of a channel. In the device with the gap of 3-$\mu$m height, most of deformed WBCs will be blocked by the filtration obstacles and RBCs with thicknesses of 1.7~2.6 $\mu$m will pass through the gaps in the focusing mode.
Fig. 4.5. (a) Blood cell separation. WBCs and RBCs were separated following the filtration and the focusing mode, respectively. The critical separation diameter of 4.0 μm set between the thickness of RBCs and the diameter of WBCs. (b and c) Blood cells before separation (b) and after separation (c) are shown in bright-field (left) and fluorescence (right) images. These images were acquired with 50× magnification.
4.4.4 Recovery of white blood cells and their subpopulations

The hydrophoretic filtration device was also tested to quantify the differential recovery of WBCs as well as their total recovery. The concentration of blood cells used in this experiment was \( \sim 6.4 \times 10^9 \) cells mL\(^{-1}\). The concentration of WBCs in the blood was \( \sim 1.1 \times 10^7 \) cells mL\(^{-1}\), which was composed of neutrophils (13.3%), lymphocytes (69.3%), monocytes (5.2%), eosinophils (0.4%), and basophils (11.7%). The blood sample was diluted 1:10 in PBS buffer for this separation experiment. The applied flow rate for blood cell separation was 1 mL min\(^{-1}\). Blood was run through the filtration device over 1 h. The filtrated sample was collected and counted by a conventional hematology analyzer. The principal leukocytes of a rat are neutrophils and lymphocytes. The differential count of the other cells such as monocytes, eosinophils, and basophils show high coefficients of variation (CVs) of 40–50% due to their lower absolute numbers [12,13]. Therefore, I could obtain reliable recovery rates only for neutrophils and lymphocytes. After the hydrophoretic filtration, neutrophils and lymphocytes were recovered up to 75 and 70%, respectively, compared with their initial concentrations. The total recovery rate for two cell types was \( \sim 71\% \). There is no significant difference in the recovery rate between the WBC subpopulations. The recovered cells can be used for correlation
studies between certain diseases and global gene expression changes in leukocytes [14].

4.5 Conclusion

The filtration obstacles as a filter play not only the role of filtering large particles blocked by the obstacles but also the role of focusing small particles able to pass through the gap in the obstacle area. From the experimental results, I showed that particles with a minute diameter difference of 7.3% were completely separated through the hydrophoretic filtration. The critical separation diameter can be easily defined by changing the height and gap of the filtration obstacle. In the presented device, WBCs were separated from RBCs with an enrichment ratio of ~210-fold at a throughput of $4 \times 10^3$ s$^{-1}$. A filtration time of ~0.3 s can minimize cell damage from shear stress. Adding the focusing process, the whole process time was ~1.3 s. The filtration device has a simple channel design and can be fabricated using rapid prototyping. This will enable extension to the device with multiple heights and the separation of particles with multiple diameters, individually.
References


CHAPTER V: *Cell cycle synchronization*

*by hydrophoresis*

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5.1 Introduction

Cell cycle synchronization is commonly performed in order to collect cells at the same phase of the cell cycle. The cell cycle is the repeated series of events that consist of four distinct phases: G₁ (G indicating growth), S (S indicating DNA synthesis), G₂, and M (M indicating mitosis) phase. The regulatory factors of the cell cycle are strongly associated with programmed cell death to prevent uncontrolled cell division and repairing genetic damages, thereby any abnormal regulation of the cell cycle is associated commonly with tumor progression [1-3]. The development of gentle, noninvasive methods for cell synchrony is essential to understand the physiological roles of regulatory molecules involved in each phase of the cell cycle and their relationship with oncogenes and tumor suppressor genes for continued advancement in the fields of cell cycle regulation and cancer.

Synchronization methods can be categorized into chemical interference and physical separation. Several chemical methods called ‘whole-culture synchronization’ have been used to synchronize cell cultures by halting the cell cycle at a specific phase [4-8]. For example, aphidicolin blocks the cell cycle at G₁ phase by inhibiting DNA polymerization. Treatment with nocodazole halts cells in M phase by interfering with the polymerization of microtubules and
thereby preventing the formation of metaphase spindles. These batch treatments allow almost the entire cell population to be transformed into a synchronized cohort. However, the major disadvantage of these chemical methods is that normal cellular progression and metabolism are often disrupted by inhibiting DNA synthesis and microtubule polymerization [9].

Physical separation methods are to select cells at a specific stage of the cell cycle from the original, asynchronous cell population typically by using the difference of nucleus size or cell mass according to cell-cycle phases, obtaining unperturbed synchronous cell cultures. Fluorescence-activated cell sorting (FACS) is routinely used to measure and sort cells at a specific phase of the cell cycle by using both fluorescent labels and light scattering [10]. Although this method provides rapid quantitative information about DNA ploidy and cell cycle distribution, it is costly, mechanically complex, and requires a trained operator. The cell size-cycle relationship also allows cell cycle synchronization by size separation of cells in different phases. Many of separation methods enable size separation by utilizing physical forces or hydrodynamic phenomena, and can be used to sort cells by size [11-13]. In particular, centrifugal elutriation has been used for cell cycle synchronization through balancing between the elutriation flow and the centrifugal force against the flow direction [12].
Dielectrophoresis (DEP) has been recently employed for the selection of cells at a specific phase by exploiting the dependence of the DEP force on a particle volume [13]. Although they showed impressive results, they require a complicated equipment setup of a centrifuge, rotor assembly, separation- and elutriation-chamber, or relatively complex fabrication processes such as metal-electrode patterning. Thus, there is a still challenge for the development of a simple, low-cost, noninvasive, and effective method for cell cycle synchronization.

In this chapter, the use of hydrophoretic size separation was demonstrated for the synchronization of mammalian cells that utilizes convective, rotational flows induced from regularly patterned anisotropic microfluidic obstacles without the need for electric or any physical fields. Its performance is characterized experimentally using standard microparticles, and a pure and chemically-interfered population of U937 cells (human leukemic monocyte lymphoma cell line). The robustness of the hydrophoretic method for practical applications was also demonstrated by sorting cells in G0/G1 and G2/M phase out of original, asynchronous U937 cells.
5.2 Principle of Cell Cycle Synchronization by Hydrophoresis

Fig. 5.1 shows the schematics of the hydrophoretic device and its size separation principle. It composes of the slanted groove patterns on the straight channel, functioning as a motive power of transverse particle motions. The anisotropy of the slanted groove patterns generated rotational flows by using a steady axial pressure gradient (Fig. 5.2). Such convective vortices make cells move along the transverse direction. Thus, the cells migrate toward the sidewall 1 following the rotational streams (Fig. 5.1b). At that time, the cell motion is governed by the physical (steric) barrier of the anisotropic microfluidic obstacles as shown in Fig. 5.1c. The cell-obstacle interaction deflects the large cells in G2/M phase downward and makes the cells diffused out of the rotational streamlines. The cells thus follow the stream 1 in Fig. 5.1b and stay near the sidewall 1 without deviation. In contrast, the smaller cells in G0/G1 phase will deviate from the sidewall 1 following the stream 2 in Fig. 5.1b, sorted from the larger cells in G2/M phase.

The height of the obstacle gap $H_{ob}$ is a design parameter that determines whether particles ($D$ in diameter) assume hydrophoretic self-ordering/sorting by the steric hindrance mechanism. As shown in the previous Chapters, the obstacles of $H_{ob} \leq 2D$ hinder the rotational flows of particles and
leads to hydrophoretic self-ordering. The ratio between the channel height $H_{ch}$ and the gap height $H_{ob}$ should be considered because the degree of the particle deflection is determined by the ratio and thereby by the induced pressure gradients. At $H_{ch}/H_{ob} \geq 2$, the magnitude of the pressure gradients is high enough to assure hydrophoretic ordering. To ensure the hydrophoretic size separation of U937 cells whose diameters vary between 11 and 22 $\mu$m, the hydrophoretic device was engineered with $H_{ob} = 24 \mu$m and $H_{ch} = 46 \mu$m.
a) Anisotropic microfluidic obstacles

b) Stream 1 and Stream 2 with sidewalls

c) Flow direction and obstacles

d) Detailed view of flow and markers

Legend:
- Flow direction
- Stream 1
- Stream 2
- Sidewall 1
- Sidewall 2
- Scale: 100 μm
Fig 5.1. Cell cycle synchronization by hydrophoretic size separation. (a) Schematic showing a hydrophoretic device with anisotropic microfluidic obstacles and its size separation of asynchronous cells. (b) Simulated streamlines in the device. The slanted groove patterns on the channel generate rotational flows by using a steady axial pressure gradient. Following these rotational flows, particles cross the channel and reach the sidewall 1. The streamlines starting at the sidewall 1 move upward, i.e., the stream 1, and suddenly traverse the channel as they reach around the bottom of the obstacles, i.e., the stream 2. (c) The larger cells in G2/M phase with a diameter that is similar to the obstacle gap will steer their position downward due to the particle-wall interaction. The cells thus follow the stream 1 and stay at the sidewall 1 without deviation. In contrast, the smaller cells in G0/G1 phase will deviate from the sidewall 1 following the stream 2. (d) A PDMS device consisting of a prefilter to block dusts with a diameter over 30 μm, 200 anisotropic microfluidic obstacles, an expanded outlet region of 1-mm width, and two outlet reservoirs.
Fig. 5.2. CFD-simulation of pressure fields in the microchannel with anisotropic microfluidic obstacles. The channel for simulation is 50 μm wide \( W_{ch} \) and 46 μm deep \( H_{ch} \) with \( H_{ob} = 24 \) μm, \( \theta = 10^\circ \), \( L_{ob} = 20 \) μm, and \( D_{ob} = 30 \) μm. The applied flow rate was 4 μL/min along the flow direction. The pressure gradient is generated in a way of counter-clockwise direction for the flow direction.
5.3 Experimental

5.3.1 Device design and fabrication

Photoresist (PR) molds for the hydrophoretic device incorporating 200 anisotropic microfluidic obstacles and inlet/outlet microchannels were defined on a silicon wafer by using two-step photolithography. As shown in Chapter 2, more than 120 obstacles are required for the complete ordering of beads ranged from 520 nm to 15 µm. To ensure the complete ordering of U937 cells, the hydrophoretic device with 200 obstacles was used. The first and second layers of the PR molds define the channel structures and the obstacles, respectively. For the first-step lithography, a 24-µm layer of SU8 2010 PR (Microchem Corp., MA) was spin-coated on a 4 in. silicon wafer and then exposed to UV light through a photomask. In the second-step, a 46-µm layer of SU8 3025 PR (Microchem Corp., MA) was spin-coated on the previously patterned PR mold and then exposed to UV light through the second photomask that was aligned with respect to the previously developed SU8 patterns. The device was microfabricated in poly(dimethylsiloxane) (PDMS) by using soft-lithography as shown in Fig. 5.1 and 5.2, in which the channel width $W_{ch}$ was 50 µm, the channel height $H_{ch} = 46$ µm, the gap height of the obstacles $H_{ob} = 24$ µm, the
slanting angle of the obstacles $\theta = 10^\circ$, the obstacle thickness $L_{\text{ob}} = 20 \, \mu\text{m}$, and the pitch distance between the obstacles $D_{\text{ob}} = 30 \, \mu\text{m}$. The PDMS microchannel was then irreversibly sealed by plasma activation on a glass slide. The deviations of all dimensions were less than 5%.

5.3.2 Material preparation

Polystyrene beads with diameters of 15 and 20 $\mu\text{m}$ were purchased from Sigma-Aldrich (St. Loui, MO). The beads were prepared in 2% pluronic F68 solution (Sigma-Aldrich) in a concentration of approximately 500 beads/$\mu\text{L}$ and had a coefficient of variation of less than 5% for particle size. Synchronization experiments were performed using U937 cells (human leukemic monocyte lymphoma cell line). The cell line was cultured in RPMI 1640 medium (JBI, Korea) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen, CA), penicillin G (100 U/mL), streptomycin (100 $\mu\text{g/mL}$), and L-glutamine (2 mM) at 37 $^\circ\text{C}$ in a humidified atmosphere containing 5% CO$_2$ and 95% air. For synchronization experiments, U937 cells were centrifuged and resuspended in RPMI medium at a concentration of 1,000 cells/$\mu\text{L}$. Cell diameters range between 11 and 22 $\mu\text{m}$. To arrest U937 cells in M phase, the cells were treated with 100 ng/mL nocodazole (SigmaAldrich) for 16 h.
5.3.3 Computational fluid dynamics simulation

The flow characteristics in the device were modeled by using a commercial computational fluid dynamics software (CFD-ACE+; ESI, Huntsville, AL). The channel for simulation is 50 μm wide \( W_{ch} \) and 46 μm deep \( H_{ch} \) with \( H_{ob} = 24 \) μm, \( \theta = 10^\circ \), \( L_{ob} = 20 \) μm, and \( D_{ob} = 30 \) μm (Fig. 5.2). The total number of cells in the three-dimensional structured grid for the channel was 143298. Upwind scheme is used in the conjugates gradient squared (CGS) and preconditioning (Pre) solvers for velocity field, while algebraic multigrid (AMG) solver is used for pressure correction. The applied flow rate was 4 μL/min along the flow direction (Fig. 5.1b and 5.2).

5.3.4 Data acquisition

The beads and cells were introduced into the hydrophoretic device using a syringe pump (Pump 11 Pico Plus; Harvard Apparatus, Inc., Holliston, MA) at a flow rate of 4 μL/min. They were imaged through a CCD camera (DS-2MBWc; Nikon, Tokyo, Japan) equipped in an inverted microscopy (TS100; Nikon). A commercial image analysis software, i-Solution (IMT i-Solution, Korea) was used to measure the lateral positions and areas of cells in the 1-mm wide outlet region of the device. This program determines the positions and areas by the
calibrated pixel information. The program measures the lines and closed areas drawn by users, and converts their pixel information into metric information.

5.3.5 Flow cytometry

The cell synchrony was confirmed by using a flow cytometry (BD LSR II System; BD Biosciences, CA). Control cells were harvested and fixed in 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 30 min. Sorted cells by the hydrophoretic device were also mixed with 4% paraformaldehyde and fixed at room temperature for 30 min. The fixed cells were washed with 1× PBS and then incubated in 0.25 mL 1× PBS containing 0.03% Triton X-100 (Sigma-Aldrich) and 80 μg/mL RNase (Solgent, Korea) at 37 °C for 30 min. PI solution (1 mg/mL in distilled water) of 12.5 μL was added to each tube and mixed gently. After cell staining at room temperature for 30 min, the cell cycle distribution was determined by the flow cytometry.
5.4 Results and Discussion

5.4.1 Device characterization with standard beads

The hydrophoretic microchannel ($H_{ob} = 24 \, \mu m$ and $H_{ch} = 46 \, \mu m$) is designed so that cells whose diameters range between 11 and 22 $\mu m$ assume the hydrophoretic self-ordering/sorting. The size-separation performance of the hydrophoretic device is first characterized with polystyrene microparticles with diameters of 15 and 20 $\mu m$, to confirm whether the device follows the aforementioned design rules. The applied flow rate was 4 $\mu$L/min. Fig. 5.3 shows that 15- and 20-$\mu$m beads completely maintain their distinct trajectories at the respective lateral positions of $587.9 \pm 33.7$ and $333.1 \pm 18.1 \, \mu m$ in the 1-mm wide outlet region, satisfying the design rules. As shown in Fig. 5.1c, the 20-$\mu$m beads with a diameter that is similar to the obstacle gap stay near the sidewall 1 due to the particle-wall interaction, while the smaller 15-$\mu$m beads deviate from the sidewall 1 following the stream 2 in Fig. 5.1b.

The rotational streamlines and convective flow characteristics are not significantly affected by the operating flow speed. Thus, an increase of the operating flow speed would provide a significant increase in the separation throughput. However, this operational parameter must be considered in the
practical point of view that flowing particles suspended in fluids are subjected
to inertial lift forces such as shear-gradient and wall-induced lift forces. The
particle Reynolds number $Re_p$ is defined as the ratio of the particle inertia to the
viscous force; $Re_p = (\rho D^2 U)/(\mu D_h)$, where $\rho$ is the fluid density, $D$ is the particle
diameter, $U$ is the maximum fluid velocity, and $\mu$ is the dynamic fluid viscosity.
$D_h$ is the hydraulic diameter, defined as $D_h = 2wh/(w + h)$, where $w$ and $h$ are
the width and height of the channel, respectively. At higher $Re_p$ of order 1, the
inertial lift forces would affect particle behaviors even in microchannels. For
the flow rate of 4 $\mu$L/min, the particle Reynolds numbers are 0.21 and 0.38 for
15- and 20-$\mu$m beads, respectively. These relatively low particle Reynolds
numbers allow the hydrophoretic sorting without the significant effect of the
inertial lift forces. Thus, the following experiments were performed under the
operational condition of a flow rate of 4 $\mu$L/min.
Fig. 5.3. Size separation of 15- and 20-μm beads. The optical micrographs (top and middle) and measured position profiles (bottom) show the separated streams of the beads in the 1-mm wide outlet region. The experiments were conducted in the same device, at the same flow rate of 4 μL/min without any alteration of the separation parameters.
5.4.2 Device characterization with mammalian cells

Fig. 5.4a is a representative image obtained after cells passed through the
device having 200 anisotropic microfluidic obstacles. As shown in the figure, I
observed the cell size-dependent distribution that ranged from ~200 to ~600 μm
in the 1-mm wide outlet region. The asynchronous, control cells with areas of
175.8, 215.7, and 282.0 μm² (right-to-left order of the arrow-pointed cells in
Figure 5.4a) were separated into the distinct positions of 541.0, 404.4, and
251.4 μm, respectively. The corresponding diameters of the cells were 15.0,
16.6, and 19.0 μm, respectively. To determine the exact relationship between
the cell area and lateral distribution, the cell area and lateral position was
plotted for the asynchronous cells measured (n = 309). As shown in Figure 3b,
there exists a linear correlation between the two-paired data. In linear regression
analysis, the regression coefficient of the cell area is -1.6 μm/μm². The mean
values for the lateral position and cell area are 444.9 ± 86.4 μm and 197.5 ±
41.8 μm², respectively. The corresponding diameters of the cells are 15.8 ± 1.6
μm. The scatter plot shown in Fig. 5.4b strongly suggests that the hydrophoretic
device is able to sort cells by size that would be in different phases of the cell
cycle. To experimentally confirm that the device indeed differentiates cell cycle
arrest in a specific phase, I then measured and compared M-phase-arrested cells
by nocodazole with the control, asynchronous cells. Nocodazole is a synchronizing agent that arrests cells at or prior to mitosis and then results in an increase of cell size. Fig. 5.4c is a scatter plot resulting from our cell area and position measurements ($n = 238$). The mean values for the lateral position and cell area are $349.6 \pm 85.8$ μm and $237.8 \pm 50.2$ μm$^2$, respectively. The corresponding diameters of the cells are $17.3 \pm 1.8$ μm. Comparing with the control cells, the M-phase-arrested cells measured have an average change in both the cell area of $40.3$ μm$^2$ and lateral position of $-95.3$ μm. The nocodazole-treated cells become larger than the asynchronous, control cells and can be discriminated from the control cells in the device. Truly synchronized cells should have a narrower size distribution than asynchronous, control cells, reflecting the cell size of a normal cell at a specific cell cycle. However, the nocodazole-treated cells are still in a wide size distribution similar to the control cells. This may be attributed to the chemical interference and its effect on the cell physiology. Cooper et al. have revealed that cells treated with nocodazole do not reflect the sizes of cells seen during normal $G_2$ phase of the cell cycle, while they have the DNA content that is comparable with that of $G_2$ phase [14]. These results support that the hydrophoretic device well reflects the physiological change of cells by chemical interference.
Fig. 5.4. Size separation of U937 cells. (a) Optical micrograph showing asynchronous, control cells distributed according to their size in the 1-mm wide outlet region. The applied flow rate is 4 μL/min. (b) Scatter plot of the lateral position in the 1-mm wide outlet region versus the area of the asynchronous cells. (c) Scatter plot of the lateral position in the 1-mm wide outlet region versus the area of nocodazole-treated cells. The horizontal and vertical dashed lines in (b) and (c) denote the mean values for the lateral position and cell area, respectively.
5.4.3 Cell cycle synchronization

The cell size-dependence of the position distribution in the hydrophoretic device described above suggests that the hydrophoretic method can also allow a continuous sorting of cells in target phases by exploiting the relationship between a cell’s size and its phase in the cell cycle (i.e., proliferating cells grow in all phases of the cell cycle but M phase, and tend to double in cell mass before M phase). Cells at different stages of the cell cycle can be distinguished by the amount of DNA they contain. Cells in $G_2/M$ phase have two copies of DNA and accordingly have two times higher fluorescence intensity than cells in $G_0/G_1$ phase. Therefore, the fluorescence intensity range of $G_0/G_1$ phase can be de-convoluted by fitting the $G_0/G_1$ peak, the large peak of the cell cycle histogram as a Gaussian curve. I, then, set the intensity range approximately two times higher than the $G_0/G_1$ range as $G_2/M$ phase. The synchronization performance of the hydrophoretic device was characterized by sorting target cells in $G_0/G_1$ and $G_2/M$ phases out of asynchronous, control cells. After harvesting U937 cells from culture, I resuspended them in fresh culture medium and injected the cells into the hydrophoretic device at a throughput of approximately $2.4 \times 10^5$ cells per hour per microchannel. After the hydrophoretic size separation, I collected cells from the range of 0 to 300 $\mu$m
and of 400 to 1000 μm of the 1-mm wide outlet region for target cells in G₀/G₁ and G₂/M phases, respectively. Fig. 5.5a and 5.5b are histograms resulting from flow cytometry analysis of initial, asynchronous cells and sorted cells in G₀/G₁ phase. As shown, there is a significant decrease of the cell count in G₂/M phase after the hydrophoretic size separation. Based on this histogram, the ratio of G₀/G₁ to G₂/M cells increases to 22.1:1 from their initial ratio of 5.2:1, and the cell synchrony in G₀/G₁ phase reaches approximately 95.5%. Fig. 5.4c and 5.4d are histograms that verify the successful depletion of cells in G₀/G₁ after the hydrophoretic size separation. When comparing the histograms before and after the hydrophoretic separation, the ratio of G₂/M to G₀/G₁ cells increases to 5.8:1 from their initial ratio of 0.25:1, and the cell synchrony in G₂/M phase reaches 85.2%.
Fig. 5.5. Sorting of U937 cells in G₀/G₁ and G₂/M phases. (a) Histogram of the DNA content of control, unsorted cells. The initial asynchronous cells have a G₀/G₁:G₂/M ratio of 5.2:1. (b) Histogram of the DNA content of sorted cells in G₀/G₁ phase. After the size separation by hydrophoresis, the collected cells from the range of 400 to 1000 μm in the 1-mm wide outlet region have a G₀/G₁:G₂/M ratio of 22.1:1. (c) Histogram of the DNA content of control, unsorted cells. The initial asynchronous cells have a G₂/M:G₀/G₁ ratio of 0.25:1. (d) Histogram of the DNA content of sorted cells in G₂/M phase. After the size separation by hydrophoresis, the collected cells from the range of 0 to 300 μm in the 1-mm wide outlet region have a G₂/M:G₀/G₁ ratio of 5.8:1.
5.5 Conclusion

In summary, a hydrophoretic method have been described for sorting cells in target phases of the cell cycle entirely based on a hydrodynamic principle, the hydrophoretic size separation that uses convective, rotational flows induced from regularly patterned anisotropic microfluidic obstacles without the need for electric or any physical fields. With this method, I have demonstrated a linear relationship between a cell size and its position distribution in the hydrophoretic device and its use for sorting cells by size that were in different phases of the cell cycle, achieving a high level of cell synchrony. From a practical point of view, the hydrophoretic device is simple, noninvasive, effective, and does not require any external power for cell cycle synchronization. While I demonstrated cell cycle synchronization in the experiments reported here, this device could be used in tumor cell detection and in tumor cell studies of the effects of pharmacological agents on cell cycle and cell death.
References


CHAPTER IV: Conclusion

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6.1 Summary

Hydrophoresis started with an effort to solve the size matters of biology in purely hydrodynamic environments. The separation processes are driven by convective, rotational flows across a microchannel, which result from the anisotropy of a fluidic resistance. The hydrophoretic separation principles are in fact governed by the physical (steric) barrier of the anisotropic obstacles. The equilibrium position in hydrophoretic ordering then depends only on particle size. The resulting devices operate at high-resolutions. One percent difference in particle size at minimum was resolved in a sorting time of ~0.2 s. The DNA mixture of 49 and 115 kb was separated into two streams for a separation time of ~0.12 s over the channel length of 5 mm. The separation throughput was \(1.7 \times 10^6\) molecules/s. Further, I showed that the relative difference between the particle diameter and the obstacle height played a critical role in the operation of the hydrophoretic devices. When engineering hydrophoretic devices, the diameter of particles should be over a critical particle diameter, the half of the obstacle gap to ensure hydrophoretic ordering of the particles. The hydrophoresis mechanism was verified by measuring hydrophoretic particle ordering in three dimensions, and by conducting a computational simulation and comparing the simulation results with the experimental measurements.
The robustness of the hydrophoretic method for practical applications has been proved by sorting blood cells, and mammalian cells according to their cell-cycle phases. In the hydrophoretic filtration device, RBCs are aligned parallel to the filtration obstacles of 4.0 μm-height due to their small thickness of 1.7–2.6 μm and deformability and thus pass through the obstacles, separating from WBCs. In the presented device, WBCs were separated from RBCs with an enrichment ratio of ~210-fold at a throughput of $4 \times 10^3 \text{ s}^{-1}$. A filtration time of ~0.3 s can minimize cell damage from shear stress. The recovery rate of WBCs was 71–85 %. There is no significant difference in the recovery rate between the WBC subpopulations. The recovered cells can be used for correlation studies between certain diseases and global gene expression changes in leukocytes. I have also demonstrated the use of hydrophoretic size separation to sort cells in target phases of the cell cycle. With this method, I found that there is a linear relationship between a cell’s size and its position distribution in the hydrophoretic device. I finally demonstrated the robustness of the hydrophoretic method for practical applications by sorting cells in $G_0/G_1$ and $G_2/M$ phase out of original, asynchronous cells with a high level of synchrony of 95.5% and 85.2%, respectively.
6.2 Outlook, Challenges, and Future Works

In the field of cell separation and preparation, this thesis provides a high degree of sophistication that allows easy size separation of biological objects in high-resolution manner. The separation principle described throughout this thesis, *hydrophoresis*, has been realized by solving real biological issues as well as providing proof-of-concept forms. Nonetheless, the ultimate usefulness of the hydrophoresis has yet to be established, remaining several significant challenges.

Efforts to extend separation range are required for many other biological and environmental applications. The current design of hydrophoresis was not optimized with respect to separation range. The hydrophoretic design for size separation have shown to be sensitive to individually separate particles more than the half height of the obstacle gap in diameter, but not to resolve particles less than its half height as shown in the experimental results of Chapter 2. Thus, the separation range is restricted between the half height of the gap and its full height in particle diameter. In addition, the device has to be newly designed and fabricated with appropriate dimensions, as changing separation targets with ones of new size. These issues could be overcome by using elastic deformation of polymer microchannels that tune the height of the
obstacle gap, the criterion for hydrophoretic particle sorting. The partial channel tuning can also change the microchannels into many different heights that can broaden the separation range of hydrophoresis.

Improvements of separation resolution in submicron and nano-scales need to be achieved for biomolecular applications. As shown in the experimental results of Chapter 2, the ordering trajectories of submicron beads were not resolved according their size and overlapped each other. As mentioned, I can presume that this phenomenon results from low size selectivity that is insufficient to resolve submicron beads by their size. However, even in submicron and nano-scales, the hydrophoretic device can separate large particles in hydrophoretic ordering from relatively smaller particles in free flow. Efforts to improve the separation resolution of hydrophoretic devices should be continued for multiple size separation in submicron and nano-scales by optimizing the hydrophoretic design such as obstacle shapes.

The throughput issue of hydrophoretic size separation needs to be addressed to solve real biological problems that have not been solved with conventional separation methods. Conventional fluorescence-activated cell sorters are capable of processing from thousands to tens-of-thousands of particles per second. However, the current hydrophoretic devices have been
limited in throughput rate of tens to thousands of particles per second. Thus, continued efforts should be addressed to solve this problem by incorporating multiple separation channels in a single device.

Finally, more research needs to extend the hydrophoresis application to real biological and environmental issues and to be best used for a given application. The proposed applications discussed in Chapter 4 and 5 provide that the hydrophoresis can achieve comparable results with conventional separation methods in more effective, viable and easier ways. However, it still works as a single, individual component in whole biochemical studies. For complete biochemical analysis, it is required to optimize interfaces among separation, lysis, mixing, and detection units incorporating on-chip pumps and valves. This integration will be helpful to extend the hydrophoresis application. The advantages of hydrophoresis such as a sheathless method, passive operation, and single channel can facilitate its integration into multi-functional devices for biochemical analysis. Once fully developed, these devices will offer new opportunities for personalized diagnostics and point-of-care testing with advantages of low sample and reagent volumes, and short analysis time.
Appendix A: Fabrication process flow

Starting material: 4 inch-diameter Silicon wafers.

The process flow listed below is for two-step lithography.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Machine</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SiO₂ removal</td>
<td>acid-hood</td>
<td>Dipping in BOE more than five times</td>
</tr>
<tr>
<td>2</td>
<td>Dehydration</td>
<td>oven</td>
<td>200°C, 30 min</td>
</tr>
<tr>
<td>3</td>
<td>SU8 spin</td>
<td>spin coater</td>
<td>SU8 2010 (20 µm thickness) Ramp to 500 rpm and hold for 10 seconds, and then ramp to 1000 rpm and hold for 30 seconds</td>
</tr>
<tr>
<td>4</td>
<td>Soft bake</td>
<td>oven</td>
<td>Slow ramp from 65°C to 95°C, hold at 95°C for 3 min</td>
</tr>
<tr>
<td>5</td>
<td>UV expose</td>
<td>UV aligner</td>
<td>10 sec at 20.3 mW/cm²</td>
</tr>
<tr>
<td>6</td>
<td>Post-expose bake</td>
<td>oven</td>
<td>Slow ramp from 65°C to 95°C, hold at 95°C for 2 min</td>
</tr>
<tr>
<td>7</td>
<td>Develop</td>
<td>hood</td>
<td>3 min soak in SU8 developer and then wash with IPA and water</td>
</tr>
<tr>
<td>8</td>
<td>SU8 spin</td>
<td>spin coater</td>
<td>SU8 3025 (40 µm thickness) Ramp to 500 rpm and hold for 10 seconds, and then ramp to 2000 rpm and hold for 30 seconds</td>
</tr>
<tr>
<td>Step</td>
<td>Process</td>
<td>Equipment</td>
<td>Details</td>
</tr>
<tr>
<td>------</td>
<td>---------------</td>
<td>-----------</td>
<td>---------</td>
</tr>
<tr>
<td>9</td>
<td>Soft bake</td>
<td>oven</td>
<td>Slow ramp from 65°C to 95°C, hold at 95°C for 15 min</td>
</tr>
<tr>
<td>10</td>
<td>UV expose</td>
<td>UV aligner</td>
<td>20 sec at 20.3 mW/cm²</td>
</tr>
<tr>
<td>11</td>
<td>Post-expose bake</td>
<td>oven</td>
<td>Slow ramp from 65°C to 95°C, hold at 95°C for 3 min</td>
</tr>
<tr>
<td>12</td>
<td>Develop</td>
<td>hood</td>
<td>5 min soak in SU8 developer and then wash with IPA and water</td>
</tr>
<tr>
<td>13</td>
<td>Silanize</td>
<td>hood</td>
<td>Put 10 µL of HMDS into a cup in a vacuum jar. Place the wafer in the jar for 1 hr.</td>
</tr>
</tbody>
</table>
Appendix B: Computational modeling of hydrophoresis

Matlab 7.0 was used to perform the simulation. This modeling program calculates the trajectory of particles in the assumption that the particles follow the instant velocity vector on the particle center and their position exists at a distance from channel walls that is larger than their radius. The code is listed below.

```
% clear all

% load velocity data to current workspace
% variables: DX DY DZ DU DV DW
load velocity
X = DX; Y = DY; Z = DZ; U = DU; V = DV; W = DW;

% load obstacle geometric information by manual (in mm)
x_temp = 192*10^-6;
x0 = 192*10^-6 - x_temp;
x1 = 209*10^-6 - x_temp; %
x2 = 238*10^-6 - x_temp; % for the leading edge of the obstacle
x3 = 255*10^-6 - x_temp; %%
x4 = 284*10^-6 - x_temp; % for the back edge of the obstacle
x5 = 300*10^-6 - x_temp;

x5_temp = x5;
x0_temp = x0;
x0 = x0 - (x5_temp - x0_temp);
x1 = x1 - (x5_temp - x0_temp);
x2 = x2 - (x5_temp - x0_temp);
```
\[ x3 = x3 - (x5_{\text{temp}} - x0_{\text{temp}}); \]
\[ x4 = x4 - (x5_{\text{temp}} - x0_{\text{temp}}); \]
\[ x5 = x5 - (x5_{\text{temp}} - x0_{\text{temp}}); \]

\[ y_{\text{max}} = \max(\max(DY{\{1\}})); \]
\[ z_{\text{max}} = \max(\max(DZ{\{1\}})); \]
\[ y_{\text{min}} = \min(\min(DY{\{1\}})); \]
\[ z_{\text{min}} = \min(\min(DZ{\{1\}})); \]

\[ zm = 1.9000e-005; \]
\[ x_{\text{index}} = \text{length}(DX); \]

for \( m = 1:1:x_{\text{index-1}} \)

\[ \Delta X(m) = X(m+1) - X(m); \]
end

\[ \Delta X(x_{\text{index}}) = x5_{\text{temp}} + x_{\text{temp}} - X(x_{\text{index}}); \]

\% load particle radius and initial positions (in mm)
\( r = 2 \times 10^{-6} \); \% particle radius
\( \text{width}_{\text{pt}} = 2 \); \% the number of positions in which particles are injected along width
\( \text{height}_{\text{pt}} = 2 \);
\( \text{total}_{\text{pt}} = \text{width}_{\text{pt}} \times \text{height}_{\text{pt}} \); \% the total number of positions in which particles are injected

\( \text{width}_{\text{count}} = 0; \)
\( \text{height}_{\text{count}} = 0; \)

for \( i = 1:1:\text{total}_{\text{pt}} \)

if \( \text{width}_{\text{count}} == \text{width}_{\text{pt}} \)

\( \text{width}_{\text{count}} = 0; \)

\( \text{height}_{\text{count}} = \text{height}_{\text{count}} + 1; \)

\[ y = (y_{\text{max}} - y_{\text{min}} - 2 \times r)/(\text{width}_{\text{pt}} - 1) \times \text{width}_{\text{count}} + y_{\text{min}} + r; \]

\[ z = (z_{\text{max}} - z_{\text{min}} - 2 \times r)/(\text{height}_{\text{pt}} - 1) \times \text{height}_{\text{count}} + z_{\text{min}} + r; \]

\( \text{width}_{\text{count}} = \text{width}_{\text{count}} + 1; \)

else

\[ y = (y_{\text{max}} - y_{\text{min}} - 2 \times r)/(\text{width}_{\text{pt}} - 1) \times \text{width}_{\text{count}} + y_{\text{min}} + r; \]

end
\[ z = (z_{\text{max}} - z_{\text{min}} - 2*r)/(\text{height}\_\text{pt}-1)*\text{height}\_\text{count} + z_{\text{min}} + r; \]

\[ \text{width}\_\text{count} = \text{width}\_\text{count} + 1; \]

end

particleY(1,i) = y;
particleZ(1,i) = z;
particleX(1,i) = 0;
end

n\_obstacles = 20; % obstacle number

% stream\_line generation of the injected particles passing through obstacles
nx\_plane = y_{\text{max}} - y_{\text{min}}; % normal vector against obstacle plane
ny\_plane = x1 - x2;

marching\_index = 1; % index for particleX/Y/Z

for i=1:n\_obstacles
    x0\_old = x0;
    x5\_old = x5;

    x1 = x1 + (x5\_old - x0\_old);
    x2 = x2 + (x5\_old - x0\_old);
    x3 = x3 + (x5\_old - x0\_old);
    x4 = x4 + (x5\_old - x0\_old);
    x0 = x5;
    x5 = x5 + (x5\_old - x0\_old);
    alignment\_index = i;

    marching\_index\_temp = marching\_index;

    for k=1:total\_pt
        marching\_index = marching\_index\_temp;

        % code

    end

end
for j=1:1:n_index % generation of streamlines along x-direction
    marching_index = marching_index + 1;
    x_new = particleX(marching_index-1, k);
    x_new = x_new + deltaX(j);
    particleX(marching_index, k) = x_new;
    y_new = particleY(marching_index-1, k);
    z_new = particleZ(marching_index-1, k);
    t_new = interp2(Z(j):1:, Y(j):1:, U(j), z_new, y_new, 'spline');
    w_temp = interp2(Z(j):1:, Y(j):1:, W(j), z_new, y_new, 'spline');
    u_temp = interp2(Z(j):1:, Y(j):1:, U(j), z_new, y_new, 'spline');
    v_temp = interp2(Z(j):1:, Y(j):1:, V(j), z_new, y_new, 'spline');
    t_temp = deltaX(j)*u_temp;
    y_new = y_new + v_temp*t_temp;
    z_new = z_new + w_temp*t_temp;
    QP3 = [x_new-x1 y_new-ymin z_new-zm]; % the vector between the particle position and
    QR3 = [x2-x1 ymax-ymin 0]; % the vector for the leading edge of the upper obstacle
    Q3 = cross(QP3, QR3);
    D3 = (dot(Q3,Q3)/dot(QR3, QR3))^0.5; % the distance between the particle and the leading
    edge
    % before the upper obstacle
    if (nx_plane*(x_new - x1) + ny_plane*(y_new - ymin) < 0) & (abs(nx_plane*(x_new - x1)
        + ny_plane*(y_new - ymin)) < r*(nx_plane^2 + ny_plane^2)^0.5) & (z_new <= zm) & (D3 < r) % below the obstacle surface but the particle collides
    with the obstacle
        y_count = (ymax - y_new)/(0.4*10^-6);
    for delta=1:1:y_count
        y_search = y_new + delta*0.4*10^-6;
    end
\[ QP = [x_{\text{new}}-x_1 \ y_{\text{search}}-y_{\text{min}} \ z_{\text{new}}-z_m]; \]
\[ QR = [x_2-x_1 \ y_{\text{max}}-y_{\text{min}} \ 0]; \]
\[ Q = \text{cross}(QP, QR); \]
\[ D = \text{dot}(Q, Q)/\text{dot}(QR, QR)^{0.5}; \]

if \( D \geq r \)
\[ y_{\text{new}} = y_{\text{search}}; \]
break;
end
end

alignment(alignment_index) = 1;

elseif \( z_{\text{new}} > zm \) % above the obstacle surface
\[ y_{\text{count}} = (y_{\text{max}} - y_{\text{new}})/(0.4*10^{-6}); \]

for \( \delta = 1:1:y_{\text{count}} \)
\[ y_{\text{search}} = y_{\text{new}} + \delta*0.4*10^{-6}; \]

if \( \text{abs}(nx_{\text{plane}}*(x_{\text{new}} - x_1) + ny_{\text{plane}}*(y_{\text{search}} - y_{\text{min}})) \geq r*(nx_{\text{plane}^2} + ny_{\text{plane}^2})^{0.5} \)
\[ y_{\text{new}} = y_{\text{search}}; \]
break;
end
end

alignment(alignment_index) = 1;
end
end

% position correction by alignment effects induced from boundaries
if \( y_{\text{new}} < y_{\text{min}} + r \);
\[ y_{\text{new}} = y_{\text{min}} + r; \]
elseif \( y_{\text{new}} > y_{\text{max}} - r \);
\[ y_{\text{new}} = y_{\text{max}} - r; \]
end

if z_new < zmin + r
    z_new = zmin + r;
elseif z_new > zmax - r
    z_new = zmax - r;
end

particleY(marching_index, k) = y_new; % time marching for Y position
particleZ(marching_index, k) = z_new; % time marching for Z position
end
end

save position particleX particleY particleZ r alignment n_obstacles

% % % % % % % % % % % % % % % % % % % % % % % % % % % % % % % % % % % % % % %
Summary in Korean

미세입자 및 세포 분리를 위한 유체영동기술

생체시료는 대개 혼합물 형태로 존재하며 특정 세포 및 분자의 분석을 위해서 표적 세포 및 분자의 정체는 필수적인 전처리 과정이다. 표적 세포 및 분자의 정체를 위해서, 세포나 분자마다 다르게 갖는 고유의 표현형 (phenotype) 을 이용하게 되는데 이는 세포의 크기나 형태 또는 세포막 단백질일 수 있다. 본 논문에서는 여러 표현형 중 생체시료의 크기문제에 집중하며, 이를 효과적으로 해결하기 위한 기술로써 유체영동을 개발하여 DNA분자, 혈액세포의 분리 및 세포주기의 동기화에 응용함으로써 그 유용성을 검증하였다.

유체영동은 순수하게 유체역학적인 원리만을 이용해 생체시료의 크기문제 해결하기 위한 기술로 개발되었다. 따라서 기존의 분리기술이 갖고 있던 문제점인 복잡한 제작공정, 세포의 생리활성도에 영향을 줄 수 있는 분리환경, 정교한 유동제어의 필요성을 없앤으로써 누구나 손쉽게 생체시료의 크기별 분리를 위해 유체영동을 이용할 수 있다. 유체영동을 위한 미세유체소자는 미세 가공 기술로 제작된 경사구조물을 주요한 구성요소로 갖는다. 경사구조물은 구조물의 측면
에 비해 아랫면이 좀더 불균일한 유체저항을 갖고 있으며 이로 인해 유체유동방향의 수직으로 2차 대류유동을 유도한다. 이때 대류유동은 유체유동의 반시계방향으로 형성되며, 이러한 회전유동을 따라 입자가 외부의 물리력 없이 채널 내를 이동할 수 있게 된다. 이후 크기별 분리는 입자의 크기로 의한 입체 장해 (steric hindrance) 현상에 의해 발생한다. 회전유동 중 입자는 경사구조물의 천장면으로 정렬한다. 이때 입자의 크기가 작을수록 천장면 가까이에 위치하며 더 큰 측면 유동에 노출된다. 이 때문에 크기가 작은 입자일수록 측면유동에 더 많이 노출되어 이동하며, 유체유동에 의해 자기정렬하는 입자의 크기별 측면위치가 달라지게된다. 유체유동에 의한 입자의 자기정렬 및 크기분리는 500 nm에서 15 μm 사이의 크기를 갖는 미세입자를 이용해 검증하였다. 또한 49 kb 와 115 kb 크기를 갖는 DNA분자의 분리 를 통해 유체유동에 의해 생체분자의 크기별 분리가 가능함을 확인하였다. 이러한 입자정렬실험을 통해서 입자의 크기와 경사구조물높이의 상대적인 차이가 입자의 자기정렬을 결정함에 있어 중요한 역할을 한다는 것을 밝혔다. 주어진 입자의 자기정렬을 위해서 경사구조물의 높이를 입자지름의 두배보다 작게 설계해야 한다.

경사구조물에 의해 유도된 대류유동은 3차원으로 변화하는 특성을 갖고 있다. 따라서 3차원 대류유동을 입자의 분리에 이용하는 유
체형동의 원리를 규명하기 위해 업자의 유동계적을 3차원으로 측정 및 예측하는 과정은 필수적이라고 할 수 있다. 유체형동의 3차원 측정을 위해 거울이 내장된 미세채널을 미세 제작 공정을 통해 제작하였다. 거울이 내장된 미세채널은 채널 내에 내장된 거울을 통해 반사된 측면위치와 정면위치를 동시에 측정할 수 있기 때문에 복잡한 광학장비없이 미세입자의 3차원 측정을 가능하게 한다. 이를 이용해 4 μm와 15 μm 크기를 갖는 미세입자의 유동계적을 3차원으로 측정하였으며 이를 통해 유체형동의 원리를 증명할 수 있었다. 또한 입자의 크기에 의한 입체장해현상이 입자의 유동계적에 어떠한 차이를 가져 오는지 수치해석을 통해 확인해 보았으며 채 유체형동의 원리를 추가적으로 검증하였다.

이러한 이해를 바탕으로 유제형동을 이용해 실제 생체시료의 크기문제를 해결하는 실험을 수행하였다. 혈액세포의 분리는 면역세포 검사, 수혈, 백혈구의 기능연구 등의 다양한 목적을 위해 필요로 되는 기술이다. 혈액세포의 분리는 세포의 크기 차이를 이용하는데 실제로 적혈구의 지름은 백혈구의 지름과 유사한 6.2~7.3 μm이다. 이 때문에 멜브레인필터를 이용할 경우 혈구의 분리효율이 낮을 수 밖에 없다. 따라서, 유체형동을 이용한 혈액세포의 분리는 적혈구가 큰 지름과는 달리 1.7~2.6 μm의 얇은 두께를 갖는다는 점과 백혈구
보다 변형률이 크다는 점을 이용하였다. 적혈구는 유체영동소자를 구성하고 있는 4 \( \mu \)m 높이의 여과구조물에 평형하게 정렬되고 통과하는 반면 이보다 큰 크기를 갖는 백혈구의 경우 여과구조물을 통과하지 못하고 적혈구로부터 분리된다. 이러한 여과원리를 이용하여 210배의 높은 농축율을 갖고 백혈구를 분리할 수 있었다. 또한 초당 4000개의 세포를 분리하여 유체영동소자내로 주입된 백혈구 중에 71~85%를 출구에서 회수하였다. 이렇게 분리된 백혈구는 면역세포검사, 수혈, 백혈구의 기능연구 등에 유용하게 이용될 수 있을 것이다.

세포주기와 관련된 연구도 생물학에 있어서 중요한 크기문제 중에 하나이다. 동물세포의 경우 DNA복제나 세포분열이 세포주기에 의해 역제하게 조절되고 있다. 이러한 세포조절의 균형이 깨지게 되면 유전적 변형 등이 유발되어 양세포로 발전할 수 있다. 이 때문에 각 세포주기에 관여하는 조절인자의 생리학적 역할 및 발암유전자와의 관계를 밝히는 연구가 중요하다. 또한 이러한 연구를 위해 특정 주기의 세포를 분리하는 기술이 필요하다. 이를 세포주기동기화 (cell cycle synchronization) 기술이라 한다. 유체영동을 이용한 세포주기동기화 기법개발에는 M-phase의 세포가 G1-phase의 세포보다 2배 정도 크다는 점을 이용하였다. 실험에는 11~22 \( \mu \)m의 크기를 갖는 U937세포를 이용하였으며 세포의 크기범위를 포함하도록 경사구조물
의 높이를 24 μm로 설계하였다. 유체영동소자를 이용한 유체영동에 의해 크기별로 분리된 세포의 분리위치와 세포의 크기 사이에 선형적 상관관계가 있음을 밝혔다. 또한 17 μm보다 작은 세포와 19 μm보다 큰 세포를 분리함으로써 특정주기의 세포를 분리하는 실험을 수행하였다. 분리 후에는 형광염료로 세포핵을 염색한 후 형광세기를 유세포분리기를 이용해 측정하였다. 17 μm보다 작은 세포를 분리한 경우, 대부분의 세포가 G1-phase에 속했으며 분리 후에 G1-phase 세포의 비율이 83.8%에서 95.5%로 높아졌다. 19 μm보다 큰 세포를 분리한 경우, 대부분의 세포가 G2/M-phase에 속했으며 분리 후에 G2/M-phase 세포의 비율이 19.7%에서 85.2%로 높아졌다. 이를 통해 유체영동기술이 세포주기동기가 화 실험에 응용될 수 있음을 확인하였다. 이는 앞으로 발암유전자와 세포주기와의 관계, 세포주기와 사멸에 의약품이 미치는 영향 등에 유용하게 이용될 수 있을 것이다.

본 연구를 통해서 마치 엑셀에 숫자를 붙이고 분류 버튼을 누르면 숫자가 정렬되며 분리되는 것처럼 생체시료를 미세채널에 주입하는 것만으로도 쉽고 효과적으로 크기별분리가 가능한 기술인 유체영동을 개발하였다. 앞으로 유체영동 기술이 더 완벽한 분리기술이 되기 위해서 보완해야할 연구과제는 다음과 같다. 우선 유체영동에 의한 크기별 분리범위를 넓히는 연구를 수행해야할 것이다. 현재 유체
영동소자의 크기별 분리범위는 경사구조물의 높이와 그 높이의 절반으로 확정된다. 나노미터에서 마이크로미터에 이르는 다양한 입자의 크기별 분리에 유체영동을 이용하기 위해서는 유체영동소자의 분리범위를 넘히는 연구를 수행해야할 것이다. 두번째로 분리량을 들 수 있 다. 현재 유효영동에 의한 세포 분리량은 최대 초당 수천개로 제한되 다. 기존의 유세포분석기가 초당 수만에서 수십만개의 세포를 측정, 분리할 수 있음을 감안할 때, 유효영동의 분리량을 높이는 연구가 수행되어야할 것이다. 마지막으로 단순히 생체입자의 크기별 분리에 그 치는 것이 아니라 이를 기반으로 실제 생물학 또는 환경공학에 관련된 문제를 해결하는데 그 노력을 기울여야 할 것이다. 이러한 문제점들을 개선 보완한다면 유체영동이 생체시료의 크기별분리에 있어 최고의 기술이 될 수 있을 것이라고 생각한다.