Efficient Embryoid Body Formation from Human iPS Cells on Novel Microfabric Vessels

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Abstract

Human iPS (induced pluripotent stem) cells have high potential applications in regenerative medicine and drug discovery with their ability of differentiating into a wide variety of cell types. Suspension culture of iPS cell aggregates, named as embryoid bodies (EBs), is one of effective methods for propagation and differentiation of the iPS cells. Furthermore, size and uniformity of the EBs are known to be one of critical factors affecting the differentiation efficiency. However, there are still technical limitations in the generation method of large-number of EBs with uniform size by simple and easy handling. To solve such problems, we attempted to apply novel micro-fabricated culture wares (named EZSPHERE), on which large-number of micro-wells are solely created by laser, followed by low-cell-adhesive coating. The diameter and depth of each micro-well can be altered around 200-1,000 and 100-400 micrometer, respectively. We confirmed that the EZSPHERE is very useful for generating large-number of uniformly-sized EBs, when we inoculate 2.3 x 10^5 iPS cells into a standard type of the EZSPHERE (35 mm dish with approximately 2,400 micro-wells) in differentiation medium. After cultivation for 4 days, a typical Gaussian distribution was obtained for diametric size (108±33 micrometer) of the generated EBs with the total number of over 2,200 It was found that shape of the micro-wells is suitable for gathering inoculated cells and most of the EBs were formed within 3-6 hours. In addition, it was also confirmed that the obtained EBs could propagate at a good rate and maintain uniformity in growth medium. Differentiation tendency of the EBs was also confirmed by induction into cardiomyocyte or nerve cells. These results indicate that EZSPHERE is a useful tool for the controlled large-scale generation of EBs with uniform size and the differentiation capacity in a reproducible manner.

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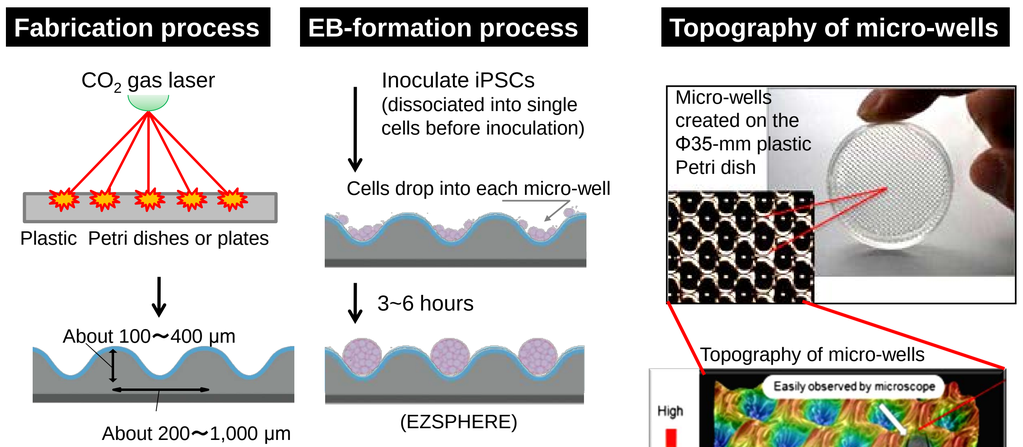
Introduction

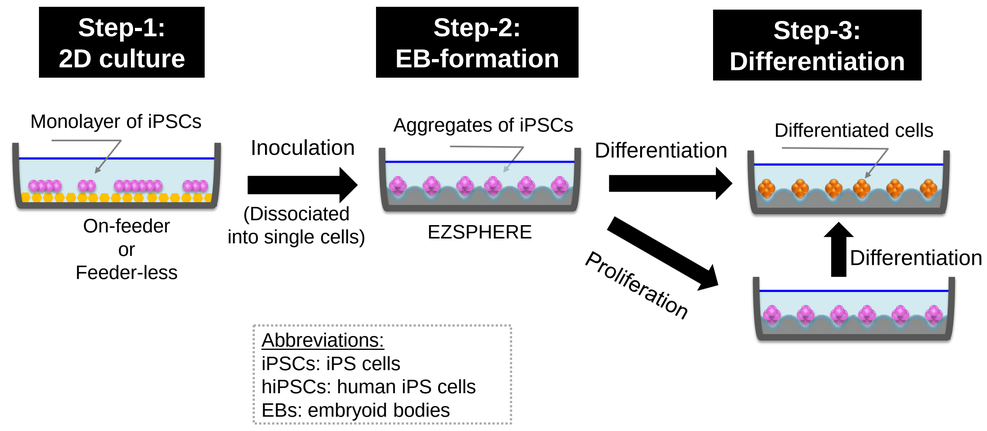
Novel micro-fabricated vessels: EZSPHERE



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

Methods and Materials

EB formation, proliferation & differentiation method



Abbreviations:
iPSCs: iPS cells
hiPSCs: human iPS cells
EBs: embryoid bodies

- **Step-1 :** Proliferate hiPS cells with on-feeder or feeder-less condition.
- **Step-2 :** Dissociate hiPS cells into single cells and inoculate them into EZSPHERE, where inoculated cells equally drop into each micro-wells and form aggregate “EBs” in 3 to 6 hours.
- **Step-3 :** Proliferate hiPS cells in the EB form with un-differentiation medium. Differentiate the EBs with using differentiation medium.

iPS cells & media

◆iPS cells:
Human iPS cell lines 201B7 and 253G1 (passage until 50), purchased and licensed from the iPS Academia Japan , Inc.

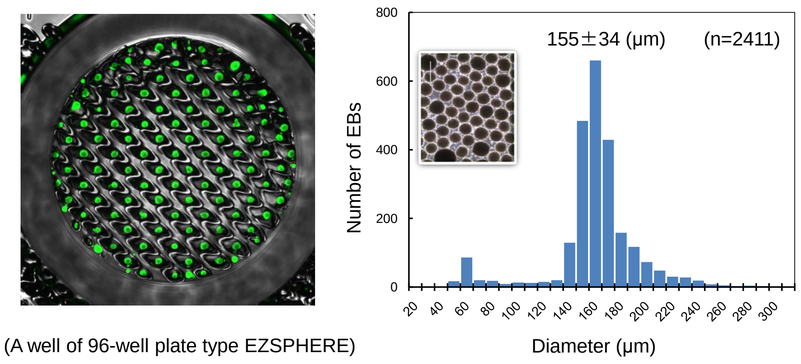
◆On-feeder culture medium:
Primate ES medium (Reprocell)., which used for iPS cell culture on SNL feeder cells treated with mytomycin C for inactivation.

◆Feeder-free culture medium:
mTeSR1 medium (STEMCELL Technologies), which used for iPS cell culture on the Matrigel (CORNING) or Laminin-521 (BioLamina) .

Results

Figure 1. High efficient generation of EBs with uniform size on the EZSPHERE

(A) EBs formed in EZSPEHERE **(B) Histogram of EB size (diameter)**

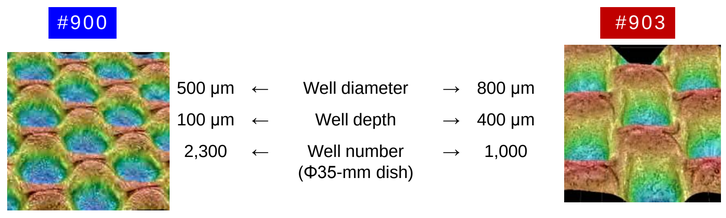


(A well of 96-well plate type EZSPHERE)

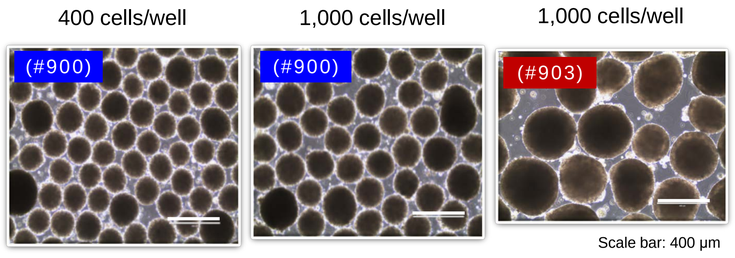
Fluorescence microscopy image of EBs obtained on the EZSPHERE (A). 2D-cultured iPS cells were inoculated into the EZSPHERE after dissociating into single cells and stained with Calcein AM (green for living cells) next day. Histogram of EB size (diameter) distribution (B). EBs created on the 35-mmΦDish type EZSPHERE were imaged and analyzed with the digital image analyzing software “Image J” to determine size distribution. The Gaussian distribution of EB size indicated uniformly sized EBs in the EZSEHERE.

Figure 2. EB size control with micro-well sizes or inoculating cell densities

(A) Micro-well size and topography of two types of EZSPHERE



(B) EB sizes affected by the micro-well size or cell density



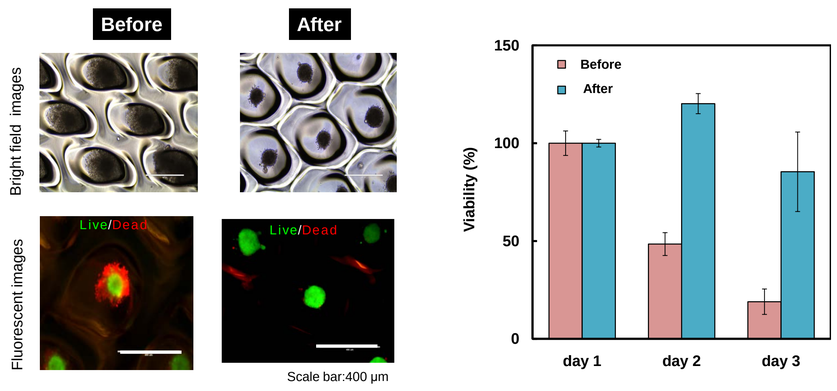
Two EZSPHERE models (code #900 & #903) and their micro-well sizes and densities (A). EBs obtained by changing micro-well sizes (#900→#903) or cell densities(400→1000 cells/well) (B) . EB sizes increased by enlargement of the micro-well size or increasing cell number inoculated into each micro-well.

Figure 3. Improvement of EB viability by optimizing culture condition on the EZSPHERE

The following two approaches on the culture condition optimization are found to be effective for improving formation efficiency and viability of EBs on the EZSPHERE.

(1) Supplementing the basal medium with Knockout Serum Replacement (KSR).
(2) Exchanging of a half volume of culture medium during the EB culture process.

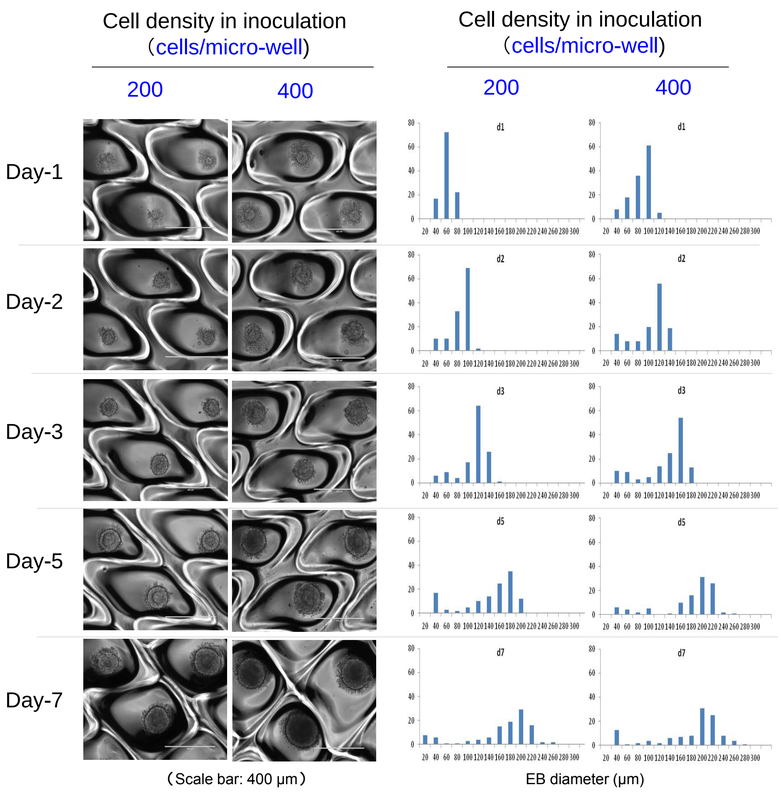
(A) EB viability **(B) Ratio of survival cells**



Comparison of EB morphology & viability before and after optimizing the culture conditions with KSR-supplementation and half-exchange of the culture medium (A). Time course of survival cell ratio determined at day 1-3 for the EBs obtained in A (B).

Figure 4. Efficient proliferation of EBs in un-differentiating condition on the EZSPEHERE

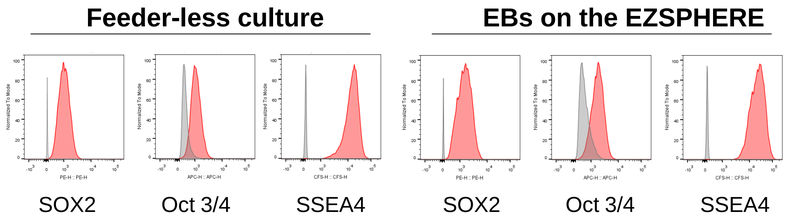
(A) Morphology of EBs **(B) Diameter of EBs**



（Scale bar: 400 μm）

Time courses of morphology (A) and diameter (B) of EBs created on the EZSPHERE with using the proliferation medium mTeSR1. For this analysis, expanded iPS cells in feeder-less culture condition with mTeSR1 medium were dissociated into single cells and inoculated at density of 200 or 400 cells/micro-well into EZSPHERE. Then, cultured for 7 days by half-exchange of culture medium (mTeSR1) everyday for the analysis. The results indicated that EB size increased effectively by time with remaining their smooth round shape and size-uniformity.

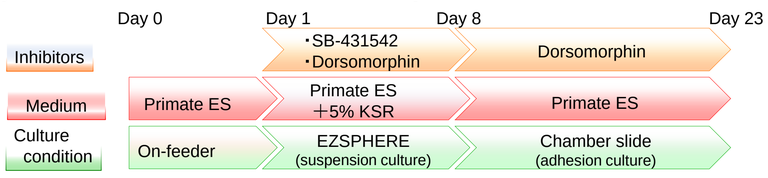
Figure 5. High performance of maintaining undifferentiation state on the EZSPHERE



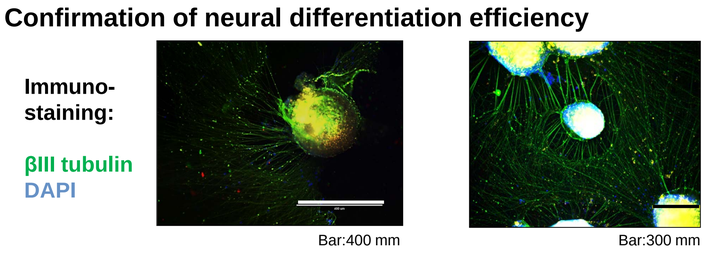
FACS analysis was performed for the iPS cells proliferated with mTeSR1 medium in the conditions of feeder-less 2D culture on the dish and EB formation on the EZSPHERE for 4 days, respectively, in order to compare their maintaining levels of undifferentiation state. In the FACS analysis, the pluripotency-associated transcription factors Sox2, Oct3/4 and SSEA4 were uses as undifferentiation markers. It was found that no significant difference between two culture conditions, indicating high capacity of maintaining undifferentiated state on the EZSPHERE.

Figure 6. Differentiation potency of EBs evaluated by neural cell induction

(A) Schematic of the differentiation protocol



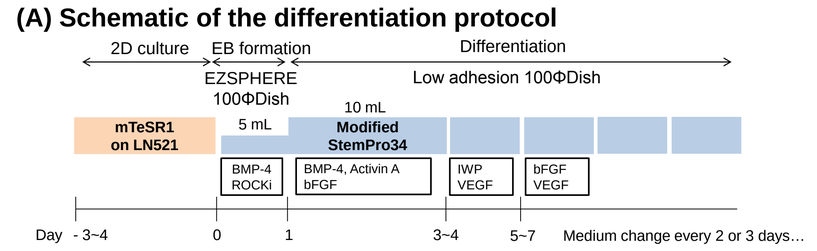
(B) Confirmation of neural differentiation efficiency



Differentiation efficiency of EBs created on the EZSPHERE were determined by induction of βIII tubulin-positive neural cells. Using the differentiation protocol (A), EBs were induced to neural cells and confirmed by Immunofluorescence staining (B). The result demonstrated differentiation ability of the EBs into βIII tubulin-positive neurites.

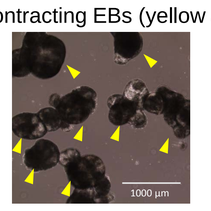
Figure 7. Differentiation potency of EBs evaluated by cardiac differentiation

(A) Schematic of the differentiation protocol

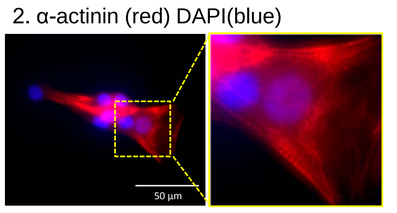


(B) Confirmation of cardiac differentiation efficiency

1. Contracting EBs (yellow arrow)



2. α-actinin (red) DAPI(blue)



EBs created on the EZSPHERE were differentiated to cardiomyocytes in above scheme (A). Most of EBs were contracting at day 15 (B)-1. EBs were dissociated and plated on gelatin coated slide at day14, followed by α-actinin and DAPI stains of cardiomyocytes after 1 day culture (B)-2. Sarcomere alignment (high magnification) was observed.

Summary

- The novel microfabric vessels EZSPHER, produced by laser-fabrication of usual plastic dishes and plates, are useful for mass-production of EBs with uniform size.
- Sizes of EBs created on the EZSPHERE are able to be controlled by choosing micro-well size of the EZSPHERE and/or changing cell number to be inoculated.
- EBs created on the EZSPHERE maintained their pluripotency state and possible to be efficiently growth with uniform size.
- It was demonstrated by inductions of nerve cells and cadiomyocytes that EBs created on the EZSPHERE kept their high differentiation potency.

Considering utilization of iPS cells for regenerative medicine, developing large-scale and efficient iPS cell producing techniques are required. In this study, we demonstrated that the novel micro-fabric culture vessels, EZSPHERE, enable to culture of EBs for both cell expansion and differentiation processes as well as useful tool for the controlled large-scale generation of EBs with uniform size in a reproducible manner by simple and easy handling. Furthermore, we are trying to attempt EZSPHERE techniques to more large-scale culture in the future.