Application of the unique micro-fabric vessels EZSPHERE for efficient generation, expansion and differentiation of human iPS cell aggregates

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Abstract

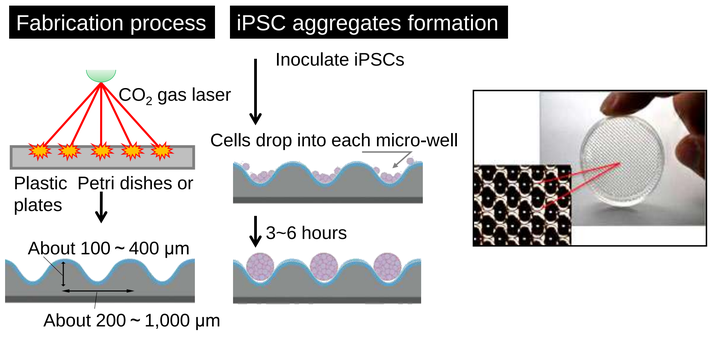
For the early realization of regenerative medicine using human induced pluripotent stem cells (hiPSCs), it is necessary to develop large-scale, reproducible and low-cost stem cell culture techniques. In such direction, suspension culture of iPSCs as aggregates or embryoid bodies (EBs) has been known to be a effective method for expansion and differentiation processes. So far, we verified that the unique microfabric vessels "EZSPHERE", in which a large number of micro-wells are solely created on the plastic dishes or plates by laser beams and followed by coating with low-cell-attachment reagents, permit high-throughput generation of uniformly-sized EBs in high density. The diameter and depth of each micro-wells on the microfabric vessels can be easily and flexibly altered respectively around 200-1,400 μm and 100-400 μm by tuning exposure time or intensity of the laser beam.

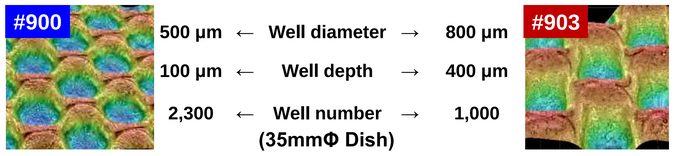
When hiPSCs dissociated to single cells were inoculated into a 35 mm dish-type EZSPHERE, on which approximately 2,400 micro-wells were created, over 2,000 uniformly-sized EBs were generated within only 3-6 hours. In addition, the size of EBs could be controlled by changing the inoculated cell number and/or size of the micro-wells. It was identified by culturing the EBs on the EZSPHERE with maintenance medium for several days that the size of each EB and the total cells number were increased significantly with keeping their undifferentiation state. Furthermore, to confirm pluripotency of the generated and expanded EBs on the EZSPHERE, a cardiomyocyte differentiation test was performed on them and as a result over 85% cTnT positive cardiomyocyte was obtained.

In addition, it was also demonstrated by a neural differentiation assay that the EBs have a very high differentiation capacity to dopaminergic neuron on the same EZSPHERE that continuously used throughout from proliferation to differentiation by only changing medium. These results indicated that high-throughput generation and culture of EBs by using the novel microfabric vessels EZSPHERE have a high potential of application for not only basic or clinical research, but also for regenerative medicine.

Materials and Methods

I. About micro-fabric vessels EZSPHERE





Micro-wells of EZSPHERE are solely created by CO_2 gas laser on the plastic dishes or plates, followed by coating with low-adhesive reagents (MPC polymer or our original polymer).

Because micro-wells are closely positioned each other in the plastic culture ware, inoculated cells equally drop into each well.

Six variations of micro-well size EZSPHERE (#900 ~ #905) are commercially available (Nacalai USA, REPROCELL). In this study We use #900, #903, and #905 type .

II. Cells and Regents

Cells

Human iPS Cell line 201B7 and 253G1 were maintained on feeder or feeder-less and dissociate into Cells Singles with Accutase or TrypLE select before inoculation into EZSPHERE

Medium

For Generation and Expansion of iPSC aggregates: mTeSR1 (feeder-free cell culture medium)

Results

I. Generation of hiPSC Aggregates

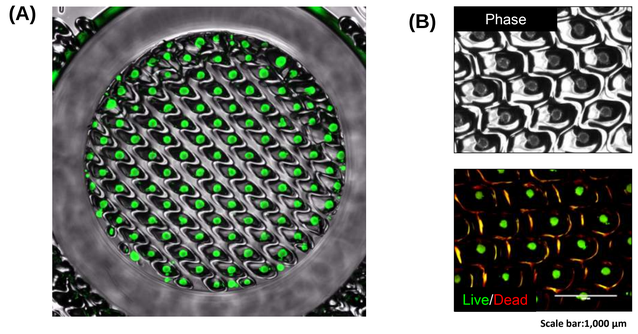


Figure 1. Efficient generation of hiPSC aggregates in each well
(A) Fluorescence microscopy image of iPSC aggregates obtained on the 96 well-plate type EZSPHERE#900. iPSC aggregates were generated in each micro-well. (B) Live Dead Cell Staining image shows high viability of generated iPSC aggregates in EZSPHERE.

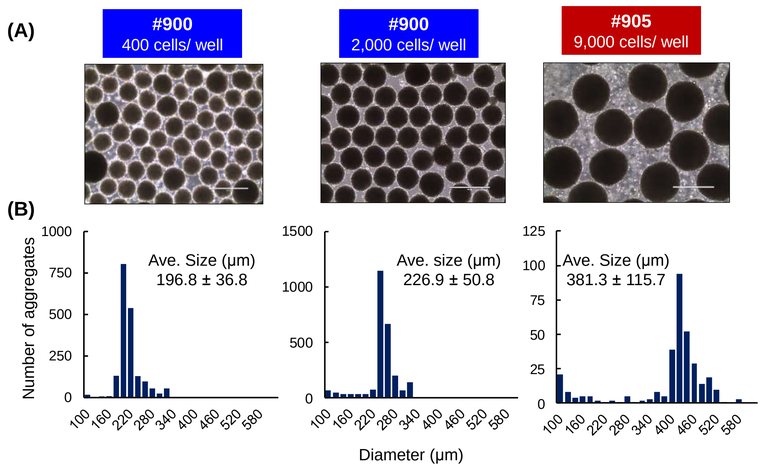


Figure 2. Size control of hiPSC aggregates
(A) Phase contrast image of iPSC aggregate generated from different number of iPSCs as 400 or 2,000 cells per micro-well in EZSPHERE#900 or 9,000 cells per micro-well in Larger size of micro-well EZSPHERE#905 (diameter = 1,400 μm). Scale Bar = 400 μm (B) The size of iPSC aggregate was uniform and could be controlled by inoculation number of iPSCs and/or micro-well size.

II. Expansion of hiPSC Aggregates

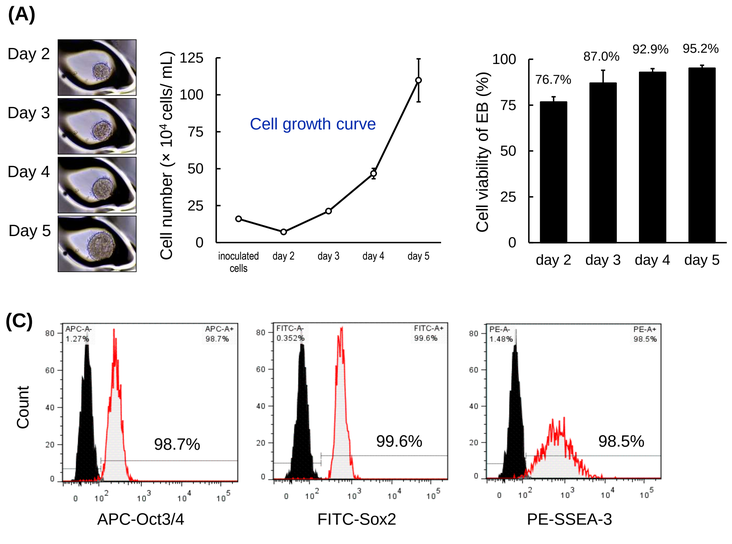


Figure 3. Proliferation of hiPSC aggregates with maintain undifferentiation state
(A) hiPSC aggregate were cultured in EZSPHERE with feeder-free cell culture medium (mTeSR1) with proliferate at a good rate. with high viability. (C) Flow cytometry analysis shows these cells maintained high capacity of undifferentiated state.

III. Differentiation of hiPSC aggregates

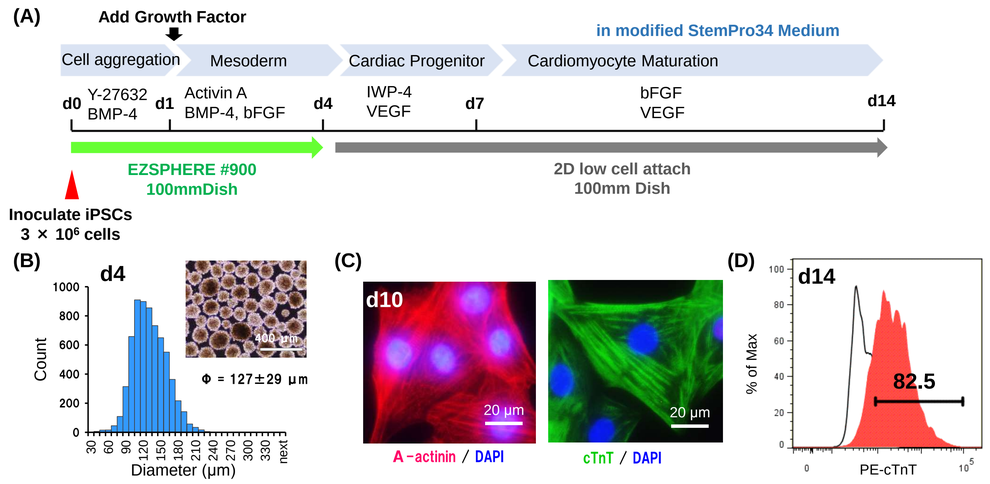
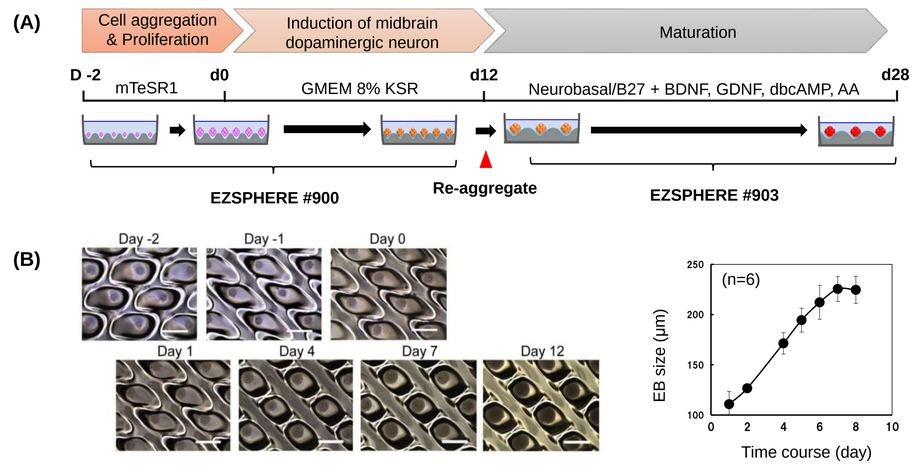


Figure 4. Cardiomyocyte differentiation in EZSPHERE
(A) Culture scheme. Cell aggregates were cultured in EZSPHERE#900 (100mm Dish) for 4 days, and transferred 2D low cell attach dish after medium change. (B) The size and microscopic image of Cell aggregates harvested at day4. (C) Immunostaining of , dissociated and re-plated cells at day10 after day1 for cardiomyocyte specific maker α-actinin and Cardiac Troponin T, cTnT. The majority of cells had cardiomyocyte Specific structures, sarcomere. (D) Flow cytometry analysis show over 80% cells were positive for cTnT.



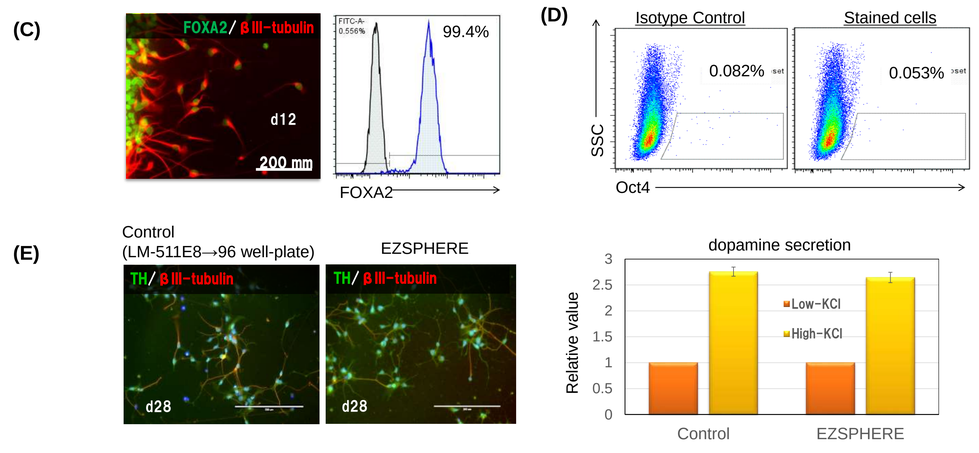


Figure 5. Dopaminergic neuron differentiation in EZSPHERE
(A) Differentiation of hiPC aggregates into dopaminergic neuron was attempted by using the EZSPHERE continuously throughout a series of steps from the hiPSC aggregate-formation to induction of midbrain dopaminergic neuron. (B) Phase-contrast images of a time course of 12 days. (C) Immunostaining for the midbrain progenitor marker FoxA2 and neural marker βIII-tubulin at day 12. (D) Flow cytometry analysis with Oct3/4 antibody indicated that there was almost no iPSCs remained without differentiation. (E) Immunostaining for the TH, Tyrosine hydroxylase and relative value of dopamine secretion in Low and High-KCl measurement by ELISA.

Summary

- **Uniform size iPSC aggregate can be easily generated in abundance by EZSPHERE**
- **Robust and efficient expansion, and differentiation / maturation of iPSC aggregate were achieved in the EZSPHERE.**

Future Plan

- **For further application of EZSPHERE, We are trying to apply these EZSPHERE techniques to large-scale culture ware ex. T225 flask and incorporate EZSPHERE to automated culture systems .**

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