Electronic Supplementary Information For

# Masked Plasma Oxidization: Simple and Scalable Micropatterning of Extracellular Matrix in a Closed Microchamber Array

Koji Hattori,[a] Ryosuke Yoshimitsu,[b] Shinji Sugiura,*[a] Atsushi Maruyama,[b] Kiyoshi Ohnuma[b] and Toshiyuki Kanamori[a]

[a] Research Center for Stem Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), Central 5th, 1-1-1 Higashi, Tsukuba, Ibaraki, 305-8565 Japan.
Fax: +81-29-861-6286; Tel: +81-29-861-6278; *E-mail: shinji.sugiura@aist.go.jp

[b] Top Runner Incubation Center for Academia-Industry Fusion, Nagaoka University of Technology, 1603-1 Kamitomiokamachi, Nagaoka, Niigata 940-2188, Japan.

## 1. Materials

Chinese hamster ovary (CHO)-K1 cells and mouse embryonic fibroblasts (NIH3T3) cells were obtained from the Riken Bioresource center (Tsukuba, Ibaraki, Japan). SU-8 negative photoresists were obtained from MicroChem (Products number: 50, 2002, 2050, 2075, 3025, Newton, MA, USA). PDMS prepolymer and its curing agent were obtained from Dow Corning (Product name: Sylgard 184, Midland, MI, USA). Tridecafluoro-1,1,2,2-tetrahydrooctyl- 1-trichlorosilane was obtained from Gelest (Morrisville, PA, USA). Fluorescein isothiocyanate (FITC), tetramethyl rhodamine isothiocyanate (TRITC), collagen type I from calf skin (MW: 300 kDa), fibronectin from bovine plasma (MW: 450 kDa), nutrient mixture F-12 HAM, and Dulbecco's phosphate buffered saline solution (PBS, pH 7.1-7.5) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Life Technologies (Gibco®, Carlsbad, CA, USA). All other reagents were obtained from Wako Pure Chemical (Osaka, Japan). All aqueous solutions were prepared with water purified by a Milli-Q Water System (Millipore, Billerica, MA, USA). All reagents were used without further purification.

## 2. Microfabrication

### 2-1. Mask-3

Mask-3 was fabricated by photolithography on a microcover glass (thickness 150 μm, Matsunami Glass Ind., Ltd., Osaka, Japan) using negative photoresist SU-8 3025.[1, 2] A SU-8 coating of 25 μm thickness was patterned in accordance with the manufacturer's recommended procedure, which consisted of spin-coating, soft baking, exposure, and post-exposure baking.

### 2-2. Microfluidic network layer

We used the microfluidic network layer that has the same dimensions reported in our previous study.[3, 4] The microfluidic network layer has the medium-inlet chamber, the 8 × 8 array of the microchamber (diameter: 1.43 mm, depth: 250 μm, pitch: 2.25 mm), the terrace structure, and the connecting microchannels with different depths (Fig. S1). A master template with a multi-thickness pattern of the microchamber and the microchannels was created by multilayer photolithography with modifications.[3] In the multilayer photolithography, the SU-8 2002, 2050, 2075 and the photomasks of each layer pattern were used. The sequence of the process, including spin-coating, soft-baking, exposure, and post-exposure baking, was repeated for three cycles to fabricate the multi-thickness photoresist pattern. In the first cycle, SU-8 2002 was spin-coated, and a pattern of the medium-inlet branch channel was created with the depth of approximately 5 μm. In the second cycle, SU-8 2050 was spin-coated over the prior photoresist layer, and a pattern of the medium-inlet main channel, the cell-inlet/medium-outlet main channel, the cell-inlet/medium-outlet branch channel and the terrace structure was created with the depth of approximately 50 μm. In the final cycle, SU-8 2075 was spin-coated over the prior two photoresist layers, and a pattern of the cell culture microchamber was created with the depth of approximately 250 μm. The development of the photoresist patterns was carried out by the above-described methods. After being washed, the master template was treated with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane at 25 °C for 3 h. PDMS prepolymer and its curing agent were thoroughly mixed with 10:1 and poured onto the master template. After curing in an oven at 120 °C for 2 h, the micropatterned PDMS plate was peeled off from the master template.

**3. Labeling of ECM proteins with fluorescent dyes**

To evaluate the ECM micropattern on the PDMS flat plate or the microfluidic network layer, collagen and fibronectin were labeled with FITC and TRITC fluorescent dyes.[5] Collagen (5 mg) was dissolved in 1 mM HCl (50 μL), and the resulting solution was added to 450 μL of 0.1 M sodium bicarbonate buffer (pH 9.0). Separately, fibronectin (5 mg) was dissolved in 500 μL of 0.1 M sodium bicarbonate buffer (pH 9.0). FITC and TRITC (5 mg each) were dissolved in separate 500-μL portions of DMSO. To synthesize FITC-labeled collagen and TRITC-labeled fibronectin, the respective dye solutions were mixed thoroughly with the corresponding ECM solution at a 20:1 molar ratio by a rotator for 1 h at room temperature. After the reaction, the dye-protein conjugates were isolated from the reaction mixtures using centrifugal filter units with a microporous membrane (MW>5,000, Ultra-free® MC, Milipore, Billerica, USA).

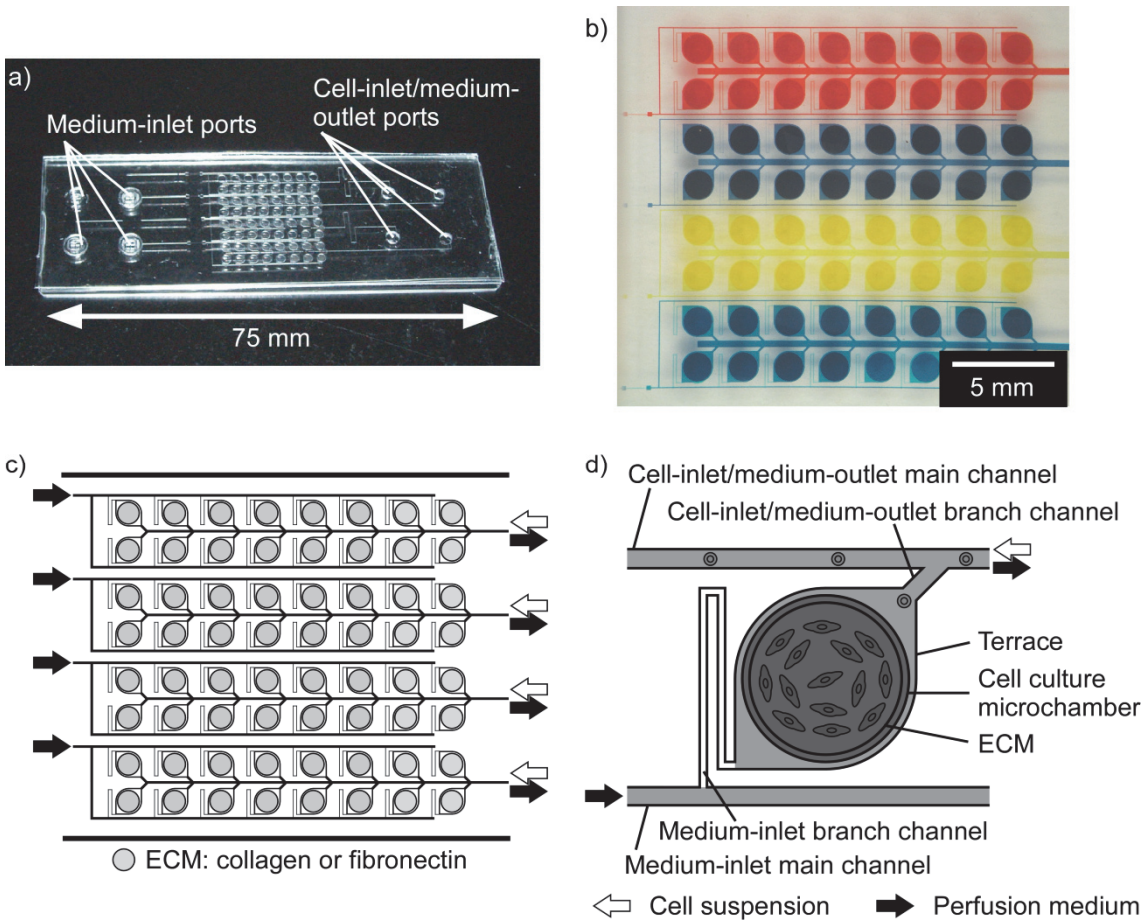


**Fig. S1** Structure of the microfluidic network layer. (a) Overview of the microchip. (b) Overview of the microfluidic network. (c) Microfluidic network design for the 8 × 8 array of the microchamber. (d) Enlargement of each microchamber. The dark gray cell culture microchamber is 250 μm deep; light gray microchannels and terrace are 50 μm deep; and white microchannel is 5 μm deep.

### 4. ECM coating of flat PDMS plate or microfluidic network layer

To form hydrophilic regions on the hydrophobic PDMS surface and thus facilitate ECM coating, a flat PDMS plate and microfluidic network layer were oxidized by $O_2$ plasma in a plasma reactor (PR500, Yamato Scientific Co., Tokyo, Japan; oxygen flow rate: 100 mL/min, pressure: 7 Pa, power: 100 W, processing time: 3 s). After the plasma oxidation, a spacer (opening space: 20 mm × 20 mm, 1 mm thickness) was placed on the PDMS flat plate and microfluidic network layer. FITC-labeled collagen solution or TRITC-labeled fibronectin solution (400 μL) was poured into the formed space and incubated for 12 min at 37 °C for physical adsorption. After the physical adsorption, the spacer and the solution were removed. The surface was rinsed with Milli-Q water and dried at 37 °C for 2 h under vacuum.

**5. Use of firm frame to ensure tight contact of physical mask with microfluidic network layer**

In masked plasma oxidation, the ECM coating on the bottom of the microchamber array must be protected by the physical mask. To place the physical mask in tight contact with the microchamber array, we used a retainer plate and firm frame made of polymethylmethacrylate (Figure S2). The physical mask and the microfluidic network layer were fastened between the retainer plate and the firm frame with two screws. To maintain a tight, level contact between the physical mask and the ECM coating on the bottom of the microchamber array, the holding torque of each screw was maintained at 2 cNm with a torque driver.

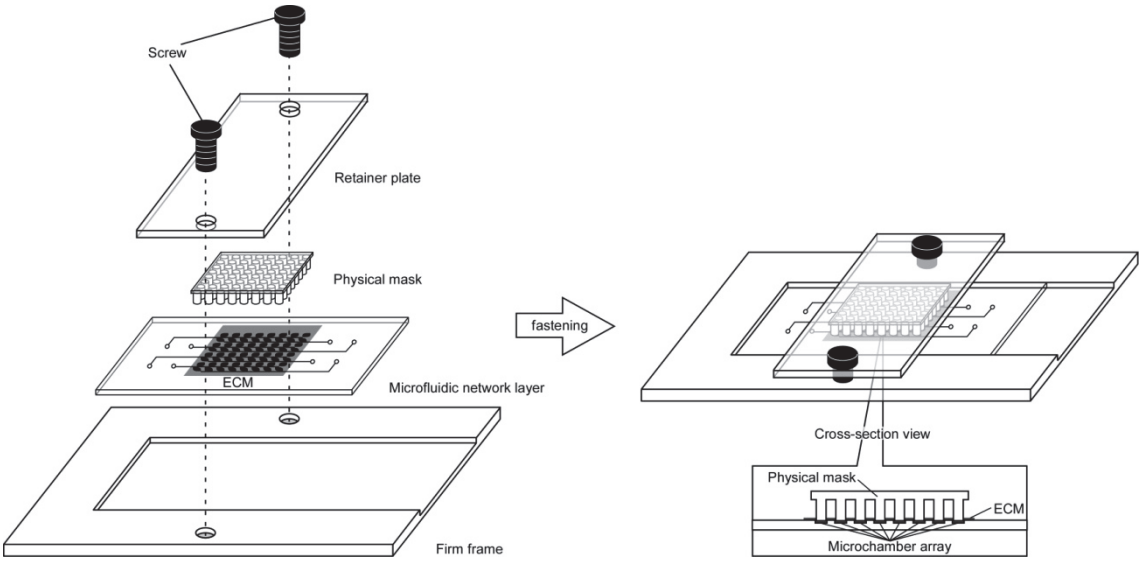


**Fig. S2** Use of firm frame to ensure tight contact of physical mask with microfluidic network layer.

**6. Fluorescence microscopy**

After masked plasma oxidation, the resulting micropatterns of FITC-labeled collagen and TRITC-labeled fibronectin were observed by a fluorescence imaging device consisting of a fluorescence filter block (Olympus, Tokyo, Japan), a CCD colour digital camera module (DFW-SX910, Sony Corp., Tokyo, Japan), and a light source (Lightningcure LC6, Hamamatsu Photonics Co., Shizuoka, Japan). Fluorescence images were recorded using the commercial software Vision Freezer VFS-42 (ver. 3.0, Chori Imaging Corp., Kanagawa, Japan).

**7. Cell culture**

CHO-K1 cells and NIH3T3 cells were maintained on tissue culture polystyrene (TCPS) dishes (BD Falcon$^{TM}$, Becton, Dickinson and Co., NJ, USA) in F-12 HAM and the DMEM, respectively,

supplemented with 10% FBS, penicillin/streptomycin, and nonessential amino acids at 37 °C in a humidified atmosphere containing 5% $CO_2$. These cells were harvested from the TCPS dishes by trypsin/EDTA treatment and suspended in the culture medium at 3.5 x $10^5$ cell/mL before being loaded onto the microfluidic cell culture chip. The cell suspension was injected into the cell-inlet/medium-outlet chamber via micropipette, and the cells were loaded into the microchambers by applying 20 kPa of pressure to the cell-inlet/medium-outlet chamber through a sterile air-vent filter (Acrodisc PTFE, Pall Corp., Port Washington, NY, USA). The cell-loaded microchip was first incubated under static culture conditions to induce cell adherence to the ECM on the bottom of the microchamber array. After 1 d, the medium was added to the medium-inlet chamber, and continuous perfusion culture was carried out for 2 d by applying pressure of 8 kPa to the medium-inlet chamber in a $CO_2$ incubator. The pressure was applied with an S100 air pump (Atem Corp., Tokyo, Japan), controlled with a PR-4102 pressure regulator (GL Science, Tokyo, Japan), and measured with a handheld manometer (PG-100, Nidec Copal Electronics Corp., Tokyo, Japan). The cultured cells were observed with a phase-contrast microscope (IX71, Olympus Corp., Tokyo, Japan) equipped with a VB7010 cooled CCD camera (Keyence, Osaka, Japan).

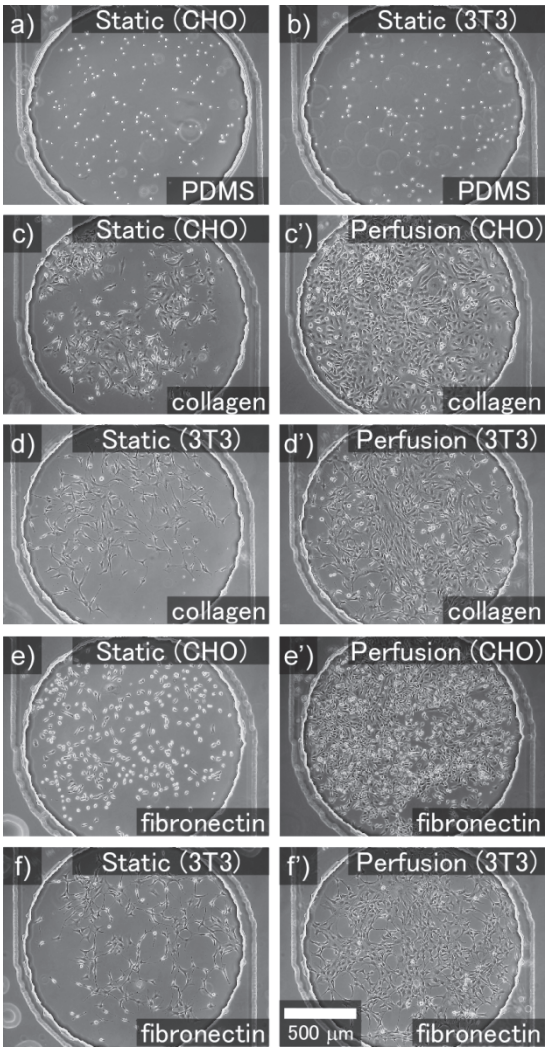


**Fig. S3** Low magnification images of microchambers after static cell culture for 24 h (a)-(f) and perfusion culture for 48 h (c')-(f'). (a) CHO and (b) NIH3T3 on bare PDMS; (c) CHO and (d) NIH3T3 on PDMS coated with collagen; (e) CHO and (f) NIH3T3 on PDMS coated with fibronectin. (c')-(f') are the corresponding samples after further perfusion culture for 48 h.

## References

1. D. C. Duffy, J. C. McDonald, O. J. A. Schueller and G. M. Whitesides, *Anal. Chem.*, 1998, **70**, 4974-4984.
2. T. Deng, H. K. Wu, S. T. Brittain and G. M. Whitesides, *Anal. Chem.*, 2000, **72**, 3176-3180.
3. S. Sugiura, J. Edahiro, K. Kikuchi, K. Sumaru and T. Kanamori, *Biotechnol. Bioeng.*, 2008, **100**, 1156–1165.
4. K. Hattori, S. Sugiura and T. Kanamori, *Lab Chip*, 2011, **11**, 212-214.
5. R. Pankov and A. Momchilova, in Methods in molecular biology, ed. N.J. Clifton, Springer, USA, 2009, vol. 522, pp. 261-274.