DISPOSABLE VS REUSABLE: PRACTICAL ASPECTS FROM END USERS AND REUSABILITY TEST OF THE BIOCHIP FOR ELECTROSTATIC MICROWELL-BASED SINGLE CELL TRAPPING AND CULTURING

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Abstract

Microfluidic technology has come in handy in single cell studies. Recently we have successfully constructed an alternative on-chip platform for single cell trapping and culturing using an electrostatic microwell trap. Although this type of device is considered disposable, it is of a great interest if, in practice, the biochip could be used multiple times to reduce cost. Here, we report our and our end users' experiences in attempts to reuse the device, as well as the experimental results to demonstrate the extent of the reusability. It is found that flushing the device vigorously with water is an effective means to purge away trapped cells from the biochip. Autoclaving is a possible method to decontaminate the device. However, the single cell capturing efficiency of the biochip slightly declines but does not significantly deteriorate with the number of times it is repetitively autoclaved. The percentage of the single cells trapped goes down from 28.5 to 25.0 and 21.3, after sterilizing 5 and 10 times, respectively. It cannot be generally concluded as to whether the device is reusable. For instance, cleaned devices may acceptably be used in a classroom demonstration and for preliminary studies but perhaps not for experimental research. A further long term study on the biological effect on cell culture in a reused device may be required.

Keywords: Electrostatic microwell based biochip, single cell trapping, microfluidic device, reusable microfluidic device, algae

Introduction

For decades, microfluidics has been involved in modern biological studies. The technology employs a variety of physical phenomena to manipulate nano-picoliter scale liquid and the

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particles inside in networks of microchannels. Application of the technology such as 'lab on a chip' allows numerous multiple biological experiments such as biomolecules detection, separation of proteins, and cell culture to be conducted in a small area using much less of an amount of a sample and time compared to the conventional methods (Beck and Goksör, 2012).

One of the applications in which microfluidics has become of great use is single cell studies (Weibel *et al*., 2007). In the past, tracking the biological phenomena of single cells, especially of those of the non-adherent nature such as phytoplankton, was nearly impossible with conventional microbiological techniques (Brehm-Stecher and Johnson, 2004). Ability to handle and grow single cells in a controllable culture environment permits scientists to monitor the cell activities in real time and the behavioral inhomogeneity of individual cells among the population (Pan *et al*., 2011; Dewan *et al*., 2012). These pieces of information are highly crucial as they provide an insight into the incomplete story of cell biology and underlay a vast spectrum of current biological studies such as evolution, ecology, environmental biology, biotechnology, and the utilization of microbes.

Recently, we have successfully fabricated an electrostatic-based biochip for single phytoplanktonic cell trapping and culture (Kuntanawat *et al*., 2014). It is basically a transparent cell culture chamber of a microscope slide size with an array of 200 microwells (width × length × depth of 1×1×0.5 mm) at the base of the device. The cells are electrostatically attracted to and captured in the wells by the positively charged bottom of the wells.

Unlike other previous designs in which cells are captured by some of the following techniques, such as channel constrictions (Männik *et al*., 2009), microdam (Yang *et al*., 2004), electrodes (Suscillon *et al*., 2013), vacuum (Zhu *et al*., 2012), and droplet-based encapsulation (Clausell-Tormos *et al*., 2008; Dewan *et al*., 2012), the biochip employs an electrostatic microwell mechanism. One of the advantages of the invented approach is that it allows trapping and long term culturing of phytoplanktonic cells of different sizes and shapes. As demonstrated by our users, it is also possible to trap and culture motile species of phytoplankton such as Volvox using the biochip. This implies that the invention could also be applied in the study of zooplankton and other motile microorganisms.

In addition, well-defined chemical gradients can be added or removed from the device at any time allowing different modes of culture with a variety of cell treatments with chemicals to be performed.

Besides its simplicity and versatility in terms of operation, the straightforward design makes fabrication and assembly of the parts of the device possible in any general laboratory with a minimal skills' requirement. These advantages together with its potential in single cell studies of various organisms attract our potential users and increase demand for the device.

Currently, our laboratory offers a small number of supplies of the biochip to our collaborators. With this kind of partnership, it is possible for us to assess the feasibility of (parts of) the device and the practical problems in different real situations. This information is crucial for the larger scale production and commercialization of the device to which we are looking forward.

One of the users' reflections is regarding the reusability of the device. The biochip is initially intended for single use similar to other polydimethylsiloxane (PDMS)-based biochips for cell culture. Although the theoretical production price of the device is affordable to the users, reusing the device would potentially reduce the cost per experiment. Reusability of the device is, therefore, an interesting aspect to study.

Besides glass, silicon-based PDMS is another frequently used material with excellent physical and chemical properties for fabrication of biomedical and biological devices (McDonald and Whitesides, 2002). Considering its mechanical property and its chemical inertia, the material is generally durable for multiple uses in microfluidics and micromolding. In fact, PDMS can be used in

microcontact printing where a piece of micropatterned PDMS is repetitively used as a stamp to transfer molecules of interest such as proteins, DNA, water, metal, and even cells to a target surface (Ruiz and Chen, 2007; Wang *et al*., 2012). It was also demonstrated that PDMS can be made as a microarray spotter that can be reused (Lamberti *et al*., 2015).

In reusing a PDMS-based device for cell culture, sterilization of the device before the second use is of great concern. In theory, this is possible since it has been shown that well-known methods of sterilization such as exposure to UV, soaking in ethanol, and autoclaving do not alter the material's favorable properties (Skaalure, 2008). In fact, autoclaving was a practical means to sterile the device even before its use in cell cultivation (Kim *et al*., 2007). One particular study showed that a PDMS-based microfluidic device for mesenchymal stem cells separation can be reused after ethanol rinsing and treating with oxygen plasma (Geng *et al*., 2011).

From previous studies, it can be concluded that PDMS can be reused in work in which the material is in contact with cells and biomolecules. However, we could not find in the literature any previous attempt to reuse a device that is really for cell culture, or more strictly planktonic cell culture. The positively charged glass slide, the base on our device, makes the PDMS-based device even far more delicate than other devices made purely of PDMS since improper sterilization may destroy the modified surface.

Considering the reuse of the device, there are 4 aspects to take into account which are: the possible ways to dislodge the captured cells, the method to sterilize the used biochip, the cell capture ability after reuse, and the optics (transparency) of the used device. In other words, as long as the trapped cells can be washed away from the wells and the device can be thoroughly sterilized without reducing the cell capturing ability and the optics, the device should be capable of multiple reuse.

In this paper, we report the experiences of our group and other users (more than 5 users in total) in experimenting with ways to prepare the used device for reuse. Different procedures to dislodge the fixed cells from the electrostatic traps are discussed. In addition, we also report our extended experiment on the effect of cyclic sterilization of the device by conventional autoclaving to the cell capturing and optics of the device. The extent to which the device could be reused is discussed at the end.

Materials and Methods

Design and Fabrication of the Device

The detailed design and construction of the device are given in our previous publication (Kuntanawat *et al*., 2014). In brief, the device is composed of 3 layers which, from bottom to top, are a positively charged microscope slide, a 500 mm thick microwell-patterned PDMS sheet, and the fluidic layer containing a diamond shaped flow channel. The formation of this device creates a cell culture space with 200 electrostatic microwells ($1000 \times 1000 \times 500$ μm; width × length × depth) that acts as the cell trap.

Figure 1 displays the schematic cross-section of the biochip. The cell can be loaded through the channel as well as the culture medium. Suspension is discarded through the outlet.

Fabrication of the PDMS layers (the micropatterned layer and the fludic layer) was done using standard soft lithography. The micropatterned layer was made using the metal mold in Figure 2(a)-2(b)obtained from the s tandard X-ray lithography technique. The acrylic mold with the pattern of a diamond shaped island was used for casting the fluidic layer. The seal between the PDMS-glass contacts was achieved with prior plasma treatment of the PDMS layer before sticking onto the microscope slide. An additional PDMS liquid was used to fill the gap between the micropatterned and fluidic PDMS layers. An image of the finished device is shown in Figure 3.

Cell Culture

Spirulina platensis (Figure 4(a)) and P*ediastrum simplex* (Figure 4(b)) were the model organisms in this study. The phytoplankton were isolated by the Applied Algal Research Unit, Department of Biology, Faculty of Science, Chiang Mai University, Thailand. JM medium

and Zarrouk's medium were used to culture *S. platensis* and *P. simplex,* respectively. Preparation of these media are described elsewhere (Thompson*et al*., 1988; Zarrouk, 1966). The cells were cultured at 25-30°C and under fluorescent illumination of about 4 klux.

Cell Loading

The enclosed fluidic channel of the device was treated with a stream of 70% ethanol, sterile reverse osmosis water (RO water), and sterile cell culture medium. The serial flushing sterilizes and removes the air pockets trapped inside the microwells. After that, cell suspension (either 200 µl of 2500 filaments/ml or 500 µl of 1000 colonies/ml, for *S. platensis* and *P. Simplex,* respectively) was introduced to the device using a syringe pump (NE-1000, New Era Pump Systems Inc., Farmingdale, NY, USA) or by hand at an average flow rate of 200 µl/min. In the case of, at least, *P. simplex,* it was found that hand injection also gave a satisfactory result. To purge away the remaining cell suspension, the sterile cell culture medium was injected into the device until all the old liquid was totally replaced. Cell suspension could be discarded at the higher flow rate of up

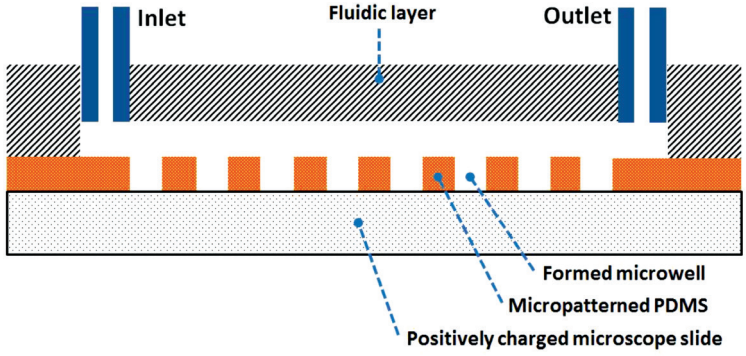

Figure 1. Schematic diagram of the biochip is shown. The layering of the positively charged microscope slide, the micropatterned PDMS and the fluidic layer creates the cell chamber with electrostatic microwell traps. The flow of the medium of cell suspension is introduced through the inlet. All the liquid can be discarded though the outlet

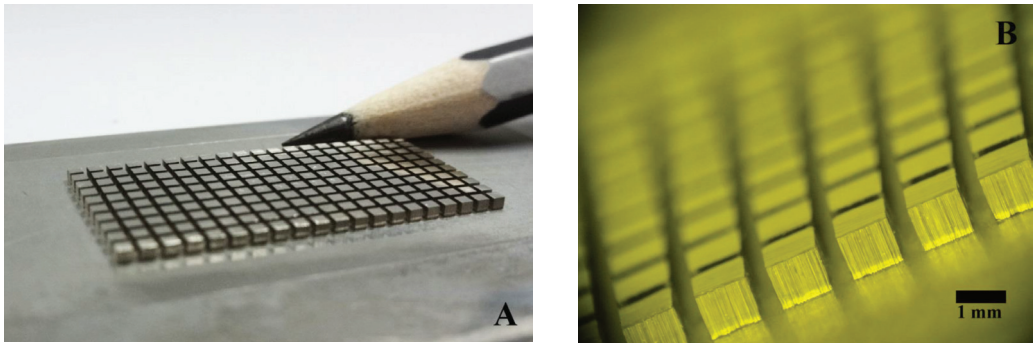

Figure 2. Micromold used in fabricating the micropatterned layer of the device is displayed (A). This Nickel micromold was obtained with the X-ray lithography technique. The close-up image of the micropatterned area reveals the well-defined rectangular structures that give the microwell pattern to the casted PDMS (B). The scale bar represents a length of ~1 cm

to 900 µl/min.

Procedures for Dislodging Fixed Cells from the Device

Variations of the techniques to flush away captured cells in the device and their efficiency are to be discussed in the Results and Discussion section. However, in general, either 70% ethanol or sterile RO water was used as an injection medium in the dislodgement. The alternatives involved submerging the ethanol-filled devices in the sonicator bath.

Cyclic Sterilization of the Device and Quantification of Cell Loading Efficiency

After cell loading, the number of microwells trapping a single cell in the newly fabricated device was counted under an inverted microscope. The device was then subjected to the cell dislodging process until all the captured cells were flushed away. The thoroughly flushed device was put in a plastic bag and sterilized with a standard autoclaving procedure (temp = 121°C, pressure = 15 psi above atmospheric pressure, time = 15 min). This cyclic sterilization was repeated 10 times. The single cell capturing ability of the device was monitored after autoclaving 5 and 10 times.

Results and Discussion

Efficiency of Cell Dislodging Methods

We found that there are 3 ways to dislodge the attached cells. The first and the most effective way is to peel of the top layer (fluidic layer) of the device and then inject RO water vigorously several times onto the exposed microwells. The device is tilted to let the rinsed water run down into the waste container. We tried this method with the attached *S. platensis* in the wells and found that it worked effectively. All

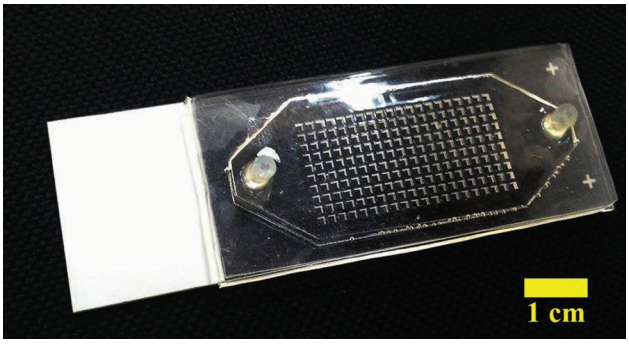

Figure 3. The image of the fabricated biochip is shown. The device is microscope slide size and clear for cell observation through a microscope. The scale bar represents a length of ~1 cm

Figure 4. *Spirulina platensis* (A) and *Pediastrum simplex* (B) are the model organisms used in the cell loading and dislodging experiment. Scale bars represent lengths of ~100 and ~10 *µ*m in (A) and (B), respectively

the attached cells can be washed away in a couple of minutes.

As the fluidic layer of the device must be detached to perform this procedure, this method is therefore suitable for users who find that they are able to reassemble the device. Although sealing the top layer back onto the device is not too technically difficult, untrained end users are not comfortable in handling this process.

As a result, alternative flushing techniques were developed. The second method to dislodge the cells is simply done by manually injecting into the device either RO water or 70% ethanol. Ideally, the more forceful the injection, the better the cell detachment should be. However, water injection that is too strong may either deform the device or make it leak. It was found that the optimum average water injection rate was around ≤5 ml/min. Figure 5 shows an image of a successful cell flushing using the procedure. The experiment done with *P. simplex* showed that injection of the device with ≤5 ml RO water/ethanol 5 times, is usually sufficient to detach all living cells in the wells. It took around 5 min to clear up the device with this procedure.

However, it was realized that the major trouble in cleansing the disclosed cell chamber and wells is the cell debris. Some algal species like *P. Simplex* asexually reproduce via autocolony production. Basically, protoplast of the parental cell undergoes division generating multiple numbers of zoospores inside the cellular cavity. These later arrange themselves into a colonial organization. Once matured, these autocolonies are released from the cell, leaving only the empty parental cell's exterior.

The following may explain why the debris is hard to get rid of. Without containment, the leftover 3D membrane structure may be collapsed and flattened by the electrostatic pull down. The debris thus becomes even more tightly attracted to the surface. Trying to move this flattened structure is similar to attempting to lift up a piece of sticky paper which is laid on a floor with an air blower. The lower thickness minimizes the interaction between the leftover cells with the dislodging flow. Experimental tests suggest that even after flushing 10 times with the abovementioned procedure, the cell debris was still stuck in the wells.

We later found that in order to wash away this debris it is necessary to enhance the detachment with ultrasonic application. This is the third method to dislodge the attached cells. Prior to performing the regular flushing, the device was filled with either ethanol or RO water

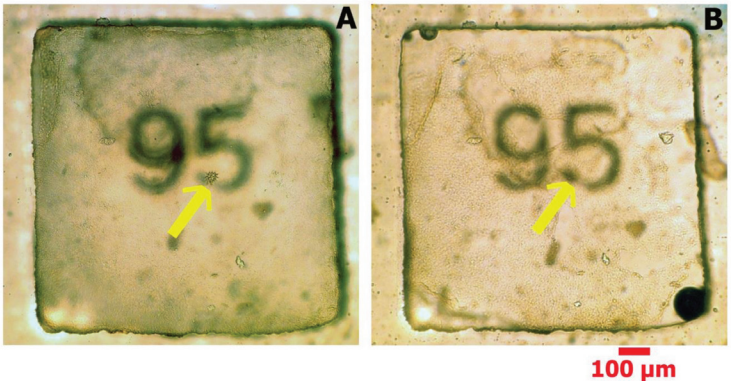

Figure 5. The microwell was capable of trapping a single P. simplex colony after cell loading (A). The arrow indicates the location of the colony. The number '95' is the running number of the well patterned at the other side of the base microscope slide. After flushing the disclosed fluidic channel with the developed protocol, the cells could successfully be detached and purged away from the device. (B) displays the image of the exact same microwell as (A) but with the cell washed away with the flush. With the number of times of reusing the device, the base of the microwell became less smooth, as seen in the pictures. The scale bar represents a length of 100 μm

before transferring to an ultrasonic bath. The device was left inside the ultrasonic application for 10 min before being brought back to the RO/ethanol injection. Cell debris was found to be removed with this procedure.

Taking the cell shape into consideration, these two species of model organisms represent two different types with different levels of difficulty to dislodge: the easy *S. platensis* and the harder *P. simplex*. When sitting in the wells, the spring-like *S. platensis* cell's contact to the electrostatic glass surface is therefore much less in comparison with the *P. simplex* that has a planar shape, almost like a thin coin. The attracting surface area/volume of the latter cell is therefore much greater. In addition, the larger and elongated filament of *S. platensis* should also be moved more easily by the stream of water compared with the circular *P. simplex*. In fact, across the microalgal species, *P. simplex* is probably one of the toughest in terms of dislodging. It is one of a type that is relatively small in size and has a radially symmetrical thin coin shape. Therefore, it is a good model to test the effectiveness of the dislodging protocol. In other words, the methods that appeared effective with *P. simplex* should also work well with the majority of the others.

Optics of the Used Device

The device, after flushing of the cells, was found to be fairly transparent and clear. Although dust-liked particles were found to have accumulated with the number of times the device was used, the wells area was clear enough for cell observation and imaging, as seen in Figure 5. The optics of the device remained acceptable to users for at least 5-10 times of reuse.

Cell Capturing Efficiency of the Device After Sterilization

Normally, the inside of the newly fabricated device is sterilized by rinsing with 70% ethanol. However, in this experiment, the used devices were sterilized with standard autoclaving. As it is exposed to biological substances, a used device is likely to accumulate contaminants. Therefore, in this case, autoclaving was considered a more proper technique to use with a used device.

After the device was autoclaved, its cell capturing efficiency was slightly decreased. Figure 6 displays the decline in the efficiency of capturing a single cell with the number of times the device is autoclaved. Based on the experiment with 3 different devices (n=3), the

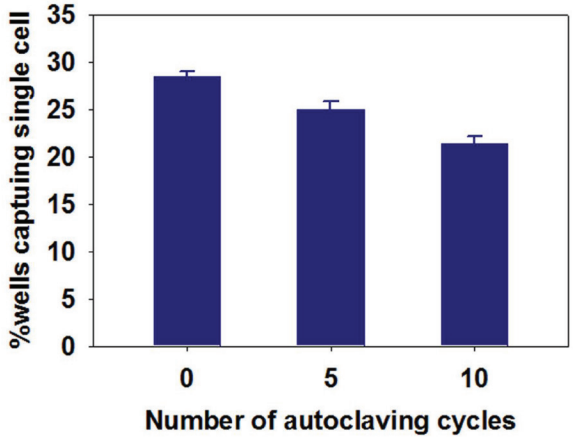

Figure 6. The percentange of single cell capturing of the device declined with the number of times the device was sterilized with autoclaving. Based on 3 different devices (n=3), after autoclaving 5 and 10 times, the percentage of single cell capturing dropped from 28.5±0.5 to 25.0±0.9 and 21.3±0.8, respectively. Bars with error bars display means±SD. The differences among the means are statistically significant ($p<0.001$)

average percentage of microwells that successfully captured a single *S. platensis* filament went down from 28.5±0.5 to 25.0±0.9 and 21.3±0.8, after 5 and 10 times of autoclaving, respectively. The reductions in the capturing efficiency were statistically different ($p<0.001$).

We hypothesize that the reductions in the capturing efficiency after the cell was detached and autoclaved were caused by 2 different factors. First, every time the device is reused there is a certain amount of particles such as cell debris and chemical precipitates that, perhaps, are negatively charged and electrostatically stuck in the wells. The tinier the particles, the greater the electrostatic attracting surface area/volume gets, and so the more firmly they stick to the base. It is therefore impossible to clear them. These unavoidable trapped particles not only caused the poorer optics of the device, but also competitively interacted with the charged surface of the device weakening the attraction between the cells and microscope slide surface.

Interestingly, it was reported by a user that a repetitively used device, even without autoclaving, has a lower capturing efficiency with the length of time it is used. This probably highlights the effect of the debris accumulation in lowering the cell capturing capability.

Secondly, wet heat under high pressure may also alter the chemical structure of the charged molecule on the microscope slide surface. The altered chemical structure may lose its capability to attract cells as a result. However, it is not clear if this hypothesis is relevant because the functional chemical species on the microscope slide surface is not known – it is not revealed by the manufacturer.

According to our findings, the device's efficiency (i.e. capturing capability and optics), was clearly reduced by the treatment. The next question would be if this device is still considered reusable. The answer is very subjective. It really depends on whether the users find the rate of decline in both optics and the cell capture capability acceptable. So far, we realize that our end users do. In practice, the increasing flaw does not appear significant enough that it disturbs observation in the experiment. In fact, based on experience with 5-6 devices, one of the users reflected that, by using methods 2 and 3 (rinsing and ultrasonication + rinsing), it is possible to reuse the device up to 5-10 times.

Although there have not been any reports regarding reusing the device in the cell culture experiment, a further study on the biological effect of culturing cells using the used device would let the reusability of the device to be discussed to a greater extent.

In addition to the protocol explained here, the use of bleaching agents and detergents to enhance removal of the attached cells and debris should be a topic of further study. However, the reverse effects of the treatment must be thoroughly investigated. If the bleach is too concentrated, it may destroy the modified chemical surface of the positively charged microscope resulting in reduction of the cell capturing efficiency. Using a detergent containing phosphate should be a concern as the remaining phosphate could change the growth pattern of the algae.

Conclusions

It is possible to dislodge the attach cells from the biochip: rinsing the exposed microwells with RO water/ethanol, injecting several times the enclosed device with RO water/ethanol, and ultrasonic bathing of the device filled with RO water/ethanol and followed by the RO water/ethanol injection. All the procedures are suitable for dislodging microalgal cells in general. The third protocol is better for removing dead cells.

The cell capturing efficiency and optics (transparency) of the device slightly decline with the number of cycles of cell loading-dislodging and sterilization. However, the majority of the end users find the drop in efficiency acceptable and that it does not significantly interfere with the experiment.

A longer term study of the effect on the cells of reusing a used device should be conducted. However, an adverse effect of such has not been observed in experiments by the users. In practice, a repetitively reused device is acceptable for use in long term algal cell culture experiments.

According to the available evidence, it

could be rather subjective to label the biochip either disposable or reusable. However, at this stage the biochip may be considered reusable and confidently used at least in a number of certain applications such as classroom demonstration and preliminary experiments.

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