Integrated Microfluidic Cell Culture and Lysis on a Chip†

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We present an integrated microfluidic cell culture and lysis platform for automated cell analysis that improves on systems which require multiple reagents and manual procedures. Through the combination of previous technologies developed in our lab (namely, on-chip cell culture and electrochemical cell lysis) we have designed, fabricated, and characterized an integrated microfluidic platform capable of culturing HeLa, MCF-7, Jurkat, and CHO-K1 cells for up to five days and subsequently lysing the cells without the need to add lysing reagents. On-demand lysis was accomplished by local hydroxide ion generation within microfluidic chambers, releasing both proteinacious (GFP) and genetic (Hoescht-stained DNA) material. Sample proteins exposed to the electrochemical lysis conditions were immunodetectable (p53) and are able to retain their enzymatic activity (HRP).

Introduction

Scientific progress is often associated with the invention of a new experimental apparatus. New tools can increase the ease and efficiency of routine experiments as well as provide the means to make new discoveries by making possible novel experiments. The development of Lab on Chip (LOC) devices is playing an important role in the progression of many different areas of research ranging from point of care diagnostics to the search for life on Mars [1, 2]. LOC devices hold promise to replace existing techniques with processes that are not only more automated and consistent but also require less time and valuable reagents [3].

Cultured cells are used in a variety of contexts ranging from drug development to synthetic biology. Microfluidic devices for cell culture studies offer numerous advantages over plate based cell culture and, because of this, are being increasingly used in laboratory settings [4, 5]. Microfluidic devices better mimic in vivo conditions by allowing for constant perfusion and 3D tissue-like structure [6-9]. Additionally, time and costs are reduced due to decreased reagent volumes and automated handling. Higher surface to volume ratios can also offer improved and novel detection schemes [10].

Existing microfluidic systems for cell-based lysate studies require the addition of lysis buffers and subsequent washing steps, increasing the complexity of such devices and reducing their ease of use [11-13]. We have developed an integrated microfluidic cell analysis system that allows for continuous perfusion cell culture with on-demand cell lysis. Lysis is achieved by applying a DC voltage to electrochemically generate hydroxide inside the device. This lysis method differs from other electrical lysis techniques [14, 15]. Rather than relying on high electric fields to electroporate the cells, electrochemically generated hydroxide ions permanently disrupt the cellular membrane by cleaving fatty acid groups, thereby releasing intracellular material.

By combining two BASICs (Biological Application Specific Integrated Circuits) previously developed in our lab, we introduce an integrated cell analysis package that minimizes the need for external reagents and manual procedures [7, 9, 16]. Here we demonstrate the practical use of this device by examining the culture and lysis of 4 different cell lines (HeLa, Jurkat, CHO-K1, and MCF-7). Additionally, we investigate the effects of this lysis technique on two biological molecules, Horseradish Peroxidase (HRP) and p53. HRP is an enzyme (derived from the plant of the same name) that is oft-used in molecular biology. P53, the 1993 ‘molecule of the year’, is a transcription factor that plays a central role in many cancer mechanisms [17]. We show that the immunodetection of p53 is not compromised by the lysis procedure within the device. The enzymatic activity of HRP is diminished as applied DC voltage increases. However, we also show that it is possible to lyse cells using a voltage with minimal effect on HRP enzymatic activity. Given the many applications that require a combination of cell culture and lysis, we believe this integration of microfluidic devices is a valuable advancement in the field of biological research and diagnostics.

Materials and Methods

Chip Design

The cell chambers were designed to have four fluid-permeable cell holding structures with 11nl of volume each (Fig 1): this equates to roughly 12,500 cells per trap and 50,000 cells per chamber. Each device has six chambers (Fig. 1a), and each chamber is individually addressable via polymer tubing connections. Independent lysing electrodes are placed on either side of the trapping region.
Filters were added in multiple locations to help keep debris from entering the trapping region as well as to break up cell clumps into individual cells during loading (Figure 1). We found the filters to work quite well at keeping out debris, but over time debris and cells would accumulate at the filters. This potential clogging was mediated by adding filters to the interfacing chip (Figure 2a) that was used to load the cells, as most of the debris-related problems arise during cell loading. After loading cells, media can be perfused through a different interfacing chip or directly into the device in order to bypass the occluded filters. The filter located upstream of the cell trapping region also improves cell loading by producing a more uniform flow profile and even loading [18].

Another structural component used was the high resistance region upstream of the cell culture/lysis chamber (Figure 1a). This component was added after initial devices developed leaks around the electrodes. The glass substrate and the PDMS device are unable to bond permanently wherever there is metal between the two. The high resistance region was added to reduce the pressure in the region where the metal lines enter the fluidic channels, which eliminated most leakage issues. These regions had the additional benefit of ensuring a more even loading from device to device when an interfacing chip was used to address multiple chambers simultaneously.

**Device Fabrication**

The microfluidic component of the device is cast in PDMS (Sylgard 184, Dow Corning) from a two layer silicon mold using standard soft lithography techniques [19, 20]. The PDMS trapping structures are separated from the glass bottom by approximately 2 microns (SU8 2002, Microchem), and the rest of the channels are approximately 40 microns high (SU8 2035, Microchem). The spacers (Fig 1b) help prevent the traps from bonding to the glass as well as reduce the risk of releasing trapped cells due to fluctuations in pressure. All photolithography was performed in the UC Berkeley microfabrication facility. The inputs of the chip were formed with a mechanical punch (Technical Innovations). The chip was permanently affixed to a glass slide by exposing both components to oxygen plasma at 1 Torr and 50 Watts for 10 seconds (Plasmod, Tegal Inc.) [21]. In some cases, the chips were bonded to a plain glass slide when cell culturing was the sole purpose of the chip. In most cases, the chips were bonded to a glass or pyrex slide with patterned metal (see below). The alignment of the chip to the patterned slide was done by hand under a dissecting microscope (Olympus SZX7). This manual alignment method is less than ideal, but efficiency was high (>95%) due to steady hands and some built in tolerance for misalignment. In most cases, an interfacing or multiplexer chip was used to split a single line into multiple lines so that multiple devices could be used simultaneously (Figure 2a). In other cases, tubing (Cole Parmer) was used to directly connect the device to a syringe pump (74900, Cole Parmer).

A simple lift off process was used to pattern the metal electrodes onto either a pyrex wafer or a standard 2” x 3” glass slide. A positive photoresist (S1818, Shipley) was lithographically patterned onto the substrate, the metal was deposited using an electron beam evaporator (Edwards EB3), and the photoresist was stripped in acetone under sonication. Both gold (with a chrome adhesion layer), and platinum (with a titanium adhesion layer) were tested. Adhesion and metal layer thicknesses ranged from 20-30 nm and 60-150 nm.
respectively. The pyrex wafer or glass slide was then cut into four separate devices using either a resin blade (Automatic Dicing Saw, Disco) or tungsten wheel glass cutter (Fletcher Framing Supply), respectively.

A simple platform was created in acrylic to hold the chip, secure the tubing connections, and make electrical contacts to the patterned electrodes (supplemental Figure 1). A CO2 laser (Versalaser, Universal Laser, Inc.) was used to fabricate the platform. The electrical connections were made with a modified plastic leaded chip carrier test socket (Zif, Digi-Key).

**Cell Loading, Culture, and Lysis**

All cell lines (HeLa, MCF-7, Jurkat, and CHO-K1) and the appropriate media were procured through the UC Berkeley cell culture facility. All media was supplemented with 10% of FBS and 1% of both penicillin and streptomycin. Petri dishes (either 100 mm or 6 well plates) with cells 50-90% confluent were trypsinized (0.25% Trypsin EDTA, Gibco) under sterile conditions. If necessary, cells were spun down and concentrated before being loaded into a disposable 3ml syringe (Becton Dickenson). Typical concentrations used ranged from 1 to 5 million cells/ml.

The chip was prepared for use by first flushing the chip with isopropanol, followed by roughly 3 ml of PBS buffer. During the PBS wash the devices were viewed under the microscope to assure that the device was free of air bubbles before cell loading. A four way stopcock (EW-30600-04, Cole Parmer) was used with two syringes: the cell solution syringe and a media syringe (Figure 2a). Loading was observed under a microscope, and performed either by hand with a syringe or with a syringe pump. When using a syringe pump, cells would tend to settle in the bottom of the syringe and not make it into the device. To alleviate this problem, a small stainless steel ball bearing was sterilized and placed inside the cell syringe prior to loading. This ball bearing could be rocked back and forth manually or with an external magnet, which acted to keep the cells mixed and suspended. We used this technique with great success, and believe this ball bearing tactic could be useful for many other microfluidic applications [22].

**Fig. 2** Cell loading. (a) Schematic of how the chip is loaded using a four way valve connected to a syringe with cells and a syringe with media or buffer. (b) Images taken from a movie of cells loading into the traps. Flow rate was 80 µl/min and cell density in the syringe was 3.02 x 10^6 cells/ml. Scale bar is 150 microns.

**Fig. 3** Cell culture. (a) Schematic of setup for cell culturing experiments. (b) Live/dead stain of HeLa cells at Day 5. Scale bar is 75 microns.

**Fig. 4** Images of breast cancer cells (MCF-7) cultured inside a device. After 72 hours, the cells have formed spheroids. Scale bar is 75 microns.
Once the cells were sufficiently loaded, the stopcock was switched over to the media syringe. The cell syringe was discarded, and media was perfused through the device at a flow rate of 1-2 µl/min for each chamber. In most cases, the device was placed inside of an incubator (Shel Lab 2310, VWR), with tubing connected to a syringe pump located outside of the incubator (Figure 3a). In other cases, the device was rested on an heated microscope stage (WIS1, Carel) to observe time lapse cell growth (Figure 4). Cell viability was determined with a live/dead assay (L3224, Invitrogen). A solution of 8 µM Calcein AM and 2 µM Ethidium homodimer-1 was pushed through the device at a flow rate of 25 µl/min for approximately 20 minutes. Fluorescent images were captured with an inverted microscope (TE-2000, Nikon) and Digital camera (Q-fire, Q Imaging). Time lapse images of cell culture were captured using a small inverted microscope (MIC-D, Olympus).

Electrochemical lysis was generated by applying a DC voltage across the electrodes on either side of the trapping region (Figure 5a). In most cases, the media syringe was replaced with a syringe filled with PBS buffer, and the buffer was pushed through the chip at a flow rate of 7.5 µl/min. A DC power supply (Agilent E3611A) provided the voltage. Current measurements were made with a multimeter connected in series with the circuit (HP 972A). Lysis was confirmed in various ways. Videos were taken of the cells during lysis using brightfield microscopy (Figures 5b, 6a, and supplemental videos 4-6). Additionally, cells were fluorescently stained and videos were recorded (Figure 6b and supplemental videos 7-8). For the results shown in figure 6b (and supplemental video 8), we used CHO-K1 cells stably transfected with Clathrin-GFP (obtained with permission from the David Drubin lab at UC Berkeley). Additionally, the genetic material of these live cells was stained using Hoescht (33342, Invitrogen) at a concentration of 5 µg/ml for 30 minutes before trypsinization. Additional HeLa cells were transiently transfected with a GFP expression plasmid using FuGENE 6 (Roche) so that the cells would express intracellular GFP. These cells were also loaded and observed under a microscope during lysis (supplemental video 7).

Lysate Analysis

To investigate the biological viability of molecules subjected to the electrochemical lysis conditions within the device, two proteins were passed through the device at different voltages, collected, and then analyzed off chip. A solution of Horseradish peroxidase (HRP, Sigma) in PBS (30 µg/mL) was flowed through the device at a flow rate of 7.5 µl/min under varying voltages. This solution was then serially diluted. 50 µL of each dilution was combined with 50 µL of TMB (3,3',5,5'-tetramethylbenzidine) in a 96-well plate. Color was allowed to develop for 20 minutes at which time optical density (OD) measurements were taken for each well using a microplate reader (BioRad Model 680) set to 405 nm. A 32 ng/ml stock solution of p53 (R&D Systems) was prepared and pumped through the device at a range of voltages. Once through the device, this solution was serially diluted, and an off-chip ELISA was performed as to specifications (DuoSet IC Human Total p53 Sandwich ELISA).

Fig. 5 Characterization of electrochemical cell lysis. (a) Schematic of electrochemical lysis via hydroxide generation at the cathode upstream of the cell traps. (b) Images taken from video of cells being electrochemically lysed in the region outlined in red in (a). Scale bar is 150 microns. (c) Percentage of trapped cells lysed over time under different voltages.
Kit, R&D Systems, Catalog Number DYC1043-2). For both the HRP experiment and the p53 experiment, two negative controls were tested: 1) an aliquot of either HRP or p53 solution that was passed through the device with no voltage applied, 2) an aliquot of either HRP or p53 solution that was never passed through the device.

During loading, the majority of the cells go around the traps, taking the path of least fluidic resistance. Based on a 3D numerical simulation (Comsol Multiphysics), the trapping efficiency (quantified as the ratio of flow in the trap to that outside the trap) begins at approximately 1%, decreasing to 0.2% as the trap fills (Supplemental Figure 2). If working with a small number of cells, this would be an obvious disadvantage. However, for immortal cell lines – which are abundantly available – trapping efficiency is not an issue. This low trapping efficiency is inherent to the design and is a result of the high fluidic resistance through the trap, which increases as the trap fills with cells. When cells are fully loaded, the flow rate through the cells is negligible. As a result, diffusion is the dominant mechanism for supplying nutrients to the cells and removing waste [7]. Not only does this mimic many tissues in the body, this also helps prevent disruption from bubbles. Care is taken to avoid bubbles from entering the device, but many times they are inevitable. When small bubbles did enter the device, they also took the path of least resistance around the traps and left the cells unperturbed.

Results and Discussion

Cell Loading

To quantify cell loading, videos were recorded of cells loaded into devices under constant flow rates. Syringe pumps were used to maintain constant flow, and the ball bearing tactic (see methods) was used to ensure even cell loading. A characteristic result is shown in Figure 2. Flow rates used varied between 5 and 100 µl/min, and complete cell loading usually occurred within five minutes. The Jurkat cells were the only cell line tested that could not be loaded to full capacity; as Jurkat cells are smaller than the adherent cells tested, the Jurkats would begin to slip under the trap as it was filling. However, even with the traps only partially filled, Jurkat cells were cultured successfully, and filled the trap after a few days. Cell loading videos can be seen in the supplemental material (supplemental videos 1-2). A Matlab script (Mathworks) was employed to analyze the videos and quantify the loading process (supplemental figure 3).
7 cells begin to form spheroids after 72 hours of culture (also see supplemental video 3), which indicates good cell viability.

Cell Lysis

The dominant mechanism for cell lysis within our device is the cleaving of fatty acid groups of cell membrane phospholipids by hydroxide ions that are electrochemically generated at the cathode upstream of the cell chamber [16]. Higher voltages, to some extent, provide faster production of hydroxide. However, increasing the voltage above 3V did not seem to have a significant decrease in lysis time (Figure 5c). We attribute this to degredation of the electrode and side reactions that begin to take over. (Incidentally, the use of titanium as an adhesion layer instead of chromium provided for much more robust electrodes). Additionally, the generation of hydrogen bubbles can cover the electrodes and decrease hydroxide production. Visible accumulation of bubbles at the electrodes tended to cause a decrease in current. At voltages lower than 3 volts, with flow rates of 7.5 µl/min, hydrogen bubbles are not generated and lysis proceeds efficiently. Simulations suggest that there is potential to optimize the lysis further by either increasing lysis voltage or decreasing flow rate (Supplementary Figure 2). As mentioned, increasing voltage too high may cause bubble formation on the electrode, and decreasing flow rate too low could insufficiently feed the cells or even allow backflow of hydronium ions from the downstream electrode.

The time to lyse observed under the experimental conditions used was on the order of 5 minutes. Simple brightfield microscopy was used to observe lysis. Upon lysis, cells seem to disappear as the membrane is compromised and the intracellular material is replaced by buffer with the same refractive index as the surrounding environment (Figures 5b, 6a, and supplemental videos 4-6). The inherent optical access of our device allows for visual insight into the mechanisms of this electrochemical lysis. As the cells lyse, severe blebbing occurs until the membrane is completely compromised and the cell is completely lysed, as defined by the intracellular material being no longer physically contained.

Biological Activity of the Lysate

By using fluorescently labeled materials we investigated the location of intracellular proteins, genetic material, and membrane-bound proteins before, during, and after lysis. Cells transfected with GFP (intracellular proteins) were loaded and lysed (supplemental video 7). Observation of the fluorescent material confirmed that intracellular proteins will leave the cell upon lysis. We also observed the lysis of stably transfected Clathrin-GFP cells in which we stained the genetic material using a live DNA stain (Hoesht 33342) (Figure 6b and supplemental video 8). These experiments confirmed that genetic material and membrane bound proteins were present in the lysate. However, not everything comprising the cells moves downstream with the lysate. Leftover material can be seen within the traps; we believe this leftover material is likely insoluble cellular debris.

In order to gauge which assays would be best suited for possible integration with our device, we have analyzed the effect of electrochemical lysis on two proteins: HRP and p53. HRP was chosen to show the effects of this electrochemical lysis on enzymatic activity, and p53 was chosen to study the effects of this lysis on immunodetection. The results of this analysis of HRP and p53 are shown in Figures 7 and 8, respectively. The immunodetection of p53 was shown to be completely unaffected by the lysis conditions created within the device. The enzymatic activity of HRP, however, was
compromised. HRP exposed to a voltage of 2.5 volts within the device had ~78% of the activity of HRP exposed to 0 volts within the device. This suggests that this method of lysis could be used to produce lysate that is largely active for downstream enzymatic assays.

Conclusions

We demonstrate the functionality of an integrated microfluidic cell analysis platform for culturing and electrochemically lysing cells on demand. Cells exposed to the lysis conditions released both protein and DNA. Proteins exposed to the lysis conditions maintained significant biological activity and were able to be immunodetected; this demonstrates that it is possible to lyse cells with electrochemically generated hydroxide without compromising downstream lysate analysis. We believe this device has great potential as a tool for cell-based analysis. We are currently developing a novel on-chip assay in order to prototype a completely integrated cell analysis lab on a chip device.

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Notes and references

† Author contributions. JTN: device design and fabrication, experimental design and execution, data analysis, manuscript writing. RC: device design and assembly, experimental design and execution. MD: experimental design and execution, manuscript writing. DNB: finite element simulation. LPL: experimental design and manuscript writing.