Protocol

iPS spheroid formation in EZSPHERE™ devices

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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.

1. Required materials (cells, media, reagents)

	Product name/#	Provider	Comments
Cells			
Human iPS Cells (iPSCs)	(201B7 strain)	Kyoto University	
Feeder cells	(SNL 76/7)	ECACC	Inactivated by Mitomycin C
Media & solutions			
Medium for maintenance	Primate ES cell medium (#RCHEMD001)	ReproCELL	Addition of 4 ng/ml bFGF
Medium for spheroid formation and cultivation	Primate ES cell medium (#RCHEMD001)	ReproCELL	Addition of 5% KSR and 50 μM Y-27632 (Y-27632 is only for Spheroid Formation)
Washing solution	DPBS (-) (#045-29795)	WAKO	
Dissociation solution	CTK solution (#RCHETP002)	ReproCELL	For removing feeder cells
Dissociation solution	Accutase (#A6964)	Sigma-Aldrich	For generating single cells
Additives			
Serum replacement	KSR (# 10828028)	Life Technologies	For spheroid formation and cultivation medium
ROCK inhibitor	Y-27632 (#253-00513)	WAKO	For spheroid formation medium

2. Protocol

Protocol for formation of Spheroid from human iPS Cells on a feeder layer using EZSPHERE™ 35 mm dish (#4000-900)

Remove feeder cells

1. Aspirate medium from iPSCs on 100mm Dish and wash with 9 ml PBS
2. Add 1 ml of CTK solution, leave at 37℃ for 1 min to detach feeder cells
3. Wash with 9 ml DPBS (-) , remove feeder cells

Prepare Single-Cell Suspension

4. Add 2 ml of Accutase (containing 50 μM Y-27632). Make sure Accutase spreads over the whole Dish. Incubate at 37℃ for 5 min
5. Add 8 ml of Spheroid formation medium. Gently pipette the iPSC colonies and Break up into single-cells.
6. Transfer the single-cell suspension into a 15 ml tube. Centrifuge for 3 min at 190 × *g*, 4℃
7. Aspirate the supernatant. Add 3 ml spheroid body formation medium. Determine cell count.

Spheroid formation

8. Calculate the number of cells required for each micro-wells in order to form desired size of spheroids*

   *ex. 1000 cells/micro-well, 1000 cells ×2,300 micro-wells (#4000-900) = 2.3×10^6 cells/ 2.7ml / 35mmDish
9. Seed the cells into an EZSPHERE™ 35 mm
10. Change the medium as described in 9. every other day by slowly aspirating and discarding half the culture medium and adding an equivalent amount of fresh medium

ATTENTION

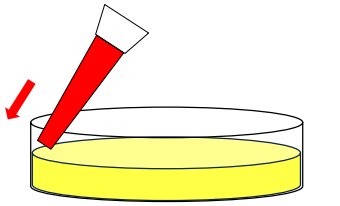
After seeding, do not disturb the culture device. Keep observation under microscope to a minimum.

Protocol: addendum

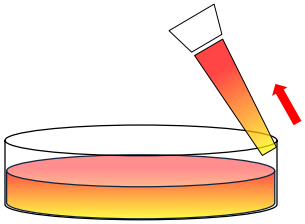
Medium change

Due to the high number of micro-wells on the culture surface of an EZSPHERE™ device containing huge numbers of iPSC, spheroids can float up from their well and enter other wells if the EZSPHERE™ container is disturbed. Please apply proper care when changing the culture medium. Keep the EZSPHERE™ device level at all times. For further details, refer to the instructions as below.

(A) Changing total medium (2.7 ml)



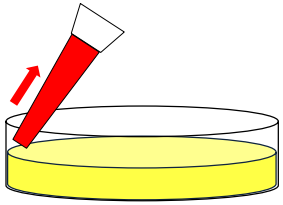
Slowly add 0.9 ml of fresh medium by micropipette against the wall of the dish



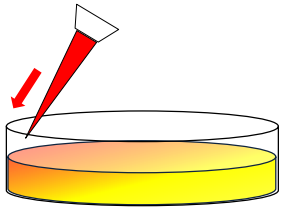
From the opposite side of where you added the fresh medium to the culture plate, aspirate 0.9 ml medium

repeat 3 times

(B) Changing half medium (1.4 ml)



Slowly aspirate 1.4 ml medium from the culture plate



With a P200 micropipette, add 200 µl of fresh medium against the wall of the culture plate (repeat 7 times)

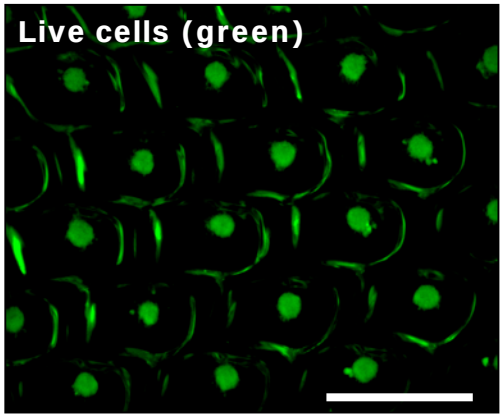
3. Case study #1: spheroid formation

Materials and Methods

■**Human iPS cell strain**: hiPSC 201B7 (on feeder layer)
■**EZSPHERE™ culture device**: 35 mm dish (#4000-900)
■**iPSC numbers**: seed 9.2×10^5 cells / 2.7ml /Dish (approximately 400 cells/micro-well)
■**Medium**: spheroid formation medium (Primate ES Cell Medium + 50 μM Y-27632 + 5% KSR)
■**Culture conditions**: incubate the Cells at 37℃ with 5% CO_2 for 3 days. The following day after seeding, exchange half volume of the medium.

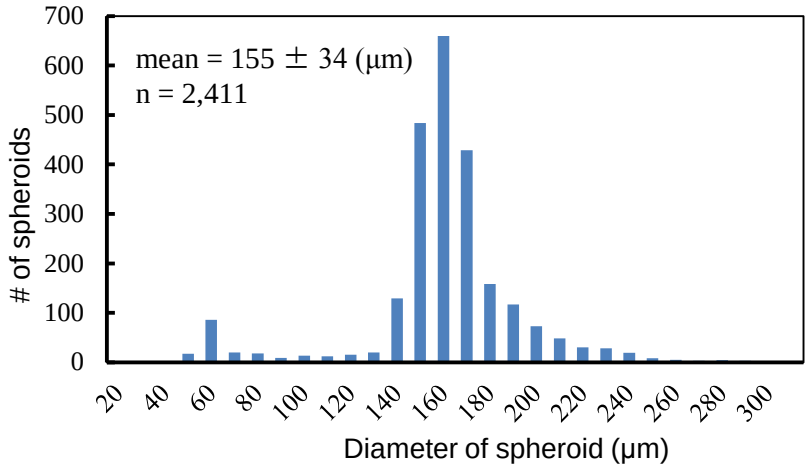
Spheroids in EZSPHERE™ 35 mm dish (brightfield microscope view)

Live cells in EZSPHERE™ 35 mm dish (fluorescent microscope view)



Scale bar: 1,000 μm

Spheroid size



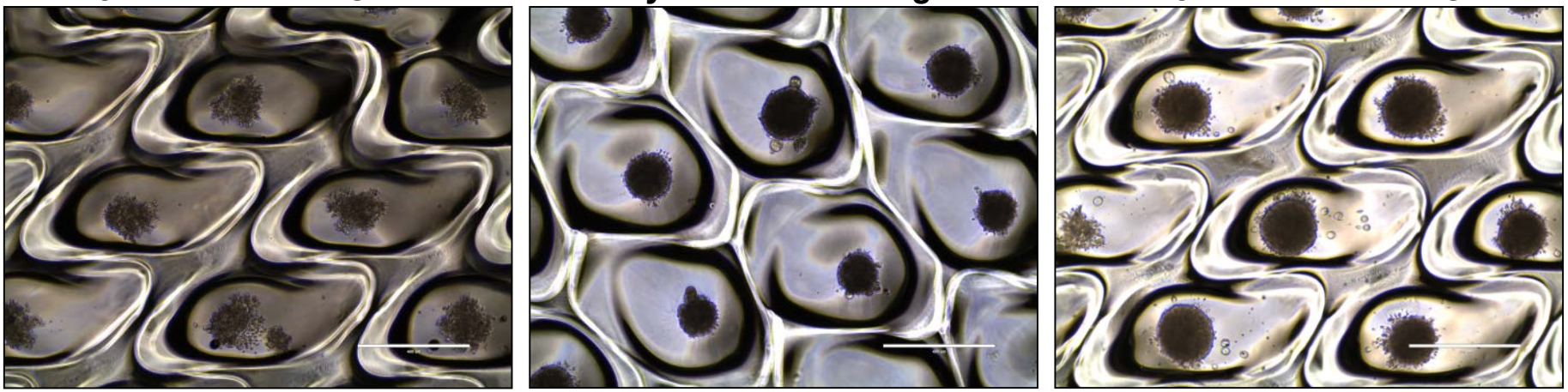
Uniform spheroids could be obtained after 3 days of cultivation (left, brightfield microscopic view). Assaying the viability of the spheroids with the Live/Dead Cell Staining Kit II (PromoKine), the majority of the cells was found to be alive (center, fluorescent microscope view; cells shown in green are alive). Analysis of the distribution of the spheroids' sizes peaked at 160 μm (right).

3. Case study #2: spheroid maintenance

Materials

■**Cells**: : hiPSC 201B7 (on feeder Layer)
■**EZPHERE™ product**: 96-well micro-well plate (#4860-900)
■**Cell numbers**: : seed 2.4×10^4 cells / 200 µl / well (approximately 400 cells/micro-well)
■**Medium**: spheroid formation medium (Primate ES Cell Medium + 50 mM Y-27632 + 5% KSR)
■**Culture conditions**: Change medium every other day after seeding and photographically document the state of the spheroids under the microscope.

1 day after seeding | **6 days after seeding** | **8 days after seeding**



Scale bar: 400 µm

iPS cell clumps were observed under the microscope the following day after seeding (left). 6 days of cultivation and medium changes every other day resulted in the formation of delicate spheroid bodies (center). 8 days after seeding, the spheroids had grown to 200-250 µm in diameter.

ATTENTION

- When culturing the cells for approx. 1 week, the frequent medium changes can lead to spheroids leaving their micro-wells, so apply proper care when handling.
- The EZSPHERE™ 96-well plate prevents spheroid spillage between wells better than the 35 mm dish.