

# Continuous Perfusion Microfluidic Cell Culture Array for High-Throughput Cell-Based Assays

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**Abstract:** We present for the first time a microfluidic cell culture array for long-term cellular monitoring. The 10 × 10 array could potentially assay 100 different cell-based experiments in parallel. The device was designed to integrate the processes used in typical cell culture experiments on a single self-contained microfluidic system. Major functions include repeated cell growth/passage cycles, reagent introduction, and real-time optical analysis. The single unit of the array consists of a circular microfluidic chamber, multiple narrow perfusion channels surrounding the main chamber, and four ports for fluidic access. Human carcinoma (HeLa) cells were cultured inside the device with continuous perfusion of medium at 37°C. The observed doubling time was  $1.4 \pm 0.1$  days with a peak cell density of  $\sim 2.5 \times 10^5$  cells/cm<sup>2</sup>. Cell assay was demonstrated by monitoring the fluorescence localization of calcein AM from 1 min to 10 days after reagent introduction. Confluent cell cultures were passaged within the microfluidic chambers using trypsin and successfully regrown, suggesting a stable culture environment suitable for continuous operation. The cell culture array could offer a platform for a wide range of assays with applications in drug screening, bioinformatics, and quantitative cell biology. © 2004 Wiley Periodicals, Inc.

**Keywords:** microfluidics; microbioreactor; cell culture; high-throughput screening; cell-based assay

## INTRODUCTION

Cell culture is an essential tool in biological science, clinical science, and biotechnology. For the past decade, a major focus of cell culture technology has been developing high-throughput cell-based assays capable of providing valuable information on potential drug targets as well as advancing cell biology (Khandurina and Guttman, 2002). There is tremendous interest in the pharmaceutical indus-

try for improved high-throughput cell-based screening platforms to expedite target validation as well as for use in preclinical trials (Sundberg, 2000). Currently, cell-based drug screening assays have been implemented for numerous molecular interactions, including ion channel activity (Denyer et al., 1998; Gonzalez et al., 1999), G-protein-coupled receptor response (Conway et al., 1999), and induction of apoptosis (Lee et al., 2003). Multiplexed devices such as microtiter plates and array bioreactors have been developed for large-scale screening applications (Kostov et al., 2001; Maffia et al., 1999). While such advances have greatly improved throughput, limitations include high cost of operation and inability to continuously monitor cells over long periods of time. Furthermore, an inexpensive integrated laboratory-scale cell culture and analysis system has yet to be realized. Recently, microfabrication and microfluidic technologies have attracted a lot of attention in this area, with promising applications in cell-based biosensors and drug screening (Park and Shuler, 2003). Microfabrication techniques offer the advantages of increased fluid control, ability to address the cellular length scale, approximating the physiologic culture environment, improved culture efficiency, and batch fabrication of high-throughput arrays. Some of the important technical aspects regarding culturing cells inside a microfluidic channel have recently been described (Walker et al., 2004). Microfabricated eukaryotic cell culture devices have previously been demonstrated on silicon and PDMS substrates with hepatocytes (Leclerc et al., 2003; Powers et al., 2002), lung (Viravaidya et al., 2004), and insect cells (Walker et al., 2002), but never for the purpose of realizing an integrated assay system.

Our current work addresses the development of a novel self-contained microfluidic cell culture array capable of conducting long-term high-throughput cell-based assays. Device functionalities include the ability to maintain and monitor cells continuously while providing a stable microenvironment. A key aspect of the device design is to

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combine a sterile cell culture microenvironment, preparation of multiple assay conditions, and long-term continuous monitoring methods in an integrated device.

## MATERIALS AND METHODS

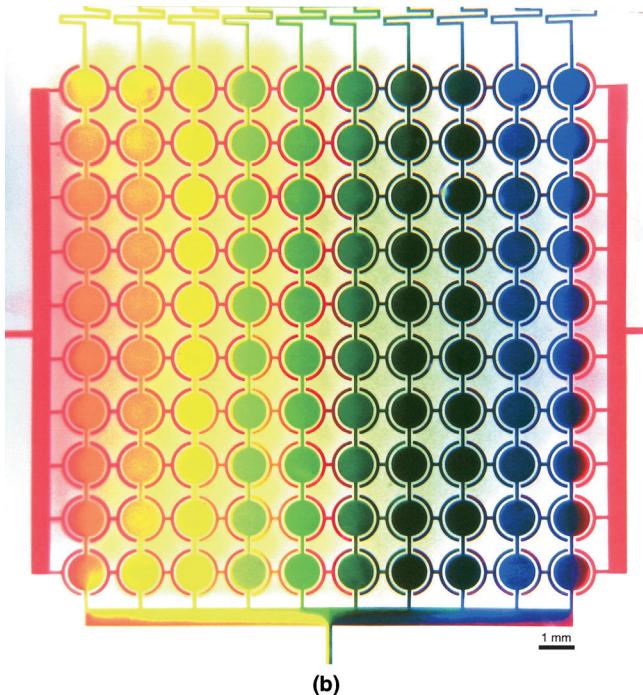
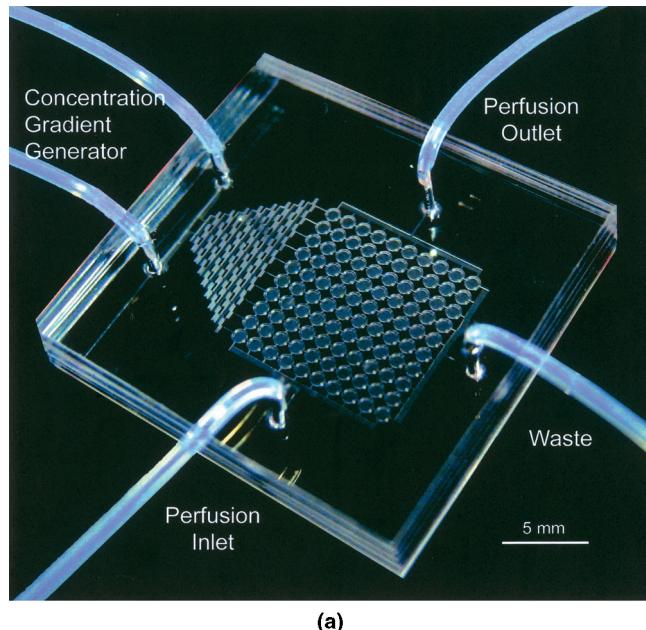
### Device Fabrication

The microfluidic cell culture array was fabricated by using soft-lithography technology and replicate molding. SU-8 negative photoresist (Microchem, Newton, MA) was used as the mold. First, the SU-8 2002 was patterned on a silicon substrate to define the 2- $\mu\text{m}$  high-perfusion channels. A 50- $\mu\text{m}$  SU-8 2050 layer was then spin-coated on top of the perfusion channels. The cell culture chamber and other channels were then photolithographically defined. PDMS (Sylgard 184, Dow Corning, Midland, MI) was prepared with a 10:1 ratio between the PDMS and the curing agents. The mold was degassed in a vacuum chamber for 10 min before curing in a 70°C oven for 4 h. The devices were then cut by a razor blade and the fluidic connection ports were punched using an 18-gauge flat-tip needle. The device was then irreversibly bonded to a precoated coverglass after oxygen plasma treatment (PlasmaTherm Etcher, 50 W, 2 torr, 40 sec) on both the bottom of the device and the glass

slide. Two different arrays were fabricated: a 1  $\times$  5 array and a 10  $\times$  10 array. The 1  $\times$  5 array was used for cell culture and cell assay characterization to reduce the complexity of data processing and the time of optical monitoring. One 1  $\times$  5 array was operated for each flow rate. The 10  $\times$  10 array was used to demonstrate the functionality of an integrated concentration gradient generator with the microfluidic cell culture array.

### Cell Culture

All culture components were sterilized with UV light prior to use. The device was capable of maintaining a sterile environment while being continuously handled in a non-sterile manner because all fluidic connections were sealed with epoxy, isolating the microfluidic cell culture array from the outer environment. HeLa cells were suspended from culture dishes at  $1 \times 10^6$  cells/ml and loaded into the microfluidic culture chambers using a sterile syringe. Cells were cultured with continuous perfusion of CO<sub>2</sub> Independent Medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, and 1% penicillin/streptomycin. During perfusion, the device was placed inside a 37°C incubator. Cell passage was achieved by flowing trypsin into the culture chambers at the standard 0.2% concentration. Perfusion was controlled with a



**Figure 1.** Microfluidic cell culture array for high-throughput cell-based assays. **a:** Photograph of the microfluidic cell culture array. A 10  $\times$  10 array of microchambers was fabricated on a 2  $\times$  2 cm device. The port at the left provided continuous perfusion of medium uniformly across the array. The port at the right was the outlet for the medium. Reagents and cells were loaded from the top and flow out through the bottom port. **b:** Concentration gradient across 10 columns. Red dye was initially perfused from left to right to fill all of the chambers. Blue and yellow dye was then loaded from the two separate ports at the top of the gradient generator, demonstrating the capability of conducting cell-based assays with multiple concentrations of reagents.

programmable syringe pump (Cole Parmer, Vernon Hill, IL, 74900). Calcein AM (Molecular Probes, Eugene, OR) was used at a final concentration of 2  $\mu$ M to fluorescently label viable cells for demonstration of the assay functionality. Optical and fluorescence microscopy was performed with a ZEISS Axiovert 200 equipped with a CCD camera. Cell number was manually determined from digital images. The specific growth rate was determined based on a simple exponential model:

$$\frac{dN}{dt} = \mu N,$$

where  $N$  was the cell number,  $t$  was time (days), and  $\mu$  was the specific growth rate ( $\text{day}^{-1}$ ). Growth rates were calculated as the slope of the best fit line to  $\ln(N)$  vs.  $t$  during the exponential phase of growth.

## RESULTS

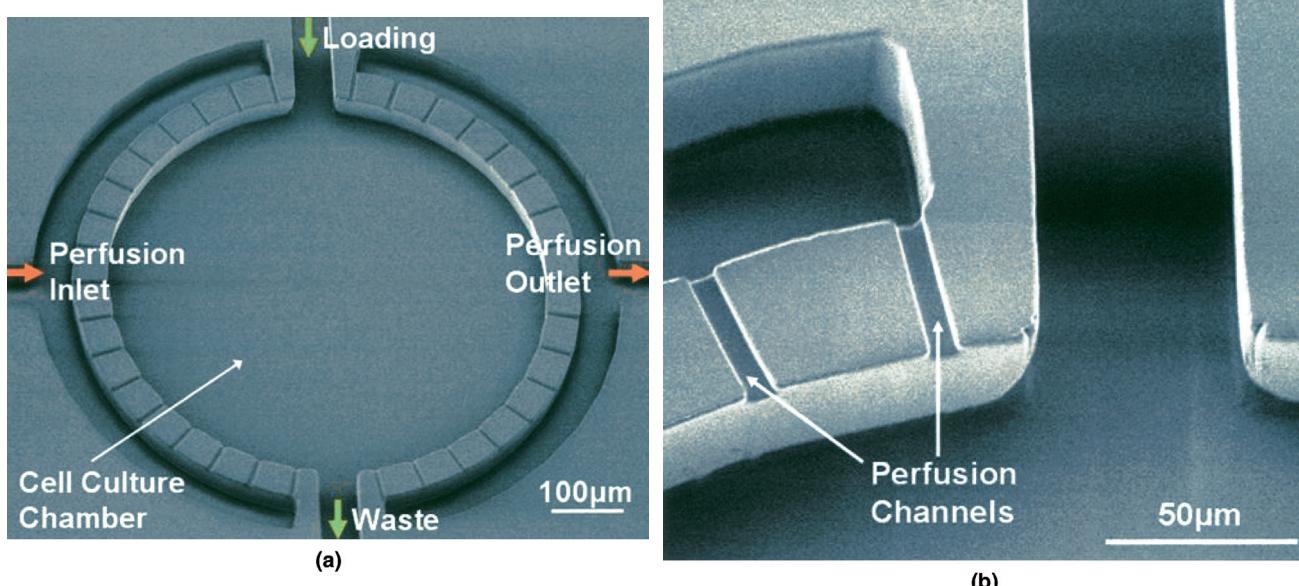
### Device Design

Multiple culture units can be combined in a two-dimensional array for high-throughput applications (Fig. 1). The addition of a microfluidic concentration gradient generator (Jeon et al., 2002) enables multiplexing cell-based assays under different conditions without increased sample preparation time. Using only two inlet concentrations, a linear gradient is

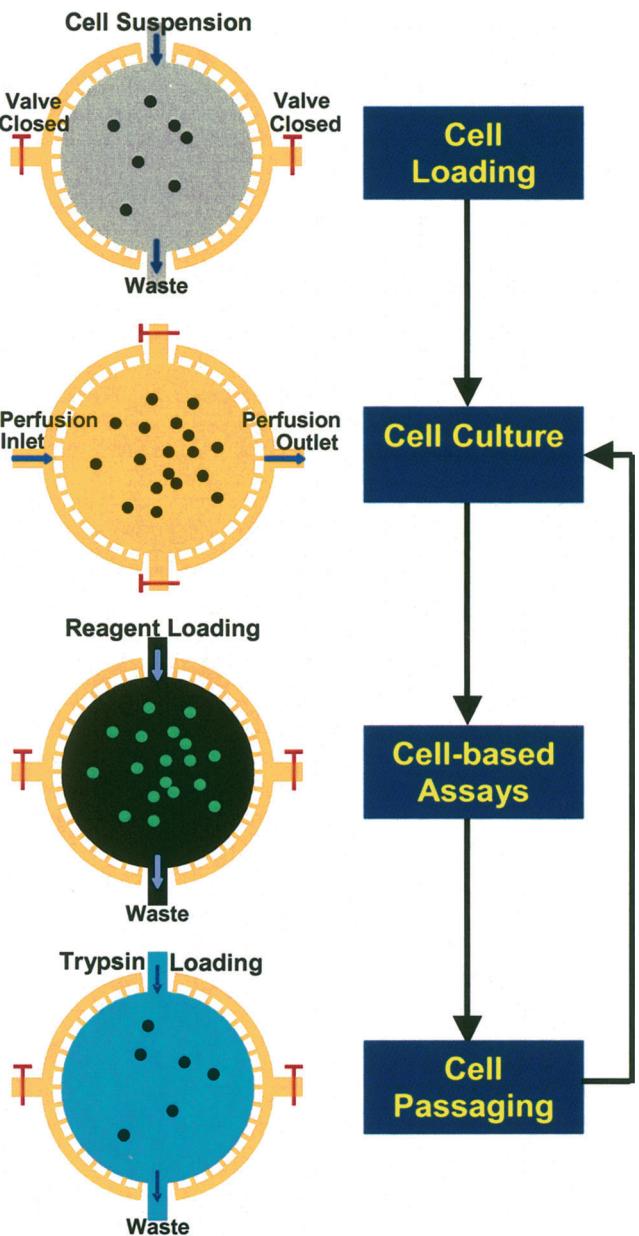
generated such that each column is exposed to a different reagent concentration. Due to the large fluidic resistance of the perfusion channels compared to the loading channels, there is minimal lateral flow between columns during reagent introduction. Each microfluidic culture chamber is designed to consist of a circular microfluidic chamber, a set of perfusion channels surrounding the main chamber, and four ports for fluidic access to the chamber (Fig. 2). The microchambers are designed to have the same cell growth area as a typical well in a 1,536-well microtiter plate. The left and right ports are designed to provide continuous perfusion of medium to the chamber to sustain cell growth and are referred to as “perfusion inlet” and “perfusion outlet,” respectively. The top and bottom ports are used to load cells and reagents for cell-based assays, and are referred to as “loading” and “waste,” respectively. Each perfusion channel is 2  $\mu\text{m}$  high and 5  $\mu\text{m}$  wide compared to the main culture chamber, which is 1 mm in diameter and 40  $\mu\text{m}$  in height. Since the perfusion channels are much smaller than the size of a typical cell (>10  $\mu\text{m}$  in diameter), they effectively localize cells within the chambers. The multiple perfusion channels also provide uniform nutrient access throughout the microchamber.

### Device Operation

The main stages of device operation are: load cells into the device, continuous perfusion of medium for cell culture, conduct cell-based assay, and passage of cells (Fig. 3).



**Figure 2.** Single microfluidic culture unit. **a:** SEM picture of a single unit of the arrayed device before bonding to a coverglass. Multiple perfusion channels surround the main culture chamber. The microchamber was 40  $\mu\text{m}$  in height with a diameter of 1 mm. Each culture unit had four fluidic access paths (left, right, top, and bottom) for “perfusion inlet,” “perfusion outlet,” “loading,” and “waste,” respectively. **b:** SEM image of perfusion channel dimensions. Each perfusion channel had a width of 5  $\mu\text{m}$  and height of 2  $\mu\text{m}$ , compared to the loading channel which had a width of 50  $\mu\text{m}$  and height of 40  $\mu\text{m}$ .



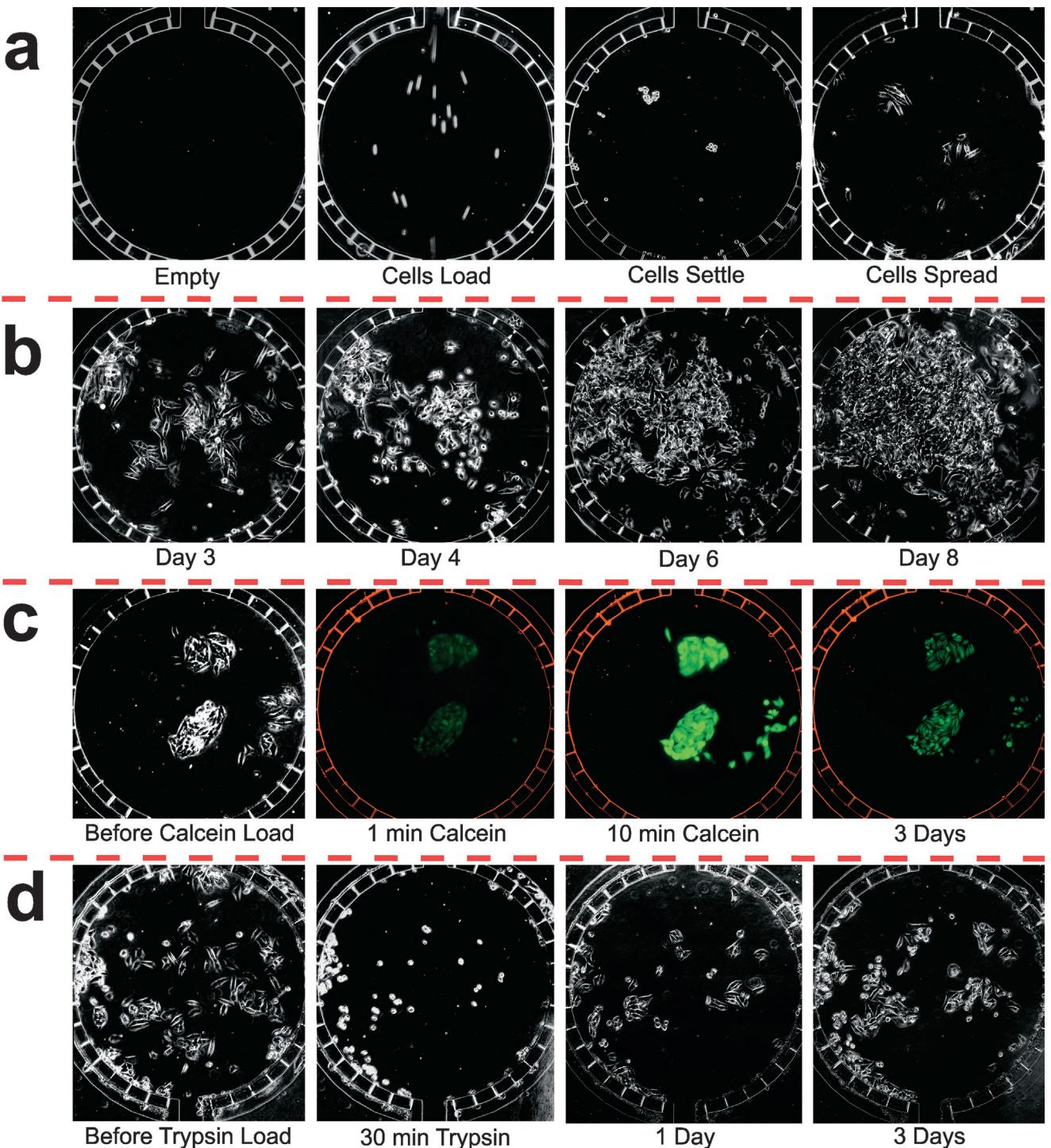
**Figure 3.** Schematic representation of device operation. The four main stages of device operation were cell loading, cell culture, cell-based assay, and cell passage. Fluid flow (top/bottom or left/right) was controlled using valves on the corresponding tubing. After cell passage, the culture and assay cycle can be repeated for long term operation. Closed valves are represented with the “T” symbol.

Each of the four fluidic ports are controlled with a valve on the corresponding tubing. Demonstration of each functionality was performed using the human HeLa cell line (Fig. 4). The device was initially prepared by filling with PBS to remove dead volume and bubbles inside the device. HeLa cell suspension was obtained at a concentration of  $10^6$  cells/mL and introduced into the microfluidic device via the “loading” port, typically resulting in 20–40 cells per chamber. The cells were then allowed to settle to the bottom of the chamber for 2 h at 37°C. After confirming

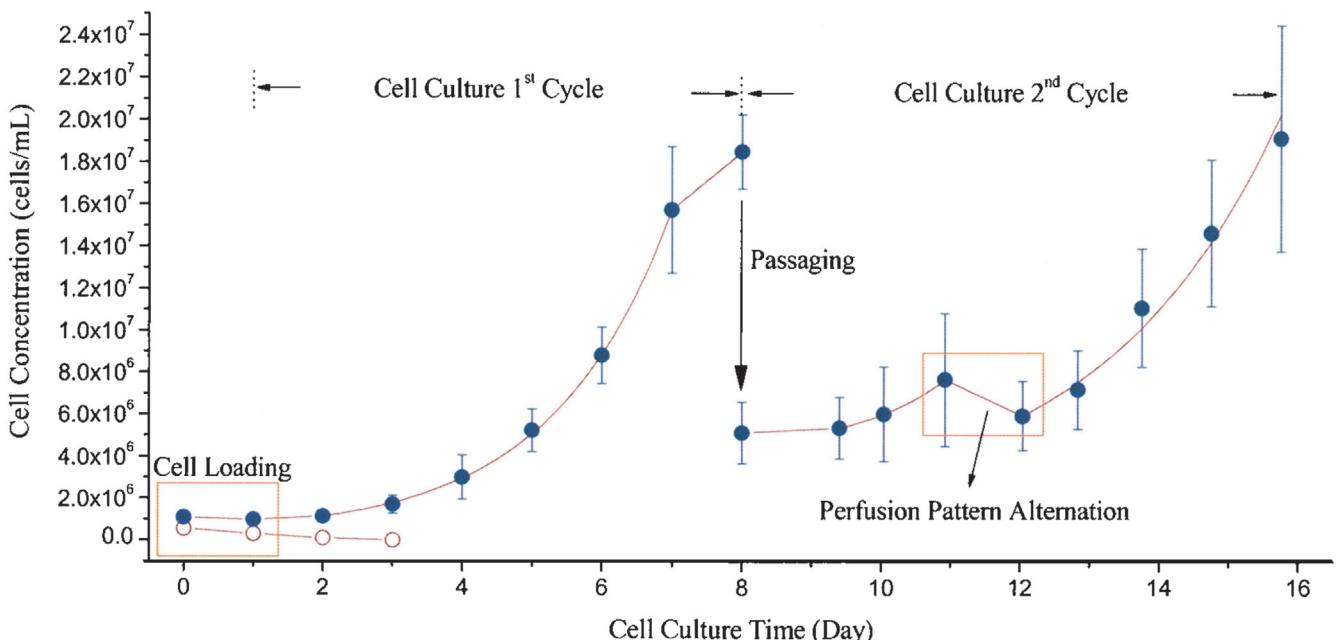
that cells were attached to the bottom of the chamber, the “perfusion inlet” and “perfusion outlet” valves were opened. A syringe containing fresh CO<sub>2</sub> independent medium was connected to the “perfusion inlet” port and operated with a syringe pump. During the continuous perfusion of the medium, the device was placed inside an incubator for humidity and temperature control (37°C). Since the PDMS was gas permeable (Charati and Stern, 1998), O<sub>2</sub> diffusion from the air was sufficient for cell culture. Due to the continuous flow of fresh medium into the culture chambers, a CO<sub>2</sub> buffering system for pH was not necessary for cell survival. Cell growth was monitored daily using a phase-contrast microscope. To demonstrate a live cell assay in the device, calcein AM was introduced from the “loading” port and the fluorescence intensity of labeled cells was monitored over time. Calcein AM is fluorescent only in viable cells after activation by intracellular enzymes. The calcein AM fluorescence intensity inside the cells reached steady state after about 10 min and gradually faded over 10 days. A similar approach could be used to test the effect of various molecules on long-term behavior of cultured cells. To replicate the processes applied to traditional cell culture, we developed a method for passaging cells. After the cells began to fill the chambers, trypsin was flowed from the “loading” port to detach cells and remove them through the “waste” port until the desired dilution was achieved. After restoring perfusion, the cells began to spread and divide, validating long-term operation of the device.

### Cell Growth

Cell growth kinetics were monitored to verify successful operation of the device. The cell growth and passage cycle was recorded over 16 days to confirm reproducibility (Fig. 5). In the control experiment where the perfusion rate was zero, all of the cells were dead and detached from the bottom of the chamber by day 4. For continuous perfusion, after loading cells (day 0), we observed a lag phase of ~2 days followed by an exponential growth. At higher cell densities (~ $1.5 \times 10^7$  cells/ml), growth was constrained by space limitation in the microchamber. We have been able to split cells within the device between a 1:2 and 1:10 dilution by controlling trypsin reaction times. Optimizing this process should reduce the relatively large variation of cell numbers observed after splitting. When we altered the medium flow orientation to be directed through the larger channels (top-down) on day 11, a reduction in cell number was observed within 24 h. This appeared to be a result of flushing newly divided cells from the chambers. We observed that newly formed cells were initially round in morphology and had weaker adhesion to the surface. Comparison of phase contrast images indicated that these cells had been removed between day 11 and 12 (data not shown). The difference between the observed cell number on day 12 and the expected value based on



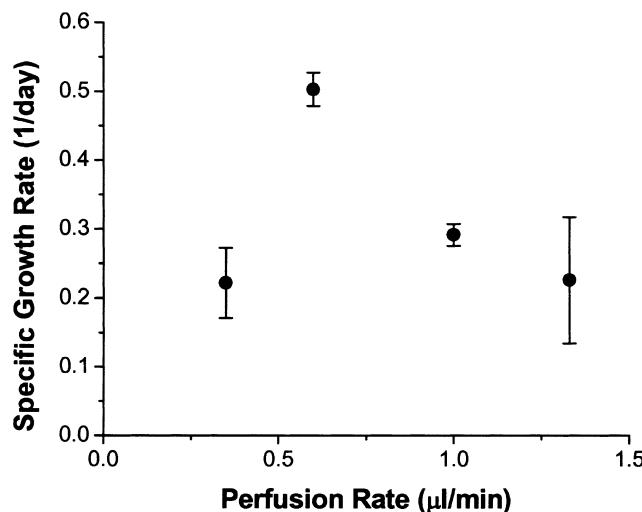
**Figure 4.** Demonstration of device functionalities. **a:** Cell loading. The device was filled with PBS prior to loading cells. Cells in suspension were then introduced from the “loading” port with the perfusion inlet and outlet valves closed. Path lines of cells indicated flow through the chambers. The loading and waste valves were then closed to stop the flow of cells and to allow them to attach to the underlying coverglass. Continuous perfusion of medium resulted in cell spreading within 24 h. **b:** Continuous growth. The perfusion inlet and outlet valves were opened and CO<sub>2</sub> independent medium was introduced continuously from the perfusion inlet at 37°C. Cell numbers were recorded daily using phase-contrast microscopy. The series of pictures shown here were at a perfusion flow rate through the chamber of 0.2 µl/min at 3, 4, 6, and 8 days after loading. **c:** Cell-based assay. Calcein AM was introduced to cultured cells via the loading port to demonstrate the cell-based assay capability of the microdevice. Short-term monitoring (<20 min) as well as long-term cellular response (>10 days) was recorded via fluorescence microscopy. The pictures shown were a phase contrast image of cells prior to labeling, after 1 min of calcein AM exposure, after 10 min of exposure, and after 3 days culture following removal of calcein AM. Fluorescence images were overlaid onto brightfield images of the channels for reference. **d:** Passaging of cells. Trypsin was flowed through the loading port to detach and remove cultured cells. Controlling the flow rate and reaction time under real-time monitoring allowed selective dilution of cells between 1:2 and 1:10. Once the desired dilution was achieved, PBS was used to remove trypsin from the chambers. The cells were subsequently cultured under continuous perfusion of medium. The pictures show a 60% reduction in cell number after trypsin treatment, cell spreading after 1 day of perfusion, and regrowth to initial density after 3 days.



**Figure 5.** Cell growth and passage cycles. Cell concentration was monitored for a control array that had no medium flow (○) and of an array with continuous per chamber perfusion rate of  $0.12 \mu\text{l}/\text{min}$  (●). Error bars represent the standard deviation within the array. As the cells approached confluence, a reduction in the growth rate was observed. The cells were split  $\sim 1:4$  on the eighth day of culture. Normal growth kinetics were observed over the following 3 days. To test the importance of the perfusion channel design for cell growth, we changed the perfusion direction from left-right to top-down on day 11. A reduction in cell number was observed after 24 h. Switching the perfusion flow back to the original configuration (day 12) restored normal cell growth. Specific growth rates (exponential fit) between days 2–7, 8–11, and 12–16 were statistically equivalent.

exponential growth from days 8–11 was found to be statistically significant ( $P < 0.05$ ). Utilizing the  $2 \mu\text{m}$  by  $5 \mu\text{m}$  channels during medium perfusion prevented this cell loss and enabled normal cell growth. The specific

growth rate of HeLa cells in the culture array was characterized under various perfusion flow rates (Fig. 6). Cells were cultured at a per chamber flow rate of  $0.07 \mu\text{l}/\text{min}$ ,  $0.12 \mu\text{l}/\text{min}$ ,  $0.20 \mu\text{l}/\text{min}$ , and  $0.27 \mu\text{l}/\text{min}$  (turnover rates of  $1.8 \text{ min}^{-1}$ ,  $3.0 \text{ min}^{-1}$ ,  $5.1 \text{ min}^{-1}$ , and  $6.8 \text{ min}^{-1}$ , respectively). We observed HeLa cells exhibited the highest growth rate at a flow of  $0.12 \mu\text{l}/\text{min}$  (doubling time of  $1.4 \pm 0.1 \text{ days}$ ).



**Figure 6.** Specific growth rate dependence on perfusion rate within the cell culture array. HeLa cell growth rates were calculated from cell numbers based on an exponential growth model. Exponential growth typically occurred between days 2 and 6 after loading. Error bars represent the standard deviation within the array.

## DISCUSSION

The development of a microfluidic cell culture array offers many potential applications in biotechnology research such as drug screening, bioreactor optimization, and quantitative cell biology. The nature of microfabrication and microfluidics allows rapid and inexpensive production of high-density arrays. This is ideally suited for high-throughput experimentation, where a large number of conditions need to be assayed in parallel with minimal reagent consumption. We envision the microfluidic cell culture array to enable high-throughput experimentation at the laboratory scale. A major focus of the device design was to make it adaptable for a large number of research investigations. Once the cells are seeded, culture within the device does not require the presence of a sterile cell culture facility. A  $\text{CO}_2$  incubator was not needed to maintain physiologic pH due to the high turnover of medium during perfusion.

Because the device is mounted on a standard coverglass, it is compatible with most types of optical microscopy, including fluorescence, confocal, and phase contrast. Efficient optical monitoring of cells for high-throughput applications can take advantage of current automated array scanner technology (Miraglia et al., 1999). The concentration gradient generator allows the creation of a large number of reagent concentrations using only two fluidic inlets. The second dimension of the array could be used to load various cell lines or cells transfected with different proteins. For the  $10 \times 10$  array design, this potentially enables performing 100 experiments in parallel with a unique condition in each microchamber.

This approach can also be valuable for optimizing mammalian cell bioreactor performance. Bioreactors are becoming increasingly important for the production of new protein pharmaceuticals, but development is limited due to poor characterization of culture parameters (Molowa and Mazanet, 2003). The microfluidic array can be applied to characterize the effect of culture medium components, pH, cell density, and perfusion rate on protein expression. In order to realize a fully automated microbioreactor array, it is important to uniformly load the cells in each of the culture chambers. While our current approach of using flow to randomly distribute cells in the array creates reasonable uniformity, we are currently investigating methods to utilize the perfusion channel design to precisely control cell number within each microchamber. Our initial results indicate that cell growth is dependent on medium flow rate. At the lower flow rate, cell growth might have been limited by mass transfer, whereas at higher flow rates, increased shear stress and hydrostatic pressure may have inhibited growth. A more thorough investigation is under way to characterize cell metabolism and model mass transfer parameters to optimize operation of the cell culture array.

Cell culture on the microscale may also offer advantages for studies of tissue behavior (Andersson and van den Berg, 2004). An ex vivo tissue array could have tremendous impact on biotechnology, such as the development of functional tissue engineering (Martin et al., 2004). The fluidic control and continuous perfusion provided by the microfluidic cell culture array may create a more "tissue-friendly" microenvironment than traditional culture methods. The small scale of the culture chambers produced a cell-volume-to-fluid ratio that more closely simulated physiologic values. At higher cell densities, it was observed that the HeLa cells formed multilayered clumps with varied morphology. While cell numbers were difficult to quantify in this state, we estimated a maximum viable cell density of  $\sim 5 \times 10^7$  cells/ml, roughly 10-fold larger than typical values reported for mammalian cell bioreactors. Confluent cultures at this density were maintained for days without any indication of device fouling or cell death. While the cell cultures in these experiments were arbitrarily terminated after 14 days, we believe continued perfusion of medium can sustain cell viability for much longer periods of time. Further char-

acterization of cells at these densities may yield interesting results for long-term bioreactor studies and three-dimensional cell culture techniques.

Currently, we are applying this device for high-throughput cell-based biological experiments as well as integrating temperature control, fluid handling, and optical detection into a self-contained system. The addition of on-chip enzymatic electrodes for metabolic monitoring (Cosnier, 2003) and microfluidic valves for fluidic control (Thorsen et al., 2002) could also further enhance the device functionalities. While the focus of this article was on a cell culture array for cell-based assays, this device could potentially benefit many areas of cell-based biomedical research.

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