Micro-imaging techniques for evaluation of plastic microfluidic chip

Jung Kyung Kim, Hyunwoo Bang, Yongku Lee, Chanil Chung, Jung Yul Yoo, Sang Sik Yang, Jin Seung Kim, Sekwang Park, and Jun Keun Chang

Abstract – **The Fluorescence-Activated Cell Sorter (FACS) is a well-established instrument used for identifying, enumerating, classifying and sorting cells by their physical and optical characteristics. For a miniaturized FACS device, a disposable plastic microchip has been developed which has a hydrodynamic focusing chamber using soft lithography. As the characteristics of the spatially confined sample stream have an effect on sample throughput, detection efficiency, and the accuracy of cell sorting, systematic fluid dynamic studies are required. Flow visualization is conducted with a laser scanning confocal microscopy (LSCM), and three-dimensional flow structure of the focused sample stream is reconstructed from 2D slices acquired at 1 μm intervals in depth. It was observed that the flow structure in the focusing chamber is skewed by unsymmetrical velocity profile arising from trapezoidal cross section of the microchannel. For a quantitative analysis of a microscopic flow structure, Confocal Micro-PIV system has been developed to evaluate the accelerated flow field in the focusing chamber. This study proposes a method which defines the depth of the measurement volume using a detection pinhole. The trajectories of red blood cells (RBCs) and their interactions with surrounding flow field in the squeezed sample stream are evaluated to find optimal shape of the focusing chamber and fluid manipulation scheme for stable cell transporting, efficient detection, and sorting**

Index Terms – **FACS, microfluidics, microchip, soft lithography, hydrodynamic focusing.**

I. INTRODUCTION

The Fluorescence-Activated Cell Sorter (FACS) is one of the most useful technologies in clinical laboratories. Cells can be identified, enumerated, classified and sorted by their physical and optical characteristics using the FACS. In a typical FACS machine, cells are arranged in a single file and illuminated by a light beam progressing orthogonal to the flow axis. Scattered light is sensed by detectors and converted to a form suitable for computer storage and subsequent analysis. Hydrodynamic focusing is used to place the cells in a single file as they pass through a laser beam for detection and evaluation. The width of the focused stream can be controlled by varying the relative flow rates or pressures of sample and sheath fluids [1].

Miniaturization of analytical biochemical systems using microfabrication and microfluidic technologies can reduce the necessary sample volume, analysis time, and the consumption of expensive reagents. Within the past decade, a variety of miniaturized devices have been developed to replace the routine biochemical processes performed with conventional analytical instruments. Recent efforts are concentrated on the integration of these microdevices on a single chip, called Lab-on-a-

Manuscript received August 12, 2001; revised September 7, 2001.

J. K. Kim are with Biomedlab Co.

H. Bang, Y. Lee, and J. Y. Yoo are with School of Mechanical and Aerospace Engineering, Seoul National University.

C. Chung is with Digital Bio Technology Co.

S. S. Yang is with School of Electronics Engineering, Ajou University.

J. S. Kim is with School of Science and Technology, Chonbuk National University.

S. Park is with School of Electronics and Electrical Engineering, Kyungpook National University.

K. Chun, and J. K. Chang are with School of Electrical Engineering and Computer Science, seoul National University.

(e-mail : jkchang@amed.snu.ac.kr) Tel : +2-880-1766

chip (LOC), which have all types of analytical functions such as sample preparation, chemical reaction, analysis, and detection. The traditional FACS machine composed of fluidics, optics, and electronics is one of the promising candidates that will be realized on integrated microfluidic circuits by LOC technologies [2, 3].

Microfluidics is an essential part of LOC technologies, which focuses on the handling and transportation of nanoliter or picoliter sample volumes by fluid flows on microchips. A low Reynolds number flow is a typical characteristic of these small-scale devices. The fluid dynamics in this regime is very different from the macroscopic phenomena [4]. In order to evaluate the microscale flow physics, as well as build a quantitative measurement tool for microfluidic devices, microscale particle image velocimetry (Micro-PIV) has been developed by several research groups. Since the first design of the Micro-PIV system by Santiago et al. [5], it has been utilized for the measurements of a microchannel flow [6], the flow structure inside an inkjet printhead [7], deformation rate of hydrogel microstructures [8], and a microflow past an obstacle array and bubble [9].

In light of technical aspects compared with conventional PIV, two important issues should be resolved in Micro-PIV. One is selecting the method to determine measurement plane in a volume-illuminated test section, and the other is eliminating the Brownian effect of seed particles. As addressed by Olsen and Adrian [10], the Brownian random motion of seed particles limits the accuracy of PIV significantly and this problem can be reduced by an ensemble averaging technique adopted by Santiago et al. in their studies [5]. The current methods of selecting and defining the depth of the measurement plane are carried out by the image processing technique, which considers the focusing characteristics of the recording optics after acquiring particle images [9, 11, 12].

A disposable plastic microchip was developed, and fabricated using a micromachining technology called soft lithography [13], which enables the design of inexpensive and flexible miniaturized fluidic devices. The microchip, which will be integrated later with other optical and electronic modules for micro-FACS device, contains the hydrodynamic focusing chamber where the sample and sheath fluids are driven by volume injection method using syringe pumps. A precisely controlled sample and sheath fluid volumes would guarantee that the width of the focused stream is stable and predictable by a simple continuity equation. The characteristics of the focused sample flow can affect sample throughput, detection efficiency, and the accuracy of cell sorting. An alternative to this hydrodynamic focusing is the electrokinetic focusing technique [14], which can be easily implemented in microscale devices. However, it requires high voltage power supply for increasing throughput, and multiple samples have different velocities according to their electrokinetic mobilities, which can result in unwanted sample separation.

Flow visualization is conducted with a laser scanning confocal microscopy (LSCM). Hitt and Lowe first proposed confocal imaging of flows in microfabricated tubes [15]. Three-dimensional flow structure of spatially confined sample stream is reconstructed from depth-wise slices acquired at 1 μm intervals. For a quantitative analysis of a microscopic flow structure, Micro-PIV system is designed to evaluate the hydrodynamic focusing characteristics on the plastic microfluidic chip. The depth of the measurement volume is defined from recording particle images using the pinhole technique adopted from confocal microscopy. The trajectories of the red blood cells (RBC) and their interactions with surrounding flow fields in the squeezed sample stream are observed to find optimal geometric shape of the focusing chamber and also the operating scheme for reliable cell transporting and efficient cell detection by optics.

II. Experimental Methods

The cross-channel was fabricated in poly (dimethylsiloxane) (PDMS) by soft lithography [13] which is based on replica molding. Negative master devices were fabricated in silicon by photolithography and etching processes, and were used as molds for PDMS. PDMS and silicone elastomer curing agent were mixed together and pumped in an evacuated chamber for 30 minutes to remove air bubbles. Then the liquid PDMS was poured on the mold and cured in an oven at 85°C for 3 hours. After this procedure, the device could be peeled from the silicon master and would bond reversibly or irreversibly to glass. Air plasma was used to seal a

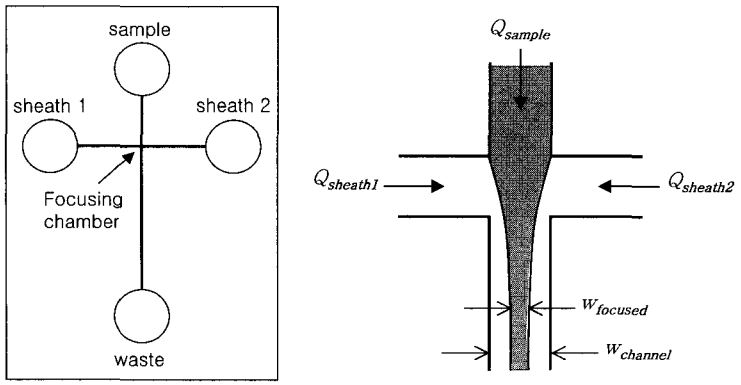
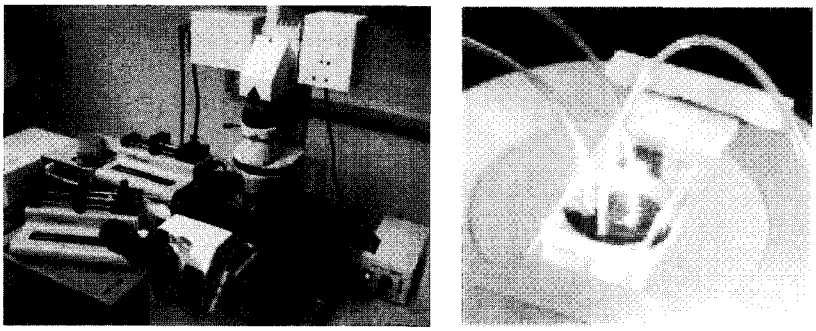


Fig. 1. Schematic of PDMS microchip for hydrodynamic focusing, where arrows depict direction of fluid transport. ($w_{channel}$ = 50 μm).



(a) (b)

Fig. 2. Experimental set-up: (a) Laser scanning confocal microscope with flow injection system for flow visualization using Rhodamine B, (b) Illuminated PDMS microchip interconnected with teflon tubes.

PDMS replica to a glass slide irreversibly.

The seal between the two substrates was strong enough to withstand high pressures induced by the syringe pumps. Teflon tubes (Upchurch Scientific, WA, USA) for fluid injection were tightly interconnected with the microchip after the fabrication process. All channels are 100 μm wide and 50 μm deep with slight variations. Fig. 1 shows the schematic of the microchip and the flow directions of sample and sheath fluids.

Because constant flow rates are supplied by the syringe pumps, the width of the hydrodynamically focused stream can be calculated roughly by the following equation derived from the continuity theory;

$$\eta = \frac{Q_{sheath}}{Q_{sample}} = \frac{\alpha - 1}{2} \quad (\alpha = \frac{w_{channel}}{w_{focused}}) \tag{1}$$

Fig. 2 represents an experimental setup for three-dimensional flow visualization. Two syringe pumps (Kd Scientific, PA, USA) were used to provide the predetermined constant flow rates of sample and sheath fluids. Rhodamine B (Sigma-Aldrich, WI, USA) was continuously injected into the focusing chamber to visualize the hydrodynamically focused sample stream. The width of the confined sample stream was controlled by varying the sheath flow rate relative to the sample flow rate. A laser scanning confocal microscopy (LSM5 Pascal, Carl Zeiss, Germany) was used with a Zeiss Axiovert 100 inverted microscopy equipped with a 20×/0.4 NA objective. Sliced images were taken at 1 μm vertical increments. Each slice is 512 × 512 pixels and acquired within 0.2 seconds. Three-dimensional shape of the squeezed sample flow was reconstructed from horizontal image slices using a volume rendering software. By filling all channels with the fluorescent dye, three-dimensional geometry of the channels could be obtained.

For quantitative velocity measurements of accelerated flow field in the focusing chamber, a Confocal Micro-PIV system was developed using pinholes to define the depth of the measurement volume. Fig. 3 shows schematic diagram of the Confocal Micro-PIV system developed by this study, which is designed to observe two different fluorescent dyes simultaneously. 500 nm diameter fluorescent microspheres (Molecular Probes, OR, USA) with a density of 1.055 g/cm^3 are used as seed particles. These microspheres fluoresce at excitation wavelength of 495 nm and emit at 515 nm. The microchip is mounted on a three-dimensional translation stage with 1.5 μm resolution. Excitation light from a 25 mW air-cooled argon ion laser (488 nm, Lasos, Jena, Germany) is transmitted through a pinhole (Melles Griot, CA, USA), a neutral density filter (Omega Optical, VT, USA) and a beam expander (Melles Griot, CA, USA). These rays of light are reflected by a dichroic mirror (Chroma Technology, VT, USA) and focused through a 40×/0.6 NA objective (Olympus, NY, USA) into microchip test section, where they illuminate a volume of the focusing chamber. The fluorescence emission from the microspheres is collected by the same objective lens, passed through the dichroic mirror, and is focused with a tube lens (Melles Griot, CA, USA) onto a pinhole located at the focal point. The emitted light is detected by a 12 bit cooled digital CCD camera (PCO, Kelheim, Germany) with a barrier filter (Chroma Technology, VT, USA). The CCD camera can capture up to 10 multiple exposed images in a single frame with time intervals down to 100 ns. It can also take single exposed double frame images and minimum time between the two images is 200 ns.

The passages of in-focus and out-of-focus rays of light are depicted in Fig. 4. Out-of-focus images are eliminated by the pinhole. The smaller the diameter of the pinhole, the less light is detected from areas outside the depth of focus. However, reducing the pinhole diameter also diminishes the intensity of the signal detected by a CCD sensor. Therefore, a trade-off exists between the minimum thickness of the measurement plane and the maximum intensity of the particle images. Relations between the thickness of the optical section and the detection signal are calculated considering a ray tracing diagram. To verify the theoretical calculations, a series of 1 μm diameter fluorescent microsphere images are acquired at 1.5 μm steps in vertical direction using pinholes of various diameters.

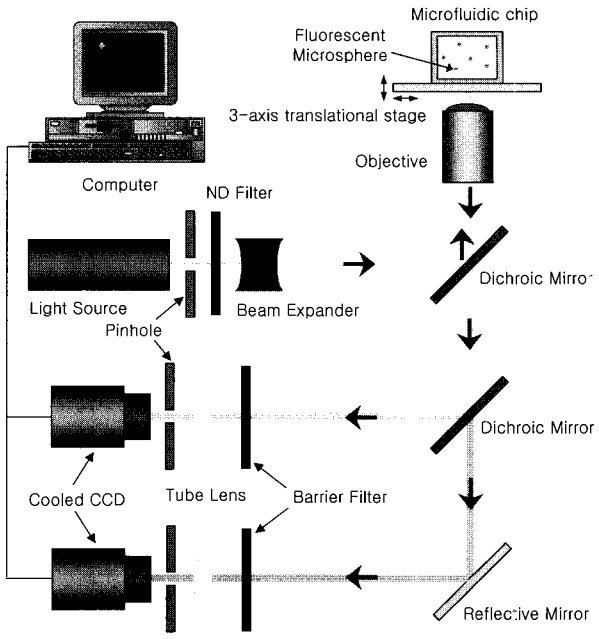


Fig. 3. Schematic of Confocal Micro-PIV system.

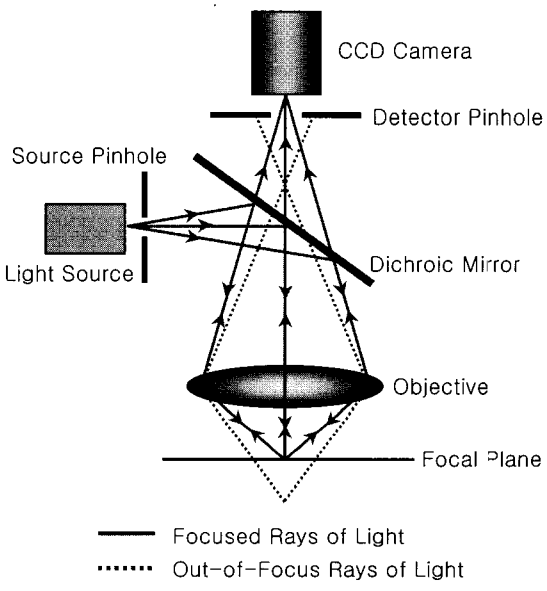
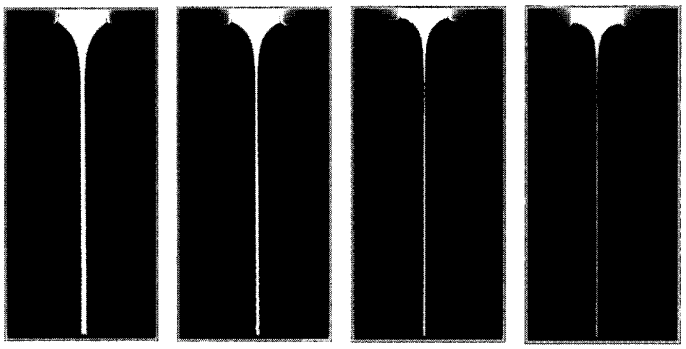


Fig. 4. Effect of pinhole on focusing characteristic of optics.

After acquiring 1280 × 1024 pixel particle images, average displacement of the particles within 32 × 32 pixel interrogation area is determined by the cross-correlation analysis using FFT (Fast Fourier Transform) with a 50% overlap. A polynomial interpolation method



(a) (b) (c) (d)

Fig. 5. CCD images of hydrodynamic focusing for Rhodamine B with sample flow rate of 10 μl/min and varying the flow rate of sheath fluids (a) $\eta = 1$, (b) $\eta = 2$, (c) $\eta = 3.5$, (c) $\eta = 7$.

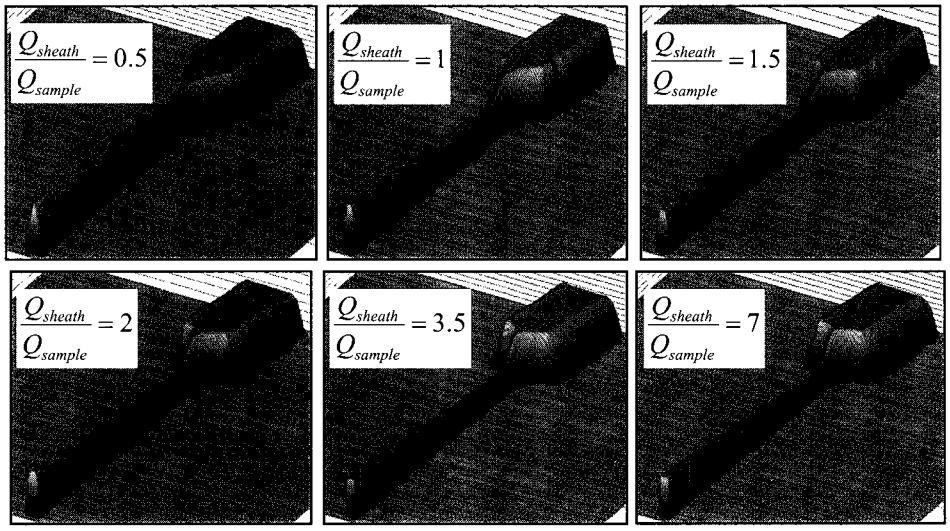


Fig. 6. Confocal scanning microscopy images of hydrodynamic focusing for Rhodamine B.

is adopted to search for a peak of the cross-correlation function with a subpixel accuracy. Erroneous vectors are eliminated by both range validation and peak-height validation methods. Two-dimensional instantaneous velocity vector fields determined from the 200 image pairs are ensemble-averaged to calculate the mean velocities.

10 cc of blood is taken from a healthy adult and diluted to 5% in PBS (Phosphate Buffered Saline) after repeated centrifugations. Diluted red blood cells (RBC) are injected into the sample port and the trajectories of the RBCs are obtained by the CCD camera using a multiple exposure technique.

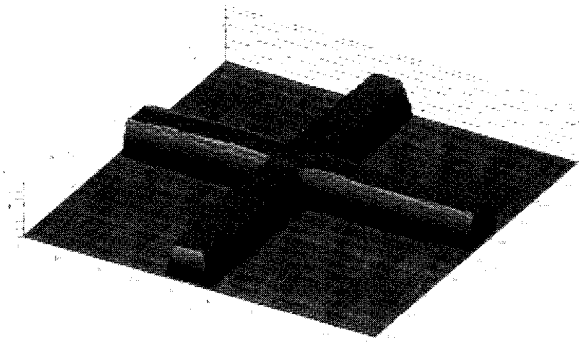


Fig. 7. Three-dimensional channel shape of microchip used for hydrodynamic focusing.

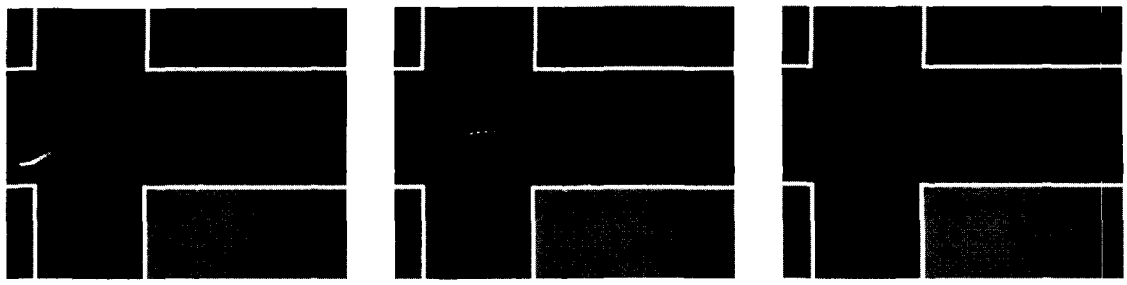


Fig. 8. Multi-exposed fluorescent microsphere images in the focusing region where squeezed sample flow is accelerated by sheath flows from the side channels.

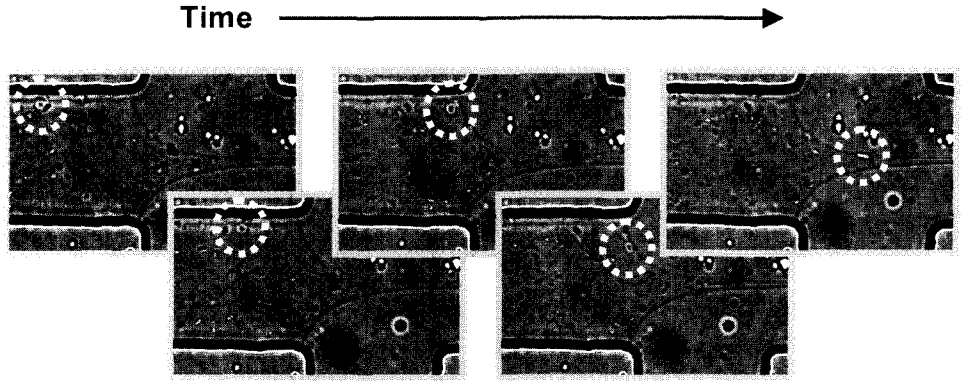


Fig. 9. CCD images of red blood cell motions in hydrodynamically focused flow stream.

III. Results and Discussion

CCD images of focusing from the flow visualization test are shown in Fig. 5. As the flow rate of sheath fluids increases, the degree of focusing increases.

For accurate three-dimensional reconstructions of the slice images obtained in LSCM, a stretch factor should be carefully considered. The stretch factor is the ratio of an increment in the actual focus position to the increment in the stage focus position. It is not equal to 1 due to the refractive effects between the sample and the objective lens. In the case of this study, the stretch factor is 1.33, which is equal to the ratio of the refractive index of the water to that of the air.

From the three-dimensionally rendered confocal images, it is demonstrated that the flow structure in the focusing chamber is skewed in vertical direction by unsymmetrical velocity profiles arising from trapezoidal cross sections of the main and the side microchannels as shown in Fig. 6 and Fig. 7. Fig. 6 shows that the skewness increases with η.

Fig. 8 shows multi-exposed fluorescent microsphere images in the focusing region. It can be clearly observed that the sample flow is abruptly accelerated by the sheath flows from the side channels and then maintains constant speed downstream of the focusing chamber.

In RBC experiments, it was observed that RBCs move along the interfaces between the sample and the sheath fluids and they are aligned in the direction of their longest dimension before entering the focused sample stream as described sequentially in Fig. 9. RBCs with a mean diameter of 8 μm can be transported in a single file downstream of the focusing chamber, when the width of the sample stream is about 10 μm.

Hydrodynamic focusing technique, which includes fast mixing of nanoliters of samples in microseconds by diffusion, can also be applied to study liquid-phase chemical and biological processes where reaction kinetics is faster than the mix times of conventional mixers [16]. The focusing width can be controlled down to as small as 50 nm, and molecules from the side flow

rapidly diffuse across the inlet stream resulting in fast mixing. It may be possible to implement this function in Micro-FACS devices to treat the samples with biological or chemical reagents and then analyzed in series on a single microchip.

IV. CONCLUSIONS

Fluid dynamic studies were conducted to characterize the hydrodynamically focused sample stream in the focusing microchip fabricated by plastic micromachining technology. In Micro-FACS device, spatially confined sample stream affects the sample throughput, detection efficiency and the accuracy of cell sorting. From the three-dimensional visualizations of flow and microfabricated structure with a laser scanning confocal microscopy, it was observed that unsymmetrical velocity profiles arising from trapezoidal cross sections of the microchannels make the flow structure skewed in the focusing chamber. The microscopic flow structure in the accelerated flow region is quantitatively analyzed using the Confocal Micro-PIV system developed by this study. A detection pinhole is used to define the depth of the measurement volume. The trajectories of red blood cells (RBC) and their interactions with surrounding flow field in the squeezed sample stream are evaluated to find the appropriate shape of the focusing chamber and the method of fluid handling for stable cell transporting and efficient detection and sorting.

Microfabrication permits integration of cell sorting with other techniques. Another advantage is that multiple cell sorters can be fabricated in parallel on a single chip, allowing increased throughput. Micro-FACS will be able to improve the reliability and the sensitivity compared with the conventional FACS machines.

ACKNOWLEDGMENTS

The authors would like to thank Junha Park and Seok Chung at Seoul National University for their efforts in fabricating the silicone molds for PDMS microchips. This work was supported by the Nano Bioelectronics and Systems Research Center of Seoul National University, which is an ERC supported by the Korea Science and Engineering Foundation (KOSEF), and also by the Brain Korea 21 Project in 2001.

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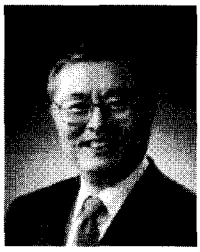
Jung Kyung Kim received the B.S. degree in mechanical engineering in 1996 and the M.S. degree in biomedical engineering in 1998, both from Seoul National University, Seoul, Korea. In 1998, He joined the Biomedlab Company, Seoul, Korea. He is currently working toward the Ph.D. degree at Seoul National University. His current research interests include microfluidic systems for biomedical applications and micro-imaging techniques for flow diagnostics.

Hyunwoo Bang was born in Korea on June 2, 1978. He received the B.S. degree in 2001 and is working toward the M.S. degree in Mechanical and Aerospace Engineering at Seoul National University, Seoul, Korea. His current research interests include plastic micro-machining techniques and microfluidics-based Lab-on-a-chip devices.

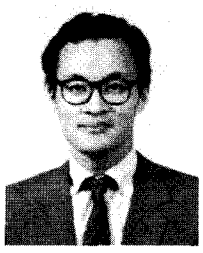
Yongku Lee was born in Korea on June 11, 1974. He received the B.S. degree in 2000 and is a candidate for the M.S. in Mechanical and Aerospace Engineering at Seoul National University, Seoul, Korea. His current major activity is focused on studying the flow characteristics in plastic microchannel.

Chanil Chung was born in Korea on December 24, 1969. He received the B.S. degree in mechanical engineering from Seoul National University, Seoul, Korea in 1992 and the M.S. and Ph.D. degrees in biomedical engineering at Seoul National University in 1994 and 1997, respectively. From 1997 to 2000, he was a research engineer on military duty at the Institute of Medical and Biological Engineering, Seoul National University. Prior to joining the Digital Bio Technology Company as a Research Director in 2001, he was a Post-Doctoral Researcher in the Center for Biomedical Engineering at Massachusetts Institute of Technology, MA between 1999 and 2000 and a Post-Doctoral Associate at the Institute of Advanced Machinery and Design, Seoul National University between 2000 and 2001. His current research interests include the microfluidics-based Lab-on-a-chip systems and the cellular application to MEMS.



Jung Yul Yoo received the M.S. degree in mechanical engineering from Seoul National University in 1969 and M.S. and Ph.D. degrees in fluid mechanics from University of Minnesota in 1973 and 1977, respectively. In 1978, he joined Department of Mechanical Engineering at Seoul National University as an assistant professor. He was promoted to an associate professor and a professor in 1983 and 1988, respectively.

Sang Sik Yang was born in Korea in 1958. He received his B.S. and M.S. degrees in mechanical engineering from Seoul National University in 1980 and 1983, respectively. In 1988, he received his Ph.D. degree in mechanical engineering from University of California, Berkeley. He was then a research assistant professor at New Jersey Institute of Technology between 1988 and 1989. In 1988, he joined Department of Control and Instrumentation Engineering at Ajou University, Korea as an assistant professor. He was promoted to an associate professor and a professor in 1992 and 1997, respectively, in School of Electronics Engineering at Ajou University. He is currently a visiting professor in University of Cincinnati between 2001 and 2002. His research interests include micro sensors, micro actuators, micro cooling devices and micro fluidic devices in microelectromechanical systems (MEMS) area. He has published over 140 technical papers in journals and conference proceedings. He has been serving as a chairman of MEMS Committee in Korean Institute of Electrical Engineers since August 1999. He received The Paper Award from Korean Institute of Electrical Engineers in 1998.



Jin Seung Kim received BS in physics from Seoul National University in 1978, MS and PhD in physics in 1980 and 1983 from KAIST, Korea. Since 1983 he has been with Chonbuk National University, Jeonju, Korea. He has been working on optical metrology systems, liquid crystal display, and light scattering. He is currently involved in the development of desktop FACS system, especially on the design and performance testing of compact optical systems.

Sekwang Park was born in Korea on October 25, 1954. He received the BS degree in electrical engineering from Seoul National University, Seoul, Korea, in 1976, and MS and PhD degrees in electrical engineering from Case Western Reserve University, Cleveland, OH, USA, in 1984 and 1988, respectively. He was project leader of LVAD Technology, USA, from 1988 till 1989. He is currently a professor in the School of Electronic and Electrical Engineering at Kyungpook National University. His research interests are sensors and actuators using micromachining technology, especially pressure sensors, flow sensors, temperature sensors, humidity sensors and optical sensors.

Jun Keun Chang received the B.S. degree in mechanical engineering from Seoul National University, Seoul, Korea, in 1990, and M.S. and Ph.D. degrees in biomedical engineering from Seoul National University in 1992 and 1995, respectively. In 2000, he joined School of Electrical Engineering and Computer Science at Seoul National University as an assistant professor. He is also a Founder and CEO of Digital Bio Technology, Co. and a director of Biomedlab, Co., Seoul, Korea. His research interests include BioMEMS and BioNEMS for portable biomedical diagnostics and microfluidic Lab-on-a-Chip (LOC) systems, as well as biomedical disposables.