Cell Micropatterning inside a Microchannel and Assays under a Stable Concentration Gradient

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Running title: Control of cell microenvironments in a microchannel

1 **Abstract**

2 We describe the use of a microfluidic device to micropattern cells in a microchannel and
3 investigated the behavior of these cells under a concentration gradient. The microfluidic
4 device consisted of 3 parts: a branched channel for generating a stable concentration
5 gradient, a main channel for culturing cells, and 2 side channels that flowed into the
6 main channel. The main channel was coated with a cross-linked albumin that was
7 initially cell-repellent but that could become cell-adherent by electrostatic adsorption of
8 a polycation. A sheath flow stream, which was generated by introducing a polycation
9 solution from the branched channel and a buffer solution from the 2 side channels, was
10 used to change a specific region in the main channel from cell-repellent to cell-adhesive.
11 In this way, cells attached to the central region along the main channel. The remaining
12 surface was subsequently changed to cell-adhesive, thereby facilitating cell migration
13 from a fixed location under a concentration gradient. We demonstrated that with this
14 device, the gradient generator could be used to conduct simultaneous cytotoxic assays
15 with anticancer agents; further, by combining this device with cell micropatterning,
16 migration assays under a concentration gradient of biological factors could be
17 conducted.

18

1 **Keywords**: Microfluidics, Concentration generator, Albumin, Micropatterned coculture,

2 Migration assay

3

1 **Introduction**

2 Microfluidic cell assays have the potential to revolutionize methodologies in diverse
3 fields ranging from fundamental biological studies to drug discovery (1–3).
4 Microfluidics technology has already provided some unprecedented approaches to
5 scientific studies, such as conducting assays in controlled microenvironments to the
6 subcellular level and high-throughput analyses that test many factors simultaneously
7 (4–7). Gradient generators have been proposed to form a stable concentration gradient
8 of biological factors by using a branched microchannel; these generators have been used
9 to investigate cell responses (8–10). This approach is expected to improve our
10 understanding of cell behaviors in vivo because concentration gradients govern a
11 diversity of biological processes including embryonic development, tissue formation,
12 and wound healing (11–13). In a typical culture system using a gradient generator, cell
13 behaviors are evaluated microscopically by continuously tracking cells that are
14 randomly distributed at the entire bottom surface of a microchannel (10), making this
15 approach relatively labor-intensive, expensive, and complicated. In addition, in most
16 previous approaches, assays have been conducted using a single cell type. Cells in vivo,
17 however, are surrounded by other cell types. Thus, much attention has recently been
18 given to approaches that spatially localize the cells of one or more cell types in a

1 microchannel. These approaches are advantageous because cell microenvironments
2 such as heterogeneous cell-cell interactions can be controlled and because the distance
3 in migration assays can be evaluated simply by measuring cell displacement from an
4 initially adhered region. The approaches have been based on a number of fabrication
5 strategies, including cell seeding or detachment from a selective surface with multiple
6 laminar flows (14), switching of cell adhesion properties by specific stimuli such as
7 light and electrical potential (15, 16), use of dielectrophoresis (17, 18), and use of
8 geometrical configurations to create a low-shear region (19). These approaches may
9 offer a promising method of examining cell behavior under a concentration gradient.
10 Some of these approaches, however, require specialized materials and extensive
11 expertise to achieve good edge definition of cell patterns or are difficult to couple with a
12 gradient generator and are limited to predetermined topographical configurations. Thus,
13 an approach that is easily applicable, provides high resolution, and uses commercially
14 available materials is of benefit.
15 In this study, we describe an innovative method for preparing micropatterned single
16 cultures and cocultures in a microchannel coupled with a gradient generator. A
17 cross-linked albumin-coated surface is used to generate a spatially and temporally
18 switchable surface for selective adhesion of cells. Albumin can be coated in a

1 microchannel by simply introducing a mixed solution containing albumin and a
2 cross-linking agent, resulting in a cell-repellent surface. The subsequent electrostatic
3 adsorption of a positively charged polymer onto the surface switches the cell adhesion
4 property from nonadherent to adherent. The switching can be localized by using the
5 sheath flow stream of a positively charged polymer solution, thereby facilitating the
6 micropatterned cultures. We demonstrate in this study that the microsystem can be used
7 to evaluate cell migration in a micropatterned culture under a concentration gradient.
8

9 **Materials and Methods**

10 Materials and reagents

11 Swiss 3T3 murine fibroblasts (RCB1642), hepatoblastoma cells (HepG2, RCB1618),
12 and pheochromocytoma cells (PC12, RCB0009) were purchased from Riken Cell Bank,
13 Japan. The reagents used for cell culture were purchased from the following commercial
14 sources: Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS)
15 from Invitrogen, USA; bovine serum albumin (BSA) from Sigma, USA; ethylene glycol
16 diglycidyl ether (EGDE), 50% polyethyleneimine (PEI) aqueous solution, fluorescein
17 diacetate, and ethidium bromide from Wako, Japan; and type I collagen from Nitta
18 Gelatin, Japan. The materials used to fabricate the culture substrates were purchased

from the following commercial sources: negative photoresist SU-8 2050 from MicroChem Corporation, USA; poly(dimethyl siloxane) (PDMS) from Shin-Etsu Silicones, Japan; and silane-coated glass slides from Dako, USA. All other chemicals were purchased from Sigma, unless otherwise indicated.

Cell preparation

Fibroblasts and Hep G2 cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin and were passaged every 3 and 6 days, respectively. PC12 cells were maintained in low-glucose DMEM containing 15% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin and were passaged every 5 days. The media were changed every other day. All cell cultures were conducted at 37°C, 5% CO_2 in a humidified incubator.

Device fabrication

The microfluidic device shown in Fig. 1 is composed of 2 PDMS substrates and 1 glass substrate. The PDMS structures were fabricated using a replica molding process. Briefly, corresponding master molds were fabricated with negative photoresist (SU-8 2050) using photolithography. A PDMS replica of the master was molded by casting a liquid

prepolymer PDMS solution composed of a mixture of 10:1 silicone elastomers and a curing agent. The mixture was cured overnight at room temperature, and the PDMS replica was then peeled off from the master. The 2 PDMS substrates and the glass substrate were treated with oxygen plasma, which created an irreversible bond, and were used to form the microchannels (Fig. 1B). The microchannels in the upper PDMS trapped and removed air bubbles. The microchannels in the lower PDMS consisted of a branched channel for generating a concentration gradient, 2 side channels for patterning cells, and a main channel for culturing cells. The height of all the channels in the lower PDMS was 50 µm, and the widths of the branched channel, the side channels, and the main channel were 100 µm, 200 µm, and 1000 µm, respectively. The 5 inlets and 1 outlet were connected to a multi-syringe pump (AS One Corp., Japan) via silicone tubes. A stable concentration gradient was generated by mixing and dividing 2 solutions in the branched channel (20). Figs. 1C and 1D show the stream at the entrance of the main channel and 6 mm downstream and were visualized using 2 fluorescent solutions—resorufin (red) and fluorescein (green)—introduced from inlets 1 and 2, respectively. Fluorescent micrographs were taken with a microscope (IX71; Olympus, Japan).

1 Cytotoxic assays in a concentration gradient

2 Prior to cell seeding, the device was filled with 70% (v/v) ethanol to sterilize it and
3 remove air bubbles from the channels. Then, the channels were washed with phosphate
4 buffer saline (PBS) solution and replaced with culture medium. A cell suspension of
5 fibroblasts or Hep G2 cells at a density of 1×10^7 cells/ml was injected into the main
6 channel from inlet 3 to the outlet. The cells in the channel were left undisturbed for 3 h
7 at 37ºC in a CO_2 incubator, which allowed them to attach to the surface of the channel.
8 Then, the culture medium was perfused into the main channel from inlets 1 and 2 at 0.2
9 µl/min, and unattached cells were discarded from the device.
10 To generate a concentration gradient of a drug, culture media with and without the drug
11 (0.02% Triton X-100 or 20µg/ml mitomycin C) were injected from inlets 1 and 2 at 0.5
12 µl/min. Fibroblasts and Hep G2 cells were exposed to Triton X-100 for 10 min and
13 mitomycin C for 24 h, respectively. Cell viability after exposure was evaluated with a
14 live/dead fluorometric assay with fluorescein diacetate and ethidium bromide (21).
15 Similar experiments were conducted with conventional culture dishes by preparing a
16 series of drug concentrations.
17
18 Micropatterning cells in a microchannel

1 To localize 2 cell types or investigate migration of a single cell type from a
2 predetermined region in the main channel, we used a cross-linked albumin-coated
3 surface, which is initially cell-repellent but can become cell-adherent by electrostatic
4 adsorption of a polycation PEI. The preparation procedures are schematically shown in
5 Fig. 1E. The entire surface of the channel was coated with a solution containing 20
6 mg/ml albumin, 215 mM EGDE, and 0.5% glycerol for 2 h. The solution was
7 subsequently aspirated through the outlet and dried overnight. Then, a PEI sheath flow
8 was generated by introducing PBS and 10 µg/ml PEI solutions through inlets 5 and 1,
9 respectively, which changed a specific region in the channel from cell-repellent to
10 cell-adhesive. PEI adsorbs onto the albumin substrate within 5 min (22). Fibroblasts or
11 PC12 cells were seeded into the channel at a density of 2×10^7 cells/ml through inlet 3.
12 Although fibroblasts adhered directly onto the PEI-adsorbed surface, an additional
13 collagen coating (30 µg/ml for 2 h) was required on the PEI surface to adhere the PC12
14 cells. The cells in the channel were left undisturbed in a CO_2 incubator for 2 h at 37ºC,
15 which allowed them to attach to the surface of the channel. Unattached cells were
16 washed with culture medium. The remaining cell-repellent surface was changed to
17 cell-adhesive by treatment with the PEI solution through inlet 4 for 5 min, thereby
18 facilitating the adhesion of secondary cells or cell migration from the initially adhered

1 region. To validate the patterns in the coculture, the primary cells (PC12) and the

2 secondary cells (fibroblasts) were fluorescently visualized with a cytoplasmic tracer

3 (CFSE-green; Invitrogen) and a membrane-labeling dye (PHK26-red; Invitrogen),

4 respectively, before seeding.

5

6 Cell growth and migration under a concentration gradient

7 Fibroblasts were localized in the central region of the main channel, and the remaining

8 region became cell-adhesive as mentioned above. The cells were then cultured under a

9 concentration gradient of 0–10% FBS or a constant concentration of 10% FBS. The

10 growth and migration of the cells were monitored for 4 days. We further examined

11 quantitatively whether the cells migrate against the concentration gradient. The region

12 in which cells are localized can be changed by altering the flow ratio of PBS solution in

13 the 2 side channels. By using a flow ratio of 1:0 at inlets 3 and 4, fibroblasts were

14 localized near the sidewall of the channel. Under the concentration gradient of 0–10%

15 FBS, the distance that the cell migrated against the gradient was evaluated by measuring

16 the width of the expanded cell layer for 48 h of perfusion culture. The results were

17 compared to a constant concentration of 5% FBS.

18

1 **Results and discussion**

2 Viable conditions of cells cultured in the microsystem

3 To evaluate the culture condition in the microsystem, fibroblasts were seeded at a low
4 density of 3×10^6 cells/ml and their growth in a perfusion culture was observed.
5 Fibroblasts grew vigorously and covered the entire surface of the main channel at 4 days
6 of perfusion culture (Figs. 2A and 2B), which was similar to that observed in a
7 conventional stationary culture. Estimated from the medium flow (0.4 μl/min) the cells
8 were subjected to a shear stress of 0.16 dyne/cm^2, which is relatively low compared
9 with physiological shear stress in the venules (1–5 dyn/cm^2) (23). The live/dead staining
10 shows that the cells are viable (green) and few cells died (red) (Figs. 2C and 2D).
11 The difficulty in handling cells in the microsystem is most frequently owing to the
12 inadvertent introduction of air bubbles, as also described by others (24, 25). Bubbles are
13 sometimes formed at tubing connections or by the reduction of gas solubility in the
14 medium and silicone because of the increase in temperature from room temperature to
15 37°C. When a bubble passed through the main channel, the experiment was completely
16 fouled. To address this issue, we built an air vent into the inlets of the microsystem (Fig.
17 1A). When unexpected air bubbles entered the inlets, they became trapped in the air
18 vent in the upper PDMS layer. The bubbles remained in the air vent for a while and

were then physically removed through the microchannel. This is a technically simple but important modification especially when a device has many connections and when maintaining long-term cultures. In our microsystem, cells were cultured for at least 10 days without interruption.

Cytotoxic assays under a concentration gradient

Solutions with 6 different concentrations of a drug were injected into the branched channel and flowed into the main channel, resulting in the formation of a smooth concentration gradient followed by molecular diffusion (26). In the main channel, fibroblasts were exposed to the concentration gradient of Triton X-100 for 10 min. The live/dead staining clearly shows that the cells died in a concentration-dependent manner (Fig. 3A). The threshold concentration was determined on the basis of fluorescence images and numerical calculation of the concentration profile of the drug (Fig. 3E). The profile is a function of several variables, such as observation position, flow rate, and diffusion coefficient.

Convection-diffusion models have previously been proposed to estimate the profile in microfluidic devices (27). The optimum location at which a linear gradient is formed has been mathematically estimated (28, 29). In our study, the optimum location was

1 estimated to be 780 µm downstream from the entrance of the main channel; however,

2 that location was found to be too close to the side channels to obtain a highly

3 reproducible cell density. Therefore, the observation position was set to 6 mm

4 downstream, and the profile was calculated using the following equations, which were

5 previously reported by others (28) and modified for this experiment.

6

7 $$C^*(x^*) = \frac{1}{2} + \sqrt{2}\sum_{n=1}^{\infty} A_n \cos(n\pi x^*) \exp(-0.015 n^2 \pi^2) \quad (1),$$

8

9 where C* is a dimensionless concentration divided by the maximum concentration and

10 x* is the dimensionless coordinate to the cross-stream divided by the width of a flow

11 channel. A_n is defined as follows:

12

13 $$A_n = \frac{\sqrt{2}}{n\pi}\sum_{i=1}^{N-1}(C_i^* - C_{i+1}^*)\sin(\frac{n\pi i}{N}) \quad (2),$$

14

15 where N is the number of discrete streams generated in the branched channel (N = 6 in

16 this experiment). Fig. 3E shows the calculated result from equation 1 and the

17 experimental results obtained by flowing 70 mM fluorescein solution and taking

18 fluorescence microphotographs. The calculation was determined using the diffusion

coefficient for fluorescein—0.425×10^{-9} m^2/s (30). Estimated from the data shown in Fig. 3E, the approximation error between the calculation and the experiment was within 5%. Fig. 3F shows the threshold concentration estimated from the live/dead staining in the channel and equation 1. Figs. 3B–D show the results from the conventional approach using a culture dish. These results indicate that the microfluidic device can be used to estimate a threshold in a single experiment. In the same manner, mitomycin C, a representative anti-cancer drug, was exposed to Hep G2 cells, and the threshold could be estimated with a good correlation (Fig. 3G).

Cellular micropatterning inside the microchannel

The tendency of albumin to avoid adsorption of other proteins and cell adhesion on its coated surface is well known (31). We previously prepared a water-insoluble, cross-linked albumin film and demonstrated that the cell adhesion property can be switched from cell-repellent to cell-adhesive by using a positively charged polymer such as PEI (22, 32). The film appears to be slightly negatively charged through the cross-linking process accompanying the reduction of the amine group of albumin. The switching was achieved within 5 min (Figs. 4A and 4B).

This short switching time encouraged us to use the same approach to deposit cells in a

1 specific region of a microchannel. After the surface of the main channel was coated
2 with the albumin film, a PEI solution was flowed through only the central region of the
3 main channel by hydrodynamically focusing 2 streams of PBS through the side
4 channels; therefore, only the central region was switched from cell-repellent to
5 cell-adhesive. In this design, PC12 cells were initially attached to the selective region
6 (Figs. 4C and 4D). The remaining surface was subsequently switched to cell-adhesive,
7 thereby allowing the adhesion of fibroblasts and the formation of a micropatterned
8 coculture in the microchannel (Figs. 4E and 4F). Although many micropatterned
9 coculture approaches have been provided in stationary cultures (33, 34), approaches that
10 can be used in a microchannel are inadequate. The approach developed in our study
11 could potentially help us understand and visualize heterotypic cell-cell interactions and
12 cell migration in other cell layers in more biomimetic environments.
13 The edge definition of micropatterns depends on pairs of cell types. In the pair used in
14 this study, the 3T3 cells covered up the culture area of PC 12 cells, which made the
15 micropattern unclear. We have fabricated micropatterns using pairs of PA 6/3T3 cells,
16 Neuro-2a/L929 cells, and Hep G2/3T3 cells, and have found that many factors such as
17 the binding affinities between cell-to-substrate and cell-to-cell, roundness of adhered
18 cells, seeded cell density, and timing to seed secondary cells appear to influence the

1 distribution of cells in micropatterns. A detailed investigation of the cell-adhesive

2 behavior onto other types of cells is required, and this is our next subject.

3

4 Cell growth under an FBS concentration gradient

5 The micropatterning technique can also be used to examine cell migration from a fixed

6 location under a concentration gradient (Fig. 1E). In general, polycations such as PEI

7 and poly(L-lysine) have a time- and concentration-dependent cytotoxic effect because of

8 membrane damage (35). Fibroblasts, however, remained stably viable after the

9 switching process using PEI because of the low concentration (10 μg/ml) and short time

10 treatment (5 min) (Figs. 5A and 5B). Estimated from the live/dead staining, the viability

11 after the PEI treatment was greater than 90%. The deposited fibroblasts were cultured

12 using 2 concentrations of FBS and showed a clear difference in their behaviors. Under

13 the constant concentration of 10% FBS, the cells proliferated, migrated slowly, and did

14 not reach either sidewall after 96 h (Figs. 5C and 5D). In contrast, under the gradient,

15 the cells actively migrated and proliferated in response to the gradient and reached the

16 sidewall of the higher-concentration side after 48 h (Fig. 5E). Migration toward the

17 lower-concentration side was much slower, and the cells did not reach the wall of this

18 side even after 96 h (Fig. 5F).

1 To examine migration against the gradient more quantitatively, fibroblasts were
2 localized near the sidewall of the channel and the length of cell mobilization was
3 evaluated (Fig. 6). Interestingly, fibroblasts under the gradient hesitated moving into the
4 free space against the gradient and formed a dense cell layer in the initially adhered
5 region, whereas fibroblasts under the 5% constant concentration actively moved into the
6 free space (Fig. 6A). The change in the position of the boundary line of the expanding
7 cell layer under the gradient was less than one-fourth of that under the constant
8 concentration after 48 h (Fig. 6B).
9 Fibroblasts play a critical role in wound healing and scar tissue formation and in the
10 formation of fibrotic tissues and organs such as the heart, lung, and liver. Migration and
11 mitosis of fibroblasts are modulated by diverse biological factors including fibroblast
12 and epidermal growth factors. Our microfluidic device may be useful for examining the
13 effects of these biological factors on fibroblast behaviors, finding a key to
14 understanding disease mechanisms, and developing an innovative therapeutic approach.
15 As is well known, FBS contains various growth factors and nutrients. The analysis
16 using a specific growth factor in single cultures or cocultures will be the subject of our
17 next investigation. In addition, because serum albumin binds to many water-insoluble
18 molecules, including drugs, and is delivered to the cells in the body through blood flow,

an additional approach may be to investigate cell behavior on the albumin layer to which drugs or biological factors were previously conjugated with the concentration generator.

In conclusion, we demonstrated a microfluidic device that can be used to fabricate cellular micropatterns in a microchannel in single cultures and cocultures and investigated cell behaviors under a concentration gradient. The cell adhesion property of a culture surface in a channel was temporally and spatially controlled using commercially available materials, including albumin and PEI. By using this unique property of the surface, it was possible to deposit primary cells in the central region along the main channel. In addition, the subsequent switching of the remaining surface enabled adhesion of secondary cells or migration of the deposited cells under a concentration gradient of FBS. With this microdevice, it was shown that fibroblasts migrated along the concentration gradient of FBS but hesitated against the gradient. Note that continuous monitoring is not required to evaluate the migration, which can be evaluated by measuring the change in positions of cells from an initially adhered region. This approach could be useful in advancing our understanding of cell behavior, especially in wound healing, tissue formation, and embryonic development.

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1 **Figure legends**

2 Fig. 1 Microfluidic device for cell micropatterning and conducting assays under
3 concentration gradients. (A) Device is composed of 2 poly(dimethyl siloxane) (PDMS)
4 substrates and 1 glass substrate. The microchannels in the upper PDMS are for trapping
5 and removing air; those in the lower PDMS consist of a main channel for culturing cells,
6 a branched channel for generating a stable concentration gradient, and 2 side channels
7 for micropatterning cells in the main channel. (B) Fabricated device. (C and D)
8 Fluorescence micrographs of a concentration gradient at the inlet of the main channel
9 and 6 mm downstream, respectively. (E) Preparation steps for coculture of 2 cell types
10 or a migration assay.

11

12 Fig. 2 Cell growth and viability in the device. (A) Fibroblasts seeded at a low density of
13 3×10^6 cells/ml in the main channel. (B) Fibroblasts grew and covered the entire
14 surface at 4 days of perfusion culture. (C and D) Live/dead staining. Almost all of the
15 cells cultured in the device were viable (C), with only a few dead cells (D).

16

17 Fig. 3 Cytotoxic assays under a concentration gradient. (A–D) Live/dead staining of
18 fibroblasts exposed to Triton X-100 under the gradient in the channel (A) or at a

1 constant concentration in a conventional culture dish (B–D). The arrow indicates the
2 direction of flow, and the dashed line indicates a live/dead boundary. (E) Concentration
3 profiles estimated from the experiment (plot) and equation 1 (line) 6 mm downstream in
4 the channel. (F and G) Cytotoxic effect of Triton X-100 (F) and mitomycin C (G)
5 evaluated in the microdevice (dashed line) and a culture dish (column). The dashed lines
6 and labeled values indicate threshold concentrations estimated from fluorescent images
7 and a theoretical concentration profile for each drug.
8
9 Fig. 4 Cell adhesion property of the cross-linked albumin-coated surface and
10 micropatterning cells in the channel. (A) Albumin-coated surface resisting cell adhesion.
11 (B) Fibroblasts attached on the surface after the polyethyleneimine (PEI) treatment. (C)
12 PC12 cells were micropatterned to the central region along the channel where the
13 surface had been previously treated with PEI. (D) Fluorescently labeled PC12 cells. (E)
14 Coculture of PC12 cells with subsequently seeded fibroblasts. (F) Fluorescent staining
15 revealed the coculture of PC12 (a cytoplasmic tracer CFSE-green) and fibroblasts (a
16 membrane-labeling dye PHK26-red) 36 h after the primary cell seeding.
17
18 Fig. 5 Cell behavior under a concentration gradient of fetal bovine serum (FBS). (A)

1 Micropatterned fibroblasts in the channel. (B) Live/dead staining showing that

2 fibroblasts were viable after the PEI treatment of the surrounding surface. (C–D)

3 Fibroblasts cultured in a constant concentration of 10% FBS for 48 h (C) and 96 h (D).

4 (E–F) Fibroblasts grew and migrated along the gradient under a concentration gradient

5 of 0 to 10% FBS for 48 h (E) and 96 h (F). The concentration increases from the top to

6 the bottom in every image.

7

8 Fig. 6 Cell migration against a concentration gradient. (A) Fibroblasts localized near the

9 sidewall of the channel were cultured in a constant concentration of 5% FBS or under a

10 gradient of 0–10% FBS. Representative images observed at 5, 24, and 48 h of perfusion

11 culture. (B) Change in the width of the cell layer was estimated from the images. The

12 cells migrated into the space constantly in 5% FBS without a gradient, and the

13 migration against the gradient was prevented. The error bars indicate SD calculated

14 from at least 3 images in 3 independent experiments for each plot.

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