A simple microwell-device

for isolation and analysis of single cell

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The graduate school

Yonsei University

INTERDISCIPLINARY PROGRAM

IN NANOMEDICAL SCIENCE AND TECHNOLOGY

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Abstract

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Circulating tumor cells (CTCs) are cells that have shed into the vasculature from a primary tumor and circulate in the bloodstream. Since individual CTCs are heterogeneous cells which have a variety of biological and physical properties such as EpCAM expression and different size, single analysis of CTCs has emerged as promising diagnostic and prognostic tool for patients with metastatic cancers. This paper describes a microfluidic-based droplet generation method, for encapsulating a single circulating tumor cells(CTCs) into a picoliter- or femtoliter-volume aqueous droplet that is surrounded by an immiscible phase. Once PBS with the target cells is injected into an inlet with a conventional micropipette. Injected cells are settled on bottom of channel because of gravity, for several minutes. Then, the exterior with cells of micro-well removed by the injection of oil, the rest of them trapped in micro well were encapsulated as well. We successfully trap a single cell in the well and generate a droplet by injecting oil. A droplet formed in each well can be used to trap and analyze a single CTCs. We also found out that the best trapping efficiency of single cells was 76% at 21um well.

Keywords : Circulating tumor cell(CTC), Heterogeneity, Single cell analysis, microwell device, microfluidics

1.Introduction

1.1. Detection of Circulating tumor cells(CTCs)

Circulating Tumor Cells (CTCs) are tumor cells that detach from the primary tumor and travel in the peripheral blood, spreading from the original tumor to other locations, leading to cancer metastasis.(Figure1.1)

Therefore, The detection of CTCs in the bloodstream of cancer patients is prognostically critical, associated with clinical stage, disease recurrence, tumor metastasis, treatment response, and patient survival following therapy.

But,The main technical challenge of CTC detection arises due to its rarity, at the level of 1 CTC in 1mL of blood (or 1 CTC against hematological cells), making efficient enrichment a prerequisite for CTC detection in most of the cases. Their identification and characterization require therefore extremely sensitive and

specific analytical methods, which are usually a combination of enrichment and detection procedures.

Aims of research on CTCs include (i) estimation of the risk for metastatic relapse or progression (i.e., prognostic information), (ii) stratification and real-time monitoring of therapies, (iii) identification of therapeutic targets and resistance mechanisms, and (iv) understanding metastatic development in cancer patients.

1.2. Heterogeneity of single cells

It is well known that individual cells differ from each other in many aspects and the way cells process input signals decides the fate of that particular cell. Heterogeneous behavior of cells, such as molecular actions and signaling, are known to occur under identical environmental conditions. This can be visualized by variations in cell division, drug efficacy, cell cycle stage or age, phenotype due to the stochastic nature of gene expression, receptor expression, concentration of a critical metabolite or ion as demonstrated in a few examples from the last decade.

There are many reasons why individual cells respond differently to a given environments. The rich information obtained from single-cell analysis can result in better understanding of the molecular machinery of a cell and its role in a complex environment. (Figure1.2)

Single-cell analysis on the other hand offers dynamic information of individual cells, such as differences in proliferation, drug response or onset time for a response, cell division etc. Subsequently, the purpose of single-cell biology is to increase knowledge and understanding at the level of individual cells, in order to better understand the entire human body.

1.3 Motivation

Heterogeneity among CTCs was significant, and cell-to-cell variations occurred even within a blood draw. Our finding of CTC variability is consistent with primary and metastatic tumor heterogeneity and suggests that single cell phenotyping of CTCs is a practical approach to exploit this variability for the effective implementation of molecular guided cancer therapy on a more comprehensive scale than possible with mutational analysis of a few known genes.

Current strategies for isolating CTCs are limited to complex analytic approaches that generate very low yield and purity. Also, several hundred clinical trials currently explore the role of circulating tumor cell (CTC) analysis for therapy decisions, but assays are lacking for comprehensive molecular characterization of CTCs with diagnostic precision.

Here we describe the development of a new device with high-throughput and

simple manipulation for isolation and analysis of single CTC regardless of properties.

Therefore, in this paper, we were focused on microfluidic platform, which can isolate and analyze single CTC, by using microwell-based droplet technology.

2.Materials and Method

2.1. Fabrication of Microfluidic Chips with microwells

A biocompatible material, poly-dimethylsiloxane(PDMS), was adopted for single-cell-based arrays in the microfluidic chip. The microfluidic chip was made two PDMS layer. The main channel, formed on the bottom PDMS layer, is 13 mm wide, 120 μm in height and 400 mm long. The channel contains $10^3 \times 10^2$ microwells, 18, 21 24 or 30 μm in diameter and 18 or 20 μm deep, on the bottom PDMS layer. The mold masters were fabricated by spinning SU-8 (SU-8 3010, 3025, MicroChem Corp., Newton, MA, USA) on a silicon wafer to define the microwells and microchannel, respectively. The mold master of the microfluidic channels was fabricated by spinning SU-8 at 1000 rpm for 20 s and then at 1500 rpm for 35 s on the silicon wafer. The resist was soft baked on a hotplate at 65 °

C for 10 min and then at 95 °C for 30 min. The resist was then allowed to cool to room temperature. The SU-8 was exposed to ultraviolet (UV) radiation at a dose of 200 mJ/cm2. The post-exposure baking was done at 65 °C for 3 min and then at 95 °C for 10 min. The exposed samples were developed with SU-8 developer for 5 min. The mold master of the microwells (around 120 μm in height) was fabricated by spinning SU-8 at 500 rpm for 30 s. The resist was developed with SU-8 developer for about 2 min after baking and exposure to UV radiation under the conditions mentioned above. PDMS prepolymer mixture was poured and cured on the mold masters to replicate the patterned structures. After peeling off the PDMS replica with the microchannel, the inlet and outlet ports were made by a puncher. The two PDMS replicas were bonded after treatment with oxygen plasma in an O2 plasma cleaner.

2.2 Cell Treatment

A MCF-7 cells (human breast adenocarcinoma cell line) was cultured for an experimental demonstration of single-cell lysis using the proposed microfluidic chips with microwells. The cells were serially passaged as monolayer cultures in RPMI medium (Gibco, Grand Island, NY, US), supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco). The cell culture dish (Falcon, Franklin Lakes, NJ, US) was incubated in a humidified atmosphere containing 5% carbon dioxide at 37 °C; the medium was replaced every 1 to 2 days. Cells grown to sub-confluence were washed with phosphate-buffered saline (PBS, Biochrome, pH 7.4) and harvested by a 5-min treatment with 0.25% trypsin. The cells were stained using a nuclear dye 4,6-diamidino-2-phenylindole (DAPI) prior to the experiment. DAPI is a blue fluorescent dye which pass through an intact cell membrane, it can be used to stain both live and fixed cells.The cell samples were then suspended in PBS.

2.3 Experimental Procedure

Prior to injecting sample, we let 70% ethanol flow by filling it for 10 min. This step was contributed to sterilization and sustenance of hydrophilicity in a microfluidic channel. Also, it is degassed in microwell.

Then, the microchannels were filled with PBS buffer including 15μm beads or MCF-7 cells using micropipette. As time passed, The beads(or cells) were settled on bottom of channel by the pull of gravity. Comparing a settlement time, there is not a great deal of difference in trapping efficiency. So, we set up 3min as the settlement time for low time consumption of experiment.

Next, injecting oil in the chip could remove external beads of micro-well and be pushed through oil flow. Finally, the cellular the cellular encapsulation in microwells was observed and recorded by an inverted fluorescence microscope.(Figure 2.1)

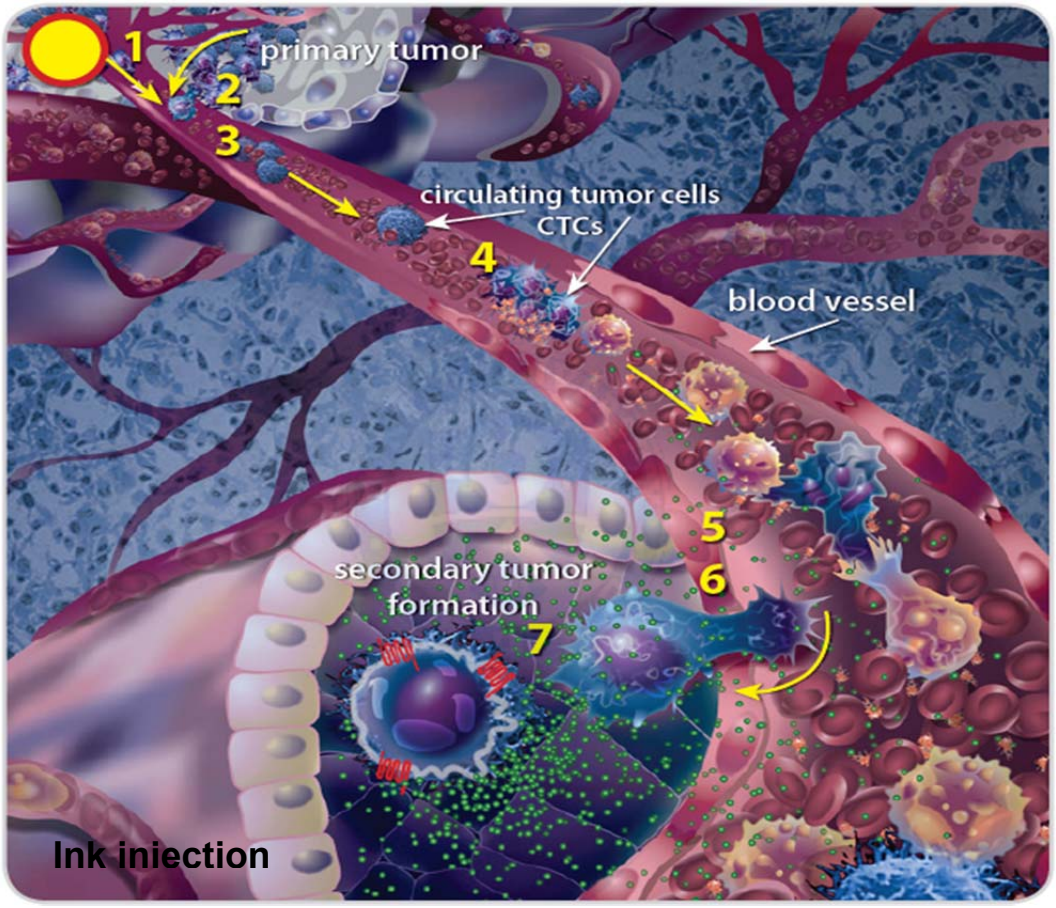

Figure 1.4. Illustration showing hematogeneous metastasis

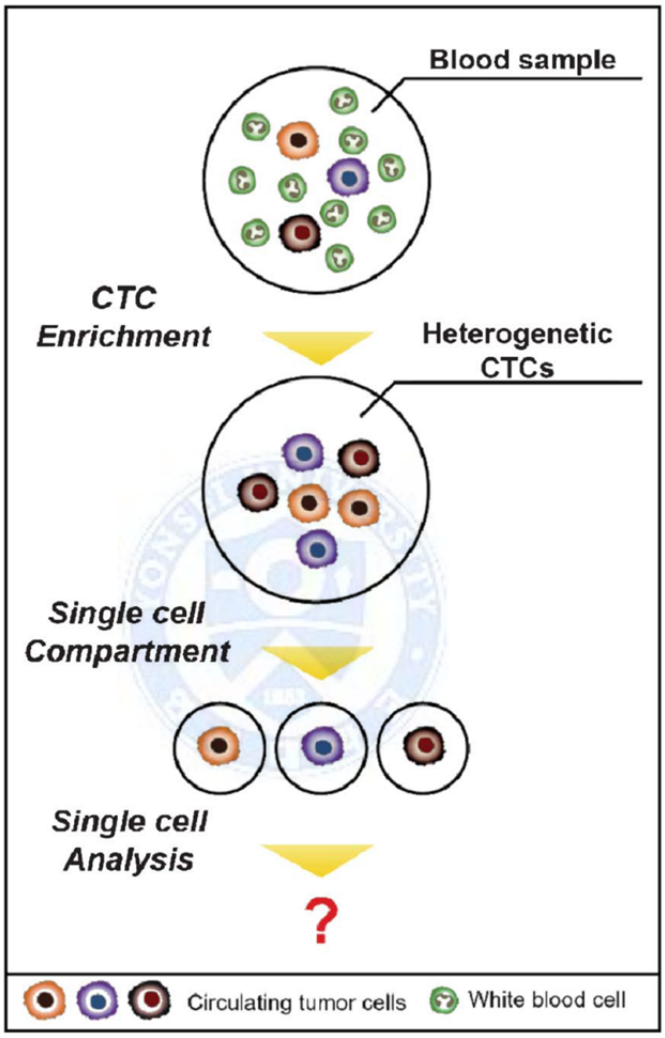

Figure 1.5. Overview for Single cell analysis of circulating tumor cell

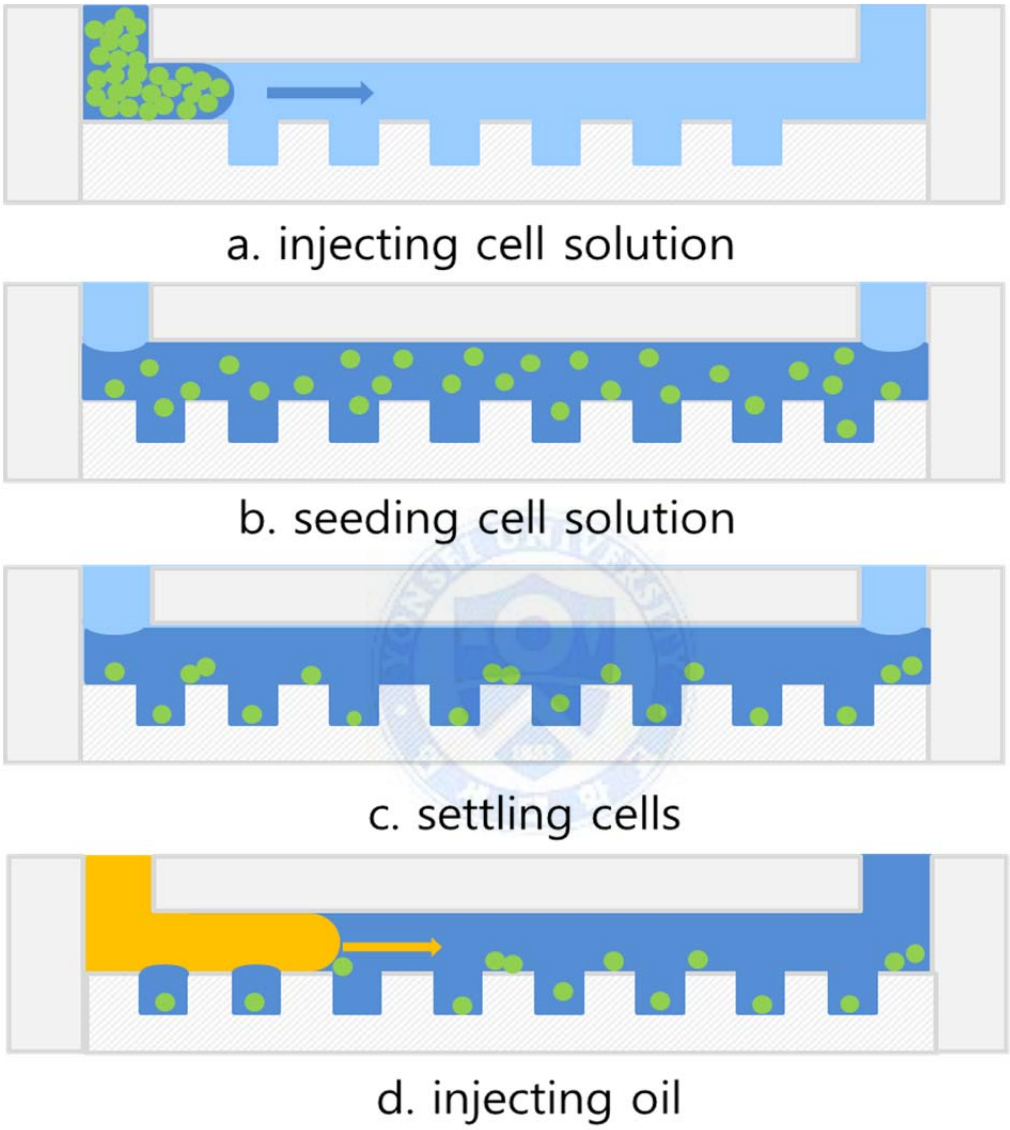

Figure 2.1. Schematic drawing of droplet generation

RESULTS AND DISCUSSION

1. Microwell-based droplet generation inside patterned microfluidic channel

In order to capture and analyze single cells, we fabricated a patterned microfluidic channel, in which the bottom of the channel was physically modified with deep-set microwells.

A droplet array is formed easily in our chip. A hydrophilic-in hydrophobic micro-patterned surface was used for the preparation of the droplets. At the first stage, the well-patterned surface is covered with an aqueous solution. The oil (Fluorinert FC 40), which has higher density than water, is then loaded into the aqueous solution (figure 3.1). More than 10^5 droplets are formed simultaneously on hollow micro-wells (figure 3.2). Red dye was diluted into aqueous solution in order to observe droplet formation to the naked eye. Since a generated droplet in micro-

wells had an inconsiderable volume of the pikoliter unit, this technique enabled the sensitive detection of CTCs. Furthermore, this experiment was able to be done with only pipettes. The present trapping mechanism, which is able to enhance cell viability by minimizing preconditioning process, is simple, and efficient.

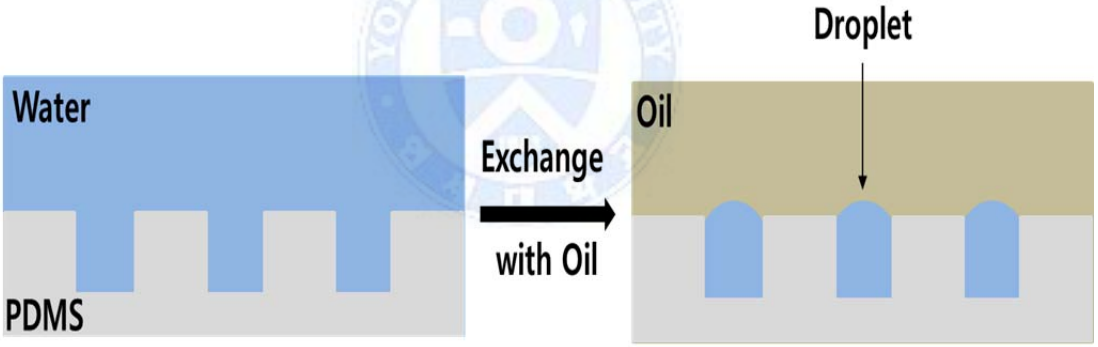

Figure 3.1. Schematics of a droplet array preparation producer.

Water on the micro-patterned surface is exchanged with oil.

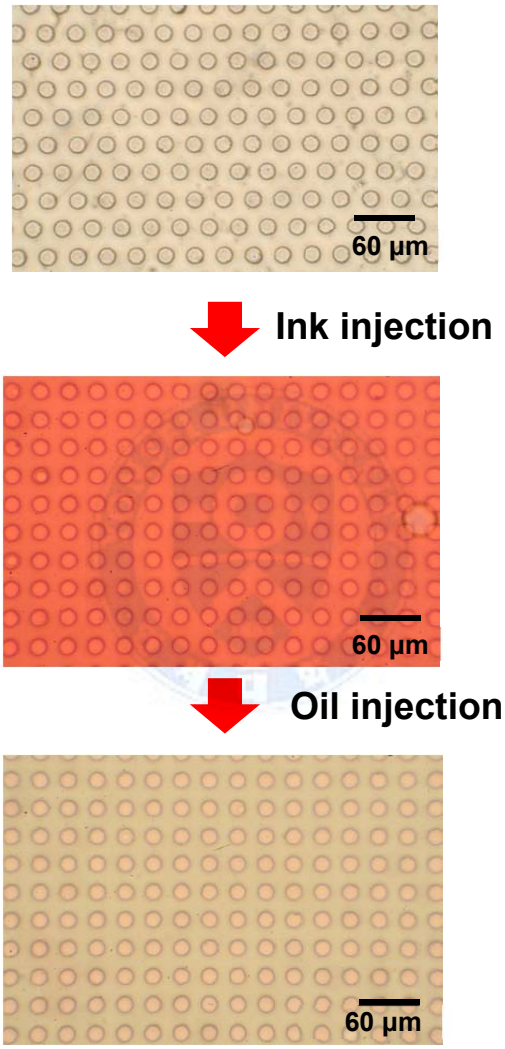

Figure 3.2. Droplet array formed on surface

2. Overview of micro-well chip

Flow lines pattern in the chamber is exactly same (figure 3.3). This is typically a case where the Stokes equations are sufficient to describe the flow.

Based on the results, we were able to prevent meniscus at the edge of micro-well chip in 1:2 aspect ratio of expanding area. So, we chose the channel dimensions of extension region for width and length at 300 and 600μm (figure 3). An overview of the chip is shown in figure 3.4. Dimension of the chip was 400mm in length, 13mm in width, and 120μm in height. This device includes $1X10^5$ micro-wells at the bottom surface. The micro-well chip was fabricated into double poly dimethylsiloxane(PDMS) layer. The bottom layer was fixed on the cover layer with cylindrical-type pillars (diameter = 60μm, height = 120μm), which blocked a bonding reaction between the two layer walls.

Using the fabricated patterned channels, a small amount of the cell solution (0.6–

0.7 mL) was introduced.

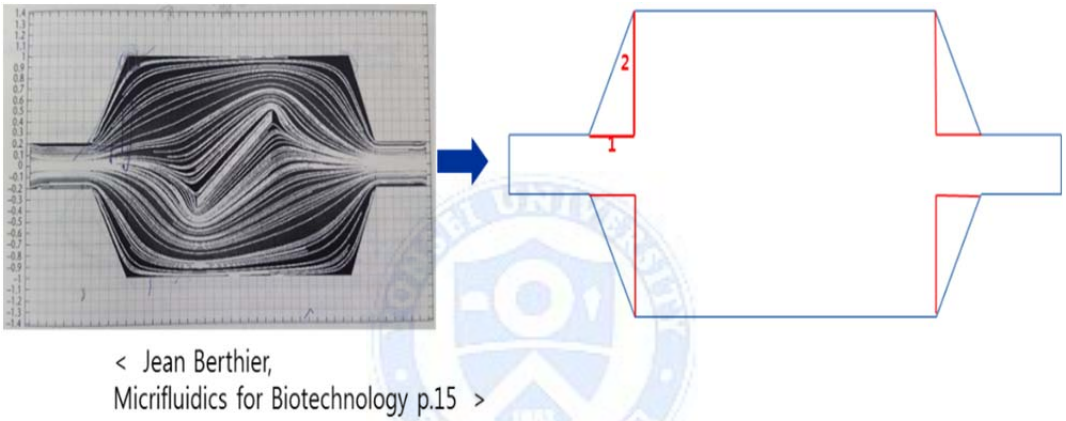

Figure 3.3. Flow line in the 2D microchamber on the left and outline of the chip on the right side

Figure 3.4. An overview of the cell chip. The cell chip has simple straight microfluidic chamber and two punched reservoirs.

3. Optimization of Microwell Dimensions.

In order to prepare a design the estimated size of well to trap single circulating tumor cell, we used 15 μm beads of a similar level for CTCs.

Also, to determine the optimal geometry of the microwells for high-efficiency cell docking and minimal flooding, various channel dimensions were tested.

So, the micro-well arrays platform adjusted micro-wells of four diameters (D=18, 21, 24 and 27μm) and fixed height (H=20 μm). The results of a representative experiment were indicated at figure 3.5. In 18 μm wells, the majority of these micro-wells were empty while micro-wells of the others were occupied by 0, 1, 2 or more than 2 beads. Since a mass of two cells in a well is hard to analyze single cell, it is important to be only captured for one's beads in a well.

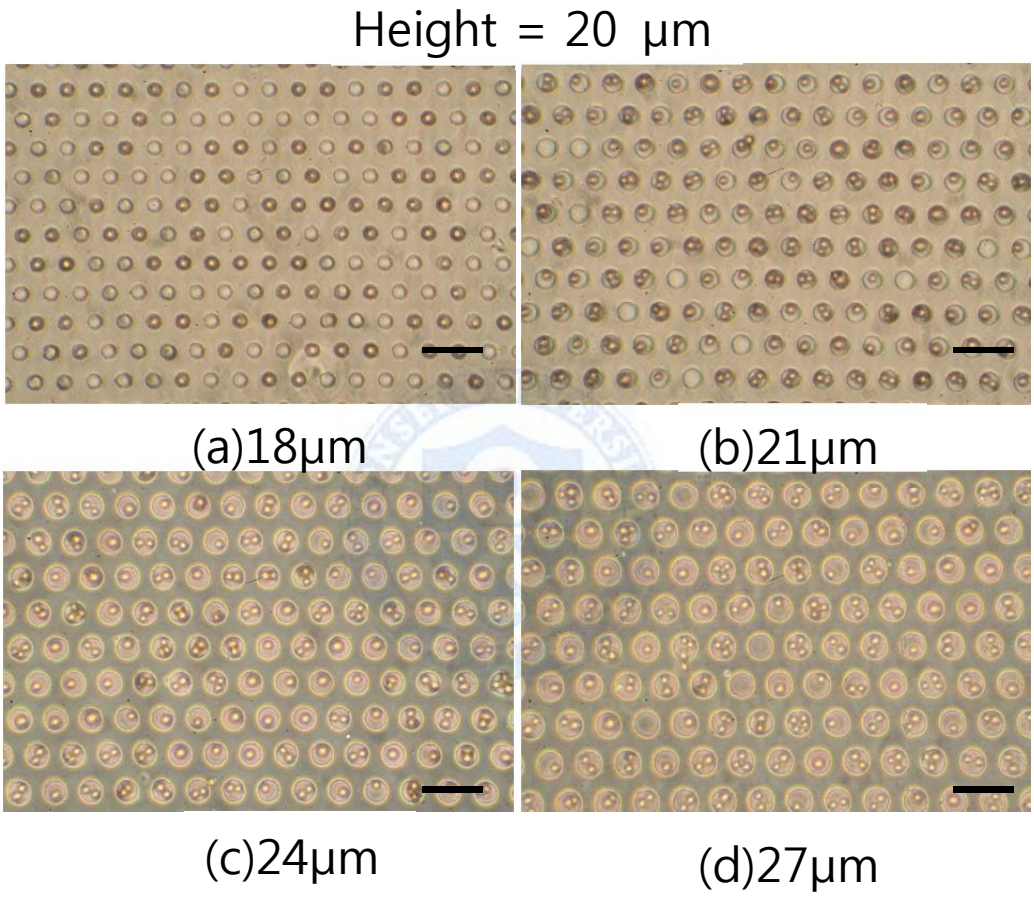

Figure 3.5. Bright field image of various microwells after bead seeding, respectively : (a) 18, (b) 21, (c)24, and (d)27μm. Scale bar: 60μm

Compared with the single bead ratio of D=21, 24 and 27μm in detail, the trapping efficiency of single bead have the highest rating on D=21μm for 58%. So, we fixed a diameter of the micro-well for it.(figure 3.6)

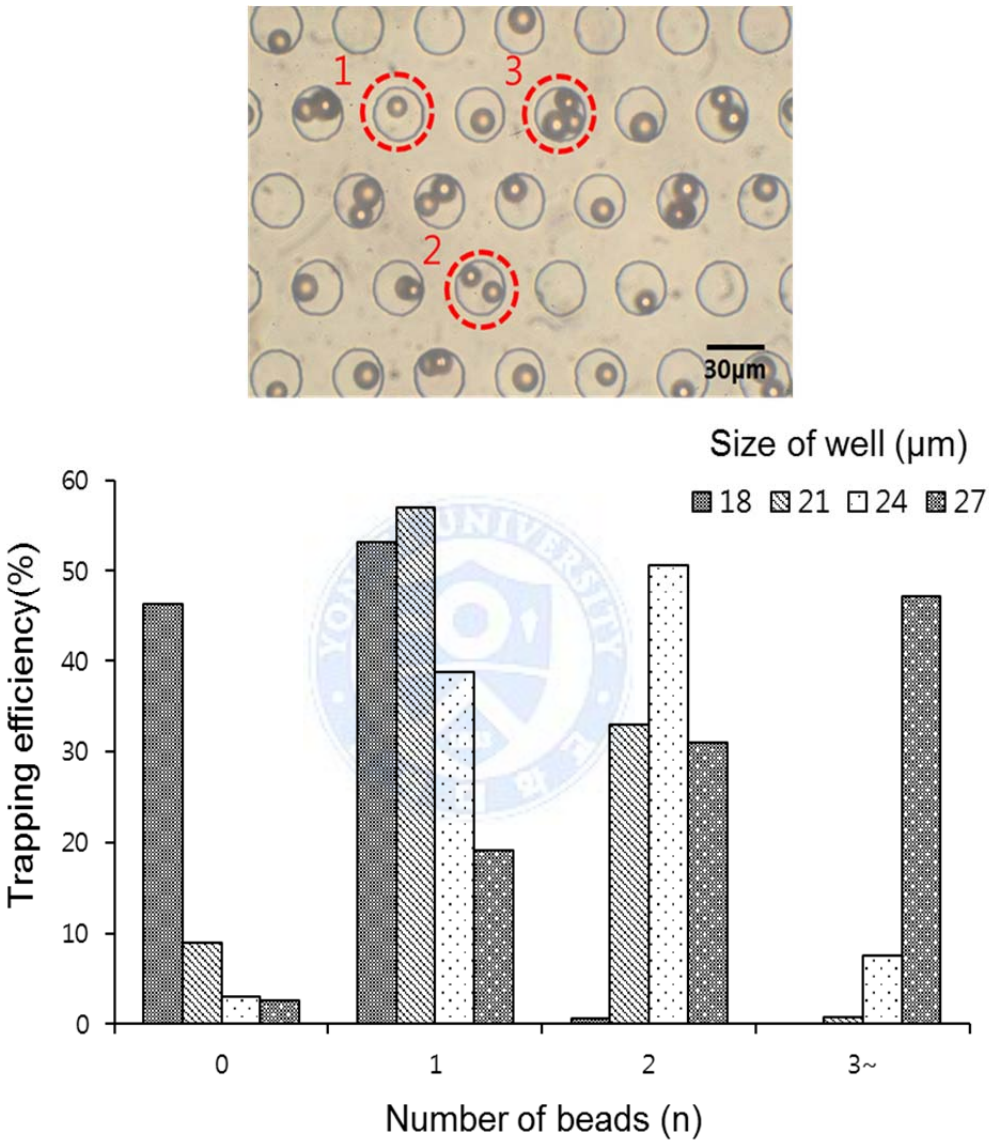

Figure 3.6. Distribution of microwell occupancies for a range of microwell diameters

Also, the height of wells was arranged to improve capability of single bead. We rarely considered testing wells deeper than H=20μm since more than two beads were already settled down at 20 um. So, even if deeper microwells resulted in a higher number of single cells, it would also likely increase the number of microwells with cells on top of each other that cannot be distinguished from single-cell microwells. As shown in the figure 3.7 (a), we were not able to focus on two beads in single well due to the difference of height level between beads. Therefore, the height of each well was adjusted on 18μm like figure 3.7(b).

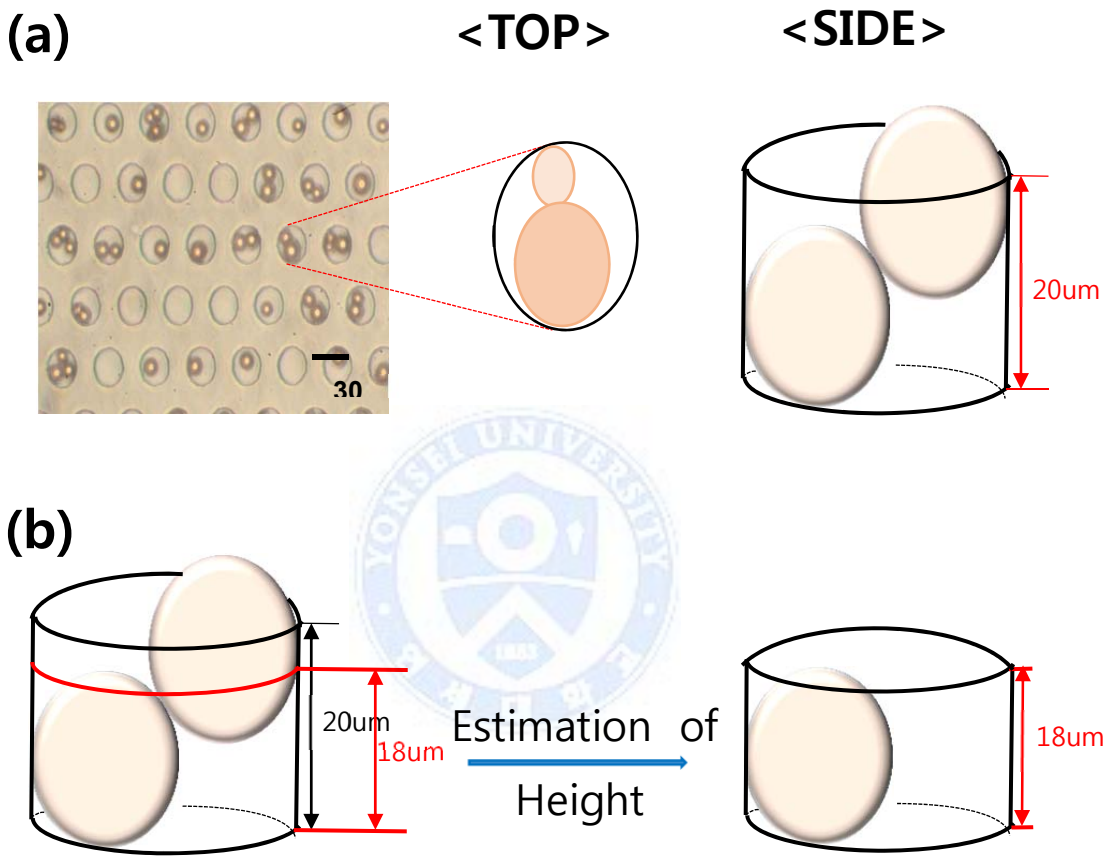

Figure 3.7. Estimation of height for trapping single bead

In order to maximize a number of micro-wells that are occupied by a cell, we used the following criteria: The micro-well should have enough space for a cell, but no more than two cells, to avoid being washed out by oil flow. Through this estimation process, we were able to conclude the final size of micro-wells(D=21μm, H=18μm) for trapping single cells. 15μm beads are injected and settled for 3 minute. Fluorescent 15μm beads in the microfluidic chips with 21μm diameters and 18μm depth micro-wells are shown in figure 3.8. This figure shows brightfield and fluorescent images of captured beads. The occupancy efficiency of the single bead in this micro-well chip is nearly 80% at $5X10^5$ concentration.(figure 3.9) In order to maximize a capture efficiency, we prepared two types of concentration beads : $1X10^5$ and $5X10^5$.

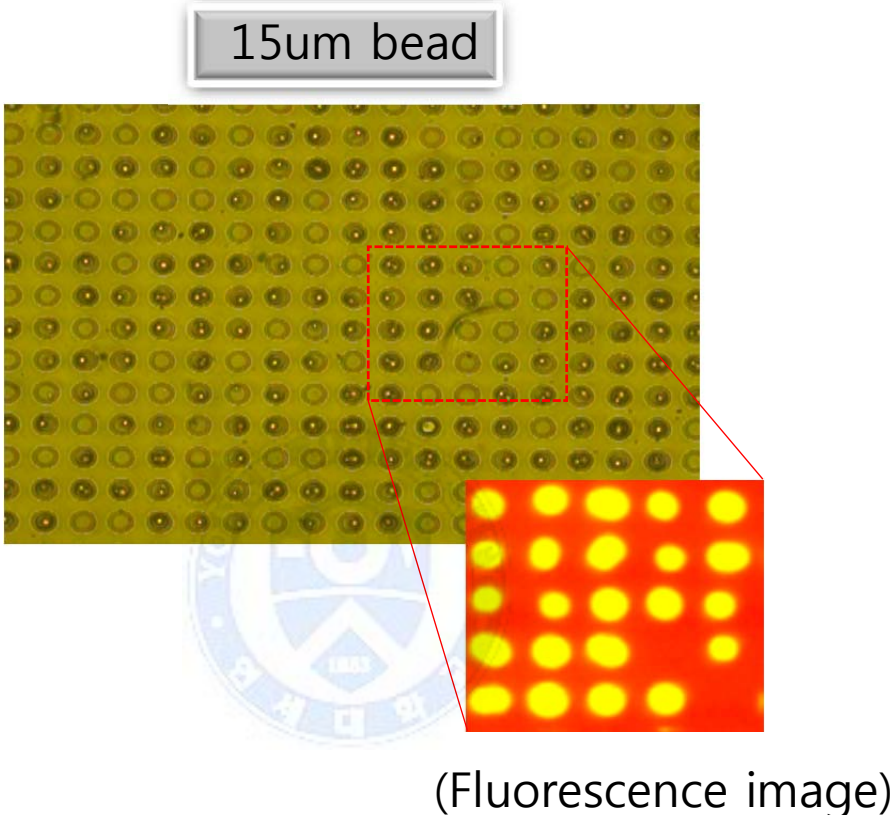

Figure 3.8. The brightfield and flourescent red images of beads

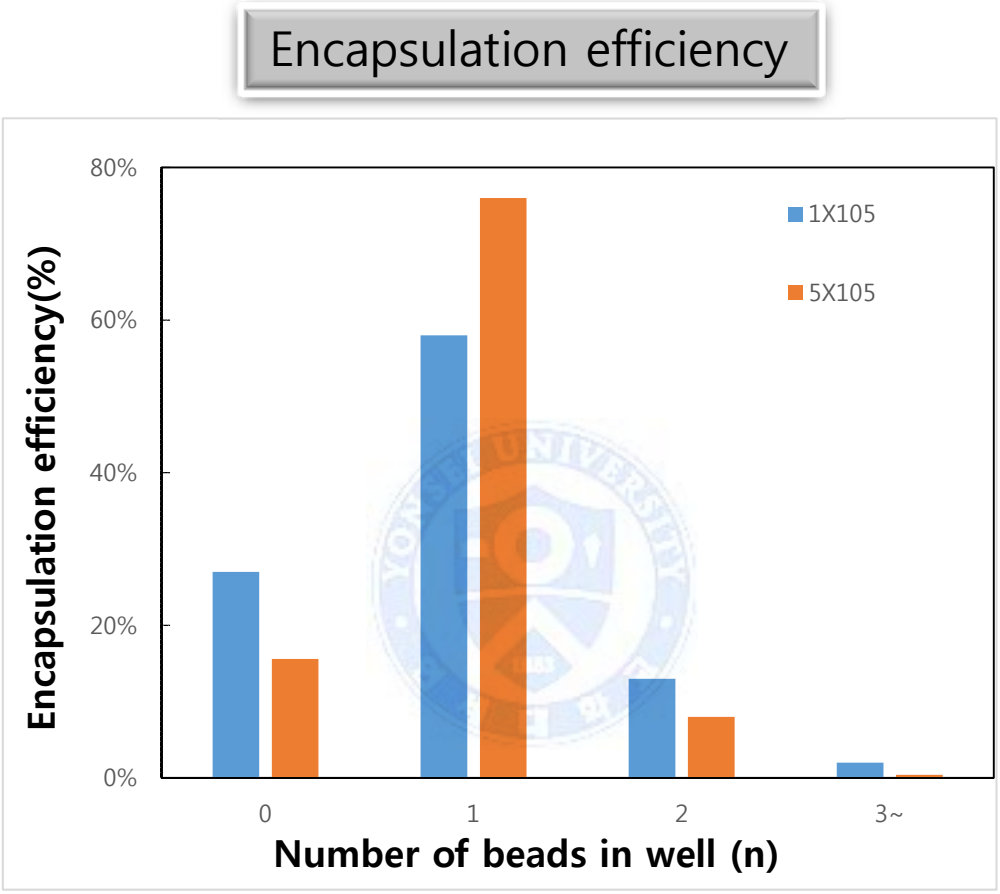

Figure 3.9. Encapsulation efficiency according to the number of beads in a well at 21μm diameter and 18μm depth

As an application of an accessible droplet array to a single cell isolation, we measured the rates for single bead efficiency.

Instead of 15μm fluorescence beads, MCF-7 cells (human breast adenocarcinoma cell line), which is 14-15μm in average, was introduced.

To show cells captured in wells, MCF-7 cells be dyed blue by DAPI staining of cellular nuclei(Figure 3.10). We identified that the best trapping efficiency of single cells was 74% at 21um wells(Figure 3.11). This result was almost the same with single bead encapsulation efficiency.

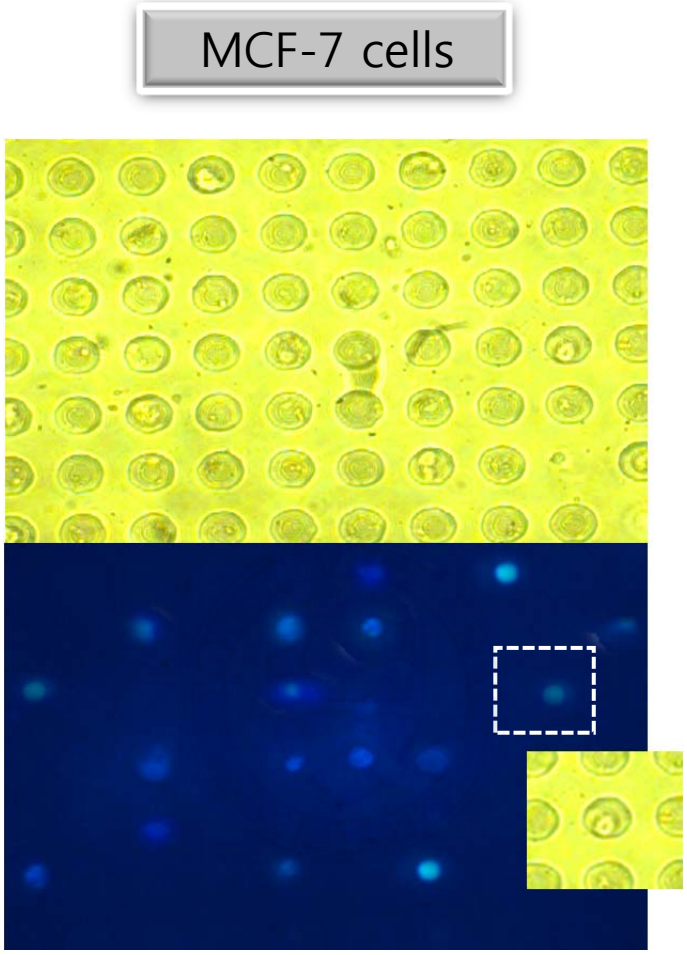

Figure 3.10. The brightfield and flourescent image showing only DAPI staining of cellular nuclei

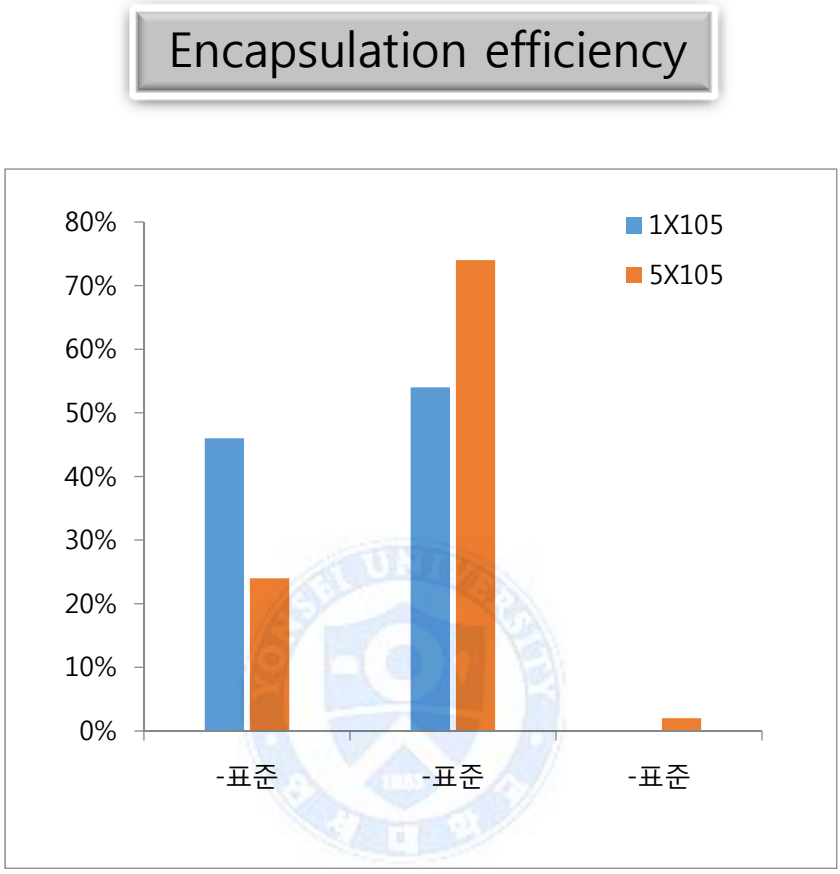

Figure 3.11. Encapsulation efficiency according to the number of cells in a well at 21μm diameter and 18μm depth

Discussion

We have developed a simple and robust microfluidic platform for analyzing single cell information in high-throughput manner. The method isolated simple microwell-based cell trapping within a patterned microfluidic channel and will enable to analyze using photothermal effect.

To optimize the cell docking scheme, the dimension of microwell sizes and different density of cell solutions. We confirmed that the 21μm microwell (18μm depth) was an optimal design for ensuring the single cell resolution and protecting the flooding effect. Also, the cell density should at least be higher than $5X10^5$ cells per mL to render a higher cell trapping efficiency (nearly 80%). We hope that this simple microwell-based cell chip potentially provides a valuable tool for analyzing of rare cells including CTC. Also, the present method should be useful for investigating the heterogeneity in cell populations.

References

[1] Kyung-A Hyun and Hyo-Il Jung., Lab on a chip. 2014, 14, 45-56

[2] Sunyoung Park, Wanjung Kim, Yongtae Kim, Young Dok Son, Sang-Chul Lee, Eunkyung Kim, Sung Ho Kim, Jung Hoe Kim, Hak-Sung Kim., Anal Chem. 2010, 82, 5830-5837

[3] Min Cheol Park, Jae Young Hur, Hye Sung Cho, Sang-Hyun Park and Kahp Y. Suh., Lab on a chip. 2011, 11, 79-86

[4] Shouichi Sakakihara, Suguru Araki, Ryota Iino, Hiroyuki Noji., Lab on a chip. 2010,10, 3355-3362

[5] Jacqueline R. Retting and Albert Folch., Anal Chem. 2005, 77, 5628-5634

[6] Chun-Ping Jen, Ju-Hsiao and Nikolay A. Maslov., Sensors. 2012, 12,347-358

[7] Kyung-A Hyun and Hyo-Il Jung., Electrophoresis. 2013, 34, 1028-1041

[8] Sara Lindstrom, Helene Andersson-Svahn., Biochimica et Biophysica Acta. 2011, 1810, 308-316

[9] Philip C. Gach, Peter J. Attayek, Rebecca L. Whittlesey, Jen Jen Yeh, Nancy L. Allbritton., Biosensors and Bioelectronics, 2014, 54, 476-473

[10] Jonathan Shemesh, Tom Ben Arye, Jonathan avesar, Joo H. Kang, Amir Fine, Michael Super, Amit Meller, Donald E. Lngber, and Shulamit Levenberg., PNAS, 2014, 111, 11293-11298

[11] Kyung-A Hyun, KihoKwon, Hyunju Han, Seung-Il Kim, Hyo-Il Jung., Biosensors and Bioelectronics

[12] Elodie Sollier, Derek E. Go, James Che, Daniel R. Gossett, Sean O'Byrne, Westbrook M. Weaver, Nicolas Kummer, Matthew Retting, Jonathan Goldman, Nicholas Nickols, Susan McCloskey, Rajan P. Kulkarni and Dino Di Carlo., Lab on a chip. 2014, 14, 63-77

[13] Bo Huang, Hongkai Wu, Devaki Bhaya, Arthur Grossman, Sebastien Granier, Brian K. Kobilka, Ricjard N. Zare., Science. 2007, 315, 81-84

[14] Chao Ma, Rong Fan, Habib Ahmad, Qihui Shi, Begonya Comin-Anduix, Thinle Chodon, Richard C Koya, Chao-Chao Liu, Gabriel A Kwong, Caius G Radu, Antoni Ribas & James R Health., Nature medicine. 2011, 17, 738-744

[15] Jon F. Edd, Dino Di Carlo, Katherine J. Humphry, Sarah Koster, Daniel Irimia, David A. Weitz and Mehmet Toner., Lab on a chip. 2008, 8, 1262-1264

[16] Haakan N. Joensson, Mathias Uhl_en and Helene Andersson Svahn., Lab on a chip. 2011, 11, 1305-1310

[17] Tania Konry, Margarita Dominguez-Villar, Clare Baecher-Allan, David A. Hafler, Martin.L. Yarmush., Biosensors and Bioelectronics. 2011, 26, 2707-2710

[18]Baiyan Qu, Young-Jae Eu, Won-Joong Jeong and Dong-Pyo Kim., Lab on a chip. 2012, 12, 4483-4488

[19] Min Cheol Park, Jae Young Hur, Keon Woo Kwon, Sang-Hyun Park and Kahp Y. Suh., Lab on a chip. 2006, 9, 988-994

[20] K. Eyer, S. Stratz, P. Kuhn, S. K. Kuster, and P.S. Dittrich., Analytical chemistry. 2013, 85, 3280-3287

[21] Song-Bin Huang, Min-Hsien Wu, Yen-Heng Lin, Chia-Hsun Hsieh, Chih-Liang Yang, Hung-Chin Lin, Ching-Ping Tseng and Gwo-Bin Lee., Lab on a chip. 2013, 13, 1371-1383

[22] Hui-Sung Moon, Kiho Kwon, Seung-Il Kim, Hyunhu Han, Joohyuk Sohn, Soohyeon Lee and Hyo-Il Jung., Lab on a chip. 2011, 11, 1118-1125