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An Innovative Method of Embryoid Body Formation Using Novel Microfabric Vessels

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Abstract

Human induced pluripotent stem cells (iPSCs) have high potential application, such as therapeutic application, examining congenital disorders and drug screening, because of their capability of differentiation into variety cell types. Suspension culture of iPSCs induces the formation of multicellular aggregates called as embryoid body (EB). EB formation is known to be a general step during the differentiation process of the iPSCs. In addition, the differentiation efficiency is highly dependent upon the size and uniformity of the formed EBs.

Although there are several methods for the EB formation including hanging drop, static suspension culture and using bioreactor, it is necessary to develop easier, more size-controllable, scalable and reproducible methods for their practical usage. In such a purpose, we attempted to apply novel microfabric culture vessels (named "EZSPHERE"), in which a large number of micro-wells are solely created on culture surface of the plastic dishes or plates by laser beam, followed by coating with low-adhesion reagents. The diameter and depth of each created micro-well can be easily altered around 200-1,000 μm and 100-400 μm, respectively, by tuning exposure time or intensity of the laser beam in the microfabric process.

When iPSCs were inoculated into a standard type of the EZSPHERE (approximately 2,400 micro-wells per 35 mm dish), these cells aggregated immediately and formed uniformly-sized EBs (155±34 μm in diameter) with the total number of over 2,000. In addition, we confirmed that the size of EBs is controlled by choosing the number of inoculation cells and size of micro-wells. We examined their differentiation into nerve cells or cardiomyocytes to evaluate whether the obtained EBs maintained their multilineage potential. Flow cytometric analysis and immunofluorescence staining demonstrated the followings;

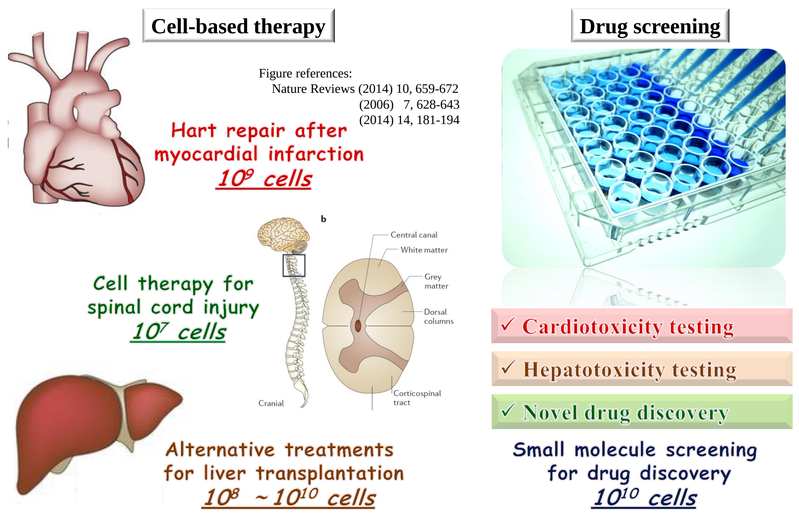
1. The obtained EBs maintained their pluripotency in a high level.
2. Moreover, when iPSCs were cultured on EZSPHERE with feeder-free cell culture medium (mTeSR1), the formed EBs in EZSPHERE could proliferate at a good rate with maintaining their undifferentiated state.
3. These results suggest that the novel microfabric culture vessels, EZSPHERE, enable to culture of EBs for both cell expansion and differentiation processes at the same culture ware.

EZSPHERE is a useful tool for the EB formation with uniform size in a reproducible manner by simple and easy handling. This study was performed as a part of the AMED (Japan Agency for Medical Research and Development) project "Research Center Network for Realization of Regenerative Medicine".

Introduction

The number of required differentiated cells

Reference: Dai *et al.* Brain Research (2013) 1533, 73-79
Lei *et al.* Proc. Natl. Acad. Sci. USA (2013) E5039-E5048

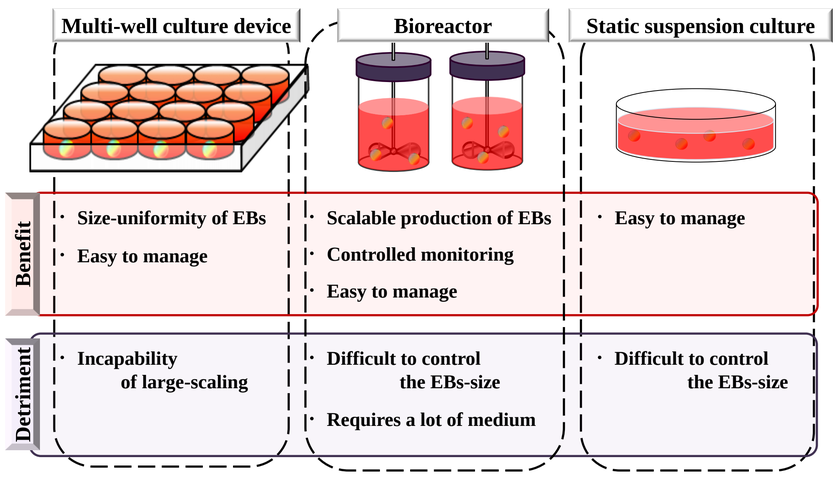


The production of large masses of pluripotent stem cells (PSCs) represents one of the major hurdles to be overcome in developing cell-based therapies, because it has been estimated that, at least, 10^9 cells would be required for clinical applications. Additionally, ～10^{10} cells may be needed to screen a million-compound library once.

For the early realization of regenerative medicine, it is problems how to production of large-scaling of iPSCs and their-derived differentiated cells

Aim of Study

Conventional methods of embryoid bodies (EBs) formation



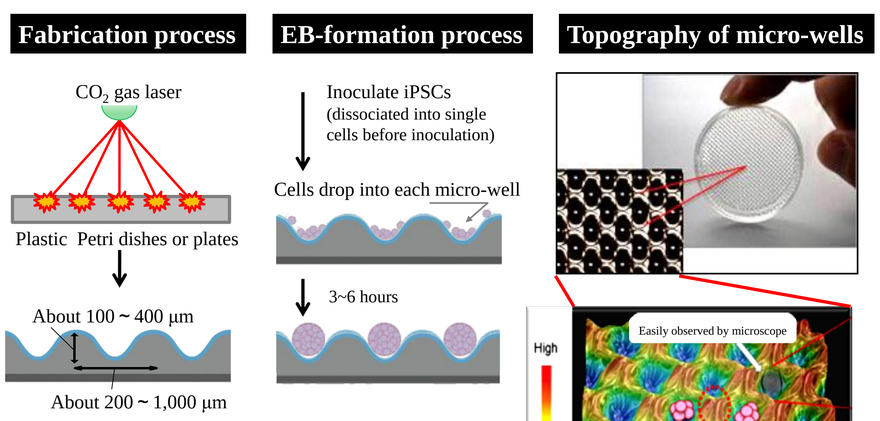
Reference: Rungarunlert *S et al.* World Journal of Stem Cells (2009) 1, 11-21

Traditional methods are known as useful tools for embryoid body (EB) formation in laboratory-scale. These protocols, however, are unsuitable for creating of the uniform EBs in large-scaling production. In such reason, development of the novel culture vessels are necessary.

We attempted to develop the new EB-formation methods using the novel microfabricated vessels (named as EZSPHERE)

Materials & Methods

Novel micro-fabricated vessels: EZSPHERE

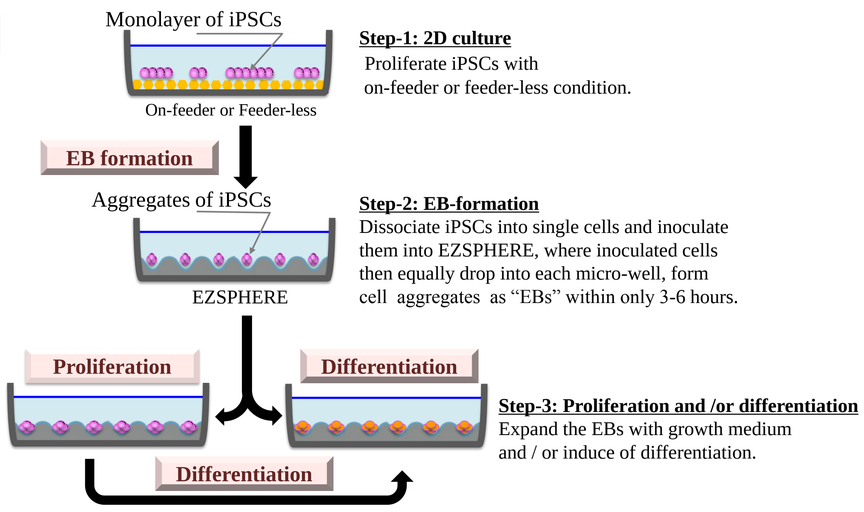


Micro-wells of EZSPHERE are solely created by CO_2 gas laser on the plastic dishes or plates, followed by coating with low-adhesive reagents (MPC polymer).

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

EZSPHERE is specifically designed for creating a large number of spheroids and EBs with uniform size

EB formation, proliferation and differentiation protocols



Considering utilization of iPSCs for regenerative medicine, developing large-scale and efficient iPSCs producing techniques are required. To solve such problems, we attempted to apply novel micro-fabricated culture wares: EZSPHERE.

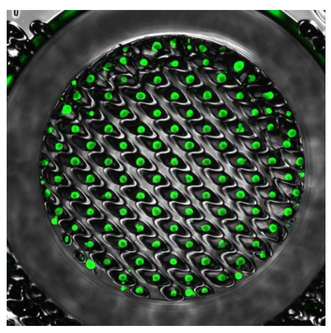
Here, we show that innovative culture methods for iPSCs by using the EZSPHERE

Results

Fig.1 High efficient generation of EBs with uniform size

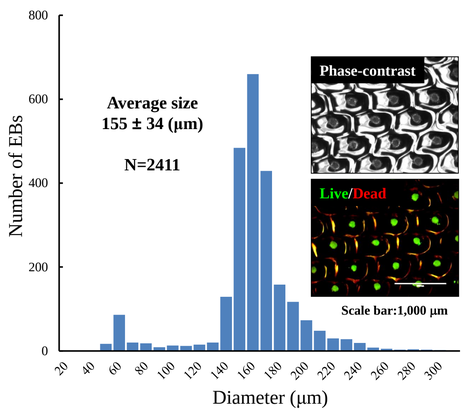
(A) EBs formed in the EZSPEHERE

Two-dimension-cultured iPSCs were inoculated into the EZSPHERE after dissociating into single cells and stained with Calcein AM (green for living cells) next day.



(A well of 96-well plate type EZSPHERE)

(B) EB size (diameter) distribution and cell viability on the EZSPHERE

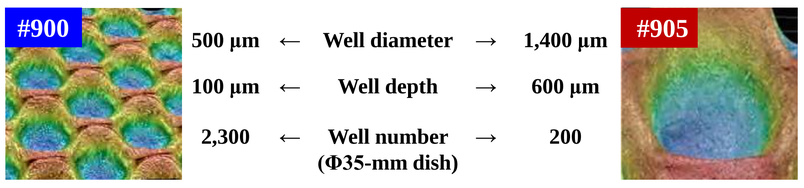


Fluorescence microscopy image of EBs obtained on the EZSPHERE (A). EBs created on the 35-mm dish type EZSPHERE were imaged and analyzed with the digital image analyzing software "Image J" to determine size distribution. Histogram of EB size (diameter) distribution. Fluorescence microscopy revealed that the almost EBs were alive with uniform size (B).

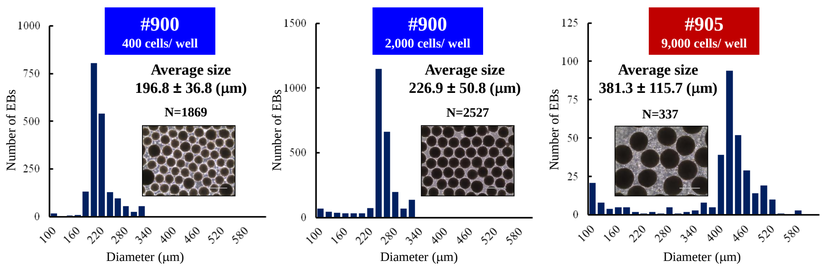
EZSPHERE is useful tool for the controlled large-scale production of EBs with uniform size by simple and easy handling

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

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



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

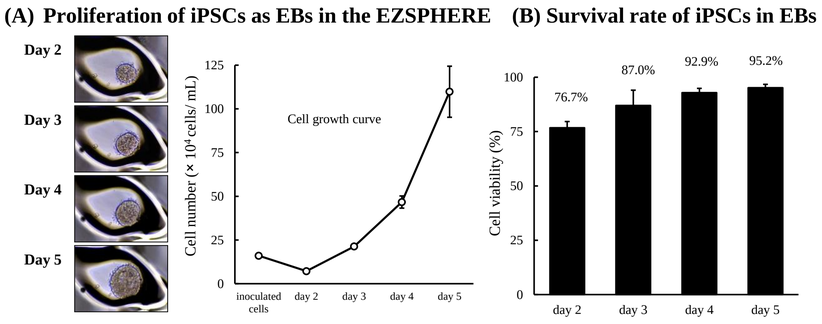


Two types of EZSPHERE #900 and #905 with micro-wells about 500 μm and 1,400 μm in diameter, respectively, were used to analysis the effect of the micro-well size or inoculated cell density on the EB sizes (A). Inoculation of iPSCs as 400 or 2,000 cells per micro-well on the same type of EZSPHERE #900 resulted in the formation of EBs with different sizes, while inoculating iPSCs as 9,000 cells per micro-well on the another EZSPHERE #905 resulted larger size of EBs (B). (Scale bars: 400 μm)

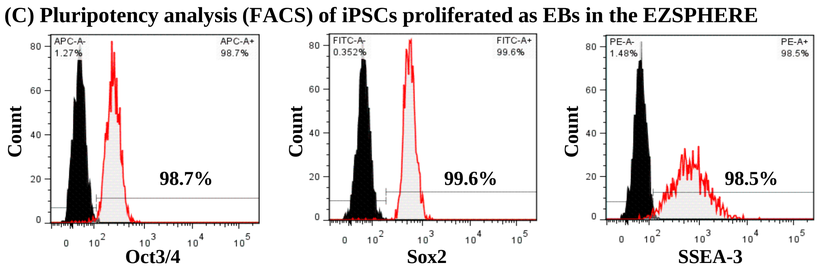
EB size is controlled by changing the inoculation cell number and/or micro-well size of the EZSPHERE

Fig.3 Proliferation of iPSCs with maintaining pluripotency

(A) Proliferation of iPSCs as EBs in the EZSPHERE (B) Survival rate of iPSCs in EBs



(C) Pluripotency analysis (FACS) of iPSCs proliferated as EBs in the EZSPHERE

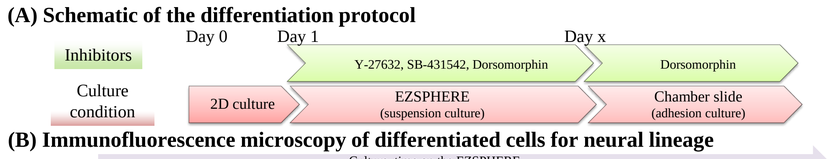


When iPSCs were cultured on EZSPHERE with feeder-free cell culture medium (mTeSR1), the formed EBs could proliferate at a good rate (A) with high viability (B). In the flow cytometry, these cells maintained high capacity of undifferentiated state (C).

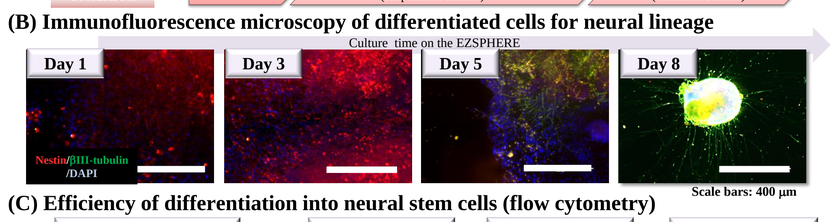
iPSCs grown as EBs with feeder-free cell culture medium on the EZSPHERE maintained their pluripotency

Fig.4 High efficient induction of neural lineage cells from the EBs

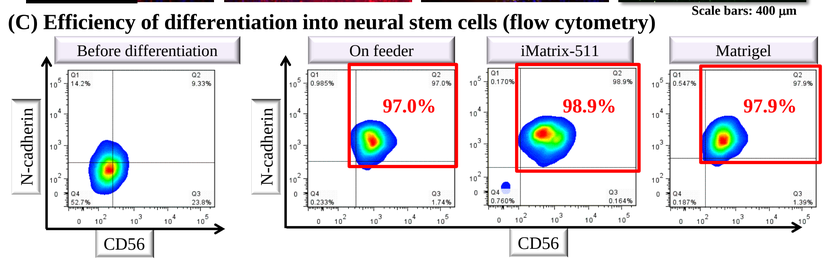
(A) Schematic of the differentiation protocol



(B) Immunofluorescence microscopy of differentiated cells for neural lineage



(C) Efficiency of differentiation into neural stem cells (flow cytometry)

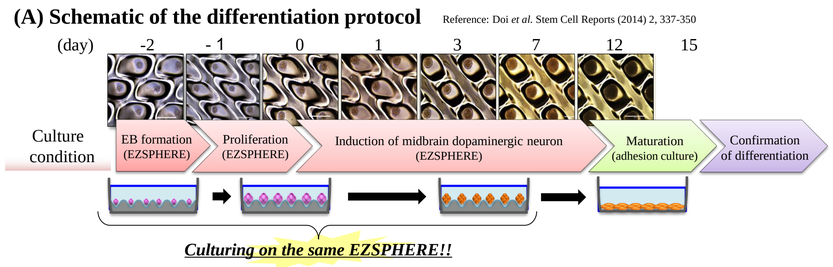


Neural lineage differentiation was performed on the EBs created by the EZSPHERE in order to confirm their differentiation potency (A). Immunofluorescence staining demonstrated neural stem cell markers (Nestin and βIII tubulin)-positive neurites with high ratio (B). Moreover, flow cytometry revealed that EBs, which were created on the EZSPHERE from iPSCs cultured on different matrices, differentiated into neural stem cells only for 3 days with very high efficiency (C).

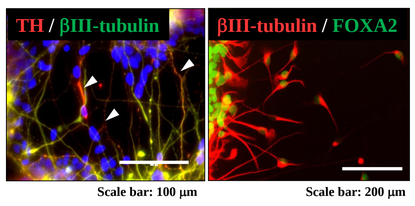
By using the EZSPHERE, iPSCs differentiate neural stem cells with high efficiency and in a short period

Fig.5 Induction of dopaminergic neurons from the EBs

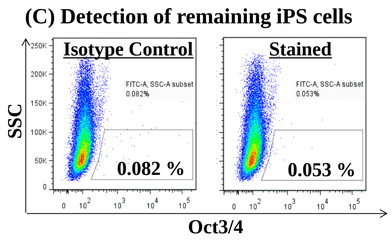
(A) Schematic of the differentiation protocol Reference: Doi *et al.* Stem Cell Reports (2014) 2, 337-350



(B) Characterization of DOPA neurons



(C) Detection of remaining iPS cells

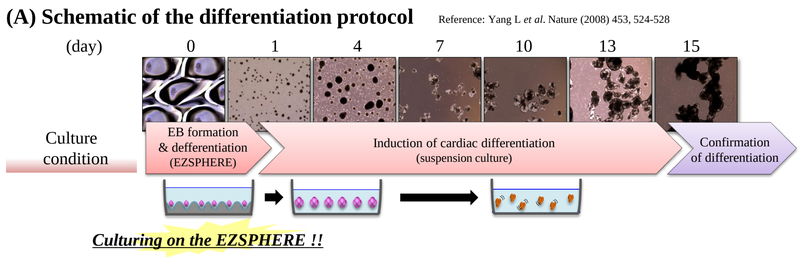


Differentiation of EBs into dopaminergic neuron was attempted by **using the EZSPHERE continuously throughout a series of steps** from the EB-formation to induction of midbrain dopaminergic neuron (A). Immunofluorescence staining revealed differentiation of the EBs into midbrain neuron, which tyrosin hydroxylase (TH: white arrows) and FOXA2 positive (B). Flow cytometry analysis (FACS) with Oct3/4 antibody indicated that there was almost no iPSCs remained without differentiation (C).

A series of steps from EB-formation and proliferation to differentiation (for dopaminergic neurons) could be accomplished on the same EZSHPERE

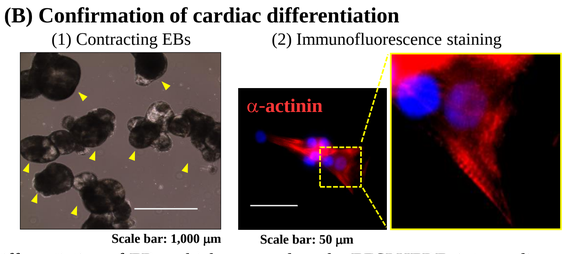
Fig.6 Induction of cardiomyocyte from EZSPHERE

(A) Schematic of the differentiation protocol Reference: Yang L *et al.* Nature (2008) 453, 524-528

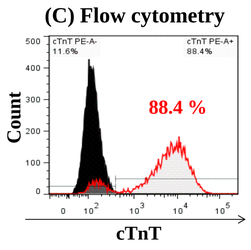


(B) Confirmation of cardiac differentiation

(1) Contracting EBs (2) Immunofluorescence staining



(C) Flow cytometry



Differentiation of EBs, which prepared on the EZSPHERE, into cardiomyocytes was attempted (A). It was observed that most of EBs showed contracting (indicated with yellow arrow) at day 15 (B)-(1). EBs were dissociated and plated on gelatin coated slide, followed by α-actinin staining and sarcomere alignment (high magnification) was observed (B-2). Flow cytometry for cardiac troponin T (cTnT) positive cells revealed high differentiation efficiency (C).

EBs obtained on the EZSPHERE differentiated into cardiomyocyte with high efficiency

Conclusions

- ◆ EZSPHERE is a unique micro-fabricated plastic vessel and very useful for mass-production of EBs with uniform size. EB size is also controllable by changing micro-well size and/or seeding cell number.
- ◆EZSPHERE is available not only for EBs formation, but also for expansion of iPSCs with maintaining their high pluripotency and/or induction of differentiation into other cell types (e.g. neural lineage cells) with high efficiency in a short period.

This study clearly demonstrated that EZSPHERE is appropriate tool for large-scaling production of EBs.

In addition, EZSPHERE will provide the innovative culture methods for iPSCs.

Furthermore, EZSPHERE is applicable to larger-scale culture and the automated culture equipment in the future.

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