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Paper-based Microreactor Integrating Cell Culture and Subsequent Immunoassay for the Investigation of Cellular Phosphorylation

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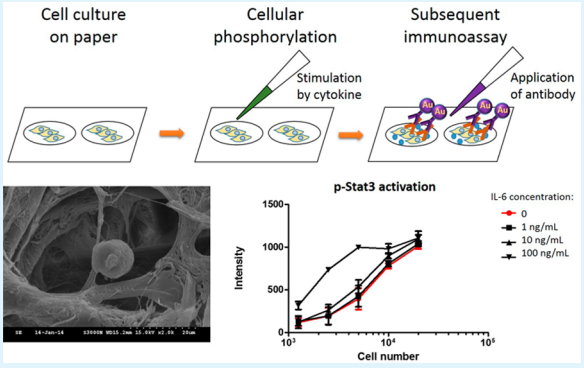
Supporting Information

ABSTRACT: Investigation of cellular phosphorylation and signaling pathway has recently gained much attention for the study of pathogenesis of cancer. Related conventional bioanalytical operations for this study including cell culture and Western blotting are time-consuming and labor-intensive. In this work, a paper-based microreactor has been developed to integrate cell culture and subsequent immunoassay on a single paper. The paper-based microreactor was a filter paper with an array of circular zones for running multiple cell cultures and subsequent immunoassays. Cancer cells were directly seeded in the circular zones without hydrogel encapsulation and cultured for 1 day. Subsequently, protein expressions including structural, functional, and phosphorylated proteins of the cells could be detected by their specific antibodies, respectively. Study of the activation level of phosphorylated Stat3 of liver cancer cells stimulated by IL-6 cytokine was demonstrated by the paper-based microreactor. This technique can highly reduce tedious bioanalytical operation and sample and reagent consumption. Also, the time required by the entire process can be shortened. This work provides a simple and rapid screening tool for the investigation of cellular phosphorylation and signaling pathway for understanding the pathogenesis of cancer. In addition, the operation of the paper-based microreactor is compatible to the molecular biological training, and therefore, it has the potential to be developed for routine protocol for various research areas in conventional bioanalytical laboratories.



KEYWORDS: *paper-based microfluidics, cell culture, immunoassay, cellular phosphorylation*

1. INTRODUCTION

Cellular phosphorylation and signaling pathway play an important role in the pathogenesis of cancer.[1,2] Dysregulation of cellular phosphorylation is critical for tumor growth and progression for many reasons. Aberrant cell survival mechanisms allow cells that harbor activated oncogenes or are genetically unstable to resist cell death, and thus allow aggressive clones to arise. Such mechanisms allow survival in a nonadherent state and thereby permit metastases. Moreover, these mechanisms contribute to tumor cell resistance to hypoxia, immune surveillance, chemotherapy, radiation therapy, growth factor deprivation, and other selective environmental pressures. Knowledge of the mechanisms of cellular phosphorylation is thus essential for understanding the pathogenesis of cancer and for developing effective therapeutic strategies.

Studying the role of cytokine is one of the interesting topics of the investigation of the mechanism of cellular phosphorylation. For example, an increase of the cytokine interleukin-6 (IL-6) leads to hepatobiliary inflammation and cancer. It was reported that this cytokine plays an important role in the pathogenesis of both cholangiocarcinoma and hepatocellular carcinoma.[3−5] Inflammation due to various chronic hepatobiliary diseases including hepatitis B, hepatitis C, alcoholic liver injury, and primary sclerosing cholangitis has been associated with increased levels of IL-6 and with increased rates of malignancy. When IL-6 engages its receptor, phosphorylation of specific kinases is triggered. Knowledge of IL-6 survival signaling in cancers is essential for the development of effective therapeutic strategies. Based on classic lab techniques, investigation of cellular phosphorylation involves a series of biological operations including cell culture using Petris dish, cell lysis, centrifugation, and Western blotting, which are time-consuming and labor-intensive. All these operations are independent (the samples has to transfer to one vessel to another vessel). Therefore, the time of cytokine stimulation to cells is difficult to control precisely. There is a need for the development of simple and rapid tool to screen the cellular phosphorylation of various specific kinases after stimulation of cytokine.

In the past decades, microfluidic systems have been extensively developed, and many excellent biomedical demonstrations have been reported.[6,7] Although these systems are

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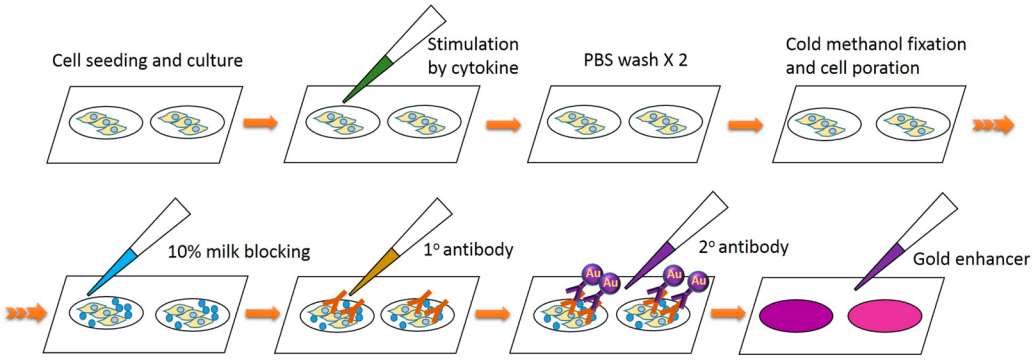

Figure 1. Methodology of the paper-based microreactor integrating cell culture and subsequent immunoassay for the study of cellular phosphorylation of cancer cells stimulated by cytokine.

much more simplified than traditional bioanalytical instruments, they are still not appropriate for rapid screening tools in conventional biological research laboratories.[8] Most recently, paper-based microfluidics has been proposed for a new class of bioanalytical technique.[9] Fabrication of paper-based microfluidics can be realized by patterning sheets of paper using different methods such as photolithography,[9,10] wax printing,[11,12] and plasma treatment.[13] The patterned barriers define the shape (e.g., width and length) of the channels, and the thickness of the paper defines the height of the channels. Aqueous solutions can be transported along the channels by wicking through the hydrophilic fibers of paper. A number of biomedical analyses have been demonstrated, including colorimetric bioassays,[14,15] electrochemical bioassays,[16−18] and paper-based enzyme-linked immunosorbent assay (ELISA).[19,20] The use of paper for the development of bioanalytical devices has the advantages of light weight, ease of use, and low cost, and they are appropriate to apply for rapid screening. For example, paper-based ELISA can be completed within an hour, whereas conventional ELISA requires at least 6 h to complete. Also, the usage of reagents is significantly reduced in paper-based ELISA because only 3 μL is required for each test zone. That means great reduction of analytical cost compared with conventional ELISA. Moreover, paper-supported 3D cell culture was reported to study cell responses to molecular gradients and to mimic tissue- and organ-level functions.[21] Cells were encapsulated in hydrogel and cultured in stacked papers. Based on this work, various investigations of cell responses and cell-based assays were performed.[22−24] The ease of fabrication of paper microzone plates also opens opportunities for a wide range of nonstandard formats for customized assays.[25]

In this work, a paper-based microreactor integrating cell culture and subsequent immunoassay is proposed and demonstrated by the investigation of cellular phosphorylation of liver cancer cells stimulated by cytokine. The paper-based microreactor was a filter paper with an array of circular zones for running multiple cell cultures and subsequent immunoassays. Cancer cells were directly seeded in the circular zones without hydrogel encapsulation and cultured for 1 day. Then, stimulation treatment (i.e., IL-6 cytokine) was applied to the circular zones and incubated for the required stimulation time. Cells were treated for the initiation of the signaling pathway. Cellular phosphorylation was activated and detected by subsequent immunoassay. Then, cells were fixed and cell poration was conducted on the circular zones. Because cells were not encapsulated in hydrogel, antibodies could directly capture the target molecules in cells. Cellular phosphorylation of the interested kinases or transcription factors was detected by specific molecules (i.e., antiphosphate primary antibodies). Secondary antibodies conjugating to gold nanoparticles were added for signal amplification. The colorimetric result of immunoassay was quantitatively measured by a commercial flatbed scanner. Study of the correlation between the stimulation treatment and phosphorylation level was demonstrated by the paper-based microreactor. The advantages of the proposed system include (1) integration of cell culture and subsequent immunoassay on a single paper; (2) reduction of tedious biological operations leading to eliminate human failure, and (3) shortening of the time required by the entire process. This work provides a simple and rapid screening tool for the investigation of cellular phosphorylation and signaling pathway, and has the potential to be developed for routine protocol in conventional biological research laboratories.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. Rabbit anti-GAPDH antibody and mouse antiactin antibody were purchased from Sigma, Taiwan. Mouse anti-Erk1/2 antibody, mouse anti-Stat3 antibody, and rabbit antiphospho-Stat3 antibody were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-influenza H1 antibody was purchased from Sino Biological Inc., China. The secondary antibodies, that is, antimouse IgG and antirabbit IgG, conjugated to gold nanoparticles were purchased from Sigma, Taiwan. Gold enhancement solution for the development of gold nanoparticles was purchased from Nanoprobes, (Yaphank, NY). Buffer used in this study was phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4). The blocking buffer consisted of 0.05% v/v Tween 20 in 1× PBS with 10% w/v fat-free milk. The washing buffer consisted of 0.05% v/v Tween 20 in 1× PBS. The antibody solution contained 0.05% v/v Tween 20 in 1× PBS with the antibody in 1:100 dilution. All experiments were performed at room temperature (22−25 °C) unless otherwise stated.

2.2. Cell Culture. Human liver cancer cells (cell line: Huh7) and nasopharyngeal cancer cells (cell line: Tw06) were used in this study. Culture medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco-RBL Life Technologies, Grand Island, NY) 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, Grand Island, NY). 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 further experiments.

2.3. Fabrication of the Paper-based Microreactor. The paper-based microreactor was a filter paper (No. 2, Advantec, Dublin, CA) with an array of 6 × 6 circular zones for running multiple cell cultures and subsequent immunoassays. The circular zones were printed on the filter paper by the wax-printing method.[11,12] An image of circular zone

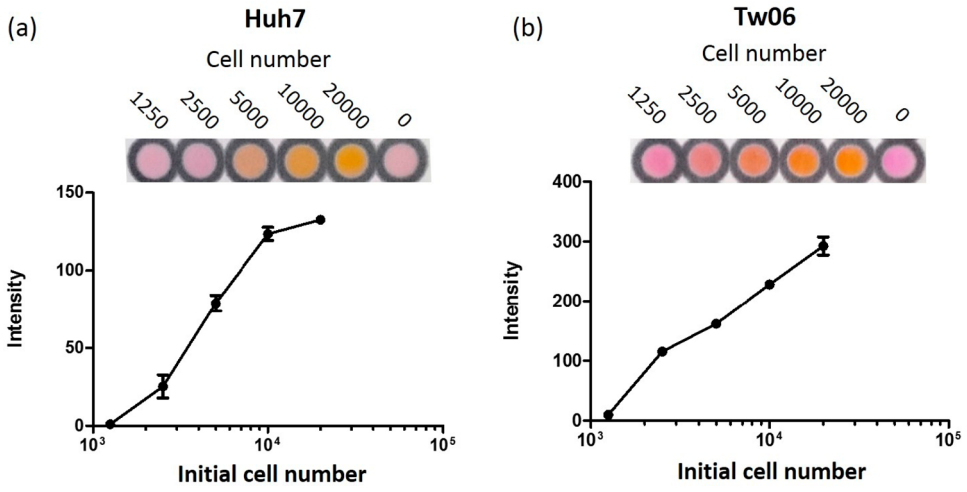

Figure 2. Correlation between colorimetric intensity and cell number measured by the paper-based microreactor. Cells at different initial cell numbers were respectively seeded and cultured in the circular zones of the paper-based microreactor for 1 day. The number of viable cells was quantified by WST-1 assay. (a) Huh7 cells and (b) Tw06 cells. Error bars represent standard deviation of at least three repeated experiments.

array was first designed by computer drawing software. The inner diameter of each circular pattern was 6 mm. The wax pattern of circular zone array was then printed onto the filter paper by a solid wax printer (ColorQube 8570N, Xerox, Japan). Subsequently, the printed filter paper was placed onto a hot plate at 100 °C for 10 min to allow the wax to fully melt through the entire paper. Therefore, the wax became hydrophobic "wall" to trap the aqueous solution in the circular zones. Only 5−10 μL solution was required to completely wet each zone and this design was a good compromise between convenience and conservation of samples and reagents. Finally, the paper was cooled and can be used immediately or stored at room temperature for later use. The fabrication of the paper-based microreactor is simple and flexible. That is appropriate for rapid screening tools in conventional biological research laboratories.

2.4. Investigation of Cellular Phosphorylation by the Paper-based Microreactor. Investigation of cellular phosphorylation of liver cancer cells stimulated by cytokine was demonstrated by the paper-based microreactor. Liver cancer cells (cell line: Huh7) were used in this study. The methodology of the paper-based microreactor integrating cell culture and subsequent immunoassay is described here and shown in Figure 1. The paper-based microreactor was first sterilized by ultraviolet (UV) light overnight. Then, each zone was wetted by PBS containing collagen (Sigma, St. Louis, MO) of 1 μg/mL in order to enhance the suitability of culture substance. Next, 20 μL of medium suspension with cells in certain cell number was pipetted to the circular zones, respectively. The paper-based microreactor was then placed in a 37 °C, 5% CO_2, and high-humidity incubator (370; Thermo Scientific, Waltham, MA). Cells were seeded in the paper zones without hydrogel encapsulation and became stable after 1 day of culturing. Next, stimulation treatment was performed by applying 10 μL of IL-6 solution in certain concentration to each zone. Cells were treated for the initiation of the signaling pathway. After incubation for the required time, the stimulation was stopped by washing twice with buffer. Cellular phosphorylation was activated and detected by subsequent immunoassay. The paper-based microreactor was placed to be suspended in the air horizontally in order to prevent the solution from wicking through the circular zones. Cells were fixed and cell poration was conducted by soaking the paper-based microreactor in cold methanol for 20 min. After 10 min of blocking treatment, cellular phosphorylation of the interested kinases or transcription factors was detected by specific molecules, namely, antiphosphate primary antibodies. Primary and secondary antibodies were sequentially added to each circular zone and incubated for 10 min each. Between each application of solution, the washing procedure was performed to wash away the unbound antibodies. Ten microliters (10 μL) of washing buffer was added to the zone twice and allowed a blotting paper to absorb the excess solution. Finally, gold nanoparticles conjugated to secondary antibody were amplified by gold enhancement solution for 20 min. The concentration of target antigen (i.e., phosphorylated kinases or transcription factors) was correlated to the aggregation of gold nanoparticles, leading to the change of the color intensity within the zone. The immunoassay results could be quantitatively measured by a commercial flatbed scanner and analyzed by ImageJ computer software. The signal was represented by intensity that was the grayscale subtracting the background grayscale.

2.5. Analysis of Cell Number in the Paper-based Microreactor. Cell number within the circular zone in the paper-based microreactor can be measured by the WST-1 assay (Roche Applied Science, Indianapolis, IN). The stable tetrazolium salts, WST-1, are cleaved to a soluble formazan by the succinate-tetrazolium reductase system that belongs to the respiratory chain of the mitochondria and is only active in metabolically intact cells. The intensity of the yellow-orange colored product, formazan, directly correlates to the number of viable cells. The operation of analyzing the cell number within the circular zone in the system is described here. Two microliters (2 μL) of reagent of WST-1 assay was simply added to each zone and incubated at 37 °C for 1 h. The colorimetric signal of the zones can be captured by a regular camera and analyzed by ImageJ computer software. The signal was represented by intensity that was the grayscale subtracting the background grayscale and was proportional to the cell number within the circular zone.

2.6. Cell Morphology Characterization. Cell morphology was investigated using scanning electron microscopy (SEM). Cells were cultured on the microzones of the paper-based microreactor for 1 day. Because cells were not encapsulated in hydrogel, hydrogel removal was not required for SEM imaging. Cells were prefixed by glutaraldehyde (Sigma, St. Louis, MO) for 2 h and then washed by PBS three times for 5 min each. Then, 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 for SEM imaging. The SEM images showed cell morphology in the paper-based culture microenvironment.

3. RESULTS AND DISCUSSION

3.1. Paper-based Cell Culture. Cellulose filter papers used in this work are reticulated structures of cotton linters and suitable for cell seeding and culture. They are biocompatible, inexpensive, disposable, and compatible to the existing culture facility. In this study, Huh7 and Tw06 cells were used to show

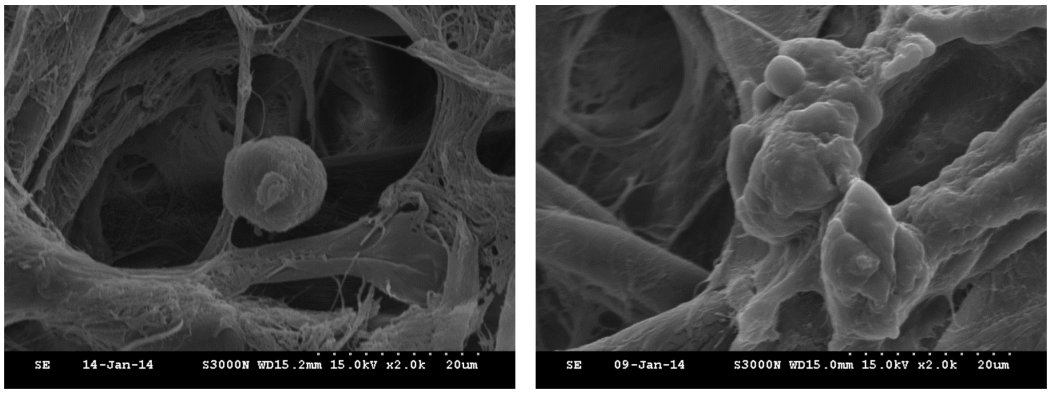

Figure 3. SEM image of cell morphologies after (left) 1 day and (right) 5 days of culturing in the paper-based microenvironment.

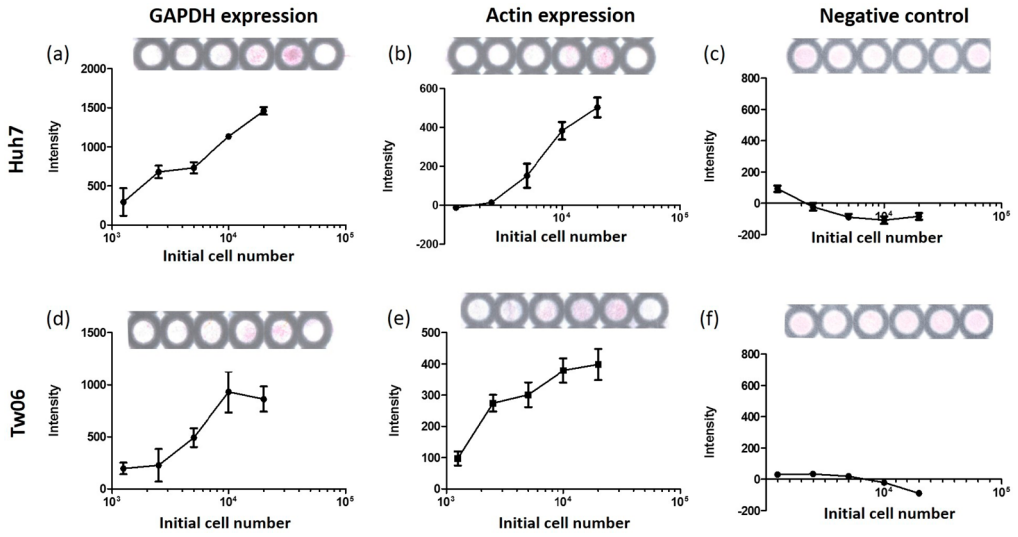

Figure 4. Detection of structural proteins of cells GAPDH and actin. GAPDH expression, actin expression, and negative control on Huh7 and Tw06 cells at different initial cell numbers were quantified by the paper-based microreactor. (a) GAPDH expression, (b) actin expression, and (c) negative control of Huh7 cells; (d) GAPDH expression, (e) actin expression, and (f) negative control of Tw06 cells. Error bars represent standard deviation of at least three repeated experiments.

the suitability of the paper-based culture microenvironment. Cells at different initial cell numbers were respectively seeded and cultured in the circular zones of the paper-based microreactor for 1 day. The number of viable cells was quantified by WST-1 assay, and the correlation between colorimetric intensity and cell number is shown in Figure 2. After 1 day of culturing, the respiratory chain of the mitochondria of the cells was still functional, and cells could be successfully cultured in the paper-based microenvironment. Intensity of both Huh7 cells and Tw06 cells showed linear correlation (R^2 = 0.7455 for Huh7 and R^2 = 0.8056 for Tw06) along the cell number from 1250 to 20 000. Moreover, cell at the initial cell number of 2500 were seeded and cultured in the circular zones of the paper-based microreactor up to 3 days to show cell proliferation in the paper-based microenvironment. Number of viable cells was respectively quantified by WST-1 assay and result is shown in Figure S1 (Supporting Information). It revealed cell proliferation at a normal rate. In addition, cell morphologies after 1 day and 5 days of culturing were respectively investigated by SEM characterization, and the images are shown in Figure 3. It revealed that cells were successfully seeded and cultured on the paper fibers. After 1 day of culturing, cells became stable and were observed to have spherical morphology. After 5 days of culturing, cells multidirectionally proliferated and aggregated on the paper fibers. Compared with standard cell culture on microplate, cells are seeded on a surface as a monolayer format and become spread morphology. The current observation indicated the paper fibers were providing not only two-dimensional scaffolds with curvature but also a three-dimensional culture microenvironment. In summary, these results confirmed the cell seeding efficiency, cell proliferation, and cell viability in the paper-based microreactor without hydrogel encapsulation.

3.2. Paper-based Microreactor Integrating Cell Culture and Subsequent Immunoassay. Two structural proteins, namely, GAPDH and actin, of cells were selected to demonstrate the feasibility of the technique of the paper-based microreactor integrating cell culture and subsequent immunoassay. GAPDH is an enzyme that serves to break down glucose for energy and carbon molecules. Because it is stable and constitutively expressed at high levels in most cells, it is considered a housekeeping gene. Actin is a globular multifunctional protein that forms microfilaments in cytoskeleton. It is present constitutively in cells and is suitable for the protocol development. In this study, Huh7 and Tw06 cells were used to confirm the feasibility of the technique. Cells at different initial

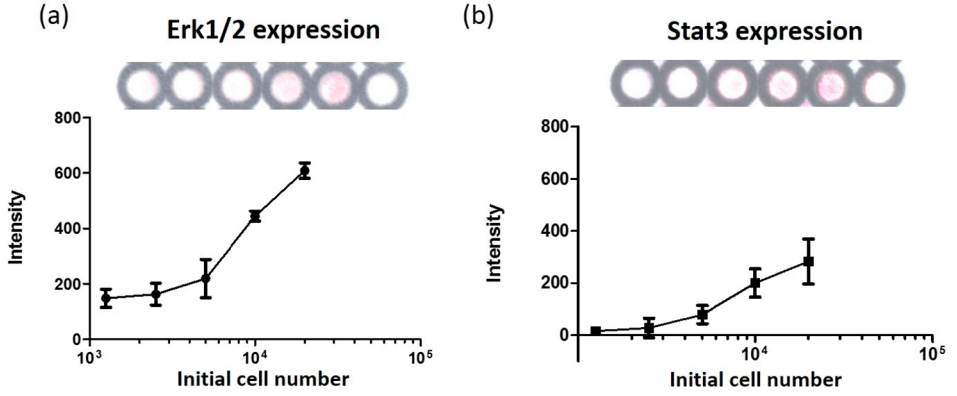

Figure 5. Detection of functional proteins Erk1/2 and Stat3. Erk1/2 expression and Stat3 expression on Huh7 cells at different initial cell numbers were quantified by the paper-based microreactor. (a) Erk1/2 expression and (b) Stat3 expression. Error bars represent standard deviation of at least three repeated experiments.

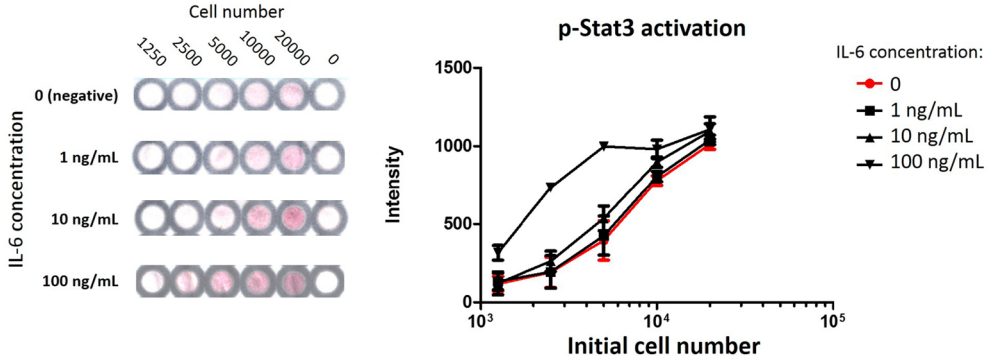

Figure 6. Activation level of phosphorylated Stat3 (p-Stat3) of Huh7 cells stimulated by IL-6 cytokine for 30 min. Cells at different initial cell numbers were cultured for 1 day and then treated with different concentrations of IL-6 cytokine for 30 min. The p-Stat3 activation was quantified by the paper-based microreactor. Error bars represent standard deviation of at least 3 repeated experiments.

cell numbers were respectively seeded and cultured in each zone for 1 day. Without the stimulation treatment, cells were fixed, lysed, and analyzed based on the protocol shown in Figure 1. GAPDH and actin expressions on cells were respectively detected by their specific primary antibodies, namely, anti-GAPDH antibody and antiactin antibody. A nonhuman antibody, anti-influenza H1 antibody, was used for the negative control to show the specificity of this technique. The results are shown in Figure 4. Because GAPDH and actin are present constitutively in most cells, GAPDH and actin expressions generally showed a linear correlation to the cell number for Huh7 cells and Tw06 cells. The intensity of GAPDH expression on Tw06 cells at cell number of 20,000 showed plateau of detection. For GAPDH expression, the *R*-square values were calculated as 0.8052 for Huh7 and 0.6934 for Tw06 except the plateau region. For actin expression, the *R*-square values were calculated as 0.8390 for Huh7 and 0.4877 for Tw06. Although the statistical values for Tw06 were fair, the signal intensity still showed reasonable correlation to the cell number. Results revealed and confirmed the feasibility of the technique. Interestingly, Tw06 cells showed higher actin expression than Huh7 cells at low cell number. Because actin is a protein that forms microfilaments in cytoskeleton, that might suggest that Tw06 cells have higher migration capability than Huh7 cells. High metastatic potential of human nasopharyngeal carcinoma was known from the clinical reports.[26,27] For the negative control, because anti-influenza H1 antibody is a nonhuman antibody and does not bind with the proteins expressed from human cells, results showed a nonspecific signal and confirmed the specificity of the technique of the paper-based microreactor integrating cell culture and subsequent immunoassay.

3.3. Detection of Cellular Phosphorylation Stimulated by Cytokine. Development of a simple and rapid tool for the detection of cellular phosphorylation of cancer cells stimulated by cytokine is very important to accelerate the understanding of pathogenesis of cancer. In this study, to show a practical application, we used the paper-based microreactor to investigate cellular phosphorylation of liver cancer cells (cell line: Huh7) stimulated by IL-6 cytokine. The signaling pathway of liver cancer cells stimulated by IL-6 cytokine is illustrated in Figure S2 (Supporting Information).

Before the investigation of cellular phosphorylation, two functional proteins, i.e., Erk1/2 kinase and Stat3 transcription factor, of Huh7 cells were detected by the paper-based microreactor to show the detection capacity. The Erk1/2 kinase is involved in functions including the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells. The Stat3 transcription factor is phosphorylated in response to cytokine stimulation and translocates to the cell nucleus, where acts as transcription activator. Both functional proteins are expressed at low levels, compared with GAPDH and actin. By using the paper-based microreactor, we could successfully detect Erk1/2 and Stat3 expressions by their specific primary antibodies, namely, anti-Erk1/2 antibody and anti-Stat3 antibody, as shown in Figure 5. Linear correlation

(R^2 = 0.8701 for Erk1/2 expression and R^2 = 0.6359 for Stat3 expression) between protein expressions and cell number was shown and the functionality of cells was confirmed. Next, activation level of phosphorylated Stat3 of Huh7 cells was investigated by the stimulation of IL-6 cytokine. From recent studies, constitutively activating IL-6/Stat3 signaling pathway has been detected in liver cancer and is considered an important factor for cancer initiation, development, and progression.[28,29] In this study, cells at different initial cell numbers were respectively seeded and cultured in each zone for 1 day. Then, cells were treated with different concentrations of IL-6 cytokine for 30 min. Then, activation level of phosphorylated Stat3 was detected by the antiphospho-Stat3 primary antibody and the results is shown in Figure 6. It revealed that higher activation level of phosphorylated Stat3 was triggered by higher concentration of IL-6 stimulation. In order to detect the low activation level, there was a trade-off of the saturation of high activation level. Detection signal showed plateau at 10 000 and 20 000 cells under high IL-6 stimulation of 100 ng/mL, which is not usually used in the cancer study.[30,31] In addition, study of the activation level of phosphorylated Stat3 under different IL-6 stimulation durations was performed. Cells at the initial cell number of 5000 and IL-6 in the concentration of 10 ng/mL were used for this study. Activation level of phosphorylated Stat3 showed to be increased by the increase of the IL-6 stimulation duration, as shown in Figure 7. The activation level was linearly correlated

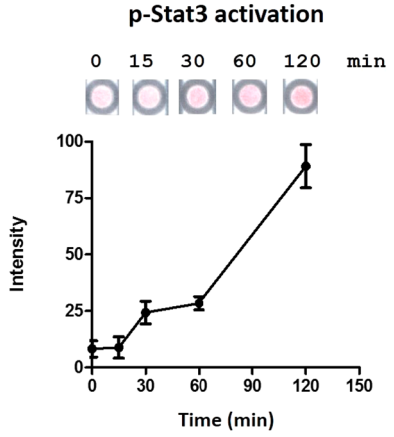

Figure 7. Activation level of phosphorylated Stat3 (p-Stat3) of Huh7 cells stimulated by IL-6 cytokine for various stimulation durations. Cells at the initial cell number of 5000 were cultured for 1 day and then treated with IL-6 cytokine in the concentration of 10 ng/mL. The p-Stat3 activation was quantified by the paper-based microreactor. Error bars represent standard deviation of at least 3 repeated experiments.

to the stimulation duration up to 120 min. The *R*-square values were calculated as 0.8719. Compared to the conventional bioanalytical technique (i.e., Western blotting), the signal showed a plateau above the stimulation duration of 20 min, as shown in Figure 8. The proposed paper-based cell culture and subsequent immunoassay technique is a promising quantitative bioanalytical method and showed to have higher detection dynamic range and comparable detection limit.

4. CONCLUSIONS

A paper-based microreactor integrating cell culture and subsequent immunoassay was developed, and a practical

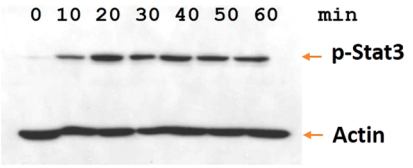

Figure 8. Activation level of phosphorylated Stat3 (p-Stat3) of Huh7 cells stimulated by IL-6 cytokine in the concentration of 10 ng/mL for various stimulation durations. The p-Stat3 activation was quantified by Western blotting technique.

application of the study of cellular phosphorylation of liver cancer cells stimulated by IL-6 cytokine was demonstrated. Results revealed that cells could be seeded and cultured in the paper-based microenvironment. Subsequently, protein expressions including structural, functional, and phosphorylated proteins could be detected by the developed technique. The entire process including cell culture and subsequent immunoassay was integrated on a single paper. Therefore, tedious bioanalytical operations could be highly reduced to eliminate human failure. Sample and reagent were conserved for each analysis. Also, the time required for the entire process could be shortened. This work provides a simple and rapid screening tool for the investigation of cellular phosphorylation and signaling pathway for understanding the pathogenesis of cancer. In addition, it has the potential to be developed for routine protocol for various research areas in conventional bioanalytical laboratories.

■ ASSOCIATED CONTENT

Supporting Information

Cell proliferation in the paper-based microreactor; experiment of cell proliferation study in the paper-based microreactor; signaling pathway of liver cancer cells stimulated by IL-6 cytokine; illustration of IL-6 signaling pathways. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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