Microenvironment array chip for cell culture environment screening†

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We have developed a microarray of cell culture environments composed of a combination of soluble factors and extracellular matrices for screening of cell culture environment.

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Cellular events (*e.g.*, extension, proliferation, apoptosis and differentiation) depend on extracellular stimuli from surrounding environment composed of soluble factors and scaffolds, *i.e.* extracellular matrixes (ECMs).[1–3] For example, growth factor signal response can be diminished in the absence of integrin binding.[4] In turn, integrin binding can alter the dynamics of growth factor signal transduction events at multiple points along the mitogen-activated protein (MAP) kinase pathway[5] and transactivate growth factor receptor pathways.[6] Therefore, the environment including both soluble factors and ECMs is important for cultivating cells. Especially, the screening of appropriate differentiation environment of the stem cells is urgent issue in the fields of regenerative medicine and drug discovery.

ECM microarray is an advantageous method for parallel analysis of the cell behaviors on the different ECMs.[7–10] However, in the previous works, cells on the different ECM spots were cultured in the continuous liquid phase, *i.e.* culture media, which are possibly contaminated with soluble factors secreted from the cells on the different ECM spots. In order to investigate the effect of each ECM without the cross-talk between neighboring spots, each ECM spot should be isolated in the discrete compartment.

Recently, microfluidic cell culture chips have been recognized as potentially advantageous research tools to realize rapid and reproducible assays of small-volume samples without labor-intensive routine works and expensive robotics.[11,12] We and other researchers have previously reported perfusion culture microchamber array chips, which can perform the parallel on-chip cell-based assays in the array-formatted microchambers.[13–17]

In this paper, we report a new-concept of the perfusion culture microchamber array chip, "microenvironment array chip", where cells are cultured in the discrete microchambers with the different environment composed of combination of soluble factors and ECMs. We demonstrated the screening of the cell culture environment for cultivating Chinese Hamster Ovary (CHO)-K1 without serum by using the microenvironment array chip.

The microenvironment array chip contains an 8 × 8 array of the perfusion culture microchambers, and was fabricated by assembling different two of PDMS layers: "microchamber array layer" and an "ECM array layer" (Fig. 1a). The microchamber array layer provides four different soluble factor conditions in four sets of two rows of the microchamber array. The ECM array layer provides four different ECM conditions in four sets of two columns of the array. The microenvironment array chip provides 16 different microenvironment conditions as combination of four different soluble factor conditions and four different ECM conditions.

The microchamber array and ECM array were designed with mutual components: an 8 × 8 array and alignment keys "+", which were marked on the center of each side for the mutual alignment. The microchamber array layer has microstructures with multi-depth: the 8 × 8 array of the microchambers, the terrace structures, the four medium-inlet/cell-outlet chambers, the cell-inlet/medium-outlet chambers and the connecting microchannels (Fig. S1 and Table S1, the detailed structure, dimension and hydrodynamic parameter of the microenvironment array are available in the ESI†).

ECM array layer was fabricated by using a "microfluidic patterning chip" by means of physical adsorption on the PDMS flat plate with alignment keys (Fig. 1b). The microfluidic patterning chip has an 8 × 8 array of through-holes, which is correspondent with the

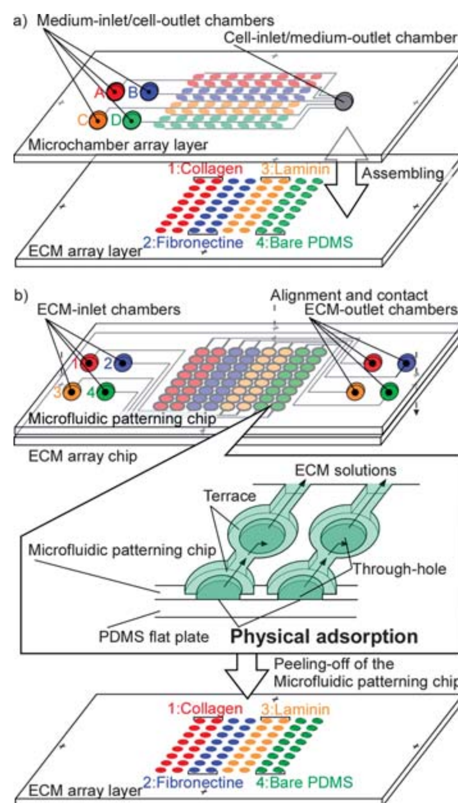

Fig. 1 Fabrication of microenvironment array chip and the ECM array layer. (a) Fabrication of microenvironment array chip by assembling the microchamber array layer and ECM array layer. (b) Fabrication of ECM array layer by using microfluidic patterning chip.

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8 × 8 array of the microchambers on the microchamber array layer, alignment keys, four ECM-inlet chambers, the four ECM-outlet chambers and the connecting microchannels. The microfluidic patterning chip was attached to the PDMS flat plate with the alignment and fastened by an acrylic resin holder. The different ECM solutions were introduced into the microchambers through the microchannels by applying pressure to the ECM-inlet chamber and the ECMs were immobilized on the surface of the PDMS flat plate by means of the physical adsorption.

The microchamber array layer, the ECM array layer, the microfluidic patterning chip and the mask were fabricated by replica molding[18,19] or microfluidic replication[8,20] and the bonding process with O_2 plasma irradiation.[16,19] The master templates of the replications were fabricated out of photoresist by multilayer photolithography[16,21] (Fig. S2 and S3, the details of the microchip fabrication are available in the ESI†).

The microenvironment array chip was fabricated by bonding of the microchamber array layer and the ECM array layer under the alignment after treatment with O_2 plasma irradiation.[16,19] During the O_2 plasma irradiation, the ECM array was protected by a mask made out of PDMS with the alignment,[22] since ECM proteins are possibly damaged by the O_2 plasma irradiation (Fig. S4, the details of the bonding process are available in the ESI†). After bonding, the array of microchambers with ECMs on the bottom was formed.

To visualize the microfluidic networks of the microfluidic patterning chip, dye solutions (red: new coccine, green: fast green, blue: gardenia blue, yellow: gardenia yellow) were added to the ECM-inlet chamber using a micropipette and the solutions were introduced into the microfluidic patterning chip attached on the ECM array layer by applying 15 kPa of pressure to the ECM-inlet chamber (Fig. 1b). During introduction of solutions, no leakage of the solutions was observed, which indicates that the microfluidic patterning chip and the PDMS flat plate were sufficiently sealed by the acrylic resin holder (Fig. 2a).

Four different solutions of the ECMs (0.5 mg mL^{-1} collagen or FITC-labeled collagen, 0.2 mg mL^{-1} fibronectin or TRITC-labeled fibronectin, 0.1 mg mL^{-1} laminin or Alexa647-labeled laminin) including one blank milli-Q water were introduced into the microfluidic patterning chip attached on the PDMS flat plate from the ECM-inlet chambers by applying 10 kPa of the pressure. The flow of the solutions was stopped after filling the microfluidic patterning chip with the solutions. The microchips containing the ECM solutions were incubated at 37 °C for 1 h under the saturated humidity. The ECMs were then immobilized on the PDMS flat plate by means of physical adsorption through the through-holes on the bottom of the microfluidic patterning chip. After immobilization, the solutions in the microfluidic patterning chip were flowed out to the ECM-outlet chambers. After peeling off from the microfluidic patterning chip, the ECM array layer was rinsed with milli-Q water and dried at 37 °C for 1 h under a vacuum.

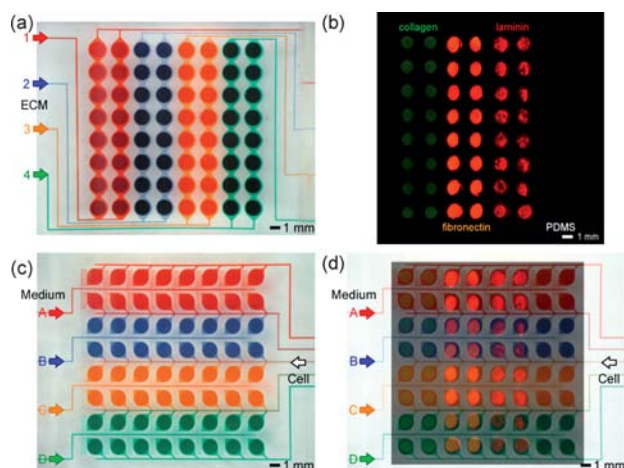

Fig. 2 Microscope images of (a) microfluidic patterning chip, (b) ECM array layer (fluorescent image), (c) microchamber array layer, (d) microenvironment array chip. Color densities in (a) and (c) indicate that the microfluidic network with different depths.

The ECM array layer was visualized by the fluorescently labeled ECMs (Fig. 2b). All of ECM spots were individually formed with approximately 1.5 mm diameter and 2.25 mm pitch, whereas the physical adsorption of the ECMs on the interspace between the spots was protected by the microfluidic patterning chip. These results indicate that the ECM proteins were successfully immobilized on the PDMS flat plate and the geometry of ECM microarray was precisely defined by the through-hole on the microfluidic patterning chip.

The microfluidic networks of the microenvironment array chip were visualized with dye solutions (Fig. 2c). During the introduction of solutions by applying 15 kPa of pressure, no leakage of the solutions from the microenvironment array chip was observed. These results indicate that the bonding by the O_2 plasma irradiation with masking the ECM array was successfully completed.

The microenvironment array can provide the 4 × 4 combinatorial microenvironments composed of four different soluble factors and four different ECMs in the microchamber array (Fig. 2d).We demonstrated the on-chip screening of the microenvironment for cultivating CHO-K1 cells without serum from 4 × 4 combinatorial microenvironments created on the microenvironment array chip.

Cell suspension of 3.5 × 10^5 cells per mL in the medium without serum was added to the cell-inlet/medium-outlet chamber using a micropipette and the cells were loaded into the microchambers with different four types of the ECMs (Table 1, 1–4) 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 the cell adherence.

The loaded CHO-K1 cells adhered on the surface of the microchamber with all ECMs and did not adhere on the PDMS surface of the microchamber after the static culture for 12 h. The results were consistent with the adhesion characters of CHO-K1 cells in our previous papers.[8,22] These results indicate that the immobilized ECM microarray on the PDMS surface successfully worked for cell adhesion.

After 12 h of static culture, four culture media containing different soluble factors (Table 1, A–D) were added into the

Table 1 Soluble factors and ECMs in the microenvironment array chip

	ECM		Soluble factor
1	Collagen	A	Fetal bovine serum, non-essential amino acids, L-glutamine, penicillin, streptomycin
2	Fibronectin	B	Human transferrin, bovine insulin
3	Laminin	C	Bovine insulin
4	PDMS	D	—

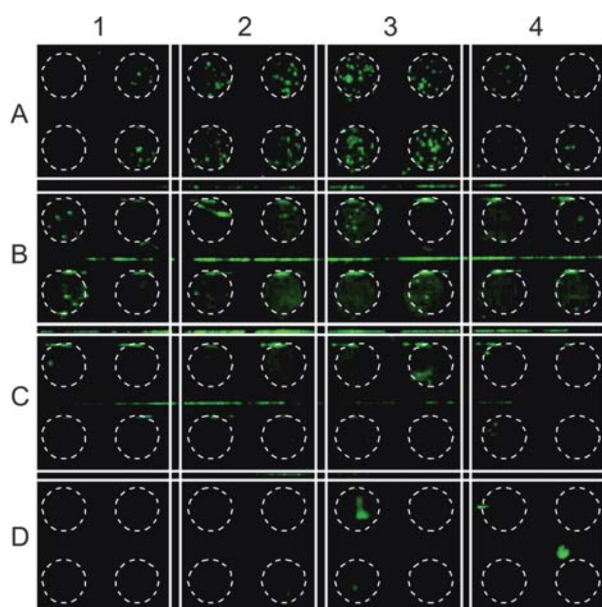

Fig. 3 Screening of the cell culture environment from 4 × 4 combinatorial microenvironment array chip.

medium-inlet/cell-outlet chambers using a micropipette, and continuous perfusion culture was carried out for total 2 days by applying 8 kPa of pressure to the medium-inlet/cell-outlet chamber in a CO_2 incubator. The growth of the CHO-K1 cells was fluorometrically analyzed by staining with calcein-AM[16] (the details of the staining procedure are available in the ESI†).

The perfusion culture was successfully performed in the microenvironment array chip and cell growth was analyzed (Fig. 3). The CHO-K1 cells grew in the microenvironment array with the serum, and cell growth depended on the ECM type. The growth of CHO-K1 cells was better in the microchamber array with laminin, fibronectin and collagen in that order. On the other hand, most of the cells died in the microenvironments without serum. However, CHO-K1 cells in the microenvironment array of B-1, B-2 and B-3 were survived without the serum. The amount of viable CHO-K1 cells was larger in the microchambers with the laminin than in the microchambers with the collagen and fibronectin. These results indicate that the viability of the CHO-K1 cells depended on the extracellular stimuli from the microenvironment composed of the combination of soluble factors and ECMs.

In this study, the microenvironment array chip was successfully demonstrated to create combinatorial cell culture environments composed of soluble factors and ECMs. The cultivable environments for the CHO-K1 cells without the serum were successfully detected from 4 × 4 combinatorial microenvironments. The microenvironment array chip is expected to be applied to high-throughput screening of the cell culture environment for cultivation of primary cells and stem cells.

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