Published in final edited form as:

Microsc Res Tech. 2011 June ; 74(6): 496–501. doi:10.1002/jemt.20937.

A microfluidic positioning chamber for long-term live-cell imaging

Lindsey Hanson¹, Lifeng Cui², Chong Xie², and Bianxiao Cui^{1,*}

¹Department of Chemistry, Stanford University, Stanford, California, 94305

²Department of Material Science and Engineering, Stanford University, Stanford, California, 94305

Abstract

We report a microfluidic positioning chamber (MPC) that can rapidly and repeatedly relocate the same imaging area on a microscope stage. The “roof” of the microfluidic chamber was printed with serials of coordinate numbers that act as positioning marks for mammalian cells that grow attached to the “floor” of the microfluidic chamber. MPC cell culture chamber provided a simple solution for tracking the same cell or groups of cells over days or weeks. The positioning marks were used to register time-lapse images of the same imaging area to single-pixel accuracy. Using MPC cell culture chamber, we tracked the migration, division and differentiation of individual PC12 cells for over a week using bright field and fluorescence imaging.

Keywords

microfluidic; micro-positioning; time-lapse imaging; live-cell imaging; microscopy

Introduction

Many important biological processes including cell cycle, differentiation and migration take long periods of time from days to weeks. For example, induction of cellular differentiation is a possible treatment of cancers (Miller and Waxman, 2002; Wainwright and others, 2001), and clinical trials are underway on several such inducers. To monitor the differentiation process in cell culture, morphological changes in living cells were often monitored for weeks after induction with chemical agent (Wainwright and others, 2001). Thus, following cellular activities and morphology over a long period of time is an important goal in cancer, stem cell, developmental biology and tissue engineering. However, time-lapse live-cell imaging for days and weeks is hindered by the difficulties to maintain cells in their natural environment on a microscope stage (Hinchcliffe, 2005; Stephens and Allan, 2003).

Mammalian cells require sensitive and controlled culture environment, and most mammalian cells are grown and maintained at an appropriate nutrients, temperature, pH and gas mixture (typically, 37°C, 5% CO₂) in a cell incubator. Culture conditions vary for each cell type, and variation of conditions for a particular cell type can result in different phenotypes being expressed. In order to follow the cell fate using non-invasive optical imaging, sophisticated environmental chambers are needed to control the temperature, pH, gas mixture, and humidity around the microscope stage (Dvorak and Stotler, 1971; Hing and others, 2000; Ince and others, 1983; Moogk and others, 2007; Rieder and Cole, 1998; Squirrel and others,

*Corresponding author: Bianxiao Cui Address: 333 Campus Dr., Stanford, CA 94305 bcui@stanford.edu Phone: (650)725-9573 Fax: (650)723-4817.

1999; Wetzels and others, 1991). Commercially available environmental systems (Nikon, Leica and Olympus) are generally composed of an inverted microscope enclosed by a specially designed incubator chamber, a heating insert, a temperature regulator, a CO₂ regulator, a CO₂ tank and a humidifying unit. Those chambers have been designed to duplicate incubator conditions on the microscope stage. However, besides being cumbersome, complicated and often with substantial price tags of more than ten thousand dollars, those environmental chambers perform rather poorly in maintaining a stable environment as compared with a regular incubator, thus limiting the imaging time to a few days. In addition, days-long imaging experiment using an environmental chamber will fully occupy the microscope, making the expensive setup inaccessible for other users during the period. Finally, stage drift and focus drift induce systematic errors in long-term time-lapse imaging (Burglin, 2000; LeSage and Kron, 2002).

In most instances, long-term imaging is a “time-lapse” imaging with a frame rate of one frame per few hours to one frame per few days. A solution to the problem of maintaining environmental conditions during time-lapse imaging is to keep cells in a regular lab incubator and place cells on the microscope stage only for active imaging. However, for high magnification imaging, this is prohibited by the impossible task of precisely locating the same imaging area for each time the culture is taken off and put back on the microscope stage. Microfluidic platform is becoming an increasingly useful tool for live cell culture (Faley and others, 2008; Kim and others, 2007; Lin and Butcher, 2006), large due to its precisely controlled microenvironment, efficient liquid exchange, its ability to generate reagent gradient over space, and minimized reagent use. Microfluidic devices have been also used for manipulating and imaging live worms such as *C. elegans* (Chung and others, 2008). In this work, we designed an innovative microfluidic positioning chamber (MPC) that can precisely and repeatedly relocate the same imaging area on the microscope stage, which would make any microscope suitable for live cell tracking over days and weeks.

Materials and Methods

Chemicals and materials

PDMS composed of silicone elastomer and curing agent (Sylgard 184 elastomer kit, Dow Corning, Corning, NY) was purchased from Fisher Scientific (Pittsburgh, PA). Poly-L-Lysine (PLL, molecular weight 70,000–150,000) and trichloromethyl silane (TCMS) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other cell culture reagents were purchased from Mediatech Inc. (Herndon, VA). Negative photoresist SU8–5 and SU8–50 were purchased from Microchem (Newton, MA). NGF was purified from mouse submaxillary glands following a published protocol (Mobley and others, 1976).

Device fabrication

The MPC chamber is made of polydimethylsiloxane (PDMS) silicone elastomer that automatically sealed against a flat substrate (either glass or plastic) by conformal contact (Fig. 1a). The master mold is a silicon wafer patterned by SU8 photoresist using standard photolithography method. Step-by-step procedures to make the MPC device were illustrated in supplementary figure 1S. Clean room fabrication was carried out at the Stanford Nanofabrication Facility Center. SU8–5 negative photoresist was spin coated at 2000rpm onto the silicon wafer to achieve the final thickness of 10μm. The photoresist layer was patterned by exposing to 365nm UV light through a high-resolution transparency mask. The first layer of the device was a PDMS chip fabricated by replica-molding (Hansen and Quake, 2003; Rhee and others, 2005)-- curing a liquid mixture of degassed silicone

elastomer and curing agent (weight ratio 10:1) on the master wafer patterned with photoresist. After curing at 70°C for 2 h, the pattern was molded into the PDMS layer, which was peeled off from the master mold. The second layer of the device was a PDMS film of 200 μm thickness, which was made by spinning liquid elastomer mixture on a new silicon wafer at 200rpm for 1min. A square opening with the size of the pattern was cut into the PDMS film, which will serve as the cell culture chamber. The first PDMS layer was aligned on top of the second PDMS layer and the combined device was cured overnight at 70°C. Two $14 \times 8 \text{ mm}^2$ wells separated by 8mm distance were cut into the PDMS device, serving as cell loading inlets and culture medium reservoirs for nutrient exchange. Both coverglass slides and polystyrene tissue culture dishes can be used as substrates for MPC device.

After sealing the PDMS chip to the substrate by slightly pressing at the edges, a covered compartment was formed that interconnected the two wells. Each reservoir well holds approximately 300 μl and the covered compartment holds only 11 μl . PC12M cells were cultured in DMEM culture medium containing 10% fetal bovine serum. Cultures were maintained according to standard protocols at 37°C in a 95% humidified incubator with 5% CO₂. Bright field and fluorescence imaging experiments were carried out on an inverted Nikon ECLIPSE Ti microscope equipped with CFI 20 $\times$ objective and a CoolSNAP HQ CCD camera.

Results and discussion

The key aspect of MPC chamber is the positioning marks printed on the “roof” of the covered compartment (Fig. 1a). The positioning marks are alternating lines of numbers, depicting Cartesian coordinates of the area. Lines are designed to be parallel to the x-axis of a reference frame. The odd-number lines are composed of increasing numbers corresponding to x-coordinate. The even-number lines are composed of repeats of the same number corresponding to the y-coordinate. The positioning mark on MPC chamber resembles the alignment marks for nanofabrication, which allows accurate positioning and alignment of substrates. However, unlike alignment marks that is located on the substrate itself, the positioning marks is located on the “roof” of the MPC chamber, which does not affect cells attached on the substrate and can be re-used with different substrates.

Mammalian cells are cultured inside the covered compartment in MPC chambers (Fig. 1b). Because mammalian cells grow attached to the glass or polystyrene substrate, cells on the “floor” and positioning marks on the “roof” of the chamber are residing on different microscope focal planes, which can be imaged independently. Figure 1c and 1d show the bright field images of the coordinate marks and the PC12 cells cultured inside a MPC chamber at the same imaging area. With the help of positioning marks, we can quickly (<10s) re-locate the same imaging area or the same cell after the culture is taken off and put back on the microscope stage. Without the positioning marks, it is close to impossible to find the same cell under high magnification imaging.

Cell survival inside a well-prepared MPC chamber is comparable to those cultured on bare substrate. MPC chamber is made of cross-linked PDMS which is biocompatible. Freshly prepared PDMS surface is found to be slightly toxic to cells, likely due to leaking of uncrosslinked monomers or oligomers into the culture medium. To address this problem, the first step we took was to increase PDMS polymerization reaction to a higher degree by baking at 80°C for 24 hrs. Then, PDMS chips were soaked in soap water for 12hrs followed by ethanol alcohol for 12 hrs to extract the remaining uncross linked monomers. Finally, PDMS chips were sterilized by dry-autoclave at 120°C for 30 min, which further increased the crosslinking degree and completely gassed out any remaining diffusive monomers and

organic solvent molecules trapped inside crosslinked PDMS. After all the baking-soaking-autoclaving steps, cell survival rate inside MPC chamber is comparable to that of bare substrate. The silicone elastomer chip can be reused by peeling off the substrate followed by cleaning and autoclaving.

MPC chamber is suitable for fluorescence imaging as PDMS is largely transparent to visible light. PC12 cells were transfected with green fluorescence protein (GFP) before plated inside the MPC chamber. Figure 1e shows the fluorescence image of GFP expressing PC12 cells in the same imaging area as showed in Fig. 1c and 1d. After superposing images with the positioning marks showing in a false magenta color (Fig. 1f), the position of green cells can be readily seen – e.g. two green cells residing near 41 of x-coordinates and 43 of the y-coordinates. Those coordinates can be used to identify and track individual cells over days or weeks. If GFP are tagged with important cellular proteins, the fate of genetically different cells can be monitored over long period of time.

Image registration using the positioning marks allows superposition of cell images at different days with single pixel accuracy. Images of the same area taken at different times often show slight translational and rotational shifts, which in part is due to the accuracy limit of the microscope stage. As displayed in Fig. 2c, direct overlap of two images of position marks show a misalignment of several micrometers. We used the automated image registration function in Matlab to overlay two images of positioning marks (Fig. 2d). The same registration parameters were employed to overlay the two cell images as displayed in Fig. 2h, in which single pixel accuracy can be achieved.

The size of positioning marks and the height of the covering chamber can be adjusted for different magnification and working distance of the microscope objective. For 10× and 20× phase objective, the size of positioning numbers is 100×100 μm^2 with a line width of 10 μm . The height of the covered compartment shown in Fig. 1 is 200 μm . A higher “roof” is desired if one would like to minimize the out-of-focus positioning marks shadowing on the cell image. For sub-cellular imaging, a 100× oil immersion objective is often used, for which the size of positioning marks was reduced to 10×10 μm^2 with a line width of 3 μm . For a 100× oil-immersion objective, the height of the covered compartment is restricted to 100 μm because of the limited focal depth of the objective.

Long-term imaging capability of MPC chamber is demonstrated by tracking the cell division and neuronal differentiation of PC12 cells. Application of nerve growth factor (NGF) to PC12 cells is known to induce morphological and biochemical changes in the cell, with extension of neurites and differentiation to a sympathetic neuron-like phenotype (Cui and others, 2007; Li and others, 1998; Mobley and others, 1976). When exposed to NGF, PC12 cells respond to the stimulation over a period of a few days by ceasing cell division and growing protruding neurite-like structures (Li and others, 1998; Mobley and others, 1976). Using a MPC chamber, the cell division and neurite extension processes of GFP-transfected PC12 cells were captured over 4 days at 1 frame /8 hours. For each frame of image, the MPC cell culture chamber was retreated from the lab incubator, placed on the microscope stage, moved to the desired area using positioning marks, and immediately put back to the lab incubator after imaging. The process took 1–2min for one frame (8hrs interval), which did not show any effect on the cell growth (as compared with the cultures that were not taken out for imaging) over a course of a week.

Figure 3 displays the bright field images focused on the positioning marks and the overlay images focused on the cultured cells. Two hours after plated into the MPC chamber, PC12 cells were seen as round cells and 11 cells in the field of view were expressing GFP. Sixteen hours after plating, 7 of the 11 green cells and also many non-transfected cells had divided.

Individual dividing or non-dividing cells can be clearly followed as indicated by the arrow head. NGF was added to the cell culture 16hrs after plating. Cell division was shown to slow down and short cell protrusions were visible 30hrs after plating. Neurites continue to elongate and branch over the next day. After three days, many neurites were longer than their cell bodies and cell division has significantly slowed down. As expected, the positioning marks do not change over a period of 3 days.

The time period for PC12M cells to double its number increased considerably upon NGF treatment. In normal culture conditions without NGF, PC12 M cells doubled their number in 40hrs. With NGF treatment, this time period increased to 85hrs, which indicated a reduced cell division activity (Fig. 4a). In the mean time, cell mobility was measured by following nucleus locations of 46 cells. For cells that divided during the 10hr counting period, they were excluded. Fig. 4b shows that PC12 cell mobility was slightly slowed down NGF stimulated cell differentiation.

MPC chamber made it possible to carry out intermittent time-lapse imaging of live cells on any regular microscope over the period of days to weeks. Compared with the commercially available environmental chambers, MPC chamber is cheaper and better suited for intermittently tracking cell morphology, mobility and proliferation with low frame rate (1frame per few hours or days) for very long period of time (weeks or months). However, automated microscope/software equipped with environmental chamber would be better suited for experiments that require shorter imaging duration but faster frame rate, such as fast cell migration and cell dynamics. The PDMS chip is made through a master pattern by replica molding. Once the master mode is fabricated, considerable amount of MPC chambers can be produced with no requirement of any capital equipment, thus making MPC chambers easy-to-use and affordable for general biological applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Yi Cui for his generous assistance and helpful discussions. This work was supported by National Institute of Health (NIH) grant NS057906, Bio-X interdisciplinary initiatives program at Stanford, Searle Scholar award and Packard Fellowship to B. Cui.

References

- Burglin TR. A two-channel four-dimensional image recording and viewing system with automatic drift correction. *J Microsc.* 2000; 200(Pt 1):75–80. [PubMed: 11012831]
- Chung K, Crane MM, Lu H. Automated on-chip rapid microscopy, phenotyping and sorting of *C. elegans*. *Nat Methods.* 2008; 5(7):637–43. [PubMed: 18568029]
- Cui B, Wu C, Chen L, Ramirez A, Bearer EL, Li WP, Mobley WC, Chu S. One at a time, live tracking of NGF axonal transport using quantum dots. *Proc Natl Acad Sci U S A.* 2007; 104(34):13666–71. [PubMed: 17698956]
- Dvorak JA, Stotler WF. A controlled-environment culture system for high resolution light microscopy. *Exp Cell Res.* 1971; 68(1):144–8. [PubMed: 4939849]
- Faley S, Seale K, Hughey J, Schaffer DK, VanCompernelle S, McKinney B, Baudenbacher F, Unutmaz D, Wikswo JP. Microfluidic platform for real-time signaling analysis of multiple single T cells in parallel. *Lab Chip.* 2008; 8(10):1700–12. [PubMed: 18813394]
- Hansen C, Quake SR. Microfluidics in structural biology: smaller, faster em leader better. *Curr Opin Struct Biol.* 2003; 13(5):538–44. [PubMed: 14568607]

- Hinchcliffe EH. Using long-term time-lapse imaging of mammalian cell cycle progression for laboratory instruction and analysis. *Cell Biol Educ.* 2005; 4(4):284–90. [PubMed: 16353401]
- Hing WA, Poole CA, Jensen CG, Watson M. An integrated environmental perfusion chamber and heating system for long-term, high resolution imaging of living cells. *J Microsc.* 2000; 199(Pt 2): 90–5. [PubMed: 10947901]
- Ince C, Ypey DL, Diesselhoff-Den Dulk MMM, Visser JA, De Vos A, Van Furth R. Micro-CO₂-incubator for use on a microscope. *J Immunol Methods.* 1983; 60(1–2):269–75. [PubMed: 6406605]
- Kim L, Toh YC, Voldman J, Yu H. A practical guide to microfluidic perfusion culture of adherent mammalian cells. *Lab Chip.* 2007; 7(6):681–94. [PubMed: 17538709]
- LeSage AJ, Kron SJ. Design and implementation of algorithms for focus automation in digital imaging time-lapse microscopy. *Cytometry.* 2002; 49(4):159–69. [PubMed: 12454979]
- Li R, Kong Y, Ladisch S. Nerve growth factor-induced neurite formation in PC12 cells is independent of endogenous cellular gangliosides. *Glycobiology.* 1998; 8(6):597–603. [PubMed: 9592126]
- Lin F, Butcher EC. T cell chemotaxis in a simple microfluidic device. *Lab Chip.* 2006; 6(11):1462–9. [PubMed: 17066171]
- Miller WH Jr, Waxman S. Differentiation induction as a treatment for hematologic malignancies. *Oncogene.* 2002; 21(21):3496–506. [PubMed: 12032784]
- Mobley WC, Schenker A, Shooter EM. Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry.* 1976; 15(25):5543–52. [PubMed: 999827]
- Moogk D, Hanley S, Ramunas J, Blaylock A, Skorepova J, Rosenberg L, Jervis E. Design and analysis of a long-term live-cell imaging chamber for tracking cellular dynamics within cultured human islets of Langerhans. *Biotechnol Bioeng.* 2007; 97(5):1138–47. [PubMed: 17243145]
- Rhee SW, Taylor AM, Tu CH, Cribbs DH, Cotman CW, Jeon NL. Patterned cell culture inside microfluidic devices. *Lab Chip.* 2005; 5(1):102–7. [PubMed: 15616747]
- Rieder CL, Cole RW. Perfusion chambers for high-resolution video light microscopic studies of vertebrate cell monolayers: some considerations and a design. *Methods Cell Biol.* 1998; 56:253–75. [PubMed: 9500142]
- Squirrel JM, Wokosin DL, White JG, Bavister BD. Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability. *Nat Biotechnol.* 1999; 17(8):763–7. [PubMed: 10429240]
- Stephens DJ, Allan VJ. Light microscopy techniques for live cell imaging. *Science.* 2003; 300(5616): 82–6. [PubMed: 12677057]
- Wainwright LJ, Lasorella A, Iavarone A. Distinct mechanisms of cell cycle arrest control the decision between differentiation and senescence in human neuroblastoma cells. *Proc Natl Acad Sci U S A.* 2001; 98(16):9396–400. [PubMed: 11481496]
- Wetzels JF, Kribben A, Burke TJ, Schrier RW. Evaluation of a closed perfusion chamber for single cell fluorescence measurements. *J Immunol Methods.* 1991; 141(2):289–94. [PubMed: 1880433]

Figure 1.

Demonstration of microfluidic positioning chamber (MPC). (a) Illustration of MPC scheme and the positioning marks on the “roof” on the covered compartment. (b) Illustration of mammalian cells growing attached at the “floor” of a MPC chamber imaged through an inverted microscope. (c) Bright-field image of positioning marks when focused at the “roof” of the MPC chamber. (d) Bright-field image of cultured PC12 cells when focused at the “floor” of the MPC chamber at the same field position. (e) Green fluorescence imaging of GFP transfected PC12 cells. (f) Superposing images (c–e) with positioning marks showing in a false magenta color. Scale bar: 100 μm .

Figure 2.

Image registration using MPC positioning marks. (a) and (b) display images of the positioning marks at 2hrs and 16hrs after PC12M cells were plated inside the microfluidic chamber. (c) displays overlay image of (a) and (b) that show an obvious misalignment of the positioning coordinates. (d) displays the registered image of (a) and (b) after translational and rotational adjustments. (e–h) show PC12 cells growing on the “floor” of the MPC chamber. Bright field images were false-colored to cyan and red for illustration purpose. The same adjustment parameters were used to construct the registered image (f). Scale bar: 50 μm .

Figure 3.

Tracking cell division and differentiation of GFP transfected PC12 cells using MPC chamber on a standard fluorescence microscope. (a), (c), (e), and (g) display images of the positioning marks focused at the “roof” of the MPC chamber. (b), (d), (f) and (h) display overlay images of the PC12 cells at the same field position over a period of 72 hrs. NGF was added to the culture 16hrs after plating. As pointed by the arrows, cell division of individual cells can be clearly followed over time. NGF-stimulated cell differentiation and neurite outgrowth is extensive at 68hrs. Scale bar: 100 μm .

Figure 4.

Comparison of PC12M cell division and motility with and without NGF. (a) Upon NGF treatment, cell doubling time for PC12M cells increased significantly from 40hrs to 85hrs. (b) NGF treatment slightly decreased the mobility of PC12M cells.