

A directly stackable microsystem onto the cultured cells for cytotoxicity tests

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Abstract Today's pharmaceutical industry is facing several challenges resulting from a vastly increasing number of samples through a high-throughput screening. In addition, the increased demand for cytotoxicity tests have caused bottlenecks, which in turn is causing serious problems. Here we present a novel approach to performing the cytotoxicity test. This new method uses a directly stackable microsystem on the cultured cells. It also enables us to perform cytotoxicity tests with more reliability by providing exactly the same cell-culture environment for all experiments. The new approach consists of two fascinating modules: First, a serial dilution module can linearly dilute one drug solution into several diluted ones in a serial manner, and equally distribute them into independent microchambers. Secondly, a microcompartment module can firmly attach itself onto any cultured cells and divide the directly-covered cell surface into multiple well-type microchambers instantaneously. This microsystem has a strong feasible advantage. It hardly needs to modify the established cell culture protocols, and at the same time it can eliminate some repetitive and laborious processes. A quick flexible integrated microsystem would reduce many redundant efforts during on-chip cytotoxicity tests.

1 Introduction

Pharmaceutical companies spend more money on compounds that subsequently fail because of their toxicity in another area. Traditional methods are too slow, labor-intensive and far too costly to meet the current increasing demands of optimization and thus *in vitro* cytotoxicity testing is very important (Slater 2001). The fundamental principal behind the cytotoxicity test is that cell is killed by a toxic drug treatment and by conducting this test (Freshney 2001). In recent decades, the measurement of cell proliferation and cell viability with a special drug treatment has been of a great importance and has become a key research topic of life science and biotechnology. Despite its importance, the conventional cytotoxicity testing has several problems that has a fundamental inefficiency in that it uses repetitive procedures still being used. Recently, we developed a serial dilution microchip which could make a serial dilution and then dispense drugs into multiple microchambers accurately (Bang et al. 2004). In addition to that, we devised a reversible bonding technique, called active sealing, enabling a soft polymer (elastomer) microchip reversibly to be attached onto another plate (Bang et al. 2006).

In the present study, we integrated these techniques into a multi-layered poly (dimethylsiloxane) (PDMS) microsystem which can easily perform the cytotoxicity test on a single petri dish. Interestingly, this device can stick firmly onto the cell cultured surface and make the surface to instantly divide itself into multiple well-type microchambers. More interestingly, this approach can dilute a drug solution in a serially increasing manner and equally distribute each diluted solutions into several independent microchambers. In our experiment, the overall procedure was regulated by the sophisticatedly designed

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microchannels (Chang et al. 2003a, b). Subsequently, we performed a reliable on-chip cytotoxicity test under the same cell culture environment, and also conducted many rapid, easy experiments without modifying the established culturing protocols in a more reproducible manner.

2 Materials and methods

2.1 Preparation of cell line and cytotoxic material

To evaluate on-chip cytotoxicity tests, we obtained the human lung carcinoma cells, HeLa cell line from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained at 37°C in Dulbeccos modified Eagles medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U l⁻¹ penicillin, and 100 mg l⁻¹ streptomycin (GIBCO BRL, Grand Island, NY, USA). Then the cells were cultured in a humidified 5% CO₂ atmosphere culture incubator and subcultured twice a week using trypsin-EDTA (0.25%, 1 mM) solution. In our experiments, a hydrogen peroxide (H₂O₂) solution was used while proceeding cancerous cell based cytotoxicity tests. Note that H₂O₂ is one of the main toxic oxygen metabolite for various cellular injuries (Weiss et al. 1981; Suttorp and Simon 1982; Cantin et al. 1987).

2.2 Fabrication of a serial dilution module

The basic idea of a serial dilution module can be described as follows. First, let x represent the number of microchannels (for a drug) and y represent the number of microchannels (for a dilution buffer) into a well. Then, the dilution ratio of the drug can be calculated from the value of x divided by the sum of x and y . As seen in Fig. 1, the main microchannels from the inlet ports are split into sub-microchannels. These microchannels have the same cross-section square of 50 μm in width. After arranging these

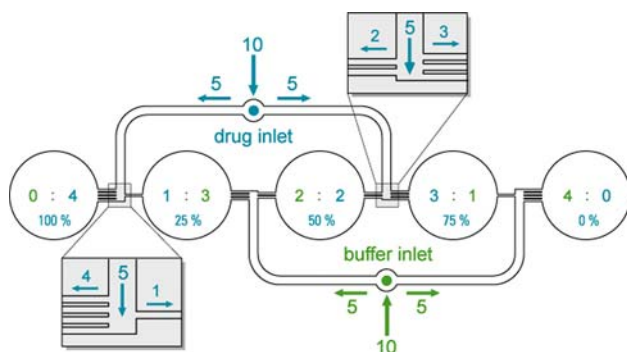


Fig. 1 Scheme of a microfluidic network for a linear scale serial dilution

microchannels in a serial fashion, we got five serially-increasing sample concentrations. A laminar flow, one of the well-known characteristics of microchannels, can ensure exactly equal distribution of the sample solution in each sub-microchannel (Jeon et al. 2000). In our experiments, the serial dilution module goes to a top layer of the integrated microsystem, and then acts as a preparation module for the serial dilution of a cytotoxic drug sample (Chang et al. 2003a, b).

2.3 Fabrication of a microcompartment module

As mentioned above, the idea of active sealing is very simple, but powerful for a flexible attachment to several cell culture environments. Normally, negative pressure taken in the sealed area can help a microchip to strongly attach itself to its bottom plate (Bang et al. 2006). This is because the pressure increases a conformal contact force of the elastomeric microchip and seals its contacting faces like a suction disk (Ng et al. 2002). Using this concept, we made a PDMS microchip that could stick onto the cell cultured surface. The microcompartment module, mainly consisting of the active sealing element, can form several well-type microchambers. This module goes to a bottom layer of the integrated microsystem. A functional integration of microsystem is described in detail in the following section.

2.4 Preparation of a functionally integrated microsystem

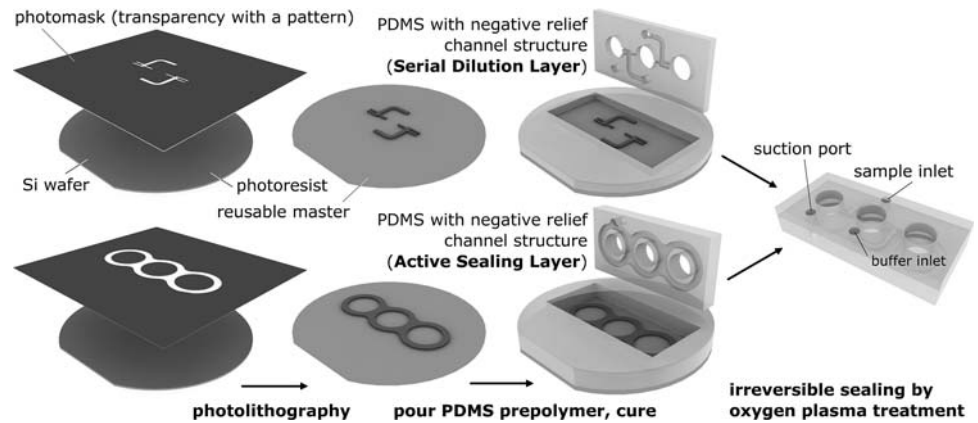
In our experiments, the microsystem was fabricated by soft-lithography using an elastomeric polymer replica molding that, in general, enables rapid prototyping of microfluidic devices (Xia and Whitesides 1998). Two types of the PDMS molds were fabricated for both the top and bottom layers of the microsystem. For the microcompartment module, some functionalized holes for active sealing were drilled using a precision end-mill, the diameters of which were regulated. Then, finally, the integration was completed by irreversibly closing both the top and bottom layers after oxygen plasma treatment as illustrated in Fig. 2.

3 Results

3.1 Serial dilution test: a linear-scale dilution of a Trypan blue solution

To evaluate the serial dilution module, we diluted a water-soluble dye, Trypan blue solution with the device and

Fig. 2 Fabrication scheme of the functionally integrated microsystem



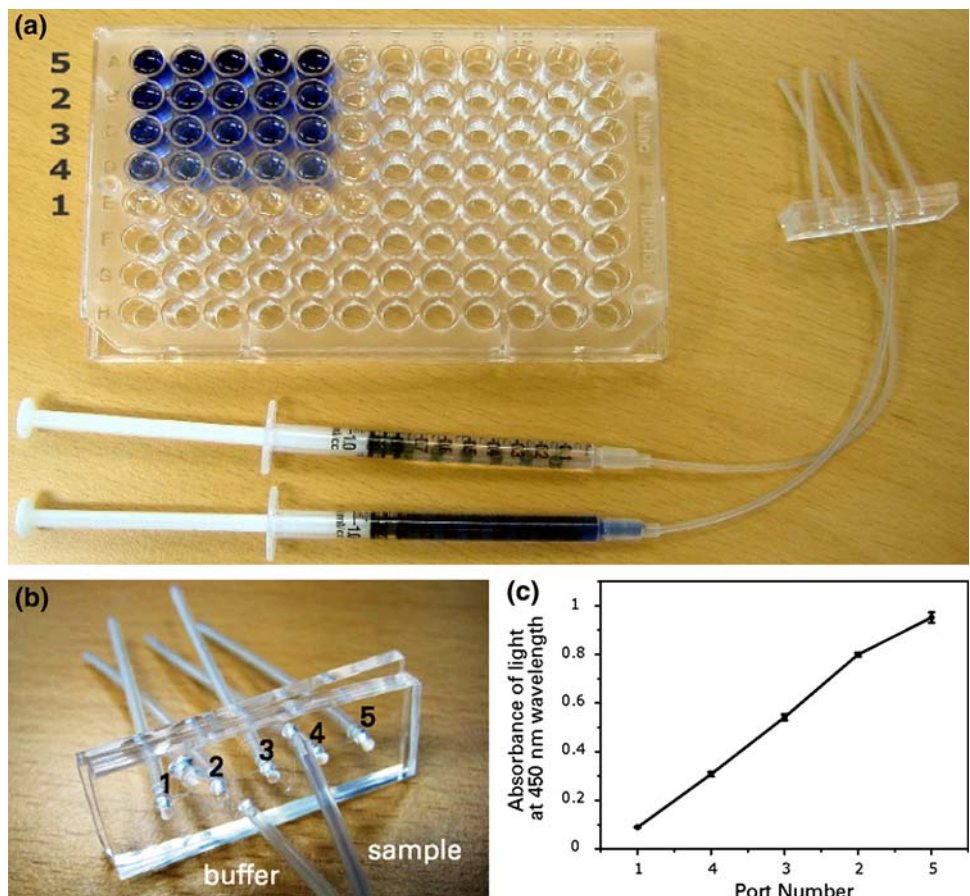
validated its performance as seen in Fig. 3a. First, 8 ml of the Trypan blue and de-ionized water were prepared. By activating the syringes, a linear-scale dilution was completed for 5 wells with linearly increasing concentrations through the module as shown in Fig. 3b. The concentration of the solution was measured by a spectrophotometer at 450 nm wavelength. These data given in Fig. 3c were acquired by repeating experiments five times with the same microchip. The measurement values of data have an

average linear deviation of $\pm 1\%$. The overall process of serial dilution took less than a minute.

3.2 Microcompartment test: a direct bonding onto the cultured cells

Directly onto the cultured cells in a single petri dish, a microcompartment module was easily attached using the

Fig. 3 Experimental results of the serial dilution of Trypan blue. **a** Photograph of serial dilution experiments, **b** photograph of this module and its port numbering, and **c** the measurement graph of light absorbance at 450 nm wavelength according to each well port



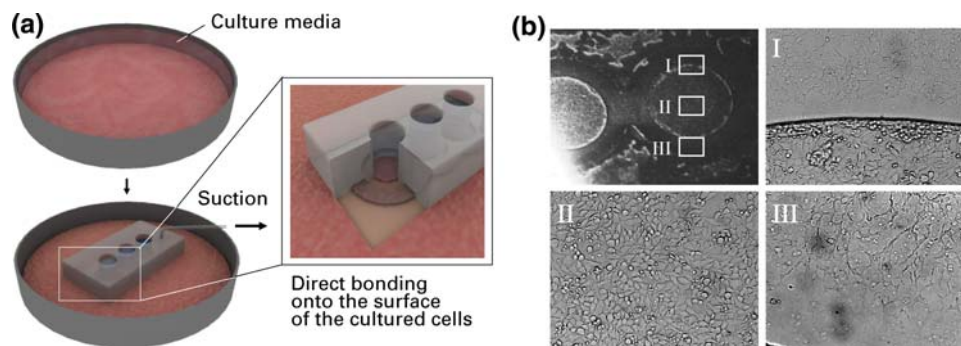


Fig. 4 Experimental results of the direct bonding of the microcompartment module onto the surface of the cultured cells. **a** Schematic illustration of the overall process (1) to culture the cells in a petri dish under the same conditions and (2) to bond directly this module onto the cultured cells directly by the active sealing technique through the suction pressure. A close-up inner view of the directly bonded module

active sealing technique as illustrated in Fig. 4a. This module was soaked into the culture media, but it was still tightly sealed onto the cultured cell surface. Then, the cell surface covered with HeLa cells was instantly divided into five different areas of microchambers. Surely, in some areas, the cells were selectively squeezed, but these cell debris played a role of a sealant for the instantly formed well-type chambers (Dittrich and Schwille 2003). Figure 4b shows bright-field images of the patterned cells after detaching the directly bonded module. Interestingly, we found that three regions were instantly divided with the inner geometry of this module under the same cell-culture condition: (I) a boundary region, (II) a cytotoxicity test region within the instantly-formed microchambers including still viable cells, and (III) a strictly contacted region where most cells suffered cell lysis (Chen et al. 1997; Thomas et al. 1999; Bang et al. 2006).

shows several possible zones through the instant microcompartment. **b** Bright field images of the patterned cells after detaching the directly bonded module. Here, three regions are instantly divided by several microchambers within this module under the same cell-culture condition. (I) a boundary region, (II) a cytotoxicity test region, and (III) a strictly contacted region for active sealing

3.3 On-chip cytotoxicity tests: cellular responses to hydrogen peroxide

With both serial dilution and microcompartment modules, we achieved on-chip functional integration and finally fabricated a directly stackable microsystem onto the cultured cells for cytotoxicity tests. The total size of the integrated microsystem was so small that this device could be easily put into a CO₂ incubator as shown in Fig. 5a. Prior to the experiment, we washed the channel twice with phosphate buffered saline (PBS, pH 7.4). Then, a quantity of 2×10^6 cells were seeded on the slide glass. To achieve the induced apoptosis, we treated HeLa cells independently with five increasing concentrations of H₂O₂ solutions using the microcompartment module. By performing this procedure repeatedly, six experimental sets of five increasing H₂O₂ treatments were completed. After 3 h of treatment,

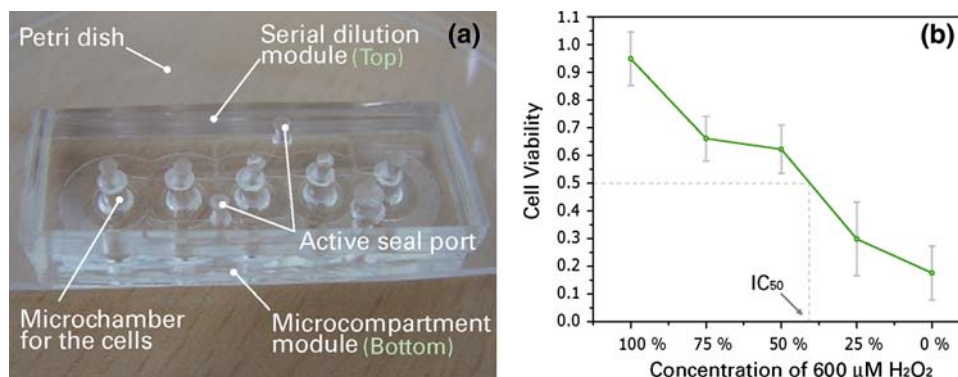


Fig. 5 Experimental results of the fabrication of microsystem and its performance evaluation. **a** Photograph of the fabricated multi-layered PDMS microsystem for cytotoxicity tests and **b** experimental results for the cell viability by the cytotoxicity test using the proposed

method. Here, we treated HeLa cells with a propidium iodide (PI) after H₂O₂ pretreatment to obtain the cell viability. These data also indicate the value of IC₅₀ under different H₂O₂ concentrations

the cytotoxicity has been measured by analyzing the cell viability obtained from an imaging flow cytometer device, C-Reader™ (Digital Bio Technology Co., South Korea). To observe the cell viability, one of cell membrane-impermeant dyes, propidium iodide (PI), was treated after H₂O₂ treatment with serially increasing concentrations. The experimental results of cell viability, including the value of IC₅₀ under different H₂O₂ concentrations, are shown in Fig. 5b.

In summary, we have presented a novel method for on-chip cellular assays, especially for the cytotoxicity test. First, we miniaturized and automated a serial dilution process by using microchannel networks. Secondly, we minimized the redundant processes of experimental preparation into a single petri dish by directly bonding the microsystem onto cell culture environments. This approach enabled us to demonstrate the feasibility of the integrated microsystem for cytotoxicity tests.

4 Discussions

Keeping pace up with a low-cost, compact miniaturization for clinical tools and a high-throughput diagnosis and detection for human diseases, many pharmaceutical companies are still striving to improve the cost effectiveness of their drug-discovery programs. It is an established fact that a considerable amount of money is being lost and will continue to be lost on compounds if they fail the toxicity test later in the process (Slater 2001). This is the first reason why the cytotoxicity test must be consistently improved.

In this study, we attempted to introduce our recently developed devices and its usefulness, where unpleasant, hazardous drug treatments should be performed more rapidly and efficiently. Our technique has salient features in that it does not modify the conventional cell culture protocols, but can reduce their repetitive and laborious processes. Here we hope that these kinds of the integrated microsystems will improve the currently tedious manual work that is done in biology or chemistry laboratories, where a mountain of serial dilution processes are waiting. If it requires a carefully defined concentrations, this method would be a versatile gadget that will guarantee both reproducibility and reliability. More importantly, this method is expected to enable many engineers or researchers to design and fabricate a cheap, flexible microsystem, including a disposable plastic microchip. In our experiment, we were able to demonstrate that the concept of the microcompartment module, in particular, can eliminate all the problems arising from the fact that target cells are not from a single microchamber or well. One basic idea could cause a new paradigm shift, simplifying a laborious

cell culture process to a simple handling with only a single petri dish or well. Testing the cytotoxicity within such a single platform obviously means having to use the same intrinsic cells under the same extrinsic condition. From this viewpoint, our proposed method is expected to help a potential researcher obtain more reliable data. Furthermore, this type of a functional integration can be effectively applied to different bottom-plates or various cell culture environments.

5 Conclusions

In this study, we developed a directly stackable microsystem onto the cultured cells for cytotoxicity tests under the same experimental conditions. In particular, we were able to conduct the cytotoxicity test within a single petri dish without modifying the current cell culture protocol. Based on the recently developed serial dilution and active sealing methods, we performed a linear-scale dilution of drug solution precisely and obtained more reliable data from the same environment. In sum, we strongly believe that this method will help overcome the growing problems relating to the cost-effectiveness of the cytotoxicity tests in a high-throughput fashion.

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References

- Bang H, Lim SH, Lee YK, Chung S, Chung C, Han D-C, Chang JK (2004) Serial dilution microchip for cytotoxicity test. *J Micro-mech Microeng* 14:1165–1170
- Bang H, Lee WG, Park J, Yun H, Lee J, Chung S, Cho K, Chung C, Han D-C, Chang JK (2006) Active sealing for soft polymer microchips: method and practical applications. *J Micromech Microeng* 16:708–714
- Cantin AM, North SL, Fells GA, Hubbard RC, Crystal RG (1987) Oxidant-mediated epithelial cell injury in idiopathic pulmonary fibrosis. *J Clin Invest* 79:1665–1673
- Chang JK, Heo YS, Bang H, Cho K, Chung S, Chung C, Han D-C (2003a) Functional integration of serial dilution and capillary electrophoresis on a PDMS microchip. *Biotech Bioprocess Eng* 8:33–239
- Chang JK, Bang H, Park S-J, Chung S, Chung C, Han D-C (2003b) Fabrication of the PDMS microchip for serially diluting sample with buffer. *Microsyst Technol* 9:555–558
- Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE (1997) Geometric control of cell life and death. *Science* 276:1425–1428
- Dittrich PS, Schuille P (2003) An integrated microfluidic system for reaction, high-sensitivity detection, and sorting of fluorescent cells and particles. *Anal Chem* 75:5767–5774
- Freshney I (2001) Application of cell cultures to toxicology. *Cell Biol Toxicol* 17:213–230

- Jeon NL, Dertinger SKW, Chiu DT, Choi IS, Stroock AD, Whitesides GM (2000) Generation of solution and surface gradients using microfluidic systems. *Langmuir* 16:8311–8316
- Ng MKJ, Gitlin I, Stroock AD, Whitesides GM (2002) Components for integrated poly (dimethylsiloxane) microfluidic systems. *Electrophoresis* 23:3461–3473
- Slater K (2001) Cytotoxicity tests for high-throughput drug discovery. *Curr Opin Biotechnol* 12:70–74
- Suttorp N, Simon LM (1982) Lung cell oxidant injury: enhancement of polymorphonuclear leukocyte-mediated cytotoxicity in lung cells exposed to sustained in vitro hyperoxia. *J Clin Invest* 70:342–350
- Thomas CH, Lhoest JB, Castner DG, McFarland CD, Healy KE (1999) Surfaces designed to control the projected area and shape of individual cells. *J Biomech Eng* 121:40–48
- Weiss SJ, Young J, Lobuglio AF, Slivka A, Nimeh NF (1981) Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells. *J Clin Invest* 68:714–721
- Xia Y, Whitesides GM (1998) Soft Lithography. *Annu Rev Mater Sci* 28:153–184