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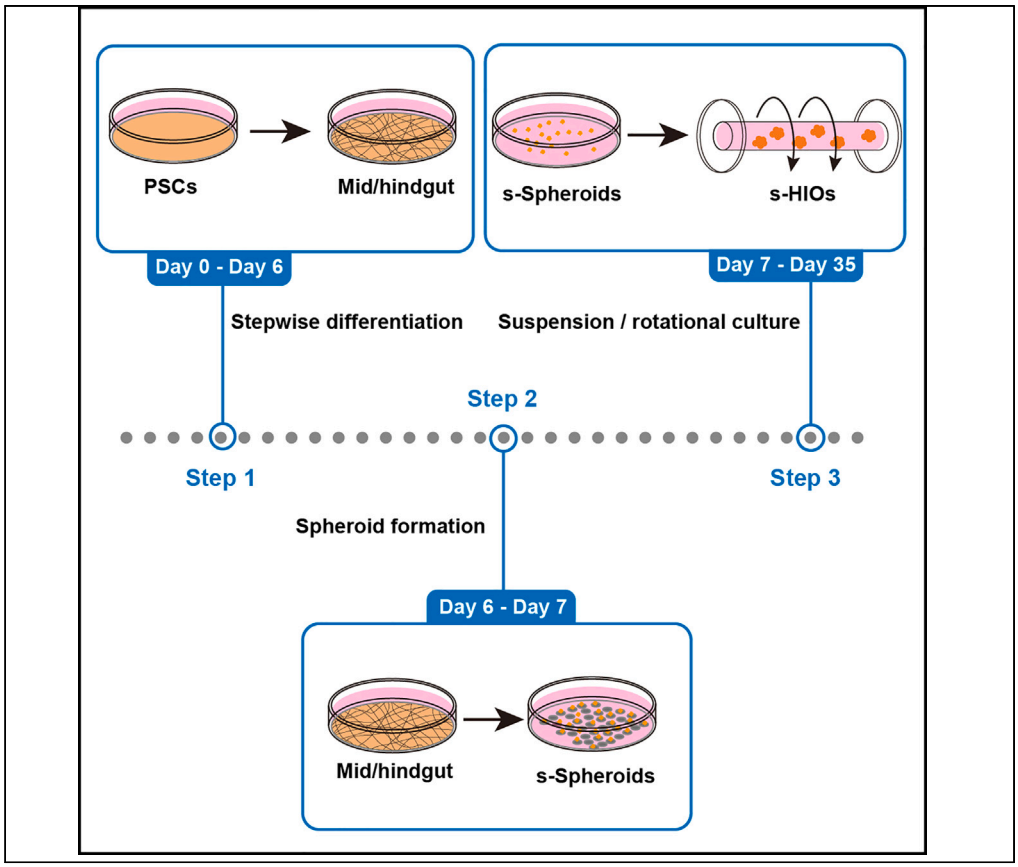
Protocol

Protocol to generate large human intestinal organoids using a rotating bioreactor



Current techniques for producing induced-pluripotent-stem-cell-derived mid/hindgut spheroids have faced major hurdles in consistency and reproducibility. Here, we present a protocol that uses mid/hindgut cells to generate homogeneous spheroids that subsequently mature into human intestinal organoids (HIOs). We describe steps for stepwise differentiation and spheroid formation using a 96-well plate. We then detail cell maturation in a suspended state and the implementation of a rotational bioreactor platform to maximize the culture efficiency of larger HIOs.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Junichi Takahashi, Hady Yuki Sugihara, Shu Kato, Sayaka Nagata, Ryuichi Okamoto, Tomohiro Mizutani

jtakahashi.gast@tmd.ac.jp (J.T.)
tmizutani.gast@tmd.ac.jp (T.M.)

Highlights

iPSC-derived mid/hindgut cells aggregate into intestinal spheroids in suspension

Large s-Spheroids are formed by using an EZ-BindShut® 96-well plate

Differentiation of s-Spheroids into s-HIOs

Rotational bioreactor supports growth of larger s-Spheroids into s-HIOs

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Protocol to generate large human intestinal organoids using a rotating bioreactor

Junichi Takahashi,[1,2,3,*] Hady Yuki Sugihara,[1,2] Shu Kato,[1,2] Sayaka Nagata,[1] Ryuichi Okamoto,[1] and Tomohiro Mizutani[1,4,*]

[1]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University (TMDU), 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

[2]These authors contributed equally

[3]Technical contact

[4]Lead contact

*Correspondence: jtakahashi.gast@tmd.ac.jp (J.T.), tmizutani.gast@tmd.ac.jp (T.M.)
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SUMMARY

Current techniques for producing induced-pluripotent-stem-cell-derived mid/hindgut spheroids have faced major hurdles in consistency and reproducibility. Here, we present a protocol that uses mid/hindgut cells to generate homogeneous spheroids that subsequently mature into human intestinal organoids (HIOs). We describe steps for stepwise differentiation and spheroid formation using a 96-well plate. We then detail cell maturation in a suspended state and the implementation of a rotational bioreactor platform to maximize the culture efficiency of larger HIOs.
For complete details on the use and execution of this protocol, please refer to Takahashi et al.[1]

BEFORE YOU BEGIN

The protocol below provides step-by-step instructions on iPSC culture, differentiation into mid/hindgut cells, generation of mid/hindgut spheroids, and spheroid culture and maturation into HIOs. The entire process of spheroid generation and culture takes place in suspension, ridding the requirement of mechanical passaging and the spatial limitations inherent in conventional Matrigel-embedding method.

Two alternative methods of mid/hindgut spheroid generation are described; EZSPHERE® method (steps 10–18) allows the production of large numbers (200–9200 per well) of relatively small spheroids, and EZ-BindShut® 96-well plate method (steps 19–24) allows the production of much larger spheroids. The generated spheroids can then be cultured and grown into HIOs in two ways; Static suspension (steps 32–39) requires no bioreactors and produces viable HIOs for down-stream experimentation, and Rotational suspension (steps 40–48) employs the bioreactor platform to enhance the growth and gas/factor exchange, enabling culture of larger HIOs generated by BindShut® 96-well plate method.

By day 35, HIOs produced through this protocol should have clear intestinal epithelial cells surrounded by a mesenchymal layer, and be suitable for *in vivo* maturation through mouse transplantation, producing complex small intestinal tissue with differentiated epithelial cells and *LGR5*-positive intestinal stem cells.[1]

The bioreactor platform used in this protocol is the CELLFLOAT CellPet 3D-iPS® culture system. When using a different bioreactor, the parameters may require optimization and the results may vary. Similarly, some deviations and variability in differentiation and growth efficiencies may arise based on the iPSC lines employed.

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Preparation of reagents

1. Prepare stock reagent in an aseptic condition. A list of stock concentrations and solvents is described below. Aliquot the stocks and store them in −20°C. Avoid repeated thaw and freeze cycles.

Reagent	Stock concentration	Solvent
Activin A	100 μg/mL	N/A
A83-01	500 mM	DMSO
CHIR99021	5 mM	DMSO
EGF	20 μg/mL	0.1% BSA-PBS
FGF4	500 μg/mL	dH_2O
Noggin	20 μg/mL	0.1% BSA-PBS
R-spondin1	100 μg/mL	0.1% BSA-PBS
Y-27632	10 mM	PBS
GF-1	N/A	N/A
GF-2	N/A	N/A
GF-3	N/A	N/A

GF-1, GF-2, and GF-3 are additives of Cellartis DEF-CS kit.

Maintenance and expansion of human induced pluripotent stem cells (iPSCs)

Note: Culture of iPSCs using the Cellartis DEF-CS culture system is described here. Research groups may apply their preferred iPSC culture protocol.

2. iPSCs are cultured in a feeder-free condition using DEF-CS culture system on 6-well plates. iPSC medium (maintenance) is changed daily.
3. Passage iPSCs when cells reach approximately 90% confluency.
   a. Pre-coat a 6-well plate with COAT-1 coating medium.
   b. Aspirate medium and wash the cell surface with 1 mL of pre-warmed PBS.
   c. Add 300–500 μL TrypLE Select and incubate the plate at 37°C for 5–7 min.
   d. Gently pipette the well for single-cell dissociation.
   e. Add 3 mL of iPSC medium (passage) to stop the enzymatic reaction.
   f. Transfer the cell-suspension to 15 mL STEMFULL tube.
   g. Seed iPSCs at a ratio of 1:15–1:10 for maintenance, or at desired cell number for differentiation.
   h. Incubate the plate at 37°C, 5% CO_2.

⚠ CRITICAL: Avoid over-confluency of iPSCs as it causes spontaneous differentiation.

⚠ CRITICAL: For consistent differentiation, avoid using iPSCs immediately after thawing or change of culture conditions. Acclimatize iPSCs to the culture system for at least 3 passages.

⚠ CRITICAL: Check for mycoplasma contamination of iPSCs when iPSCs are cultured for a long time.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Recombinant murine EGF	PeproTech	Cat#31509
Recombinant mouse Noggin	R&D	Cat#1967

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant mouse R-spondin1	R&D	Cat#3474-RS
A83-01	Tocris Bioscience	Cat#2939
Matrigel Growth Factor Reduced	Corning	Cat#354234
N-2 supplement (100×)	Thermo Fisher	Cat#17502-048
B-27 Supplement (50×), serum free	Thermo Fisher	Cat#17504-044
Activin A	Nacalai Tesque	Cat#18585-81
CHIR99021	Cayman Chemicals	Cat#13122
Recombinant human FGF4	PeproTech	Cat#100-31
Y-27632	Fujifilm Wako Pure Chemical	Cat#253-00513
Cellartis DEF-CS™ 500 culture system	Takara Bio	Cat#Y50101
Advanced DMEM/F12	Thermo Fisher	Cat#12634010
DMEM, high glucose	Thermo Fisher	Cat#11965-092
RPMI 1640 with L-Gln, liquid	Nacalai Tesque	Cat#30264-85
Embryonic stem-cell Fetal Bovine Serum	Thermo Fisher	Cat#16141079
GlutaMAX™ Supplement	Thermo Fisher	Cat#35050-061
HEPES buffer	Nacalai Tesque	Cat#17557-94
Penicillin-streptomycin mixed solution	Nacalai Tesque	Cat#26253-84
TrypLE Select Enzyme	Thermo Fisher	Cat#12563011
D-PBS(-) without Ca and Mg, liquid	Nacalai Tesque	Cat#14249-95
D-PBS(+) Preparation Reagent (Ca, Mg Solution)	Nacalai Tesque	Cat# 02492-94
Experimental models: Cell lines		
Human induced pluripotent stem cell line: HiPS-RIKEN-2F	RIKEN BRC Cell Bank (Japan)	HPS0014
Human induced pluripotent stem cell line: PB001	Kindly gifted by Dr Hideki Masaki (Institute of Medical Science, the University of Tokyo)	N/A
Software and algorithms		
Cell^3 iMager duos Software version 1.6	SCREEN Holdings	N/A
Other		
EZSPHERE® 6-well plate	AGC Inc.	Cat#4810-900SP
EZ-BindShut® 96-well plate	AGC Inc.	Cat#4870-800SP
Ultra-low attachment 6-well plate	Corning	Cat#3471
CellPet 3D-iPS® system	JTEC Corp.	CELLPET iPS/3S/MA-2.1
Culture vessel syringe (10 mL)	JTEC Corp.	CELLPET VES/S10/6
Culture vessel syringe (30 mL)	JTEC Corp.	CELLPET VES/S30/6
Culture vessel syringe (50 mL)	JTEC Corp.	CELLPET VES/S50/6
STEMFULL Centrifuge tube	Sumitomo Bakelite Co., Ltd	Cat#MS-90150
All-in-one fluorescence microscope	KEYNCE	BZ-X710
Cell^3 iMager duos	SCREEN Holdings	N/A
Stereomicroscope system	OLYMPUS	SZX7

Alternatives: The spheroid formation is optimized using EZSPHERE® 6-well plate and EZ-BindShut® 96-well plate. Other types of 6-well spheroid-forming plates are commercially available (i.e., AggreWell from Veritas) and may be used, though we have not tried them. As for EZ-BindShut® 96-well plate, spheroid forming plate (Corning, Cat #4515) can be an alternative.

MATERIALS AND EQUIPMENT

COAT-1 coating medium

Reagent	Final concentration	Amount
COAT-1	5%	50 μL
D-PBS(+) Ca^+ Mg^+ (100×)	1×	10 μL
D-PBS(-) Ca^- Mg^-	N/A	To 1 mL
Total	**N/A**	**1 mL**

Coating medium should be freshly prepared.

iPSC medium (maintenance)		
Reagent	Final concentration	Amount
GF-1	N/A	3 μL
GF-2	N/A	1 μL
DEF-CS basal medium	N/A	To 3 mL
Total	**N/A**	**3 mL**
Medium should be freshly prepared.		

iPSC medium (passage)		
Reagent	Final concentration	Amount
GF-1	N/A	3 μL
GF-2	N/A	1 μL
GF-3	N/A	1 μL
DEF-CS basal medium	N/A	To 3 mL
Total	**N/A**	**3 mL**
Medium should be freshly prepared.		

Definitive endoderm (DE) day 0 medium		
Reagent	Final concentration	Amount
Activin A (100 μg/mL)	100 ng/mL	3 μL
CHIR99021 (5 mM)	3 μM	1.8 μL
Pen/Strep (100×)	1×	30 μL
RPMI1640	N/A	To 3 mL
Total	**N/A**	**3 mL**
Medium should be freshly prepared.		

DE day 1 medium		
Reagent	Final concentration	Amount
Activin A (100 μg/mL)	100 ng/mL	3 μL
FBS	0.2%	6 μL
Pen/Strep (100×)	1×	30 μL
RPMI1640	N/A	To 3 mL
Total	**N/A**	**3 mL**
Medium should be freshly prepared.		

DE day 2 medium		
Reagent	Final concentration	Amount
Activin A (100 μg/mL)	100 ng/mL	3 μL
FBS	2%	60 μL
Pen/Strep (100×)	1×	30 μL
RPMI1640	N/A	To 3 mL
Total	**N/A**	**3 mL**
Medium should be freshly prepared.		

Mid/hindgut medium		
Reagent	Final concentration	Amount
CHIR99021 (5 mM)	3 μM	1.8 μL
FGF4 (500 μg/mL)	500 ng/mL	3 μL
FBS	2%	60 μL
Pen/Strep (100×)	1×	30 μL
RPMI1640	N/A	To 3 mL
Total	**N/A**	**3 mL**
Medium should be freshly prepared.		

Spheroid-forming medium		
Reagent	Final concentration	Amount
CHIR99021 (5 mM)	3 μM	1.8 μL
FGF4 (500 μg/mL)	500 ng/mL	3 μL
FBS	2%	60 μL
Y-27632 (10 mM)	10 μM	3 μL
Pen/Strep (100×)	1×	30 μL
RPMI1640	N/A	To 3 mL
Total	**N/A**	**3 mL**
Medium should be freshly prepared.		

HIO basal medium		
Reagent	Final concentration	Amount
N-2 supplement (100×)	1×	5 mL
B-27 supplement (50×)	1×	10 mL
GlutaMAX (100×)	1×	5 mL
HEPES (1 M)	15 mM	7.5 mL
Pen/Strep (100×)	1×	5 mL
Advanced DMEM/F12	N/A	500 mL
Total	**N/A**	**532.5 mL**
HIO basal medium should be prepared in a sterile condition. HIO basal medium can be stored at 4°C for 2–3 weeks.		

HIO d 7 medium		
Reagent	Final concentration	Amount
EGF (20 μg/mL)	50 ng/mL	7.5 μL
Noggin (20 μg/mL)	100 ng/mL	15 μL
R-spondin1 (100 μg/mL)	1000 ng/mL	30 μL
A83-01 (500 mM)	500 nM	3 μL
Matrigel	10%	300 μL
Pen/Strep (100×)	1×	30 μL
HIO basal medium	N/A	To 3 mL
Total	**N/A**	**3 mL**
HIO d 7 medium should be freshly prepared and kept on ice before use.		

HIO medium		
Reagent	Final concentration	Amount
EGF (20 μg/mL)	50 ng/mL	7.5 μL
R-spondin1 (100 μg/mL)	1000 ng/mL	30 μL
A83-01 (500 mM)	500 nM	3 μL
Matrigel	10%	300 μL
Pen/Strep (100×)	1×	30 μL
HIO basal medium	N/A	To 3 mL
Total	**N/A**	**3 mL**
HIO medium should be freshly prepared and kept on ice before use.		

Note: Ensure Matrigel is kept on ice before use as it starts to solidify at higher than 20°C.

STEP-BY-STEP METHOD DETAILS

Seeding iPSCs for stepwise differentiation

Timing: Day -1, 1 h

These steps describe iPSC preparation for stepwise differentiation. The aim is to passage the iPSC line specifically to reach 80%–90% confluency on the next day to start the differentiation. Thus, the seeding cell number may differ depending on the cell line, and require optimization. To ensure reliable and consistent results, iPSC culture should be stable (at least 3 passages after thawing), proliferative without over-confluency stress, and devoid of mycoplasma contamination.

1. Pre-coat a 6-well plate with COAT-1 coating medium.
   a. Add 1 mL coating medium to each well.
   b. Incubate the plate for 30 min to 2 h at 37°C before use.
2. Single-cell dissociation of iPSCs.
   a. Aspirate the DEF-CS medium and rinse the cell surface with pre-warmed 2 mL PBS.
   b. Aspirate the PBS and add 500 μL TrypLE Select.
   c. Incubate the plate at 37°C for 5–7 min.
   d. Gently pipette the well to dissociate iPSCs to single cells.
   e. Add 3 mL of pre-warmed iPSC medium (passage) to stop the enzymatic reaction.
   f. Transfer the cell-suspension medium to 15 mL STEMFULL tube.
   g. Aliquot 20 μL of cell-suspension medium for cell count.
3. Seed iPSCs to the plate.
   a. Aspirate coating medium and gently add pre-warmed 3 mL of iPSC medium (passage).
   b. Seed the required cell number from the cell-suspension medium to each well.
   c. Gently shake the plate to disperse cells evenly.
4. Incubate the plate in a humidified 37°C, 5% CO_2 incubator for 16 h.

⚠ CRITICAL: Cell density prior to differentiation is an important factor for efficient differentiation. We typically seed 3.6 × 10^5–4.0 × 10^5 cells per well, which leads to approximately 80%–90% confluency on the next day. Seeded cell numbers should be optimized according to the iPSC strains.

Stepwise differentiation of iPSCs to mid/hindgut cells

Timing: Day 0 to Day 5, 20 min per day

These steps describe the stepwise differentiation towards mid/hindgut cells, largely based on a previously established protocol.[2,3] Briefly, the protocol involves Activin-induced definitive endoderm formation from days 0–2, then Wnt/FGF4-induced posteriorization towards mid/hindgut patterning from days 3–5 (Figure 1).

5. **Day 0**: Aspirate iPSC medium (passage) and add 3 mL of pre-warmed DE day 0 medium.
6. **Day 1**: Aspirate DE day 0 medium and add 3 mL of pre-warmed DE day 1 medium.
7. **Day 2**: Aspirate DE day 1 medium and add 3 mL of pre-warmed DE day 2 medium.
8. **Day 3**: Aspirate DE day 2 medium and add 3 mL of pre-warmed mid/hindgut medium.
9. **Day 4-5**: Change mid/hindgut medium daily.

Note: Cell death and detachment sometimes occur during DE differentiation, especially from day 0 to day 1. Cells typically start to proliferate from day 1 and should be confluent on day 3 (Figure 1). If excessive cell death occurs from day 0 to day 1 and cells are not confluent on day 3, it is unlikely that the following differentiation is successful. In this case, seeded cell numbers or cell conditions (i.e mycoplasma infection or spontaneous differentiation of iPSCs during maintenance) should be checked. Medium volume can be increased in case of over-acidification.

Optional: Aggregates of mid/hindgut cells sometimes start to detach from the mid/hindgut cell sheet, especially from day 5. These aggregates can be used for HIO differentiation in a three-dimensional culture system as previously described.[2]

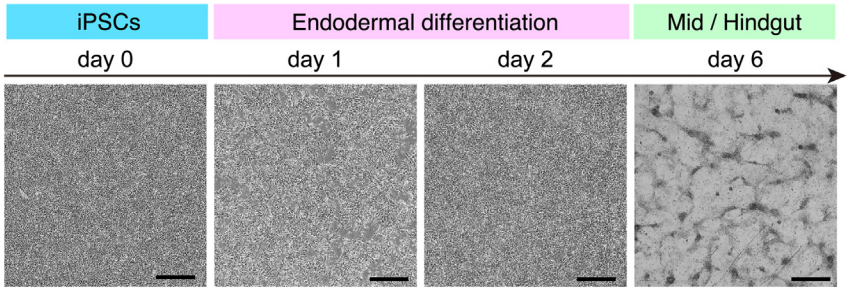

Figure 1. Stepwise differentiation of iPSCs to mid/hindgut

Representative images of cells during differentiation. Cell death is typically observed from day 0 to day 1, but typically cells start proliferating from day 1. Scale bar, 500 μm.

Mid/hindgut spheroid formation using an EZSPHERE® 6-well plate

Timing: Day 6, 1 h

These are critical steps for the generation of homogeneous mid/hindgut spheroids in suspension (suspension-Spheroids; s-Spheroids). Mid/hindgut cells differentiated from iPSCs are dissociated into single cells and seeded onto an ultra-low attachment spheroid-forming plate with microwells, which generates homogeneous spheroids.

For the generation of larger spheroids, proceed to step 19.

Note: The size of spheroids can be controlled by changing the microwell diameter of EZSPHERE® plates (Figure 2A). Microwell diameters of 200 μm, 500 μm, 800 μm, and 1400 μm produces spheroids of 100 μm, 180 μm, 230 μm, and 330 μm respectively (Figure 2B). Only 500 μm microwell is commercially available in 6-well plate format, while the rest is available as 35 mm dishes. The protocol describes the use of 6-well plates; however, it is largely transferrable to the 35 mm dish format.

10. Prepare spheroid-forming medium (3 mL per well) and warm it to 37°C.
11. Prepare an EZSPHERE® 6-well plate.
    a. Add 2.5 mL of the spheroid-forming medium.
    b. Gently pipette the medium to remove bubbles within microwells.
    c. Place the plate in humidified 37°C, 5% CO_2 incubator before use.
12. Dissociate mid/hindgut cells to single cells.
    a. Aspirate mid/hindgut medium and rinse the cell surface with pre-warmed 2 mL PBS.
    b. Aspirate PBS and add 700 μL TrypLE Select.
    c. Incubate the plate at 37°C for 7 min.

⚠ CRITICAL: Optimize incubation time according to cell density. Make sure cells are loosely detached from the well. Avoid harsh mechanical detachment as it will lead to low cell viability.

    d. Gently tap the plate to detach the cells.
    e. Gently pipette mid/hindgut cells for single-cell dissociation.
    f. Add 3 mL of pre-warmed DMEM to stop the enzymatic reaction.
    g. Transfer the cell-suspension to 15 mL STEMFULL tube.
    h. Aliquot 20 μL of cell-suspension medium for cell count.
13. Calculate the total cell number and required well numbers.

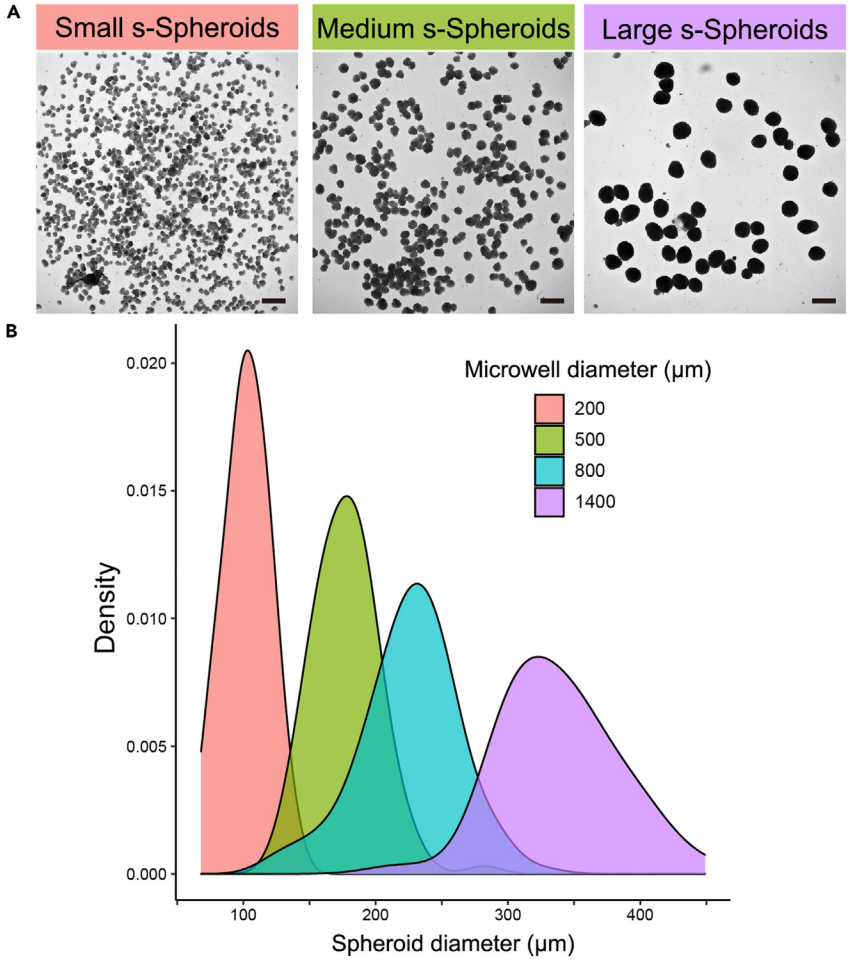

Figure 2. s-Spheroid size design by various size EZSPHERE® plates

(A) Bright-field images of small, medium, and large spheroids formed in microwell diameters: 300, 500, and 1,400 μm, respectively. Scale bar, 500 μm.

(B) Diameter distribution of spheroids formed in each microwell condition. (A) is modified from the original Figure 2 of Takahashi et al. (2022).[1]

Note: We typically seed 1.5 × 10^6–2.0 × 10^6 cells per well for spheroid formation. Required well numbers can be calculated as follows.

Required well numbers = total cell count / seeded cell numbers per well.

14. Centrifuge the tube at 200 *g* for 3 min and aspirate the supernatant.
15. Add the spheroid-forming medium to the tube and mix gently. The volume of added medium is calculated as follows.

Medium volume = 500 × n μL (n = seeded well number).

16. Add 500 μL of the cell-suspension medium to each well of the EZSPHRE® 6-well plate.

⚠ CRITICAL: Ensure that no bubbles remain in the microwells (Figure 3). Residual bubbles prevent efficient spheroid formation. If bubbles are present, remove them by gently pipetting the medium.

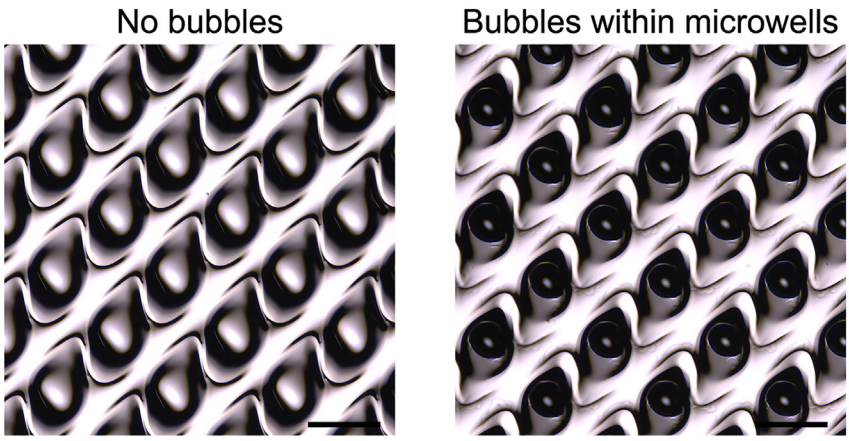

Figure 3. Preparation of EZSPHERE® plate
Images of microwells with trapped air (left) and without air (right). Residual air in microwells hampers homogeneous spheroid formation. Scale bar, 500 μm.

17. Gently shake the plate to disperse cells evenly.
18. Incubate the plate in a humidified 37°C, 5% CO_2 incubator for 24 h (Figure 4).

⚠ CRITICAL: Avoid agitating the EZSPHERE® plates during spheroid formation as mid/hindgut cells and spheroids can easily be displaced from the microwells (Figure 5).

Optional: Spheroid formation can be monitored by time-lapse imaging using the BZ-X710 microscope to observe aggregation and self-organization.

Large spheroid formation using an EZ-BindShut® 96-well plate

Timing: Day 6, 1 h

An alternative to the EZSPHERE® method. These steps produce fewer, but much larger spheroids using 96-well spheroid forming plates (Figure 6A). The size of the spheroids can further be micro-adjusted by the seeded cell numbers (Figure 6B). Skip this part when large spheroids are unnecessary.

19. Prepare mid/hindgut spheroid-forming medium (24 mL per plate) and warm it to 37°C.
20. Dissociate mid/hindgut cells to single cells.
    a. Aspirate mid/hindgut medium and rinse the cell surface with pre-warmed 2 mL PBS.
    b. Aspirate PBS and add 700 μL TrypLE Select.
    c. Incubate the plate at 37°C for 7 min.

⚠ CRITICAL: Optimize incubation time according to cell density. Make sure cells are loosely detached from the well. Avoid harsh mechanical detachment as it will lead to low cell viability.

    d. Gently tap the plate to detach the cells.
    e. Gently pipette mid/hindgut cells for single-cell dissociation.
    f. Add 3 mL of pre-warmed DMEM to stop the enzymatic reaction.
    g. Transfer the cell-suspension to 15 mL STEMFULL tube.
    h. Aliquot 20 μL of cell-suspension medium for cell count.
21. Calculate the total cell number and required well numbers.

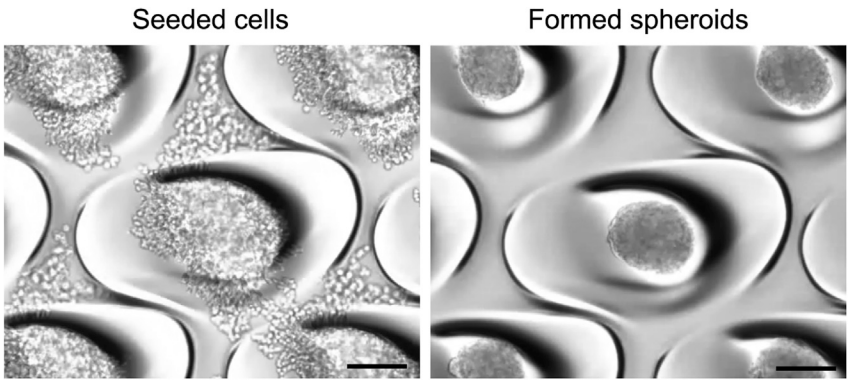

Figure 4. Spheroid formation of dissociated mid/hindgut cells on an EZSPHERE® plate
Seeded cells equally spread around the well and fall into microwells (left), forming homogeneous spheroids within 24 h (right). Scale bar, 200 μm.

Note: Seeded cell number per well is determined by the desired spheroid size. A clear relationship between seeded cell numbers and the spheroid diameters is observed (Figure 6B). Required well numbers are calculated as follows.

Required well numbers = total cell count / seeded cell numbers per well.

22. Centrifuge the tube at 200 *g* for 3 min and aspirate the supernatant.
23. Add spheroid-forming medium to the tube and mix gently.

Note: Medium volume per well depends on the seeded cell numbers. We typically use 200 μL of medium per well when making spheroids from 1 × 10^5 cells per well.

24. Place the plate in a humidified 37°C, 5% CO_2 incubator for 24 h.

Note: When making larger spheroids, sometimes a longer incubation period is required for true sphere formation. If spheroids fail to form a sphere, incubate the plate for an additional 24 h.

s-Spheroid collection from EZSPHERE® 6-well plate

Timing: Day 7, 20 min

If proceeding from step 24, skip to step 29.

These steps describe the collection of spheroids from EZSPHERE® 6-well plates for subsequent culturing. After 24 h of incubation, mid/hindgut cells should have aggregated in the individual microwells to form one spheroid per microwell (~9200 spheroids per well). The structure is still a relatively loose aggregate; thus, aggressive pipetting or movements should be avoided.

25. Gently shake the plate and pipette the medium so that spheroids float out from the microwells.
26. Collect the spheroid containing medium to 15 mL STEMFULL tube.
27. Add 1 mL of pre-warmed DMEM to each well and repeat steps 25–26 to maximize spheroid collection.
28. Centrifuge the tube at 80 *g* for 3 min and aspirate the supernatant. Proceed to step 32.

Note: When spheroids are in physical contact for a period, they tend to fuse, which doesn't pose a problem for further culture and growth. In order to have s-Spheroids with homogeneous shape, size and inner structure, it would be better avoiding fusion. To that end,

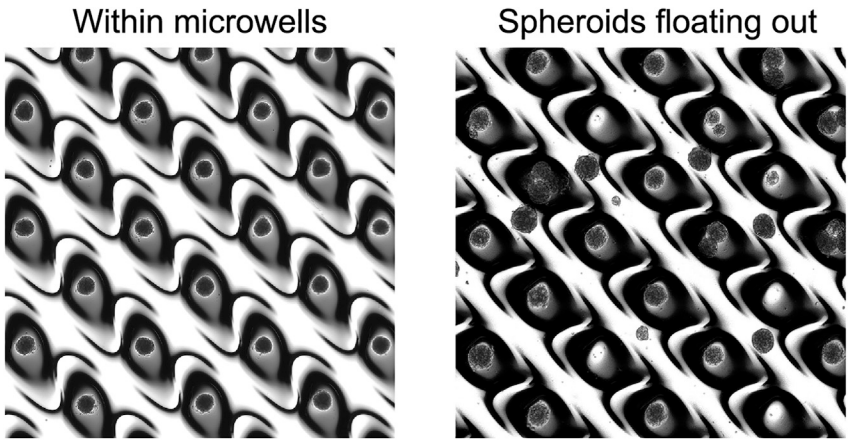

Figure 5. Representative images of spheroids in EZSPHERE® plate
Spheroids are confined to each microwell (left). Spheroids easily float from microwells upon agitating the plate (right), which causes spheroid fusion. Scale bar, 500 μm.

spheroid collection and transfer to suspension culture should be performed quickly (Figure 7A).

s-Spheroid collection from EZ-BindShut® 96-well plate

Timing: Day 7, 20 min

These steps describe the collection of spheroids from EZ-BindShut® 96-well plates for subsequent culturing. After 24–48 h of incubation, mid/hindgut cells should have aggregated into one spheroid per well. These spheroids are much larger than those formed on EZSPHERE® 6-well plates, and so may require longer period to form. Typically, larger spheroids are more prone to structural damage and disintegration; thus, special care is warranted when handling.

29. Pick up the s-Spheroids from individual wells into a 15 mL STEMFULL tube with a wide tip pipette.

Note: Use the appropriate pipette tip that is wide enough to safely pick up the generated spheroids. For larger spheroids, we recommend cutting the tip of a 1000 μL pipette tip in an aseptic condition. This should be adequate to safely pick up even millimeter-scale spheroids.

30. Place the tube vertically for 30–60 s to allow gravity sedimentation of s-Spheroids.
31. Aspirate the supernatant. Proceed to step 32.

Differentiation of s-Spheroids into human intestinal organoids (HIOs) in a suspended state (s-HIOs)

Timing: Day 7 to Day 35, Day 7: 10 min, Day 10: 20 min per medium change

These steps describe the differentiation of s-Spheroids into HIOs through suspension culture. This protocol describes two suspension methods; static suspension culture and rotational culture. For smaller s-Spheroids (i.e., s-Spheroids generated by EZSPHERE® 6-well plate), static suspension culture is sufficient for s-HIO differentiation (steps 32–39). For larger s-Spheroids (i.e., s-Spheroids

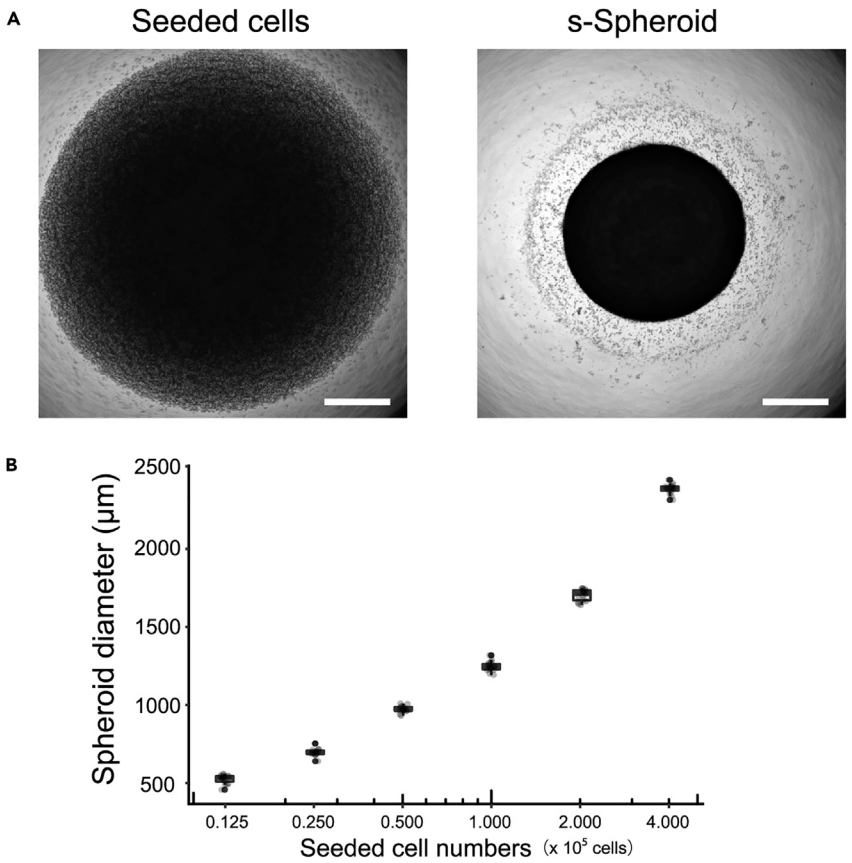

Figure 6. Spheroid formation of dissociated mid/hindgut cells on an EZ-BindShut® plate

(A) Dissociated mid/hindgut cells are seeded on each well (left), forming a spheroid within 24–48 h (right). Scale bar, 500 μm.

(B) The relation between seeded cell numbers and formed s-Spheroid diameter. (B) is modified from the original Figure 2 of Takahashi et al. (2022).[1]

generated by EZ-BindShut® 96-well plate), we recommend static suspension culture for three days, followed by rotational culture (steps 40–48) for optimal growth and differentiation.

32. **Day 7**: Prepare HIO d 7 medium.

Note: medium should be kept on ice to avoid Matrigel polymerization.

33. Add HIO d 7 medium to spheroid-collecting tube (step 28, step 31).
34. Transfer spheroid-suspension medium to a 6-well ultra-low attachment (ULA) culture plate. 3 mL of HIO d 7 medium is used for each well.

Note: We typically culture approximately up to 900 small s-Spheroids (i.e. s-Spheroids generated from EZSPHERE® 6-well plate) per 1 well of the ULA culture plate. For larger s-Spheroids (i.e. s-Spheroids generated from EZ-BindShut® 96-well plate), up to 16 s-Spheroids are applied to each well. Medium volume can be reduced to 2 mL depending on the s-Spheroid concentration.

Note: Culturing individual spheroids in 96-well low attachment plate would eliminate the potential of spheroid fusion, but medium change would be laborious.

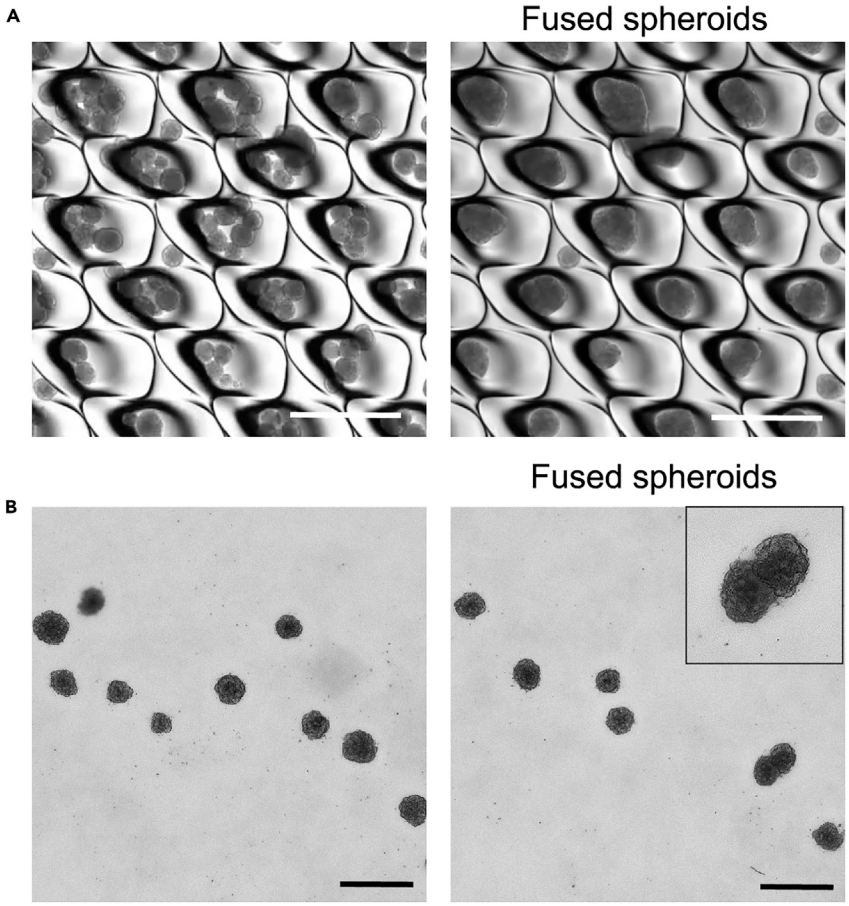

Figure 7. Spheroid fusion during culture

(A) Nearby spheroids quickly assemble to form homogeneous spheroids on an EZSPHERE® plate.

(B) Representative images of spheroids without fusion (left) and with fusion (right, inset). Scale bar, 500 μm.

⚠ CRITICAL: Excessive spheroid concentration increases the risk of spheroid fusion. Culture concentrations should be optimized accordingly.

35. Gently shake the ULA plate to disperse the s-Spheroids.

Note: Even distribution of spheroids within the well is important for preventing s-Spheroid fusion (Figure 7B).

36. Culture the spheroids for 3 days in a humidified 37°C, 5% CO_2 incubator.
37. **Day 10**: Collect the s-Spheroids.
    a. Prepare HIO medium and keep on ice.
    b. Prepare ice-cold DMEM.
    c. Collect the spheroids into a 15 mL STEMFULL tube with a wide tip pipette.
    d. Add ice-cold DMEM to the tube (up to 12 mL).
    e. Gently rotate the tube to dislodge the spheroids from Matrigel scaffold.
    f. Large Spheroids: Place the tube vertically to allow gravity sedimentation of s-Spheroids.
    Small spheroids: Centrifuge 40 *g* for 3 min.
    g. Aspirate the supernatant.

Note: Depending on the amount of spheroids collected, steps 37 d-g may need to be repeated 2–3 times. If there are considerable Matrigel residuals, repeat those steps.

Note: For rotational culture of large spheroids, proceed to step 40.

38. **Day 10**: Add HIO medium to the tube and transfer the spheroid-suspension medium back to the ULA culture plate.
39. **Day 13 - Day 35**: Change HIO basal medium every 3–4 days (Repeat 37–38).

Note: Mesoderm gradually develops around the spheroids and through the culture period spheroids gradually differentiate into HIOs. The culture period can be shortened or expanded depending on the developmental stage desired.

Rotational culture and differentiation of s-Spheroids into s-HIOs

Timing: Day 10 to Day 35. Day 10: 20 min, Day 13: 20 min per medium change

For optimal growth and differentiation of larger s-Spheroids, we recommend rotational culture from Day 10[1]. This section provides step-by-step instructions for transitioning from static to rotational culture, and culture maintenance. The bioreactor employed is the CELLPET 3D-iPS® bioreactor, in which the s-Spheroids are exposed to a constant flow of the medium, enhancing active gas exchange and penetration of the medium.

Note: Before starting rotational culture, we recommend three days of static suspension culture, as s-Spheroids are initially vulnerable to mechanical stress.

40. **Day 10**: Prepare 10 mL rotational culture vessel syringe (Figure 8A).
    a. Prepare 8 mL of ice-cold HIO medium.
    b. Close the cap of the vessel. Stand the vessel vertically on a 1.5 mL tube stand.
    c. Add 7 mL of HIO medium to the vessel (Figure 8C).
41. Add 1 mL of HIO medium to the spheroid containing tube (step 37) (Figure 8D).
42. Transfer the spheroids to the vessel using a 1000 µL pipette (with cut tip) (Figure 8E).

Note: Less than 20 spheroids are cultured in a 10 mL vessel. 10 mL, 30 mL, and 50 mL vessels are currently commercially available. The size of the vessel can be adjusted depending on the number and sizes of spheroids.

Note: HIO medium used per culture vessel must be less than 80% of the total vessel volume (i.e. 24 mL medium or less for a 30 mL vessel). Using more medium increases the risk of over-pressurization and spillage.

⚠ CRITICAL: Culturing too many s-Spheroids in one vessel will cause spheroid fusion and medium over-acidification. For each iPSC strain and culture system, optimization is necessary for the number of spheroids per vessel, vessel size, and frequency of medium changes.

43. Set the plunger to the 10 mL scale.

Note: The positive pressure within the vessel will push back on the plunger. Thus, constant pressure needs to be applied on the plunger to maintain its position. Be prepared to loosen the cap and release the pressure when setting the plunger.

⚠ CRITICAL: Ensure that no spheroids are present at the tip of the syringe culture vessel (the very bottom where the cap is placed). It endangers the spheroids when the positive

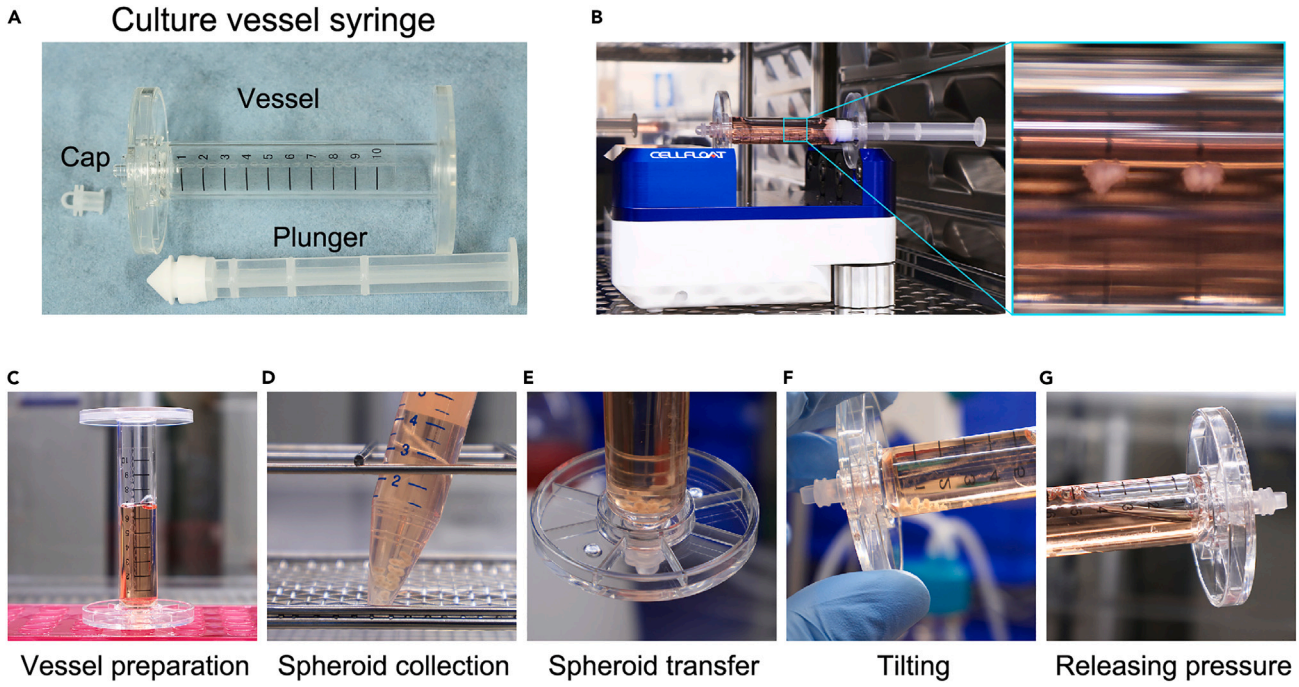

Figure 8. Overview of the rotational culture

(A) Image of a culture vessel syringe.

(B) Representative image of spheroids cultured in a rotational bioreactor; CELLFLOAT 3D-iPS system (Corresponding with Methods video S1).

(C) Medium is applied to the vertically stood vessel.

(D) Spheroids were collected in a 15 mL tube.

(E) Spheroids are transferred to the vessel. In this step, spheroids typically sink to the bottom.

(F) Vessel is tilted before setting the plunger to minimize the risk of spheroids sinking to the very bottom.

(G) Release the positive pressure by loosening the cap, then closing the cap (Corresponding with Methods video S2).

pressure is released. If a spheroid can be seen close to the tip of the vessel, gently pipette the medium, tilt the vessel 45 degrees, then set the plunger (Figure 8F).

44. Release the positive pressure within the vessel by loosening the cap and close the cap (Figure 8G).

Note: Some HIO medium will leak from the vessel cap. Loosen the cap with an absorbent paper in hand.

45. Put the vessel on the CELLPET 3D-iPS® bioreactor placed within a humidified 37°C, 5% CO_2 incubator and start rotation (Figure 8B, Methods video S1).

Note: The optimum rotational speed is such that the cultured tissue is static relative to the viewer and rotating relative to the vessel (Methods video S1). This speed differs depending on the size and density of the tissue. Typically, we start rotational speed from 8 rpm and as s-Spheroids grow the speed is gradually increased to maximize the nutrient/oxygen exchange of the spheroids.

⚠ CRITICAL: When the rotation is set too fast, it generates high shear stress and destroys the s-Spheroids/HIOs. Optimize the rotational speed depending on the size of s-Spheroids and HIOs.

46. **Day 13**: Collect s-Spheroids from the vessel.

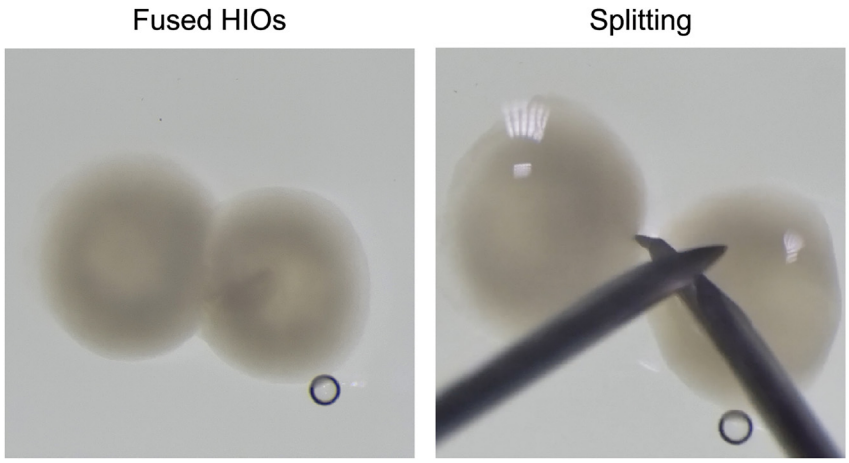

Figure 9. Mechanical dissociation of fused s-HIOs

Under a microscope, fused s-HIOs can be split along the demarcation line using 25G needles.

a. Prepare ice-cold DMEM.
b. Collect s-Spheroids from the vessels with a 1000 μL pipette tip (with cut tip) and transfer them to a 15 mL STEMFULL tube.
c. Add ice-cold DMEM to the tube (up to 12 mL).
d. Gently rotate the tube to dislodge the spheroids from Matrigel scaffold.
e. Place the tube vertically to allow gravity sedimentation of spheroids.
f. Aspirate the supernatant.

Note: Depending on the amount of spheroids collected, steps 46 c-f may need to be repeated 2–3 times. If there are considerable Matrigel residuals, repeat those steps.

47. Transfer s-Spheroids to the vessel (Repeat steps 40–45) (Figures 8C–8G, Methods video S2).
48. **Day 16 - Day 35**: Change HIO medium every 3–4 days (Repeat steps 46–47).

Note: Mesoderm gradually develops around the spheroids and through the culture period spheroids gradually differentiate into HIOs. The culture period can be shortened or expanded depending on the developmental stage desired.

Note: Sometimes nearby s-Spheroids/HIOs fuse. Fused HIOs can be separated mechanically along the demarcation line using 25G needles under a stereomicroscope in a sterile condition (Figure 9).

EXPECTED OUTCOMES

Following the above protocol, iPSCs are differentiated and aggregated into mid/hindgut spheroids (s-Spheroids), then matured into HIOs (s-HIOs) in suspension culture. Using different EZSPHERE® plates, between 200-9200 structurally homogeneous s-Spheroids of 100–330 μm diameter can be generated per well (Figure 2), stably expressing the mid/hindgut marker CDX2. On the other hand, using EZ-BindShut® 96-well plates, seeded cell number can be tweaked to control s-Spheroid diameter, ranging from 500 μm (1.25×10^4 cells per well) to 2000 μm (4×10^5 cells per well) (Figure 6B).

The generated spheroids can be cultured in suspension with Matrigel-containing medium, eliminating the necessity of periodic passaging, and allowing continuous culture for 35 days. Spheroids generated on EZ-BindShut plates can be transferred to rotational suspension culture on a bioreactor

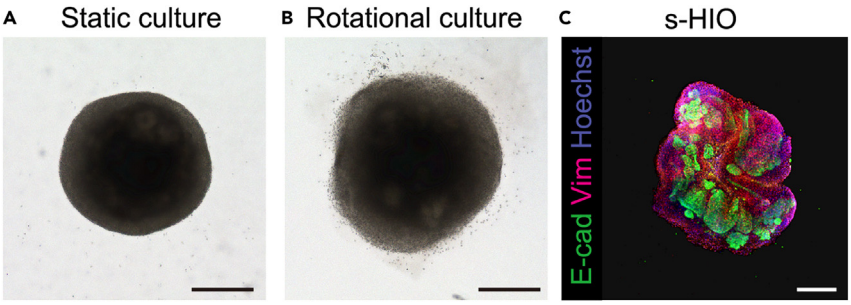

Figure 10. Representative images of s-HIOs

(A and B) A bright field image of a large s-HIO cultured in static suspension (A) and rotational suspension (B).

(C) Whole-mount immunostaining of Vimentin and E-cadherin in an s-HIO cultured in a rotational bioreactor. E-cadherin-positive intestinal epithelial layer is surrounded by vimentin-positive mesenchymal layers. Scale bar, 500 μm.

after Day 10, which accelerates s-Spheroid/HIO growth (Figure 10). By Day 35, HIOs grown in rotational suspension are structurally complex with E-cadherin-positive epithelial core surrounded by Vimentin-positive mesenchymal layer, and diameters reaching around 2500 μm.

Although the described protocol is reliable at producing viable and uniform spheroids, the details regarding the rate of growth and the proportion of mesenchymal components tend to vary widely between iPSC lines. Therefore, we highly recommend using the protocol as a base, and optimize the details, such as the seeded cell number, length of aggregation, length of culture, etc., according to each cell line and experimental set-up.

QUANTIFICATION AND STATISTICAL ANALYSIS

Spheroid enumeration and size measurement:

Spheroid counting and size measurements can be useful to ascertain, for example, the effect of a parameter on spheroid size and/or growth. The measurements can be done manually using any image processing software such as ImageJ with limited throughput and consistency. This section will describe in detail the use and setting of Cell^{3}iMager Duos, a high-throughput imaging scanner which automatically detects and measures the 2-dimensional characteristics of spheroids.

We recommend training an AI model using an in-built software for spheroid detection as opposed to image parameter-based detection. If trained properly with a training set containing a reasonable variation of spheroid size/shape, backgrounds, brightness, etc., AI-based detection tends to be more flexible and accurate than parameter-based detection (Figures 11A and 11B). Training an AI model is straight forward, involving the annotation of spheroids on several images (training set), and running a deep learning software (Figure 11C). If using several iPSC lines, the training set should contain a balanced set of images from all lines. In our experience, training for 30–40 min using training sets containing 5–10 images with 30–80 annotated objects produces adequate AI models.

All detected spheroids are included in data analysis except fused spheroids, which are excluded in most cases. As mentioned previously, spheroids have an epithelial core surrounded by a mesenchymal layer. If the epithelial cores of the fused spheroids are visibly separate, the detected object is split at the demarcation line, and the resulting two objects are included in the analysis (Figure 11D).

LIMITATIONS

Mid/hindgut spheroid generation relies on a differentiation protocol that mainly directs the differentiation towards endodermal cells; however, some mesodermal cells co-develop which turn into mesenchymal cells. Each iPSC line has distinct inclinations for the proportion of endodermal to

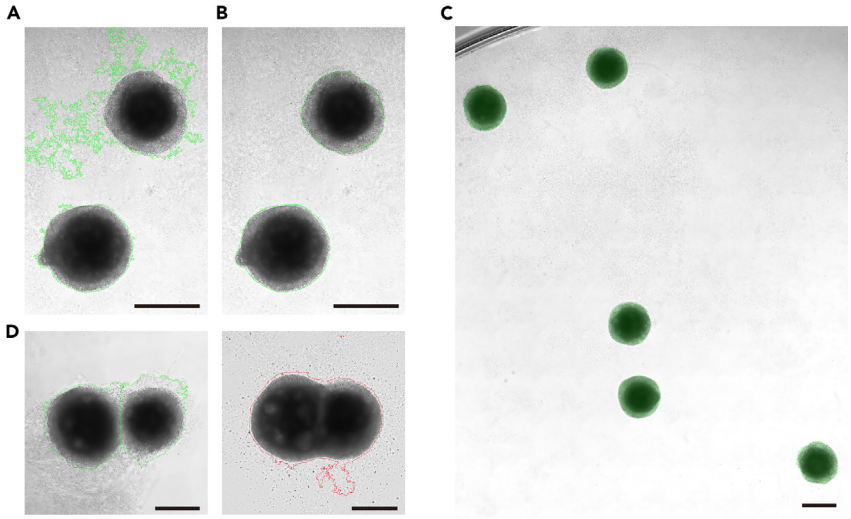

Figure 11. Spheroid measurement on Cell3iMager Duos

(A and B) Detected spheroids outlined in green using image parameter-based detection (A), and AI model-based detection (B).

(C) Image annotation for AI model training showing annotated spheroids in green.

(D) Example images of fused spheroids to include (left) and exclude (right) from further analysis. Scale bar, 1000 μm.

mesodermal developments, leading to noticeable variability in HIO morphology and cell populations. Specifically, certain iPSC lines may develop minimal mesenchymal components.

The suspension culture protocol described above requires Matrigel containing medium, which is changed twice weekly. Compared to conventional Matrigel-embedding culture, suspension culture will consume substantially more Matrigel for the culture duration.

Rotational suspension culture is mainly intended for the culture of large spheroids. We have not validated the rotational culture method for small spheroids generated on EZSPHERE® plates for two reasons. First, diffusion is likely sufficient for gas/factor exchange in small tissues. Second, preliminary trials led to considerable spheroid fusion. This lack of optimization presents a limitation for the above protocol.

TROUBLESHOOTING

Problem 1

Excessive cell death and detachment from day 0 to day 1, related to steps 5–6.

Potential solution

Cell death is a common observation in the initial phase of DE differentiation, especially from day 0 to day 1. However, excessive cell death may indicate a sub-optimal pre-conditioning of iPSCs, and culture should be aborted.

- Check the quality of iPSCs (i.e., mycoplasma contamination, loss of pluripotency, karyotype change). If the iPSCs are compromised, thaw a new batch and redo after 3 passages.
- Optimize the seeded cell density.
- Change the medium gently to avoid detachment of the well-coating.

Problem 2

Poor aggregation of mid/hindgut cells into spheroids, related to steps 10–24.

Potential solution

Low viability of mid/hindgut cells after dissociation may cause poor aggregation. Ensure cell viability before seeding. Avoid harsh mechanical dissociation as it will cause cell death. Over-acidification of the medium during differentiation also affects cell viability.

- Check the cell viability before seeding (using Trypan Blue or other viability assays).
- Avoid harsh mechanical dissociation and optimize the single-cell dissociation procedure (i.e., adjust the amount of TrypLE Select and/or the incubation time depending on the cell density).
- Increase the medium volume if over-acidification is observed.

Problem 3

Large spheroids forming biconcave structures, related to step 19–24.

Potential solution

When making large spheroids using the EZ-BindShut® 96-well plate, 24 h of incubation may be insufficient for adequate aggregation depending on iPSC lines and cell numbers. Longer incubation time may allow cells to aggregate into a true sphere.

- Allow longer incubation time (48 h at most).

Problem 4

Spheroids/HIOs fuse to form heterogeneous structures during culture (related to steps 32–48).

Potential solution

Spheroids/HIOs in physical contact easily fuse. When cultured in high concentration, spheroids are more likely to fuse. Therefore, lower the concentration of spheroids to minimize the risk. If necessary, fused HIOs can be separated using needles.

- When splitting fused HIOs do so with a 25G needles under a stereomicroscope.

Problem 5

Lack of visible Matrigel surrounding every spheroids/HIOs in suspension culture, leading to impaired growth (related to steps 32–48).

Potential solution

Spheroids/HIOs require physical contact with Matrigel for mechanical and signaling support. It is crucial to ensure even mixture of Matrigel in HIO medium and prevent the polymerization process before its application to the wells/vessels. Premature polymerization leads to clumps of Matrigel within the medium which becomes inaccessible to the spheroids/HIOs.

- Make sure the HIO medium is prepared and kept ice cold.

Problem 6

Structural damage to the mesenchymal and/or epithelial components of spheroids/HIOs during suspension culture (related to steps 32–48).

Potential solution

High mechanical stress to spheroids/HIOs endangers their structural integrity. Physical damage could be caused by the usage of inappropriate tips for collection of spheroids/HIOs, and over-zealous shaking during Matrigel removal steps (steps 37 e and 46 d). Make sure to minimize the physical stress places on spheroids/HIOs.

During rotational culture, another possible cause could be excessive shear stress placed upon the spheroids/HIOs. The optimal rotational speed differs according to spheroid/HIO size. We typically start from low speed as early-stage spheroids are more vulnerable, and then gradually increase the speed as spheroids/HIOs grow. Aim to keep the spheroids/HIOs static from the viewer's perspective during rotation.

Problem 7

Gradual reduction of spheroid/HIO numbers during rotational culture (related to steps 40–48).

Potential solution

The most common cause of loss during rotational culture is overlooking the spheroid stuck at the vessel tip when releasing the pressure after changing the culture medium (step 44). This tends to lead to the complete loss of the spheroid, possibly stuck within the cap.

- Before pushing the plunger into the vessel, gently pipette the medium so that spheroids float out from the bottom.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tomohiro Mizutani (tmizutani.gast@tmd.ac.jp).

Materials availability

This protocol did not generate new unique reagents.

Data and code availability

This study did not generate datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102374.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.T., T.M.; investigation, J.T., H.Y.S., S.K., S.N., T.M.; writing – original draft, J.T., H.Y.S., S.K., T.M.; writing – review and editing, J.T., H.Y.S., S.K., S.N., R.O., T.M.; supervision, T.M., R.O.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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