Separation of Human Breast Cancer and Epithelial Cells by Adhesion Difference in a Microfluidic Channel

Keon Woo Kwon*, Sung Sik Choi, Byungkyu Kim***, Se Na Lee**, Sang Ho Lee**, Min Cheol Park*, Pilnam Kim*, Sukho Park ****, Youngho Kim***, Jungyul Park ****, and Kahp Y. Suh*†**

***Abstract*—A simple, label-free microfluidic cell purification method is presented for separation of cancer cells by exploiting difference in cell adhesion. To maximize the adhesion difference, three types of polymeric nanostructures (50nm pillars, 50nm perpendicular and 50nm parallel lines with respect to the direction of flow) were fabricated using UV-assisted capillary moulding and included inside a polydimethylsiloxane (PDMS) microfluidic channel bonded onto glass substrate. The adhesion force of human breast epithelial cells (MCF10A) and human breast carcinoma (MCF7) was measured independently by injecting each cell line into the microfluidic device followed by culture for a period of time (e.g., one, two, and three hours). Then, the cells bound to the floor of a microfluidic channel were detached by increasing the flow rate of medium in a stepwise fashion. It was found that the adhesion force of MCF10A was always higher than that of MCF cells regardless of culture time and surface nanotopography at all flow rates, resulting in a label-free detection and separation of cancer cells. For the cell types used in our study, the optimum separation was found for 2 hours culture on 50nm parallel line pattern followed by flow-induced detachment at a flow rate of 300 μl/min.**

***Index Terms*—Cell separation/Cell purification, Microfluidics, Cell adhesion**

I. INTRODUCTION

The distinction between cancer and normal cells is the fundamental step of diagnosis and further biological analysis of cancer. Current methods for detecting and separating cancer cells rely on observing morphological features or labeling with specific biomarkers.[1-5] Use of a biomarker is highly specific and accurate but requires laborious, time-consuming preparation for staining prior to separation and a post process such as removal of labeling. Also, a limited number of effective biomarkers (e.g., aptamers [6]) have been reported for cancer probing and diagnosis.

In contrast, label-free methods typically utilize difference in physical properties such as cell size, [7] density, [8] cell adhesion, [9-11] and dielectric properties.[12-14] A potential shortcoming in label-free methods is that the physical difference is not high enough for efficient separation in many cases, which limits the widespread use of the label-free methods. Nonetheless, the efficiency of separation could be enhanced under a controlled micro/nanoenvironment by amplifying the physical differences. Cell adhesion is one of the physical markers that can be used to detect and separate cells of interest. Currently, most adhesion-assisted cell sorting methods like cell affinity chromatography (CAC) have used specific proteins to enhance the adhesion of one cell type than those of other cell types.[9-11] It is noted in

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* School of Mechanical and Aerospace Engineering, Seoul National University, Seoul, Korea.
** School of Life Sciences and Biotechnology, Korea University, Seoul, Korea.
*** School of Aerospace and Mechanical Engineering, Hankuk Aviation University, Kyonggi-Do, Korea.
**** Microsystem Research Center, Korea Institute of Science and Technology, Seoul, Korea
† Corresponding author: Kahp Y. Suh
E-mail: sky4u@snu.ac.kr

this regard that the cell adhesion is also easily modified on a surface with different nanotopography without specific proteins; it could be increased or decreased depending on the material and geometry used to construct the surface structure by controlling binding forces between integrin receptor and extracellular matrix protein (ECM).[15] Recently, extensive efforts have been made to understand/control cell adhesions on various surface nanotopographies using conventional or unconventional lithographic techniques.[16-18]

Microfluidic system is of advantage for analysis of cell adhesion in various aspects because of small sample consumption, study of multiple parameters, generation of large shear stress.[19-21] By integrating nanostructured surfaces with a microfluidic system, we herein a simple cell separation method using standard soft lithography and microfluidics protocol. In this device, three polymeric nanostructures were sequentially included inside a microfluidic channel to increase the difference in cell adhesion and the cell adhesion was measured for each cell type by controlled detachment of human breast epithelial cells (MCF10A) or human breast carcinoma (MCF7) from the floor of the channel. As a consequence, a simple and efficient cell separation/purification device was created by exploiting the adhesion difference that was amplified on the nanopatterned surface, which is described below.

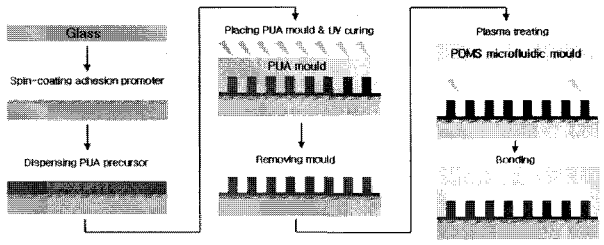
II. METHODS AND MATERIALS

1. Fabrication of PUA Mould

The ultraviolet (UV)-curable polyurethane acrylate (PUA) mould material consists of a functionalized precursor with an acrylate group for cross-linking, a monomeric modulator, a photoinitiator and a radiation-curable releasing agent for surface activity. Details of the synthesis and characterization of the material have been published elsewhere.[22] To fabricate a sheet-type mould, the liquid mixture was drop-dispensed onto a silicon master and then a flexible, transparent polyethylene terephthalate (PET) film was brought into contact with the liquid mixture. Subsequently, the mould was exposed to UV light (λ = 200–400 nm) for 20 s through the transparent backplane (dose = 100 mJ/cm^2). After UV curing, the mould was peeled off from the master and additionally cured overnight to terminate the remaining active acrylate groups prior to use as a first replica. The resulting PUA mould used in the experiment was a thin sheet with a thickness of ~50 μm.

2. Fabrication of Microfluidic Channel with PUA Nanopatterned Substrate

PDMS microfluidic moulds were fabricated by curing PDMS pre-polymer (Sylgard 184 Silicon elastomer, Dow Corning) on silicon masters prepared by photolithography. The masters used for microfluidic moulds had protruding (positive) features with the impression of microfluidic channel (600 μm in width and 50 μm in height). To cure the PDMS pre-polymer, a mixture of 10:1 silicon elastomer and the curing agent was poured on the master and cured at 70 ℃ for 1 h. The PDMS replicas were then peeled from the silicon masters and cut prior to use. For the PDMS microfluidic moulds, holes were punched through the inlet and the outlet as a reservoir. Glass substrate was rinsed with isopropyl alcohol (IPA) in an ultrasonic bath for 30 min and washed in a flow of distilled water and dried in a stream of nitrogen. Three types of nanostructures were fabricated onto glass substrate using UV-assisted capillary moulding as shown in Scheme 1.[23-26] A small amount of the PUA precursor (~0.1-0.5 mL) was drop-dispensed on the substrate and a first-replicated PUA mould (same material but without active acrylate groups) was directly placed onto the surface. The PUA precursor spontaneously moved into the cavity of the mould by means of capillary action and was cured by exposure to UV for ~ 20 s. After curing, the mould was peeled off from the substrate using a sharp tweezer. To increase the adhesion at the PUA nanostructure/glass interface, an adhesion promoter (phosphoric acrylate: propylene glycol monomethyl ether acetate = 1:10, volume ratio) was coated onto glass substrate. For channel bonding, the PDMS microfluidic mould and glass substrate were treated in oxygen plasma for 60 s (60 W, PDC-32G, Harrick Scientific, Ossining, NY). After plasma treatment, the channel mould was brought in conformal contact with the substrate with careful alignment and firmly pressed to form an irreversible seal. The device was heated on a hot plate for several tens of minutes for further strengthen the sealing.



Scheme 1. A schematic diagram of the fabrication of a microfluidic channel integrated with a nanopatterned substrate where PUA nanostructures were fabricated using UV-assisted capillary moulding onto glass substrate and the patterned substrate was bonded to the channel through treatment with oxygen plasma.

3. Scanning Electron Microscopy

High-resolution scanning electron microscopy (SEM) images of the PUA nanostructures were obtained using a HITACHI S-4800 microscope (Hitachi, Japan) operating at an accelerating voltage of 5 kV. To avoid charging effects, substrates were sputter-coated with Au to the thickness of 20 nm prior to measurements.

4. Cell Culture and Measuring Cell Contact Area/ Cell Adhesion

Both MCF10A and MCF7 cells were obtained from the American Type Culture Collection (ATCC). The human normal breast epithelial cell line, MCF10A, was cultured in DMEM/F-12 medium containing 5% horse serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 100 ng ml^{-1} cholera toxin, 10 ng/ml epidermal growth factor, 0.5 mg/ml hydrocortisone, 10 mg ml^{-1} insulin, and 1% (w/v) L-glutamine at 37°C under an atmosphere of 5% CO_2 (all from Sigma Chemical, St Louis, MO). The human breast cancer cell line, MCF7, was similarly cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg ml^{-1} streptomycin, 10 mg ml^{-1} insulin, and 1% (w/v) L-glutamine. Both cells were grown at a concentration of 1.0×10^5 ml^{-1} and sub-cultured when the cells are confluent at about 70%. For cell culture in the microfluidic chamber, cells were harvested by treating with 0.025% trypsin-EDTA, washed three times to remove the trace of trypsin. Cell density was controlled at ~2.5×10^6 ml^{-1} to provide consistent experimental conditions. When the mixed cells of MCF10A and MCF7 lines were used, the medium was made by mixing two different culture media at a ratio of 1:1 to facilitate two cell lines together.

To measure cell contact area and cell adhesion, each cell solution was injected inside a microfluidic channel by surface tension driven capillary flow and the cells were left adhered to the floor of the channel in an incubator for 1-3 hour. Then, bright-field images of cells were taken under optical microscope (Olympus, IX71). Contact area of MCF10A and MCF7 cells was measured by analyzing bright-field images of cells using image analysis program (MediaCybernetics, Image-Pro PLUS). Adhesions of MCF10A and MCF7 cells on 50nm pillars, 50nm perpendicular and 50nm parallel lines, and glass substrate was measured with varying incubation times to find an optimum condition of separation. To apply a uniform shear stress, cells were distributed uniformly with a sufficient spacing between cells. After cell culture, each microfluidic channel was connected to a syringe pump and then the medium solution was flowed into the channel with increasing flow rate in a stepwise manner. For each step, the medium was flowed for the time span of 1 min and the number of adherent cells was counted under an optical microscope.

5. Separation of MCF7 Cells

10% of MCF7 cells were mixed with MCF10A cells at a total density of ~2.5×10^6 ml^{-1} as described above. The nucleus of MCF7 cells was stained with Hoechst 33342 (10 μg/ml) before mixing two cell lines. Both cell types were introduced inside a microfluidic channel having a patterned substrate with 50nm perpendicular and parallel lines by surface tension driven flow, followed by culture for 2 hrs in an incubator. After the microfluidic device was installed in an optical microscope, the unviable cells were initially washed away by flowing the medium at a flow rate of 10 μl/min for 1 min and the locations of MCF7 cells were marked under a fluorescent microscope. Subsequently, the medium was flowed into the channel at a flow rate of 300 μl/min for 1 min and the adhered cells were counted under an optical microscope.

III. Results and Discussion

1. Integration of a Microfluidic Device with Nanopatterned Substrate

Fig. 1(a-c). shows SEM images of three types of PUA

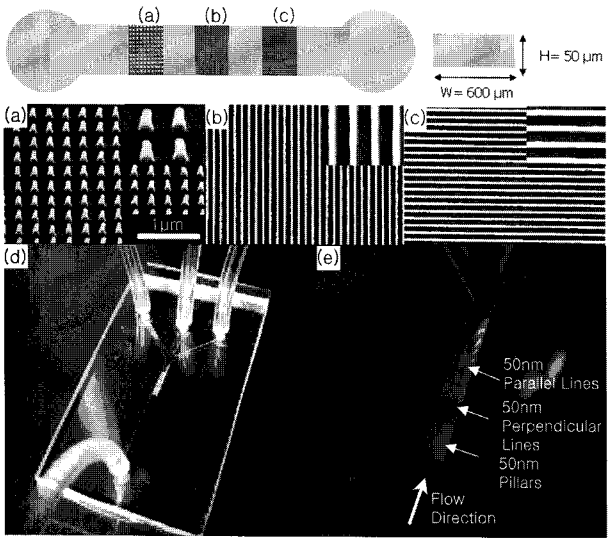


Fig. 1. SEM images of the three PUA nanostructures sequentially included inside a microfluidic channel (600μm width, 50μm height, and 15mm length): in order of appearance in the direction of flow, (a) 50nm pillars with 400nm height and 450nm spacing, (b) 50nm perpendicular lines with 200nm height and 150nm spacing, and (c) 50nm parallel lines with 200nm height and 150nm spacing. (d) A photo image of the fabricated microfluidic channel combined with a patterned substrate. (e) An enlarged image of the nanopatterned region inside the channel.

nanostructures that were included inside a microfluidic channel (600 μm width, 50 μm height, and 15 mm length). In order of appearance in the direction of flow, the geometric parameters were: 50 nm pillars with 400 nm height and 450 nm spacing, 50 nm perpendicular, and 50 nm parallel lines (with respect to the direction of flow) with 200 nm height and 150 nm spacing. The patterned area for each nanostructure was 0.55 × 1.1 mm^2. A simple three-branched microfluidic channel was used to test the cell adhesion as shown in Fig. 1(d). A magnified image in Fig. 1(e) indicated that the above-mentioned nanostructures were sequentially included in the direction of flow. Since the flow is fully developed (the entrance length was 0.154 μm at 500 μl/min) and the height of the nanostructures was much small compared to the channel height, the hydrodynamic conditions for each nanostructure were assumed to be constant.

2. Morphology of MCF10A and MCF7 Cells

We first investigated the morphology of MCF10A and MCF7 cells that were bound to the floor of a microfluidic

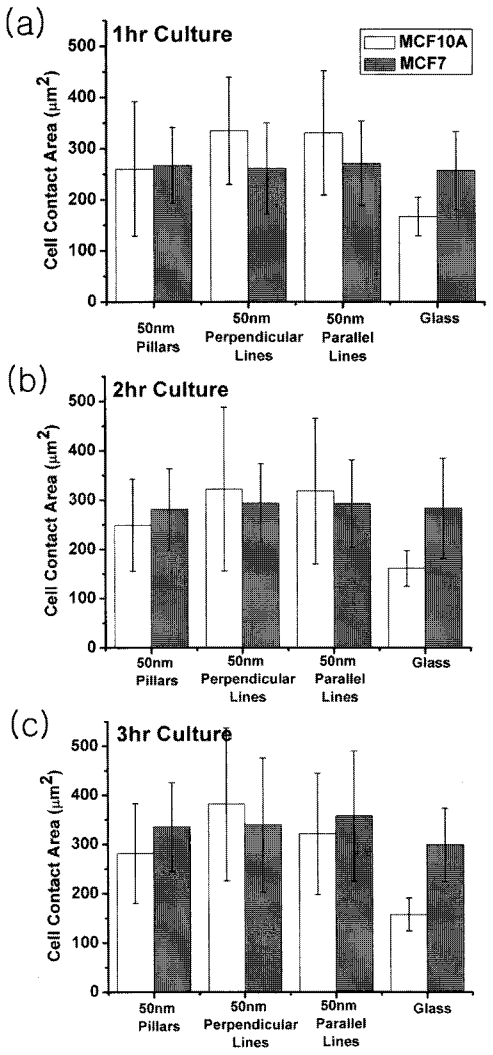


Fig. 2. Contact area of MCF10A and MCF7 cells on 50nm pillars, 50nm perpendicular lines, 50nm parallel lines, and glass substrate for different culture times: (a) one hour, (b) two hours, and (c) three hours

channel. Each cell solution was injected inside a microfluidic channel by surface tension driven capillary flow and the cells were left adhered to the floor of the channel in an incubator for one, two, and three hours, respectively. Such a short time of culture ensured fast and reliable detection of cancer cells. A culture time longer than 3 hrs turned out to give relatively strong adhesion of MCF7 cells, which is not desirable for efficient cell sorting. As shown in Fig. 2, when the cells were cultured on glass substrate, the contact area of MCF10A cells (~150 μm^2) was much smaller than that of MCF7 cells (~270 μm^2) with more uniform spherical shape for all culture times. When the cells were cultured on nanopatterned substrates, a number of notable differences

were found. First, the contact area of MCF7 cells did not show an appreciable change, suggesting that these cells are not sensitive to the patterned substrate. On the other hand, the MCF10A cells were larger and more spreaded on nanostructured surfaces for all the nanopatterns tested, suggesting that these cells can fast recognize and interact with surface nanotopography. The contact area of the MCF10A cells was measured to be ~ 250 μm^2 on 50nm pillars and ~330 μm^2 on perpendicular and parallel 50nm line patterns, which is more than twice the value on glass substrate. It appears that the orientation of line patterns facilitates focal adhesions and growth of the adhered MCF10A cells. Fig. 3 is the image of morphology of MCF10A and MCF7 cells on three different nanopattern and glass surface cultured for 2 hrs. MCF10A cells onto the nanopattern were more spread than cells onto the glass surface. And MCF10A cells cultured on the 50nm perpendicular and parallel lines were elongated and aligned along the direction of the pattern. But MCF7 cells onto the nanopattern were not spread compared to MCF10A, and MCF7 cells onto the line pattern were less elongated than MCF10A cells but some cells were shown long and irregular lamellipodium. It showed that MCF10A interacted with nanopattern more actively than MCF7 cells.

3. Adhesions of MCF10A and MCF7 Cells

Difference in cell adhesion is a key parameter to enable diagnosis and separation of cancer cells for an adhesion difference-assisted cell sorting system presented here. The adhesion force was evaluated for each cell type by flowing the medium with a stepwise increase of flow rate. The step increase of flow was adjusted to the culture time such that it was 25, 50, and 100 μl/min for 1, 2, and 3 hrs of culture, respectively. This is because the adhesion force would increase with increasing culture period. The duration of each step was 1 min throughout the experiment.

A number of notable findings were derived from Fig. 3. First, the adhesion strength of MCF10A was always higher than that of MCF7 irrespective of culture period and surface nanotopography at all flow rates. In Fig. 3(a-c), the fraction of adherent cells was plotted, showing that the fraction of MCF7 decreased drastically whereas that of MCF10A was decreased slightly. Second, the adhesion force of MCF10A cells was relatively lower on glass substrate. It was found that more than 50% of the cells were

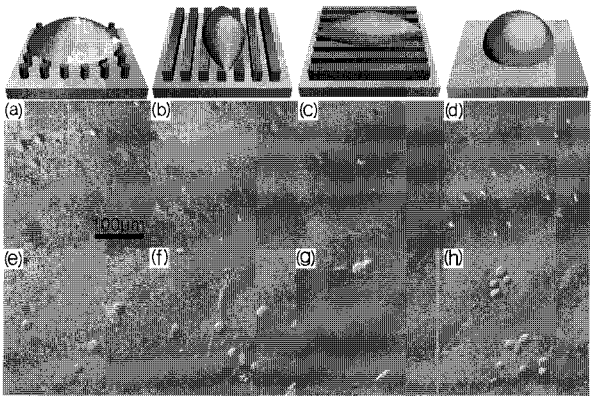


Fig. 3. Bright-field images of MCF10A (a-d) and MCF7 (e-f) cells after 2hours culture. (a, e) 50nm pillars. (b, f) 50nm perpendicular lines. (c, g) 50nm parallel lines. (d, h) Glass.

detached from glass surface as opposed to only 2-3% of cells detached from the nanopatterned surface. It was thought that the polymeric nanostructure could create a favorable environment for the adhesion of MCF10A, which was already confirmed by the increased contact area in Fig. 2. In this regard, the merger of a nanopatterned substrate with a microfluidic device could enhance the performance of the adhesion difference-assisted cell sorting. Third, the effects of the geometry of nanostructure turned out to be less as can be seen from Fig. 3(a-c). The fraction of adhered cells was expected to be highest for the 50nm parallel lines since the hydrodynamic force (or shear stress) would be smallest with the cells aligned parallel to the direction of flow. For MCF10A cells, it seems that the culture period used in this experiment was not long enough for fully recognizing the surface nanoenvironment. This is well represented in Fig. 2 in that the spherical geometry was maintained with the increased contact area (low proliferation) and the contact area became similar for all the nanopatterns after 1 hr. Also, the flow rate was not high enough for detecting the adhesion difference of MCF10A cells on different nanopatterns, as the adhesion of MCF10A cells appeared to be much higher than the hydrodynamic force that was applied to the cell surface. For MCF7 cells, the differences in adhesion on different nanostructures would be small since the interactions at the cell/substrate interface were observed to be weak, as verified in Fig. 2. In this light, the fractional difference would increase further with a longer culture period, which is supported by a slightly increased difference for culture of 3 hrs in Fig. 3(c).

Shown in Fig. 3(d-f) are the plots for the fractional difference of adherent cells as a function of flow rate. One notable finding is that the adhered MCF10A cells were robust, rendering a larger difference with increasing flow rate up to the maximum flow rate of the syringe pump used in our experiment (~500 μl/min). In contrast, the fractional difference showed a maximum peak at a certain flow rate for the cells cultured on glass substrate, suggesting that the MCF10A cells started to detach at higher flow rates. The maximum fractional difference was measured to be ~0.97 when the cells were bound onto nanopatterned substrate over 2 hrs of culture and then detached at a flow rate of > 300 μl/min. In terms of detection speed and minimally invasive environment (shear damage) to cells, the optimum condition for the separation would be 2 hrs of culture on 50nm parallel line pattern followed by detachment at a flow rate of 300 μl/min.

4. Separation of MCF7 Cells from a Mixed Phase of MCF10A and MCF7 Cells

To demonstrate potential applications to label-free separation of cancer cells, a simple separation scheme is presented here. First, MCF7 cells were mixed with MCF10A cells at a fraction of ~10 % providing easily recognizable numbers in the mixed cell population although the population would be far less in reality. To distinguish MCF7 and MCF10A cells, the nucleus of MCF7 cells was stained with Hoechst 33342 before mixing and both cell types were introduced inside a microfluidic channel having a patterned substrate with 50nm perpendicular and parallel lines, followed by culture for 2 hrs. Subsequently, the medium was flowed into the channel at a flow rate of 300 μl/min and the adhered cells were counted under an optical microscope. As mentioned earlier, this condition was assumed to be the optimum for the cell types used in this study. As shown in Fig. 4, there were initially three MCF7 cells on the 50nm parallel lines in a screen but all the cells were detached from the surface within 1 min of flow. In contrast, all the MCF10A cells were still adhered onto the substrate as shown in Fig. 4. Similarly, all the MCF7 cells on the 50nm perpendicular lines were detached from the substrate while only one MCF10A cell was detached from the surface (images not shown). It is noted that the cells were separated at an enough distance for the cell sorting experiment since the dye Hoechst 33342 readily diffused to the adjacent cells and then destroyed the selective fluorescent signal from the adhered MCF7 cells. Also, such sparse distribution of cells could suppress or minimize potential cell-to-cell communication and change of flow conditions induced by the adhered cells.

IV. CONCLUSIONS

We have presented a simple microfluidic device for label-free detection and separation of cancer cells using adhesion difference as a physical marker for breast cancer cells. To enhance the adhesion difference, three types of nanostructures (50 nm pillars, 50 nm perpendicular and 50 nm parallel lines with respect to the direction of flow) were included inside a microfluidic channel without disturbing flow conditions. The results demonstrated that MCF10A cells were more sensitive to the surface topography than MCF7 cells. The enhanced adhesion was evaluated by the increased contact area of the adhered MCF10A cells where the contact area was increased by 1.7 times for the 50nm pillars and by 2.2 times for the 50nm lines after adhesion. By contrast, the contact area of the adhered MCF7 cells did not show an appreciable change. As a consequence, the adhesion strength of MCF10A cells turned out to be always higher than that of MCF7 cells regardless of culture time and surface nanotopography at all flow rates. Also, it was found that the fractional difference between the adhered cells increased with increasing flow rate on the nanostructured surface while the glass substrate showed a maximum peak at a certain flow rate. For the cell types used in our study, the optimum separation was carried out for 2 hrs culture on 50 nm parallel line pattern followed by flow-induced detachment at a flow rate of 300 μl/min. While the 50 nm parallel lines were selected for the optimum condition, the effects of different nanostructure were not significant as the culture time was long enough for fully recognizing surface nanoenvironment. It is hoped that this microfluidic device could serve as an alternative for separating and purification of cancer cells with a minimal volume of body fluids containing mixed population of normal and cancer cells obtained by a biopsy. A future study would be directed towards capturing the detached cancer cells for drug screening with a higher throughput.

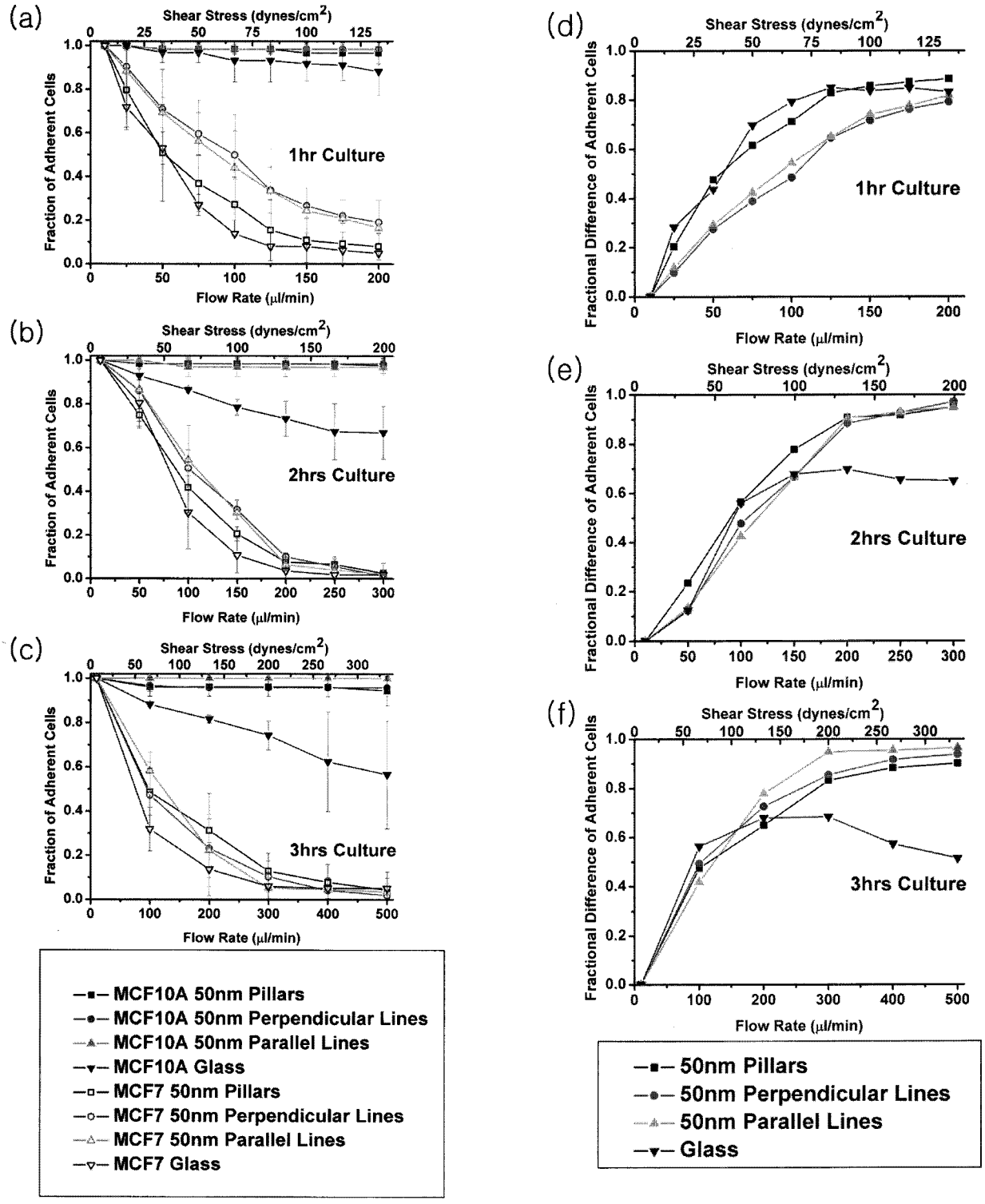


Fig. 4. (a-c) Fraction of adherent MCF10A and MCF7 cells and (d-f) the corresponding fractional difference from (a-c) for different culture times of (a, d) 1 hr, (b, e) 2 hrs, and (c, f) 3 hrs, respectively.

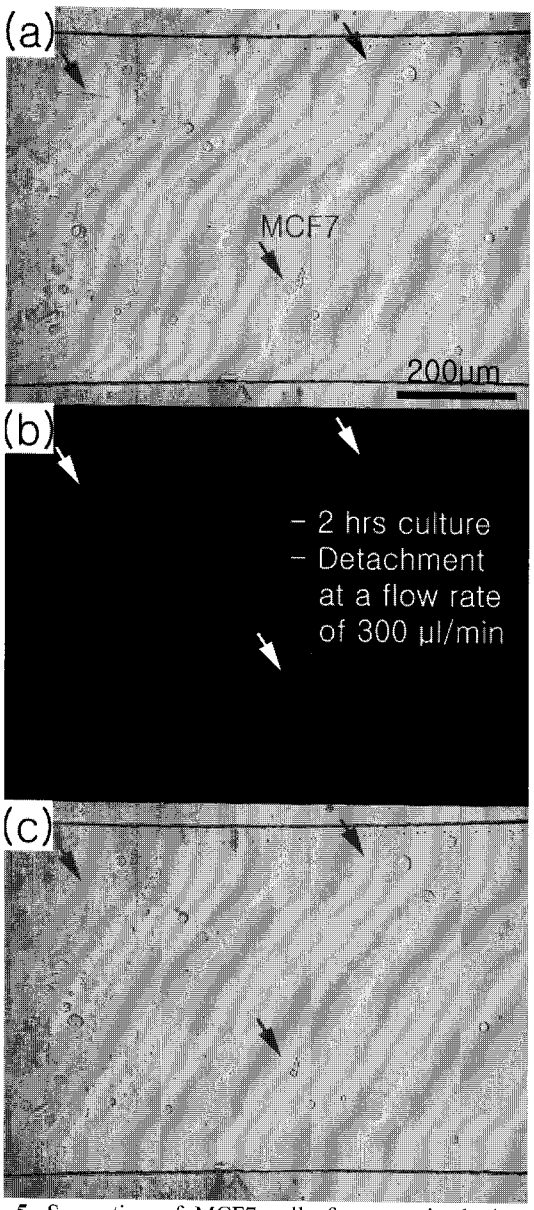


Fig. 5. Separation of MCF7 cells from a mixed phase of MCF10A and MCF7 cells (MCF7: 10 %). The cell sorting was performed based on the optimum protocol established in the experiment (2 hrs culture on 50nm parallel lines followed by detachment at a flow rate of 300 μl/min: (a) A bright-field image of the channel after flowing the medium at 10 μl/min for 1 min. (b) A corresponding florescence image of the channel. Three MCF7 cells are seen from the figure. (c) A bright-field image of the same channel after flowing the medium at 300 μl/min for 1 min. As shown, all the MCF7 cells were detached from the nanopatterned substrate while all the MCF10A cells were still adhered.

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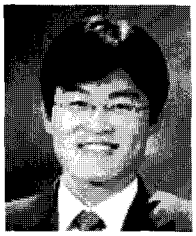
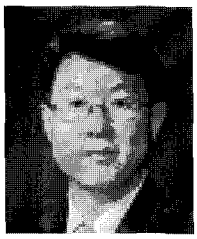
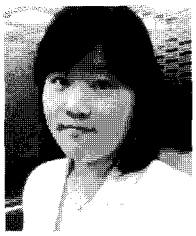


Keon Woo Kwon received his B.S. degrees in Agricultural Machinery and Process Engineering (2005) and the M.S. degrees in Mechanical and Aerospace Engineering (2007) from the Seoul National University, Korea. Currently, He is a Ph.D. candidate under supervision of Prof. Kahp Y. Suh in the School of Mechanical and Aerospace Engineering, Seoul National University, Korea. His current research interests include nano/micro fabrication and microfluidics to develop lab-on-a-chip devices for cell research.

Sung Sik Choi received the B.S. degree in Biotechnology from Korea University, Korea, in 1995, and M.S. degree in Biochemistry from Korea University, Seoul, Korea, in 2002. He is currently working as a PhD student in Korea University. He is interested in the neuro-developmental biology and stem cell biology.



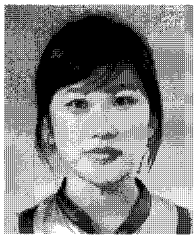
Byungkyu Kim received his Ph.D. in Mechanical Engineering from the University of Wisconsin, Madison, in 1997. From 1990 to 1993 he was a research scientist of the Robotics Center at KIST (Korea Institute of Science and Technology), where he worked on the robot application for automobiles automation. From 1997 to 2000, he was a technical staff member of CXrL (Center for X-ray Lithography) in the University of Wisconsin where he developed a computer code for thermal modeling of a mask membrane and wafer during beam exposure. From 2000 to 2005, he worked for Microsystem Center of KIST as a senior research scientist. He was in charge of the developing the microrobot for microcapsule-type endoscope. Currently, he is an assistant professor at the School of Aerospace and Mechanical Engineering, Hankuk Anivation University. His research interest includes microelectromechanical systems (MEMS) actuator, micro/nano-manipulator and bio/medical application microrobots.



Se Na Lee received the B.S. degree in Food and Nutrition from Soonchunhyang University, Chunan, Korea, in 2006, and M.S. degree in Food and Nutrition from Duksung Women's University, Seoul, Korea, in 2007. She is currently working as a research scientist in industry. She is interested in the nutrigenomics and nutrigenetics.

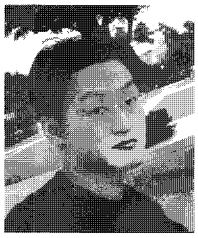
Sang Ho Lee received the B.S. and M.S. degrees from Korea University, Seoul, Korea in reproductive biology, 1978 and 1980, respectively, and then Ph.D. in developmental biology from University of London, U.K. in 1989 and has worked in the MRC Experimental Embryology and Teratology Unit, St. George's Hospital Medical School, London, U.K. as a visiting scientist and non-clinical research scientist for 7 years. Currently, he is the professor of Department of Molecular and Cellular Biology, Korea University. His main interests are molecular and cellular mechanisms involved in early embryonic development and stem cell researches for cell replacement therapy.

Min Cheol Park was born in South Korea in 1979. He received his B.S. in Wood Science from the Seoul National University (SNU) in April 2005 and M.S. in Mechanical and Aerospace Engineering from the same university in April 2007. He is presently working towards his Ph.D. degree under supervision of Prof. Kahp Y. Suh in the School of Mechanical & Aerospace Engineering, Seoul National University, Korea. His current research interests include fabrication of lab-on-a-chip devices and micro/nano/bio fusion technology.



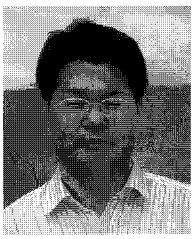
Pilnam Kim was born in Korea in 1979. She received his B.S. in Chemical engineering from the Kyungpook National University in 2003 and M.S. Mechanical and Aerospace Engineering from the Seoul National Univerity in 2006. She is presently working towards his Ph.D. degree under supervision of Prof. Kahp Y. Suh in the School of Mechanical and Aerospace Engineering, Seoul National University, Korea. Her current research interests include fabrication of Micro- and Nanochannel and development of biological-platform for cell focal adhesion.

Sukho Park received his B.S., M.S. and Ph.D. degrees in Mechanical Engineering from Korea Advanced Institute of Science and Technology (KAIST), Korea. From 2000 until 2004 he worked as a senior research engineer at LG Electronics Production Research Center, Korea. In 2004, he moved to Korea Institute of Science and Technology (KIST) where he was a senior research scientist of Microsystem Research Center. Currently, he is an assistant professor at the school of Mechanical System Engineering, Chonnam National University. His research interests are microactuator/robot and micromanipulation for biomedical instrumental applications.



Youngho Kim received the B.S. degree in bioengineering from Korea University, Korea, in 2000 and the M.S. degree in electrical engineering from Korea University, Korea, in 2002. From 2002 to 2004, he worked for the Microsystem Research Center (MRC) of Korea Institute of Science and Technology (KIST), Seoul, Korea, where he engaged in researching "Development of Micro manipulation technology" and "Development of Bio Cell Processor." In 2005, he joined the Nanomechatronics Research Center of Korea Electronics Technology Institute (KETI), Seongnam, Korea. From 2006 to 2007, he was a member of the Technical Research Staff at Nano-Bio-Robotics Laboratory (NBRL) of Korea Aviation University, Goyang, Korea, where he worked on the design of Dielectrophoretically Activated Cell Sorter (DACS). His research interests are in microfluidics, cell mechanics, cell manipulations based on MEMS technology, and Integrated Microsystems (for cell separation and culture) such as: DACS system, Fluorescent Activated Cell Separation (FACS) system and Magnetically Activated Cell Separation (MACS) system based on droplet technology.

Jungyul Park received his B.S. and M.S. degrees in Mechanical Design and Production Engineering from Seoul National University in 1998 and 2000, and received his Ph.D. degree from School of Mechanical and Aerospace Engineering at Seoul National University, Korea in 2005. Currently he has worked as a postdoctoral associate in the department of biomedical engineering, John Hopkins University, USA. His research interests are design, fabrication and analysis of bioMEMS, manipulation, characterization and identification of cells/bio-molecules using micro/nano-technology, and ultra precise control for micro/nano-manipulation.



Kahp Y. Suh received his B.S. (1996), M.S. (1998), and Ph.D. (2002) degrees from the School of Chemical Engineering at Seoul National University. His Ph.D. thesis was focused on developing an unconventional nanolithography using capillary force as an alternative to photolithography. After graduation, he moved to Massachusetts Institute of Technology (MIT) as a postdoctoral associate and carried out research on bioMEMS, microfluidics and cell-based biosensor. Currently, he is an assistant professor at the School of Mechanical and Aerospace Engineering, Seoul National University. His current interests include nanofabrication, bioMEMS, and biomimetics.