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Microplatform for Intercellular Communication

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***Abstract*— A microplatform was designed, fabricated, and tested for demonstrating the propagation of molecular signals through a line of patterned HeLa cells expressing gap junction channels (HeLa Cx43 cells). The microplatform was capable of patterning cells onto a predefined design with lithography and surface chemical treatment. Lucifer Yellow was first used as a fluorescent marker to demonstrate the formation of functional gap junction channels between patterned HeLa Cx43 cells. The cells at one end of the cell line were next chemically stimulated to induce the propagation of intercellular calcium waves along the cell line, which was successfully monitored with Fluo4. The designed microplatform allowed intercellular communication over an arbitrary network topology of cells, which may provide new insight into mechanisms of intercellular communication.**

Keywords: microplatform, photolithography, cell-patterning, intercellualr calcium waves

I. INTRODUCTION

Experimental studies of intercellular communication are often performed in the randomly seeded *in vitro* environment (e.g., see [1,2,3,4] for experimental studies of intercellular calcium waves), although in the *in vivo* environment mammalian cells form a complex structure to communicate and coordinate their activities. As demonstrated in a recent theoretical study [5], the cellular network topology is an important factor influencing the nature of intercellular communication in tissues and organs, motivating the need of more sophisticated experimental methods for intercellular communication.

In this paper, we report the design, fabrication, and application of a microplatform that is capable of patenting mammalian cells onto a predefined geometry and that allows observation of intercellular communication between patterned cells through fluorescence microscopy. The designed microplatform allows intercellular communication over an arbitrary cellular topology, and therefore it may help provide new insight into mechanisms of intercellular communication. In addition, controlled intercellular communication demonstrated by means of the designed platform is potentially useful to design and engineering of novel biological devices such as cell-based bio-sensors [6] and synthetic biological systems [7, 8].

The microplatform for intercellular communication was designed by utilizing photolithography and surface chemical treatment [9]. A gold layer with specific geometry was first deposited on a glass substrate, and the resultant Au/SiO_2 substrate was treated with polyethylene glycol-5000' thiol (PEG-5000' thiol). PEG has known to exhibit low toxicity and poor binding of proteins [10], and the thiol links allow PEG to attach to only the Au layer of the substrate, thereby making the gold layer protein-resistant while the glass surface remains protein-adhesive. HeLa cells (human cervical cancer cells) expressing gap junction channels [11] (HeLa Cx43 cells) were then cultured on the microplatform and successfully patterned on the Au surface, so that a desired spatial pattern of HeLa Cx43 cells was established. A patterned HeLa Cx43 cell was microinjected with fluorescent molecules that propagated from cell to cell, indicating that functional gap junction channels were present among patterned HeLa Cx43 cells. Finally, a line of patterned HeLa Cx43 cells was chemically stimulated at one end, resulting in the propagation of intercellular calcium waves along the line to the other end.

II. EXPERIMENTAL DESIGNS

Microplatform Fabrication

Figure 1 illustrates the designed single mask micro-fabrication process by utilizing lift-off technique. Three and half micrometers thick Shipley 1827 positive photoresist was spun on a 0.17 mm thick glass substrate. After ten minutes soft back, the line patterns were defined by photolithography method with soft-contact exposure and MF319 developer. A 100 Å-thick titanium (Ti) layer was then deposited over the photoresist patterns through E-beam evaporation followed by the evaporation of a layer of about 400 Å-thick gold (Au). The Ti was used as an adhesion layer between Au and glass. The overall thickness of this metal film was controlled lower than 500 Å to serve as a semi-transparent thin film. This provided an optical window that helped to monitor and confirm the successfulness of controlling the distribution of cells. The final pattern of Au/Ti layer was created by lift-off technique in acetone solution, yielding 7 sets of gold lines with line widths of 10, 20, 30, 45, 60, 75, and 90 um. The fabricated glass slides were cleaned and autoclaved before applying self-assembly monolayer.

Self-Assembled Monolayer (SAM) Preparation

A 0.1 mM PEG-5000' thiol solution was prepared by diluting *O*-[2-(3-Mercaptopropionylamino)ethyl]-*O*'-methyl-poly-ethylene glycol 5000 (Sigma-Aldrich, 11124) with pure

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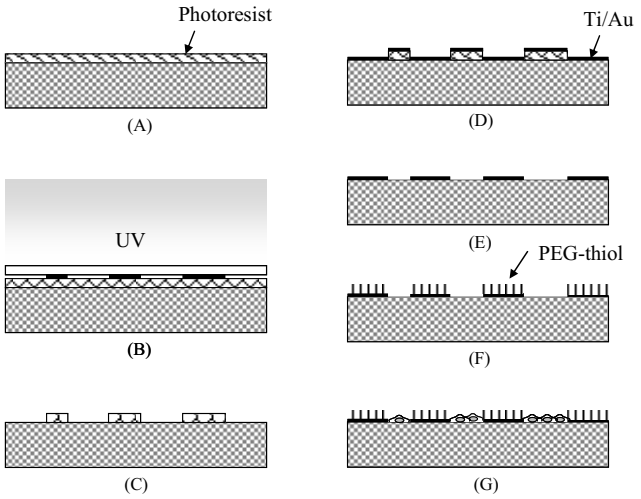

Figure 1. Cross section drawings of the process flow: (A) Photoresist is spun onto a glass substrate, (B) line patterns are defined by photolighography technique, (C) a photoresist pattern is developed, (D) Ti and Au thin films are deposited by a E-beam evaporator, (E) the final pattern is created by lift-off technique, (F) PEG-5000' thiol SAM layers is formed on the metal film, (G) distribution of cells is controlled by the patterned and modified Ti/Au metal lines (see Figure 2).

ethanol. The fabricated glass slides with Au patterns were immersed in the solution in a small tube to allow the formation of SAM on the Au patterns. A paraffin film was used to cover the tube to minimize exposure to atmospheric oxygen, which would otherwise result in oxidizing the PEG. The tube was also covered with aluminum foils to eliminate light exposure, which would accelerate PEG oxidization. After 48 hours, the glass slides were removed from the solution and washed with pure ethanol. The slides were then dried and placed inside a culture dish for cell culture.

Cell Culture

HeLa Cx43 cells (source: Dr. K. Willecke of Institut für Genetik, Germany) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (Gibco), 100 i.u./ml penicillin and 100 mg/ml streptomycin (Sigma). The cells were incubated in 10 cm culture dishes at 37°C under 5% CO_2. For fluorescence imaging of patterned cells, a fabricated slide treated with the PEG-5000' thiol solution was first placed in a 35 mm glass-bottom dish (Iwaki, 3910-035). Cells were next transferred to the dish and cultured on the fabricated slide under the same culture conditions. After 3 hours, the cells were washed with the supplemented DMEM, and unattached cells were removed. Cells were then grown for 2 to 3 days prior to fluorescence imaging of intercellular Ca^{2+} waves.

Microinjection of Lucifer Yellow

Cells were microinjected with Lucifer Yellow (Molecular Probe, L453) as a 4% solution in distilled water. The molecular weight is 443 and it has two negative charges. This molecular probe has high fluorescence efficiency and is capable of propagating from cell to cell in the presence of functional gap junction channels [11].

Calcium imaging

Intercellular calcium levels were monitored using a calcium indicator, Fluo4. Cells were first loaded with 2.0 μM of Fluo4/AM (Molecular Probes, F14201) in Hank's Balanced Salt Solution buffered with 25 mM N-2-Hydroxyethylpiperazine- N'-2-Ethanesulfonic Acid containing Ca^{2+} [HBSS-HEPES (+)] for 1 hour at room temperature. The cells were then washed twice and the culture media was replaced with 25 mM N-2-Hydroxyethylpiperazine- N'-2-Ethanesulfonic Acid containing no Ca^{2+} [HBSS-HEPES (-)]. To allow de-esterification of AM esters, cells were left 30 minutes prior to fluorescence measurements.

Flash-photolysis of caged-ATP

Caged-ATP (Dojindo, 349-05501) was introduced into the HBSS-HEPES (-) buffer to photo-release ATP to locally initiate intercellular Ca^{2+} waves. Prior to fluorescence measurements, caged-ATP was added to the buffer to make the final concentration of 5mM. In fluorescence measurements, ATP was photo-released by laser spot illumination with 406 nm wavelength. A selected spot was exposed for 0.5~1.0 sec.

Fluorescence measurements

Fluorescence measurements were performed using a fluorescence microscope (the DeltaVision microscope system, Applied Precision, Inc., Seattle, WA). For imaging of propagating Lucifer Yellow, 430/528 wavelengths were used for excitation/emission, and a fluorescence image was obtained 3~5 minutes after microinjection. For imaging of Fluo4, 488/528 wavelengths were used for excitation/emission, and fluorescence images were obtained every 1~2 sec.

III. RESULTS

Cell Patterning

HeLa Cx43 cells were cultured on the platform about 4 days. 3 hours after cells were seeded, cells on the gold surface, which were deemed cell-resistant by PEG, were found rounded and easily removed by gentle wash out, while cells on the glass surface, which were cell adhesive, remained attached. (See Figure 2, 4 hours of cell culture). After 1~2 days of cell culture (see Figure 2, 24 and 48 hours of cell culture), remaining cells proliferated on the glass surface, and little or no cells were found on the gold surface. In our experiments at this point of time (1~2 days), about 1 cell was fit into a 10 um line width, 1~2 cells into a 20 um line width, 2 cells into a 30 um line width, 2~3 cells into a 45 um line, and more cells into 60, 75, 90 um lines. The cellular pattern remained stable for another day. After 4 days, however, cells started to grow and move onto the gold surface (see Figure 2, 100 hours of cell culture) and eventually became semi-confluent on both gold and glass surfaces. The breakdown of cellular patterns can be caused by oxidization and degradation of PEG. Alternatively,

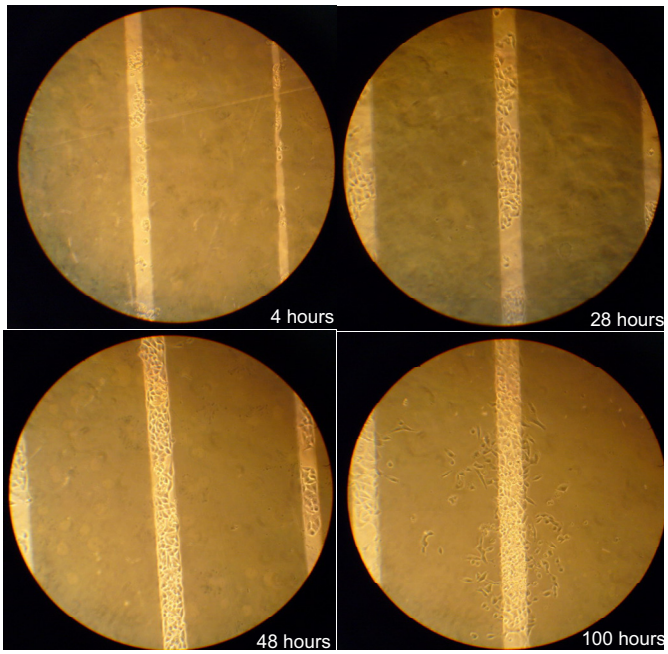

Figure 2: HeLa Cx43 cells cultured for 4, 28, 48, and 100 hours on the designed platform. 4 hours: Cells on the glass surface (bright area) remained attached while little cells were found on the PEG-coated gold surface (dark area). 28 and 49 hours: Cells proliferated on the glass surface while little cells were still found on the PEG-coated gold surface. 100 hours: cells started to migrate and proliferate on the gold surface.

HeLa cells may secrete ECM proteins to enhance adhesion to the gold areas and/or secrete enzymes that can degrade PEG [12]. (It is noted that HeLa cells are highly adhesive and its long-term patterning is not easy [13].)

Propagation of Lucifer Yellow

Figure 3 top shows cells patterned into a straight line with 30 μm of line width. (The picture was obtained after 2 days of cell culture.). Figure 3 bottom shows an experimental result of microinjection of Lucifer Yellow into the marked cell. The photograph showed that Lucifer Yellow has propagated from cell to cell, indicating that functional gap junction channels were formed between patterned cells on the designed microplatform. Cell-cell propagation of Lucifer Yellow between patterned cells was observed 6 times out of a total of 8 microinjection experiments. This result is highly comparable with those from a conventional coupling assay using Lucifer Yellow. For example, it is reported in [14] that about 70% of Lucifer Yellow microinjection into HeLa Cx43 cells resulted in cell-cell propagation.

Propagation of Calcium Waves

Figure 4 shows successful imaging of intercellular Ca^{2+} waves between patterned HeLa Cx43 cells on the designed microplatform. The Ca^{2+} waves were initiated and propagated in response to photo-release of ATP. The five images shown in Figure 4 were obtained using a fluorescence microscope before flash photolysis of caged-ATP and 2, 8, 15, 20 seconds after flash photolysis of caged-ATP. Figure 5 shows the time

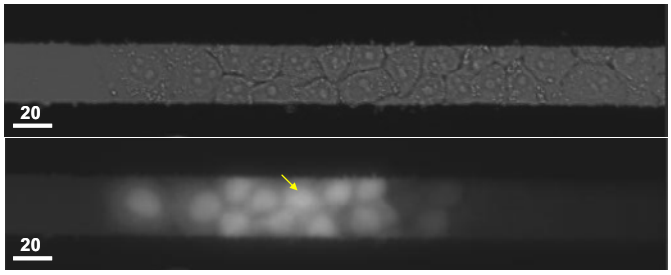

Figure 3: Propagation of Lucifer Yellow over patterned HeLa Cx43 Cells. Dark areas represent gold surfaces and gray areas glass surfaces. Top: a transmitted image before Lucifer Yellow microinjection. Bottom: Lucifer Yellow was microinjected into the cell indicated by the arrow, which propagated cell-cell through gap junction channels (Cx43 channels).

course of fluorescence intensity of six randomly selected cells (cells A, B, C, D, E, F) in Figure 4. In this experiment, the center of cell A was flashed by a laser spot illumination, and therefore ATP was photo-released locally around cell A. The cell A instantly increased its cytosolic Ca^{2+}. The increase in Ca^{2+} level then propagated along the cell line (e.g., cells A, B, C, D, E) at a rate of about 5 μm/sec, and reached the cell that was 10 cells away from the flashed cell (cell A). As shown, imaging of intercellular communication on the designed platform was successful.

The mode of intercellular Ca^{2+} wave propagation in our experimental setup is not determined, and further experiments are needed to discuss possible mechanisms of intercellular Ca^{2+} wave propagation. In the literature, several models of intercellular Ca^{2+} wave propagation were proposed. In one model [15], IP_3 (i.e., Ca^{2+} mobilizing molecules) is generated in response to stimulating agents (chemical or mechanical stimulus), diffuses through gap junction channels from cell to cell, and stimulates Ca^{2+} stores (ER: Endoplasmic Reticulum) of each cell, which triggers Ca^{2+} release from the stores at each cell. As IP_3 diffuses cell-cell, intercellular Ca^{2+} waves propagate. In the other model [16], ATP is produced by stimulus, released into the extracellular environment, and reacts with membrane receptors of each cell. Each cell then produces Ca^{2+} mobilizing molecules (e.g., IP_3) and triggers Ca^{2+} release from Ca^{2+} stores. As ATP diffuses in the extracellular environment, intercellular Ca^{2+} waves propagate. In some other model, Ca^{2+} itself propagates through gap junction channels and causes Ca^{2+} release at each cell (calcium induced calcium release), thereby generating self-regenerative Ca^{2+} waves. Further, Ca^{2+} may positively or negatively interact with IP_3 [17], and ATP may be regenerated [18]. In order to determine the mode of intercellular communication in our experimental setup, further experiments need to be conducted. Using the designed microplatform, it's now possible to examine how different network connectivity and topology may affect intercellular communication between cells. Experimental data acquired from the designed microplatform can be used to test various hypotheses as well as to verify theoretical models to determine the mode of intercellular Ca^{2+} wave propagation.

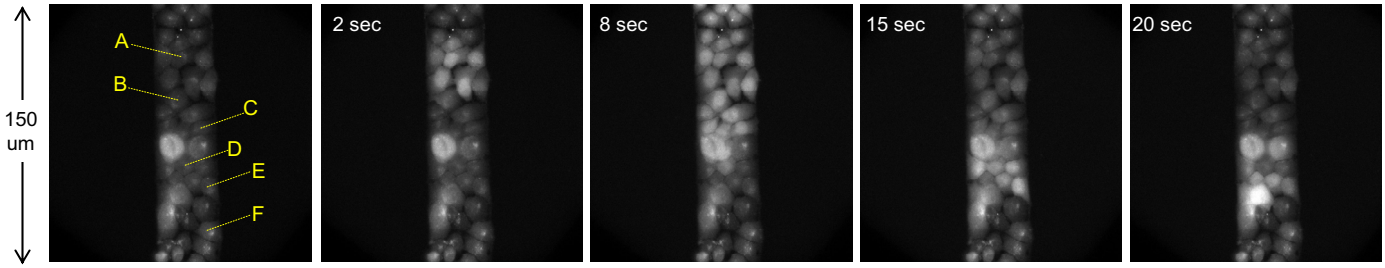

Figure 4: Intercellular Ca^{2+} waves propagating over patterned HeLa Cx43 cells. Flash photolysis of caged-ATP was used to initiate intercellular Ca^{2+} waves. Cell A was flashed, which increased the Ca^{2+} level. The increased Ca^{2+} level propagated along the straight line about 5 um/sec, and reached the cell that was 10 cells away from the flashed cell (cell A). The images shown were obtained before flash photolysis of caged-ATP and 2, 8, 15, 20 seconds after flash photolysis of caged-ATP using a fluorescence microscope.

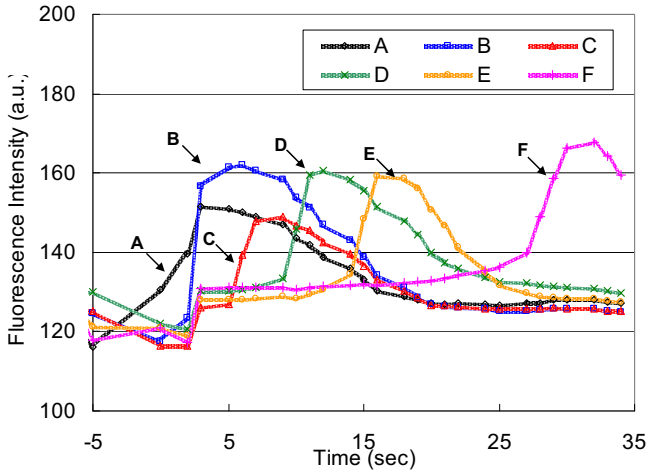

Figure 5: Fluorescence intensity of cells A-F in Figure 4

IV. Conclusions

We have designed and demonstrated a microplatform with a single-mask photolithographic process that allowed cells to be patterned in a pre-defined geometry. The microplatform was successfully used to study propagation of signal molecules along a line of cells patterned on the platform. The designed microplatform allowed signaling over an arbitrary network topology of cells, which may provide new insight into mechanisms of intercellular communication.

References

[1] S.O. Suadicani, C. E., Flores, M. Urban-Maldonado, M. Beelitz M, and E. Scemes E, "Gap junction channels coordinate the propagation of intercellular Ca^{2+} signals generated by P2Y receptor activation," *Glia*, vol. 48, issue 3, pp. 217-29, 2004.

[2] K. Paemeleire, P. E. M. Martin, S. L. Coleman, K. E. Fogarty, W. A. Carrington, L. Leybaert, R. A. Tuft, W. H. Evans, and M. J. Sanderson, "Intercellular calcium waves in HeLa cells expressing GFP-labeled connexin 43, 32, or 26," *Mol Biol Cell*, vol. 11, issue 5, pp 1815-1827, 2000.

[3] K. Braet, W. Vandamme, P. E. Martin, W. H. Evans, and L. Leybaert, "Photoliberating inositol-1,4,5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26," *Cell Calcium*. vol. 33, issue 1, pp. 37-48, 2003.

[4] C. Giaume and L. Venance, "Intercellular calcium signaling and gap junctional communication in astrocytes," *Glia*, vol. 24, issue 1, pp. 50 – 64, 1998.

[5] I. V. Dokukina, M. E. Gracheva, E. A. Grachev and J. D. Gunton, "Role of mitochondria and network connectivity in intercellular calcium oscillations," Physica D (2007).

[6] J. J. Pancrazio, J. P. Whelan, D. A. Borkholder, W. Ma, D. A., Stenger, "Development and application of cell-based biosensors," *Ann Biomed Eng*., vol. 27, pp. 697-711, 1999.

[7] T. Nakano, T. Suda, M. Moore, R. Egashira, A. Enomoto, and K. Arima, "Molecular communication for nanomachines using intercellular calcium signaling," *Proc. IEEE NANO 2005*, June 2005.

[8] S. Hiyama, Y. Moritani, T. Suda, R. Egashira, A. Enomoto, M. Moore and T. Nakano, "Molecular communication," *Proc. 2005 NSTI Nanotechnology Conference*, 2005.

[9] A. Folch and M. Toner, "Microengineering of cellular interactions," *Annu. Rev. Biomed. Eng*, vol. 2, pp. 227-256, 2000.

[10] B. Zhu, T. Eurell, R. Gunawan, D. Leckband, "Chain-length dependence of the protein and cell resistance of oligo(ethylene glycol)-terminated self-assembled monolayers on gold," *J Biomed Mater Res*., vol. 56, pp. 406-16, 2001.

[11] C. Peracchia (Ed), *Gap junctions: molecular basis of cell communication in health and diseases*, Academic Press, 2000.

[12] D. I. Rozkiewicz, Y. Kraan, M. W. T. Werten, F. A. de Wolf, V. Subramaniam, B. J. Ravoo, D. N. Reinhoudt, "Covalent microcontact printing of proteins for cell patterning," *Chemistry*, vol. 12, pp. 6290-6297, 2006.

[13] M. Nishizawa, A. Takahashi, H. Kaji, and T. Matsue, "Micropatterned HeLa Cell Culture on PEG Monolayer-Coated Glass Substrates," *Chem. Lett.*, vol. 9, pp. 904-905, 2002.

[14] C. Elfgang, R. Eckert, H. Lichtenberg-Frate, A. Butterweck, O. Traub, R.A. Klein, D. F. Hulser and K. Willecke, "Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells," *J. Cell Biol.*, vol. 129, pp. 805-817, 1995.

[15] M. J. Sanderson, "Intercellualr waves of communication," *News Physiol. Sci.*, vol. 11, 1996.

[16] M. V. Bennett, J. E. Contreras, F. F. Bukauskas, and J. C. Sáez, "New roles for astrocytes: gap junction hemichannels have something to communicate," *Trends Neurosci*, vol. 26, no. 11, pp. 610-617, 2003.

[17] R. W. Tsien and R. Y. Tsien, "Calcium channels, stores, and oscillations," Annu. Rev. Cell Biol. Vol. 6 , pp 715-760, 1990.

[18] C. M. Anderson, J. P. Bergher, and R. A. Swanson, "ATP-induced ATP release from astrocytes," *J Neurochem*, vol. 88, pp 246-256, (2004).