Volume 55 Number 4 April 2012 451–642

ISSN 1674-7291 CN 11-5839/O6

中国科学：化学

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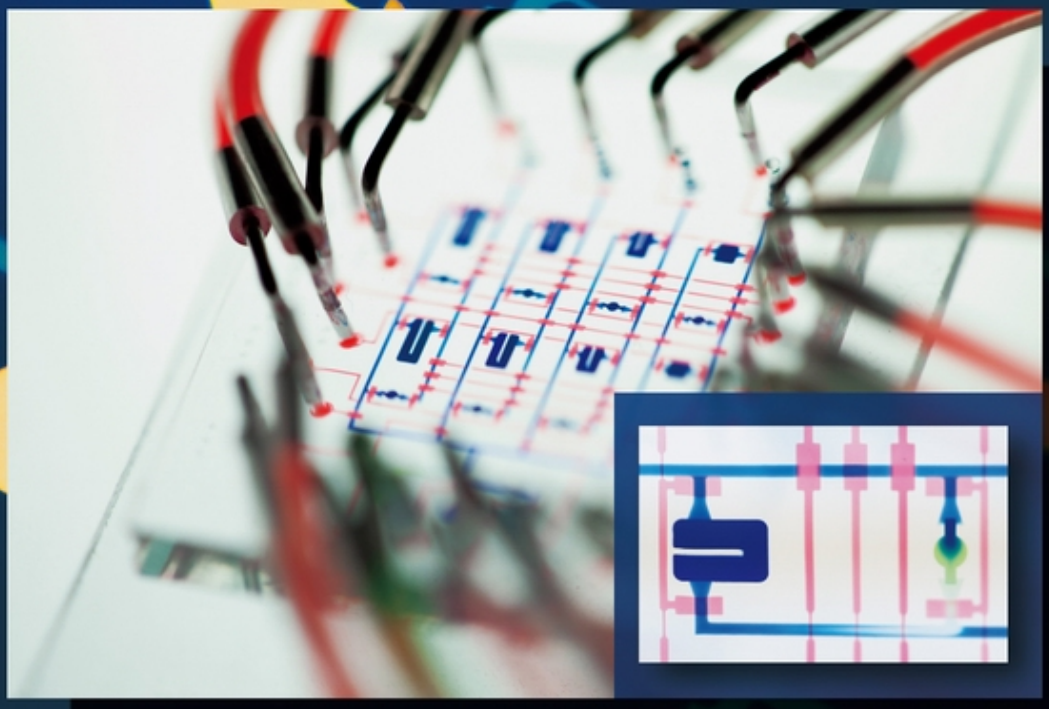

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April 2012 Vol.55 No.4: 502–507
doi: 10.1007/s11426-012-4493-1

An integrated microfluidic device for long-term culture of isolated single mammalian cells

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Received December 5, 2011; accepted December 14, 2011; published online January 24, 2012

We developed an integrated microfluidic chip for long-term culture of isolated single cells. This polydimethylsiloxane (PDMS) based device could accurately seed each single cell into different culture chambers, and isolate one chamber from each other with monolithically integrated pneumatic valves. We optimized the culture conditions, including the frequency of medium replacement and the introduction of conditioned medium, to keep the single cells alive for 4 days. We cultured a few hundred cells in a separated chamber on the same chip to continuously supply the conditioned medium into the culture chambers for single cells. This approach greatly facilitated the growth of single cells, and created a suitable microenvironment for observing cells' autonomous process *in situ* without the interference of other adjacent cells. This single cell colony assay is expandable to higher throughput, fitting the needs in the studies of drug screening and stem cell differentiation.

microfluidics, single cell culture, conditioned medium

1 Introduction

Cell studies at the single cell level, including stimulation, gene expression, protein content quantification, secretion, inter-cell communication, pluripotency verification, and cell cytometry, are becoming more and more critical to understand the complex biological processes [1–5]. Single cell's fate or decision-making, such as the stem cell differentiation or somatic cell mutation, has proven the essential events to embryo development and disease formation [6, 7]. Single-cell colony assay is also the gold standard to verify cell autonomous processes. Recently, newly developed technologies allow us to observe and to quantitatively analyze individual cells, further revealing cells' heterogenic behaviors and stochastic responses that are typically obscured in ensemble measurements.

Microfluidic devices have been intensively applied to tackle the challenges of single cell analyses. Many methods, such as optical tweezers, optical lattice, positive-dielectrophoretic cell sorting arrays, single cell electroporation set-up, and micro-patterned devices, have been reported to couple with microfluidics and to precisely handle single cells in the experiments that are difficult to perform using conventional approaches [8–14]. Microfluidic technology also offers many advantages to isolate a single cell from a group for further manipulation or measurement. Isolation has been done by many designs, for example, multi-phase liquid droplets, micro-fabricated hydrodynamic trapping structures, and microwells, etc. [15–19]. However, in most approaches, although the single cells can be recognized, tracked, and analyzed individually, they are still cultured as a whole group and share the medium during the entire culture process, or even during the analyses. Therefore, inter-cell interaction is inevitable since many secreted chemokines will diffuse from one cell to another, and there may be cell communication through physical contact in case of higher density. Thus it would be impossible to monitor the true single-cell response. One way to solve this problem is to culture many cells together and then divide them into

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individual cells for further studies. However, many analyses require long-term cell culture while single-cell colony culture is still challenging in most cases. A better and optimal technical approach is to culture single cells individually for long-term and each cell is isolated from others.

Here we present a new method to culture isolated single cells for 4 days. Thanks to the intrinsic properties of polydimethylsiloxane (PDMS), we can fabricate gas and moisture permeable devices with monolithically integrated PDMS pneumatic micro-valves [20]. These valves ensure complete isolation between cell culture chambers and each single cell maintained in one chamber neither contacts nor diffusively interacts with another cell. We monitored the single cell's activities, such as migration and proliferation, in each culture chamber by time-lapse image acquisition, demonstrating the great potential of this novel approach and its possible applications to other biological studies.

2 Experimental

2.1 Chip fabrication

We used a photolithographic process to fabricate the molds for fluidic channels and control channels, and the soft lithography method to fabricate all chips. The mold of control channels was made by negative photoresist (SU8-2025, MicroChem, Newton, MA, USA), a 25 μm-thick feature patterned on the silicon wafer. The hybrid mold for fluidic layer was made by negative photoresist (SU8-2025) for cell culture chamber with 25 μm-thick features, and by positive photoresist (P4620, AZ Electronic Materials, Branchburg, NJ, USA) for the other fluid channels with 25 μm-thick round profiles. PDMS (GE, RTV 615) was used to make the chip. All molds were exposed to chlorotrimethylsilane (TMCS) vapor for 10 min. RTV 615 A and B component were mixed at a ratio of 5:1 and poured onto the control channel mold. And for the fluidic channel mold, the RTV 615 A and B component were mixed at a ratio of 23:1 and spin-coated onto the mold at 2000 r/min for 60 s. Both of the two layers were cured at 80 °C for 30 min. Holes were punched for the access to control channels and the two layers were aligned and cured at 80 °C for 35 min. After the assembly two bonded layers were peeled off from the mold, holes for the access to fluidic channels were punched. The chip was then placed on a glass slide, which was spin-coated with PDMS at 1400 r/min for 60 s with a ratio of RTV 615 A:B at 10:1 and cured at 80 °C for 30 min. Finally, the whole device was put in an 80 °C oven overnight.

2.2 Cell culture

The CHO, Hela, and NIH 3T3 cells were obtained from the Cell Culture Center of the Peking Union Medical College. The NRK cell line was a gift from Professor Wensheng Wei at Peking University. NIH 3T3, CHO, and NRK cells were cultured in DMEM (Invitrogen) with 10% FBS and 1% PS. Hela cells were cultured in 1640 medium (Invitrogen) with 10% FBS and 1% PS. Other supplements were purchased from Invitrogen. Cells were cultured in a 5% CO_2 humidified atmosphere at 37 °C.

2.3 Chip operation

All fluidic channels were coated with fibronectin (200 μg/mL, Invitrogen), an extracellular matrix protein, for 2 h, before the cells were loaded. The confluent cells were detached from Petri dishes using 0.25% trypsin with 0.1% EDTA (Invitrogen), and centrifuged at 1000 r/min for 3 min. The supernatant was discarded and the cells were re-suspended with fresh medium at a proper density. For single cell culture experiment, the cell density was 10^6/mL and the old medium was replaced by fresh one every 4 h. For single cell-mass cell coculture experiment, the loaded single cell density was also set at 10^6/mL to ensure that the cell number was low enough to be easily isolated in the culture chambers. The loading density for mass cell culture chamber was set at 10^8/mL, thus the chamber may host enough cells to generate sufficient amount of the conditioned medium. The conditioned medium was driven from the mass cell culture chamber to the single cell culture chamber by a peristaltic pump composed from a serial of pneumatic valves embedded on-chip. The frequency of the valve actuation was set at 10 Hz and the pumping time was 10 s for each medium change.

3 Results and discussion

We designed and fabricated an integrated microfluidic device to screen the optimal condition for isolated single cell culture. This device has two layers of microfluidic channels: the fluidic layer and the control layer. To clearly indicate the channel routing and the layered structure of micro-channels on-chip, we loaded blue dye into the fluidic channels and red dye into the control channels, as shown in Figure 1(a). Fluidic network was composed of a 5 × 9 array of chambers for culturing single cells and the connective fluid channels for medium transportation. With cell suspension of proper density, single cells were sequentially loaded into the culture chambers, exact one cell per chamber, by controlling the horizontal and longitudinal valves. The whole loading process took less than 30 min if the cell density was properly adjusted. Take the first row of culture chambers as an example, at first we kept all horizontal valves closed, and then opened the first horizontal valve and the 9th longitudinal valve to allow medium with low-density cells to load from the inlet port. Observing under the microscope, once a single cell flowed into the 9th

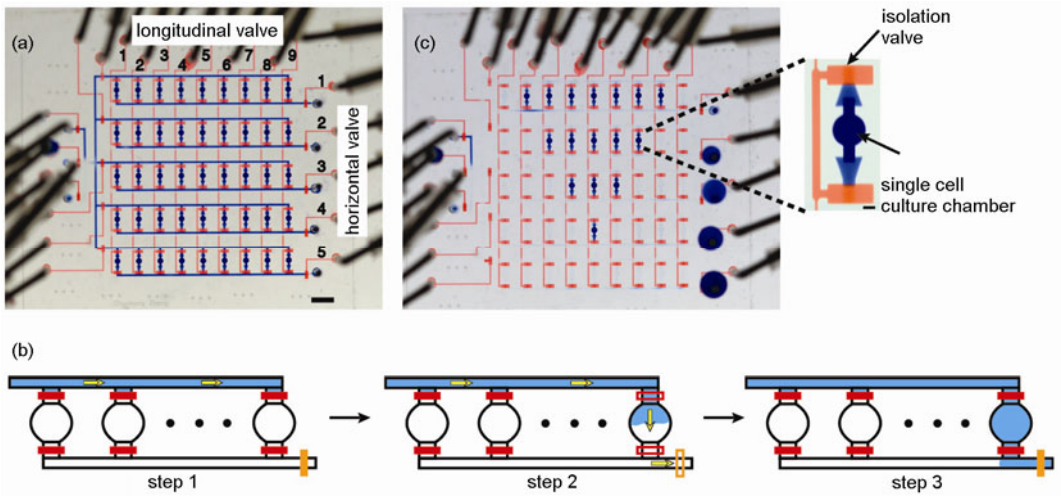

Figure 1 The integrated microfluidic chip for long-term single cell culture. The chip was filled with dyes to illustrate different layers of channels: blue for the fluidic layer and red for the control layer, respectively. (a) Most micro-valves, labeled as longitudinal valves and horizontal valves, were used to isolate the single cell culture chambers by dividing all culture chambers into columns and rows, respectively. Scale bar is 2 mm; (b) with combinatorial actuations of these two groups of valves, any individual culture chamber was addressable. The fluid was directed into a single chamber (arrow) through multi-step operation of valves; (c) each chamber was addressable. By repeating the previous process, multiple chambers could be precisely loaded with blue dye. Scale bar is 200 μm.

culture chamber, we closed the 9th longitudinal valve immediately to lock in this chamber and capture the cell (Figure 1(b)). We used a phase contrast microscope to verify that there was only one cell in the chamber. Then we opened the 8th longitudinal valve and locked another single cell into the 8th culture chamber through the same procedure. This operation did not affect the previously loaded single cell in the 9th culture chamber because it had been completely isolated and protected by the 9th longitudinal isolation valve. This method was capable to address each single chamber individually. Figure 1(c) shows that we could fill certain chambers with blue dye while others with colorless solution through the previously described procedure. By repeating this procedure we finally loaded all the chambers and guaranteed that each chamber had only one cell.

The height of the culture chamber (Figure 1(c)) was 25 μm and the diameter was 550 μm. The volume was ~ 6 nL, which was much larger than the volume needed to hold a single cell (a few pL). We determined the volume of the culture chamber under the following considerations. First, the volume of the medium for each cell should be comparable to that of conventional Petri-dish-based cell culture, which is typically in the range of 10–100 nL/cell. Second, the chamber should be big enough for cell to proliferate. Third, the surface area of each chamber should be large enough for efficient CO_2 diffusion through the chip. For adherent mammalian cells, a shallow chamber with big surface area to facilitate cell attachment and gas permeability was a rational choice.

We performed multiple experiments in parallel in a single device by placing different numbers of cells in each individual chamber to observe the difference in cell behaviors. The normal rat kidney (NRK) cells were cultured on-chip for 24 h. The chip was partially immersed in water at 37 °C to prevent the evaporation of medium in the chambers. To supply sufficient nutrition and remove metabolic waste, we replaced the culture medium every 4 h by controlling the integrated micro-valves. The fresh medium driven by compressed air flushed away the old medium thoroughly within a few seconds. Although the cell may be robust enough to handle high shear [21], long-time exposure to shear greater than 100 dyne/cm^2 may cause significant difference in gene expression and other physiological changes [22]. The flow velocity of the medium was kept relatively low (< 500 dyne/cm^2) and the flush has to be finished within a very short period to eliminate shear damage or detachment of the cells. Phase contrast microscopic images of every chamber were taken at 6, 12, and 24 h (Figure 2(a)). We found that at the first 24 h cell division never happened if cells were cultured individually in the micro chambers, confirming our knowledge from conventional culture methods with Petri dishes. We carefully checked the proliferation rate in single-cell culture chambers and found that this reduction of proliferation was not stochastic behavior. All singly cultured cells were kept single. However, in contrast to the chambers with single cells, when a small group (> 10) of cells were seeded together in a chamber there was always noticeable proliferation and the cell number increased ~ 50% (Figure 2(a)). For comparison, we did a few extra cell culture experiments on another chip-based design (Figure S1) with open microstructures. We confirmed the observation that when cells were not isolated from each other they were more likely to proliferate (Figure S2), and the proliferation

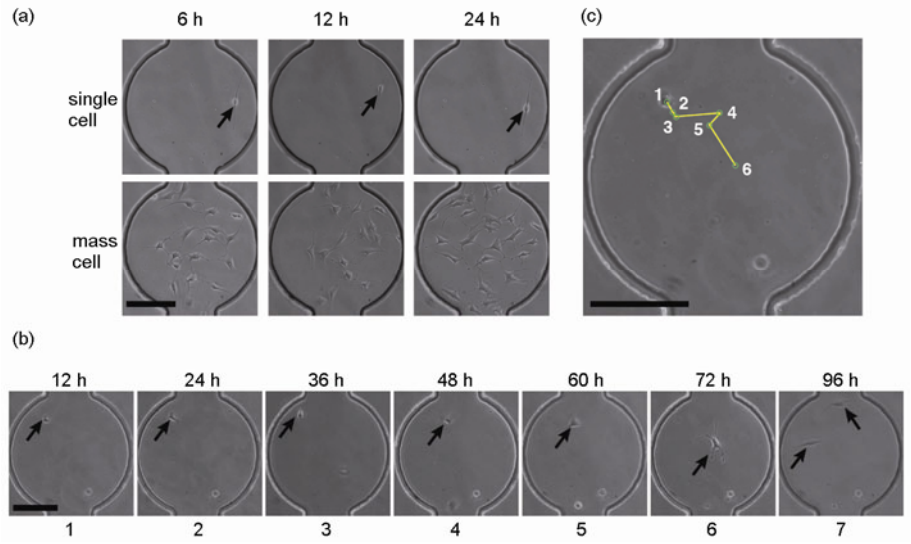

Figure 2 (a) Time-lapse phase contrast microscopic images of cell chambers with different cell densities. No cell division occurred in a single cell but obvious cell proliferation was observed in mass cell culture in one chamber; (b) time-lapse images of a single cell (arrow) cultured in the microfluidic cell culture chamber. Cell division occurred at 72 h time point; (c) the trajectory of the single cell was over 72 h. Numbers are corresponding to panel (b). Scale bar is 200 μm.

usually happened less than 24 h after cell loading.

We optimized the culture condition on-chip and achieved long-term single-cell culture for up to 96 h. The key to keep single cell alive was to feed fresh medium every 4 h. This refreshment frequency was higher than conventional culture in dishes. We tried to extend the interval between refreshments but cells' growth and motion slowed down when the interval was 8 h or longer, leading to low viability. From the microphotographs of every 12 h (Figure 2(b)), we could dynamically monitor single cell's behavior in each chamber (arrows). For the sake of demonstration, a single cell's migration trajectory in the micro chamber was also marked in Figure 2(c). We noticed that a single cell's morphological behaviors in an isolated chamber could be divided into three major phases. In the first phase, the cell tried to strengthen the adhesion to the extra cellular matrix coated on the bottom. In this phase the cell did not move much and eventually spread itself. After complete spreading, the cell entered the second phase, during which the cell randomly migrated within the chamber. The cell began to contract at ~70 h and then started the third phase, in which it was divided into two cells. With the completion of the whole process, we have demonstrated that a single normal mammalian cell, with properly optimized condition control, can survive by itself with normal phenotype and motility for a long period of time and slowly proliferate. However, the whole process has to be performed very carefully and the cells are very sensitive to the change of microenvironment.

If a cell is closely surrounded by many cells, it would be impossible to perform single-cell colony assays or to distinguish the cell-autonomous effects from the non-cell-autonomous ones. However, the vulnerability of single living cells creates many technical hurdles for the control of microenvironments. To solve this dilemma we designed a new device (Figure 3(a)) to continuously supply the freshly generated conditioned medium to support the single cells, which were seeded in a different chamber. In this device, each single cell culture chamber was the same as the previous device, connecting to a bigger chamber for mass cell culture. The density of the loaded cell suspension controlled the final cell-population in the chambers. The cells in the bigger chamber could not physically contact the single cells but the cytokines and growth factors secreted from them were brought to the single cell chamber by active pumping via the microfluidic channel. As shown in Figure 3(b), each chip had two parallel groups of chambers, and each group had four different configurations. All these 8 experiments were carried out automatically through home-developed programs. We designed 4 different sizes for the mass cell culture chamber to screen the best feeder/receiver ratio for the single cell culture. We found that the peristaltic pump, constructed with three pneumatic valves, is critical for conditional medium transfer since through the microfluidic channel the passive quasi-2D diffusion would not be sufficient for biomolecules. In Figure 3(b), the chambers were filled with color dyes to illustrate the conditioned medium transfer. The single cell culture chambers were completely filled with conditioned medium within 10 s by pumps (see arrows in the figure).

During the experiment, the conditioned medium was pumped into the single cell chambers every 4 h, then the mass cell chambers were refreshed with new medium. We used NIH 3T3 fibroblast cells in this system and controlled the cell density to make sure each single cell chamber was loaded no more than two cells to easily identify their cell-autonomous effects. We kept the cells alive for 42 h,

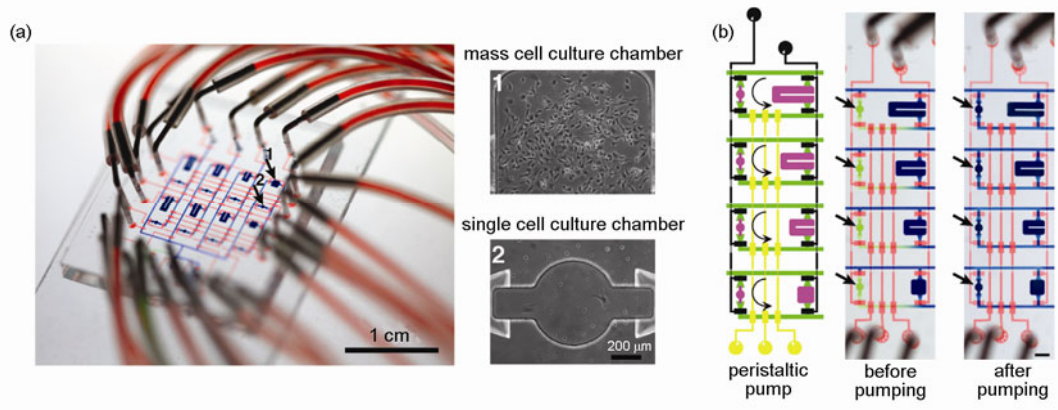

Figure 3 (a) An integrated microfluidic chip for long-term single cell culture. This chip had two sets of cell culture chambers, one for single cells and one for mass cells; (b) introduction of the conditioned medium generated from the mass cell culture chambers into the single cell culture chambers by integrated pneumatic pumps. Scale bar = 1 mm.

and Figure 4 shows the phase contrast microscopic images of a single chamber with two cells. With the help of conditioned medium these two cells (identified with arrows) quickly attached to the bottom surface of the chambers. Within 15 h, the cells fully spread and the cell division started at around 40 h, much faster than the previous approach and was comparable to those experiments of single cell colonies through conventional culture processes. Our observation clearly indicates that this approach will greatly facilitate the isolated single cell culture, and this method also fits the requirement of long-term culture. This method holds great potential to be scaled up for higher throughput screening and assaying, especially for single-cell-colony-based studies.

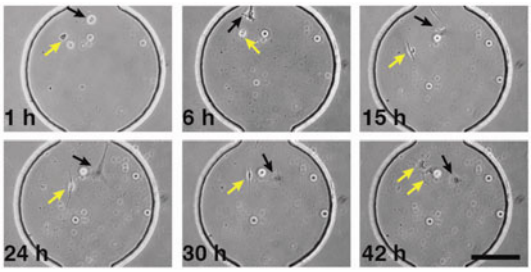

Figure 4 The time-lapse images of a culture chamber on-chip. Two cells were fed by the conditioned medium generated from an adjacent mass cell culture chamber. Dividing of one of them has been identified at t = 42 h. Scale bar = 200 μm.

4 Conclusions

We have developed a novel microfluidic-based method to perform long-term colony culture of isolated single cells. Thanks to the continuously supplied conditioned medium pumped from mass cell chamber, in which hundreds of cells are cultured together, the single cells are exposed to much favorable microenvironment for long-term culture and proliferation. This method has great potential to be applied to single-cell based drug screening and the studies of stem cells and induced pluripotent cells.

This work was financially supported by the National Natural Science Foundation of China (20733001, 20890020, 90913011, 20905004), the Ministry of Science and Technology of China (2011CB809106), the Ministry of Education of China, and the Fok Ying Tung Education Foundation.

1 Zhong JF, Chen Y, Marcus JS, Scherer A, Quake SR, Taylor CR, Weiner LP. A microfluidic processor for gene expression profiling of single human embryonic stem cells. *Lab Chip*, 2008, 8: 68–74
2 Adjaye J, Daniels R, Bolton V, Monk M. cDNA libraries from single human preimplantation embryos. *GENOMICS*, 1997, 46: 337–344
3 Kamme F, Salunga R, Yu JX, Tran DT, Zhu J, Luo L, Bittner A, Guo HQ, Miller N, Wan J, Erlander M. Single-cell microarray analysis in hippocampus CA1: Demonstration and validation of cellular heterogeneity. *J Neurosci*, 2003, 23: 3607–3615
4 Quake SR, Marcus JS, Anderson WF. Microfluidic single-cell mRNA isolation and analysis. *Anal Chem*, 2006, 78: 3084–3089
5 Zare RN, Huang B, Wu HK, Bhaya D, Grossman A, Granier S, Kobilka BK. Counting low-copy number proteins in a single cell. *Science*, 2007, 315: 81–84
6 Lanza R, Chung Y, Klimanskaya I, Becker S, Marh J, Lu SJ, Johnson J, Meisner L. Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. *Nature*, 2006, 439: 216–219
7 Monuki ES, Chung BG, Flanagan LA, Rhee SW, Schwartz PH, Lee AP, Jeon NL. Human neural stem cell growth and differentiation in a gradient-generating microfluidic device. *Lab on a Chip*, 2005, 5: 401–406
8 El Haj AJ, Walker LM, Holm A, Cooling L, Maxwell L, Oberg A, Sundqvist T. Mechanical manipulation of bone and cartilage cells with "optical tweezers". *FEBS Letters*, 1999, 459: 39–42
9 MacDonald MP, Spalding GC, Dholakia K. Microfluidic sorting in an optical lattice. *Nature*, 2003, 426: 421–424
10 MacDonald MP, Neale S, Paterson L, Riches A, Spalding GC, Dholakia K. Microfluidic optical sorting: particle selection in an optical lattice. *P Soc Photo-Opt Ins*, 2004, 5514: 1–14
11 Voldman J, Taff BM. A scalable addressable positive-dielectrophoretic cell-sorting array. *Anal Chem*, 2005, 77: 7976–7983
12 Lee LP, Khine M, Lau A, Ionescu-Zanetti C, Seo J. A single cell electroporation chip. *Lab on a Chip*, 2005, 5: 38–43
13 Toner M, Revzin A, Tompkins RG. Surface engineering with poly (ethylene glycol) photolithography to create high-density cell arrays on glass. *Langmuir*, 2003, 19: 9855–9862

14 Toner M, Folch A, Jo BH, Hurtado O, Beebe DJ. Microfabricated elastomeric stencils for micropatterning cell cultures. *J Biomed Mater Res*, 2000, 52: 346–353
15 Brouzes E, Medkova M, Savenelli N, Marran D, Twardowski M, Hutchison JB, Rothberg JM, Link DR, Perrimon N, Samuels ML. Droplet microfluidic technology for single-cell high-throughput screening. *Proc Natl Acad Sci USA*, 2009, 106: 14195–14200
16 Chiu DT, He MY, Edgar JS, Jeffries GDM, Lorenz RM, Shelby JP. Selective encapsulation of single cells and subcellular organelles into picoliter- and femtoliter-volume droplets. *Anal Chem*, 2005, 77: 1539–1544
17 Lee LP, Di Carlo D, Wu LY. Dynamic single cell culture array. *Lab on a Chip*, 2006, 6: 1445–1449
18 Cooper JM, Wlodkowic D, Faley S, Zagnoni M, Wikswo JP. Microfluidic single-cell array cytometry for the analysis of tumor apoptosis. *Anal Chem*, 2009, 81: 5517–5523
19 Whitesides GM, Ostuni E, Chen CS, Ingber DE. Selective deposition of proteins and cells in arrays of microwells. *Langmuir*, 2001, 17: 2828–2834
20 Quake SR, Unger MA, Chou HP, Thorsen T, Scherer A. Monolithic microfabricated valves and pumps by multilayer soft lithography. *Science*, 2000, 288: 113–116
21 Leverett LB, Hellums JD, Alfrey CP, Lynch EC. Red blood cell damage by shear stress. *Biophysical J*, 1972, 12: 257–273
22 Chisti Y. Hydrodynamic damage to animal cells. *Crit Rev Biotech*, 2001, 21: 67–110