BioChip J. (2015) 9(2): 97-104
DOI 10.1007/s13206-015-9202-7

Original Article

Paper-based Cell Culture Microfluidic System

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Received: 28 October 2014 / Accepted: 07 January 2015 / Published online: 10 March 2015

Abstract In the past decades, glass/PDMS-based microfluidic systems have been rapidly developed to provide homogenous and stable microenvironment for culturing cells. Although these excellent demonstrations involve much simplified operations than traditional cell culture protocol, but they are still not readily accessible to untrained personnel and not appropriate to operate in conventional biological laboratories. In this work, cellulose filter papers were used for the substrates of the cell culture microfluidic system, which provides a convenient tool for cell-based assay. A paper was patterned with culture areas and channels by wax printing technique. Medium or tested substance can be passively perfused to the culture areas. Analyses of cyto-compatibility, cell proliferation, cell morphology, and cell chemosensitivity were performed to confirm the possibility of the paper-based system. The culture system could provide a platform for a wide range of cell-based assays with applications in drug screening and quantitative cell biology. This work demonstrated a paper-based cell culture microfluidic system and the system is inexpensive, disposable, and compatible to the existing culture facility.

Keywords: Paper-based microfluidics, Filter paper, Cell culture, Cell proliferation, Chemosensitivity

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Introduction

Cell-based assay is a cost efficient and high biological relevant technique to study the cell proliferation and chemosensitivity under the tested conditions. In conventional cell culture (e.g., Petri dish or multi-well microplate) practices, cells are attached on a surface in a monolayer format, which is referred to two-dimensional (2D) cell culture. However, animal cells inhabit in very three-dimensional (3D) environment. The 2D cell culture model may not faithfully predict the *in-vivo* cellular response[1,2]. Recently, 3D cell culture model has received great attention and this model is to encapsulate and culture cells within extracellular matrix (ECM). Expression of differentiated functions and tissue organization were reported to be different[3]. For example, differences in the phenotype or cellular response were found when cells are cultured under 2D versus 3D environments. It was reported that chondrocytes lost their phenotypic natures to synthesize surrounding ECM when they were cultured in 2D environment, whereas the phenotype can be restored when transferred to 3D environment[4]. That indicated that 3D cell culture model provides a more physiologically-meaningful culture condition for cell-based assays[1,2].

In the past decades, microfluidic technology has been rapidly developed and is often interpreted as a miniaturized and automated laboratory[5]. There are a number of advantages to their use, such as less sample/reagent consumption, reduced risk of contamination, less cost per analysis, lower power consumption, and higher reliability. A number of demonstrations have been reported for culturing cells in microfluidic systems[6]. For example, a cost-effective and high-throughput microfluidic device was reported for culturing cells inside an array of high aspect ratio microchambers with continuous perfusion of medium[7]. Human carcinoma (HeLa) cells were cultured inside the device and was grown

to confluency. In this work, cells were attached on 2D surfaces in the microchambers and considered as 2D cell culture format. Alternatively, a microfluidic cell culture chip has been developed to investigate cell proliferation and chemosensitivity in 3D cell culture format[8]. Human oral cancer cells were encapsulated in 3D agarose scaffold and cultured in a miniaturized chamber under perfusion of tested substance. Besides, gel-free microfluidic systems have been developed to provide alternative 3D cell culture models. Mammalian cells were successfully cultured three-dimensionally in a microfluidic channel with micro-fabricated pillar array[9]. Cells showed 3D cellular morphology, cellular functions, and differentiation capability. Although these excellent microfluidic culture systems involve much more simplified operations than traditional cell culture protocol, but they are still not readily accessible to untrained personnel and not appropriate to operate in conventional biological laboratories.

More recently, paper-based microfluidics has been proposed for various biomedical applications[10-13]. Aqueous solution can be passively transported along the hydrophilic paper fibers by wicking phenomenon. It is realized by patterning sheets of paper into hydrophilic channels, i.e., paper, bounded by hydrophobic barriers, which can be fabricated by the techniques of photolithography[14], wax printing[15-17], or plasma treatment[18]. The use of paper for the development of microfluidic systems has the advantages of lightweight, ease-of-use, and low cost. To date, various biomedical analyses have been demonstrated including colorimetric bio-assays[19-23], electrochemical bio-assays[24-27], and paper-based enzyme-linked immunosorbent assay (ELISA)[28-30]. Moreover, paper-supported 3D cell culture was reported to study cell responses to 3D molecular gradients and to mimic tissue- and organ-level functions[31]. Cells were encapsulated in hydrogel and cultured in stacked papers. Based on this work, various investigations of cell responses and cell-based assays were reported[32-35].

In this work, a paper-based cell culture microfluidic system is proposed to study cell proliferation and chemosensitivity in 3D microenvironment. Cells were directly seeded in the paper without hydrogel encapsulation. The advantage of paper-based cell culture without hydrogel encapsulation is the ease of further assays, such as scanning electron microscope (SEM) characterization. A paper was patterned with fluidic channels and culture areas by wax printing technique. Medium or tested substance can be passively perfused to the culture areas. Investigation of cytotoxicity on paper with wax was performed to confirm the cyto-compatibility of the paper-based cell culture system. Then, cell morphologies after culture of 1 and 5 days were investigated by SEM. Five-days cell proliferation was studied using the paper-based system and the results were compared with the standard 3D cell culture model using methyl cellulose (MC) hydrogel. Finally, tests of cell chemosensitivity under tested substance for 2 days were performed to demonstrate a practical application. This work demonstrated a paper-based cell culture microfluidic system and is inexpensive, disposable, and compatible to the existing culture facility.

Results and Discussion

Cyto-compatibility of filter paper with wax

Since mammalian cells are sensitive to extracellular microenvironment, study of cell proliferation and the release amount of LDH is performed to confirm the cyto-compatibility of filter paper with wax. In this study, wax was printed on circular filter papers of 15 mm in diameter with different coverage percentages, i.e., 0, 15, 50, and 100% of wax. The patterns of wax printed on circular filter papers are shown in Figure 1 (a). Next, the papers were placed onto a hot plate at 120˚C for 5 min to allow the wax to fully melt through the entire paper. After collagen application and sterilization, the papers were respectively laid on the bottom of the wells of the standard 24-wells microplates. Cells at the cell number of 3×10^4 suspending in medium were respectively added to each well. Cells were seeded and cultured on the papers for up to 6 days in the incubator. Cell proliferation analyzed by WST-1 assay and release amount of LDH analyzed by LDH activity assay were investigated in the following days.

Study of cell proliferation on filter papers with different coverage percentages of wax is shown in Figure 1(b). Importantly, results revealed that cells could proliferate during the 6 days culture and there is no significant difference between filter papers with different coverage percentages of wax. Moreover, release levels of LDH from the collected supernatant liquids on day 1, 3, and 5 were analyzed and is shown in Figure 1(c). A standard 2D culture on microplate was also conducted for the control group. It showed that the LDH levels had no significant difference between control group and filter papers with different coverage percentages of wax during 5 days culture. That indicated filter paper with wax is non-toxic and suitable for cell culture.

Cell morphology in filter paper

In order to show the morphology of cells cultured in paper substrate, SEM imaging was conducted. Cells at the cell number of 3×10^4 suspending in medium were respectively seeded and cultured in paper substrate. Cell morphologies after 1 and 5 days culture were in-

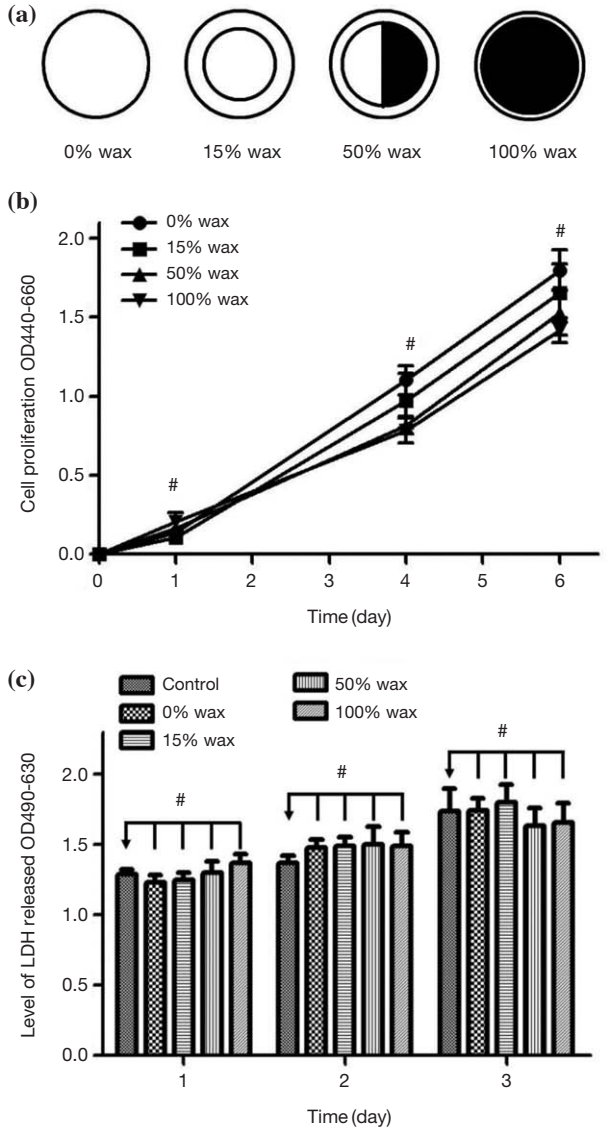

Figure 1. Study of cyto-compatibility of filter paper with wax. (a) The patterns of wax with different coverage percentages, i.e., 0, 15, 50, and 100% of wax, printed on circular filter papers. (b) Study of cell proliferation on filter papers with different coverage percentages of wax. Cells were seeded and cultured up to 6 days and cell proliferation was quantified by WST-1 assay. (c) Cytotoxicity study of filter paper with different coverage percentages of wax. Release levels of LDH from the collected supernatant liquids were analyzed on day 1, 3, and 5. Error bars represent the standard deviation of 3 repeated experiments.

vestigated and the SEM micrographs are respectively shown in Figure 2. Results revealed that cells were successfully seeded on the paper fibers and spherical morphology was observed after 1 day of culture (Figure 2(a)). After 5 days of culture, cells multi-directionally proliferated and aggregated on the paper fibers

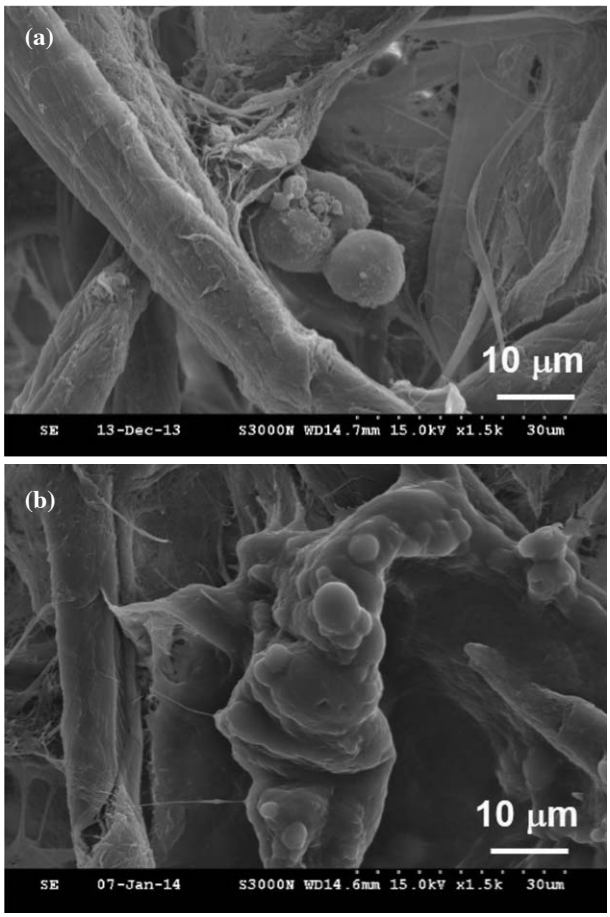

Figure 2. SEM micrographs of cell morphology in filter papers. (a) Cells were cultured in filter paper after 1 day. (b) Cells were cultured in filter paper after 5 days.

(Figure 2(b)). Therefore, cells proliferated in 3D space, but not only along the paper fibers. Compared with 2D cell culture model, cells only proliferate on a surface as a monolayer format. It was suggested that the paper fibers were not just providing 2D scaffolds with curvature, but 3D culture microenvironment.

Cell proliferation on paper-based cell culture microfluidic system

The design of the paper-based cell culture microfluidic system for the study of cell proliferation is shown in Figure 3. Four culture areas in the system were designed for culturing cells. There are 2 fluidic channels connecting between each culture area and 2 absorbing areas. The width of the channel was 1 mm and the diameter of the culture area was 15 mm. Fresh medium was passively transported from the medium reservoir to the culture areas. Wettability of the culture area could be ensured and perfusion-based cell culture on paper

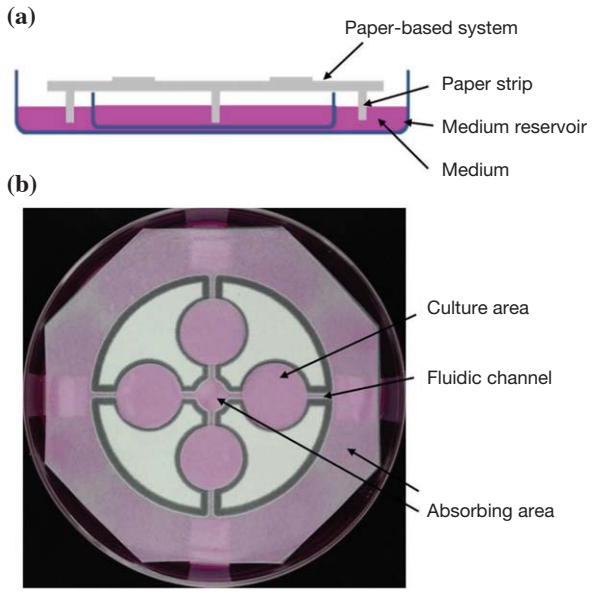

Figure 3. Design of the paper-based cell culture system for the study of cell proliferation. Four culture areas in the system were designed for culturing cells. There are 2 fluidic channels connecting between each culture area and 2 absorbing areas. Fresh medium was passively transported from the medium reservoir to the culture areas. Wettability of the culture area could be ensured and perfusion-based cell culture on paper could be realized.

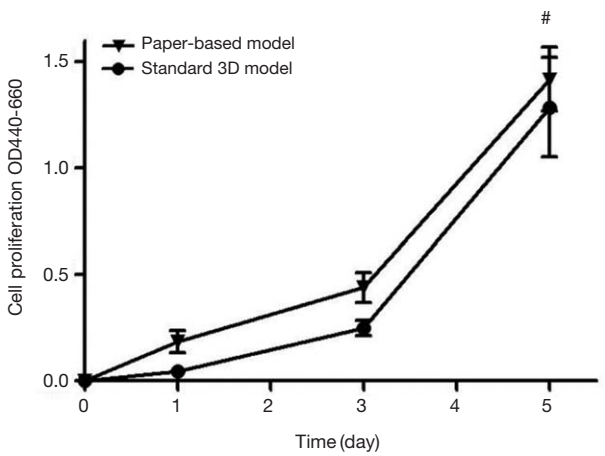

Figure 4. Study of cell proliferation in standard 3D and paper-based cell culture models. Cells were respectively cultured in MC hydrogel (standard 3D model; control group) and the paper-based system. Cells were seeded and cultured up to 5 days and cell proliferation was quantified by WST-1 assay. Error bars represent the standard deviation of 3 repeated experiments.

could be realized.

To study cell proliferation between standard 3D and paper-based cell culture models, cells were respectively cultured in MC hydrogel (standard 3D model, control group) and the paper-based system. Cells were seeded and cultured up to 5 days and cell proliferation quantified by WST-1 assay is shown in Figure 4. Results revealed that cell proliferation rate between both models had no significant difference. Recently, 3D cell culture technique has been received great attention and hydrogel is the most widely used material to encapsulate cells[36,37]. In this study, hydrogel-free cellulose-based culture model was proposed to provide a versatile and experimentally convenient solution for cells growing in 3D space. Cellulose filter paper is thin ($<$ 200 μm) and mechanically strong and has large void space among the paper filters. Cell proliferation was studied and the proposed paper-based cell culture system was confirmed. Paper-based cell culture system is flexible because paper is commercially available in a wide range of pore size, thickness, and diameter. This work demonstrated a paper-based cell culture microfluidic system and is inexpensive, disposable, and compatible to the existing culture facility.

Cell chemosensitivity on paper-based cell culture system

Studying of the chemosensitivity of cancer cells to anti-cancer drugs is necessary for effective and personalized chemotherapy. This can assist medical doctors to select effective chemotherapy regimens for individual patient. In this work, study of cell chemosensitivity was demonstrated using paper-based cell culture system. The design of the paper-based microfluidic system for the study of cell chemosensitivity is shown in Figure 5. Sixteen culture areas in the system were designed for culturing cells. Medium and anti-cancer drug were respectively applied to each end of the system. In order to illustrate the diffusion of drug, red colored solution (representing drug) and transparent solution (representing medium) were respectively applied to each end. Red colored solution passed through the first column of the culture areas after 2 hr. Because of the hydraulic pressure, diffusion progress of the red colored solution was slow after 2 hours and the solution took 24 hours to pass through the second column of the culture areas. When passing through the second column, the red colored solution stayed at the second column until the end of this experiment (48 hours). That indicated that the distance of the culture areas determined the time lag of drug application. Based on the geometric design of the culture areas, time lag of drug application can be adjusted.

Study of cell chemosensitivity was conducted in the paper-based system for 2 days. The first and second column of the culture areas were respectively diffused

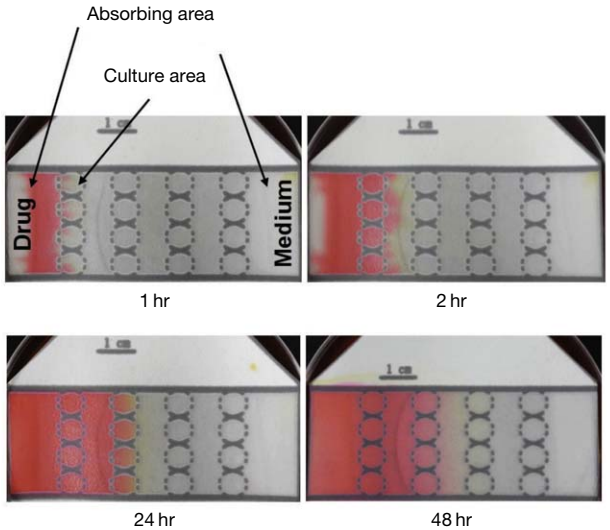

Figure 5. Design of the paper-based cell culture system for the study of cell chemosensitivity. Sixteen culture areas in the system were designed for culturing cells. Medium and anti-cancer drug were respectively applied to each end of the system. Images showed the diffusion of red colored solution from 1, 2, 24 to 48 hours.

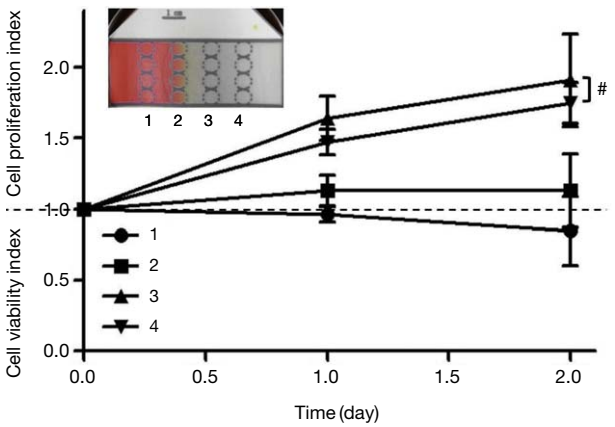

Figure 6. Study of cell chemosensitivity in the paper-based system. The first and second column of the culture areas were respectively diffused by anti-cancer drug. The third and fourth column of the culture areas were cultured in medium. Cell proliferation/viability index was quantified by WST-1 assay on day 1 and 2. Error bars represent the standard deviation of 3 repeated experiments.

by anti-cancer drug. The third and fourth column of the culture areas were cultured in medium. Cell proliferation/viability index was quantified on day 1 and 2 and the results are shown in Figure 6. Generally, cell death was observed when drug diffused to the culture areas. Cells in the first and second columns showed apoptosis starting respectively from the first and second day. In contrast, cells in the third and fourth columns showed proliferation during cell culture. This preliminary results showed that the paper-based system provides a new platform for cell-based assays.

Conclusions

A paper-based cell culture microfluidic system has been proposed to provide a 3D cell culture technique. Studies of cell proliferation and chemosensitivity were demonstrated and the paper-based system showed promising results. Cellulose filter paper was selected as culture substrate because it is biocompatible, inexpensive, disposable, and compatible to the existing culture facility. Cyto-compatibility of filter paper with wax was studied and confirmed the non-toxicity and suitability for cell culture. From the cell proliferation study, results indicated that paper-based system provides 3D culture microenvironment. Finally, tests of cell chemosensitivity were performed to demonstrate a practical application. This work provides a 3D cell culture microfluidic system and has great potential to develop tools for cell-based assay.

Materials and Methods

Cell culture

Human liver cancer cells (cell line: Huh-7) at an initial cell number of 3×10^4 were used throughout this study. Culture medium was Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco-RBL Life Technologies) and antibiotic/antimycotic (100 U/mL of penicillin G sodium, 100 mg/mL of streptomycin, and 0.25 mg/mL of amphotericin B; Gibco-BRL Life Technologies). Cells were amplified by standard cell culture technique and trypsinized using 0.05% trypsin for 3 min, centrifuged at 1000 rpm for 5 min, and resuspended in the medium for the experiments. Grade 4 (size of particle retention: 20-25 μm) cellulose filter paper (Whatman) was used as a substrate of the paper-based cell culture system. Washing buffer used in this study was phosphate-buffered saline (PBS; 50 mM phosphate, 150 mM NaCl, and 10 mM EDTA; pH 7.6).

Lactate dehydrogenase (LDH) activity assay

In order to investigate the cyto-compatibility of filter paper with wax, LDH activity assay was performed to study the level of cell damage, which can be indirectly indicated by the release amount of LDH into the culture medium. Because LDH is an intracellular enzyme

released from cells when they are injured or damaged, it provides an accurate and sensitive marker to determine the cyto-compatibility of culture substrates and suitability of cell survival. In this analysis, supernatant liquids during culture at day 1, 3, and 5 were respectively collected and analyzed by cytotoxicity detection kit (Roche Applied Science). The procedure follows the instruction provided by the manufacturer and is briefly described here. The supernatant liquid was incubated with the reaction mixture from the kit for 30 min. LDH content was assessed by ELISA and read at an absorbance of 490 nm in an ELISA plate reader (Synergy^{TM} HT, BioTek) with a reference wavelength of 630 nm.

Scanning electron microscopy (SEM) characterization

SEM was used to investigate the cell morphology in the paper-based microfluidic system. The operation of SEM characterization is briefly described here. Cells were pre-fixed by 2.5% glutaraldehyde (Sigma) for 2 hours and then washed by PB buffer (0.2 M KH_2PO_4, 0.2 M Na_2HPO_4) triple times for 5 min each. Next, cells were in dehydration by series of different concentration of ethanol from 50% to 100%, and immersed twice in pure ethanol for 15 min each. Then, cells were dried in a critical point drying machine with dry carbon dioxide. Finally, the samples were coated with a thin conducting gold layer with a thin carbon layer by vacuum evaporation. SEM images were respectively captured in the top view.

WST-1 assay

Because cyto-compatibility of culture environment, cell proliferation, and chemosensitivity are highly related to cell viability, WST-1 assay (Roche Applied Science) can determine cell viability to carry out these investigations. The principle of WST-1 assay is described below. The stable tetrazolium salts, i.e., WST-1, are cleaved to a soluble formazan by the succinate-tetrazolium reductase system which belongs to the respiratory chain of the mitochondria, and is only active in metabolically intact cells. The intensity of the yellow-orange colored product, i.e., formazan, directly correlates to the number of viable cells. The operation of WST-1 assay is described below. Reagent of WST-1 assay was diluted in medium in a volume ratio of 1 : 10. The paper of culture area was cut from the paper-based system and placed in the well of standard microplate. Then, 300 μL diluted reagent was simply added to the well and incubated at 37˚C for 2 hours. The optical density (OD) of the reacted solution was read on an ELISA plate reader at an absorbance of 440 nm with a reference wavelength of 660 nm. The absorbance was proportional to the number of viable cells.

Paper-based cell culture microfluidic system

The paper-based microfluidic system was fabricated based on the reported wax printing method[15-17]. The pattern of culture areas and fluidic channels was designed by computer drawing software. In this study, two different patterns were respectively used for the studies of cell proliferation and cell chemosensitivity in the paper-based system. The designed pattern was then printed onto a cellulose filter paper by a commercial solid wax link printer (ColorQube 8570N, Xerox). Next, the paper was placed onto a hot plate at 120˚C for 5 min to allow the wax to fully melt through the entire paper. The wax barrier defined the shape, e.g., width and length, of the channels and the thickness of the paper defined the height of the channels. Aqueous solutions can be transported along the channels by wicking phenomenon through the hydrophilic fibers of paper. Also, paper strips were attached to the absorbing areas of the filter paper and soaked into the medium for the medium perfusion. Before cell culture experiment, the paper-based system was sterilized by ultraviolet light (UV) overnight. The culture areas were then wetted by 20 μL PBS with collagen (Sigma) in the concentration of 10 μg/mL for the promotion of cell seeding. Collagen was deposited on the paper fibers and can help to anchor the receptors on the cell surface[1]. Subsequently, UV sterilization is performed for another 2 hr. The paper-based system was then placed on the medium reservoir for the passive perfusion of medium. Until wetting of the entire culture areas, cells suspended medium was respectively pipetted to each culture area. Cell seeding efficiency and uniformity were studied and the results are shown in Figure S1 in the supplementary material. Finally, the entire system was placed in the incubator in humidified atmosphere with 5% CO_2 at 37˚C (370; Thermoscientific). Cell proliferation in the culture area after 1, 3, and 5 days of culture was determined by WST-1 assay.

Cell culture using methyl cellulose (MC) hydrogel

Cells cultured in MC hydrogel is one of the standard 3D cell culture methods and is used as control group in this study. MC powder was obtained from Fluka (646 30; Methocel MC, Switzerland). Aqueous MC solution was prepared by dispersing the MC powder in PBS solution at a concentration of 1.8% (w/v) at room temperature. Next, the MC hydrogel was sterilized by using autoclave at 121˚C under 100 kPa for 20 min. On the other hand, bottom of the well of the standard microplate was coated with a layer of agar gel (mixture of 250 μL 0.7% agar solution and 250 μL 2 × DMEM).

This agar layer could eliminate cell attachment to the bottom surface of the well. After solidification, a mixture of 250 μL cells suspended medium and 250 μL MC hydrogel was poured into the well. Cells suspended in the MC hydrogel were cultured in the incubator. The cell proliferation was quantified by WST-1 assay.

Exposure of cells to anti-cancer drug

Doxorubicin (MW: 579.98, Sigma), i.e., anti-cancer drug, was dissolved into dimethyl sulfoxide (DMSO) as a stock solution. First, the paper-based microfluidic system was placed on the medium reservoir for the passive perfusion of medium. Until wetting of the entire culture areas, cells suspended medium was respectively added to each culture area after wetted by collagen solution for the promotion of cell seeding. The anti-cancer drug in the final concentration of 10 μg/mL was added to one side of the medium reservoir. Then, the entire system was placed in the incubator for 2 days to study the cell chemosensitivity in the paper-based system. Cell proliferation or viability after 1 and 2 days of exposure to medium or drug was determined by WST-1 assay. The OD outputted from the assay was proportional to the cell number of the culture area in the paper-based system. Cell proliferation or viability was represented by an index which was the successive OD divided by the starting OD of the initial seeding cell number.

Statistical analysis

Experimental data were presented as mean ± standard deviation (SD) from more than 3 independent experiments. Results were analyzed by one way analysis of variance (ANOVA). The notation of # indicates data without statistical significance ($p > 0.05$).

Acknowledgements This work is partially supported by the Ministry of Science and Technology, Taiwan (project no. MOST103-2221-E-182-004-MY3) and Chang Gung Memorial Hospital (project no. CMRPD2C0142).

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