Inverting microwell array chip for the cultivation of human induced pluripotent stem cells with controlled aggregate size and geometrical arrangement

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(Received 18 December 2013; accepted 31 March 2014; published online 16 April 2014)

We present a novel cell culture chip, namely, “inverting microwell array chip,” for cultivation of human induced pluripotent stem cells. The chip comprises a lower hydrogel microwell array and an upper polystyrene culture surface. We demonstrate the formation of uniform cellular aggregates in the microwell array, and after inversion, a culture with controlled aggregate size and geometrical arrangement on the polystyrene surface. Here, we report effects of cell concentrations on a cultivation sequence in the chip. © 2014 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4871036>]

I. INTRODUCTION

The potency of Human induced pluripotent stem (hiPS) cells¹ promises huge changes in a variety of fields such as cell biology,² drug discovery,^{3–5} and clinical therapy.⁶ In these fields, the maintenance of homogeneous cell populations and the reproducible control of differentiation are important issues, because behavior of hiPS cells is severely affected by the surrounding microenvironment, which includes the size and shape of the cellular aggregates during passage and differentiation, the geometrical arrangement of the adhered cellular aggregates (i.e., colonies), the timing of medium change as well as addition of differentiation factors, and composition of the extracellular matrix.^{7,8} The development of processes, ideally involving programmed perfusion^{9–11} and automated cultivation,^{12–14} to control the culture microenvironment would overcome these problems and allow this approach to be applied to industrial applications.

The conventional method for cultivating hiPS cells involves the handling of cells as cellular aggregates; however, obtaining uniformly sized aggregates is technically difficult. Pipetting, which is generally the preferred method of handling liquids in biological laboratories, inevitably causes a broad distribution of cellular aggregate sizes, even in automated systems; the size of cellular aggregates after pipetting depends on the shear stress, the size, and shape of the original aggregates, and the strength of the cell–cell and cell–extracellular matrix interactions of each individual aggregate.

Cell-assembling of single hiPS cells offers a solution for producing uniform cellular aggregates. Although the break-down of cellular aggregates into single cells induces cell death, the formation of cellular aggregates promotes cell survival. In order to evaluate the effects of cellular aggregate size on differentiation, many previous studies have reported various methods for the formation of size-controlled cellular aggregates of hiPS and embryonic stem cells and the use of microwell structures, in particular, produced favorable results in the size-control.^{15–23} Some groups have reported a subsequent adherent culture after formation of uniform cellular

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aggregates in microwells.^{21–25} In addition, some groups have reported microfluidic devices using gravity for spheroid formation.^{26–28} Couple of these methods realized retrieval of spheroids from the devices using medium flow.^{26,27} However, the production of a geometrical arrangement of adhered cellular aggregates is still challenging; previous reports adopted inoculation of cellular aggregates by pipetting, which resulted in adhesion at random positions. Voldman *et al.* reported the BioFlipChip, a microfabricated micro well array chip, to trap small number of cells to pattern cells in a predetermined geometrical arrangement.²⁹ However, the application was limited to single cell or small number of cells, and applicability to cellular aggregate is unclear.

Here, we describe an inverting microwell array chip that is capable of producing uniform cellular aggregates and of controlling the position of the cellular aggregates in the adhesion culture. Fig. 1 illustrates the procedure for cultivating hiPS cells in the inverting microwell array chip. A microwell array with sloped walls made out of poly(ethylene glycol) (PEG) based hydrogel is placed in the bottom of the chamber, into which a single-cell suspension is loaded. The cells loaded into the chamber settle to the bottom of the microwells and form cellular aggregates. During this process, PEG based hydrogel with the sloped walls enhances the sedimentation of cells at the center of the microwells. After cellular aggregates have formed, the chip is inverted, which results in the cellular aggregates being plated onto a polystyrene (PS) culture surface in the same geometrical arrangement as the microwells. During this process, PEG based hydrogel with the sloped walls enables detachment of cellular aggregate from the microwells and sedimentation onto the PS surface. Further incubation allows the cellular aggregates to attach to the culture surface and start spreading and proliferating. Ideally, the adhered cellular aggregates will have a uniform size distribution and an array-formatted geometry. Finally, the proliferated cells are collected from the chip as a single-cell suspension by enzymatic treatment.

II. EXPERIMENTAL

A. Chip assembly

A negative template made of polydimethylsiloxane (PDMS, Silpot 184, Dow Corning Toray Inc., Tokyo, Japan) for microwell imprinting was formed by repeated replica molding cycles from a negative brass template, which had a 4×24 array of cones with a pitch of 2.7 mm on a flat surface. One cone had a height of 1.25 mm and a top angle of 90° . The negative PDMS template was coated with the small amount of mixed prepolymer solution of PDMS followed by curing for smoothing its surface and forming gradual curved slopes around the top of cones and was then treated with n-butyltrichlorosilane vapor under reduced pressure. A chamber block was prepared by curing PDMS on a polytetrafluoroethylene master template, which has a negative structure of the chambers and was originally fabricated by milling machine: One PDMS block ($108 \times 67 \times 5$ mm) had four chambers ($65 \times 12 \times 4$ mm). Silicone tubes were connected to both ends of the chamber via through-holes fabricated at both ends of each chamber (Fig. 2(a)).

FIG. 1. Schematic illustration of the cultivation process of hiPS cells in an inverting microwell array chip. PS, polystyrene; PDMS, polydimethylsiloxane.

The PDMS chambers were temporary filled with a solution of benzophenone in acetone (10%) for 1 min. The negative microwell templates were placed in the PDMS chambers; the tops of cones were set 0.5 mm apart from the chamber surfaces. A mixture solution of PEG diacrylate (average Mn 700, Sigma-Aldrich Japan, Tokyo, Japan) (4.40%), poly(ethylene glycol) methacrylate (average Mn 360, Sigma-Aldrich Japan) (3.08%), sodium acrylate (Nakalai Tesque, Kyoto, Japan) (1.32%), and Irgacure 2959 (BASF Japan, Tokyo, Japan) (0.5%) in water was filled into opening spaces between the PDMS chambers and negative templates. The solutions were irradiated with an UV light at 90 mJ/cm² for 300 s by use of an UVE-251S UV light source equipped with an UVC-251S UV lamp controller (San-Ei Electric Co., Tokyo, Japan). Prepared hydrogels were soaked three times in PBS to wash and were then exposed to an UV light for sterilization.

The PDMS block for chambers equipped with the hydrogel layers was combined with a flat plate of PS (110 × 68 × 1 mm) coated with Matrigel (BD Bioscience Japan, Tokyo, Japan) and was fastened by use of an aluminum holder and screws. The holder had windows on chambers for observations and connection of transfusion tubes.

B. Cell cultivation

hiPS cells (201B7) were obtained from RIKEN Bioresource Center (Tsukuba, Japan). All media and reagents were purchased from commercial sources. The cells were maintained on Matrigel-coated dish in ReproFF2 (ReproCELL, Tokyo, Japan) containing bFGF (5 ng/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml) under feeder-free conditions. A Rho-associated protein kinase (ROCK) inhibitor (Y-27532, Mitsubishi Pharma, Osaka, Japan) (10 µM) was added to the medium used in a passage process (Kurosawa, 2012).

Adhered hiPS cellular aggregates on a culture dish were washed with PBS and were then treated with Accutase (Innovative Cell Technologies Inc., San Diego, California) at 37 °C for 6 min. The cellular aggregates were dispersed into single cells by gentle pipetting after dilution of the enzyme solution with a medium. The cells were collected by centrifugation (1000 rpm, 3 min) and resuspended with a fresh medium to prescribed concentration. The media used in this process contained the ROCK inhibitor.

Cultivation of hiPS cells in the inverting cell culture chip was typically performed as follows. The chambers were washed three times with a fresh medium containing the ROCK inhibitor to replace PBS in hydrogels. Hydrogel layers were placed at the underside of the chip and a single hiPS cell suspension was loaded into the chambers. The chip was statically kept in an incubator for 20 h except for intermittent tapping during early 4 h. The medium was mostly removed from the chambers and that in the microwells was left as it was. The chip was touched on a vortex mixer. The chambers were filled with a fresh medium containing the ROCK inhibitor and the chip was inverted to settle the cellular aggregates onto the cultivate surfaces. When only a few aggregates were settled, application of mechanical vibration was effective for accelerating settling down. The chip was kept in an incubator and medium was changed every 2 days. The inside of the chambers was washed with PBS after prescribed cultivation period and the cells were treated with Accutase at 37 °C for 8 min. The chip was gently inverted after loading small air bubbles into the chambers, which resulted in a dispersion of the adhered cellular aggregates into single cells. The cell suspension was retrieved from the chambers and cells were collected by centrifugation. Triplicate experiments were performed to evaluate adhesion rate and production yield.

C. Determination of diameters of the cellular aggregates

The diameters of the cellular aggregates were calculated by image analysis using a measurement software WinROOF version 5 (Mitani Co., Fukui, Japan) from microscope images.

III. RESULTS AND DISCUSSION

The inverting microwell array chip is composed of a PDMS block with four individual chambers, a PS plate as the culture surface, and hydrogel layers in which the microwell arrays

FIG. 2. (a) Overview of the chip without a holder. (b) Cross-section of the microwells imprinted in the hydrogel layer. Scale bar: 2 mm.

are imprinted (Fig. 2). Each chamber has a hydrogel layer with 96 microwells each with a depth of 0.8 mm. The bottom of the microwell forms a reverse cone shape with a top edge angle of 90° , meaning that each microwell wall has a slope angle of 45° . We made the microwells out of poly(ethylene glycol)-based hydrogels because of its low cell-adhesive property, easy method of preparation, and transparency.

We then cultivated hiPS cells in the inverting microwell array chip. Single-cell suspensions were prepared by Accutase treatment of hiPS cellular aggregates that had been cultivated in feeder-free culture conditions. A cell suspension corresponding to 2000 cells/microwell was loaded into the chambers of the chip. The cells were incubated for 4 h with intermittent tapping to gather the cells at the bottom of the microwells. Uniform cellular aggregates were observed after a further 16 h of static cultivation (Fig. 3(a)). While keeping some of the culture medium in the microwells, most of the medium was temporarily removed from the chambers. In this step, cells that did not settle into the microwells were washed out from chambers. The chip was then vibrated to release the cellular aggregates from the bottom of the microwells. Inversion of the chip after refilling a fresh medium resulted in settling of the cellular aggregates onto the

FIG. 3. Cultivation of hiPS cells in the inverting microwell array chip. (a) Cellular aggregates formed in the microwells (arrows). (b) Cellular aggregates on the culture surface just after inversion (arrows). (c) Cellular aggregates adhered and spread 2 days after inversion. (d) Proliferated cellular aggregates 5 days after inversion. Scale bars: 1 mm.

culture surface in an array format corresponding with the geometry of the microwell array (Fig. 3(b)), although less than half of the aggregates successfully settled. The distance from the center of the microwell to the settled position was $380\text{ }\mu\text{m}$ in average and standard deviation was $150\text{ }\mu\text{m}$, although approximately 20% of the aggregate moved out to the observed area. The cellular aggregates adhered to and spread on the PS surface during cultivation for 2 days (Fig. 3(c)). Fig. 3(d) shows the proliferated cellular aggregates at 5 days after plating. We observed that the spread aggregates kept their array formation. After 6 days of cultivation, which included the period of aggregate formation, the cellular aggregates were treated with Accutase, and most of the cultivated hiPS cells were collected as a single-cell suspension. These results demonstrate the successful passage of hiPS cells in the inverting microwell array chip.

We also investigated the effects of cell concentration on cultivation in the inverting microwell array chip. When cell suspensions were loaded at concentrations of 31, 125, 500, or 2000 cells/microwell, the diameters of the subsequent cellular aggregates were 70, 130, 160, and $250\text{ }\mu\text{m}$, respectively (Figs. 4 and 5(a)). Assuming the volume of the cellular aggregates is proportional to the number of cells loaded in each microwell, a cell concentration ratio of 31/125/500/2000 is equivalent to a diameter ratio of 1.0/1.6/2.5/4.0. However, the observed diameter ratio was 1.0/1.8/2.2/3.4. The smaller-than-expected cellular aggregates were observed under the 500 or 2000 cells/microwell conditions, although the difference between the observed ratio and the expected ratio was within the experimental error (Fig. 5(a)). At high cell concentrations, several small satellite aggregates were observed around the major aggregates (Fig. 4). These satellite aggregates likely explain the formation of smaller-than-expected cellular aggregates at 500 or 2000 cells/microwell.

Fig. 5(b) shows the adhesion rate of the cellular aggregates that had settled on the culture surface after inversion. Adhesion rate was calculated from the number of adhered cellular aggregates and of settled aggregates. We observed complete release of the aggregates from the microwells by applying mechanical shocks to the chip in addition to the vibration. However, the geometry of the aggregates was disturbed in the process. The adhered cellular aggregates were counted microscopically 2 days after inversion. We calculated the cellular aggregates that have spread to diameters more than two times larger than that of those before the settling

FIG. 4. Microscopic images of cellular aggregates formed after a 20 h incubation at concentrations of (a) 31, (b) 125, (c) 500, and (d) 2000 cells/microwell. Satellite-like aggregates were observed around major one at the higher concentrations ((c) and (d)). Scale bars: $400\text{ }\mu\text{m}$. A striped-pattern on a PDMS surface, which resulted from machining marks on a template, is seen through a hydrogel layer.

FIG. 5. (a) Average diameters of hiPS cellular aggregates calculated by eight aggregates in one chamber. (b) Average adhesion rates of the aggregates ($n = 3$). (c) Average production yields in the inverting microwell array chip after cultivation for 6 days, which included the period of aggregate formation ($n = 3$).

down; thresholds of the diameter of the counted cellular aggregates were 150, 250, 350, and 500 μm for the conditions of 31, 125, 500, and 2000 cells/microwell, respectively. We observed the maximum adhesion rate of approximately 85% at the conditions of 500 and 2000 cells/microwell. Increased numbers of cells capable of contacting the culture surface probably enhanced the interactions of the cells with the Matrigel at these higher cell concentrations.

Cells were collected 5 days after inversion. Fig. 5(c) shows the production yields for the four cell concentrations assessed, which was calculated as the number of cells collected divided by the number of cells loaded. The production yields were 193%, 203%, 221%, and 228% for the conditions of 31, 125, 500, and 2000 cells/microwell, respectively. Production yields are likely affected by the following parameters: (i) aggregate formation yield, (ii) settling rate, (iii) adhesion rate, (iv) aggregate proliferation, and (v) collection yield after Accutase treatment. The settling rate and collection yield were nearly 100% in this experiment, as determined microscopically. The aggregate formation yield was possibly lower under the 500 or 2000 cells/microwell conditions than under the 31 or 125 cells/microwell conditions, as discussed above (Fig. 5(a)). However, the adhesion rate was higher under the 500 or 2000 cells/microwell conditions than under the 31 or 125 cells/microwell conditions. These opposing effects probably resulted in the small difference in production yields between each cell concentration condition.

In this study, we have presented the inverting microwell array chip, in which hiPS cells were cultured with controlled aggregate size and geometry. It is well known that the aggregate size affects the behavior of stem cells.^{22,23} Furthermore, effect of autocrine factors is principally different in different aggregate size and spacing. Therefore, keeping the geometrical arrangement as well as aggregate size is very important to keep the quality of culture. We believe that our device is useful for maintenance culture and differentiation culture of stem cells. However, further details on pluripotency and differentiation markers need to be characterized for additional information as a future issue.

IV. CONCLUSIONS

In summary, we successfully demonstrated the cultivation of hiPS cells in an inverting microwell array chip. We were able to control aggregate size and produce an array-formatted arrangement. Cultivation using the inverting microwell array chip is very simple and includes the formation of the cellular aggregates and adhesion to the culture medium. Aggregate size was controlled in the microwell array and was dependent on initial cell concentration. The cellular aggregates formed adhered to the culture medium in an array pattern and proliferated uniformly. Production yields were approximately 200% after 6 days of cultivation, which included the period of aggregate formation. The inverting microwell array chip permits controlled and

reproducible cultivation of hiPS cells and this will provide new opportunities for controlled maintenance cultivation, efficient differentiation, and automated cultivation in basic laboratory studies and industrial applications.

ACKNOWLEDGMENTS

This research was partially supported by KAKENHI (No. 24106512).

- ¹K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka, *Cell* **131**, 861 (2007).
- ²S. Yamanaka and H. M. Blau, *Nature* **465**, 704 (2010).
- ³S. M. Wu and K. Hochedlinger, *Nat. Cell Biol.* **13**, 497 (2011).
- ⁴H. Inoue and S. Yamanaka, *Clin. Pharmacol. Ther.* **89**, 655 (2011).
- ⁵N. Zeevi-Levin, J. Itskovitz-Eldor, and O. Binah, *Pharmacol. Ther.* **134**, 180 (2012).
- ⁶D. A. Robinton and G. Q. Daley, *Nature* **481**, 295 (2012).
- ⁷R. Peerani, B. M. Rao, C. Bauwens, T. Yin, G. A. Wood, A. Nagy, E. Kumacheva, and P. W. Zandstra, *EMBO J.* **26**, 4744 (2007).
- ⁸C. L. Bauwens, R. Peerani, S. Niebruegge, K. A. Woodhouse, E. Kumacheva, M. Husain, and P. W. Zandstra, *Stem Cells* **26**, 2300 (2008).
- ⁹L. Kim, M. D. Vahey, H. Y. Lee, and J. Voldman, *Lab Chip* **6**, 394 (2006).
- ¹⁰E. Figallo, C. Cannizzaro, S. Gerecht, J. A. Burdick, R. Langer, N. Elvassore, and G. Vunjak-Novakovic, *Lab Chip* **7**, 710 (2007).
- ¹¹D. Titmarsh, A. Hidalgo, J. Turner, E. Wolvetang, and J. Cooper-White, *Biotechnol. Bioeng.* **108**, 2894 (2011).
- ¹²R. J. Thomas, D. Anderson, A. Chandra, N. M. Smith, L. E. Young, D. Williams, and C. Denning, *Biotechnol. Bioeng.* **102**, 1636 (2009).
- ¹³S. Terstegge, I. Laufenberg, J. Pochert, S. Schenk, J. Itskovitz-Eldor, E. Endl, and O. Brüstle, *Biotechnol. Bioeng.* **96**, 195 (2007).
- ¹⁴S. Haupt, J. Grützner, M.-C. Thier, T. Kallweit, B. H. Rath, I. Laufenberg, M. Forgber, J. Eberhardt, F. Edenhofer, and O. Brüstle, *Biotechnol. Appl. Biochem.* **59**, 77 (2012).
- ¹⁵J. C. Mohr, J. J. de Pablo and S. P. Palecek, *Biomaterials* **27**, 6032 (2006).
- ¹⁶E. Sinkala and D. T. Eddington, *Lab Chip* **10**, 3291 (2010).
- ¹⁷J. M. Karp, *Lab Chip* **7**, 786 (2007).
- ¹⁸M. Charnley, M. Textor, A. Khademhosseini, and M. P. Lutolf, *Integr. Biol.* **1**, 625 (2009).
- ¹⁹G. S. Jeong, Y. Jun, J. H. Song, S. H. Shin, and S.-H. Lee, *Lab Chip* **12**, 159 (2012).
- ²⁰H. Tekin, M. Anaya, M. D. Brigham, C. Nauman, R. Langer, and A. Khademhosseini, *Lab Chip* **10**, 2411 (2010).
- ²¹J. C. Mohr, J. H. Zhang, S. M. Azarin, A. G. Soerens, J. J. de Pablo, J. A. Thomson, G. E. Lyons, S. P. Palecek, and T. J. Kamp, *Biomaterials* **31**, 1885 (2010).
- ²²Y. Y. Choi, B. G. Chung, D. H. Lee, A. Khademhosseini, J. H. Kim and S. H. Lee, *Biomaterials* **31**, 4296 (2010).
- ²³Y. S. Hwang, B. G. Chung, D. Ortmann, N. Hattori, H. C. Moeller, and A. Khademhosseini, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 16978 (2009).
- ²⁴C. Kim, K. S. Lee, J. H. Bang, Y. E. Kim, M.-C. Kim, K. W. Oh, S. H. Lee, and J. Y. Kang, *Lab Chip* **11**, 874 (2011).
- ²⁵H. C. Moeller, M. K. Mian, S. Shrivastava, B. G. Chung, and A. Khademhosseini, *Biomaterials* **29**, 752 (2008).
- ²⁶B. Patra, Y.-H. Chen, C.-C. Peng, S.-C. Lin, C.-H. Lee, and Y.-C. Tung, *Biomicrofluidics* **7**, 054114 (2013).
- ²⁷K. Lee, C. Kim, J. Y. Yang, H. Lee, B. Ahn, L. F. Xu, J. Y. Kang, and K. W. Oh, *Biomicrofluidics* **6**, 014114 (2012).
- ²⁸T. Kim, I. Doh, and Y.-H. Cho, *Biomicrofluidics* **6**, 034107 (2012).
- ²⁹A. Rosenthal, A. Macdonald, and J. Voldman, *Biomaterials* **28**, 3208 (2007).