A Cell Programmable Assay (CPA) chip†

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This article describes two kinds of “Cell Programmable Assay” (CPA) chips that utilize passive pumping for the culture and autonomous staining of cells to simply common protocols. One is a single timer channel CPA (sCPA) chip that has one timer channel and one main channel containing a cell culture chamber. The sCPA is used to culture and stain cells using Hoechst nuclear staining dye (a 2 step staining process). The other is a dual timer channel CPA (dCPA) chip that has two timer channels and one main channel with a chamber for cell culture. The dCPA is used here to culture, fix, permeablize, and stain cells using DAPI. The additional timer channel of the dCPA chip allows for automation of 3 steps. The CPA chips were successfully evaluated using HEK 293 cells. In addition, we provide a simplified equation for tuning or redesigning CPA chips to meet the needs of a variety of protocols that may require different timings. The equation is easy to use as it only depends upon the dimensions of microchannel and the volume of the reagent drops. The sCPA and dCPA chips can be readily modified to apply to a wide variety of common cell culture methods and procedures.

1. Introduction

The development of microfluidic technologies has the potential to significantly impact biological and chemical studies.1-4 Yet, up to this point, devices have been developed with the primary goal of creating specific tools with advanced functionality and/or increased throughput. Next generation microfluidic devices are expected to bring these advanced functionalities together into flexible, robust, and autonomous devices.3

These integrated devices are more commonly referred to as “chips” (cell chip, PCR chip, DNA chip, etc.) and are currently being advanced by many different groups.1,6-16 Some common types of functionality that are being incorporated onto these chips include: mixing, valving, culture, treatment, selection, lysis, separation, and other analyses. A subset of these chips is aimed at cell culture for high throughput screening (HTS) research and interface with robotics and culture control systems.17-19

However, current embodiments of these and other chips for cell studies typically require equipment or methods that can make translation of the device into regular use in small biology labs challenging, such as intricate and expensive peripheral equipment, external power for fluid pumping, control valves, mixers, or time controls. Our microfluidic platform can be used to eliminate many of these complicated components and external energy sources by using a surface tension-based method to create fluid flow called passive pumping along with passive fluidic timers and logic gates. By taking this approach we hope to increase the throughput of many common laboratory procedures without requiring the machines and equipment needed for most high-throughput approaches.

Perhaps the most common procedure in cell biology research is in vitro cell culture followed by staining. This procedure is a daily occurrence in most cell biology research labs. In this paper, we describe the design, fabrication, and operation of a “Cell Programmable Assay” (CPA) chip that is able to culture cells and automate the process of staining using passive components. Fig. 1 shows the steps of using a CPA chip: (1) the culture chamber is filled with media and cells are loaded, (2) the cells are incubated/cultured, (3) droplets of reagents are deposited at the...
appropriate ports, and (4) the cells are observed/imaged on a microscope.

A CPA chip is comprised of two main parts: a main channel with a cell culture chamber and a highly resistive microchannel (timer channel) used to deliver treatments in a timed manner. The timer channel has one input and one output while the main channel has a cell culture chamber, a primary input port, switch port(s), and one output port. The output of the timer channel is placed near the switch port of the cell culture channel with the gap between them designed to act as a timer switch. In step 3 where staining takes place, all the reagents are applied one immediately after the other yet the time at which those reagents pass through the culture chamber is passively controlled through the timer channel resistance and fluidic switch(s). A fluidic switch is tripped when enough fluid builds up at the timer output such that the droplet connects with the fluid exposed at the culture channel switch port. When the droplet connects with the switch port, the fluid that has built up is then allowed to flow through the culture chamber and treat the cells. The threshold volume of the switch is controlled using the spacing between the timer channel output and culture channel switch port.

We have carried out two sets of experiments to demonstrate the use of two different CPA designs. The first experiment uses a single timer channel CPA (sCPA) to culture and stain cells using Hoechst nuclear staining dye. The second uses a dual timer channel CPA (dCPA) to culture, fix, permeabilize, and stain cells using DAPI. All fluid pumping is done with passive pumping and requires only a micropipette. The CPA chips are made from a single layer of PDMS fabricated using soft lithography and placed on a polystyrene culture substrate. Moreover, we provide a simplified equation for tuning or redesigning CPA chips to meet the needs of a variety of protocols that may require different timings. The simplified equations depend only on the dimensions of the resistive channel and the volume of the reagent drops.

2. Materials and methods

2.1 Device design and fabrication

The Cell Programmable Assay (CPA) chip has a main channel with cell culture chamber and timer channel and uses specially designed ports that can act as timer switches to “schedule” treatments of the cultured cells. The timer channel has a relatively high fluidic resistance and has one input and one output port while the culture channel has a primary input port, switch port(s), and an output port. 3D schematic diagrams of two different CPA chips and switch zone are shown in Fig. 2. A CPA chip was fabricated in poly(dimethylsiloxane) (PDMS, Sylgard 184 Silicon Elastomer, Dow Corning, USA) from masters prepared by soft lithography using SU-8 100 photoresist (MicroChem, USA). Two layers of SU-8 were spun and exposed individually and developed to generate a mold with fluidic microchannels that are 250 μm high and fluid injection ports that extend the full depth of the PDMS device (500 μm) on 3 inch silicon wafers. The PDMS was molded over the SU-8 master and sandwiched between transparency film using weights cushioned with rubber sheets to allow direct molding of the ports into the device.\(^{21}\) The PDMS-based microdevices are autoclaved and placed on tissue culture treated dishes (Omni-Tray, NUNC). As mentioned earlier, there are two kinds of CPA chips in this work, sCPA (one timing channel) and dCPA (two timing channels) that can be used for culture and subsequent staining. The sCPA chip is for simple staining that does not require prior fixation and permeation like with a Hoechst nuclear stain while the dCPA is for staining that requires an additional pretreatment step such as a combined fixation and permeation treatment followed by DAPI nucleic acid staining. The sCPA chip has one timer channel (width \(\times\) height \(\times\) length [mm]: 0.3 \(\times\) 0.25 \(\times\) 50.0) and the main channel with one cell chamber (dimension: 2.0 \(\times\) 0.25 \(\times\) 6.0, volume: 2.90 mm\(^3\)), one input port, a switch port, and an output port with a switch (or gap) area of 11.57 mm\(^2\) as shown in
Fig. 2(a). The dCPA chip has two timer channels (0.2 × 0.25 × 28.6 and 0.2 × 0.25 × 50.0) and the main channel with one cell chamber (dimension: 2.0 × 0.25 × 5.0, volume: 2.28 mm³), a primary input port, two switch ports, and an output port with switch areas of 9.14 mm² as shown in Fig. 2(b). Fig. 2(c) shows the principal of connection between output port of the timer channel and the switch port of main channel in detail.

2.2 Cell culture and loading cells in microchannel

All manipulations of cells were performed in a biosafety cabinet (Level II), and cell culture was conducted in a traditional cell culture incubator (temperature: 37 °C and CO₂ concentration: 5%). Human embryonic kidney (HEK 293, ATCC) cells were cultured in a cell culture flask in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% MEM non-essential amino acids (NEAA, 100×, Gibco), 1% MEM sodium pyruvate (100×, Gibco), and 1% antibiotics (pen/strep). The cells were washed with phosphate buffered saline (PBS, Gibco), removed from flask using 0.05% Trypsin–EDTA (Gibco), and placed in culture medium. The cells were then centrifuged (1000 rpm for 5 min) and resuspended in culture medium and prepared at 1.5 × 10⁶ cells ml⁻¹ in 15 ml conical tube (BD science) along with culture media. Cells were then loaded by micropipette-injection into the cell culture chamber of the CPA chip. After loading, the CPA chip was placed in a large bioassay dish (Corning) containing filter-sterilized reagent grade water and incubated. The large surface area of water in the bioassay dish helps keep the environment inside and around the CPA chip saturated with water vapor and thus reduces evaporation. Helped keep the environment inside and around the CPA chip.

The sCPA chip for Hoechst staining. Fig. 1 shows the concept of the CPA chip and general procedure while Table 1 contains an illustrated, step-wise description of sCPA chip operation using dyed water (McCormick food dye). The specific procedure was as follows. (1) The timer channel is filled with PBS while the cell culture chamber of main channel was filled with media containing HEK cells. (2) The sCPA chip is incubated for 48 hours. (3) PBS is placed at the outputs of the timer and main channel to establish appropriate output droplet sizes for timer operation: 150 ± 10 µl at the output port of main channel and 30 ± 2 µl at the timer output. (4) A 20 ± 2 µl droplet of Hoechst staining dye (5 µg ml⁻¹ in media, Invitrogen) is placed at the input port of the main channel (t = 0 s), and (5) a 15 ± 2 µl droplet of PBS at the input port of the timer channel to begin device operation (t = 10 s). The PBS flowed to the output port of timer channel where the output droplet grew until it overlapped with the fluid in the switch port of the main channel, creating a connection between the timer channel and the main channel. Upon connecting (t = 60 s), the fluid that had previously built up in the switch region passively pumped to the main channel output, thereby “washing” the cell chamber (t = 90 s). The cells were then observed by fluorescence microscopy.

The dCPA chip for DAPI staining. Table 2 contains an illustrated, step-wise description of dCPA chip operation using dyed water. The experimental procedure used with the dCPA was as follows. (1) The two timer channels are filled with PBS while the cell culture chamber of main channel is filled with media containing HEK cells. (2) The dCPA chip is incubated for 48 hours. (3) PBS is placed at the outputs of the timer and main channel to establish appropriate output droplet sizes for timer operation: 150 ± 10 µl at the output port of main channel and 30 ± 2 µl at the output port of timer channel 1, and 30 ± 3 µl of DAPI staining dye with PBS at the output of timer channel 2. (4) A 20 ± 2 µl droplet of mixed solution (0.1% Triton ×100 + 4% paraformaldehyde (PFA) in PBS) is placed at the input port of the main channel for fixing and permeabilization of cells (t = 0 s), and (5) a 15 ± 2 µl droplet of PBS at the input port of the timer channel 1 (t = 6 s) and the input port of the timer channel

Table 1 sCPA chip operation

<table>
<thead>
<tr>
<th>t=0, 15 µl. of Hoechst (red) placed at input port of main channel.</th>
<th>Input Port (Timer)</th>
<th>Out Port (Timer)</th>
<th>Input Port (Main)</th>
<th>Out Port (Main)</th>
<th>Cell Chamber (Main)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS (Water)</td>
<td>Hoechst (Red)</td>
<td>Media (Water)</td>
<td>Media (Water)</td>
<td></td>
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<tr>
<td>t=10, 15 µl. of PBS (green) placed at input port of timer channel.</td>
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<tr>
<td></td>
<td>PBS (Green)</td>
<td>PBS (Water)</td>
<td>Hoechst (Red)</td>
<td>Media (Water)</td>
<td>Hoechst (Red)</td>
</tr>
<tr>
<td>t=60, droplet at timer output port just prior to connection with switch port of main channel.</td>
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<tr>
<td></td>
<td>PBS (Green)</td>
<td>PBS (Green)</td>
<td>Media (Wate)</td>
<td>Hoechst (Red)</td>
<td></td>
</tr>
<tr>
<td>t=90, cell chamber has been washed with PBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBS (Green)</td>
<td>PBS (Green)</td>
<td>Media (Green)</td>
<td>PBS (Green)</td>
<td></td>
</tr>
</tbody>
</table>
2 (t = 12 s) to begin device operation. In this case, there are two timed events that occur after step (4): washing with PBS at t = 132 s and staining with DAPI at t = 210 s. The images for t = 6 and 12 s also illustrate that a relatively small volume of fluid compared to the timer output droplet is needed at the timer input to “trip” the switch and cause flow into the culture channel. For this reason, the treatment reagents intended for the culture channel are placed at the timer outlet while the timer input droplet may or may not be of the same fluid. When the fluids are different, a predictable amount of dilution occurs before the switch is tripped. In this way, PBS or some other fluid can consistently be used as the pumping fluid in the timer channel to provide repeatable timing. The cells were then observed by fluorescence microscopy.

2.4 Image acquisition

Images of nuclear staining using Hoechst and DAPI were acquired on an inverted microscope (IX70, Olympus) using a SPOT RT Monochrome digital camera (Diagnostic Instrument, Inc.).

2.5 Principal of passive pumping time and prediction of pumping times

Well known equations of fluid dynamics were used to develop a simplified equation to relate droplet volume to pumping time through a microchannel of defined dimensions (see details in ESI†). The time at which the input drop finishes pumping can then be estimated using eqn (1).

\[ T_p = \frac{RV_{out}}{2(1 - \frac{r_{out}}{r_{in}})} \times 2 \]

\[ R = \frac{12 \mu L}{m w h} \] and \[ \gamma = 0.072 \frac{N}{m} \] and \[ r_{PDMS} = \sqrt[3]{0.32 V_d} \]

where \( T_p \) is time of pumping, \( R \) is resistance of the channel, \( V_{in} \) is volume of input droplet, 2 is a calculated coefficient\(^{20,22-24} \) (ESI†), \( \gamma \) is the surface tension of water (at 25 °C), \( r_{in} \) and \( r_{out} \) are the radii of the input and output droplet, \( r_{PDMS} \) is the radius of the droplet on the PDMS surface and \( V_d \) is the volume of droplet, respectively. The variables \( m, L, w \) and \( h \) represent fluid viscosity and dimensions of the timer channel: length, width, and height, respectively.

3. Results

3.1 Estimation and measurement of pumping time

Operation of the CPA timing mechanism depends upon the difference between the volume of the output drop and threshold volume of the switch area along with the rate at which fluid enters the switch area. Thus, if the volume of the input drop is closely matched with the amount of fluid needed to trip the switch, eqn (1) (i.e., the time to pump a given input droplet) can be used to predict the time at which tripping will occur. Pumping times for various input drop volumes were measured and compared to

<table>
<thead>
<tr>
<th>Table 2  dCPA chip operation</th>
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<tr>
<td><strong>Input Port</strong> (Timer1)</td>
</tr>
<tr>
<td>t=0, 15 pl. drop of “PFA + Triton” (green) placed at input port of main channel</td>
</tr>
<tr>
<td>t=6, 15 pl. drop of PBS (red) placed at input port of timer channel 1</td>
</tr>
<tr>
<td>t=12, 15 ul. drop of DAPI (blue) placed at input port of timer channel 2</td>
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<tr>
<td>t=132, PBS of timer 1 connects with switch port to wash cell chamber</td>
</tr>
<tr>
<td>t=203, DAPI of timer 2 connects after cell chamber has been finished being washed with PBS</td>
</tr>
<tr>
<td>t=210, DAPI finishes pumping through cell chamber</td>
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predictions using eqn (1) to verify the validity of the underlying, simplified model of fluid flow for use in the CPA design process. The experiments were carried out using a straight channel with dimensions of width \( \times \) height \( \times \) length [mm] = 0.40 \( \times \) 0.25 \( \times \) 30.0. A micropipette was used to fill the channel with fluid and subsequently place a 100 \( \mu \)l drop at the output port. In volumes of 10 \( \mu \)l, 20 \( \mu \)l and 30 \( \mu \)l at the input port, Fig. 3 compares the results of estimated pumping-time with the results of measured pumping time.

### 3.2 Immunostaining test for single timer channel CPA (sCPA) chip

In the following experiment, a sCPA chip was used to culture HEK 293 cells for 2 days and stain them using Hoechst dye. For comparison, cells were also cultured and stained in a plastic multiwell device (NUNC, USA, diameter: 1 cm) under the same conditions (see Section 2.2 for culture details). Representative bright-field and fluorescent images of the cultures and results of staining are shown in Fig. 4. The timing mechanism was able to delay washing by 60 \( \pm \) 3 seconds (\( \pm \)1 std. dev., \( n = 10 \)). The timer output droplet began at 30 \( \mu \)l (radius: 2.28 mm) and increased to 45 \( \mu \)l (2.62 mm) where upon the droplet connected with the fluid of the culture channel switch port and washed the stained cells with media.

### 3.3 Immunostaining test for dual timer channel CPA (dCPA) chip

The HEK 293 cells were cultured in a dCPA chip for 2 days after which the timing mechanisms of the chip were used to permeate, fix stain (DAPI), and wash the cells to prepare them for imaging. For comparison, we cultured HEK 293 cells in plastic multiwell plate (NUNC, USA, diameter: 1 cm) under the same conditions (see Section 2.2 for culture details), the results of which are shown in Fig. 5. The timer channel outputs grew from their original size of 30 \( \mu \)l (radius: 2.28 mm) until they reached a volume of 45 \( \mu \)l (2.62 mm), at which time the fluid would be connected with that of the adjacent culture channel switch port. The time between the placing of the timer input droplets and when they proceed to connect and flow through the culture channel was 132 \( \pm \) 3 s for timer channel 1 and 210 \( \pm \) 3 s for timer channel 2 (\( \pm \)1 std. dev., \( n = 10 \)).

### 4. Discussion

The sCPA and dCPA chips are examples of devices that use passive elements to provide laboratory users an added level of automation to a common manual task yet still only require a micropipette to operate. This type of “automation” is well-suited for repeated manual tasks and can help simplify and standardize laboratory procedures. The CPA chips can be easily adapted to a variety of common cell culture methods and procedures. Importantly, while PDMS was used to fabricate the devices here, we have previously reported passive pumping in non-elastomeric materials such as polystyrene,\(^{17,25,26}\) thus, for high volume manufacturing the CPAs could be made out of standard cell culture materials lowering costs and eliminating potential biases from PDMS.\(^{27,28}\)
Although the approach we have taken here can be applied to many applications, care must be taken when translating macroscale protocols for microscale devices. For example, one must keep in mind that adsorption and absorption can significantly affect concentrations of reagents and can require optimization to achieve similar results to macroscale assays. Similar considerations can also affect cell culture.30-32

5. Conclusions

In this study, “Cell Programmable Assay” (CPA) chips capable of culturing cells and automating the process of staining using surface tension-based pumping and passive timing elements were successfully designed and fabricated, and their performance was evaluated using HEK 293 cells. The single timer channel CPA (sCPA) chip was used to automate Hoechst staining (1 treatment and 1 wash step) while the dual timer channel chip (dCPA) was used to automate DAPI staining (2 treatment steps and 1 wash step). Moreover the calculated pumping times closely match experimental data allowing the use of a simplified equation for tuning or redesigning CPA chips to meet the needs of a variety of protocols that may require different timings.

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