# Openly Accessible Microfluidic Liquid Handlers for Automated High-Throughput Nanoliter Cell Culture

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S *Supporting Information*

ABSTRACT: Cell culture is typically performed in Petri dishes, with a few million cells growing together, or in microwell plates with thousands of cells in each compartment. When the throughput of each experiment, especially of screening based assays, is increased, even using microliter solution per well will cost a considerable amount of cells and reagents. We took a rational approach to reduce the volume of each cell culture chamber. We designed and fabricated a poly(dimethylsiloxane) based liquid pipet chip to deliver and transfer nanoliter (50−500 nL) samples and reagents with high accuracy and robustness. A few tens to a few hundreds of cells can be successfully seeded, transferred, passaged, transfected, and stimulated by drugs on a microwell chip using this pipet chip automatically. We have used this system to test the cell growth dynamically, observed the correlation between the culture conditions and cell viabilities, and quantitatively evaluated cell apoptosis induced by *cis*-diammineplatinum(II) dichloride (cisplatin). This system shows great potential to facilitate large-scale screening and high-throughput cell-array based bioassays with the volume of each individual cell colony at the nanoliter level.
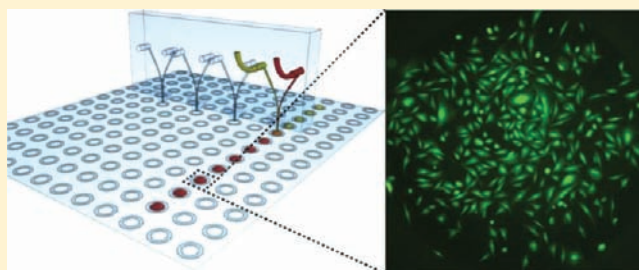
High-throughput cellular assays are becoming necessary in many situations to decipher the complex network in chemical biology.[1] They are also critical to drug screening[2] and genomic profiling.[3] These large-scale experiments would naturally prefer minute reaction volumes to reduce the waste and the total consumption of materials and reagents.[4,5] Currently, high-density microwell plates, developed decades ago, are the popular platforms for high-throughput cell experiments.[6] With the continuously increased throughput of each single experiment, the total volume consumption of well plates often becomes a bottleneck for cell assays.[7] Besides, the relatively large bottom area is not suitable for single cell culture or microscopy observation.

Recently, two promising approaches, large-scale patterned cell arrays[8,9] and highly integrated microfluidic devices,[10] have been developed for cell culture and analyses on an even smaller scale. Many reported micropatterned cell arrays can achieve ultrahigh throughput for screening large libraries or rare events, but it is difficult to isolate one clone of cells from the other.[11−13] There are also various microfluidic cell-culture chips that integrate customized microstructures inside of the channels to trap cells[14−16] or create small chambers to generate an independent microenvironment.[17] However, since the complexity and density of a single device increase enormously with the increase of integrated functional components such as valves, pumps, and necessary bypassing channels, it is still challenging to realize individual addressability. Moreover, the additional accessories for micropumps and valves, including pressurized gas supply, tubing, electronic circuits, and solenoid valves, are so cumbersome that the microfluidic chip-based cell culture has to be performed either in a customized incubation chamber[17] or inside a conventional incubator with tubing all

connected. Furthermore, the "world-to-chip" interface has been a problem for chiplike devices with many inlets for liquid introduction.[18−20] It is always desirable to have a device that is openly accessible and can perform multistep experimental procedures such as complete functions of a liquid pipet including dispensation, removal, transfer, replacement, and mixing (typically due to aspiration-dispensation cycles), with great accuracy, robustness, and scalability. Previously reported technologies which handle cell arrays have successfully carried out positional cell seeding and release,[21−23] and a digital microfluidic device also shows a promising future to culture, passage, and transfect a small amount of cells.[24] However, without suitable robotic liquid handling systems, high-throughput manipulations in the cell culture and cell-based assays, such as medium change, addition and removal of stimulus, especially in a single reactor resolution, are still difficult to perform.[25]

In this paper, we present a dynamic nanoliter liquid handling system, which performs complete functions for cell culture. This system employs a microfluidic chip as the nanoliter liquid pipet chip for liquid handling and a corresponding microwell-chip as the substrate for cell culture. This well-engineered system has surpassed our previously reported nanoliter dispenser,[4] which can only perform one single liquid-handling function. To drive the liquid, we use the peristaltic pumps, instead of the compressed air as used in our previous report. To control the motion and positioning of the components, we use electronic translational stages.
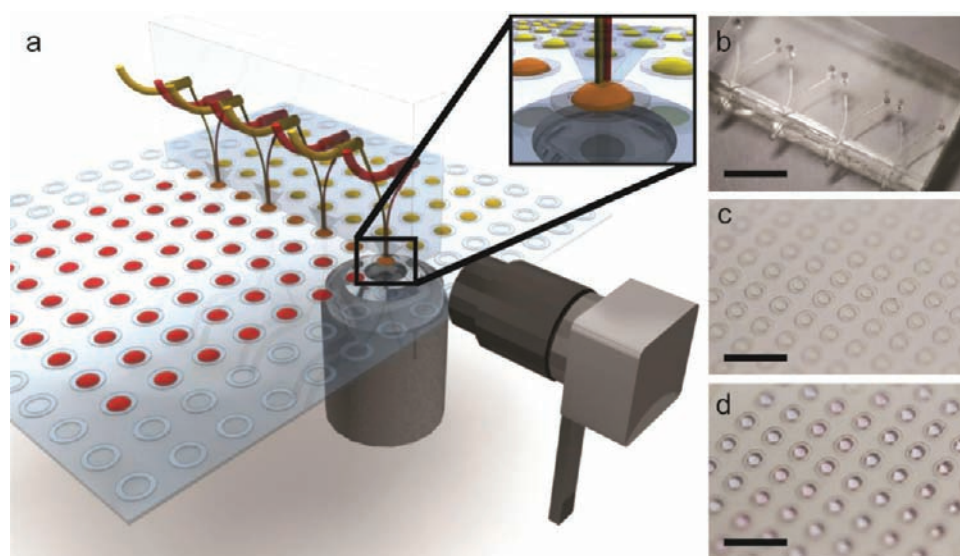
Figure 1. The microfluidic liquid handling system. (a) A microfluidic chip was aligned to the microwell chip horizontally and vertically using a microscope and a camera. Inset: a close view of the outlet of the microfluidic pipet chip. (b) A four-channel microfluidic liquid pipet chip. (c) Before and (d) after the microwell chip was filled with ~350 nL of liquid sample in each well. Scale bars are 5 mm.

The system can handle liquid from 50 to 500 nL in parallel, with a coefficient of variation (CV) < 8% at 150 nL. We demonstrate uniform cell seeding in 96 microwells in a single run, with a volume of ~350 nL for each well. All cells in these isolated wells can be maintained over 48 h with periodic medium changes every 8 h. We also successfully perform cell passage and plasmid transfection of any given microwell with efficiency comparable to conventional practices. Furthermore, we observe *cis*-diam-mineplatinum(II) dichloride (cisplatin) induced cell apoptosis with three different dosages on a single microwell chip. We believe this method has great potential to be scaled up to much higher throughput and hence to be applied to various large-scale parallel cell assays.

EXPERIMENTAL SECTION

Automation and Imaging. As shown in Figure 1a, the microwell chip was mounted on an inverted microscope (Nikon TE2000-S, Japan) equipped with a motorized $X-Y$ stage (MLS203-E-OLY, Thorlabs), the microfluidic pipet chip was mounted on a Z stage (TSA30-C, Zolix, Beijing, China) aligned on top of the $X-Y$ stage. Liquid flow was driven by two off-chip peristaltic pumps (BT100 1-F, Longer, Baoding, China) at speeds of 4.8 and 1.4 μL/s for dispensation and aspiration, respectively. The stages and peristaltic pumps were controlled by a home-built digital I/O controller with a computer. The $X-Y$ alignment between the microfluidic pipet chip and the microwells were done through the microscope objectives, while the Z displacement was monitored through a photographic macrolens (MP-E, Canon, Japan) and a CCD camera (Marlin F-033B, Allied Vision Technologies, Germany). Phase contrast images and fluorescent images of microscale transfection were taken with a 10× objective and a CCD camera (IMG 2S, QHY, Beijing, China) on the microscope. Fluorescent images for conventional transfection and apoptosis detection were taken with a 10× objective and an EM CCD camera (iXon 897+, Andor Technology, U.K.) on an inverted microscope (Nikon TE2000-E, Japan). Videos of liquid handling were recorded by a CCD camera (WAT-221S, Watex, Japan) and digital cameras (EOS 5D Mark II, and

EOS 600D, Canon, Japan). Confocal microscopic images were taken with a confocal microscope (TCS SP5II, Leica Micro-systems, Germany) for automatic cell counting.

Microfluidic Pipet Chip Fabrication. We fabricated microfluidic chips using the multilayer soft lithographic method.[10,26] The mold for fluid layers were fabricated on a 3″ silicon wafer using SU-8 1070 photoresist (Gersteltec, Switzerland) to get 100 μm thick features. Similar to our previous design,[4] the channel width was 200 μm and tapered down to 100 μm when getting close to the outlets. The chip was made from PDMS (RTV-615, GE Advanced Materials, Wilton, Connecticut). To fabricate the fluid layer, we treated the mold with trimethylchlorosilane (Sinopharm, Beijing, China) vapor for 5 min, spin-coated PDMS (10:1 elastomer to cross-linker ratio, 700 rpm, 60 s, ~180 μm) on the mold, baked the device at 80 °C for 12 min, and then got the fluid layer pattern. To fabricate the scaffold layer, we poured PDMS (15:1 elastomer to cross-linker ratio, ~3 mm) on a blank 3″ silicon wafer pretreated with trimethylchlorosilane and baked the wafer with PDMS at 80 °C for 15 min. Then we peeled the scaffold layer off the wafer, cut it into two parts, and bonded them to the fluid layer, covering the whole device except the channel outlets region. Then we peeled the two scaffolds bonded with fluid layers and punched holes in one of them for channel inlets. To fabricate the isolating layer, we spin-coated PDMS (20:1 elastomer to cross-linker ratio, 2000 rpm, 60 s, ~40 μm) onto a blank silicon wafer, baked at 80 °C for 12 min. Sequentially, we bonded the hole-punched fluid layer with scaffold to the isolating layer by baking at 80 °C for 30 min and then peeled off the bonded three-layer PDMS structure and punched other necessary inlets. We then bonded this piece with the other fluid layer with scaffold by the last baking at 80 °C overnight and then cut the nozzle part with scissors to get the final device.

Microwell Chip Fabrication. The microwell chip was fabricated on a 4.5 cm × 6 cm coverslip (Matsunami Galss, Japan) by SU-8 1075 photoresist (Gersteltec, Switzerland) through conventional photolithography. Before putting into use, the coverslip was cleaned by hot piranha solution (a 3:1

mixture of concentrated H_2SO_4 and 30% H_2O_2 solution. **Caution:** Piranha solution is a vigorous oxidant and should be used with extreme caution!) for 10 min, washed thoroughly with pure water, and baked at 90 °C for 3 h. We then spin-coated the photoresist on the coverslip (1200 rpm, 60 s, ~150 μm), waited for several hours, prebaked the photoresist (from 45 to 95 °C, increase 10 °C per hour), and exposed it to 300 mJ/cm^2 of collimated UV light. After exposure, the photoresist was postbaked (from 45 to 95 °C, increase 10 °C per hour) and developed. The microwell chip was finally baked at 150 °C for 3 h.

Conventional Macroscale Cell Experiments. CHO and U2-OS cells were maintained in cell culture flasks and cultured in an incubator with 5% CO_2 at 37 °C. The culture medium for CHO and U2-OS cells were Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Calsbad, CA) and McCoy's 5A Medium (GIBCO 16600, Invitrogen), respectively, both of which were supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen) and 1% penicillin-streptomycin (PS, Invitrogen).

CHO cells were cultured in 12-well plates (Costar 3513, Corning) for 48 h with medium replacement each 8 h. The cells were maintained with 350, 550, and 750 μL of medium in each well supplemented with 10% FBS.

For plasmid transfection, CHO cells were cultured in a 12-well plate in 360 μL of PS-free medium in each well to reach at least 90% confluence (~3 × 10^5 cells per well). Prior to transfection, we prepared the transfection solution for one well by three steps: (1) mixed 1.2 μL of lipofectamine 2000 (Invitrogen) and 59 μL of Opti-MEM (Invitrogen), incubated for 5 min at 25 °C and prepared 0.6 μg of GFP plasmid DNA (GenePharma, Shanghai, China) in 59 μL of Opti-MEM; (2) mixed these two solutions and incubated for 20 min; (3) diluted the mixture by 240 μL of PS-free DMEM supplemented with 10% FBS. After the solution was prepared, we replaced the medium by the transfection mix (1.7 ng/μL plasmid and 360 μL in total) and incubated the cells for 5 h. Then we replaced the solution by fresh medium with PS and cultured the cells for another 20 h with medium replacement each 8 h before image acquisitions.

To induce apoptosis, U2-OS cells were cultured in a 96-well plate (655180, greiner bio-one, Germany) with 100 μL of medium in each well to reach at least 90% confluence. A 50 mM stock solution of cisplatin (Sigma) was prepared in DMSO. For the experimental groups, the old medium was replaced by fresh medium supplemented with 500, 250, or 80 μM cisplatin at 8 and 16 h after cell seeding. For the control group, the old medium was replaced by fresh medium supplemented with 1% DMSO. After maintaining the cells in the drug-containing medium for 16 h, we stained the cells with an annexin V-FITC cell apoptosis detection kit (KGA106, KeyGen BioTECH, Nanjing, China) by replacing the medium with 80% binding buffer supplemented with 13% Annexin V and 7% PI and then incubated for 10 min before imaging.

Cell Manipulation Using Nanoliter Microfluidic Liquid Handler. During the dispensation, the liquid was stored in 1.5 mL vials and pumped into microfluidic pipet chips by a multichannel peristaltic pump. For aspiration, we reversed the driving direction of the peristaltic pump. All the liquid manipulations were performed in a humidified environment to prevent evaporation of liquid in the microwells. Before cell seeding, we incubated the microwells with fibronectin (Fn, 150 μg/mL) as the extracellular matrix protein for 1 h to improve cell attachment. Then we rinsed the microwell chip with PBS and dried it. When cells in the flask were harvested and rinsed in a fresh medium, we dispensed ~350 nL of cell suspension to each well, yielding ~500 cells per well. We implemented a 2 s dwell time after dispensing cell suspension to one well to alleviate the cell's inertial movement. After liquid manipulation, the microwell chip containing cells was immediately moved into a humidified chamber (diameter 15 cm) and then placed in a conventional CO_2 incubator.[27] For liquid change, eight change cycles were implemented with 45% liquid changed per cycle and 5 s interval between every four cycles if not specially mentioned. In all experiments, the volume of each well is kept the same after a medium change.

For a longtime cell culture, the medium was DMEM total medium supplemented with 10% FBS and 0.5% PS, and the time interval between each two medium changes was 8 h if not specially mentioned. For cell counting, we stained the cells at the 48th h by replacing the medium with 10 μM Hoechst 33342 (Dojindo, Japan) in DMEM and incubating for 20 min. Cells were stained only at the end of the experiment to avoid the dye's potential affection to cell growth.

For GFP plasmid transfection, CHO cells were cultured in microwells in the PS-free medium for 12 h to reach at least 90% confluence. Each microwell contained ~700 cells. We used the same transfection mix prepared for the conventional methods to perform our chip-based transfection. We added 350 nL of transfection mix into each microwell. To induce apoptosis, U2-OS cells were cultured in microwells for 12 h. The remaining procedures for both experiments were the same as the corresponding macroscale experiments except that all the liquid manipulations were performed by the liquid handler.

Cell Counting. Cell numbers were counted to evaluate the proliferation rate, transfection efficiency, as well as the apoptosis rate. Cell numbers were manually counted in the bright field images using Image J[28] with the "cell counter" plug-in. For the fluorescent images, we counted the cells with home-developed image processing scripts in Matlab (Mathworks, Natick, MA). Automatic cell counting was as accurate as manual counting, as shown in Figure S1 in the Supporting Information.

■ RESULTS AND DISCUSSION

Characterization of the Liquid Handler. For easier observation of the manipulating process of liquid at the nanoliter range, we built our system on top of an inverted microscope (Figure 1a). An $X-Y$ translational stage was used to control the receiving microwell chip to align to the vertically placed microfluidic pipet chip (Figure 1b), which was mounted on a Z-axis translational stage for accurately adjusting the distance between the pipet chip and the microwell chip. This chip-to-chip configuration, which makes arrays of liquid droplets openly accessible, is similar to commercially available liquid handling robots using multiple pipet tips (or capillary needles) with multiwell plates for microliter operations. Microfluidic devices with open outlets, such as spotters,[29,30] dispensers,[4] chemistrodes,[31,32] and circulating liquid tip pipet,[33] have been developed to carry out bioassays in confined volumes. In the current system, we further extend the functions of open-channel microfluidic chips by integrating four liquid manipulation modes: dispensation, removal, replacement, and aspiration-dispensation. Combining these four modes, complete manipulations for cell culture, including cell seeding,

medium change, cell passage, transfection, and drug stimulation, can be performed at the nanoliter range.

The microwell chip consists of arrays of open chambers to hold liquid, as shown in Figure 1c. We typically pattern 96 (12 × 8) isolated microwells on a 4.5 cm × 6 cm glass substrate for cell culture and optical observation. The wall of the wells is hydrophobic, made from SU-8 photoresist, while the bottom is hydrophilic glass substrate. Each well's inner diameter is 1.12 mm, offering the maximum volume of ~500 nL with a convex surface due to surface tension (Figure 1d).

The design of the pipet chip has been evolved from a format similar to the original dispenser[4] to an optimized form with extended nozzles (Figure 1a,b). First, we employed a microfluidic pipet chip with one channel corresponding to one microwell, as shown in Figure 2a. Using a bidirectional
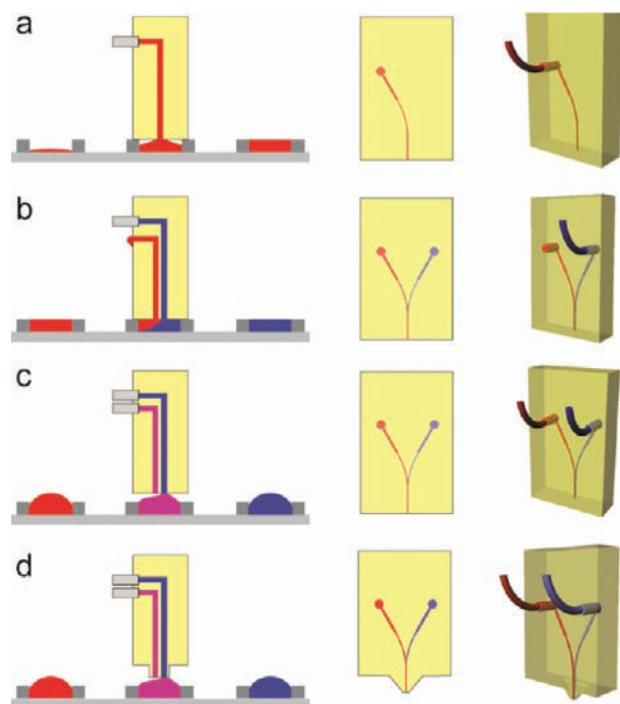

Figure 2. Four different versions of microfluidic chips we employed to perform liquid handling: (a) The first version had a flat bottom and one channel connected to a peristaltic pump. This chip is suitable for dispensation. (b) The second version had a flat bottom and two individual channels, one of which was connected to a peristaltic pump, and when injecting new liquid, the old liquid would be pushed out of the well. (c) The third version also had two individual channels, connected to two different peristaltic pumps, one for dispensation and the other for aspiration. (d) The fourth version had a nozzle-shaped outlet for easier and more efficient liquid manipulation.

peristaltic pump for liquid driving, the chip was capable of liquid dispensation or aspiration. Cell suspension can be loaded into and sucked out of the microwells with this setup. However, this one-channel-one-well configuration is difficult to perform culture medium change efficiently since the new medium and the old medium have to share the same channel, and the pipet tip has no access to the bottom of the microwells for complete liquid aspiration. One solution to this problem is to have a pair of channels, one for delivery and the other for removal, to address each microwell. To save space, we aligned two channels together, as shown in Figure 2b. The thickness of the pipet chip is greater than the microwell's diameter. We gently sealed the

microwells by covering the microwells with the end-facet of the elastic PDMS chip, and through this design the old medium inside of the microwells could be pushed out of one channel while the new replacement is driven into the microwell through the other channel by a peristaltic pump. There are two challenges associated with this approach. One is that the pipet chip and the microwell chip must be tightly sealed and the liquid surface must be lower than the well's wall. If not tightly sealed, the medium would leak through the gap between chips during liquid handling. The other challenge is that the intrinsic nature of the laminar flow will limit the efficiency of medium replacement in the microwells. To tackle this challenge, we used two peristaltic pumps to drive the liquid flow with opposite directions in the two channels, respectively (Figure 2c). This improvement not only lowers the requirement for the plateaus flatness but also allows convex droplets.

To further improve the pipet part, we created a fine nozzle at the chip's end-facet embedded with two aligned channel outlets (Figure 2d). This nozzle allows the pipet to access the bottom of the microwells, so as to remove most of the liquid inside. This design is the most efficient way to perform pipetting, compared to the three former versions. The pipet chip contains five layers of PDMS: a stack of three thin layers, two fluid layers, and one isolating layer, are sandwiched between two 3 mm thick scaffold layers, making the connection with the peristaltic pump tubing stable (Figure 1a,b). In contrast to a previously reported circulating liquid tip pipet,[33] which patterned multiple channels in a single fluid layer, we purposely placed two channels in different layers to reduce the fabrication difficulties. We found that if the two channels are placed adjacent to each other in the same layer, for the channel size we typically used (100 μm × 100 μm), the smallest gap had to be at least 50 μm to guarantee tight sealing. While placed in two different layers, the separation of two channels can be well adjusted through the thickness of the thin PDMS membrane in between, which can be made as thin as a few micrometers. This feature makes sure the pipet chip can be used for smaller microwells and that the two outlets are both close to the center of the microwells for reproducible pipetting.

To run the experiments in parallel, we fabricated a microfluidic pipet chip with four pairs of channels to fit the microwell chip with a 12 × 8 array. We set the pitch between channel outlet-pairs twice as much as that between microwells (Figure 1a). With this setup, a customized computer program can perform liquid handling efficiently, and each pipetting nozzle has to handle liquid for 24 different microwells in serial. We need to implement a cleaning step between two handling processes to prevent cross contamination. Nozzle cleaning can be performed by repeating aspiration-dispensation cycles many times using washing buffer. This step is critical for those experiments that screen the cell behavior under the different culture conditions. The main components of the system, the microfluidic chip and the microwell chip, are easy to fabricate, to operate, and to be scaled to higher throughput and 10-fold smaller size.

Quantitative Liquid Handling. The pipet chip is aligned perpendicularly to the microwell chip for liquid manipulation. On the basis of the accuracy of our peristaltic pump and its controller, liquid can be manipulated in a quantitative manner with the resolution of ~5 nL.

Dispensation. Dispensation is a basic function of the liquid handler. It includes three steps: (1) move the pipet chip to the appropriate height above the top plateau of the microwells, (2) generate droplets through the peristaltic pump, and (3) raise

the pipet chip and release the liquid from the pipet tips; the tips are moved to new locations for the next dispensation. The tip-microwell gap is critical to secure good reproducibility and uniformity and to minimize the residue of the droplets on the outlet facet. The tip-microwell gap should be small enough to allow a droplet to touch the bottom but not too small that the tip would be immersed in the droplet. The immersion can be avoided by raising the pipet chip while generating droplets in step 2. We typically set the gap within the range of 30−200 μm, depending on the size of the droplets. For cell dispensation, we added a 2 s dwell time after step 2 to make sure the cell's inertial movement stops and guarantee uniform cell density across the microwell chip. The dwell time was typically 0.5−3 s, decided by the liquid injection speed. The droplet volume depends on the duty time and the velocity of the pump.

Removal. Removal of liquid is as much important as dispensation during a pipetting cycle. The procedure includes three steps: (1) move and align the pipettes toward the top of the droplets; (2) the peristaltic pump turns reversely to aspirate liquid and the pipet chip moves down simultaneously until the scheduled amount of liquid is removed; (3) the pipet chip moves up and gets ready for the next removal. During the whole process, the descending speed of the pipets must match the aspirating speed to ensure the tips just touch the liquid. If the aspiration speed is too slow, the tips will be immersed in the liquid and hence liquid is left on the facet surface.

Replacement. Replacement is to change the liquid in a well. It is essential to keep the cell reservoir hydrated all the time to reduce cell damage. Thus we made a strategy to partially replace the liquid during one cycle, which contains removal and dispensation steps. A complete liquid replacement can be realized after several cycles, as illustrated in Figure 3a. The

removal and dispensation are performed through separated microfluidic channels/outlets, respectively. We found that the effective efficiency of liquid replacement is highly dependent on three factors: the amount of liquid replaced per cycle, the cycle number, and the dwell time between cycles which determines the diffusion completeness in the well. We used fluorescence microscopic images to quantitatively evaluate the efficiency of liquid replacement by changing the fluorescein solution with phosphate buffered saline (PBS). We performed a series of tests through 16 removal-dispensation cycles, with 5 s dwell between every 4 cycles and for each cycle the liquid replacement was set at 70%, 45%, or 20%. The fluorescent microscopic images are shown in Figure 3b, and the quantitative analysis of these images is plotted in Figure 3c. The fluorescence intensity decreased to zero as the cycle number increased, indicating the completion of liquid replacement. As expected, the larger portion of liquid is changed per cycle, and fewer cycles are needed. For 70% and 45% liquid change per cycle, it took 8 and 12 cycles, whereas for 20% liquid change per cycle, it needed more than 16 cycles.

Aspiration-Dispensation. Aspiration-dispensation cycles are commonly used during liquid handling processes to facilitate solvation and mixing, to generate homogeneous microenvironments, and to enhance the biochemical reactions. Aspiration-dispensation cycles also generate a hydrodynamic force without changing the liquid volume, thus, especially useful for cell detachment. The aspiration-dispensation cycle is similar to liquid replacement, except that in an aspiration-dispensation cycle the liquid flows in and out of the microwells through the same channel (Figure 3d), while during the liquid replacement two channels are used (Figure 3a). We typically aspirated 90% of the liquid in a microwell and then dispensed back and repeated for a few cycles to generate strong hydrodynamic force. It is critical to ensure that the amount of liquid aspirated and dispensed should be the same to prevent air bubbles.

Cell Manipulation. *Cell Seeding.* Uniform cell density across all microwells is critical for parallel screening. In most microfluidic cell culture systems, in which cells are typically put into closed chambers, it is challenging to make cells equally distributed into different chambers because the flow resistances are usually different between chambers when connected to different channels. One way to solve this problem is to perform a sequential loading procedure, which inevitably consumes more time when throughput is increased.[17] Another approach is to use open systems, in which several methods, including wiping,[34] vacuum-assist seeding,[35] and washing away unattached cells after seeding,[36] have been reported. Recently, inject printing is becoming a facile access for cell microarray generation at the single cell level.[25,37−40] Our liquid handler has but not limited to the function of inject printing. We dispensed 350 nL cell suspension to 12 by 8 microwells, resulting in 400−500 cells per well with a coefficient of variation (CV) of ∼15% in 96 wells, which is acceptable for most cell-related assays. This uniformity is achieved mainly because of the liquid driven by the peristaltic pump is not sensitive to the difference of flow resistance between the channels. The uniformity can be guaranteed as long as the tygon tubings of the pumps have similar weariness. The cells attached to the surface within 1 h and exhibited a well-adhered morphology in 4−8 h, suggesting that no cell damage was caused during the dispensation. The population ratio between the suspended cells and the attached cells in microwells (6.0 ± 0.45%) was comparable to that of the cells conventionally cultured in the flask (6.4 ± 1.1%), as shown in
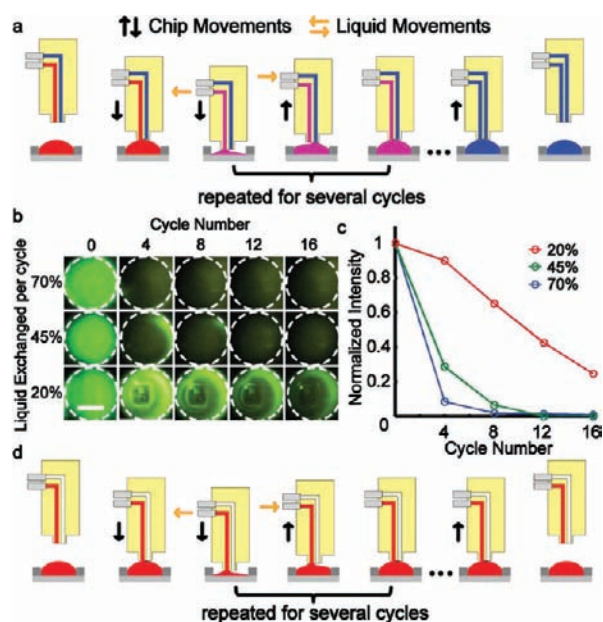

Figure 3. (a) Illustration of liquid replacement. Red solution is replaced by the blue solution after several "removal and dispensation" cycles. (b) Evaluation of efficiency of liquid replacement by changing fluorescein solution with PBS. Scale bar is 0.5 mm. (c) Fluorescent intensity of the solution in the microwells is plotted as a function of the replacement cycle number. (d) Illustration of the "aspiration-dispensation" process. The solution (red) is removed from and reinjected into the microwells for several times to facilitate mixing or to generate a hydrodynamic force.
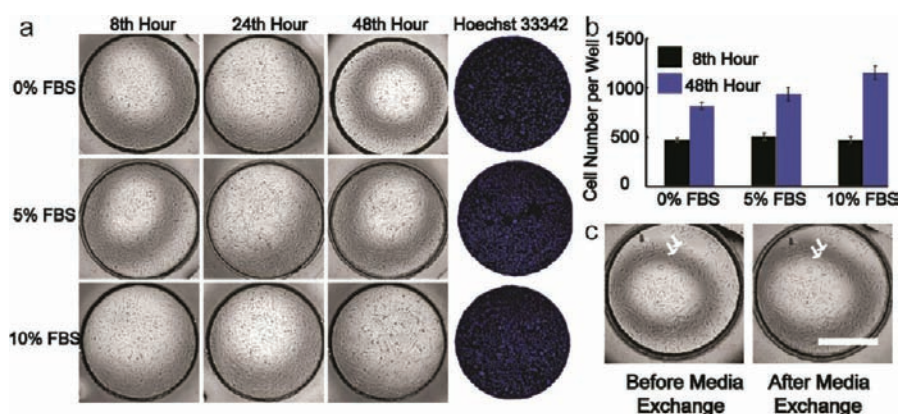
Figure 4. Demonstration of longtime cell culture using the liquid handling system. (a) Around 500 cells were seeded and cultured in each microwell in DMEM with 0%, 5%, and 10% FBS for 48 h. The medium was changed for every 8 h. Cells were labeled with Hoechst 33342 at the 48th hour for cell counting. (b) Cell numbers at the 8th and the 48th hour were counted manually and through image processing. (c) Some dead cells and debris (indicated by arrows) were taken away during the medium change. Scale bar is 0.5 mm.

Figure S2 in the Supporting Information. The cell number ranging from 50 to more than 1000 is suitable for our microwell culture chip, as long as the cells are not too crowded in the wells.

Medium Change. Medium change is necessary for longtime cell culture. If the medium is changed completely during one cycle, it is easier to cause dehydration in the nanoliter cell reservoir than at the microliter scale. Our liquid handling system keeps cells hydrated all the time because of multiple cycles of partial replacement. To do it successfully, the amount of liquid replaced per cycle should be properly determined. Although it seems to be more efficient to increase the portion of liquid replacement per cycle, the portion must be limited. The adherent cells can handle the hydrodynamic force generated by liquid removal and dispensation. When we attempted to replace all the liquid in the well in one cycle, the nozzle had to be brought to the bottom of the well. In total, 98% of the liquid would be replaced if the distance between the nozzle and the bottom of the well was as close as 5 μm. However, cells next to the nozzle would be easily detached and flushed, probably because the nozzle touched the cells and mechanically scratched some away (Figure S3 in the Supporting Information).

We carefully investigated this issue by changing 70%, 45%, and 20% of the liquid of a well during each cycle. The entire evaluation took 48 h, with the medium changed every 8 h and eight replacement cycles per medium change (Figure S4 in the Supporting Information). The eight cycles were performed in two batches, with four continuous cycles per batch and 5 s dwell time between the two batches. The liquid replacement took away loosely attached cells and created a few blanks in the microwells. Cells left in the microwells showed normal morphology and would migrate to the blank regions during culture. Cells in all three groups exhibited regular proliferation. For 70%, 45%, and 20% liquid replacement per cycle, respectively, the numbers of cells at the 48th hour were 2.4, 2.3, and 1.8 times of those at the 8th hour. Increasing liquid replacement of each cycle from 45% to 70%, cell proliferations do not accelerate significantly, because both approaches replace more than 90% of the liquid after 8 cycles (Figure 3c). Besides, the cells stop proliferating after confluence. Cell proliferation is affected not only by the amount of fresh medium in each replacement but also by the frequency of medium change. Cells cultured with the medium changed every 8 h have sufficient nutriment and showed higher proliferation rate than those with longer time intervals (16 or 24 h, see Figure S4 in the Supporting Information).

We then investigated the cells' response to different serum concentration in microwells. We replaced 45% liquid during each cycle, eight cycles per medium change, and one change every 8 h. We seeded ~500 cells and cultured for 8 h in a medium supplemented with 10% fetal bovine serum (FBS). Then we replaced the medium and cultured the cells to the 48th hour with medium supplemented with 0%, 5%, and 10% FBS, respectively. Figure 4a shows phase contrast microscopic pictures of cells cultured in microwells. Cells are well-attached and alive. From the 8th hour to the 48th hour, the cell number was increased by a factor of 1.6, 1.8, and 2.3 when cultured in 0%, 5%, and 10% FBS, respectively (Figure 4b). A high concentration FBS provides more growth factors, facilitating cells' proliferation. Comparing images before and after the medium change (Figure 4c), we observed that some dead cells and debris were taken away during liquid aspiration, without affecting the living cells.

To evaluate the performance of our nanoliter cell culture system, we performed the cell culture in 12-well plates as comparison. We found that the cells grew slightly faster with 750 μL of culture medium per well than with 350 μL per well, indicating that cell proliferation is not critically dependent on the medium volume (Figure S5 in the Supporting Information). The proliferation rate we achieved in the microwells is comparable to that from conventional culture, indicating the normal behavior of cells living in the nanoliter chambers.

Cell Passage. Passaging cells is necessary for longtime cell culture and cell lines maintenance. Combining the four liquid manipulation modes, we used our system to automatically harvest cells from four microwells, to transfer and reseed the cells into eight new microwells in parallel. The whole procedure is the same as the regular protocol for cell culture in Petri dishes, including PBS wash, trypsin incubation, trypsin neutralization, and cell transfer. We first replaced the culture medium with PBS and then replaced PBS with trypsin. The cell attachment was firm enough that the hydrodynamic force generated by the liquid flow could not detach cells without trypsin treatment. After incubation in 37 °C for 2 min, we removed 80% liquid from the microwells and replaced with the same amount of medium supplemented with 10% FBS. We usually repeated this replacement process one more time to ensure that trypsin was completely removed and neutralized. A small amount of overtrypsinized cells were taken away through this procedure. After neutralization, we detached cells from the bottom surface inside
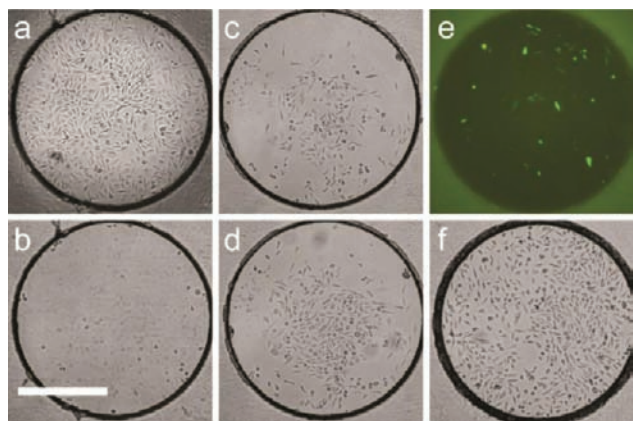
Figure 5. Demonstration of cell passage and transfection using the liquid handling system. (a) Cells in a well reached confluence after 12 h culturing. (b) Cells in the well were trypsinized, detached, and transferred to two other wells, leaving few cells in the original well. Cells adhered to the new well and proliferated for 8 h (c) and 22 h (d) after the passage. (e) Fluorescent and (f) bright field images show that 13% cells in the well expressed GFP 20 h after the transfection of GFP plasmid DNA. Scale bar is 0.5 mm.

the microwells by aspirating and dispensing 90% volume of the microwells. After five aspiration-dispensation cycles, almost all the cells in the microwells were detached (Figure 5a,b), thus cell suspension could be stored in the microfluidic channel and tubing after the final aspiration of 100% volume of the microwells. Then we moved the microfluidic chip toward the target wells and dispensed the cell suspension. Typically we divided cells from one original microwell into two new microwells and dispensed an additional 175 nL medium per well to maintain the volume at 350 nL. On average, there were about 600 cells in each original well, and after the whole procedure more than 60% of them would be recovered. Transferring only a small portion of cells from one well to another is also feasible by reducing trypsin incubation time and pipet cycles. The cells left in the original microwell could also be cultured by fresh medium dispensed immediately after the aspiration. To test the viability of the cells transferred to the new wells, we further cultured the cells for 22 h with medium changed every 8 h. Cells exhibited healthy morphology and proliferated normally, indicating the success of the cell passaging.

Transfection. Transfection is another popular cell manipulation method. We transfected plasmid into the cells through the widely used lipofection method with our liquid handling system. After the cells were seeded and cultured for 12 h, a mix of plasmid DNA and lipofectamine 2000, a liposome-based transfection reagent, were introduced into the microwells by medium change. After 5 h incubation, we replaced the medium with DMEM total medium supplemented with 10% FBS. After additional culture for 20 h with fresh medium changed every 8 h, bright field and fluorescent microscopic images were taken (Figure 5) to assess the efficiency of transfection. We used the same transfection mix for both conventional and the chip-based approaches. To avoid evaporation during the long-time cell culture, we had to add 360 μL to each container in the 12-well plate, while only 350 nL was needed for each microwell on-chip. This over 1000-fold volume reduction also resulted in the reduced plasmid/cell ratio, from 2.0 pg/cell (12-well plate) to 0.8 pg/cell (microwell chip). With this reduced plasmid/cell ratio, the GFP plasmid transfection efficiency in microwells can

still reach 13%, which is comparable to 16% achieved in conventional approaches (Figure S6 in the Supporting Information). We also observed that the transfection efficiency and cell viability varied as the transfection reagent formula changed, as shown in Figure S7 in the Supporting Information.

Drug Induced Cell Apoptosis. We use this new platform to perform the apoptosis rate analysis of U2-OS cells. Apoptosis was induced by cisplatin, a commonly used cancer drug, for 16 h. The apoptotic cells were identified by Annexin V-FITC and PI labeling. The Annexin V-FITC positive and PI negative cells were in early apoptosis, while both Annexin V-FITC and PI positive cells were in the final stage of apoptosis.[41] We noticed that some cells were taken away during liquid replacement. We believe these cells were loosely attached and belonged to the final stage of apoptosis. We counted both the missing cells and the PI positive cells and divided by the total cell number to obtain the ratio of final stage apoptosis. As shown in Figure 6,
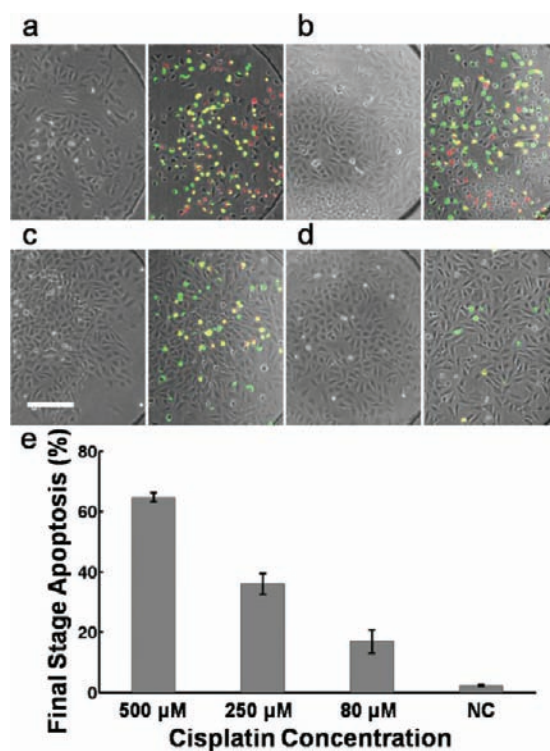

Figure 6. Cisplatin induced cell apoptosis. The phase contrast (left image of each panel) before adding cisplatin and fluorescence (merged with phase contrast image after cisplatin stimulation) microscopic images of U2-OS cells induced by (a) 500 μM, (b) 250 μM, (c) 80 μM, and (d) 0 μM cisplatin, and the ratio of the final stage apoptotic cells under each condition is shown in part e. Annexin V-FITC (green) and PI (red) are stained after 16 h of drug treatment. Some cells at the final stage of apoptosis are taken away during liquid replacement. Scale bar is 0.2 mm.

when the drug concentration was high (500 μM), over 65% cells were induced to apoptosis, and over 75% of the apoptotic cells were in the final stage; while treated with lower drug concentration, a much less numbers of cells were induced to apoptosis and most of which were in the early stage. The whole experiment is automatically performed in 40 microwells on a microwell chip, with merely 0.42 μL of drug stock solution in total. Compared to the conventional approach that easily cost 33.3 μL of solution to reach similar throughput using a 96 well

plate, this 80-fold reduction of reagent consuming, together with robust and accurate liquid handling, will greatly facilitate the cell-based high-throughput and high-content screenings.

CONCLUSIONS

We have developed a high-throughput, openly accessible, and highly automated liquid handling system to culture cells within the nanoliter range through a microfluidic approach. The cells were cultured on a chip containing 12 × 8 individual open microwells with single well addressability. Each microwell could hold a maximum of 500 nL of medium containing 50 to more than 1000 cells. The complete cell culture procedure and a few key cell manipulation experiments, including cell passage and plasmid transfection, were automatically performed on the chip. Besides, we evaluated drug-induced apoptosis on U2-OS cells in a parallel manner. The system was able to deliver and change soluble reagents to arbitrary microwells. Combined with a microscope, this system can perform fully automatic cell culture, manipulation, and real-time observation. After handling one well, the microfluidic pipet chip can be cleaned before it reaches another well to prevent cross contamination. Our method requires much less consumption of samples and reagents, provides large-scale uniformity and reproducibility, and offers great flexibility to handle multiple tasks with a single set of devices. This nanoliter cell culture system can also be easily coupled with other technologies to facilitate the high-throughput studies on cell arrays. Moreover, both the microwell chip and microfludic chip can be scaled down to fit high-throughput single-cell-based manipulation and analysis such as stem cell culture and hybridoma screening. We envision that this system could be well adapted to numerous applications for cell assays.

ASSOCIATED CONTENT

ⓢ Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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Supporting Information

Openly Accessible Microfluidic Liquid Handlers for Automated High-throughput Nanoliter Cell Culture

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E-mail: yanyi@pku.edu.cn
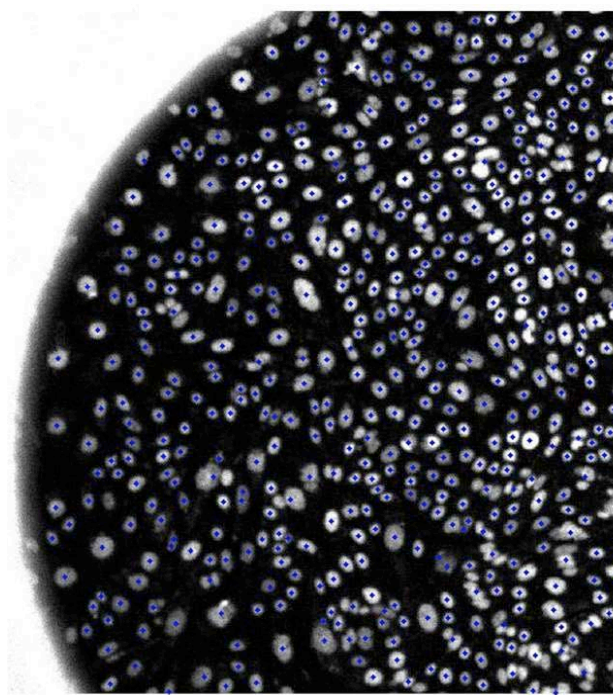
Figure S1. Cell counting with a confocal microscopic image. The cell number was 464 and 476, counted automatically by a home-developed image processing program and manually with Image J, respectively. White spots were nuclei stained by Hoechst 33342. Blue spots indicated the centroids of cells recognized automatically. Most mis-counting was caused by the connected cell nuclei.
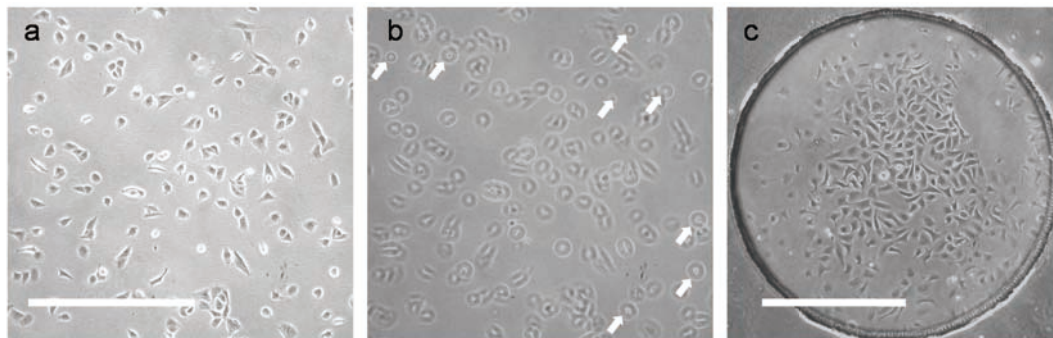
Figure S2. Evaluation of the cell damage caused by dispensation by comparing the number of cells suspended and the number of cells adhered at the 6th h after cell seeding. Images of cells cultured in the flask, focusing on the attached cells (a) and suspended cells (b). White arrows in (b) indicate suspending cells. In this field of view, the ratio of suspended cells to the attached cells is 4.8% (8/166). (c) The image of cell cultured in a microwell. The suspended/attached ratio is 5.2% (14/270). Scale bars are 0.5 mm.
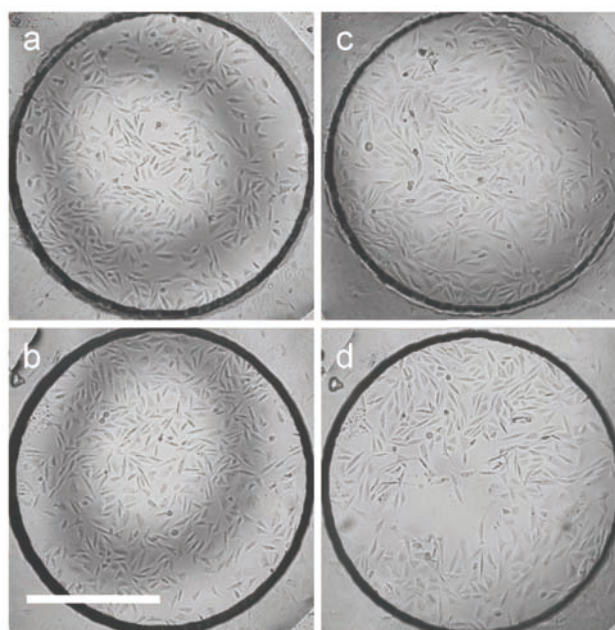
Figure S3 (a) Before and (b) after 8 medium change cycles with 90% liquid replaced per cycle. Few cells were detached. (c) Before and (d) after 8 medium change cycles with more than 98% liquid replaced per cycle. The distance between the nozzle tip and the bottom of the well was smaller than 5 μm in this case. Some cells close to the nozzle region were detached probably because the nozzles mechanically scratched them away. Scale bar is 0.5 mm.
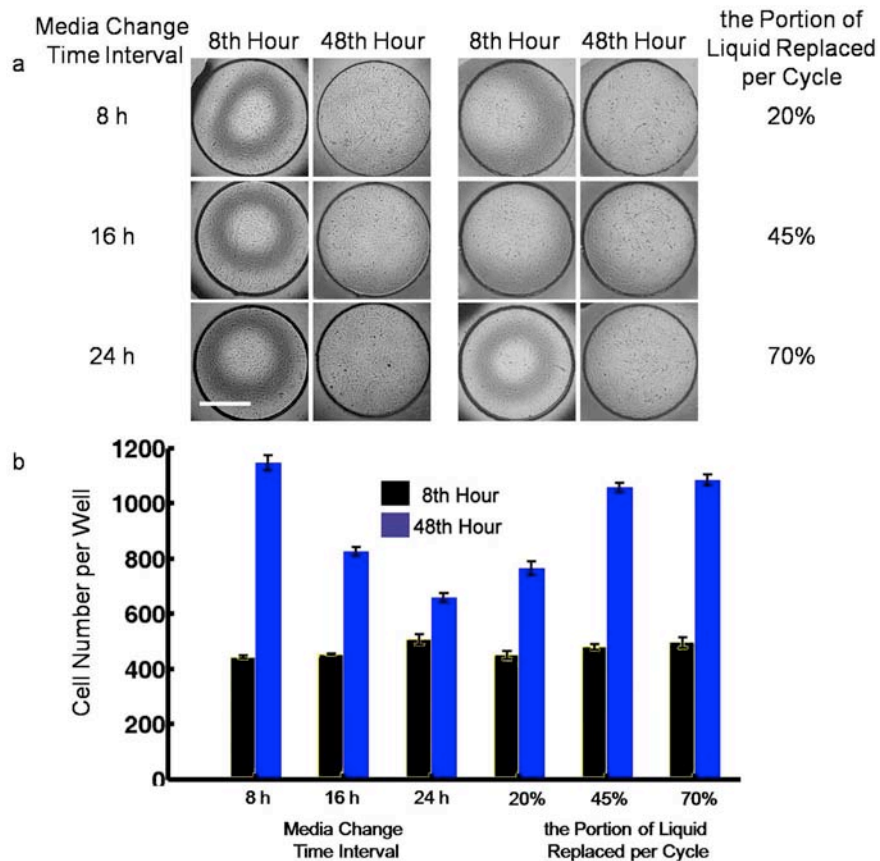
Figure S4. Investigation of the effect of different medium change time interval and the portion of liquid replaced per cycle to longtime cell culture. (a) Left panel: cells were cultured for 48 h with medium changed every 8 h, 16 h, and 24 h, respectively. 45% of the liquid of a well was replaced per cycle. Right panel: cells were cultured for 48 h with 20%, 45%, and 70% of the liquid of a well replaced per cycle. The medium was changed each 8 h. Scale bar is 0.5 mm. (b) Cell numbers at the 8th and 48th hour were counted. Although the proliferation rates obtained from different batches of experiments were slightly different depending on the cell status (for example, left panel vs. right panel), the repeatability between wells was great in the same batch of experiments.
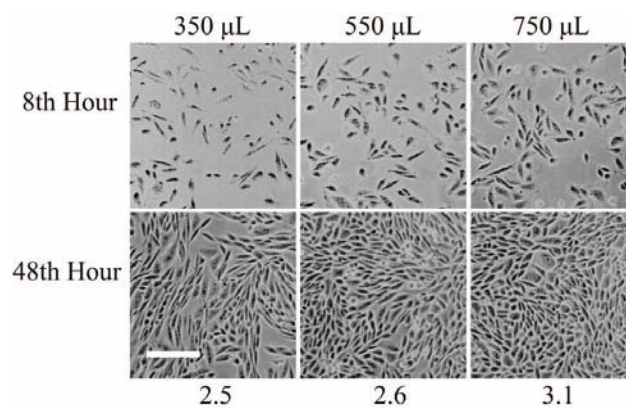
Figure S5. Proliferation rates of CHO cells cultured in the 12-well plates. The culture volume of each well is set to be 350 μL, 550 μL, or 750 μL, respectively. The corresponding increase of the cell number is 2.5, 2.6, and 3.1. Scale bar is 0.2 mm.
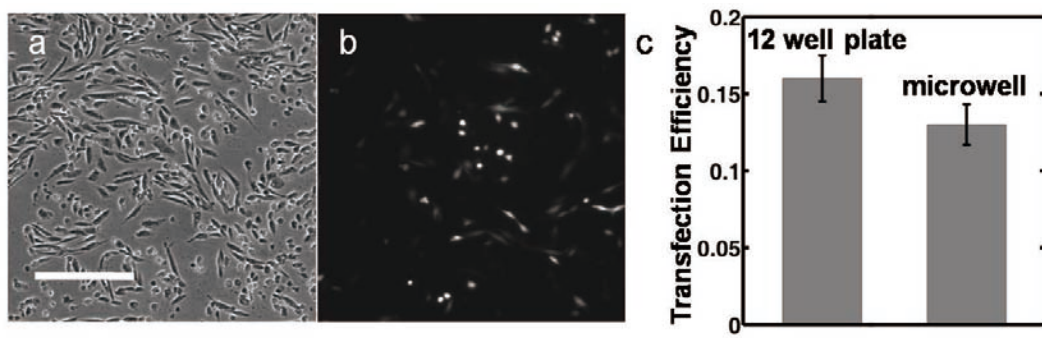
Figure S6. The GFP plasmid transfection in 12-well plates. (a) Bright field and (b) fluorescent images show that 16% cells in the well expressed GFP 20 h after the transfection. Scale bar is 0.2 mm. (c) Transfection in microwells on-chip has comparable efficiency (13%).
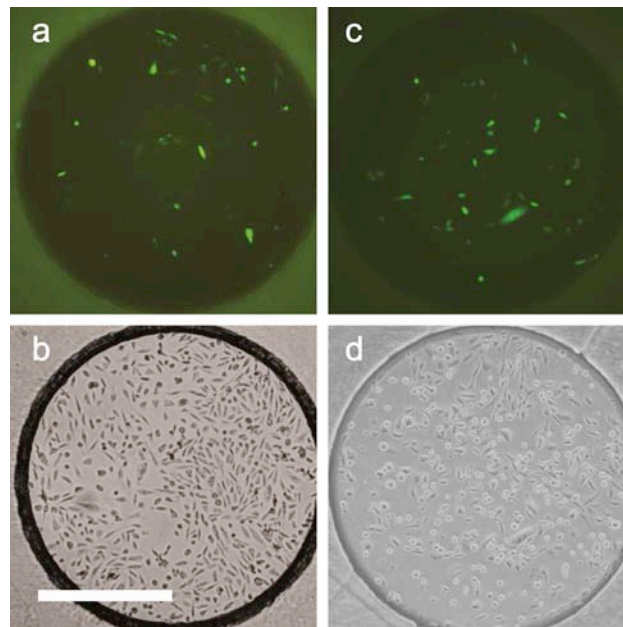

Figure S7. Fluorescent (a) and (c) and corresponding phase contrast (b) and (d) images were taken 20 h after the transfection of GFP plasmid DNA on the microwell chips. For (a) and (b), the formula of the transfection mix was the same as that in conventional experiments. For (c) and (d): the amount of lipofectamine was 3 times as much as that in (a) and (b). The additional amount of lipofectamine 2000 increased transfection efficiency but decreased cell viability. Scale bar is 0.5 mm.
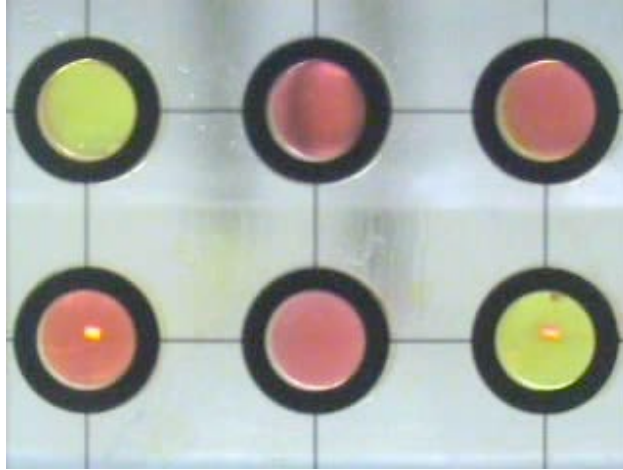
Movie S1 Demonstration of the dispensation and replacement procedures by dyes (bottom view).
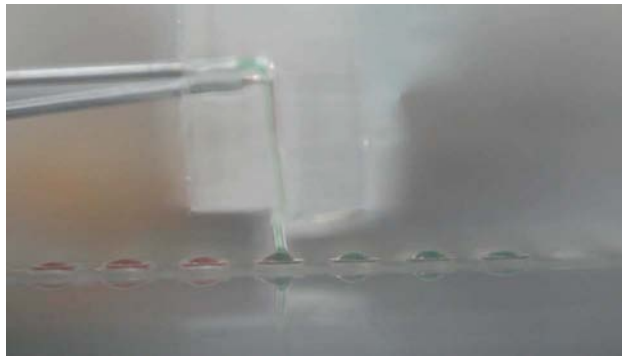
Movie S2 Dispense red dye, and then replace red dye by green dye (side view). 45% of the liquid in the well was replaced each cycle.
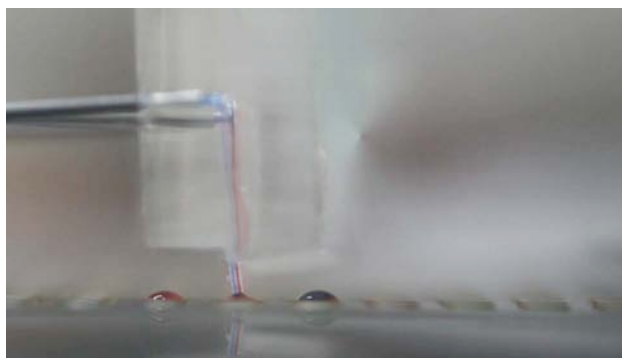
Movie S3 Dispense red dye, and then replace red dye by blue dye (side view). 20%, 45%, and 70% of the liquid in the well was replaced per cycle for the 3 microwells (from right to left), respectively.