Supplementary Material (ESI) for Lab on a Chip
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**Electronic supplementary information for**

# Microenvironment array chip for cell culture environment screening

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## 1. Materials

SU-8 negative photoresists were obtained from MicroChem (Products number: 2005, 2050, 2075, Newton, MA, USA). PDMS prepolymer and its curing agent were obtained from Dow Corning (Product name: Sylgard 184, Midland, MI, USA). New coccine as a red dye were obtained from Kanto Chemical (Tokyo, Japan). Collagen type I from calf skin (MW: 300 kDa), fibronectin from bovine plasma (MW: 450 kDa), laminin from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma (MW, A Chain: 400 kDa, B1 chain: 210 kDa, B2 chain: 200 kDa), nutrient mixture F-12 HAM, and Dulbecco's phosphate buffered saline solution (PBS, pH 7.1-7.5) were all obtained from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Calcein-AM for cell viability assay was obtained from Dojindo Laboratories (Kumamoto, Japan). 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).

## 2. Structure of the microenvironment array chip

Fig. S1 shows the overview of the microenvironment array. The microenvironment array chip provides different 16 types of the microenvironments in an 8 x 8 array of perfusion culture microchambers. The basic design of the perfusion culture microchamber array is described in our previous report[1]. The microenvironments in the microchamber array were composed of the combination of different four types of scaffolds on the bottom of the microchamber array and different four types of the soluble factors in the culture media through the microchannels. The scaffolds on the bottom of the microchamber array were different three types of ECMs (collagen, fibronectin and laminin) and the bare PDMS in each two columns. Cell suspensions are loaded into all 64 microchambers thorough five cell-inlet/medium-outlet main channels (Fig. S1a, right side) and the culture media with different four types of the soluble factors are provided thorough four medium-inlet main channels after cell loading (Fig. S1a, left side).

Fig. S1b shows enlargement of each cell culture microchamber and the connecting microchannels between the medium-inlet main channel and the cell-inlet/medium-outlet main channels. The connecting microchannels are composed of a medium-inlet branch channel and a

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cell-inlet/medium-outlet branch channel. Table S1 gives the dimensions and the hydrodynamic parameters of the microchannels and the cell culture microchamber. The medium-inlet branch channel is significantly narrower than the other microchannels. The pressure drop of the medium-inlet branch microchannel is significantly higher than the other microchannels. The flow perfusion rate of the culture media into each microchamber is therefore determined by the largest fluidic resistance of the medium-inlet branch channel and the pressure applied to the medium-inlet chamber. The narrow medium-inlet branch channel also works as a hydrophobic passive valve to introduce a predetermined volume of the cell suspension during a cell-loading process and the same number of the cells can be loaded into each microchamber[1].

The continuous perfusion culture can be performed in the cell culture microchamber by applying pressure to the medium-inlet chamber. The hydrodynamic parameters when 8 kPa of pressure is applied to the medium-inlet chamber were calculated from the dimensions of the microstructure (listed in Table S1). The share stress at the center of the cell culture microchamber is $10^{-5}$ Pa, which is $10^{-3}$ times the value affecting cell growth[2]. The Peclet numbers (*Pe*) for the medium-inlet and the medium-outlet branch channels are $10^5$ and $10^2$, respectively. It means that the convection mass transfer is significantly greater than the diffusion mass transfer. In other words, the perfusion culture is performed without cross-contamination between neighboring microchambers.

The dimensions and the calculated hydrodynamic parameters of the microenvironment array chip were similar to that on the perfusion culture microchamber array chip in our previous paper[1]. Therefore, we can speculate that the distribution of the flow velocity and homogeneity of the cell growth in the microenvironment array were consistent with that in the previous perfusion culture microchamber array. In the previous paper, we characterized the flow pattern in the cell culture microchamber by hydrodynamic simulation. The flow velocity at the center of the cell culture microchamber are slightly higher than that at the side, but the no heterogeneity of the cell growth was observed in the cell culture microchamber (refer to Fig. 3a, Fig. 6 and Fig. 7 in the previous paper). *Pe* for the cell culture microchamber is significantly lower than *Pe* for the other microchannels. It means that the soluble factors in media are transported by not only the convection but also the diffusion in the cell culture microchamber. We suppose that the manner of the mass transfer should be generated the homogeneous cell growth in the cell culture microchamber.

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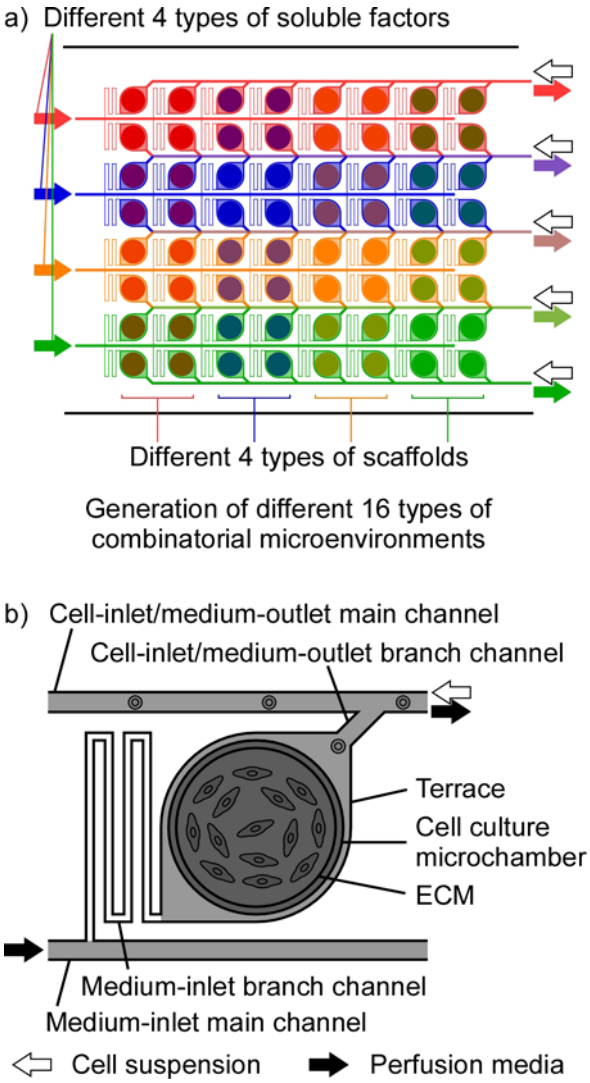


**Fig. S1** Schematic structure of microenvironment array chip, (a) Overview of microenvironment array. Different 16 types of the combinatorial microenvironments are generated in the microchambers, (b) Enlargement of each perfusion culture microchamber array. The dark grey cell culture microchamber is 271.2 μm deep; gray microchannels and terrace are 52.5 μm deep; and light grey microchannel is 4.5 μm deep.

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**Table S1** Dimensions and hydrodynamic parameters of microchannels and the cell culture microchamber.

| | Medium-inlet main channel | Medium-inlet branch channel | Cell culture microchamber | Medium-outlet branch channel |
|---|---|---|---|---|
| Depth ($H$) [μm] | 52.5 | 4.5 | 271.2 | 52.5 |
| Width ($W$) [μm] | 100 | 40 | 1,430 (= diameter) | 100 |
| Length ($L$) [μm] | >15,000 | 6,800 | 1,430 (= diameter) | 500 |
| Advantage of flow velocity ($U$) [μm/s] | 1,010 | 1,840 | 0.9 | 63 |
| Retention time [s] | >15 | 4 | 1,670 | 8 |
| Shear stress [Pa] | 0.1 | 1.8 | $10^{-5}$ | $10^{-2}$ |
| Pressure drop [Pa][a] | 100 | 8,000 | $10^{-4}$ | $10^{-1}$ |
| $Re$ ($=\rho U D_{eq}/\eta$)[b] | $10^{-1}$ | $10^{-2}$ | $10^{-4}$ | $10^{-3}$ |
| $Pe$ ($=LU/D$)[c] | $10^{5}$ | $10^{5}$ | 12[d] | $10^{2}$ |

Hydrodynamic parameters are the values obtained when 8 kPa of pressure was applied to the medium-inlet chamber. [a] Pressure drop was calculated assuming steady-state pressure-driven flow in a rectangular microchannel[3]. [b] $Re$ denotes the Reynolds number. $D_{eq}$ denotes the equivalent diameter and was calculated as $D_{eq} = 2HW/(H+W)$. [c] $D$ denotes the diffusion coefficient and was assumed to be $10^{-10}$ $m^2/s$. [d] Diameter of the microchamber was used as $L$ for calculation.

## 2. Microchip fabrication

The microenvironment array chip contains an 8 x 8 array of the perfusion culture microchambers and was fabricated by assembling two types of PDMS layers: a "microchamber array layer" and "ECM array layer" (Fig. 1a in the body text).

### 2-1. Microchamber array layer

Microchamber array layer was composed of the microstructures with different depth, the medium-inlet chamber, the microchamber array, the terrace structure, the cell-inlet chamber, the connecting microchannels and the alignment keys for the contact of the ECM array layer. A master template of the microchamber array layer with a multi-thickness pattern of the microstructures was fabricated by multilayer photolithography[4] with modifications[1]. In the multilayer photolithography, the SU-8 negative photoresists (SU-8 2005, 2050 and 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 2005 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

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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 medium-inlet chamber, the cell-inlet chamber and the cell culture microchamber was created with the depth of approximately 200 μm. After post-baking, the photoresist pattern was developed in ethyl lactate and washed with isopropanol. 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 layer was peeled off from the master template carefully.

**2-2. Mask for the protection of ECM array**

Mask (25.0 mm width x 38.0 mm length x 1 mm thickness) has an 8 x 8 array of cylindrical rods (diameter: 1.53 mm, height: 1 mm, pitch: 2.25 mm) corresponding with the ECM array and the alignment keys for the contact of the ECM array layer. The mask was used to protect the array of the ECM proteins during $O_2$ plasma irradiation used for subsequent bonding process of the microchamber array layer and the ECM array layer. The mask was fabricated by the photolithography and the replica molding of the PDMS[5, 6] A master template with an 8 x 8 array of small pits (diameter: 1.53 mm, depth: 1 mm, pitch: 2.25 mm) was created by the photolithography. In the photolithography, the SU-8 2075 and the photomask of the array pattern of cylindrical rods were used. The sequence of the process, including spin-coating, soft-baking, exposure, and post-exposure baking, was repeated for two cycles to form the array pattern of the small pits with the depth of approximately 1 mm. The development of the photoresist patterns and the replica molding of the PDMS were performed by the above-described methods.

**2-3. Microfluidic patterning chip**

The microfluidic patterning chip was fabricated by the assembling of different two layers of PDMS, the micropatterned thin PDMS film and the PDMS flat plate with eight through-holes (Fig. S2). The micropatterned thin PDMS film has the microstructures with different depth, an 8 x 8 array of through-holes (diameter: 1.53 mm, height: 415.1 μm, pitch: 2.25 mm), which is correspondent with the 8 x 8 array on the ECM array layer and the microchamber array layer, alignment keys, four ECM-inlet chambers, the four ECM-outlet chambers and the connecting microchannels (width: 100 μm; depth: 52.3 μm).

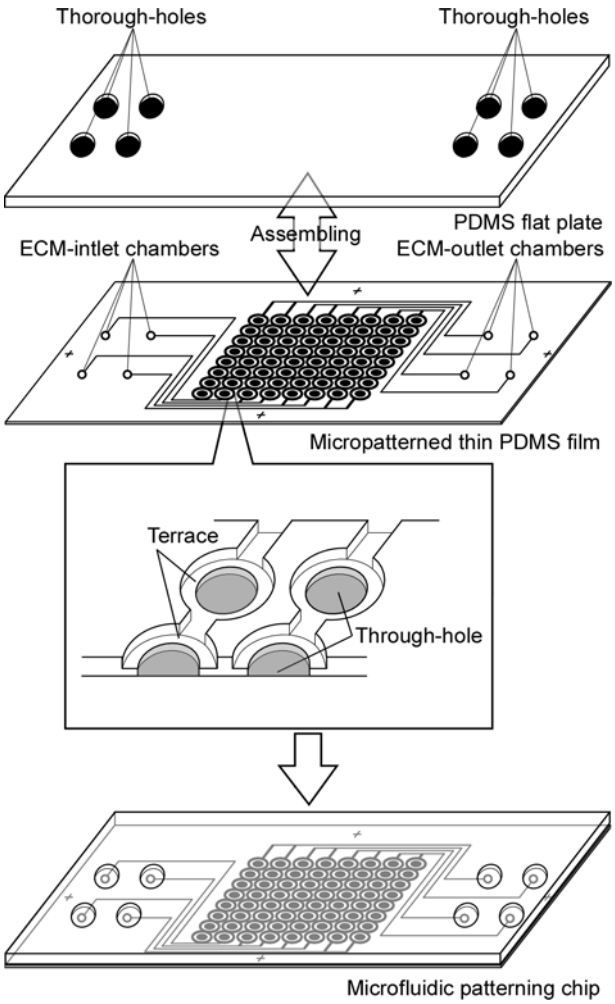


**Fig. S2** Fabrication of the microfluidic patterning chip. The microfluidic patterning chip was composed of the PDMS flat plate with eight through-holes and the micropatterned thin PDMS film with an 8 x 8 array of through-holes.

A master template of the micropatterned thin PDMS film with a multi-thickness was also fabricated by multilayer photolithography[4] with modifications[1]. In the multilayer photolithography, SU-8 2050 and SU-8 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 two cycles. In the first cycle, SU-8 2050 was spin-coated, and the pattern of the alignment keys, the ECM-inlet/outlet chambers, the terrace structure and the connecting microchannels were fabricated with the depth of approximately 50 μm. In the second cycle, SU-8 2075 was spin-coated, and the pattern of the 8 x 8 array of through-holes was fabricated with the depth of approximately 400 μm. The development of the photoresist patterns was performed by the above-described methods.

The micropatterned thin PDMS film was fabricated by the microfluidic replication[7] and modification[8] (Fig. S3). Briefly, the master template for the micropatterned thin PDMS film was

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closely attached with the thin poly (ethylene terephthalate) (PET) film using a rubber plate and a stainless weight plate (Fig. S3a). Both of the rubber plate and the stainless weight plate on the master template have an air vent port on the center of their bodies. All parts were set on the center of the dish and clamped by two stainless plates with the constant torque of 5 dNm. PDMS prepolymer and its curing agent were thoroughly mixed with 10:1 weight ratio and poured into the dish. The space between the thin PET film and the master template was evacuated and PDMS was degassed by the oil-sealed rotary pump for 20 min (Fig. S3b). PDMS mixture was introduced into the space between the master template and the thin PET film under a vacuum (Fig. S3c). The thickness of the micropatterned thin PDMS film was then determined by the thickest layer of the resist pattern on the master template. After curing in an oven at 120 °C for 2 h, resulting micropatterned thin PDMS film was carefully peeled off from the master template and the PET films. The thin PDMS film was washed in the ethanol for 10 min using ultrasonic cleaner, and dried at 70 °C for 1h under a vacuum. The microfluidic patterning chip was fabricated by assembling of the micropatterned thin PDMS film, the PDMS flat plate and macroscopic reservoirs. The oxidation of the PDMS surface by the irradiation of $O_2$ plasma is often performed to bond the PDMS microchips[5]. $O_2$ plasma was irradiated on the adherend surface of the micropatterned thin PDMS film and the PDMS flat plate in a plasma reactor (PR500, Yamato Scientific Co., Tokyo, Japan). The two oxidized PDMS surfaces of the micropatterned thin PDMS film and the PDMS flat plate were tightly contacted each other. The two macroscopic reservoirs were fabricated by the PDMS with a template made out of an acrylic resin and also bonded on the ECM-inlet chambers and ECM-outlet chambers.

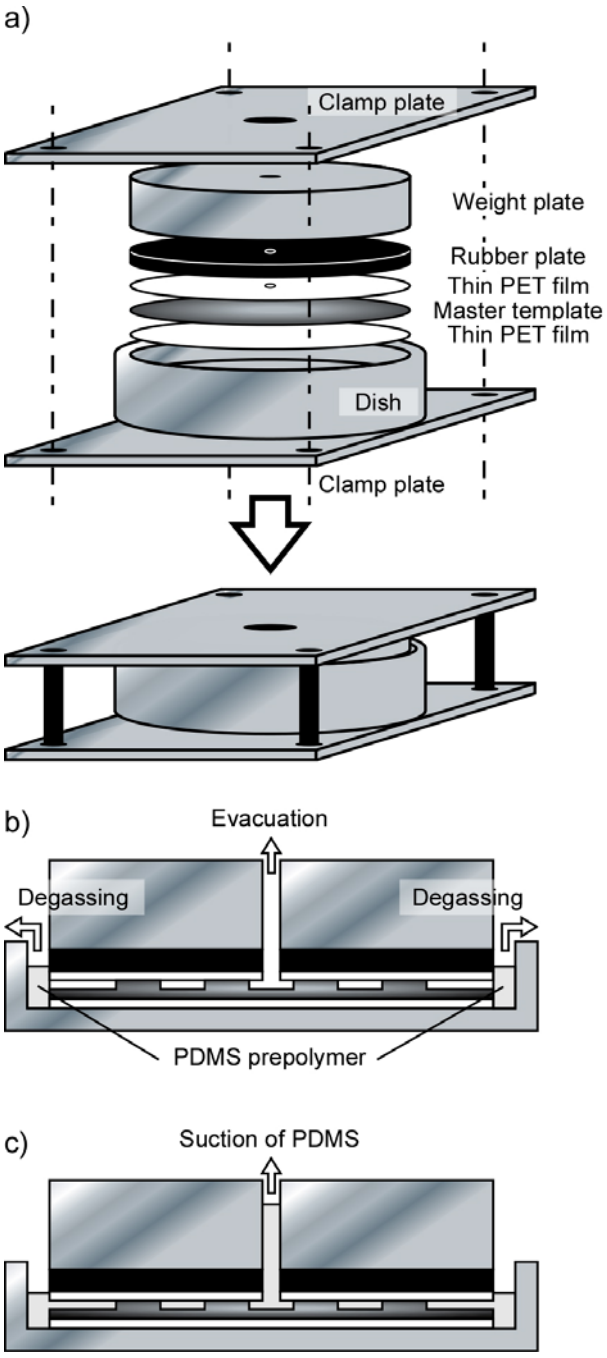


**Fig. S3** A schematic diagram depicting the fabrication of a mictopatterned thin PDMS film by vacuum suction technique, (a) Setup of the apparatus for the fabrication, (b) Evacuation in the apparatus and degassing from the PDMS around the apparatus, (c) Vacuum suction of PDMS into the space between the thin PET film and the master template.

### 3. Bonding process

The microenvironment array chip was fabricated by the bonding of the microchamber array layer

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and the ECM array layer with the alignment. We protected the ECM array from the $O_2$ plasma by using the mask, since ECM proteins are possibly damaged by the $O_2$ plasma irradiation. The 8 x 8 array of cylindrical rods on the mask was contacted to the 8 x 8 array of ECMs after alignment (Fig. S4a), $O_2$ plasma was then irradiated on the adherend surface of the microchamber array layer and the masked ECM array layer in a plasma reactor (Fig. S4b). After irradiation, the mask was peeled off from the ECM array layer and the two oxidized PDMS surfaces of the microchamber array layer and the ECM array layer were tightly contacted each other after alignment. Each ECM spot was then arranged on the bottom of the corresponding the microchamber, and the microchamber and the microchannels were then enclosed. The two macroscopic reservoirs were also bonded on the medium-inlet chamber and the cell-inlet/medium-outlet chamber.

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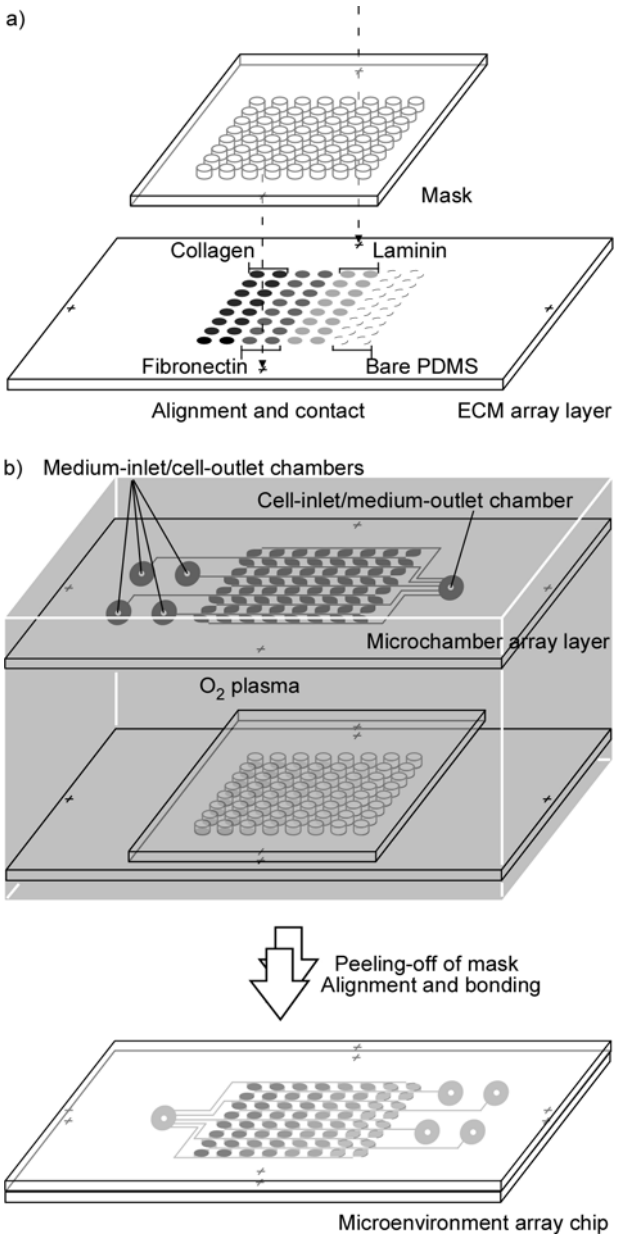


**Fig. S4** Fabrication of the microenvironment array chip, (a) Masking of the ECM array, (b) Irradiation of $O_2$ plasma on the adherend surfaces, (c) Bonding of the microchamber array layer and the ECM array layer.

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## 5. Cell culture

CHO-K1 cells were obtained from the Riken Bioresource center (Tsukuba, Ibaraki, Japan), and maintained in nutrient mixture F-12 HAM medium supplemented with 10 % fetal bovine serum (Gibco BRL, Rockville, MD, USA), penicillin/streptomycin and nonessential amino acids at 37 °C in humidified atmosphere containing 5 % $CO_2$. The CHO-K1 cells were harvested after the addition of trypsin and then suspended in the culture medium. Before loading of the cells, the microenvironment array was sterilized by the irradiation of UV from germicidal lamp for 1 h in a clean bench. The cell suspension (3.5 x $10^5$ cell/mL) was added to the cell-inlet/medium-outlet chamber using a micropipette and the cells were loaded into the microchambers by applying 15 kPa of pressure to the cell-inlet/medium-outlet chamber through a sterile air-vent filter. The cell-loaded microchip was firstly incubated under static culture conditions to induce cell adherence on the bottom of the microchamber. After 12 h, the culture medium was added to the medium-inlet chamber, and continuous perfusion culture was performed for 1 day 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).

## 6. Analytical method

After total 2 days of continuous perfusion culture, the viability of the CHO-K1 cells in each microenvironment array was fluorometrically analyzed by staining with calcein-AM[1]. A 0.4-μg/mL calcein-AM solution in Dulbecco's PBS was added to the medium-inlet chamber using a micropipette and loaded into the microchambers by applying 50 kPa of pressure for 10 min to the medium-inlet chamber through a sterile air-vent filter. After 15 min of static incubation at 37 °C, a fluorescence image was obtained with a fluorescence imaging device consisting of a U-MNIBA2 fluorescence filter block (Olympus, Tokyo, Japan), a CCD color digital camera module (DFW-SX910, Sony Corp., Tokyo, Japan), and a spotlight source of ultraviolet (UV) light (LIGHTNINGCURE LC6, Hamamatsu Photonics Co., Shizuoka, Japan). The fluorescence images were captured with the commercial software VISION FREEZER VFS-42 (ver. 3.0, Chori Imaging Corp., Kanagawa, Japan) with an exposure time of 1 s.

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