A simple PDMS-based microfluidic channel design that removes bubbles for long-term on-chip culture of mammalian cells†‡

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This report shows methods to fabricate polydimethylsiloxane (PDMS) microfluidic systems for long-term (up to 10 day) cell culture. Undesired bubble accumulation in microfluidic channels abruptly changes the microenvironment of adherent cells and leads to the damage and death of cells. Existing bubble trapping approaches have drawbacks such as the need to pause fluid flow, requirement for external vacuum or pressure source, and possible cytotoxicity. This study reports two kinds of integrated bubble trap (IBT) which have excellent properties, including simplicity in structure, ease in fabrication, no interference with the flow, and long-term stability. IBT-A provides the simplest solution to prevent bubbles from entering microfluidic channels. *In situ* time-lapse imaging experiments indicate that IBT-B is an excellent device both for bubble trapping and debubbling in cell-loaded microfluidics. MC 3T3 E1 cells, cultured in a long and curved microfluidic channel equipped with IBT-B, showed high viability and active proliferation after 10 days of continuous fluid flow. The comprehensive measures taken in our experiments have led to successful long-term, bubble-free, on-chip culture of cells.

Introduction

In recent years, polydimethylsiloxane (PDMS)-based microfluidic technology has been rapidly developed for many applications in cell biology,[1,2] including stem cell fate analysis,[3] neuronal cell peptide secretion,[4] sickle cell vasoocclusion and rescue,[5] and single cell calcium imaging.[6] Culturing cells in a microfluidic system, however, particularly in the long term (tens of days), may face many problems, including contamination, leakage, clogging, and undesired bubble accumulation in the channel. While some of these problems are manageable by careful operation or selection of materials or protocols, bubble accumulation is a frequent obstacle that is extremely difficult to avoid in most PDMS-based microfluidic systems. Bubbles have a high probability to form at the connection between the channel and tubing or are introduced when fixing and removing tubing to and from the device or transferring the device between the microscope and the incubator. Furthermore, bubbles may gradually grow in the channel due to the variation of temperature and pressure, the hydrophobicity of the channel surface, and properties of the cell culture medium. Occurrence of air bubbles can harm cells and disrupt experiments: the liquid–gas interface can spoil cell viability, rupture cell membrane, and even wash away cells (see ESI‡ Fig. S1). The accumulation of air bubbles has been a constant problem in microfluidic systems, and several approaches have been developed to solve this problem. An ultrasonic micro-degassing device,[7] hydrophobic degassing plate,[8] semi-impermeable membrane,[9] and mineral oil[10] could all help remove bubbles in a microfluidic system. These strategies, however, are not always compatible with cell culture microfluidic systems where maintaining cell viability is the key. A simple way to tackle bubbles is to make a bubble trap before the main flow system to capture bubbles before they enter the main flow system.[11] The bubble trap captures bubbles by utilizing the buoyancy of air bubbles in the liquid and provides a space for the upward floated bubbles. The advantage of the bubble trap is that device operation is maintained while the bubbles are trapped. The drawback is that, because the bubble trap does not remove bubbles from the system and the volume of the bubble trap is limited, additional bubbles would still enter the microfluidic system when the bubble trap completely fills with bubbles. A successful strategy which can remove trapped bubbles is to force bubbles out of the microfluidics through a gas-permeable PDMS membrane.[12] Alternatively, the application of vacuum and the gas-permeable PDMS membrane can actively trap and remove air bubbles maintaining continuous flow in the microfluidic device.[13] A recent report uses both buoyancy of air bubbles and physical trapping to perform long-term (4 days) cell culture experiments.[14] On-chip cell culture, however, needs mild circumstances such as continuous medium and gas supply, constant pressure, and minimal contamination possibility. Moreover, the real-time observation of cells in the microfluidic channel and the need to transfer the device between the microscope and the incubator require simple, stable, and efficient bubble-ridding strategies that the reported methods could not satisfy.

In this study, we report two kinds of integrated bubble trap (IBT), whose properties include simplicity in structure, ease in fabrication, no interference with the flow system, and excellent stability. IBT-A is simply fabricated by adding a small cylindrical chamber at the connection between the inlet tubing and microfluidic channel. Given the simplicity and practicality, IBT-A is a system suitable for short-term (3–5 h) microfluidic experiments

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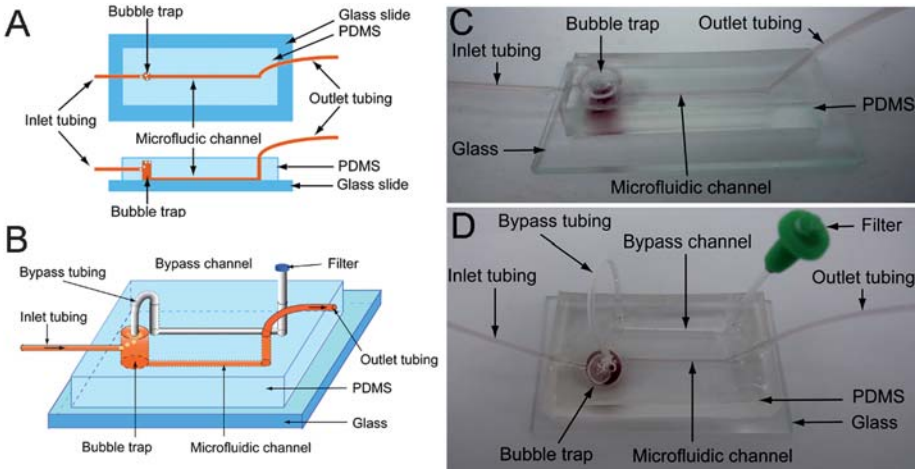

Fig. 1 Schematic diagram of a series of IBTs. (A) Top view and side view of IBT-A. (B) A three-dimensional picture of IBT-B. (C) and (D) Photographs of IBT-A and IBT-B.

(Fig. 1A and 1C). IBT-B is a double channel bubble trapping and discharging device with the bypass channel ending in a 0.2 μm filter which is highly permeable for the air bubble but prevents any bacteria from entering (Fig. 1B and 1D). Culturing MC 3T3 E1 cells for a span of 10 days in long and curved channels indicated that IBT-B is an excellent device for bubble prevention over this entire time course. In addition, we systematically studied important factors (such as medium temperature and components, hydrophilicity/hydrophobicity of the channels, and driving force) contributing to bubble generation. Measures against these factors have led to successful long-term cell culture in microfluidic channel.

Experimental

1. The fabrication and improvement of PDMS-on-glass microfluidic channel chip

Two types of built-in bubble trapping devices were devised and fabricated to improve the efficiency for eliminating bubbles in our experiments. These devices are all based on PDMS-on-glass microfluidic channels.[15,16] (see ESI‡ Text S1).

1.1. Integrated bubble trap A (IBT-A). IBT-A is designed and fabricated based on a chip that comprises a PDMS slab with engraved features of microfluidic channels and a flat glass (Fig. 1A and 1C). Briefly, the inlet of the channel is connected directly to the bottom of a cylindrical bubble trap chamber. The chamber was made by punching a PDMS mold with an appropriately sized puncher before sealing the PDMS to glass. The size of the chamber varied according to the requirements of experiments (diameter ~3 mm and height ~3 mm, or diameter ~5 mm and height ~5 mm). The inlet PE tubing is horizontally connected to the other side wall of the bubble trap and its opening is near the roof of the trap to avoid the entrance of bubbles into the microfluidic channel.

1.2. Integrated bubble trap B (IBT-B). IBT-B is a double channel bubble trapping and discharging device in which one channel is for applying shear flow over cultured cells, another serves as a bubble discharging bypass which opens to the outside (Fig. 1B and 1D). The parabolic bypass tubing connecting the bubble trap chamber and the bypass channel is designed to break big bubbles possibly generated during high-speed fluid flow. The bypass channel after the parabolic bypass tubing is used to tackle accidental spilling of medium out of the bypass tubing during long-term experiment, the resistance force generated from the spilled liquid in the bypass channel would counteract any further spilling of the liquid. Furthermore, the bypass channel can provide extra volume for cushioning accidental changes of air pressure in the bubble trap chamber. To avoid bacterial contamination, the bypass ends with a 0.2 μm filter which is permeable to air, but impenetrable to contaminants such as bacteria or dust particles. The function of the filter is three-fold: bubble discharge, bacteria contamination prevention, and immediate air exchange (5% CO_2) for the cultured cells. At the working condition, the medium reservoir is carefully adjusted to an appropriate height where the hydrostatic pressure of the medium in the inlet tubing is the same as that of the bubble trap chamber. The driving force from the pump can precisely push the medium into the microfluidic channel and the bubbles collected from the bubble trap will be drained through the bypass and finally be quickly discharged *via* the filter.

2. Testing the efficiency of IBTs

The IBTs efficiencies were tested by introducing colored phosphate buffer solution (PBS) or minimum essential medium alpha (α-MEM, Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) into the microfluidic channel equipped with IBTs. Artificial air bubbles were produced by injecting air into the inlet tubing with a micro-volume syringe quantitatively. The driving force was provided by a peristaltic pump (Longer BT100-2J, China). Time-lapse experiments were recorded by a portable digital camera (Panasonic, DMC FX 520, Japan) or a fluorescence microscopy (Leica DM 6000, Germany).

3. Cell seeding, staining, and imaging in microfluidic channels

We employed a previously described method of isolation and *in vitro* expansion of rat marrow mesenchymal stem cells (rMSCs).[17] Before the seeding of cells, the microfluidic channels were sterilized with 75% ethanol and thoroughly washed with PBS. When rMSCs or MC 3T3 E1 (mouse preosteoblast cell line) in the Petri dish reached ~80–90% confluence, they were washed twice with PBS and cells were trypsinized with 0.05% trypsin in 1 mM ethylenediaminetetraacetic acid (EDTA, Gibco) for 5 min at 37 °C. After centrifugation, cells were resuspended with serum supplemented medium and counted and adjusted to a density of about 1×10^5 cells mm^{-3} in a 1.5 mL Eppendorf tube. The cell suspension was gently transferred into a 50 μL microsyringe and injected into the channel (height: 500 μm, width: 500 μm) slowly, any bubble in the channel should be avoided during the injection process. Cells in the channel were placed into an incubator (37 °C, 5% CO_2) for about 10 h to adhere and spread on the substrate in static condition. The fluorescence staining of cells in microfluidic channels were performed before observation.[18,19] The solutions of calcein AM (5 μg mL^{-1}, Invitrogen) and Hoechst (1 μg mL^{-1}, Invitrogen) were slowly introduced into microfluidic channels *via* the inlet tubing and incubated in an incubator (37 °C, 5% CO_2) for 30 min in darkness. The real-time imaging of cell behavior in the presence of fluid flow shear was realized on an *in situ* cell culture and observation system (see ESI‡ Fig. S2, Text S2).

Results and discussion

Published reports for the prevention of bubbles mainly comprise two different approaches: trapping *versus* debubbling.[12–14] Although reported approaches are successful in dealing with the bubble problem, they either require pause of the fluid flow or the use of external vacuum or pressure source that brings interruption and inconvenience to experiments, particularly for real-time experiments. Here, we present two kinds of IBTs which are simple, automatic, and independent of external equipment. Different levels of complexity of fabrication of the devices correspond to different maximum times for preventing bubble generation (from ~3 h to ~10 days).

IBT-A

The simpler of the two devices comprises the direct addition of a built-in bubble trap before the microfluidic channel. IBT-A is built by punching a cylindrical chamber immediately before the microfluidic channel. Because the inlet PE tubing is near the roof of the chamber, the bubbles could be trapped at this position. We used cell-free experiments to evaluate the effectiveness of bubble trapping of the device. As shown in Fig. 2A (or Movie S1‡), bubble-free medium (α-MEM supplemented with 10% FBS) began to pass through the channel at a speed of 3 μL min^{-1} (Fig. 2A, 0 min). The subsequent 183 min performance of the chip was not interfered by any bubble (Fig. 2A, 183 min). At 186 min, a big bubble, squeezed by the side walls of the channel, appeared and moved forward to occupy most of the space in the channel (Fig. 2A, 186 min). In comparison, the channels without a bubble trap can work under bubble-free conditions for only about 60 min (Movie S2‡). The bubble trap, therefore, effectively delayed the appearance of bubbles. Furthermore, the

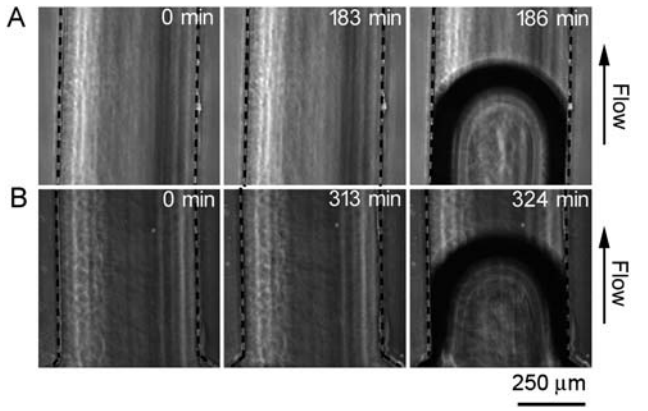

Fig. 2 *In situ* time-lapse imaging of the bubble trapping efficiency of the IBT-A in a cell free channel. (A) IBT-A with a small sized bubble trap chamber can maintain bubble-free conditions for about 3 h. (B) IBT-A with a large sized bubble trap chamber prolonged the bubble-free time to about 5 h. The dashed lines outline the borders of the channels. Black arrows indicate the flow directions of the fluid flow.

efficiency of the device could be improved by enlarging the size of the bubble trap chamber. A larger bubble trap (5 mm diameter, 5 mm height) (Fig. 2B, Movie S3‡) increased the bubble-free working time to 313 min. Moreover, with the increase of the chamber size, the time for the bubble from appearance to occupation of most of the area of the channel increased from 3 min to 11 min. Due to the limited volume of the bubble trap chamber, IBT-A is unable to avoid bubbles for long-term fluid flow experiments in microfluidic channels.

IBT-B

An ideal bubble trap system for a long-term experiment should be capable of capturing and eliminating bubbles simultaneously. To attain this objective, we developed IBT-B which has the capability to discharge trapped bubbles efficiently. IBT-B can collect bubbles by bubble trap and discharge them *via* a bypass opening to air (Fig. 1B and 1D). To avoid the spilling of medium over the bypass tubing and channel, the medium in the medium reservoir, bubble trap chamber, and medium collector are carefully adjusted to the same liquid level where the hydrostatic pressure of the medium in the inlet tubing is the same as that of the bubble trap chamber (see ESI‡ Fig. S2). This procedure can ensure not only the balance of the liquid in the system, but also the elimination of the interference of the hydrostatic pressure on the driving force from the pump. Subsequently, the pump was connected to the tubing between the medium reservoir and the microfluidic channel to precisely push the medium into the microfluidic channel. The 0.2 μm filter fixed to the outlet of the bypass tubing has three functions: discharging bubbles, preventing bacterial contamination, and exchanging air (5% CO_2) for cultured cells to maintain the pH of the medium. The efficiency of trapping bubbles in IBT-B was first tested by deliberate introduction of air bubbles into the chamber *via* the inlet tubing. As shown in Fig. 3, the series of time-lapse images show the bubble trapping and debubbling process of IBT-B (also see Movie S4‡). At $t = 0$ s the trap is filled with a blue solution, 100 μL air is

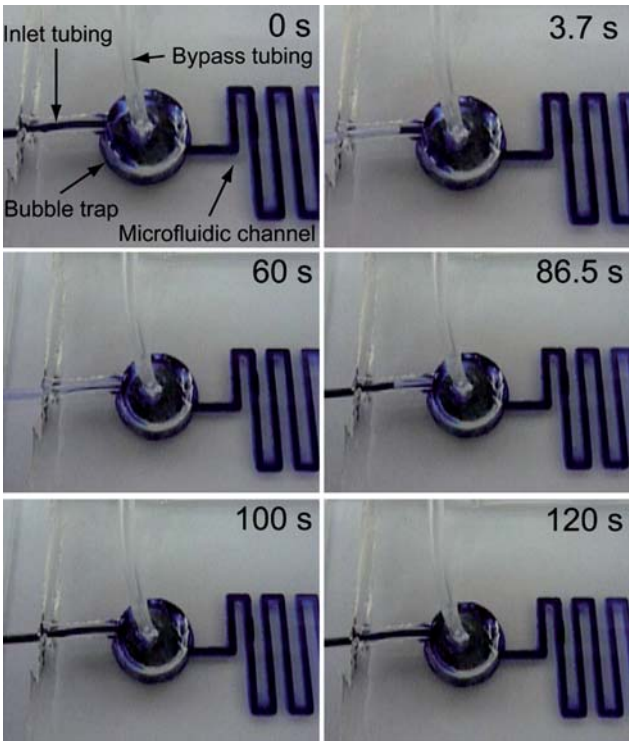

Fig. 3 Time-lapse images of the debubbling processes of IBT-B. At t = 0 s the trap is filled with blue solution. 100 μL air bubble is pumped continuously through the inlet tubing and pushed into the bubble trap chamber, the flow rate is 1.2 μL s^{-1}. At t = 3.7 s the bubble is entering the bubble trap chamber. From t = 3.7 s to t = 86.5 s, the 100 μL air bubble enters the bubble trap chamber and consecutively goes through the bypass tubing without interfering with the normal flow in the microfluidic channel. At t = 86.5 s, the end of the 100 μL air bubble is entering the bubble trap chamber. From t = 86.5 s to t = 120 s, the subsequent solution perfusion shows normal performance of the microfluidic channel and the stability of the bubble trap.

pumped continuously through the inlet tubing and pushed into the bubble trap chamber, at a flow rate of 1.2 μL s^{-1}. At t = 3.7 s, the bubble enters the bubble trap chamber. From t = 3.7 s to t = 86.5 s, the 100 μL air bubble continuously enters the bubble trap chamber and is drained *via* the bypass tubing without interfering with the normal flow in the microfluidic channel. At t = 86.5 s, the end of the 100 μL air bubble enters the bubble trap chamber. From t = 86.5 s to t = 120 s, subsequent solution perfusion shows normal performance of the microfluidic channel and the stability of the bubble trap. Next, the operation of IBT-B was evaluated by conducting cell-free flow fluid experiments using *in situ* time-lapse microscopy. As shown in Fig. 4A, 24 h of perfusion of α-MEM supplemented with 10% FBS in the chip does not show any sign of interference by any bubbles (also see Movie S5‡). Moreover, the chip loaded with rMSCs was tested by performing 24 h fluid flow experiments (flow rate 3 μL min^{-1}) using *in situ* time-lapse microscopy (Fig. 4B). The results showed that the cells in the channel kept their normal physiology during the entire time of observation. The cells kept their normal patterns of migration in the presence of the fluid shear stress without any interference from any bubbles (also see Movie S6‡).

To test the bubble elimination property of IBT-B for more complex and longer microfluidic channels, further cell culture

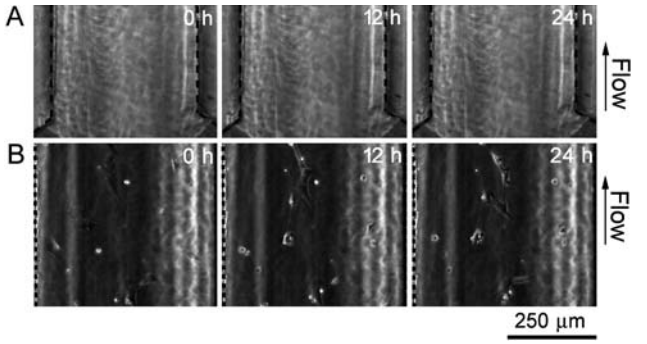

Fig. 4 *In situ* time-lapse imaging of the long-term performance of IBT-B at cell-free (A) and cell-loading (B) conditions. The dashed lines outline the borders of the channels. Black arrows indicate the flow directions of the fluid flow.

and fluid flow experiments (flow rate 3 μL min^{-1}) were performed in a curved microfluidic channel equipped with IBT-B. As shown in Fig. S3A,‡ *in situ* time-lapse images show that even MC 3T3 E1 cells adhering at the corners of the curved channel were not interfered with by any bubbles during the entire time of observation (24 h) (also see Movie S7‡). To further evaluate the bubble elimination capability of IBT-B, a subsequent 6 h fluid flow at a speed of 70 μL min^{-1} in the same channel was performed. Fig. S3B‡ shows that no bubble appeared over this period of time (also see Movie S8‡).

To demonstrate the maximum length of bubble-free operation, we cultured MC 3T3 E1 cells in curved microfluidic channels for 10 days in the presence of incessant fluid flow (flow rate, 3 μL min^{-1}). As shown in Fig. 5A and 5C, the phase contrast images indicate that the cells in the straight part of the channel appear normal on the substrate after a long period of flow stimulation. To evaluate the viability of these cells, the cells were stained with two kinds of fluorescent dyes, calcein AM and Hoechst 33342 (Fig. 5B and 5D). Calcein AM is a cell-permeanant dye that can be used to determine cell viability in most eukaryotic cells. In live cells the non-fluorescent calcein AM is converted to a green-fluorescent calcein after acetoxymethyl ester hydrolysis in intracellular esterases. The calcein AM fluorescence in Fig. 5B and 5D demonstrates that almost all cells lived well in the channel. The Hoechst staining further indicates that the nuclei of all cells were normal (Fig. 5B and 5D). Fig. 5E–5H show that cells at the corners of the curved channel, where the likelihood of retaining bubbles is high, appear normal as well. Compared with the starting time (Fig. S3A,‡ 0 h), the number of cells increased significantly, suggesting the rapid proliferation of the cells during the long-term fluid flow stimulation period. Generally, the series experiments confirmed that IBT-B is excellent for rapid, large volume bubble trapping and discharging in both short- and long-term (up to 10 days) cell culture.

By designing and fabricating a series of IBTs, we successfully solved the bubble problem that affects cell-related research based on microfluidic channels. In addition, we have taken comprehensive measures to deal with the bubble problem, because bubble trapping is a "down-stream" method dealing with pre-existing bubbles, the "up-stream" methods, *i.e.* prevention of bubble generation is more fundamental in a microfluidic channel system. In our experiments, we considered the various factors,

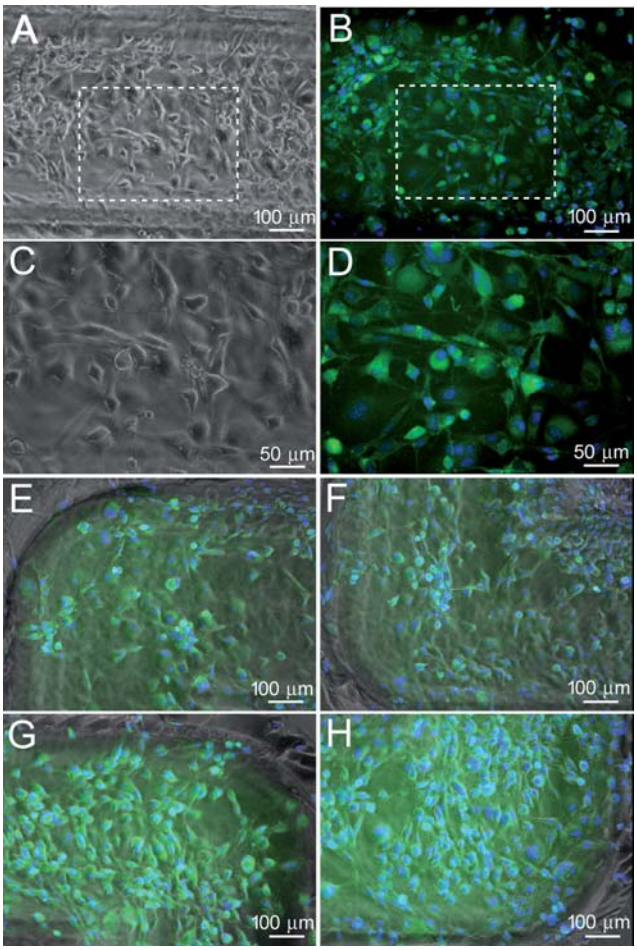

Fig. 5 Long-term (10 days) culture of MC 3T3 E1 in long and curved microfluidic channel equipped with IBT-B. (A) Phase contrast image of the cells in the straight part of the channel. (B) Fluorescent image of the cells as in (A). (C) Zoom in image of (A) (marked by white rectangle). (D) Zoom in image of (B) (marked by white rectangle). (E–H) The phase contrast–fluorescence merged images of the cells at the corners of the curved channel. The cell nuclei were stained with Hoechst 33342 (blue), the cytoplasm was stained with calcein (green).

including medium temperature and components, hydrophilicity/hydrophobicity of the channels, and driving force, that normally affect the generation of bubbles in microfluidic channels (for details see Text S3‡). Other details such as avoiding external mechanical forces, degassing medium in vacuum before use, and checking possible bubbles at the joint between PE tubing and channels are all emphasized in our experiments for the successful long-term cell culture in microfluidic channels.

Conclusions

Long-term cell culture in microfluidic channels faces a range of obstacles among which air bubble accumulation is one of the most bothersome. We not only developed successful devices for bubble trapping and debubbling, but also took comprehensive measures to avoid bubble generation. We believe these advancements in this study will benefit research in microfluidics.

Acknowledgements

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