Supplementary Information

Rapid and high-throughput formation of 3D embryoid bodies in hydrogels using dielectrophoresis technique

Samad Ahadian, Shukuyo Yamada, Javier Ramón-Azcón, Kosuke Ino, Hitoshi Shiku, Ali Khademhosseini, Tomokazu Matsue

aWPI-Advanced Institute for Materials Research, Tohoku University, Sendai 980-8577, Japan
bGraduate School of Environmental Studies, Tohoku University, Sendai 980-8579, Japan
cDepartment of Chemical and Biomolecular Nanotechnology, Advanced Chemical Research Institute of Catalonia (IQAC-CSIC), Jordi Girona 18-26, Barcelona 08034, Spain
dNanobiotechnology for Diagnostics (Nb4D) Group, IQAC-CSIC, Jordi Girona 18-26, Barcelona 08034, Spain
eDepartment of Medicine, Center for Biomedical Engineering, Brigham and Women’s Hospital, Harvard Medical School, Cambridge, Massachusetts 02139, USA; Harvard–MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA; Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, Massachusetts 02115, USA; Department of Physics, King Abdulaziz University, Jeddah 21569, Saudi Arabia; and Department of Maxillofacial Biomedical Engineering and Institute of Oral Biology, School of Dentistry, Kyung Hee University, Seoul 130-701, Republic of Korea

*Corresponding author: WPI-Advanced Institute for Materials Research, Tohoku University
2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan
Phone: +81-22-217-6140; Fax: +81-22-217-6140
E-mail address: ramonazconjavier@gmail.com
Figure S1. Fabrication of dielectrophoretically aggregated ESCs of different shapes. (A) Schematic representation of the arrangement of four electrode band elements in the DEP device. (B) Top view of the simulated electric fields within the devices. (C) Optical images of the ESC aggregate with different shapes in GelMA hydrogels. Scale bars show 50 µm.
Figure S2. Fabrication of dielectrophoretically aggregated ESCs of different sizes. (A) Optical images of the ESCs patterned by the n-DEP technique to different sizes. Scale bars show 100 μm. (B) Average diameter of ESC spherical aggregates with respect to the electrode width. (C) Number of stem cell aggregates per device as a function of electrode width in the DEP device. (D) Average circularity of fabricated cell aggregates as a measure of electrode width in the DEP device.
**Experimental section**

**Instrumentation**

A multichannel function generator (Hioki 7075, Hioki E.E. Co., Japan) was used to apply the AC voltage to ITO-IDA electrodes for the DEP manipulation of cells. Fluorescence images were observed and recorded through a fluorescence microscope (Leica DMIRE2, Leica Co. AG., Solms, Germany) equipped with a digital camera (Leica DFC565FX, Leica Co. AG., Solms, Germany). Fluorescence intensities were analyzed using Image J software package (ver. 1.42, National Institute of Health, USA). The prepolymer were exposed to 7 mW/cm² UV light (Hayashi UL-410UV-1, Hayashi Electronic Shenzen, Japan).

**Materials**

The materials and suppliers used were as follows: Hexamethyldisilazane (Tokyo Ohka Kogyo Co., Ltd., Japan); developer (MF CD-26) and positive g-line photoresist (S1818) (Shipley Far East Ltd., Japan); methacrylic anhydride, 3-(trimethoxysilyl)propyl methacrylate (TMSPMA), trichloro (1H, 2H, 2H-perfluorooctyl)silane, 3,3,4,4,5,5,6,6,6-nonafluorohexyl trichlorosilane, gelatin type A made from porcine skin, and penicillin/streptomycin (P/S) (Sigma-Aldrich Chemical Co., USA); 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959) (Ciba Chemicals, Japan); and ITO glass (Sanyo Vacuum Industries Co., Ltd., Japan).

**Cell culture**

129/SVE-derived ES mouse cell line was purchased from DS Pharma Biomedical Co., Ltd. The cells in their undifferentiated state were maintained in serum-free media...
(DS Pharma Biomedical Co., Ltd.) with 1000 U/mL mouse leukemia inhibitory factor (LIF; Millipore), 1% P/S, and 1 mM β-mercaptoethanol (Millipore). The cells were grown on the 0.1% gelatin (Millipore)-coated flasks and incubated at 37.0°C in a water-saturated atmosphere of 95% air and 5% CO₂. The cell medium was replaced every day. After the ESC aggregation by the DEP, the devices were cultured in 15% FBS, 1% P/S (GIBCO), and 1 mM β-mercaptoethanol to induce the stem cell differentiation.

**GelMA prepolymer synthesis**

GelMA prepolymer was synthesized as described elsewhere. In brief, 12 mL of methacrylic anhydride was added to 6 g of gelatin in phosphate-buffered saline (PBS) for 1 hr at 50°C, resulting in a high degree of methacrylation (~80%) of the gelatin. The mixture was dialyzed with a 12-14 kDa dialysis membrane against distilled water for one week at 40°C and then lyophilized for one week. The GelMA prepolymer was stored at -20°C until use. The GelMA prepolymer (10% [w/v]) was dissolved in the sucrose buffer with 1% (w/v) photoinitiator (Irgacure 2959), stored at 60°C to complete the process, and then used in experiments.

**Design and fabrication of ITO-IDA electrodes**

The DEP devices consisted of two overlapped ITO-IDA) electrodes. The effective electrode dimensions were 9 × 9 mm². The electrode bands of devices were fabricated on a glass slide (thickness, 1 mm; Matsunami Co., Japan) by conventional photolithography and chemical etching using an etchant solution (HCl:H₂O:HNO₃/4:2:1 by volume) for 15 min under ultrasonication, followed by removing photoresist with acetone and washing with isopropyl alcohol. The
microfluidic chamber was defined by a polyester film (100 µm thick, Lintec Co., Japan) between the bottom and top ITO-IDA electrodes.

**Stem cell patterning using DEP technique**

The DEP device was composed of two interdigitated array ITO (ITO-IDA) electrodes (Figure 1) fabricated using conventional photolithography and chemical etching techniques. The upper ITO-IDA electrode was mounted on the lower ITO-IDA electrode with a polyester film in between in order to create a microfluidic chamber (100 µm high) between both electrodes, ensuring 3D formation of stem cell aggregates. Just prior to the DEP experiments, mouse stem cells were suspended in a sucrose buffer (0.81 mM MgSO$_4$, 1.81 mM Na$_2$HPO$_4$.2H$_2$O, 1.81 mM KH$_2$PO$_4$, 44.1 mM NaHCO$_3$ with 50 mM glucose at pH 7.4) and then mixed with the GelMA prepolymer (1:1 ratio) at a density of $1 \times 10^7$ cells/mL. The final concentration of cell-laden hydrogel was 5% (w/v), which is the optimum GelMA hydrogel concentration for cell viability and growth. The mixture (20 µL) was pipetted into the microfluidic chamber, and then an AC voltage (voltage 12 Vpp and frequency 1.0 MHz) was applied to the lower and upper ITO-IDA electrodes. The dielectrophoretically-patterned cells within the GelMA prepolymer were fixed upon the exposure to 365 nm UV light for 4 min. Following crosslinking of the GelMA hydrogel, the top ITO-IDA electrode was gently removed, and 3D cell aggregates encapsulated in the GelMA hydrogel on the lower ITO-IDA electrode were cultured for up to 1 week in a standard incubator for the EB development.

**Assessment of cell viability**
The calcein AM/ethidium homodimer live/dead assay (Dojondo, Laboratories, Japan) was employed to quantify the cell viability encapsulated in the hydrogels according to the manufacturer instructions. Calcein AM is a cell-permanent dye that is changed to green fluorescent calcein in live cells through the action of intracellular esterases. Ethidium homodimer is a DNA-binding dye that enters the damaged membrane of dead cells. At least five images of two independent experiments were used for the quantification of cell viability.

**RNA extraction and cDNA synthesis**

RNA was extracted using β-mercaptoethanol and purified according to the manufacturer’s protocol (RNeasy© microkit, Qiagen, Venlo, Netherlands). Reverse transcription was performed according to the manufacturer’s instructions (Quantitech Reverse Transcription, Qiagen, Venlo, Netherlands) for up to 1 µg of total RNA. The temperature profile of the cDNA synthesis protocol was as follows: 12 µl of sample (3 µg of total RNA) was diluted with 14 µl of RNase-free water and 4 µl of gDNA wipeout buffer and incubated for 2 min at 42°C and then cooled down to 4°C. Quantiscript Reverse Transcriptase and Reverse Transcriptase primer mix were subsequently added, and the mixture was incubated for 15 min at 42°C followed by incubation for 3 min at 95°C. The samples were kept at 4°C until use for the quantitative PCR (qPCR).

**Real time PCR**

Primer sets for GAPDH and Nanog were obtained from Nihon Gene Research Laboratories, Inc. (Japan) and validated for qPCR. The primer sequences are (left primer: tgtccgtcgtggatctgac and right primer: cctgcttcaccaccttcttg) and (left primer:
ttctgttaaggggtctgc, right primer: agaggaagggcggaggaga) for GAPDH and Nanog, respectively. Real time PCR was performed on a Roche Lightcycler 1.5 (Roche, Mannheim, Germany) using 2 µl of cDNA, 2 µl of the primer set (50 µm), and 14 µl of Lightcycler FastStart DNA Master SYBR Green 1 (Roche, Mannheim, Germany). Following an initial denaturation step at 95°C for 10 min, real time PCR was performed over 45 cycles of 95°C for 10 s, 62°C for 10 s, and 72°C for 20 s, followed by a melt curve analysis. The expression of the target gene was assessed using the comparative method and the results were normalized to the mouse GAPDH gene as the internal reference. Reported gene expression levels were the average of at least two independent experiments.

Reference