Video Article

A Microfluidic Platform for High-throughput Single-cell Isolation and Culture

Ching-Hui Lin[1,2], Hao-Chen Chang[1,2], Chia-Hsien Hsu[1,2,3]

[1]Institute of Biomedical Engineering and Nanomedicine, National Health Research Institutes, Taiwan

[2]Tissue Engineering and Regenerative Medicine, National Chung Hsing University

[3]Institute of NanoEngineering and MicroSystems, National Tsing Hua University

Correspondence to: Chia-Hsien Hsu at chsu@nhri.org.tw

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Abstract

Studying the heterogeneity of single cells is crucial for many biological questions, but is technically difficult. Thus, there is a need for a simple, yet high-throughput, method to perform single-cell culture experiments. Here, we report a microfluidic chip-based strategy for high-efficiency single-cell isolation (~77%) and demonstrate its capability of performing long-term single-cell culture (up to 7 d) and cellular heterogeneity analysis using clonogenic assay. These applications were demonstrated with KT98 mouse neural stem cells, and A549 and MDA-MB-435 human cancer cells. High single-cell isolation efficiency and long-term culture capability are achieved by using different sizes of microwells on the top and bottom of the microfluidic channel. The small microwell array is designed for precisely isolating single-cells, and the large microwell array is used for single-cell clonal culture in the microfluidic chip. This microfluidic platform constitutes an attractive approach for single-cell culture applications, due to its flexibility of adjustable cell culture spaces for different culture strategies, without decreasing isolation efficiency.

Video Link

The video component of this article can be found at https://www.jove.com/video/54105/

Introduction

Currently placing single cells individually in a culture space is commonly achieved by using limiting dilution or fluorescence-activated cell sorting (FACS). For many laboratories, limiting dilution is a convenient method, as it only requires a pipette and tissue culture plates, which are readily available. In this case, a cell suspension is serially diluted to an appropriate cell density, and then placed into culture wells by using a manual pipette. These compartmented single cells are then used for cell analysis, such as genetic heterogeneity screening[1] and colony formation[2]. However, this method is low-throughput and labor-intensive, without utilizing a robotic arm for assistance, because the Poisson distribution nature of the limiting dilution method restricts single-cell events to a maximum probability of 37%[3]. FACS machines with an integrated robotic arm can overcome the limitation of Poisson distribution by accurately placing one single-cell in a culture well at a time[4]. However, the high mechanical shear stress (thus, lowered cell viability)[5] and machine purchase and operational costs have limited its usage in many laboratories.

To overcome the above limitations, microscale devices have been developed to highly efficiently load single cells into microwells[6]. However, the microwells do not provide adequate space for the loaded cells to proliferate, due to the need of making the size of each microwell close to that of a single cell to maximize the single-cell loading probability. As culture assays are required in many cell-based applications (*e.g.,* clonogenic assay[7]), larger microwells (from 90 - 650 µm in diameter or in side length) have also been utilized to allow for extended cell cultures. However, like the limiting dilution method, they also possess low single cell loading efficiencies, ranging from 10 - 30%.[8,9]

Previously, we have developed a high-throughput microfluidic platform to isolate single cells in individual microwells and demonstrate its application in clonogenic assay of the isolated cells.[10] The device was made with poly-dimethylsiloxane (PDMS), and comprises two sets of microwell arrays with different microwell sizes, which can largely improve the efficiency in loading a single cell in a microwell whose size is significantly larger than the cell. Notably, this "dual-well" concept allows the size of the culture area to be flexibly adjusted without affecting the single-cell capture efficiency, making it straightforward to adjust the design of the device to suit different cell types and applications. This high-efficiency method should be useful for long-term cell culture experiments for cell heterogeneity studies and monoclonal cell line establishment.

Protocol

Note: The photomask designs for our microfluidic device fabrication were drawn by using a computer aided design (CAD) software. The designs were then utilized to fabricate chrome photomasks using a commercial service. The PDMS devices were made using soft lithography techniques.[11]

1. Fabrication of Master Molds by Lithography

1. Before the photolithography process[12], use the 4-inch silicon wafers as a substrate and dehydrate the wafers in a conventional oven at 120 °C for 10 min.
2. Clean the dehydrated silicon wafers by using oxygen plasma treatment at 100 watts for 30 sec in a plasma cleaner.
3. Preheat two hotplates at 65 °C and 95 °C, respectively, for the following baking process.
4. Coat 5 g of negative photoresist (PR) on the cleaned silicon wafers by a spin coater; spin at 1,200 rpm (SU-8 50) for 30 sec to produce the microchannel layer.
5. Place the PR coated wafer on a preheated hotplate at 65 °C for 12 min and transfer it to another preheated hotplate at 95 °C for 33 min (for 100 µm thick patterns) to perform a soft bake process.
6. After baking, place the PR coated silicon wafer on the holder of a semi-automated mask aligner and align it to a 25,400 dpi resolution transparency photomask.
7. Expose the PR coated silicon wafer to UV light (365 nm) at a dose of 500 mJ/cm^2 to create the PR pattern on the silicon wafer.
8. Remove the wafer from the aligner and place it on a hotplate for post-baking at 95 °C for 12 min.
9. Soak the wafers in SU-8 developer (propylene glycol monomethyl ether acetate, PGMEA) solution to wash off uncrosslinked PR for 12 min and gently dry with nitrogen gas to expose the alignment marks.
10. Again, coat 5 g of negative photoresist on the wafers by a spin coater; spin at 700 rpm (SU-8 100) for 30 sec and 1,200 rpm (SU-8 10) for 30 sec for 300 µm thick pattern and 27 µm thick pattern respectively to make the microwell layer.
11. Place the PR coated wafer on a hotplate at 65 °C for 4 min and at 95 °C for 8 min (for 27 µm deep capture-well layer); and at 65 °C for 40 min and at 95 °C for 110 min (for 300 µm deep culture-well layer).
12. After cooling, place the PR coated silicon wafer on the mask aligner equipped with UV light.
13. Expose the PR coated silicon wafer to the UV light (365 nm) at a dose of 250 mJ/cm^2 (for 27 µm thick pattern) and 700 mJ/cm^2 (for 300 µm thick pattern).
14. Bake the wafers at 95 °C for 5 min (27 µm thick pattern) and 30 min (300 µm thick pattern), respectively.
15. Wash off the uncrosslinked PR by the PGMEA for 6 min (27 µm thick pattern) and 25 min (300 µm thick pattern), respectively, and then dry them with nitrogen gas.
16. Measure the height of pattern features on the wafer with a scanning laser profilometer by placing the wafer on the xy-stage of a scanning laser profilometer.
    1. Adjust the focal plane to clearly show the pattern features on the wafer by using the "camera view" observation mode under a 20X objective lens.
    2. Switch the observation mode from "camera view" to "laser view", and set the upper and lower positions of the features.
    3. Set the measurement mode, area, quality, and z-pitch up to transparent (top), 1 line (1,024 x 1), high-accuracy and 0.5 µm, respectively, and then press the start bottom to begin the measurement.

2. Preparation of PDMS Devices for Single-cell Isolation

1. Before PDMS casting, silanize the master molds with trichlorosilane to create a hydrophobic surface, which makes it easier to peel off the PDMS replicas from the master molds.
    1. Place the master molds and a weighting boat containing 200 µl of trichlorosilane in a desiccator, and apply vacuum (-85 kPa) for 15 min.
    2. Stop the vacuum and then leave the master molds in the desiccator to silanize the master molds at room temperature for at least 1 hr.
    3. Remove the master molds from the desiccator and place each master mold in a 10 cm Petri dish.
2. Mix a total amount of 17.6 g PDMS polymer kit containing a base and a curing agent at a ratio of 10:1, and then pour the PDMS onto the master mold in the Petri dish.
3. Place the Petri dish in a desiccator and apply vacuum (-85 kPa) for 1 hr to remove air bubbles in the PDMS.
4. Remove the Petri dish from the desiccator and place it in a conventional oven at 65 °C for 3 - 6 hr to cure the PDMS.
5. Remove the cured PDMS replicas from the master molds and punch two holes as an inlet and an outlet at the two ends of the microchannel on the capture-well array PDMS replica by a puncher with inner-diameter of 0.75 mm for the fluidic channel (**Figure 2A**).
6. Use tape to clean the surface of the PDMS replicas, and then place the PDMS replicas in a plasma cleaner for brief oxygen plasma treatment (100 watts for 14 sec).
7. Remove the PDMS replicas from the oxygen plasma machine.
8. Align a top (containing the capture microwells) and a bottom PDMS (containing the culture microwells) replica by hand under a stereomicroscope and bring them into contact.
9. Place the aligned PDMS replicas in an oven at 65 °C for 24 hr to achieve permanent bonding between the PDMS replicas to form the final device.
10. Soak the PDMS device in a deionized (DI)-water filled container and place the container in a desiccator under vacuum (-85 kPa) for 15 min to remove air from the microchannel of the PDMS device.
11. Place the DI water-filled PDMS device in a tissue culture hood and use UV light (wavelength of light: 254 nm) to sterilize of the device for 30 min.
12. Replace the DI water in the PDMS device with a 5% bovine serum albumin (BSA) in 1x PBS solution and incubate at 37 °C for 30 min to prevent cells from sticking to the PDMS surface.
    Note: The BSA coating is critical to improve the transfer efficiency of isolated cells from capture-well to culture-well.
13. Replace the 5% BSA solution in the PDMS device with sterilized 1x PBS solution.

3. Preparation of Single-cell Suspension

1. Culture Neural stem cell KT98, and human carcinoma cell A549 and MDA-MB-435 we in Petri dish in conventional cell culture incubator (37 °C, 5% CO_2, and 95% humidity) to prepare cells for the PDMS device cell experiment.
2. Remove and discard the spent culture medium (DMEM basal medium supplied with 10% fetal bovine serum and 1% antibiotics) from the culture dish with cells grown to 70 - 80% confluence.
3. Gently wash the cells with sterilized PBS three times.
4. Remove and discard the PBS, and add 2 ml of a recombinant enzyme mixture with proteolytic, collagenolytic, and DNase activities (please see the Materials List for the detailed information).
5. Incubate the culture dish at room temperature for 5 min, and then tap the culture dish to facilitate cell detachment.
6. Add 4 ml of sterilized PBS to disperse cells, and then transfer the cell suspension to a 15 ml conical tube.
7. Centrifuge the tube at 300 x g for 3 min and remove the supernatant.
8. Gently resuspend the cell pellet in 1 ml sterilized PBS and count the live cell number using the standard Trypan Blue exclusion method[13].
   Note: This resuspension step is critical for preparing well-dissociated single-cell suspension to improve the single-cell isolation efficiency.

4. Single-cell Isolation and Clonal Culture

1. Load 50 µl of cell suspension at a concentration of 2.2 - 2.5 x10^6 cells/ml into the microchannel of the PDMS device via the device's outlet hole with a handheld pipette.
   Note: The cell suspension loading pipette needs to be stopped and held at the first stop position to avoid introducing air bubbles into the microchannel.
2. Load another 50 µl of cell suspension through the inlet hole to evenly fill up the whole microchannel with cells.
3. Seal the outlet hole with a plug (a 3 mm long, 1 mm diameter cut nylon fishing line) to avoid flow induced by hydrostatic pressure from the droplets on inlet and outlet holes.
   Note: The plugs were UV sterilized inside a tissue culture hood prior to use.
4. Fill up a 1 ml sterile syringe with culture medium, eject air bubbles from it, and set it up on a syringe pump.
5. Connect the medium loaded syringe to the inlet hole of the PDMS device via a 23 G blunt needle and a poly-tetrafluoroethene (PTFE) tubing.
6. Remove the plug from outlet hole and allow a 2-min time interval to allow the cells to settle into the single-cell capture-wells by gravitational force.
7. Wash away the uncaptured cells with 300 µl of culture medium at a flow rate of 600 µl/min driven by the syringe pump.
8. Wait for 2 min to stabilize the device, and seal the inlet and outlet holes with plugs to form a closed culture system.
9. Flip the device by hand to transfer the captured single-cells to the culture microwells.
10. Place the PDMS device in a 100-mm tissue culture dish, and add 10 ml of sterilized PBS around the device to avoid culture medium evaporation from the microchannel.
11. Move the culture dish to a conventional cell culture incubator (37 °C, 5% CO_2, and 95% humidity) for clonal culture of the single-cells.

5. Culture Medium Replenishment

1. After 1 d of culture, replace the culture medium in the PDMS device with fresh medium to improve cell proliferation.
2. Place two droplets of culture medium on top of the PDMS device near the inlet and outlet holes areas to avoid introducing air bubbles into the microchannel while performing the next step.
3. Punch two holes from the top of the PDMS device near the two ends of the microchannel as an inlet and an outlet for the microchannel.
   Note: Do not punch through the whole two layers of the PDMS device; instead, just punch through the top layer of the PDMS.
4. Connect a 1 ml plastic syringe containing fresh medium to the inlet via a 23 G blunt needle and PTFE tubing.
5. Flow 120 µl fresh medium into the device for 5 min to replace the old medium.
6. Insert two plugs to seal the inlet and outlet holes, and return the device to the cell culture incubator.
7. Refresh the culture medium every 2 d during the period of culture by removing the sealing plugs, followed by repeating steps 5.4 to 5.5.

Representative Results

The microfluidic platform for single-cell isolation and culture comprises a microchannel (200 µm in height) with two sets of microwell arrays (**Figure 2A**). The two sets of microwell arrays are termed as capture-well (25 µm in diameter and 27 µm in depth) and culture-well (285 µm in diameter and 300 µm in depth) for single-cell isolation and culture, respectively, and each capture-well is positioned at the center of a culture-well when seen from the top-view (**Figure 2B**). For device operation (the schematic operation flow is illustrated in **Figure 1**), the required equipment (syringe pump, tissue culture incubator, and microscopes) and supplies (syringe, pipette, and tubing) are commonly available in biological laboratories. Moreover, the portability and transparency of the platform allowed the cells to be easily observed and analyzed in a device with a conventional microscope during the cell culture experiment.

The capture efficiency of cells in the capture-wells after the washing step ranges from 67.80 ± 11.38% to 85.16 ± 1.91% (depending on cell type) (**Figure 3A**). Moreover, most of the capture-wells contain only a single cell for all three cell types (89.89% - 92.98%), which was confirmed by analyzing the number of loaded cells in the culture-wells (**Figure 3B**). The final single-cell isolation efficiency in the culture-wells was more than 76% for KT98 and MDA-MB-435 cells, but only 61% for A549 cells due to cell size differences among the tested cell types.

For cancer research, clonogenic assay of single-cells can be utilized to test drug resistance and proliferation rates of individual cell colonies. The cell culture and clonogenic assay of the isolated single-cells in our platform were conducted by refreshing the culture medium every 2 d. This demonstration highlights the applicability of this device for studying cellular heterogeneity at the single cell level by measuring the differences in cell survival and proliferation rates of individual cells.

The cellular heterogeneity and proliferation rate of the isolated cells were analyzed from daily-acquired images. The results showed that the isolated single cells could be cultured for 7 d and exhibited different growth patterns. For example, 13% of the isolated cells had no cell division and remained as one single cell (**Figure 4B**), while 17.5% of the cells divided into more than 15 cells and formed cell colonies (**Figure 4A**) during the culture. Cellular heterogeneity among the proliferating cells was also evidenced by their different growth patterns of forming two cells (4.3%), three cells (2.5%), and 4 - 14 cells (15%) in the cell culture experiment (**Figure 4C**).

These results indicated that the platform can be used for long-term cell culture, clonogenic assays, and cellular heterogeneity studies. Having a large number of individual cell colonies grown in a small footprint area also makes microscopic analysis of the cells easier.

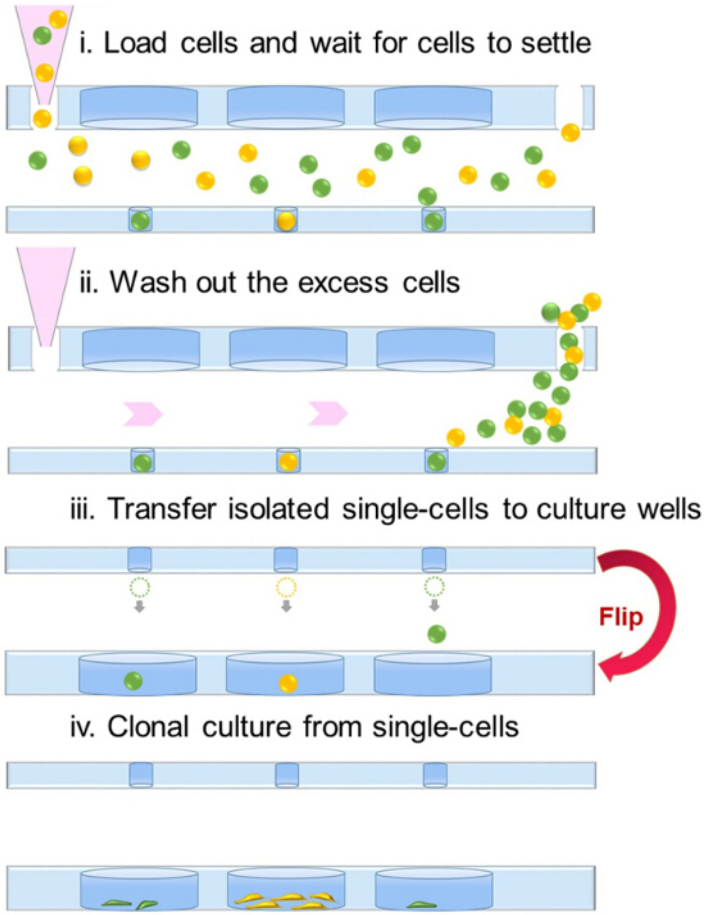

Figure 1. Schematic illustration of operation flow for high-throughput single-cell isolation and culture in the microfluidic platform. The operation procedure for single-cell isolation and clonal culture includes 4 steps: i) load cells into the microchannel by a plastic tip and manual pipette; ii) wash away the uncaptured cells with fresh culture medium driven by a syringe pump; iii) after sealing the inlet and outlet openings with plugs, the isolated cells were transferred from capture-wells to culture-wells by flipping the device; and iv) perform clonal culture (clonogenic assay) in culture-wells. Please click here to view a larger version of this figure.

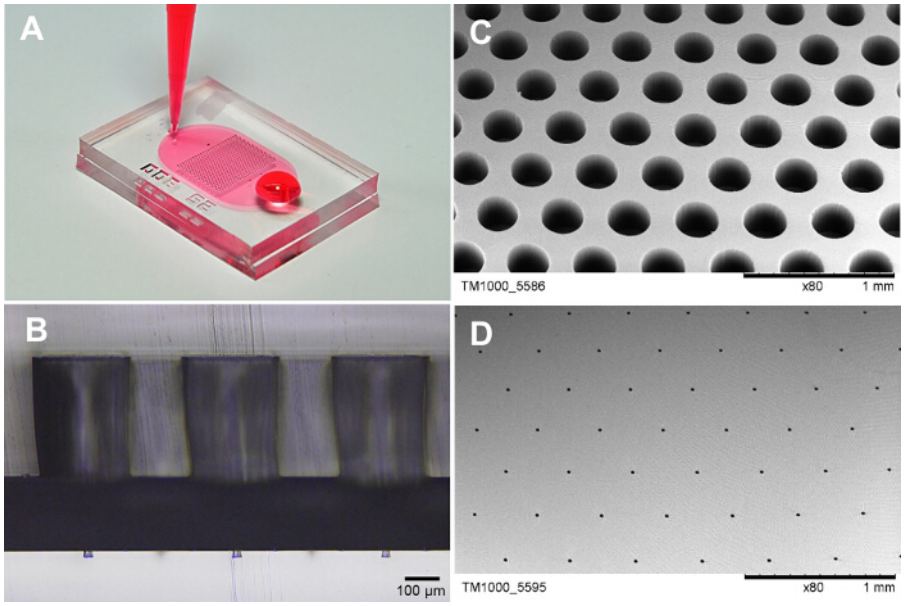

Figure 2. Photograph and SEM images of the microfluidic single-cell isolation and culture platform. (**A**) Microfluidic platform loaded with red dye that shows the microchannel and microwell structures for single-cell culture. (**B**) A representative image taken from a cut device, showing the side-view of three single-cell isolation and clonal culture units in the microfluidic platform. Scale bar: 100 µm. SEM images of single-cell culture (**C**) and capture (**D**) structures (microwells in different sizes). The sizes of the microwells can be adjusted for specific cell types and culture strategies. Scale bar: 1 mm. Please click here to view a larger version of this figure.

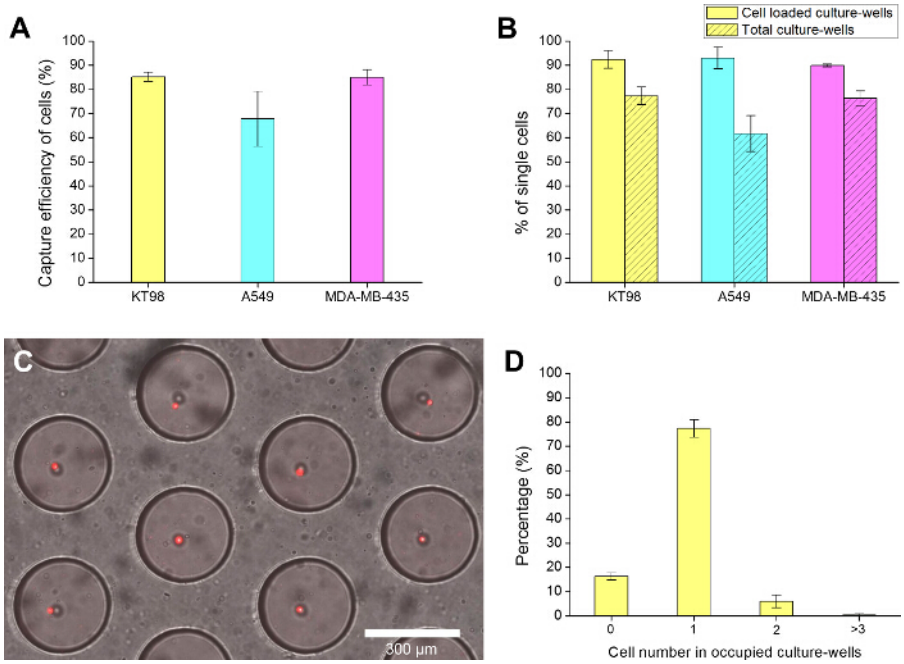

Figure 3. KT98, A549, and MDA-MB-435 single-cell isolation efficiency in the microfluidic platform. (**A**) Efficiency of KT98, A549, and MDA-MB-435 cells captured in capture-wells after washing away the uncaptured cells. The capture efficiency of cells was from 67.80% to 85.16% in the three cell types. (**B**) The single-cell ratio in total culture-wells (slash bar) was more than 76% for KT98 and MDA-MB-435, but 61.63% for A549. (**C**) A representative image of individual KT98 single-cells (red, stained with cell tracker) isolated in large culture-wells for clonal culture. Scale bar: 300 µm. (**D**) Cell number in cell occupied culture-wells of the KT98 cell model. The ratio of culture-wells was 77.3% with a single-cell, 16.31% with no cells, 5.96% with two cells, and 0.43% with more than three cells. Error bars are represented as ±SD. Please click here to view a larger version of this figure.

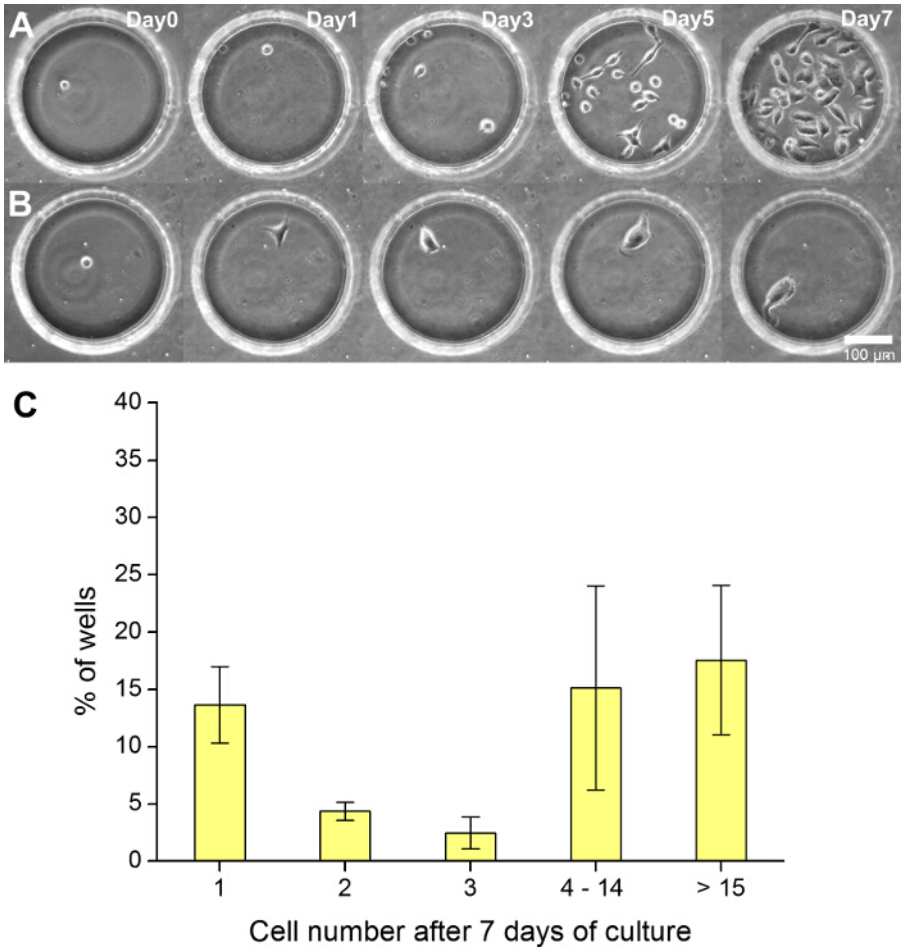

Figure 4. Cell culture, clonogenic assay, and cellular heterogeneity study of isolated A549 cells in the microfluidic platform for 7 d. (**A**) An isolated single-cell grew and proliferated to a cell colony in culture-well. (**B**) An isolated single-cell survived, but did not divide, after 7 d of culture. Scale bar: 100 µm. (**C**) The isolated single-cells exhibited heterogeneous growth patterns after being cultured for 7 d. Error bars are represented as ±SD. Please click here to view a larger version of this figure.

Discussion

Microwell-based device systems[6,14] have been utilized for single-cell manipulation and analysis, such as large-scale single cell trapping[6] and single hematopoietic stem cell proliferation[15]. Although well size, number, and shape can be adjusted for specific applications, the single-cell isolation efficiency is always compromised when the size of the well is increased.[9,15]

To overcome this limitation, Park *et al.* reported a microfluidic chip with triangular microwells that have a high single-cell trapping rate (58.34%), while the microwell size is enlarged to allow for cell spreading and growth. However, the microwells (with the length of a side ~50 µm) could only support cell proliferation for up to 2 d.[16] Our dual-well strategy allows single-cell loading in large microwells to not be limited by the probability of the Poisson distribution encountered in limiting dilution methods and open well systems[3], as demonstrated by providing high single-cell isolation efficiency (more than 75%) in large microwells whose size was sufficient for cell proliferation for at least 7 d (**Figure 4A**). Due to its ability to highly efficiently perform single-cell isolation and clonal culture with a simple device and operation procedure, we envision that our microfluidic platform could constitute a useful tool for a broad range of applications, including cancer stem cell selection by clonogenic assay[17] and high-throughput cardiotoxicity screening of drugs[18,19].

A major limitation of our single-cell isolation and culture platform is that it does not form individually closed culture-wells to prevent crosstalk between cells cultured in the culture-wells. Therefore, the behavior of the cells may be affected by the paracrine secretion of other isolated single-cells in the microfluidic device. The other limitation of our platform is the need to prepare relatively high-density cell suspension for high efficiency single-cell isolation. This high cell consumption can limit the applications of rare cell isolation and culture, such as aqueous humor cells.

Before the device operation, it is important to inject DI water from the inlet hole to fill the microchannel and carefully observe any fluid leakage from the bonded PDMS device. Fluid leakage affects the flow condition in the microchannel thus could adversely affect the cell capture and transferring outcome. In addition, although our single-cell loading efficiency study results show very low cell loss after transferring the isolated cells from capture-wells to culture-wells, indicating that most cells could be effectively transferred by flipping the device, the transferring efficiency is decreased if the BSA blocking step (2.12) is not properly performed. Users may modify the BSA blocking protocol or use alternative surface modification methods for their specific cell experiment needs (*e.g.*, increasing BSA concentration or coating time when a more adherent cell type

is used). The dimensions of the cell capture-wells are the dominant factors, which need to be optimized for the best single-cell loading efficiency in capture-wells, depending on the size of the specific cell type of interest. High multiple-cells-in-a-capture-well events are due to having the capture well size too large for the cell type of interest, whereas low cell loading efficiency in the capture well is an indication of the size of the capture well being too small. It is also important to minimize cell clusters in the prepared single cell suspension, as cell clusters can decrease the efficiency of single-cell loading in the capture-wells.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

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