Human HepatiCult™ Protocol Walkthrough

Lecture 3

Presenter

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Note: *The images in these slides depict representative morphologies. Users may see some variation in their own cultures compared to these images. Deviation from the morphology shown here does not necessarily indicate that an experiment will fail.*

Magnifications (2X, 4X, 10X, 40X) refer to the objective magnification used.

STEMCELL™ TECHNOLOGIES

Learning Objectives

After this session, you should be able to:

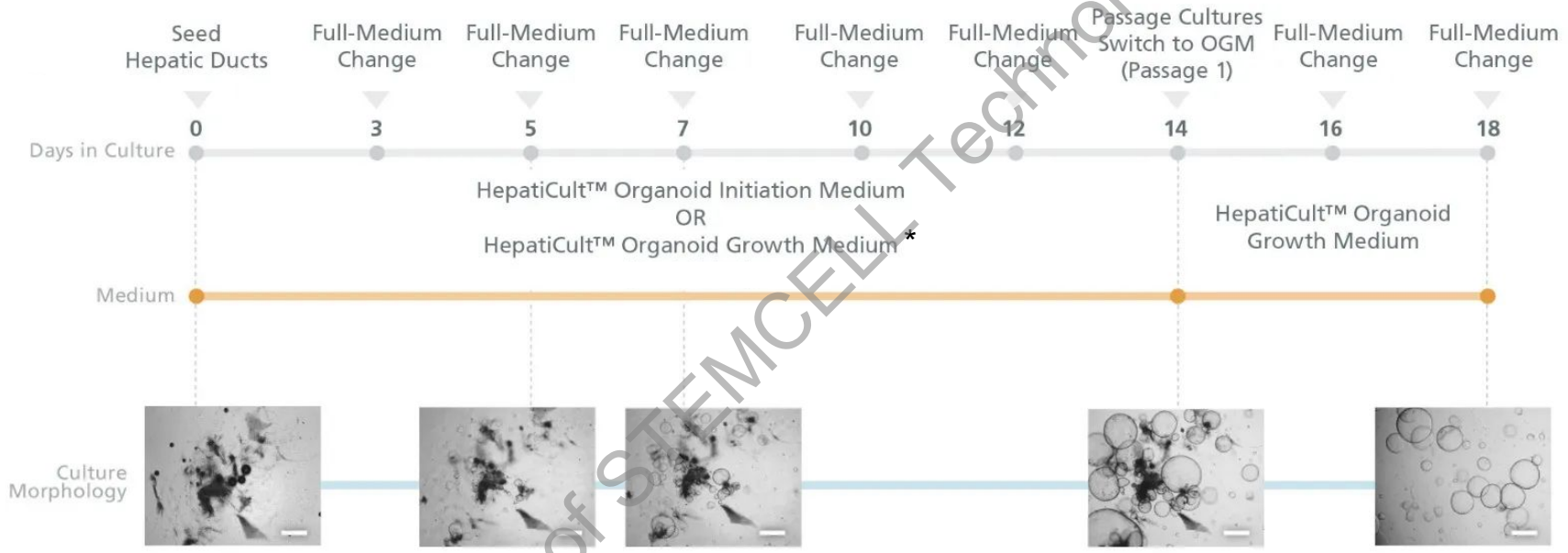
- Apply confidently the key techniques and methodological considerations for hepatic organoid cultures
- Describe morphological features of cultures through different stages of the workflow

Outline

1. Human HepatiCult™ Expansion Workflow
2. Human HepatiCult™ Differentiation Workflow

Section 1 | Human HepatiCult™ Expansion Workflow

HepatiCult™ Workflow: Initiation from Tissue-Derived Hepatic Ducts



*if looking for serum-free initiation

Matrix: Matrigel® GFR and Phenol Red-Free Basement Membrane Matrix, 10 mL, Corning® Cat: 356231

Media Preparation

Complete HepatiCult™ Organoid Initiation Medium (OIM)

- HepatiCult™ Organoid Basal Medium + Organoid Supplement + HepatiCult™ Organoid Growth Supplement + Y-27632 (final concentration 10 µM) + antibiotics (e.g. final concentration 50 µg/mL gentamicin)
- Thaw the supplements overnight (O/N) at 2 - 8°C. If not used immediately, aliquot and store at -20°C. After thawing, use immediately. Don't re-freeze
- Complete HepatiCult™ OIM can be stored at 2 - 8°C for up to 2 weeks
- Warm the complete media to room temperature (RT) before use

Complete HepatiCult™ Organoid Growth Medium (OGM)

- HepatiCult™ Organoid Basal Medium + HepatiCult™ Organoid Growth Supplement + antibiotics (e.g. final concentration 50 µg/mL gentamicin)
- Thaw the supplement O/N at 2 - 8°C. If not used immediately, aliquot and store at -20°C. After thawing, use immediately. Don't re-freeze
- Complete HepatiCult™ OGM can be stored at 2 - 8°C for up to 2 weeks
- Warm the complete media to room temperature (RT) before use

Tissue Sourcing for Liver

Considerations

- **Tissue Type - Normal vs Diseased**
  - Fresh, unprocessed, normal (ideally non-fibrotic/cirrhotic) liver tissue is preferred
  - Cadaveric tissue and biopsy samples/resections can be used for organoid establishment
  - If working with diseased samples, digestion protocol will likely be different depending on the biomechanical properties and cellular composition of the tissue
  - Some diseased samples might be easier to grow than others

- **Sources**
  - Researcher/institutional collaborations and affiliations
  - Biobanks or commercial sources (e.g. BioIVT)

- **Tissue Processing**
  - Tissue should be processed fresh, whenever possible
  - Tissue can be cryopreserved for a later use with CryoStor® CS10
  - Liver samples/biopsies are usually large, it may not be feasible to freeze down the entire section of tissue
    - Split up the tissue into small enough pieces for fitting into a cryovial for cryopreservation
  - Organoid yield is not correlated to tissue sample size - we have used tissue weighing <0.5g
  - Flash frozen tissue has not been tested

Hepatic Tissue Sources

	BioIVT	Discovery Life Sciences	US Biolabs Custom Collections	IIAM (Custom Request)	National Disease Research Interchange	ATCC
Normal Tissue	Yes	Yes	Yes	Yes	Yes	No
Cancer Tissue	Yes	Yes	Yes	Maybe	Yes	Yes
Other Diseases	No	No	• Alcoholic Steatohepatitis (ASH) • Liver Fibrosis • Nonalcoholic Steatohepatitis (NASH) • Liver Cirrhosis	• Nonalcoholic Steatohepatitis (NASH) • Hepatitis	Yes	No

Tissue Digestion

Before starting the digestion, prepare the following solutions:

a. Wash Solution = Fetal Bovine Serum (FBS) + DMEM/F-12 with 15 mM HEPES (*Store on ice)*

→ Washing and mincing tissue

b. Tissue Dissociation Cocktail = Collagenase Type IV + DNase I Solution (1 mg/mL) + DMEM/F-12 with 15 mM HEPES (*Warm to 37°C before use)*

→ Digest the tissue into ductal material DNase I = Break up the DNA from dead cells, makes samples less 'sticky'

c. TrypLE™ + BSA in water (*Store on ice)*

→ Hepatic ducts can be quite large in size, TrypLE™ breaks them into more manageable sizes (not necessarily single cells), BSA reduces stickiness

d. AdvDMEM + DNase I = DMEM/F-12 + BSA + DNase I Solution (1 mg/mL) + Y-27632 *(Store on ice)*

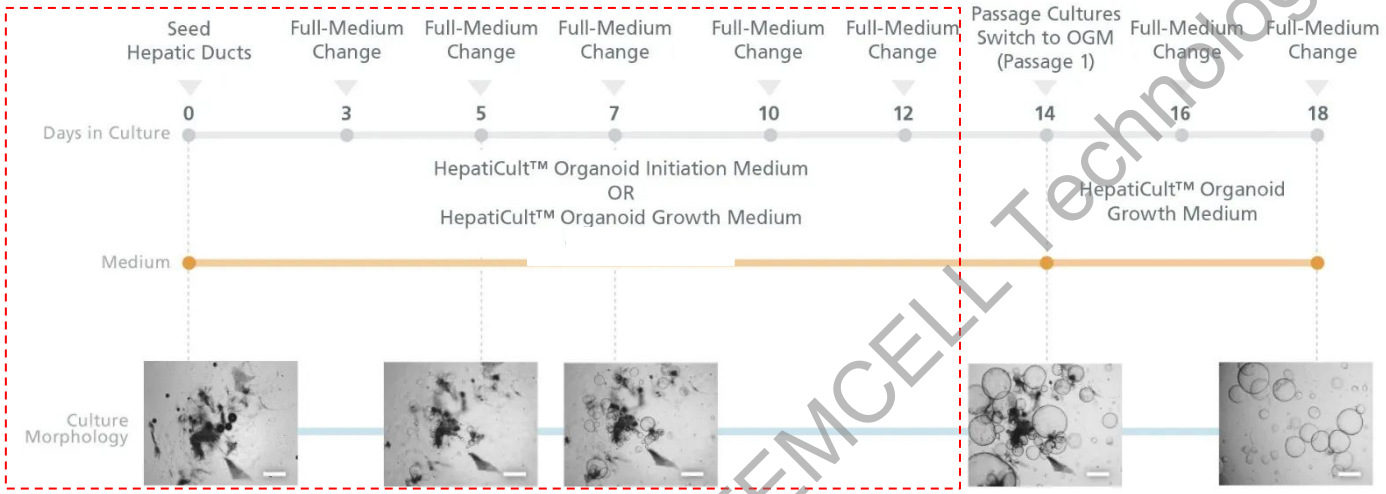
→ Dilutes the TrypLE™ enzymatic reaction, washing/resuspending pellet at the end of digestion

Critical Steps: Tissue Digestion

Key Points for Tissue Dissociation:

- Prior to enzymatic digestion → keep everything cold (on ice)
- Digestion → water bath (37°C)
  - Repeat the digestion steps (at least 4 times)
    - Assess the size of the pellet to decide if additional digestion might be required
    - If digestion is complete, there should be no tissue pieces, only white floating hepatic ducts (looks like fibres)
    - Repeat additional digestion cycle if tissue pieces still remain. Keep repeating the digestion until tissue pieces have been completely dissociated into hepatic ducts, and there are no visible tissue pieces
- Optional filtration step → using a 100 µm reversible cell strainer to separate the large ductal material from the smaller ductal material
- Optional ACK lysis step → if there is RBC contamination, a thin layer of RBCs is visible
  - If the pellet looks red, perform lysis. If the pellet looks clear, ACK lysis can be skipped
- After digestion is complete → keep ductal material cold (on ice)

Dome Culture Initiation: HepatiCult™ OIM or OGM



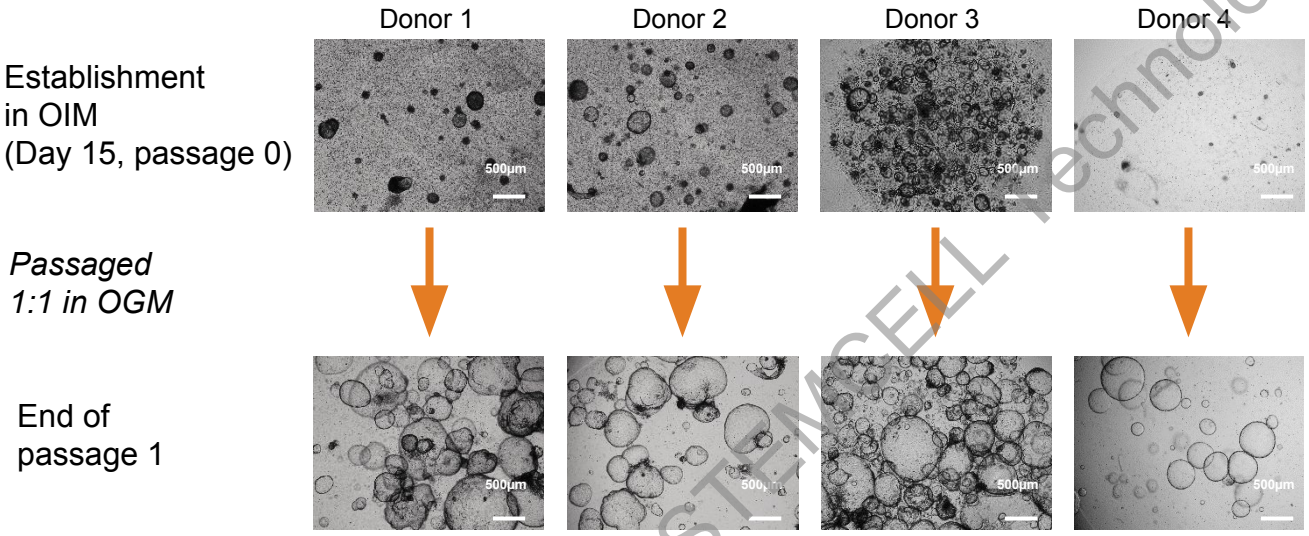
Important Points:

- Perform a **viable cell count** from the liver digestion
- Use **15 mL falcon tubes** to get a more "compact" pellet
- Leave 5 - 10 µL of supernatant behind to **avoid aspirating off the pellet**
- Important to keep the **pelleted cells/tube "cold"**
- Be careful to **not introduce bubbles** when resuspending pellet in Matrigel®
- Use **pre-cooled pipette tips** to minimize premature solidification of Matrigel®
- Only dispense to the first stop to **avoid bubbles** on the top of the dome.
- Don't pipette directly onto the domes when adding media
- Passage cultures before the lumen turns dark and organoids collapse (up to 2 weeks)

Before Passaging

- Place a 24-well tissue culture-treated plate in a 37°C incubator for at least 1 hour. Plates can be left overnight in a 37°C incubator
- Thaw ~40 µL of Corning® Matrigel® on ice for each dome to be seeded. Keep Matrigel® on ice when handling to prevent it from solidifying
- Prepare the complete OGM media as per the Technical Manual. If already prepared, leave an aliquot at RT
- Assess the morphology of hepatic organoids

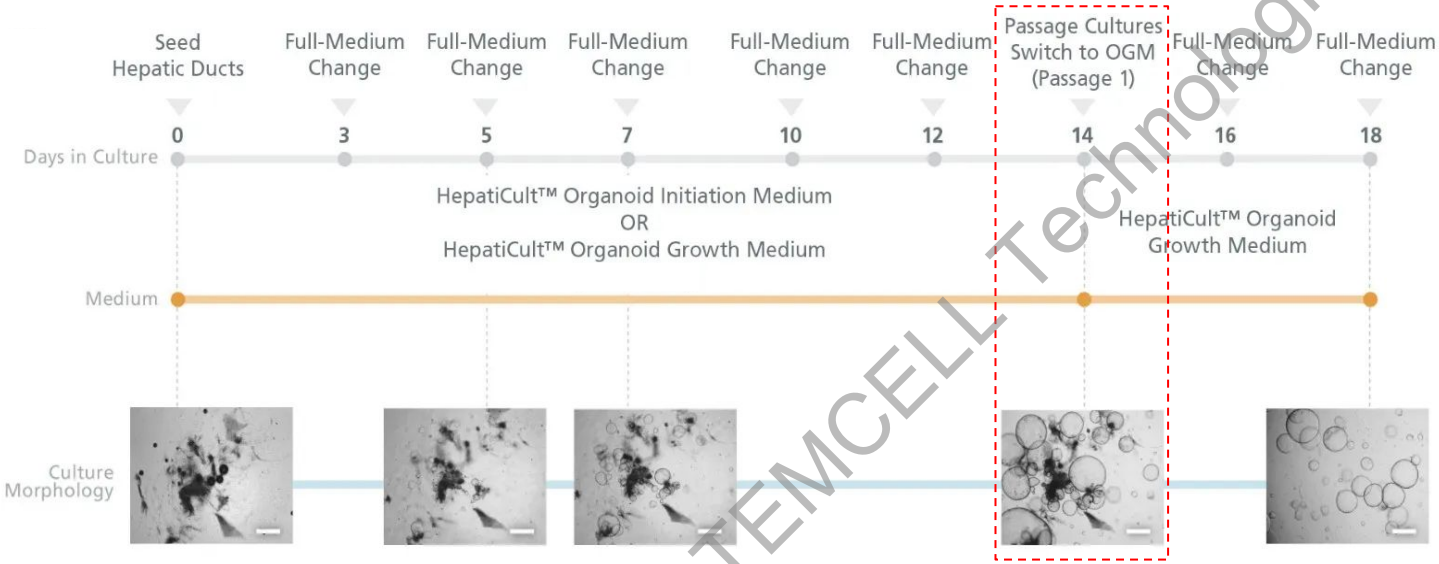
Hepatic Organoid Initiation: Morphology



Important Points:

- P0 organoid morphology can be heterogeneous
- Always passage to P1 using OGM - use 1:1 split ratio for sparse P0 wells
- 100% in-house success rate establishing organoids from fresh tissue or tissue frozen in cryoprotectant (n = 14)

Passaging: HepatiCult™ OGM

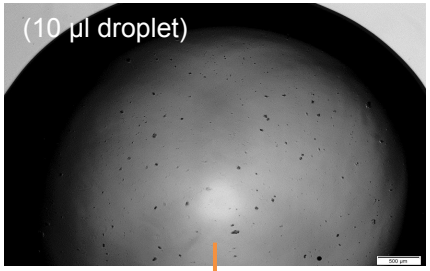


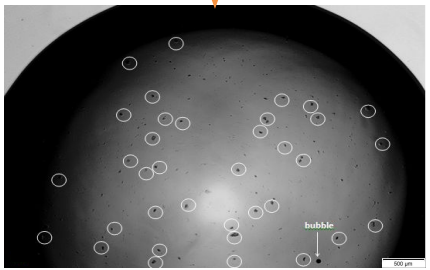
Important Points:

- Passaging involves mechanical trituration using **cold AdvDMEM + BSA**
- Ideally, aim for **30 - 100 µm fragments**
- **Single cell passaging possible** → not recommended for routine maintenance, end point assays only
- Check under the microscope, if fragments are larger than 100 µm, tritutate further (mechanical trituration)
- **Optimize seeding densities** per donor line
- Pellet is often not visible, and can be loose. Be careful when aspirating
- Monitor hepatic progenitor organoids daily; passage before the lumen turns dark and organoids collapse
- Cultures require passaging every 7 - 10 days

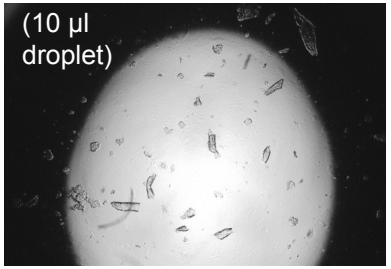
Optimal Hepatic Organoid Fragment Sizes for Passaging

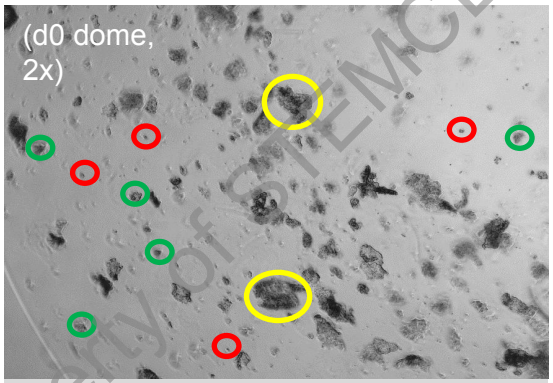
Recommended fragment sizes for passaging





Additional trituration recommended



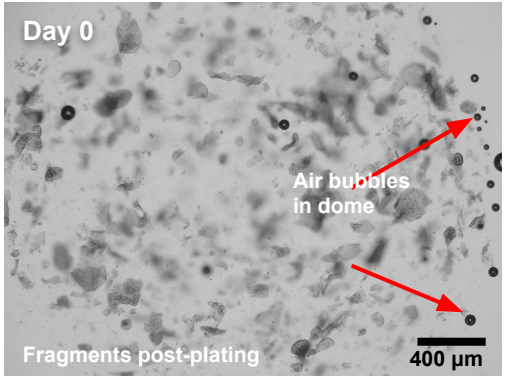


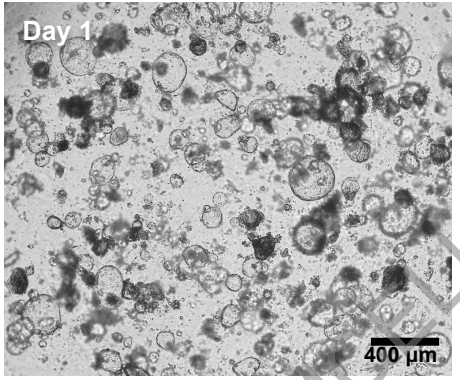
>100 µm, triturate further
<30 µm, usually single cells
30-100 µm, desired size

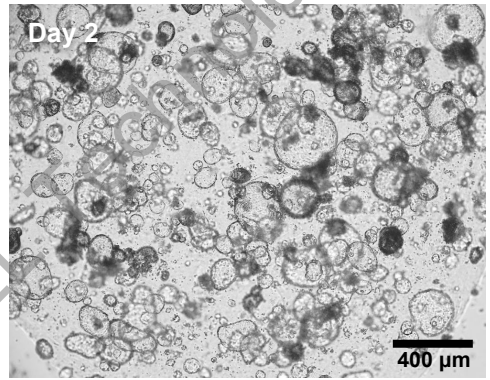
Fragment generation and counting

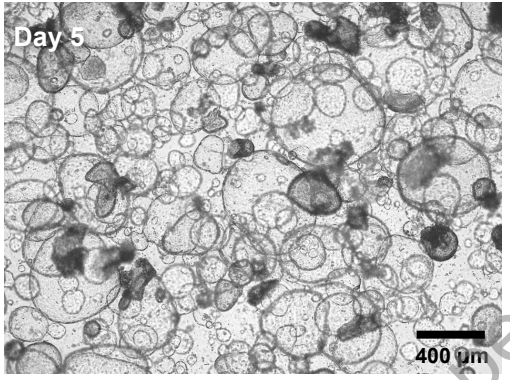
- Generate uniformly sized fragments (recommended = 30 - 100 µm)
- Use cell strainers to size-filter fragments
- Properly mix fragment suspensions before sampling - via stirring or gentle vortexing
- Dilute fragment suspensions for counts if needed - important for accurate counts
- Fragment size can influence organoid size and culture uniformity
    - Larger fragments will yield organoids earlier than smaller fragments/single cells

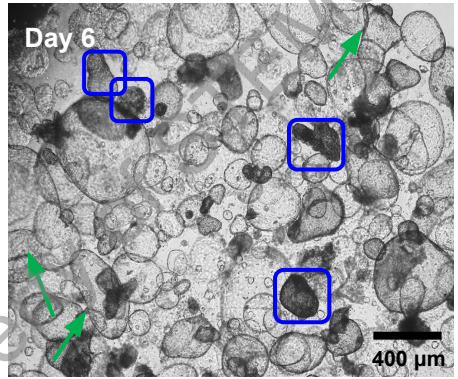
Representative Organoid Morphology: Post-thaw Time Course











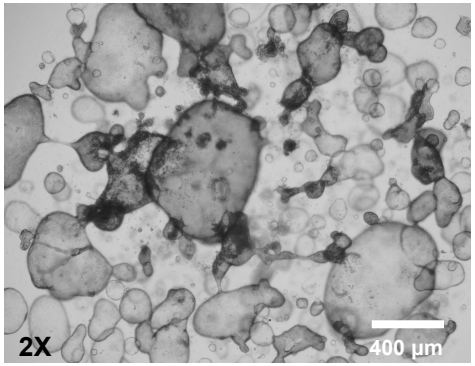
Cultures passaged again on Day 6

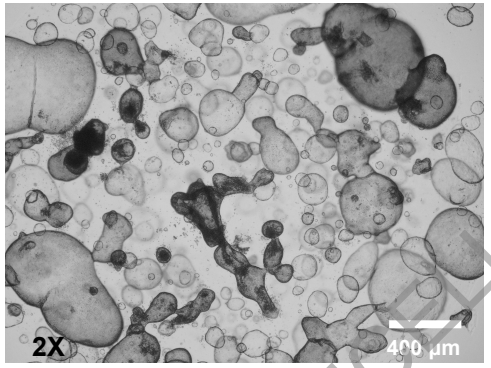
All images taken at 2X magnification

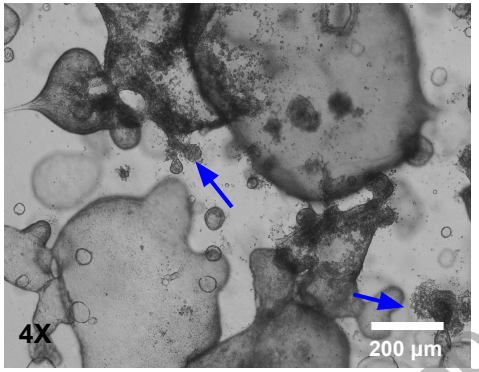
Important Points:

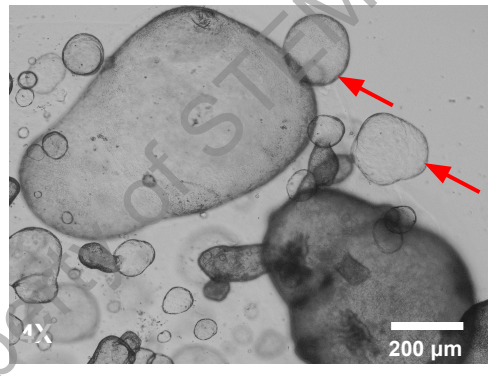
- Bubbles may form when seeding domes (red arrow), but should not impact organoid growth
- Average organoid size is influenced by the organoid density in the dome
- Later in the passage, organoids may collapse (blue box) and fuse (green arrow)
- Culture is ready to be passaged on Day 5 - 6 (the recommended density is 1000 fragments/well)

Representative Organoid Morphology: Day 8 in HepatiCult™ OGM









Single cells

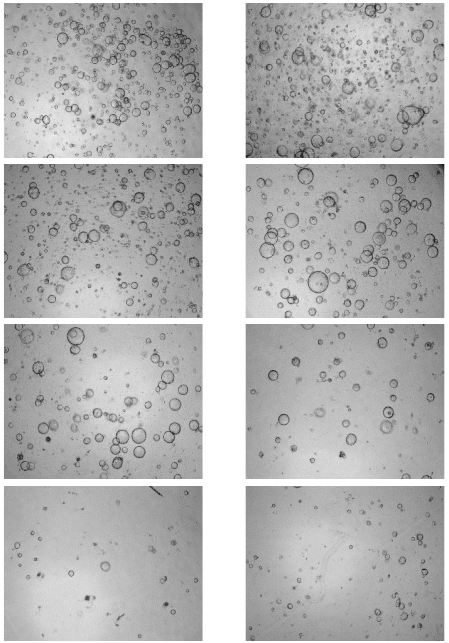
“Organoids” outside of the dome
“Large” organoids visible (overgrown)

Important Points:

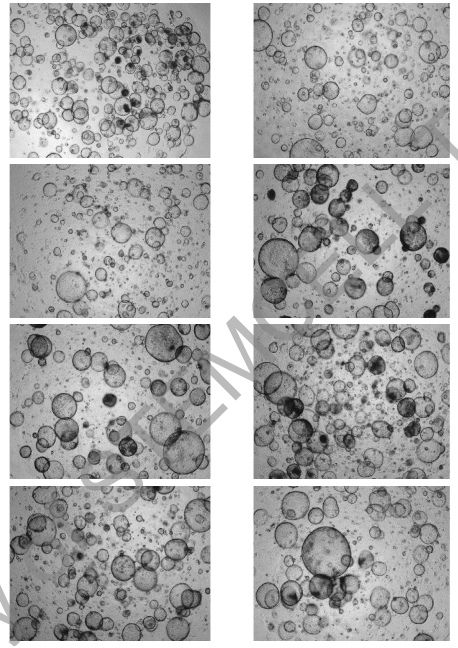
- Optimal passaging day can vary for donors and cultures - this culture should have been passaged 1-2 days earlier
- Average organoid size is influenced by the organoid density in the dome - larger organoid size likely related to a less densely seeded dome
- Organoids can ‘push out’ of the Matrigel® dome edges **(red arrows)**
- Collapsed organoids can ‘shed’ or extrude cells in addition to deflating **(blue arrows)**

Identifying When to Passage Hepatic Organoids

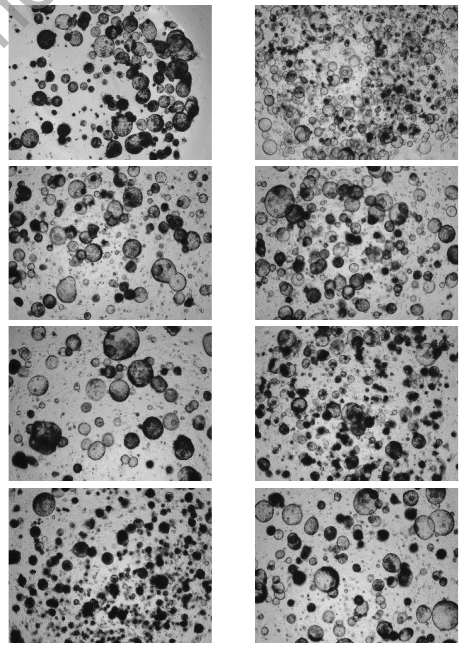
D1 - 4
Too early to passage



D4 - 8
Ready to passage



D7/8+
Should have been passaged 1-2 days earlier



When to passage

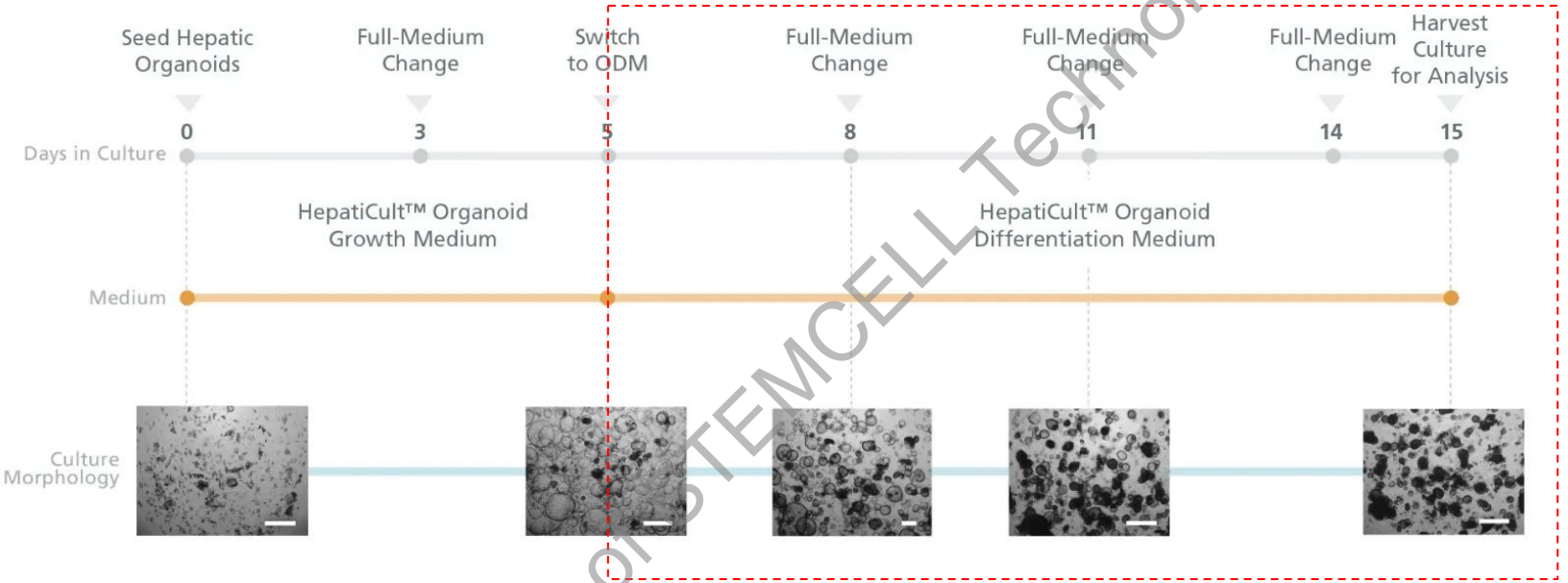
Monitor hepatic progenitor organoids daily; they should be passaged before the lumen turns dark and organoids collapse (generally 7 - 10 days)

Summary

- Hepatic progenitor organoids can be established from fresh or frozen liver tissues
- Hepatic organoids can be monitored daily; they should be passaged before the lumen turns dark and organoids collapse
- Cultures require passaging every 7 - 10 days
- Routine culture maintenance is recommended using fragment generation and counting (recommended = 30 - 100 µm)

Section 2 | Human HepatiCult™ Differentiation Workflow

HepatiCult™ Workflow: Differentiation



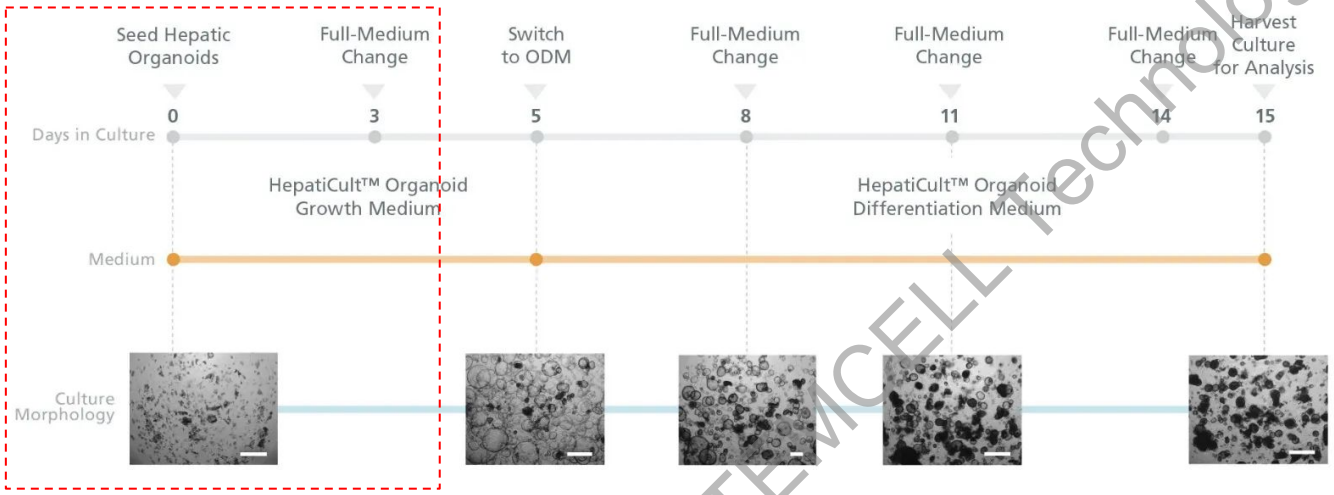
Matrix: Matrigel® GFR and Phenol Red-Free Basement Membrane Matrix, 10 mL, Corning® Cat: 356231

Media Preparation

Complete HepatiCult™ Organoid Differentiation Medium (ODM)

- HepatiCult™ Organoid Basal Medium + HepatiCult™ Organoid Differentiation Supplement + dexamethasone (final concentration 3 µM) + antibiotics (e.g. final concentration 50 µg/mL gentamicin)
- Thaw the supplement O/N at 2 - 8°C. If not used immediately, aliquot and store at -20°C. After thawing, use immediately. Don't re-freeze.
- Complete HepatiCult™ ODM can be stored at 2 - 8°C for up to 2 weeks
- Complete HepatiCult™ ODM is light-sensitive; minimize exposure to light
    - To minimize exposure to the light, on days when the medium is required, aliquot the required volume into a tube, and warm to RT before use
    - Minimize repeated exposure of the stock bottle to the light
    - The medium should ideally be warmed and used in a BSC without the lights on, and if being stored in a fridge that is opened frequently, wrapping in foil is a good suggestion

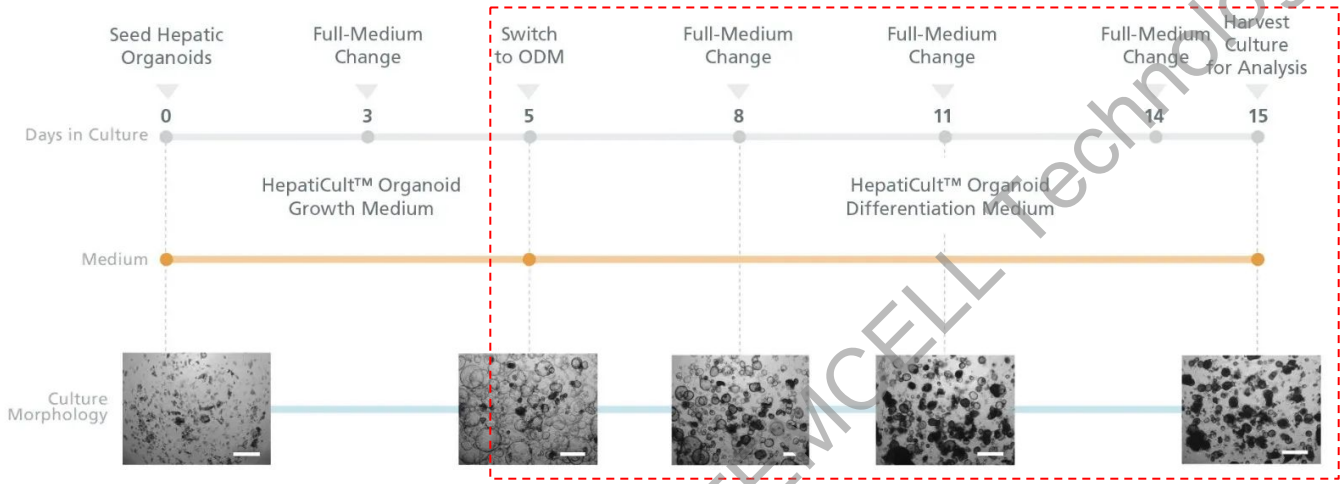
Passaging for Differentiation: HepatiCult™ OGM



Important Points:

- Perform at least one passage after initiating organoids
- For the fragment count, calculate the volume required to transfer 2000 fragments/well
- Seed additional domes in complete HepatiCult™ OGM for use as progenitor organoid controls/references in downstream analyses
- Perform a full media change on Day 3 with the complete HepatiCult™ OGM
- On Day 5 post-seeding, proceed with the differentiation step with HepatiCult™ ODM

Differentiation: HepatiCult™ ODM



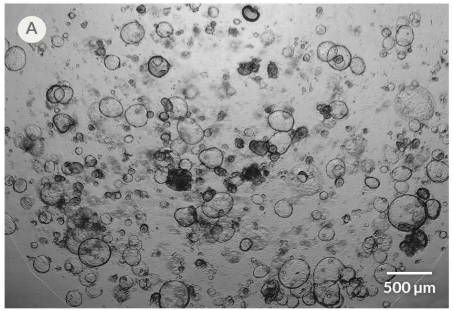
Important Points:

- Aliquot the required volume of ODM and warm to RT
- Perform complete media change on Day 5 post-seeding
  - Aspirate off OGM from well. Wash domes by adding 750 µl of RT DMEM/F-12 + 15 mM HEPES
- Perform full media change every 3 days using complete RT ODM
- Full media change is performed on Days 8, 11, and 14
  - For assays investigating secretory functions using the spent media, perform a final media change on Day 13 (i.e. at least 48 hours before final harvest on Day 15)
  - If domes are loose, perform a partial media change (e.g 500 µl instead of 750 µl)
- On Day 15, harvest/process domes for downstream characterization/functional assays
- Harvest not recommended later than Day 15

Representative Morphology: Differentiation Time Course

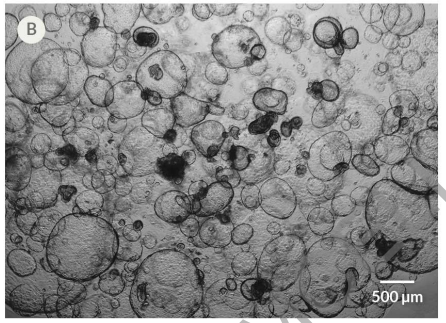
HepatiCult™ OGM

Day 2 (OGM)

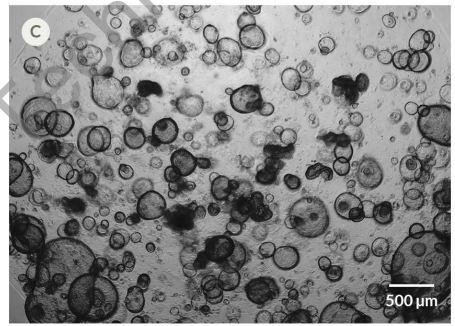


HepatiCult™ Differentiation Medium (ODM)

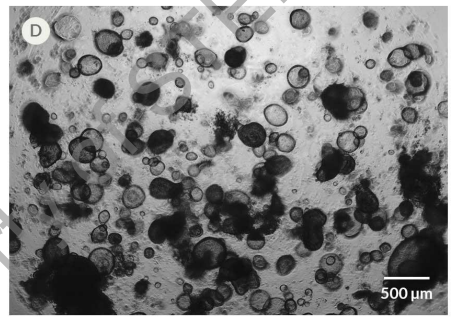
Day 5 (ODM Day 0)



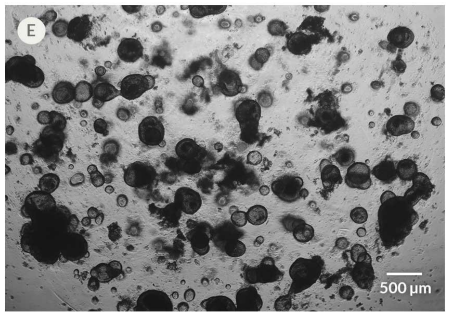
Day 7 (ODM Day 2)



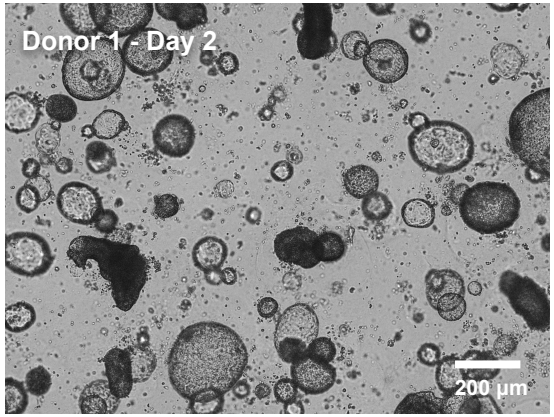
Day 10 (ODM Day 5)

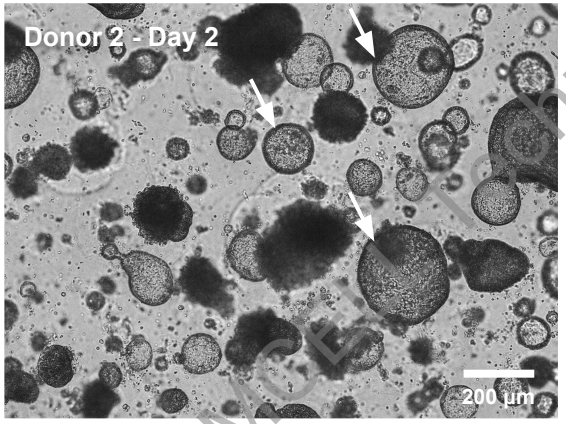


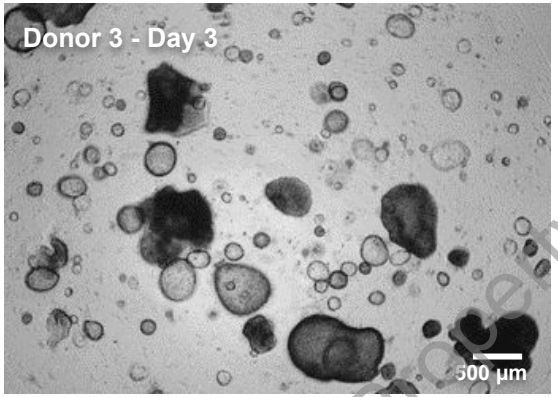
Day 15 (ODM Day 10)

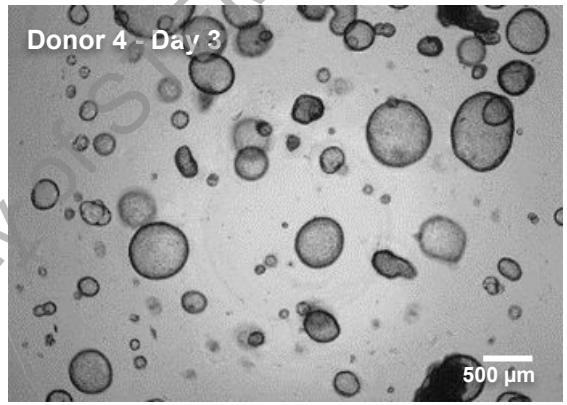


Representative Morphology: Differentiation Day 1- 3





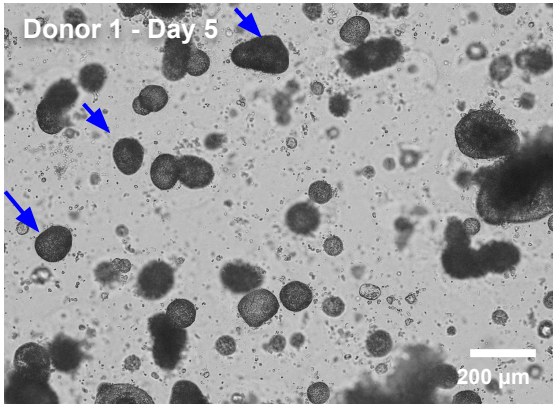


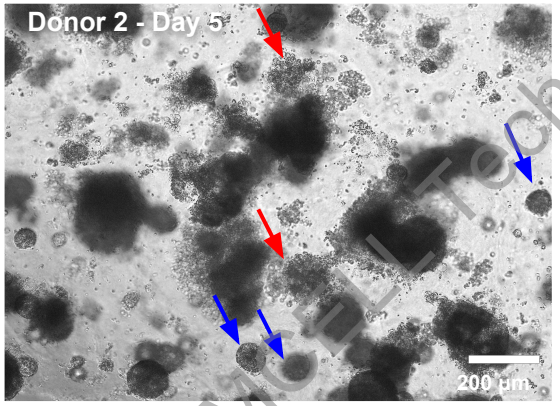


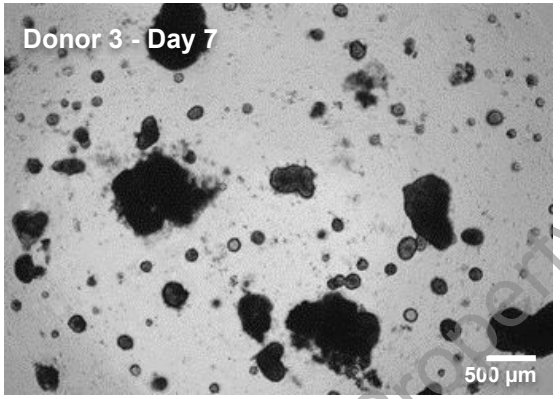
Important Points:

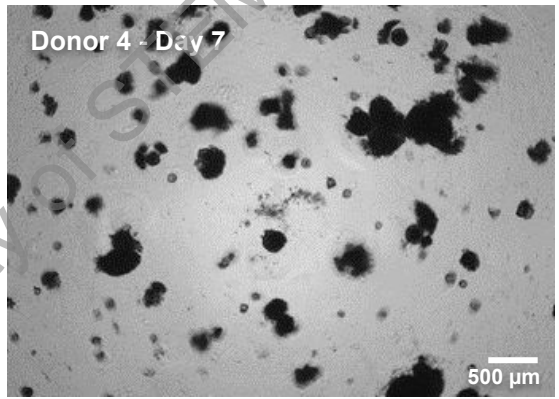
- Organoids begin to undergo morphology changes rapidly when differentiated
- Thickened epithelia (**white arrows**), compacted organoid size, and shift from cystic to dense morphologies are evident as early as differentiation day 1
- Onset and extent of morphological changes can be donor dependent

Representative Morphology: Differentiation Day 5 - 7









Important Points:

- Organoid morphologies continue to change over the course of the differentiation
- Thickened epithelia, compacted organoid size (**blue arrows**), and shift from cystic to dense morphologies are more evident
- Cell debris (**red arrow**) and organoids that appear collapsed are often observed and do not negatively affect differentiation outcome
- Differentiating organoid morphologies are heterogeneous and may vary by donor
- A further 3-5 days of differentiation is recommended to generate organoids with mature hepatic functionality

Differentiation Optimization

Seeding density:

- Recommended seeding densities to test: 1000 - 2500 fragments per dome
- Optimize organoid differentiation protocol parameters for every donor and assay of interest

Number of domes for differentiation assay:

- Recommended testing ranges: 3 - 6 wells per donor per differentiation
- Intracellular protein- and nucleic acid-based analyses require more differentiated organoid material
- Assays investigating enzymatic activity or secretory functions (spent media) can be performed on fewer wells
- Application dependent: Important to seed at appropriate density for desired downstream application

Duration of culture:

- Recommended testing range: Culture in complete HepatiCult™ OGM for 3 - 5 days
- Organoids should be differentiated once they are expanding, but before they start collapsing or deflating
- 14 - 15 days is the upper limit for differentiation duration

Differentiation Optimization - Dome Disintegration

Technical Matrigel® dome handling and media change tips:

- Ensure protein content of Matrigel® lots being used is ≥8 mg/mL
- Ensure plates are being sufficiently pre-warmed at 37°C
- Remove as much of the supernatant from pellet fragments as possible when seeding domes (i.e keep Matrigel® content as close to 100% as possible for dome stability)
- Ensure medium is warmed before performing media changes on the domes
- Ensure dome is not disturbed during media changes
    - Tilt the plate during media changes to visualize where the dome is
    - Perform slow media changes, removing and adding media to the wall of the well
    - For domes that look a little more loose, perform partial media changes, i.e. replace ~75% of the media in the well. Loose domes can still be used for analyses if media changes are performed carefully

Summary

- Protocol optimization recommended for every donor to determine:
  - Seeding densities for passaging
  - Passaging frequency before organoids collapse
  - Differentiation set-up
- A total of 1 - 2 passages is recommended prior to starting differentiation, functional applications and biobanking
- Donor-to-donor variability observed in functional assays - may be reflective of patient heterogeneity
  - Important to optimize experimental parameters for each donor and readout of interest

Thank You

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