

# 3D Cell Co-culture System on Hydrogel Micro-Patterned Surface Fabricated by Photolithography

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We present herein the 3D cell co-culture systems, which is based on the cell spheroid formation on cell-adhesion micro-patterned glass surfaces fabricated by silane coupling treatment and photoirradiation technique. The typical method for constructing cell-adhesion micro-patterned glass surface is as follows; a mixture of methacryloyl-ended PEG telechelics and Irgacure 2959 as the photoinitiator was dropped on a silanized glass surface, which was pretreated with 3-(trimethoxysilyl)propyl methacrylate, and then the irradiation with UV light (254 nm) using a patterned mask with 100  $\mu\text{m}$  aligned cavities separated by 100  $\mu\text{m}$  intervals (edge-to-edge distance) was carried out. When hepatic cells (human hepatic cancer cell line and fetal mouse primary liver cell (FMLC)) were seeded onto the constructed surface, a spheroid array of these liver cells were fabricated and these hepatic functions were higher than the hepatic cells cultured on monolayer state. In this method, the co-culture of spheroids with monolayer-state cells is easily constructed and the functions of the FMLC spheroid were significantly upregulated by co-culture with nonparenchymal liver cells as feeder-cell.

**Keywords:** photolithography, spheroid, cell adhesion, micropatterned surface, poly(ethylene glycol) (PEG), hepatic cancer cells, fetal mouse liver cells (FMLCs), co-culture

## 1. Introduction

Recent progress in cell culture and microfabrication technologies has stimulated researches on the integration of cell cultures and sensors on a chip. New high-throughput techniques based on cells and tissues microarrays will not contribute to understand fundamental cell biology but also facilitate clinical and pharmaceutical analysis of molecular targets, because living cells can monitor the targets through the physiological changes that are induced in them by exposure to drugs and environmental perturbations, such as

toxicants, pathogens or other agents [1]-[4].

Since primary hepatocytes play many important roles in various metabolic pathways *in vivo*, the possibility of using chips covered with hepatocyte arrays in cell-based assay systems as drug screening tools has been investigated [5] [6]. However, hepatocytes are well known to lose much of their hepatic functions within the first 2 days of monolayer culturing [7]. Thus, the most crucial issues in cell or tissue culturing are long-term viability, and the upregulation and retention of cell functions on the supporting

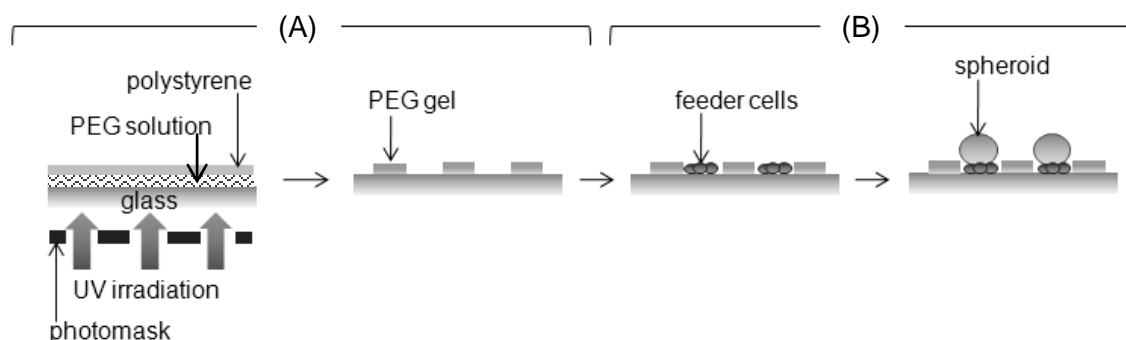


Figure 1. The experimental procedure for construction of (A) cell-adhesion micro-patterned glass surface by photolithography technique and (B) spheroid co-culture array.

surface [8]. In order to solve these problems, we focused on the well-known fact that multicellular spheroids exhibit a characteristic *in vivo*-like morphology; this is attributed to the retention of the 3-D architecture and establishment of important cell–cell contacts. Actually, the spheroid patterned array culture of rat primary hepatic cells retains cellular activity for more than one month, if bovine aorta endothelial cells (BAECs) are used as feeder-cell in 100- $\mu\text{m}$  patterned domains [5] [9]. Among the hepatic cells, fetal mouse liver cells (FMLCs) have been studied as a new material for growing artificial livers [10] [11] [12] and for liver-cell implantation [13], because they are regarded as a suitable cell source for implantation and regeneration due to their genetic normality and potentially proliferative activity *in vitro*.

In this study, we tried to fabricate FMLC spheroid arrays on PEG-gel micropatterned surface [9] [14] [15], which was constructed by silane coupling treatment and photoirradiation technique, and evaluate the activity of the FMLCs and the efficiency of the differentiation induction.

## 2. Experimental

### 2.1. Materials

Poly(ethylene glycol) diacryloyl (M.W. = 575 Da), 3-(trimethoxysilyl)propyl methacrylate, and 2-hydroxy-4'-hydroxyethoxy-2-methylpropiophenone were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and they were used as received. Dulbecco's modified Eagle's medium (DMEM) containing 4,500 mg/L of glucose, Hanks' balanced salt solution modified (HBSS), insulin, dexamethasone, trypsin-EDTA solution (0.5% trypsin, 5.3 mM sodium EDTA), and oncostatin M (OSM) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) and antibiotic-antimycotic were purchased from Gibco-Invitrogen (Grand Island, NY, USA).

Williams' medium E was purchased from MP Biomedicals Inc. (CA, USA). The water used in this study was purified using a Milli-Q system (Nihon Millipore Co., Tokyo, Japan).

### 2.2. Construction of a PEG-gel micropatterned surface

The slide glass purchased from Matsunami Glass Ind. (Tokyo, Japan) was soaked in piranha solution (1:1 volume of  $\text{H}_2\text{SO}_4$  and 30 w/v% of hydrogen peroxide) for 1h. The surface was modified with an ethanol solution of 3-(trimethoxysilyl)propyl methacrylate (4 vol%) for 3h, followed by rinsing with deionized water and heating at 120  $^\circ\text{C}$  for 12 h. 15  $\mu\text{L}$  mixture of diacryloyl-PEG (M.W. = 575 Da, 33 vol%) and 2-hydroxy-4'-hydroxyethoxy-2-methylpropiophenone (1 w/v%) as a photoinitiator in 2:1 volume of the methanol/water co-solvent was dropped onto the methacryloyl group-introduced glass surface thus prepared and expanded uniformly by pressing polystyrene chip. The micropattern of PEG-gel was prepared by the irradiation of UV light (254 nm, 3.6  $\text{mJ}/\text{cm}^2$ ) using UV irradiation system (SUPERCURE-352S, SAN-EI ELECTRIC Co., Osaka, Japan) through patterned photomask. The photomask has metallic circle patterns on quartz glass plate which were 100  $\mu\text{m}$  diameter and interval, where the metallic patterns area inhibited from exposing UV for gelation. After irradiation of UV light, the PEG-gel micropatterned surface that had 100  $\mu\text{m}$  diameter cavities was constructed by rinsing distilled water. The exterior of the cavities was modified with cell-incompatible PEG-gel, while the interior of the cavities was cell-compatible glass surface, relatively. The obtained PEG-gel micropatterned chip was soaked in PBS overnight prior to use.

### 2.3. Cell cultivation

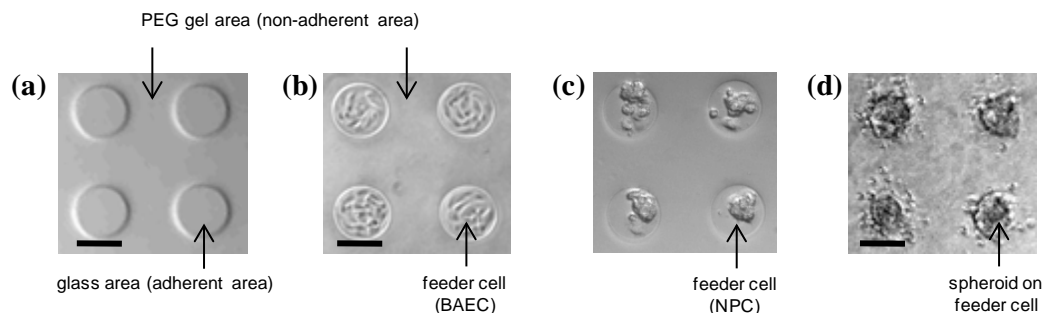


Figure 2. Phase-contrast micrographs of PEG-gel micropatterned surface, feeder-cell patterned arrays, and spheroid-feeder cell co-culture arrays. (a) PEG-gel micropatterned surface, where the cell-adherent area is glass surface, while cell non-adherent area is a PEG-gel modified surface. Feeder-cell array patterns on the constructed surface; (b) BAECs and (c) NPCs. FMLCs spheroid arrays cultured on NPCs-patterned arrays at 16 days after seeding the FMLCs. All scale bars represent 100  $\mu\text{m}$ .

Bovine aortic endothelial cells (BAECs) were purchased from the Health Science Research Resources Bank (JCRB0099, Osaka, Japan). BAECs were used under 20 passages in all cell culture experiments. Nonparenchymal cells (NPCs) was isolated from the same mice as described above.

FMLCs were isolated from C57BL/6 fetal mice (14 embryonic days) and this type of mouse was purchased from Japan SLC Inc. (Shizuoka, Japan). Mouse fetuses were extracted from the amnion and placed in HBSS and then the placenta was removed. The livers were separated from mouse fetuses by using two pairs of tweezers and the separated fetal livers were temporarily stored in HBSS containing 5% FBS and dispersed by pipetting of a syringe attached 22 gauge needle. The obtained FMLCs were filtered through a cell-strainer which is a 70  $\mu\text{m}$  nylon mesh.

As shown in Figure 1(B), FMLCs were seeded onto feeder-cell micropatterned surfaces and the FMLC spheroid array co-cultured with feeder-cell was constructed. One hundred  $\mu\text{L}$  of culture medium supernatant were carefully substituted with the same volume of fresh medium every 2 days without removing the cells. At 7th days of culture, a fully constructed spheroid array was obtained and the loose FMLCs were removed with all the medium, followed by washing in PBS. The cell morphology was monitored using a phase-contrast microscope (IX71, OLYMPUS Co., Tokyo, Japan) and differential interference contrast (DIC) microscope (Axiovert 200M, Carl Zeiss, Germany).

#### 2.4 LIVE/DEAD assay for the FMLC spheroids

The viability of FMLCs constituted spheroids was assessed using fluorescent staining SYTO<sup>®</sup> 10 and DEAD Red<sup>™</sup> (Molecular Probes Inc., CA, USA) that are nucleic acid staining reagents for live and dead cells. The micrographs were obtained by DIC microscope at 21th day of culture.

#### 2.5 Quantification of albumin release from the FMLC spheroids

The amount of albumin secretion from FMLC spheroids on the constructed surfaces was quantified by sandwich enzyme linked immunosorbent assay using a Mouse Albumin ELISA Quantitation Kit (BETHYL Laboratories, Inc., TX, USA) and LumiGLO<sup>™</sup> Chemiluminescent Substrate Kit (Kirkegaard & Perry Laboratories, Inc.). The luminescence measurements were carried out by fluorescence plate reader (ARVO<sup>™</sup> MX, PerkinElmer Japan Co., Ltd., Yokohama, Japan). The measurements were carried out at 9, 12, 16, 21 days of culture from the 7th days. The number of cells was counted by flowcytometry Guava EasyCyte Mini (Bio-medicine Inc., Hayward, CA, USA).

#### 2.6 Determination of enzymatic activity of cytochrome P450 (CYP) 1A2

CYP1A2 is enzyme that appears only in the mature hepatocytes of mice or humans. The CYP1A2 activity was determined using a P450-Glo<sup>™</sup> Assays kit (Promega, WI, USA) in accordance with the protocols. This assay is based on the measurements of luminescence from beetle luciferin derivatives, which can be converted to luciferin by the CYP1A2 enzymatic reaction and the luminescence can be observed by reaction with luciferin detection reagent. Before the

measurement, the spheroids were retrieved by incubating 200  $\mu\text{L}$  trypsin-EDTA solution under the condition of 37°C in a humidified atmosphere with 5%  $\text{CO}_2$  for 10 min. and the spheroids were dispersed as FMLCs by strong pipetting. Twenty  $\mu\text{L}$  FMLCs dispersed solution was used to measure the CYP1A2 activity. The activities were monitored at 9, 12, 16, 21 days of culture from the 7th days. The enzymatic activity was normalized with the amount of luminescence of sample solution without cells.

### 3. Results and Discussion

The PEG-gel micropatterned surface for fabricating the feeder-cell array was prepared on a glass support by silane coupling treatment and photolithography technique using a photomask (Figure 1A). The obtained PEG-gel micropatterned chip shown in Figure 2a was soaked in PBS overnight prior to use, the exterior of the cavities in the chip was modified with cell-incompatible PEG-gel, while the interior of the cavities was lined with cell-compatible glass. In order to construct the substrate-modified surface for fabricating the spheroid array, two kinds of feeder-cell arrays were prepared on the constructed PEG-gel micropatterned surface using NPCs obtained from fetal mouse and BAECs. BAECs and NPCs at a concentration of 600 cells/ $\text{mm}^2$  were seeded onto the PEG-gel patterned surface (Figure 2b and 2c, respectively). These cells were incubated at 37°C with DMEM in a humidified atmosphere with 5%  $\text{CO}_2$ . DMEM containing 4,500 mg/L glucose was supplemented with 10% FBS and 1% antibiotic-antimycotic. Another type of substrate-modified surface was constructed by micropatterning collagen on a PEG-gel micropatterned surface as control using 0.1 mg/mL collagen solution in 0.02 N acetic acid.

The FMLCs were seeded at various cell concentrations onto the constructed BAEC-, NPC-, and collagen-micropatterned PEG-gel surfaces. One hundred  $\mu\text{L}$  of culture medium supernatant were carefully substituted with the same volume of fresh medium every 2 days without removing the cells. At the 7th days of culture, a fully constructed spheroid array was obtained and the loose FMLCs were removed with all the medium, followed by washing in PBS. With any type of feeder cells, FMLC spheroids with a diameter of approximately 70  $\mu\text{m}$  were formed in about 70 % of the cavities (about 800 cavities) when FMLCs at a concentration of  $1.1 \times 10^5$  cells/ $\text{mm}^2$  were seeded

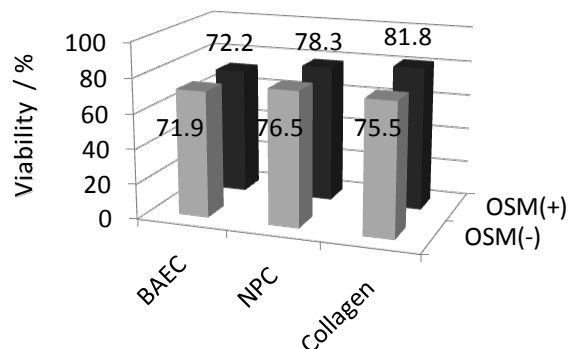


Figure 3. Cell viability in FMLC spheroids cultured on BAEC-, NPC-, collagen- micropatterned surfaces in the presence and absence of OSM.

onto the constructed surfaces (Figure 2d).

The viability of FMLCs constituting the spheroids was assessed by fluorescent staining assay using SYTO® 10 and DEAD Red™, which are nucleic-acid staining reagents for live and dead cells, respectively. Figure 3 shows cell viabilities in the constructed spheroids cultured at 21 days on BAEC-, NPC-, and collagen-micropatterned surfaces in the presence of OSM (OSM(+)) and absence of OSM (OSM(-)). As shown in Figure 3, it is concluded that the present microarray cultivation system maintained their viability about 70-80% at 21 days of culture. Since monolayer-cultured FMLCs can't maintain their viability in more than a week, these results indicate that the long-term culturing of FMLC was successful in spheroids on all substrate-modified surfaces.

The amount of albumin secretion, as one of the most important liver functions, from the constructed FMLC spheroid arrays was measured by sandwich enzyme linked immunosorbent assay (ELISA) using a Mouse Albumin ELISA Quantitation Kit according to the manufacture's protocol. In the absence of OSM, and the FMLC spheroid array on the NPCs showed a steadily large amount of albumin secretion, while that on the BAECs showed almost no secretion for the entire 21-day culture period. The FMLC spheroid array on collagen also showed a low amount of albumin secretion, at the same level as that on BAECs. The same tendency of albumin secretion level was observed in the presence of OSM. These results indicate that the amount of albumin secretion from FMLC spheroids on a PEG-gel micropatterned surface can be enhanced by using NPCs as the feeder-cell.

In order to assess the differentiation induction of the constructed FMLC spheroid arrays, the cytochrome P450 (CYP) 1A2 activity, which appears only in the mature hepatocytes of mice or humans, was evaluated using a P450-Glo™ Assays Kit and monitored at 9, 12, 16 and 21 days of culture, starting on the 7th day. Figure 4 shows the CYP1A2 activity of the FMLC spheroids on BAEC-, NPC-, collagen -micropatterned surfaces in the presence and absence of OSM. In the case of the spheroid array with the BAECs as the substrate, almost no CYP1A2 activity was observed, although the activity of the spheroids with OSM (BAECs/OSM(+)) was slightly higher than that without OSM (BAECs/OSM(-)). Interestingly, in the case of the spheroids with NPCs as the substrate, the activity of the spheroids with OSM (NPCs/OSM(+)) was extremely high, while that of the spheroids without OSM (NPCs/OSM(-)) was low, at almost the same level as the spheroids with BAECs/OSM(+). These results indicate that NPCs and OSM can upregulate the CYP1A2 activity of the FMLC spheroids on the constructed arrays, indicating that constructed FMLC spheroid arrays have undergone a high degree of differentiation. In the case of collagen/OSM(-), the P450 activity was low and nearly equal to that with BAECs (OSM- and OSM+) and NPCs(OSM-) until 15 days of culture. Then the activity significantly increased at 21 days of culture. On the other hand, the P450 activity with collagen/OSM(+) showed the liner increase in the P450 activity from 7 days to 21 days of culture and consequently reached to the same activity of FMLCs spheroid with collagen/OSM(-). We are now investigating the effect of feeder layerments in the viability of the FMLCs in spheroids to understand the mechanism of upregulation phenomena.

#### 4. Conclusions

In conclusion, we succeeded in constructing a two-dimensional array of PEG-gel micropatterned surface, where FMLC spheroids were co-cultured on feeder cells patterned surfaces with uniform size and number. The FMLC spheroids on the constructed array showed long-term viability and high hepatic activity. Especially, co-culturing of FMLC spheroid with NPCs upregulated their hepatic activity. This novel cell-chip technology and the findings in this study could provide an interesting new approach to the construction of tissues and organs for regenerative medicine.

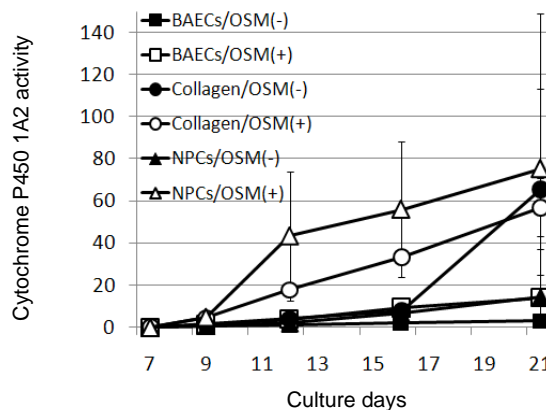


Figure 4. CYP1A2 activity of cells in FMLCs spheroid constructed on BAEC-, NPC-, collagen -micropatterned surfaces in the presence and absence of OSM.

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