Patterning Adherent Cells within Microchannels by Combination of Electrochemical Biolithography Technique and Repulsive Dielectrophoretic Force

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We describe a method to create cell-adhesive regions and to position adherent cells on the newly created regions in sequence within a microfluidic channel. One of the microelectrodes fabricated at the channel wall was used for locally electrogenerating hypobromous acid that renders the opposite face of the channel protein-adsorptive. After the fibronectin was immobilized on the treated region, an ac voltage (1 MHz, 20 Vpp) was applied to the microelectrodes array in the presence of suspended HeLa cells. Since a repulsive force of negative dielectrophoresis (DEP) directs the cells toward the weakest region of the nonuniform electric field, the cells were positioned on the fibronectin-patterned region to allow the cell adhesion, even in the presence of fluid flow (< 0.1 μL min⁻¹). By repeating the above process, two types of cells could be patterned in the microchannel.

Key Words: Cell Adhesion, Dielectrophoresis, Electrochemical Biolithography, Microfluidic Device

1 Introduction

Microfluidic systems have been widely used for cell-based assays and bioreactors since they offer several advantages including efficient delivery of drug candidates and nutrition to cells cultured in the microfluidic channel.¹² Controlling the location of adherent cells on the channel surface is necessary to exploit such advantages. Early studies addressed this matter by patterning cells³ or cell-adhesive matrices⁴⁵ before the device assembly. Recent efforts employing dynamic surface chemistry⁶⁷ for miniaturized systems enabled locally creating cell-adhesive regions in preassembled microfluidic devices.⁸ This approach is much suitable to pattern cell-adhesive proteins (subsequently cells) on the channel surface because such unstable materials are not subjected to denaturing conditions such as desiccation and heat usually accompanied with the device assembly. However, since cells introduced into the microchannel are randomly distributed, only cells that happen to settle on the adhesive regions can attach to the surface. Meanwhile, cells on nonadhesive regions are rinsed away after waiting for the cell adhesion on the adhesive regions.

To efficiently induce patterned cell adhesion on the channel surface, it will be required for cells to be placed only on the adhesive regions. There are several techniques available that can be used to manipulate living cells. Optical tweezers technique can be used to manipulate single cells, while it requires a special optical equipment which is not commonly available.⁰¹⁰ Although large numbers of cells can be manipulated by using magnetic forces, the prior binding of magnetic materials to the cells are needed.¹¹¹² Among these techniques, cell manipulation by dielectrophoresis (DEP), wherein ac electric fields from shaped electrodes produce forces by coupling to the induced electric dipole moments of the cells, is readily applicable to microfluidic system by assembly of the device with microelectrode arrays.¹³¹⁰

![Fig. 1 Schematic representation of cell patterning within a microchannel by sequentially applying electrochemical biolithography and negative DEP.](image)

(a) Microfluidic channel

(b) Pt electrode

(c) Br⁻ HBrO

(d) 1 MHz, 20 Vpp

(e) HeLa cell

Fig. 1 Schematic representation of cell patterning within a microchannel by sequentially applying electrochemical biolithography and negative DEP. (a) A microchannel equipped with a parallel array of three microband electrodes on the inner wall. (b) Generate HBrO at the central electrode to make a protein-adsorptive region on the opposite face of the channel. (c) Immobilize cell-adhesive protein, fibronectin, on the created region. (d) Manipulate cells on the cell-adhesive region by repulsive force of negative DEP. (e) Remove nonadherent cells.
In this paper, we describe a method to create cell-adhesive regions and to position adherent cells on the newly created regions in sequence within a microfluidic channel by combination of electrochemical lithography technique \(^{17}\) with negative DEP \(^{13-15}\) (Fig. 1).

2 Experimental

2.1 Materials

Polyethyleneimine (PEI, average $M_w$ 600, Wako Pure Chemical Industries Ltd.), heparin (sodium salt, Wako Pure Chemical Industries Ltd.), fibronectin (from human plasma, CHEMICON International), 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer (NOF Corporation), Cy3 reactive dye (Ab Cy3 labeling kit, GE Healthcare Ltd.), CellTracker\textsuperscript{TM} Green, CellTracker\textsuperscript{TM} Orange (Invitrogen), and all other chemicals were used without further purification or modification.

2.2 Microfluidic device fabrication

The device consists of three layers: a microelectrodes-bearing substrate, silicone rubber, and a glass slide. A parallel array of three Pt microband electrodes (20 $\mu$m band width, each separated by 50 $\mu$m) and a counter electrode were fabricated on a glass slide by a series of photolithography-based microfabrication technologies. The electrode-bearing substrate was previously coated with 0.5 wt% MPC polymer/ethanol to prevent nonspecific adsorption of proteins. The silicone rubber has a stenciled pattern that forms the channel when sandwiched between the electrode substrate and the glass slide. The resulting microchannel is 400 $\mu$m in width and 100 $\mu$m in depth. The inlet for the reservoir and the outlet for aspiration via syringe pump were provided at either end of the channel. The potential of the central electrode of the parallel array was referred to the Ag/AgCl reference electrode placed at the inlet reservoir.

2.3 Cell culture

HeLa cells were cultured in GIT medium (Wako Pure Chemical Industries) containing 50 $\mu$g mL\(^{-1}\) penicillin and 50 $\mu$g mL\(^{-1}\) streptomycin at 37°C under a 5% CO\(_2\) atmosphere. A subconfluent cell monolayer was dissociated with a 0.25% trypsin solution, then suspended in fresh medium and finally introduced into the microchannel at a cell density of $2 \times 10^6$ cells mL\(^{-1}\).

2.4 Patterning cells within microchannel

Solutions of PEI (5 mg mL\(^{-1}\)) and heparin (2 mg mL\(^{-1}\)) were passed through the microchannel, each in turn for 20 min, to form an antifouling layer of PEI and heparin (PEI/Hep) on the channel surface. Subsequently, 0.1 M phosphate buffered saline (PBS) containing 25 mM KBr was introduced into the channel and a potential pulse of 1.7 V vs. Ag/AgCl with a period of 0.5 sec was applied to the central electrode of the parallel array (Fig. 1b). This procedure resulted in electrochemical oxidation of Br\(^-\) to Br$_2$ (subsequently HBrO) at the electrode to give a protein-adsorptive region on the opposite face of the channel \(^{17}\). Then, a solution of fibronectin (25 $\mu$g mL\(^{-1}\)) was added, followed by a wash with PBS (Fig. 1c). Subsequently, a medium (13 mS cm\(^{-1}\)) containing HeLa cells was introduced into the channel, and an ac voltage (20 Vpp, 1 MHz) was applied to the electrodes array with a function generator to concentrate cells on the fibronectin-patterned region by a repulsive force of negative DEP \(^{13}\) (Fig. 1d). After the DEP operation for 5 min, cell-free medium was flowed through the channel to remove nonadherent cells (Fig. 1e).

3 Results and Discussion

Figure 2 shows HeLa cells patterned within a sealed microchannel according to the procedure shown in Fig. 1. The electrochemical lithographic treatment was first conducted to create cell-adhesive region on the channel surface by using the central electrode of the parallel array at the upper wall (Fig. 2a). As seen in Fig. 2b, line pattern of Cy3-labeled fibronectin (line width, 100 $\mu$m) was formed on the bottom wall, opposite face of the electrode. Subsequently, a suspension of HeLa cells was introduced into the channel, followed by the application of ac voltage (1 MHz, 20 Vpp) between the central electrode and the other two electrodes. Since HeLa cells experience a repulsive force in GIT medium under the 1

![Fig. 2](image-url) (a) Micrograph of a parallel array of three microband electrodes fabricated at the upper wall of the microchannel. (b, c) Fluorescence images of (b) fibronectin (labeled with Cy3) locally immobilized on the channel bottom wall and (c) HeLa cells (stained with CellTracker Green) adhered on the fibronectin region after applying the DEP force. The dotted lines in the images denote the position of the side wall of the channel. (d) Plot of the numerically calculated electric field in the horizontal plane at the channel bottom wall when the potential difference between the central electrode and the other two electrodes at the upper wall was set at 20 V. The electric field distribution was simulated using the finite element method and FEMLAB software.
MHz ac electric field, cells rapidly moved to regions of weaker electric fields within 15 sec, especially to the fibronectin-immobilized region just below the central electrode. It was observed that the cells adhere on fibronectin after incubation for 5 min under the ac voltage application (Fig. 2c). Meanwhile, cells concentrated on other regions of weaker electric fields (the foot of each peak in Fig. 2d) were removed by flowing the medium since they could not adhere to the antifouling layer of PEI/Hep. No damage was observed in a live/dead staining test on HeLa cells adhered on fibronectin, indicating that the cell manipulation by negative DEP has no significant influence on this cell line.

Figure 3 shows the plots of the capture efficiency of HeLa cells in the fibronectin-immobilized region with (●) or without (○) applying the DEP force versus the flow rate. The capture efficiency was evaluated as a rate of the number of cells attached to the number of whole cells flowing for 5 min. Without applying the DEP force, HeLa cells could hardly attach to the fibronectin-immobilized region in the presence of fluid flow. Even at a flow rate of 0.01 μL min⁻¹ (the slowest case in this study), the capture efficiency was only about 20%. On the other hand, with applying the DEP force, the cell capture efficiency was almost 100% at flow rates lower than 0.1 μL min⁻¹. As seen in Fig. 3b, a line pattern of cells was formed on the fibronectin-immobilized region just below the central electrode and its width became larger gradually as time passed. For flow rates higher than 0.1 μL min⁻¹, the capture efficiency suddenly began to decrease, since the rheological force becomes stronger and drags cells against the DEP force.¹⁰

Figure 4 shows an array of two types of HeLa cells patterned in the microchannel by repeating a set of the electrochemical lithographic treatment and the cell manipulation by the negative DEP. After the first cell type (stained with CellTracker Green) was immobilized at the right side of the channel (Fig. 4a), the second cell type (stained with CellTracker Orange) was trapped in the fibronectin-immobilized region (left side of the channel) at a fluid flow rate of 0.1 μL min⁻¹ by using another set of the microelectrodes array. Since most of the cells flowing from the upstream (left side of the channel) can be captured on the fibronectin region and, even if some cells happen to flow to the downstream, the cells hardly attach on the prepatterned region of the first cell type in the presence of the fluid flow (see Fig. 3a), the two types of cells could be localized in the single channel without cross contamination of these cell types (Fig. 4b). The pattern width of the second cell type was different from that of the first cell type, because the cell suspension of the second cell type flowing through the channel happened to be ununiform during applying the DEP force. However, by culturing cells to induce their growth, the cells can satisfactorily represent the underlying fibronectin pattern.

In summary, we have described a method to create cell-adhesive regions and to position adherent cells on the newly created regions in sequence within a pre-assembled microfluidic device by combination of electro-
chemical biolithography and negative DEP. Since the electrodes fabricated on the channel surface can be used both for electrogenerating the oxidant and for producing DEP forces, the electrode configuration is very simple enough not to sacrifice miniaturization of microfluidic systems. Also, since the application of the DEP force in the presence of an appropriate fluid flow enabled the efficient capture of HeLa cells only on the created cell adhesive regions, two types of cells could be patterned in the single channel with reducing cross-contamination of the cell types.

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