Standard Operating Procedures for the Scale-up Production of Human Liver Organoids (HLOs) on a Pillar Plate *via* Microarray 3D Bioprinting

This standard operating procedure (SOP) provides step-by-step methods for the scale-up generation of human liver organoids (HLOs) on a 36PillarPlate by microarray 3D bioprinting of foregut cells suspended in Matrigel onto a pillar plate and differentiating them in a 384DeepWellPlate for static culture (or 36PerfusionPlate for dynamic culture). Please read the protocol carefully before performing experiments.

1. Materials

1.1 Materials for maintenance and passage of iPSCs

- Costar® 6-well clear TC-treated multiple well plates, individually wrapped, sterile (Corning, Cat. no. 3516)
- EDi029-A, a male human iPSC line (Cedar Sinai Biomanufacturing Center, USA)
- Matrigel® growth factor reduced (GFR) basement membrane matrix (Corning, Cat. no. 354230)
- DPBS, no calcium, no magnesium ($dPBS^{-/-}$; Gibco, Cat. no. 14190)
- Accutase™ cell dissociation reagent (Gibco, Cat. no. A1110501)
- **Complete mTeSR™ plus medium:** mTeSR™ plus basal medium and 5x supplement (StemCell Technologies, Cat. no. 100-0276).
  Note: *Thaw supplements or complete medium at room temperature (15 - 25°C) or overnight at 2 - 8°C. Do not thaw in a 37°C water bath. Once thawed, use the supplement immediately or aliquot and store at -20°C for up to 3 months.*
- StemPro™ EZPassage™ tool (Gibco, Cat. no. 23181-010)
- ReLeSR™ human pluripotent stem cell passaging reagent (StemCell Technologies, Cat. no. 100-0483)
- CryoStor® CS10 cell freezing medium (StemCell Technologies, Cat. no. 07959)
- Mr. Frosty™ freezing container (Thermo Scientific, Cat. no. 5100-0001)

1.2 Materials for differentiation of iPSCs into HLOs on the pillar plate

- 36PillarPlate (Bioprinting Laboratories Inc., Cat. no. 36-01-00)
- 384PillarPlate (Bioprinting Laboratories Inc., Cat. no. 384-03-00)
- 36PetriLid (Bioprinting Laboratories Inc., Cat. no. 36-04-00)
- 384DeepWellPlate (Bioprinting Laboratories Inc., Cat. no. 384-03-00)
- LoadingPlate (Bioprinting Laboratories Inc., Cat. no. 384-03-00)
- Costar® 6-well clear TC-treated multiple well plates, individually wrapped, sterile (Corning, Cat. no. 3516)
- Petri dish, 90 mm x 15 mm (VWR, Cat. no. 75799-946)
- iMatrix-511 silk, recombinant laminin-511 (Elixirgen Scientific, Cat. no. NI511)
- Matrigel® basement membrane matrix, phenol red-free (Corning, Cat. no. 356237)
- ASFA® spotter, version 6 (Medical & Bio Decision Co. Ltd., Republic of Korea)
- TC20 automated cell counter (Bio-Rad, Cat. no. 1450102)
- CEPT cocktail consisting of 50 nM chroman 1 (R&D systems, Cat. no. 7163), 5 µM emricasan (Selleckchem, Cat. no. S7775), 0.1%(v/v) polyamine supplement (Sigma Aldrich, Cat. no. P8482), and 0.7 µM trans-ISRIB (R&D systems, Cat. no. 5284)
- mTeSR™ plus basal medium and 5X supplement (StemCell Technologies, Cat. no. 100-0276) (*see* **Table 1**)

- **RPMI basal medium:** RPMI 1640 (Gibco, Cat. no. 22400) supplemented with 1% (v/v) Penicillin/Streptomycin (Pen/Strep; Gibco, Cat. no. 15140)
- **Advanced DMEM basal medium:** Advanced DMEM/F12 (Gibco, Cat. no. 12634) supplemented with 2% (v/v) B27 (Gibco, Cat. no. 17504), 1% (v/v) N2 (Gibco, Cat. no. 17502), 10 mM HEPES (Gibco, Cat. no. 15630), 1% (v/v) Pen/Strep, and 1% (v/v) GlutaMAX™ (Gibco, Cat. no. 35050)
- **Day 1 medium:** RPMI basal medium supplemented with 50 ng/mL recombinant human bone morphogenetic protein-4 (BMP4; R&D Systems, Cat. no. 314-BP) and 100 ng/mL recombinant human activin A (R&D Systems, Cat. no. 338-AC) (*see* **Table 1**)
- **Day 2 medium:** RPMI basal medium supplemented with 100 ng/mL activin A and 0.2% (v/v) knockout serum replacement (KSR; Gibco, Cat. no. 10828)
- **Day 3 medium:** RPMI basal medium supplemented with 100 ng/mL activin A and 2% (v/v) KSR
- **Day 4-6 medium:** Advanced DMEM basal medium supplemented with 500 ng/mL recombinant human fibroblast growth factor 4 (FGF4; Peprotech, Cat. no. 100-31) and 3 µM CHIR 99021 (R&D Systems, Cat. no. 4423) (*see* **Table 1**)
- **Day 7-10 expansion medium:** Advanced DMEM basal medium supplemented with 5 ng/mL recombinant human FGF basic/FGF2/bFGF (R&D systems, Cat. no. 233-FB), 10 ng/mL recombinant human VEGF-165 (Gibco, Cat. no. PHC9391), 20 ng/mL recombinant human EGF (R&D system, Cat. no. 236-EG), 0.5 µM A 83-01 (R&D Systems, Cat. no. 2939), 3 µM CHIR 99021, 50 µg/mL L-ascorbic acid (Sigma Aldrich, Cat. no. A4544), and the CEPT cocktail (containing a final concentration of 50 nM chroman 1, 5 µM emricasan, 0.1% (v/v) polyamine supplement, and 0.7 µM trans-ISRIB) (*see* **Table 1**)
- **Day 11-14 retinoic acid medium:** Advanced DMEM basal medium supplemented with 2 µM retinoic acid (RA; Sigma Aldrich, Cat. no. R2625) (*see* **Table 1**)
- **Day 15-25 maturation medium:** Hepatocyte culture medium (HCM; Lonza, Cat. no. CC-3198) prepared as per manufacturer's instructions without EGF and supplemented with 100 nM dexamethasone (Dex; Sigma Aldrich, Cat. no. D4902), 20 ng/mL recombinant human oncostatin M (OSM; Peprotech, Cat. no. 300-10), and 10 ng/mL recombinant human hepatocyte growth factor (HGF; Peprotech, Cat. No. 100-39) (*see* **Table 1**)

1.3 Materials for cell viability assay of HLOs on the pillar plate

- Fisherbrand™ 384-well polystyrene plates, white (Fisher Scientific, Cat. no. 12-566-623)
- CellTiter-Glo® 3D cell viability assay (Promega, Cat. no. G9681)

1.4 Materials for immunofluorescence staining of HLOs on the pillar plate

- Petri dish, 90 mm x 15 mm (VWR, Cat. no. 75799-946)
- 36PillarPlate (Bioprinting Laboratories Inc., Cat. no. 36-01-00)
- 384DeepWellPlate (Bioprinting Laboratories Inc., Cat. no. 384-03-00)
- Fisherbrand™ 384-well polystyrene plates, clear (Fisher Scientific, Cat. no. 12-566-625)
- Phosphate-buffered saline (PBS, 1x), sterile-filtered (Fisher Scientific, Cat. no. AAJ61196AP)
- 4% Paraformaldehyde (Fisher Scientific, Cat. no. AAJ19943K2)
- Sodium borohydride (Sigma Aldrich, Cat. no. 452882)
- Triton X-100 (Fisher Scientific, Cat. no. MTX15681)
- Tween 20 (Sigma Aldrich, Cat. no. P1379)
- Normal donkey serum (Sigma Aldrich, Cat. no. S30-M)
- Primary antibodies to target protein of interest

- Secondary antibodies based on the host of primary antibodies
- DAPI (Fisher Scientific, Cat. no. D1306)
- Visikol Histo-M tissue clearing reagent (Visikol, Cat. no. HM-30)
- RapiClear 1.52 (Sunjin Lab, Taiwan)
- Fisherbrand™ premium cover glass (Fisher Scientific, Cat. no. 125485J)

Table 1. Preparation of the stock solution of additives and the final working concentrations in cell differentiation media

Additives	Solvent	Stock conc.	Working conc.	Dilution factor
Chroman 1	DMSO	10 mM	50 nM	200,000x
Emricasan	DMSO	50 mM	5 μM	10,000x
trans-ISRIB	Warm DMSO	10 mM	0.7 μM	14,286x
BMP4	$dPBS^{-/-}$ containing at least 0.1% (w/v) BSA	10 μg/mL	50 ng/mL	200x
Activin A	$dPBS^{-/-}$ containing at least 0.1% (w/v) BSA	10 μg/mL	100 ng/mL	100x
FGF4	$dPBS^{-/-}$ containing at least 0.1% (w/v) BSA	100 μg/mL	500 ng/mL	200x
CHIR 99021	DMSO	15 mM	3 μM	5,000x
FGF basic/FGF2/bFGF	$dPBS^{-/-}$ containing at least 0.1% (w/v) BSA	50 μg/mL	5 ng/mL	10,000x
VEGF-165	$dPBS^{-/-}$ containing at least 0.1% (w/v) BSA	100 μg/mL	10 ng/mL	10,000x
EGF	$dPBS^{-/-}$ containing at least 0.1% (w/v) BSA	200 μg/mL	20 ng/mL	10,000x
A 83-01	DMSO	10 mM	0.5 μM	20,000x
L-ascorbic acid	Distilled water	50 mg/mL	50 μg/mL	1,000x
Retinoic acid (RA)	DMSO	20 mM	2 μM	10,000x
Dex	Absolute ethanol	50 μM	100 nM	500x
OSM	$dPBS^{-/-}$	100 μg/mL	20 ng/mL	5,000x
HGF	$dPBS^{-/-}$ containing at least 0.1% (w/v) BSA	25 μg/mL	10 ng/mL	2,500x

2. Methods

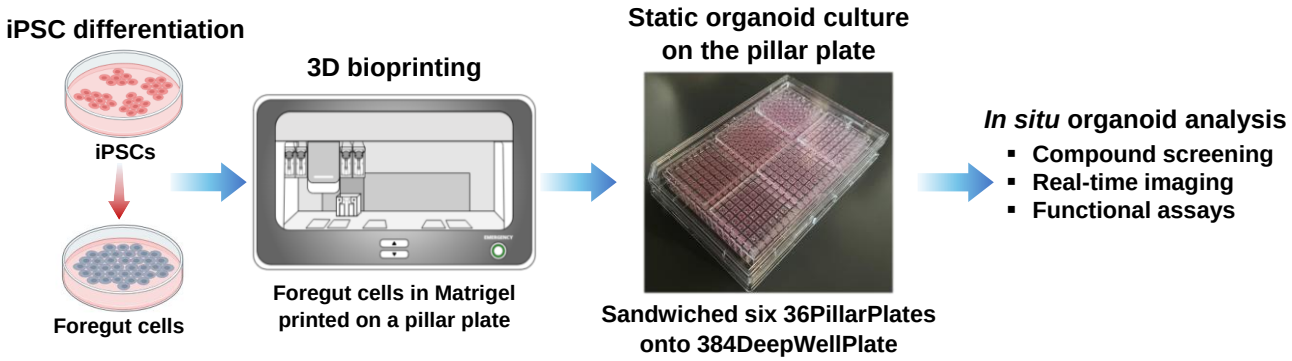

Fig. 1 Standard operating procedures (SOPs) for the scale-up production of human liver organoids (HLOs) using a pillar plate through 3D bioprinting of foregut cells in Matrigel and static culture in a 384DeepWellPlate.

2.1 Maintenance and passage of iPSCs

1. Thaw growth factor reduced (GFR) Matrigel overnight by incubating the vial in a refrigerator at 4°C.
2. Confirm the concentration of GFR Matrigel by checking the lot number of the Matrigel vial and prepare the aliquot of 0.5 mg GFR Matrigel using the following calculation:

$$\frac{0.5\ \text{mg}}{\text{Stock concentration of GFR Matrigel in mg/mL}} = \text{Volume (mL) for aliquote}$$

   Suspend each aliquot of 0.5 mg GFR Matrigel in 6 mL of cold advanced DMEM/F12 to coat 6 wells of a 6-well plate.
3. Dispense 1 mL of the diluted GFR Matrigel solution in cold advanced DMEM/F12 in each well of a 6-well plate and swirl the 6-well plate to cover the surface of each well with Matrigel evenly (**Fig. 2**). Incubate the 6-well plate with Matrigel at room temperature for at least 1 hour.
   Note: *Place the Matrigel solution on ice while handling. Make sure not to dry the Matrigel-coated wells. Matrigel-coated plates can be stored in a 4°C refrigerator for up to 1 week by wrapping it with parafilm, if not used immediately.*

Fig. 2 Coating of the 6-well plate with Matrigel for iPSC culture.

4. Prepare 50 mL of complete mTeSR plus medium by adding 10 mL of 5x supplement in 40 mL of mTeSR plus basal medium.

5. Thaw the frozen iPSCs quickly in a 37°C water bath until only a small ice crystal remains. Using a 1 mL pipette, slowly add the cells to 9 mL of complete mTeSR plus medium in a sterile 15 mL conical tube. Gently mix the cells with the medium and centrifuge the conical tube for 3 minutes at 200 rcf. Discard the supernatant and resuspend the cells in 2 mL of fresh mTeSR plus medium.
6. Remove the Matrigel solution from the 6-well plate by aspiration and then dispense 2 mL of the iPSC suspension into the Matrigel-coated wells.
   Note: *Typically, one cryovial of iPSCs is seeded into a single well of the Matrigel-coated 6-well plate.*
7. Gently rock the 6-well plate with iPSCs in all directions to ensure even distribution of the cells within the wells and then place the plate in a 5% CO_2 incubator at 37°C.
8. After a 1-day incubation, observe the plate under a bright-field microscope to confirm cell attachment on the surface. If minimal attachment is observed, continue the incubation for an additional day without changing the medium to allow more time for cell attachment.
9. After cell attachment, change the medium daily until the cells are ready for use or passage. Monitor colony formation under the microscope and remove any differentiated areas of iPSCs using a sterile cleaning tool (**Fig. 3**).
   Note: *The sterile cleaning tool can be prepared by bending a glass Pasteur pipette using an alcohol lamp.*

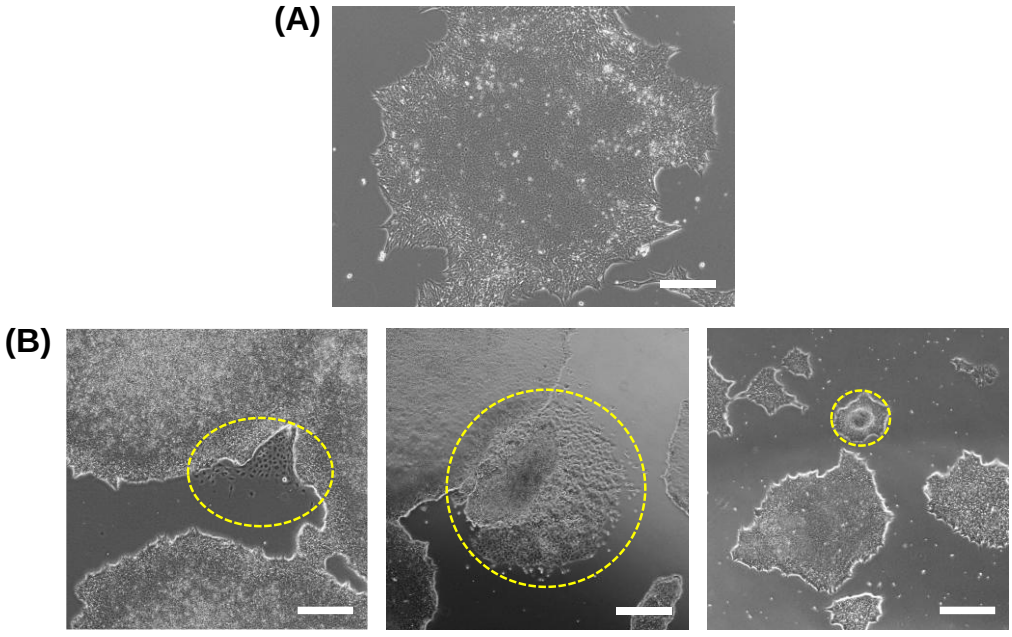

Fig. 3 Morphology of (A) an undifferentiated colony of iPSCs and (B) a differentiated colony of iPSCs, the latter encircled by a yellow dashed line. The differentiated iPSCs resemble pebbles, characterized by a dense structure and a dark color when observed under the microscope. Scale bars: 200 μm.

10. Before iPSC passage, prepare a new 6-well plate with Matrigel coating.
11. After removing differentiated iPSCs, passage iPSCs with either the EZPassage tool or the ReLeSR enzyme-free passaging reagent and seed the cell suspension in the new Matrigel-coated 6-well plate.

i. When passaging iPSCs using the EZPassage tool, first aspirate the spent medium and replace it with fresh mTeSR plus medium. Next, cut the iPSC colonies into small squares by rolling the EZPassage tool across the entire 6-well in one direction, applying uniform pressure. After rotating the 6-well plate 90 degrees, repeat the cutting process. Check the 6-well plate under a microscope to confirm that the colonies have been properly sectioned. Then, using a 5 mL serological pipette, aspirate the medium from the 6-well plate and gently scrape the surface to dislodge the colonies, slowly releasing the medium as you do so. Finally, transfer the cell suspension to a new Matrigel-coated 6-well plate containing 2 mL of complete mTeSR plus medium per well.

ii. When passaging iPSCs using ReLeSR, first remove the spent medium by aspiration and then add 1 mL of the ReLeSR reagent. After incubation for 30 - 45 seconds, aspirate the ReLeSR from the well plate and incubate the well plate for 4 - 5 minutes in a 5% CO_2 incubator at 37°C. Then, add 1 mL of complete mTeSR plus medium and pipette gently to dislodge the cells. Finally, transfer the cell suspension at a desired density (typically 100 µL of the cell suspension for a 1:10 split ratio) to a new Matrigel-coated 6-well plate containing 2 mL of complete mTeSR plus medium per well.

12. After a 1-day incubation, observe the plate under the microscope to confirm cell attachment on the surface.
13. After cell attachment, change the medium daily until the cells are ready for use or passage.
14. For cryopreservation, harvest the iPSC suspension using either the EZPassage tool or the ReLeSR reagent into a 15 mL conical tube and centrifuge the tube for 3 minutes at 200 rcf. Discard the supernatant and resuspend the cell pellet with 1 mL of CryoStor® cryoprotectant reagent. Transfer the cell suspension into a labeled cryovial, place the vial into a Mr. Frosty container, and store it in a -80°C freezer overnight (**Fig. 4**). Finally, transfer the cryovial containing the cell suspension into a liquid nitrogen dewar for long-term storage of the cells.

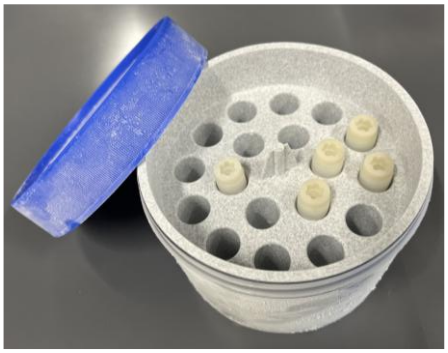

Fig. 4 Freezing cryovials containing iPSCs in the Mr. Frosty container in a - 80°C freezer.

2.2 Differentiation of iPSCs into foregut cells

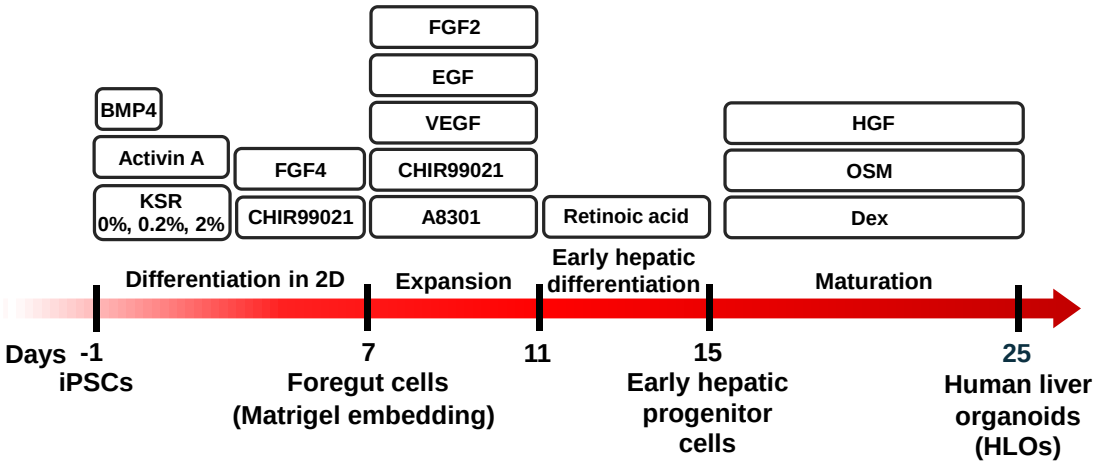

Fig. 5 The differentiation protocol of iPSCs into day 25 HLOs on a pillar plate, modified from the Ouchi protocol.

Day -1:

1. To coat a 6-well plate, use laminin iMatrix-511 silk to achieve a final concentration of 0.5 µg/cm^2. For example, to coat a single well in the 6-well plate with a surface area of 9.5 cm^2, dispense 990 µL of dPBS$^{-/-}$ and add 9.5 µL of iMatrix-511 silk, which equals 4.75 µg. Swirl the plate to ensure complete coverage with the iMatrix-511 silk. Then, place the well plate in a 5% CO_2 incubator at 37°C for 1 hour, at room temperature for 3 hours, or at 4°C for overnight for coating.
   Note: *An iMatrix-511 silk-coated 6-well plate can be stored for up to 2 weeks at 4°C. To prevent desiccation, it should be securely wrapped in parafilm. This method ensures that the plate remains hydrated and is ready for use without any compromise to its quality or integrity.*
2. Inspect iPSC colonies under a bright-field microscope and remove any differentiated iPSCs using a sterile cleaning tool (**Fig. 3**). Remove the spent medium, rinse the iPSCs with dPBS$^{-/-}$ once, and dispense 1 mL of Accutase into a single well of the 6-well plate. Incubate the plate in a 5% CO_2 incubator at 37°C for 5 minutes.
   Note: *For optimal outcomes, it is crucial to initiate the differentiation process with high-quality iPSCs. Therefore, the passage number of iPSCs should ideally range between 20 and 60. Additionally, it is important to begin cell passage at 70 - 80% confluency. This meticulous preparation is essential for successful iPSC differentiation.*
3. After iPSC detachment, transfer the cell suspension into a 15 mL conical tube containing 9 mL of complete mTeSR plus medium using a 1 mL pipette, centrifuge the tube at 200 rcf for 3 minutes, carefully discard the supernatant, and resuspend the cells in 1 mL of complete mTeSR plus medium.
4. Count the viable cells using trypan blue exclusion and calculate the number of viable cells per mL in the cell suspension. If necessary, supplement complete mTeSR plus medium with 10 µM Y27632. Seed the cells at a density of 1.3 x 10^6 cells per 2 mL of the cell suspension in each well of a 6-well plate.
   Note: *For each iPSC line, the seeding density needs to be experimentally optimized to achieve 90% confluency by day 1, which is typically 48 hours after seeding.*
5. Remove the iMatrix-511 silk solution from the 6-well plate and add 2 mL of the cell suspension in complete mTeSR plus medium containing 10 µM Y27632 in each well of the iMatrix-511 silk-coated 6-well plate. Gently swirl the plate in all directions at least three times to ensure an even distribution of cells.

6. Incubate the 6-well plate with iPSCs in the 5% CO_2 incubator at 37°C for 1 day.

Day 0:

1. Inspect the seeded cells under the microscope and check their health and growth conditions throughout the 6-well plate.
2. Warm the complete mTeSR plus medium to 37°C by placing it in a water bath.
3. Aspirate the spent mTeSR plus medium containing Y27632 and add 2 mL of fresh mTeSR plus medium without Y27632 per well in the 6-well plate.
4. Incubate the 6-well plate in the 5% CO_2 incubator at 37°C for 1 day.

Day 1:

1. Inspect the cells under the microscope to ensure that the cells reach 85 - 90% confluency (**Fig. 6**). If the cells don't reach the optimum confluency, replace the spent medium with 2 mL of fresh complete mTeSR plus medium and incubate them in the CO_2 incubator at 37°C for an additional day.
   Note: *Too low or too high cell confluency could result in complete cell death after activin A treatment, incomplete differentiation, and/or overgrowth of mesoderm.*
2. Warm the Day 1 medium to 37°C by placing it in a water bath.
3. Aspirate the spent mTeSR plus medium and add 2 mL of the Day 1 medium per well.
4. Incubate the 6-well plate in the 5% CO_2 incubator at 37°C for 1 day.

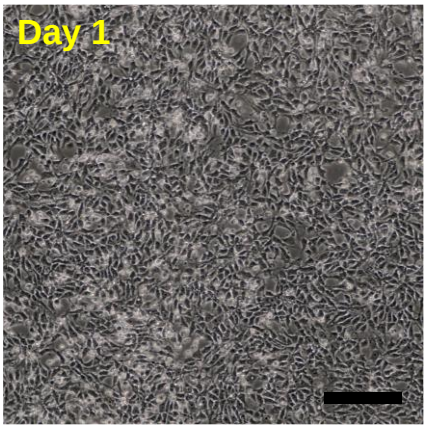

Fig. 6 Confluent iPSCs in an iMatrix-511 silk-coated well of the 6-well plate before differentiation. Scale bar: 200 µm.

Day 2:

1. Inspect the cells under the microscope to ensure cell detachment after incubation with the Day 1 medium.
   Note: *After incubation with the Day 1 medium, there will be cell death or floating cells in the medium. If there are little or no cell attachment, discard the 6-well plate and restart the cell differentiation process.*
2. Warm the Day 2 medium to 37°C by placing it in a water bath.
3. Aspirate the spent Day 1 medium and add 2 mL of the Day 2 medium per well.
4. Incubate the 6-well plate in the 5% CO_2 incubator at 37°C for 1 day.

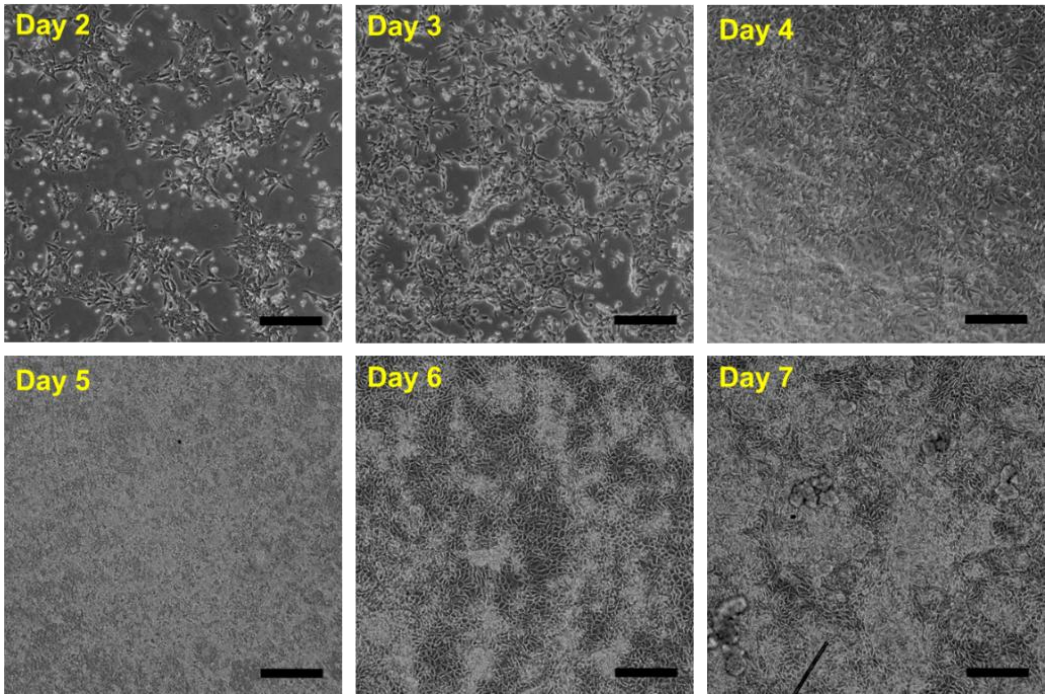

Fig. 7 Changes in cell morphology during iPSC differentiation into foregut cells in the laminin-coated 6-well plate. Scale bars: 200 μm.

Day 3:

1. Inspect the cells under the microscope and check their health and growth conditions throughout the 6-well plate.
   Note: *After incubation with the Day 2 medium, the cells should recover and proliferate rapidly.*
2. Warm the Day 3 medium to 37°C by placing it in a water bath.
3. Aspirate the spent Day 2 medium and add 2 mL of the Day 3 medium per well.
4. Incubate the 6-well plate in the 5% CO_2 incubator at 37°C for 1 day.

Days 4-6:

1. Inspect the cells under the microscope and check their health and growth conditions throughout the 6-well plate.
   Note: *On day 4, endoderm cells should be fully confluent, forming a uniform monolayer. If the cells are too dense or too sparse, differentiation efficiency will be low, resulting in a higher mesodermal population.*
   Checkpoint: *At this stage, perform immunofluorescence staining for definitive endoderm biomarkers such as Sox17 and FoxA2. Co-stain 85 - 90% confluent cells with Sox17 and FoxA2 antibodies. If 85 - 90% confluent cells are not co-stained for the biomarkers efficiently, discard the 6-well plate and restart the cell differentiation process.*
2. Each day, warm the Day 4-6 medium to 37°C by placing it in a water bath and replace the spent medium with 3 mL of fresh Day 4-6 medium per well. Confirm the formation of 3-dimensional (3D) structures from the monolayer of the cells, such as attached and floating spheroids, on days 5 and 6.

2.3 Microarray 3D bioprinting of day 7 foregut cells in Matrigel on the pillar plate

Day 7:

1. Inspect the cells under the microscope and check the formation of confluent cell monolayers with attached and floating spheroids.
   Checkpoint: *At this stage, perform immunofluorescence staining for foregut cell biomarkers such as FoxA2, FoxF1, and CDX2 with the following expected staining frequencies: FoxA2 (≥85%), FoxF1 (≥10-15%), and CDX2 (≥90%).*
2. Thaw aliquots of Matrigel on ice or in a 4°C refrigerator.
   Note: *To prepare Matrigel aliquots, thaw the whole bottle of Matrigel stock overnight in a 4°C refrigerator. Take 500 µL aliquots of Matrigel and store them in a - 20°C freezer for future use. Avoid repeated freezing and thawing of Matrigel.*
3. Gently dispense 80 µL of the Day 7-10 expansion medium per deep well in a 384DeepWellPlate to avoid big air bubble formation, cover it with a well plate lid, and place it in the 5% CO_2 incubator at 37°C for at least 1 hour to warm up the medium.
   Note: *It is critical to pop air bubbles entrapped in the 384DeepWellPlate after warming up the medium by gently tapping the plate on the surface of the biosafety cabinet. Any residual air bubbles in the deep wells will interfere with the visual inspection of cells and potentially inhibit cell growth.*
4. Hydrate the surface of the pillar plate by inserting two 36PillarPlates in the 36PetriLid on a 90 x 15 mm petri dish containing 1 mL of sterile, distilled water and placing it in the 5% CO_2 incubator at 37°C for 30 minutes (**Fig. 8**).
   Note: *Changing the surface of the pillar plate to hydrophilic by hydration in a humid environment is necessary to minimize air bubble entrapment on the pillars during cell loading in Matrigel.*

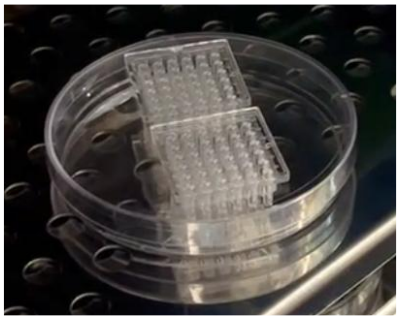

Fig. 8 Hydration of the surface of the pillar plate in a 90 x 15 mm petri dish with 1 mL of sterile, distilled water to minimize the entrapment of air bubbles.

5. Sequentially turn on the computer, the ASFA^{TM} cell spotter, the air compressor, and the chiller (**Fig. 9**).
   Note: *The air compressor is used to apply pressure for dispensing the cell suspension in Matrigel on the pillar plate. Typical air pressure is set to 40 psi. The chiller is used to maintain the deck temperature below 8°C and to prevent premature Matrigel gelation and water evaporation during printing.*
6. Run the ASFASpotter software to initialize the 3D bioprinter and set the temperature to 8°C in the Cooling unit window to prevent water evaporation during printing and to protect temperature-sensitive samples such as Matrigel.

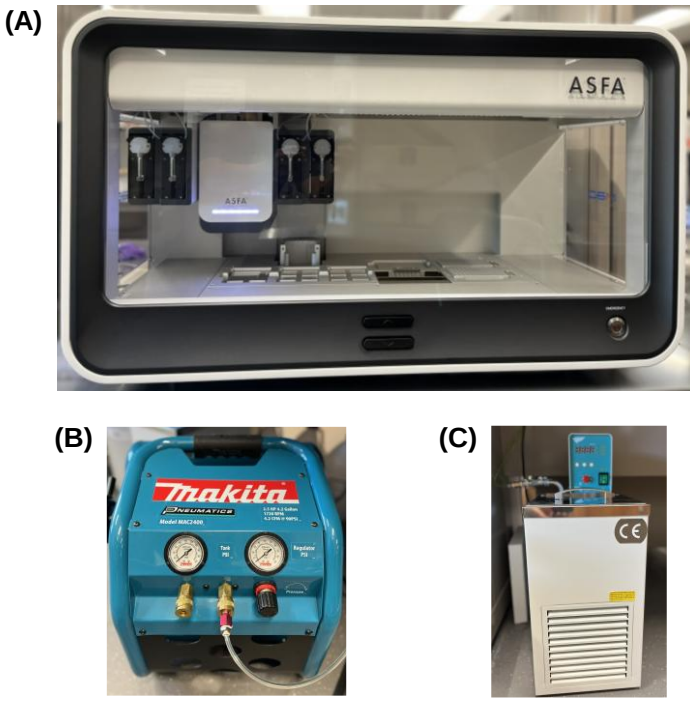

Fig. 9 (A) ASFA™ cell spotter, (B) Air compressor, and (C) Chiller.

7. On day 7, collect the spent medium containing floating spheroids in a sterile 15 mL conical tube. Wash the confluent cell monolayers with attached spheroids with $dPBS^{-/-}$ and collect it in the same 15 mL conical tube.
8. Dispense 1 mL of Accutase into each well of the 6-well plate and incubate the plate in the 5% CO_2 incubator at 37°C for 5 minutes to detach the cells. Inspect the plate under the microscope to ensure complete detachment of the cells. If the cells are not detached properly, incubate the plate for an additional 2 minutes.
   Note: *Do not incubate the cells in Accutase for longer than 10 minutes.*
9. Transfer the detached cells into the same 15 mL conical tube containing the previously collected floating cells. Add advanced DMEM/F12 basal medium to make a total volume of 10 mL. Gently mix the cells using a 1 mL pipette. Centrifuge the conical tube for 3 minutes at 300 rcf. Discard the supernatant and resuspend the cell pellet in 1 mL of advanced DMEM/F12 basal medium.
10. Count the viable cells with trypan blue exclusion and calculate the number of viable cells per mL in the cell suspension.
11. Take the aliquots of thawed Matrigel from a 4°C refrigerator and place them on ice in the biosafety cabinet.
    Note: *Matrigel should be chilled on ice during use to prevent premature gelation at temperatures above 10°C. If air bubbles are observed in thawed Matrigel, briefly centrifuge the aliquots at high speed to remove the entrapped air bubbles.*
12. Calculate the total number of cells required for seeding on the pillar plate at a density of 3,000 cells per pillar.
    Note: *Since 5 µL of the cell suspension in Matrigel is typically printed on each pillar at a density of 3,000 cells per pillar, the initial cell seeding density will be 600 cells/µL Matrigel.*

13. Based on the calculation, prepare 500 µL of the cell suspension at a density of 600,000 cells/mL by adding advanced DMEM/F12 medium. Gently mix the cell suspension using a 1 mL pipette. Centrifuge the tube for 3 minutes at 300 rcf. Discard the supernatant and resuspend the cell pellet with 500 µL of cold, undiluted Matrigel. Place the cells suspended in Matrigel on ice until use.
    Note: *Resuspend the cell pellet in Matrigel gently to avoid the formation of air bubbles. Use a 200 µL pipette to resuspend the cell pellet to better prevent air bubble formation. Any large air bubbles in Matrigel will interfere with the visual inspection of cells and potentially inhibit cell growth. It will require at least 200 µL of the cell suspension in Matrigel per 36PillarPlate.*
14. Select one of the printing programs designed for printing 5 µL of the cell suspension in Matrigel on the 36PillarPlate. Check the locations of the disposable tip on the source plate and the 36PillarPlate on the target plate.
    Note: *The disposable tip with an orifice of 400 µm diameter at the bottom is used for sample loading and dispensing. The critical parameters for printing the cell suspension in hydrogels are the open time (µs) of the solenoid valve and the pneumatic pressure (kPa) applied. To print a 5 µL droplet of the cell suspension in Matrigel, the open time and pressure are set to 13,800 µs and 6 kPa, respectively.*
15. Gently resuspend the foregut cells in Matrigel without introducing air bubbles and load at least 200 µL of the cell suspension per 36PillarPlate into the disposable tip.
    Note: *Maintain a homogeneous suspension of foregut cells in Matrigel before printing to ensure uniform cell loading on each pillar. Cells in Matrigel can settle within 5 - 10 minutes, leading to non-uniform cell printing on the pillar plate.*
16. Load the disposable tip with the cells suspended in Matrigel on the designated source plate location and the 36PillarPlate on the designated target plate location. Press the Start button to begin cell printing (**Fig. 10**).
    Note: *The sample volume required for printing is displayed on the screen and can be adjusted based on the volume of droplets and the number of pillars printed. It takes approximately 20 and 60 seconds to print the entire 36PillarPlate and 384PillarPlate, respectively. To avoid contamination, ensure to carry the pillar plate and the disposable tip in a sterile bioassay plate to the microarray spotter. If 3D bioprinters are not available, cells suspended in hydrogels can be loaded using the LoadingPlate (Bioprinting Laboratories Inc., Cat. no. 384-03-00). The video clip of the manual cell loading method can be found at https://youtu.be/JgQQ2lrfJX0. The video clip of the cell printing process can be found at https://youtu.be/0Tfmbh6fAEQ.*

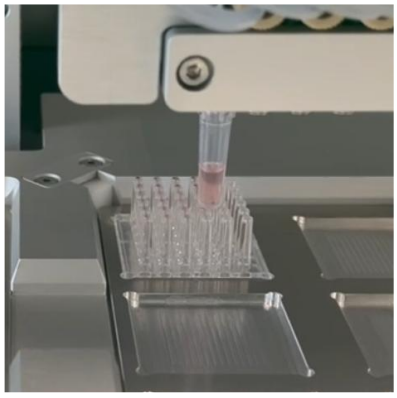

Fig. 10 Printing foregut cells suspended in Matrigel onto the 36PillarPlate using the microarray spotter.

17. After printing, insert the pillar plates into the 36PetriLid on the 90 x 15 mm petri dish containing 1 mL of sterile, distilled water and incubate in the 5% CO_2 incubator at 37°C for 10 - 12 minutes to allow Matrigel gelation (**Fig. 8**).
18. After gelation, sandwich the pillar plates within the 384DeepWellPlate filled with warm Day 7-11 expansion medium (**Fig. 11**).

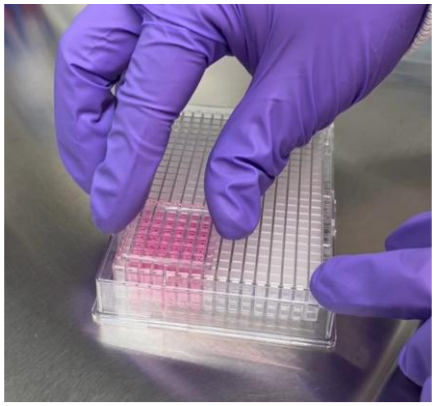

Fig. 11 Sandwiching the 36PillarPlate onto the 384DeepWellPlate containing the pre-warmed culture medium.

19. Inspect the pillar plate with bioprinted cells in Matrigel under the microscope and incubate the assembled plates in the CO_2 incubator at 37°C for 2 days.

2.4 Differentiation of foregut cells into HLOs on the pillar plate

Day 9:

1. Inspect the cells on the pillar plate under the microscope and confirm the formation of small spheroids within the Matrigel droplets.
2. Gently dispense 80 µL of the Day 7-10 expansion medium per deep well in a new 384DeepWellPlate to avoid big air bubble formation, cover it with a well plate lid, and place it in the 5% CO_2 incubator at 37°C for at least 1 hour to warm up the medium.
3. Gently tap the 384DeepWellPlate with the Day 7-10 expansion medium on the surface of the biosafety cabinet to remove big air bubbles entrapped in deep wells.
4. To replace the spent medium, separate the 36PillarPlate and sandwich it within the 384DeepWellPlate filled with warm, fresh Day 7-11 expansion medium.
   Note: *Unused deep wells in the 384DeepWellPlate can be used for dispensing fresh medium.*
5. Incubate the assembled plates in the CO_2 incubator at 37°C for 2 days.

Days 11-14:

1. On day 11, inspect the cells under the microscope and confirm the morphology of the spheroids.
2. Gently dispense 80 µL of the Day 11-14 retinoic acid medium per deep well in a new 384DeepWellPlate to avoid big air bubble formation, cover it with a well plate lid, and place it in the 5% CO_2 incubator at 37°C for at least 1 hour to warm up the medium.
3. Gently tap the 384DeepWellPlate with the Day 11-14 retinoic acid medium on the surface of the biosafety cabinet to remove big air bubbles entrapped in deep wells.

4. To replace the spent medium, separate the 36PillarPlate and sandwich it within the 384DeepWellPlate filled with warm, fresh Day 11-14 retinoic acid medium.
5. Incubate the assembled plates in the CO_2 incubator at 37°C for 2 days.
6. On day 13, repeat the steps 1 - 5.

Days 15-25:

1. On day 15, inspect the cells under the microscope and confirm the formation of liver organoids with mesenchymal cell aggregates.
2. Gently dispense 80 µL of the Day 15-25 maturation medium per deep well in a new 384DeepWellPlate to avoid big air bubble formation, cover it with a well plate lid, and place it in the 5% CO_2 incubator at 37°C for at least 1 hour to warm up the medium.
3. Gently tap the 384DeepWellPlate with the Day 15-25 maturation medium on the surface of the biosafety cabinet to remove big air bubbles entrapped in deep wells.
4. To replace the spent medium, separate the 36PillarPlate and sandwich it within the 384DeepWellPlate filled with warm, fresh Day 15-25 maturation medium.
5. Incubate the assembled plates in the CO_2 incubator at 37°C for 2 days.
6. On days 17, 19, 21, and 23, repeat the steps 1 - 5. The cells will be differentiated until day 25 to generate mature HLOs.

2.5 Assessment of HLO viability on the pillar plate

The CellTiter-Glo® 2.0 cell viability assay kit can be used to assess the viability of HLOs on the pillar plate before and after compound treatment. The cell viability assay kit allows to quantify the level of ATP in metabolically active cells. It is a ready-to-use reagent which has cell lytic capacity. The recommended storage condition is -30°C to -10°C up to the expiration date. Take 10 mL aliquots of the reagent to prevent multiple cycles of freezing and thawing.

1. Thaw one aliquot containing 10 mL of the CellTiter-Glo reagent at room temperature.
   Note: *It is crucial for the reagent to reach room temperature before use in the assay as it is temperature sensitive.*
2. Calculate the total volume of the reagent necessary in an opaque white 384-well plate for the 36PillarPlate.
   Note: *The volume of the reagent needed is 40 µL per well or 1,440 µL for 36 wells per 36PillarPlate.*
3. Mix the CellTiter-Glo reagent and advanced DMEM/F12 medium at a 3:1 ratio in a 15 mL conical tube.
4. Dispense 40 µL of the diluted reagent in each well of the opaque white 384-well plate using a multichannel pipette (**Fig. 12**).

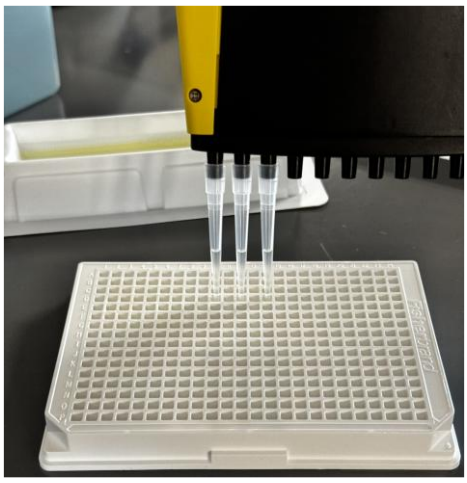

Fig. 12 Dispensing 40 µL of the diluted CellTiter-Glo reagent into the opaque white 384-well plate using a multichannel pipette.

5. Sandwich the 36PillarPlate with day 25 HLOs onto the opaque white 384-well plate containing the diluted CellTiter-Glo reagent and incubate it for 1 hour at RT on an orbital shaker at 80 rpm in the dark to ensure complete lysis of the organoids (**Fig. 13**).

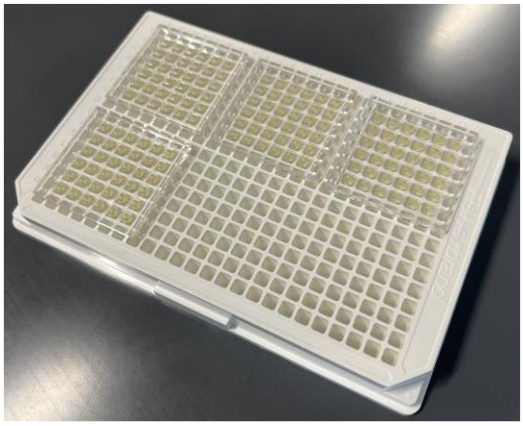

Fig. 13 Sandwiching the 36PillarPlate with HLOs onto the opaque white 384-well plate containing the diluted CellTiter-Glo reagent.

6. Separate the 36PillarPlate from the opaque white 384-well plate. Incubate the opaque white 384-well plate at room temperature for 15 - 20 minutes in the dark to stabilize the luminescence signal.
7. Record luminescence at an emission wavelength of 560 nm (gain at 135) from the opaque white 384-well plate using a microtiter well plate reader.

2.6 Immunofluorescence staining of whole HLOs on the pillar plate

The protocol provides step-by-step methods to stain whole organoids on the pillar plate without removing the cells. It is a high-throughput method of immunofluorescence staining of organoids.

The video clip of the immunofluorescence staining process can be found at https://youtu.be/tBGvF83wIew.

1. For rinsing, insert a 36PillarPlate with organoids in the 36PetriLid on a 90 x 15 mm petri dish containing 20 mL of 1x phosphate-buffered saline (PBS), and incubate for 10 minutes at room temperature (**Fig. 14**).
   Note: *Perform organoid rinsing in a static condition to avoid organoid detachment from the pillar plate. If the cells are encapsulated in alginate on the pillar plate, use Tris-buffered saline (TBS) instead of PBS due to alginate degradation in PBS.*

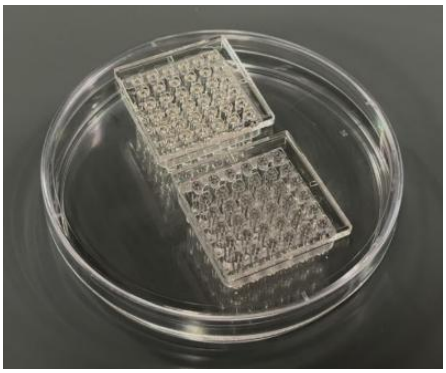

Fig. 14 The 36PillarPlate with organoids inserted in the 36PetriLid on a 90 x 15 mm petri dish containing phosphate-buffered saline (PBS) for rinsing.

2. For fixation, prepare a petri dish with 20 mL of 4% paraformaldehyde solution, insert the 36PillarPlate in the 36PetriLid on the petri dish containing 4% paraformaldehyde, and incubate for 2 hours at room temperature.
   Note: *Bring the 4% paraformaldehyde solution to room temperature for fixation as Matrigel might weaken at low temperature, resulting in spot detachment.*
3. Prepare a petri dish with 20 mL of 0.1% (w/v) sodium borohydride in PBS, insert the 36PillarPlate in the 36PetriLid on the petri dish containing 0.1% (w/v) sodium borohydride, and incubate for 15 minutes at room temperature. Repeat this step once.
   Note: *This step is important to reduce background due to residual aldehyde.*
4. For permeabilization, prepare a petri dish with 20 mL of 0.5% Triton X-100 in PBS, insert the 36PillarPlate in the 36PetriLid on the petri dish containing 0.5% Triton X-100, and incubate for 15 minutes at room temperature with rocking at 3 - 5° tilting angle and speed 5 (**Fig. 15**). Repeat this step twice to have a total permeabilization time of 45 minutes.

Fig. 15 Petri dish with the pillar plates placed on a low-speed rocker.

5. For blocking, prepare a petri dish with 5% normal donkey serum (NDS) diluted in 1x PBS with 0.25% Triton X-100 (blocking buffer), insert the 36PillarPlate in the 36PetriLid on the petri dish containing 5% NDS in the blocking buffer, and incubate 2 - 4 hours at room temperature.
   Note: *This step is critical to prevent non-specific binding of primary antibodies. If necessary, you can incubate it overnight at 4°C instead of 2 - 4 hours of incubation at room temperature.*
6. For primary antibody staining, dilute the primary antibody solution in the blocking buffer according to the manufacturer's recommendation.
7. Dispense 80 µL/well of the diluted primary antibody solutions in a 384DeepWellPlate, insert the 36PillarPlate in the 384DeepWellPlate containing primary antibodies, and incubate for 24 hours at 4°C on the rocker at 3 - 5° tilting angle and speed 5 (**Fig. 16**).

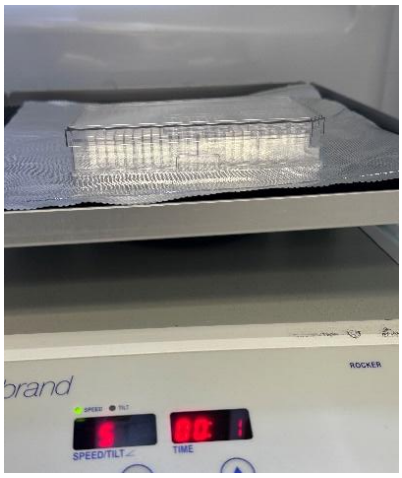

Fig. 16 The 384DeepWellPlate with primary antibody on the low-speed rocker at 4°C.

8. Separate the 36PillarPlate from the 384DeepWellPlate, insert it in the 36PetriLid on a petri dish containing 20 mL of the blocking buffer, and rinse the 36PilllarPlate for 20 minutes each at room temperature on the rocker. Repeat this step twice with fresh blocking buffer.
   Note: *This rinsing step is critical to remove unbound primary antibodies.*
9. For secondary antibody staining, dilute fluorophore-conjugated secondary antibodies 200-fold in the blocking buffer, dispense 80µL/well of the 200-fold diluted secondary antibody solutions in a 384DeepWellPlate, insert the 36PillarPlate in the 384DeepWellPlate containing secondary antibodies, and incubate for 2 - 4 hours at room temperature on the rocker.
10. Prepare a 384DeepWellPlate with 80 µL/well of 0.5 µg/mL DAPI in 1x PBS, insert the 36PillarPlate in the 384DeepWellPlate containing 0.5 µg/mL DAPI, and incubate for 25 minutes at room temperature with gentle rocking.
11. For rinsing, insert the 36PillarPlate with stained organoids in the 36PetriLid on a petri dish containing 20 mL of 1x PBS and incubate for 20 minutes at room temperature on the rocker. Repeat this step once.
12. For organoid clearing, dispense 30 µL/well of Visikol Histo-M or 1x RapiClear 1.52 in a regular 384-well plate, pre-warm the 384-well plate containing the clearing solution for 20 minutes at 37°C, insert the 36PillarPlate with stained organoids in the 384-well plate, and incubate for 1 hour at room temperature.
    Note: *The tissue clearing step is necessary to avoid light scattering of large organoids or spheroids and enable deep imaging of the large cell samples.*
13. For organoid imaging, separate the 36PillarPlate with stained and cleared organoids from the 384-well plate and attach the thin microscopic glass slide on the pillar plate (**Fig. 17**).

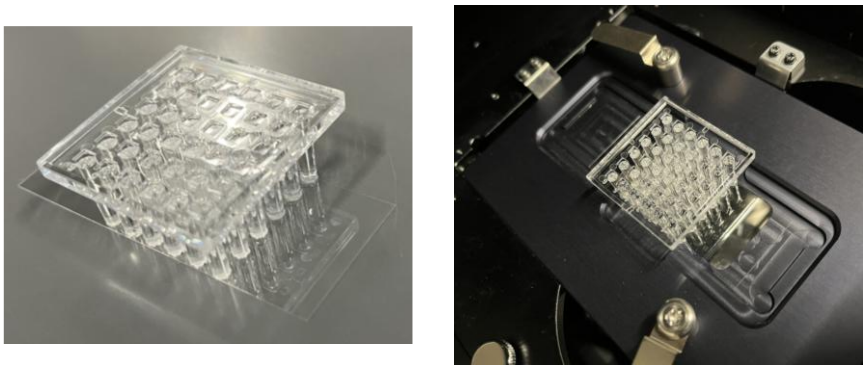

Fig. 17 The thin microscopic glass slide attached to the pillar plate with organoids.

14. Mount the 36PillarPlate with the thin microscopic glass slide and obtain fluorescence images using a confocal microscope (**Fig. 18**).

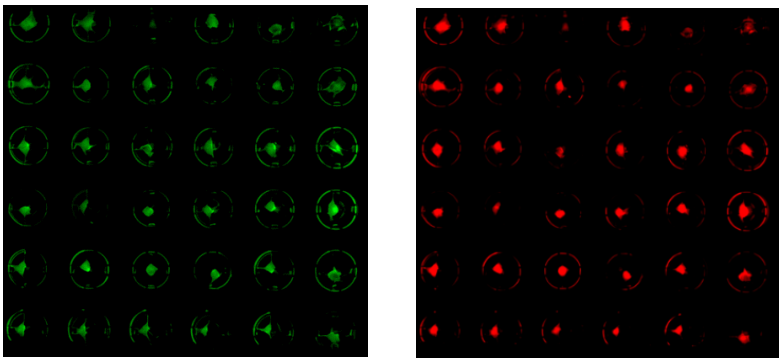

Fig. 18 Stitched images of organoids obtained from the 36PillarPlate.