TECHNICAL MANUAL

Generation of Human Pancreatic Organoids Using PancreaCult™ Organoid Medium Kits

STEMCELL™ TECHNOLOGIES

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1.0 Introduction

PancreaCult™ (Human) is a culture medium system optimized for the robust establishment, expansion, and long-term maintenance of pancreatic duct organoids from fresh and cryopreserved islet-depleted exocrine fractions as well as from dissociated tumor tissue.

PancreaCult™ Organoid Initiation Medium (OIM) provides support for the establishment of organoids from primary tissue. Subsequent maintenance, expansion, and cryopreservation is performed in serum-free PancreaCult™ Organoid Growth Medium (OGM). PancreaCult™ OGM also allows for the transient removal of epidermal growth factor (EGF) for the depletion of normal cells from K-Ras-activated pancreatic tumor tissue cultures.

Organoids established from normal tissue display a cystic morphology with hollow lumen and apical-basolateral orientation, and they maintain expression of several duct cell markers. Organoids cultured in PancreaCult™ OGM can be adapted to various culture protocols, including 2D Transwell® monolayer cultures, dilute Corning® Matrigel® suspension cultures, and high-throughput assay-compatible culture formats.

Applications of pancreatic duct organoid cultures include studying the development and function of duct epithelium, modeling pancreatic diseases such as cancer or cystic fibrosis, and performing targeted drug screens.

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2.0 Materials, Reagents, and Equipment

2.1 PancreaCult™ Organoid Media

The components listed below are sold as complete kits and are not available for individual sale.

Refer to the Product Information Sheet (PIS) for component storage and stability information; the PIS is available at www.stemcell.com, or contact us to request a copy.

PRODUCT NAME	CATALOG #	COMPONENT NAME	COMPONENT #	QUANTITY
PancreaCult™ Organoid Initiation Medium (Human)	100-0820	PancreaCult™ Organoid Basal Medium (Human)	100-0782	95 mL
		PancreaCult™ Organoid Growth Supplement (Human)	100-0783	5 mL
		Organoid Supplement	100-0191	50 mL
PancreaCult™ Organoid Growth Medium (Human)	100-0781	PancreaCult™ Organoid Basal Medium (Human)	100-0782	95 mL
		PancreaCult™ Organoid Growth Supplement (Human)	100-0783	5 mL

2.2 Additional Required Materials and Reagents

PRODUCT	CATALOG #
Human Recombinant EGF	78006.1
Prostaglandin E2	72192
Dimethyl sulfoxide (DMSO)	---
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free, LDEV-Free (≥ 8 mg/mL protein recommended)	Corning 356231
Y-27632 (Dihydrochloride)	72302
Tissue culture-treated flat-bottom plate	e.g. 38017 (24 wells)
Bovine serum albumin (BSA)	e.g. 100-0175
DMEM/F-12 with 15 mM HEPES	36254
D-PBS (Without Ca++ and Mg++)	37350
Conical tubes, 15 mL and 50 mL	e.g. 38009 and 38010
Optional: Gentamicin	---
For cryopreservation: CryoStor® CS10	07930

For a complete list of products available from STEMCELL Technologies Inc., visit www.stemcell.com.

2.3 Equipment

- Biosafety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Low-speed centrifuge with a swinging bucket rotor
- Pipette-Aid with appropriate serological pipettes (e.g. Catalog #38002)
- Sterile filtration unit
- Hemocytometer or automated cell counter
- Pipettor with appropriate tips (e.g. Catalog #38059)
- Inverted microscope
- Freezing containers
- -150°C freezer or liquid nitrogen (LN_2) vapor tank
- -80°C freezer
- -20°C freezer
- Refrigerator (2 - 8°C)

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3.0 Preparation of Reagents and Media

3.1 Epidermal Growth Factor (EGF) (100 µg/mL)

1. Briefly centrifuge vial of lyophilized EGF.
2. Add 100 µg EGF to 200 µL sterile distilled water to achieve a 500 µg/mL stock solution. Mix thoroughly.

   Note: If not used immediately, store at -20°C for up to 6 months.
3. Dilute the 500 µg/mL stock solution 1 in 5 to prepare a 100 µg/mL (2000X) solution.

   Note: If not used immediately, aliquot and store at -20°C. After thawing aliquots, use immediately; do not re-freeze.

   Note: Pancreatic ductal adenocarcinoma (PDAC) cells with KRAS-activating mutations are able to maintain EGF-independent growth. Selective removal of EGF from the medium can thus be used to deplete contaminating normal cells from the majority of PDAC cultures. For this, we recommend expanding and banking established PDAC cultures in PancreaCult™ OGM with EGF followed by three passages in PancreaCult™ OGM without EGF. While PDAC cells can maintain growth in the absence of EGF, removal of EGF might result in overall slower growth kinetics.

3.2 Prostaglandin E2 (PGE2) (1 mM)

Reconstitute 1 mg PGE2 in 3.077 mL sterile DMSO to prepare a 1 mM (1000X) solution. Mix thoroughly.

Note: If not used immediately, aliquot and store at -20°C. After thawing aliquots, use immediately; re-freeze once only. PGE2 is a cytotoxic compound and should be handled according to your institution's procedures and disposal regulations.

3.3 Wash Medium

1. For 500 mL Wash Medium, dissolve 5 g lyophilized BSA in 500 mL DMEM/F-12 with 15 mM HEPES. Mix thoroughly.
2. Sterile-filter the solution.

   Note: If not used immediately, store at 2 - 8°C for up to 1 month.

3.4 PancreaCult™ Organoid Initiation Medium (OIM)

Use sterile technique to prepare PancreaCult™ Organoid Initiation Medium (OIM) (PancreaCult™ Organoid Basal Medium + PancreaCult™ Organoid Growth Supplement + Organoid Supplement + EGF). The following example is for preparing 100 mL of medium. If preparing other volumes, adjust accordingly.

Note: PGE2 and Y-27632 are added to PancreaCult™ OIM immediately before use, as indicated in the protocols.

1. Thaw PancreaCult™ Growth Supplement and Organoid Supplement at 2 - 8°C overnight or on ice.
2. Prepare EGF (100 µg/mL; section 3.1) solution.
3. Using a serological pipette, remove 10 mL Organoid Basal Medium from the bottle.
4. Thoroughly mix PancreaCult™ Growth Supplement and add the entire volume (5 mL) to the Basal Medium bottle.
5. Thoroughly mix Organoid Supplement and add 10 mL to the Basal Medium bottle.

   Note: Aliquot remaining supplement and store at -20°C. Do not exceed the expiry date as indicated on the label. After thawing aliquots, use immediately; do not re-freeze.
6. Add 50 µL of 100 µg/mL EGF to the Basal Medium bottle.

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7. **Optional:** We recommend adding gentamicin (50 µg/mL final concentration) for the first 7 days of culture when establishing cultures with primary tissue. Gentamicin can also be added to medium aliquots prior to use, as needed.
8. Mix thoroughly.

   Note: If not used immediately, store at 2 - 8°C for up to 3 weeks. Aliquot an appropriate amount of medium and warm to room temperature before use. Avoid repeated warming of the medium. Alternatively, aliquot and store at -20°C for up to 9 months. Do not exceed the shelf life of the individual components. After thawing aliquots, use immediately; do not re-freeze.
9. Immediately before use, add 100 µL of 1 mM PGE2. Mix thoroughly.

3.5 PancreaCult™ Organoid Growth Medium (OGM)

Use sterile technique to prepare PancreaCult™ Organoid Growth Medium (OGM) (PancreaCult™ Organoid Basal Medium + PancreaCult™ Organoid Growth Supplement + EGF + PGE2). The following example is for preparing 100 mL of complete medium. If preparing other volumes, adjust accordingly.

1. Thaw PancreaCult™ Growth Supplement at 2 - 8°C overnight or on ice.
2. Prepare EGF (100 µg/mL; section 3.1) solution.
3. Thoroughly mix PancreaCult™ Growth Supplement and add to the Basal Medium bottle.
4. Add 50 µL of 100 µg/mL EGF to the Basal Medium bottle.

   Note: Pancreatic cancer cultures that carry K-Ras activating mutations can maintain growth in the absence of EGF. EGF removal can therefore be used to deplete normal epithelial duct cells from the culture. For this, we recommend first expanding and banking organoids using PancreaCult™ OGM with EGF as described. A subset of dome cultures can then be used to perform EGF removal for 3 passages. EGF supplementation can support faster growth of cancer cultures and can be re-supplemented after the depletion step for growth support.
5. **Optional:** We recommend adding gentamicin (50 µg/mL final concentration) for the first 7 days of culture when establishing cultures with primary tissue. Gentamicin can also be added to medium aliquots prior to use, as needed.
6. Mix thoroughly.

   Note: If not used immediately, store at 2 - 8°C for up to 3 weeks. Aliquot an appropriate amount of medium and warm to room temperature before use. Avoid repeated warming of complete medium. Alternatively, aliquot and store at -20°C for up to 9 months. Do not exceed the shelf life of the individual components. After thawing aliquots, use immediately; do not re-freeze.
7. Immediately before use, add 100 µL of 1 mM PGE2. Mix thoroughly.

Tip: When working with smaller experimental setups, PancreaCult™ OGM for organoid expansion can also be prepared using components from the OIM kit. Refer to Table 1 for a medium preparation example that will yield sufficient PancreaCult™ OIM to seed 8 starting domes, perform a medium change to PancreaCult™ OGM after day 3, then passage and expand domes in p1.

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Table 1. Preparation of PancreaCult™ OIM and OGM for Small Experimental Setups

COMPONENT NAME	PancreaCult™ OIM + PGE2 + Y-27632 (5 mL)	PancreaCult™ OGM + PGE2 (90 mL)
PancreaCult™ Organoid Basal Medium (Human)	4.2 mL	85.4 mL
PancreaCult™ Organoid Growth Supplement (Human)	0.25 mL	4.5 mL
Organoid Supplement	0.5 mL	---
EGF (100 µg/mL)	2.5 µL	45 µL
Gentamicin (optional) (50 mg/mL)	5 µL	90 µL
PGE2 (1 mM) (immediately before use)	5 µL	90 µL
Y-27632 (10 mM) (immediately before use)	5 µL	---

4.0 Protocol Diagrams

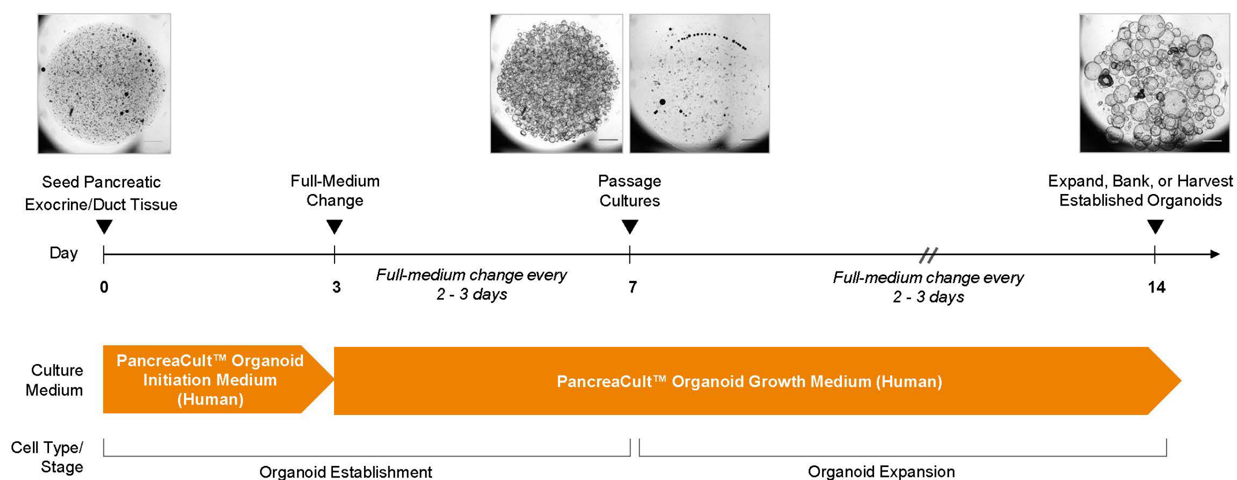

Figure 1. Establishment of Organoids from Digested Primary Tissue

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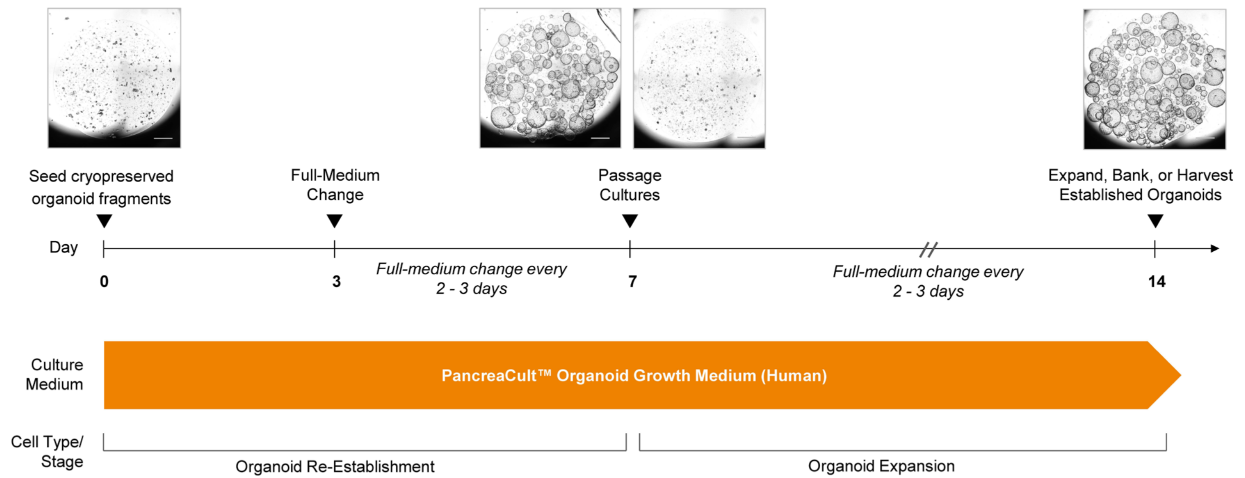

Figure 2. Re-Establishment of Organoids from Cryopreserved Fragments

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5.0 Organoid Establishment from Islet-Depleted Exocrine Fractions

Refer to Figure 1 for the protocol diagram. For **seeding and cryopreserving fresh tissue**, proceed to section 5.1. For **thawing and seeding cryopreserved tissue**, proceed to section 5.2.

5.1 Seeding and Cryopreserving Fresh Tissue

1. On the day before tissue delivery, place 24-well plates into the 37°C incubator.

   Note: Warming the plate for < 18 - 24 hours can result in spreading/flattening of Matrigel® domes. Tissue culture-treated Costar® plates are recommended.
2. On the day of sample delivery, prepare Wash Medium (section 3.3), PancreaCult™ OIM (section 3.4), 1 mM PGE2 stock solution (section 3.2), and 10 mM Y-27632 stock solution, or thaw pre-prepared aliquots.

   Note: It is strongly recommended to add gentamicin (50 µg/mL final concentration) to primary tissue culture during the first 7 days to avoid contamination.
3. Upon delivery of the sample, place the tube containing the cell suspension on ice and let tissue settle.
4. Record all information about the specimen, including, but not limited to, estimated volume of cell pellet, date of procedure, providing institution, donor, and institution ID.
5. Carefully mix the cell suspension thoroughly with a 25 mL serological pipette, then divide the cell suspension into two 50 mL conical tubes.
6. Centrifuge tubes at 200 x *g* for 5 minutes at 2 - 8°C.
7. With a 10 mL serological pipette, carefully remove the supernatant without disturbing the pellets.
8. Resuspend each pellet with 20 mL of Wash Medium.
9. For each tube, divide 20 mL of cell suspension equally between two 15 mL conical tubes (total of four tubes with 10 mL/tube).
10. Centrifuge tubes at 200 x *g* for 5 minutes at 2 - 8°C. Remove and discard supernatant.
11. Wash each pellet twice more using 10 mL Wash Medium with each wash, making sure to break up the pellets by trituration (approximately 15 times in total).
12. Remove and discard supernatant from all four tubes.
13. When seeding fresh tissue directly into domes, process three pellets from step 12 for cryostorage (steps 14 - 15), then proceed to step 16 using the remaining pellet; otherwise, proceed to step 14 for cryostorage of all four pellets.
14. Resuspend each pellet in 7.5 mL CryoStor® CS10. Aliquot the suspension from each 15 mL tube into seven cryovials at 1 mL/vial.
15. Place cryovials in a freezing container and freeze cells overnight at -80°C. After 24 hours, transfer frozen cells to a liquid nitrogen freezer for long-term storage. Primary tissue can be cryopreserved for at least 18 months.
16. Resuspend one tissue pellet from step 12 in 15 mL Wash Medium. Mix well to create an even suspension. Aliquot the appropriate amount of tissue suspension (see TIP) to seed the desired number of domes into a fresh 15 mL tube or small conical vial containing 1 mL Wash Medium. Proceed to section 5.3.

    Example: 7 x 17 µL suspension of 15 mL to seed 6 domes, including 1 additional volume to account for volume lost during pipetting.

    Note: We have observed efficient establishment of organoids with islet-depleted fractions. If desired, fractions can be enriched for duct cells or duct fragments by picking, filtration, or cell sorting. Publications

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describing these protocols are provided in section 13.0. If remaining tissue is to be cryostored, spin down the tube at 200 x g for 5 minutes at 2 - 8°C. Remove and discard the supernatant and process the leftover tissue pellet as described in steps 14 - 15.

5.2 Thawing and Seeding Cryopreserved Tissue

1. Prepare cool (not ice-cold) Wash Medium (section 3.3). Add 3 mL Wash Medium to a 15 mL conical tube, for each cryovial of tissue.
2. Thaw cryovial of tissue in a 37°C water bath until content is ~70 - 80% thawed.
3. Wipe the outside of the cryovial with 70% isopropyl alcohol and transfer to a biosafety cabinet.
4. Carefully uncap cryovial. Add 1 mL cool Wash Medium to the cryovial. Using a serological pipette, mix contents by gently pipetting up and down 4 times.
5. Immediately transfer contents to the conical tube containing 3 mL Wash Medium. Rinse cryovial with an additional 1 mL of cool Wash Medium and combine with material in the tube.
6. Centrifuge the tissue at 200 x *g* for 5 minutes at 2 - 8°C. Carefully remove and discard supernatant without disturbing the tissue pellet.
7. Resuspend pellet in 2 mL Wash Medium, then aliquot appropriate amount of tissue suspension (see TIP) to seed the desired number of domes into a fresh 15 mL conical tube or small conical vial containing 1 mL Wash Medium.

5.3 Seeding Domes with Primary Tissue

1. Centrifuge tissue suspension at 200 x *g* for 5 minutes at 2 - 8°C. Carefully remove as much supernatant as possible without disturbing the pellet and place the tube back on ice.
2. Resuspend pellet in Matrigel® (40 µL Matrigel® per dome). Place tube on ice.

   Example: 7 x 40 µL Matrigel® to seed 6 domes, including 1 additional volume for pipetting loss.
3. Distribute tissue evenly by pipetting up and down at least 5X while moving the tip below the Matrigel® surface. Do not push the plunger past the first step, to avoid introducing bubbles. Place tube on ice.

Perform the following steps quickly:

4. Retrieve the 24-well plate from the incubator.

   Note: Wells must be dry to maintain surface tension and allow for domes to form. Do not re-use plates or pre-wet with fluids.
5. Adjust pipettor to 40 µL. Pre-wet tip by pipetting up and down once without introducing bubbles, then draw up 40 µL of fragment-Matrigel® suspension. Align and gently touch the center of the well with the tip, lift it slightly up, and start dispensing. Continue to lift the tip as you dispense and stop when the plunger reaches the first stop of the pipette.
6. Repeat step 5 until all domes are seeded. If possible, use the same tip to minimize loss of Matrigel® suspension retained in the tips.

   Note: When working with precious/scarce material, adjust the pipettor for the last dome to collect all remaining material in the tube. This can result in slightly more material being seeded for the last dome, but no material will be lost. Take care not to introduce air bubbles.
7. Close lid and label plate, taking care not to mark the lid area over the well, as this will obscure imaging later. Avoid fast movements or bumps against the plate so as not to disturb domes. Carefully transfer the plate to the incubator, and let domes solidify at 37°C for 10 - 15 minutes.
8. Aliquot an appropriate amount of PancreaCult™ OIM and warm to room temperature during incubation time.

9. Shortly before the end of the dome incubation time, supplement PancreaCult™ OIM with 1 mM PGE2 and 10 mM Y-27632 stock solutions at 1 in 1000 dilutions.
10. Remove the 24-well plate from the incubator and place in the biosafety cabinet. Using a 1 mL pipettor, mix medium once, then slowly add 0.5 mL of PancreaCult™ OIM + PGE2 + Y-27632 against the wall of each well.

    Note: Rapid medium addition to the well or pipetting medium directly on top of the dome can damage or dislodge the Matrigel® dome.
11. Add D-PBS to unused wells.
12. Image Day 0 cultures.

TIP: For islet-depleted exocrine samples, it can be difficult to determine the appropriate amount of cell suspension to use per dome, as the amount and quality of material provided by islet isolation facilities can vary.

We recommend recording the initial volume of the settled exocrine pellet to estimate the amount of tissue to seed. From 2 and 2.5 mL pellets, we have seeded 1/50th and 1/80th of a cryovial (Figure 3), an equivalent to 17 and 27 µL of diluted suspension created from fresh tissue (step 15 of section 5.1) or cryopreserved tissue (step 7 of section 5.2).

The optimal amount will be influenced by the quality of cell material and is best determined by titration for each donor. Overcrowded domes result in slower organoid growth and can cause dome disintegration during p0 (Figure 4). This does not prevent organoid establishment but can result in slower culture establishment; domes with small organoids and debris at p0, as well as material in dissociated domes, can be passaged into p1 and will grow into organoids.

Sticky or rubbery islet-depleted tissue pellets indicate cell lysis and low viability. Organoids can often still be established from these samples, but they will require a larger amount of tissue to be seeded at p0.

5.4 Organoid Establishment and Expansion

1. Incubate p0 cultures at 37°C in PancreaCult™ OIM + PGE2 + Y-27632. After 3 days, perform a full-medium change with 0.5 mL/well PancreaCult™ OGM + PGE2 (Y-27632 is not required for subsequent feeds and passaging). After 3 - 4 days, passage cultures into p1. Perform a full-medium change with 0.5 mL/well PancreaCult™ OGM + PGE2 every 2 - 3 days.

    Note: Organoids established from densely seeded islet-depleted exocrine tissue often look smaller at p0 than in later passages (see Figure 3 [Day 6] and Figure 7), but they can still be passaged. We recommend expanding and cryobanking organoids during passage 1 - 2, if expansion rates permit. Prior to cryobanking organoids, it is recommended to seed at least two domes for each donor to safeguard against sample loss due to handling errors.

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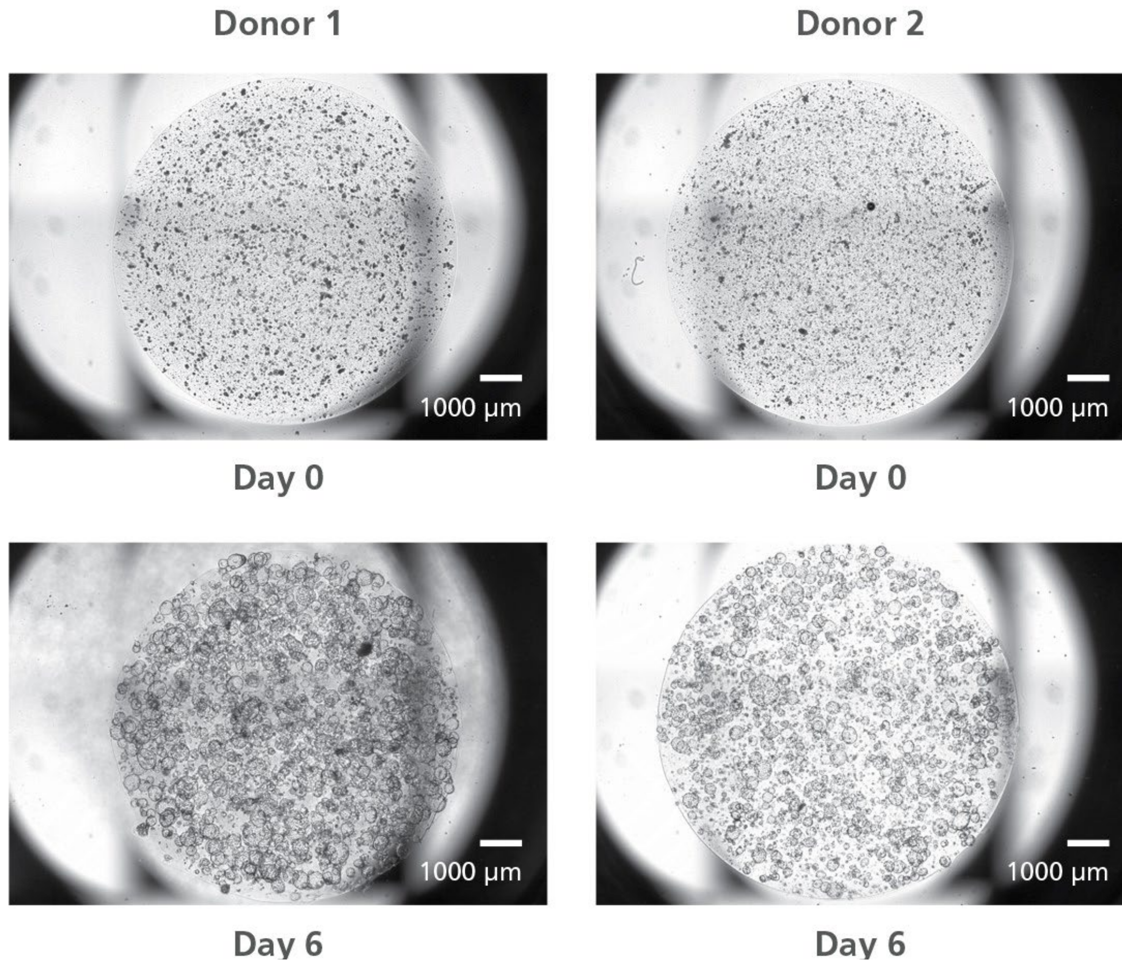

Figure 3. Seeding Examples for Two Islet-Depleted Exocrine Samples

Donor 1 seeded as 1/80th of a cryovial; Donor 2 seeded as 1/50th of a cryovial.

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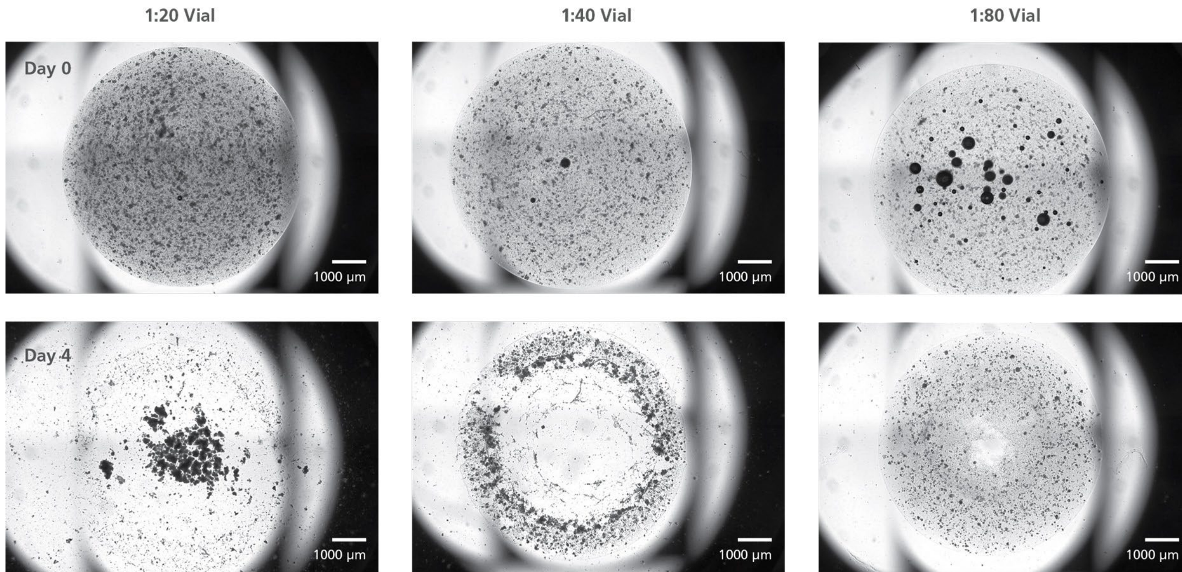

Figure 4. Overseeding Domes in p0 Can Result in Dome Disintegration

Example of how overseeding on Day 0 can result in complete or partial dome disintegration by Day 4 (as seen in the 1:20 and 1:40 images). Dark structures in the image of the 1:80 diluted tissue represent air bubbles in the dome.

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6.0 Seeding Cryopreserved Organoid Fragments

Refer to Figure 2 for the protocol diagram.

1. On the day before seeding, place tissue culture-treated 24-well plates into the 37°C incubator to warm overnight. Prepare Wash Medium (section 3.3).

   Note: Warming the plate for < 18 - 24 hours can result in spreading/flattening of Matrigel® domes. Costar® plates are recommended.

2. Thaw Corning® Matrigel® on ice.

   Note: If larger volumes of Matrigel® are required, thaw on ice overnight.

3. Prepare PancreaCult™ OGM (section 3.5), 1 mM PGE2 stock solution (section 3.2), and 10 mM Y-27632 stock solution, or thaw pre-prepared aliquots.
4. Prepare Wash Medium (section 3.3). Add 3 mL cool (not ice-cold) Wash Medium to a 15 mL conical tube, for each cryovial of organoid fragments.
5. Thaw cryovial of organoid fragments in a 37°C water bath until content is ~70 - 80% thawed.
6. Wipe the outside of the cryovial with 70% isopropyl alcohol and transfer to a biosafety cabinet.
7. Carefully uncap cryovial. Add 1 mL cool Wash Medium to the cryovial. Using a serological pipette, mix contents by gently pipetting up and down 4 times.
8. Immediately transfer contents to the conical tube containing 3 mL Wash Medium. Rinse cryovial with an additional 1 mL of cool Wash Medium and combine with material in the tube.
9. Centrifuge the organoid fragments at 300 x *g* for 5 minutes at 2 - 8°C. Carefully remove and discard supernatant without disturbing the pellet.
10. If organoid fragment numbers per thawed vial are known, proceed to step 11. If fragment numbers are unknown and recommended fragment numbers are to be seeded:
    a. Resuspend pellet in 1 mL Wash Medium and count fragments as described in section 8.0. If suspension is too dense to count, dilute with additional medium (defined volume) and repeat count.
    b. Once the fragment number is determined, use all fragments or subaliquot the desired amount of fragments into a fresh tube (see step 11). Centrifuge tube at 300 x *g* for 5 minutes at 2 - 8°C. Remove as much supernatant as possible without aspirating the fragment pellet.

       Note: If no counting is performed, we recommend seeding cryopreserved material from one densely grown dome into 2 - 4 domes. It is recommended not to overload domes with material, as this can inhibit growth.

11. Resuspend fragments in Matrigel® at 125 - 250 fragments per 10 µL (500 - 1000 fragments/40 µL dome).

    Note: Matrigel® is viscous, which causes some loss of material in the pipette. Calculate sufficient material for one or two additional domes if excess material is available. If organoid material is scarce and/or valuable, resuspend in slightly more Matrigel® (e.g. for four domes: 4 x 40 µL + 20 µL).

    Note: Optimal fragment numbers may vary depending on the growth characteristics of a specific organoid line or donor sample.

12. Distribute fragments evenly by pipetting up and down at least 5X while moving the tip below the Matrigel® surface. Do not pipette past the first step, to avoid introducing bubbles. Place tube on ice.

Perform the following steps quickly:

13. Retrieve the 24-well plate from the incubator.

    Note: Wells must be dry to maintain surface tension and allow for domes to form. Do not re-use plates or pre-wet with fluids.

14. Adjust pipettor to 40 µL. Pre-wet tip by pipetting up and down once without introducing bubbles, then draw up 40 µL of fragment-Matrigel® suspension. Align and gently touch the center of the well with the

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tip, lift it slightly up, and start dispensing. Continue to lift the tip as you dispense, and stop when the plunger reaches the first stop of the pipette.

15. Repeat step 14 until all domes are seeded. If possible, use the same tip to minimize loss of Matrigel® suspension retained in the tips.

    Note: When working with precious/scarce material, adjust the pipettor for the last dome to collect all remaining material in the tube. This can result in slightly more material being seeded for the last dome, but no material will be lost. Take care not to introduce air bubbles.

16. Close lid and label plate, taking care not to mark the lid area over the well, as this will obscure imaging later. Avoid fast movements or bumps against the plate so as not to disturb domes. Carefully transfer the plate to the incubator, and let domes solidify at 37°C for 10 - 15 minutes.
17. Aliquot an appropriate amount of PancreaCult™ OGM and warm to room temperature during incubation time.
18. Shortly before the end of the dome incubation time, supplement PancreaCult™ OGM with 1 mM PGE2 and 10 mM Y-27632 stock solutions at 1 in 1000 dilutions.
19. Remove the 24-well plate from the incubator and place in the biosafety cabinet. Using a 1 mL pipettor, mix medium once, then slowly add 0.5 mL of PancreaCult™ OGM + PGE2 + Y-27632 against the wall of each well.

    Note: Rapid medium addition to the well or pipetting medium directly on top of the dome can damage or dislodge the Matrigel® dome.

20. Add D-PBS to unused wells.
21. Image Day 0 cultures.
22. Incubate organoids at 37°C until densely grown and at least 100 µm in diameter (organoids can grow up to 1 mm). This will take 3 - 7 days on average, depending on the amount of seeded material and the donor. After 3 days, perform a full-medium change with 0.5 mL/well PancreaCult™ OGM + PGE2 (Y-27632 is not required for subsequent feeds and passaging). Perform a full-medium change with 0.5 mL/well PancreaCult™ OGM + PGE2 every 2 - 3 days.

    Note: If large organoids start to darken, become thick-walled, and/or collapse, cultures are overgrown and need to be passaged. Deteriorating cultures can be saved by passaging, but it is recommended to passage organoids before their decline to maintain optimal long-term growth kinetics.

    Note: When working with pre-established pancreatic cancer organoids, re-establishment efficiency and passaging frequency may differ from normal organoids depending on the growth characteristics of the cancer sample.

23. Proceed to section 7.0 to passage organoids.

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7.0 Passaging Pancreatic Organoids

This protocol is suitable for the mechanical passaging of organoids from normal primary tissue and pancreatic cancer organoids with cystic morphology or loosely packed spheroids.

1. On the day before passaging, place new 24-well plates into the 37°C incubator.

   Note: If larger volumes of Matrigel® are required for passaging, thaw overnight on ice.

2. On the day of passaging:
   - If fragment counting will be performed, prepare a 96-well plate as described in section 8.0.
   - Check all wells for damaged or loose domes prior to passaging.
   - Record brightfield images of domes.
   - Prepare ice-cold Wash Medium (section 3.3) and 1 mM PGE2 stock solution (section 3.2), or use pre-prepared aliquots.
   - Prepare PancreaCult™ OGM (section 3.5) and warm to room temperature (0.5 mL per dome plus 10% for pipetting error).
   - Thaw Matrigel® on ice.

3. **If domes are intact:** Transfer plate into biosafety cabinet and try to locate domes by tipping the plate toward the operator (see Figure 5). If the gel is not touching the edge of the well (spilled dome), aspirate medium clockwise starting at 12 o'clock. Move pipette tip alongside the well wall to minimize risk of damaging or aspirating the dome in the center of the well. If the dome was not visible when the plate was tipped, aspirate slowly and locate the dome as medium is aspirated. Take care not to touch the gel with the tip. Proceed to step 4.

   If any of the domes are loose or damaged: Do not aspirate medium. Instead, measure medium volume in the well with a 1 mL pipettor, then top the well up to 1 mL with ice-cold Wash Medium. Proceed to step 5.

   Note: When using this method, any dome disruption during handling is irrelevant.

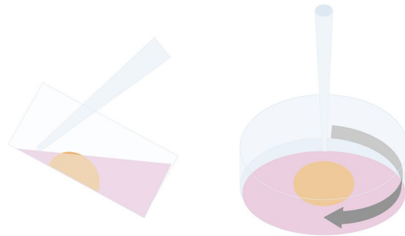

Figure 5. Medium Aspiration for Dome Cultures

Place tip just below medium surface where no dome is visible and aspirate, slowly moving along the wall of the well to avoid touching and aspirating the dome.

4. Add 1 mL cold Wash Medium directly to the well.
5. Triturate 10 - 20 times with a 1 mL pipettor to disperse Matrigel® and break organoids into fragments.

   Note: To efficiently break up organoids, increase shear force by tilting the plate and pressing the pipette tip into the corner of the well. Triturate the whole volume while avoiding bubbles. The tip is placed correctly when resistance is felt while expelling the volume.

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6. Check fragment sizes with a brightfield microscope. Most fragments should be between 50 - 200 µm (see Figure 6).

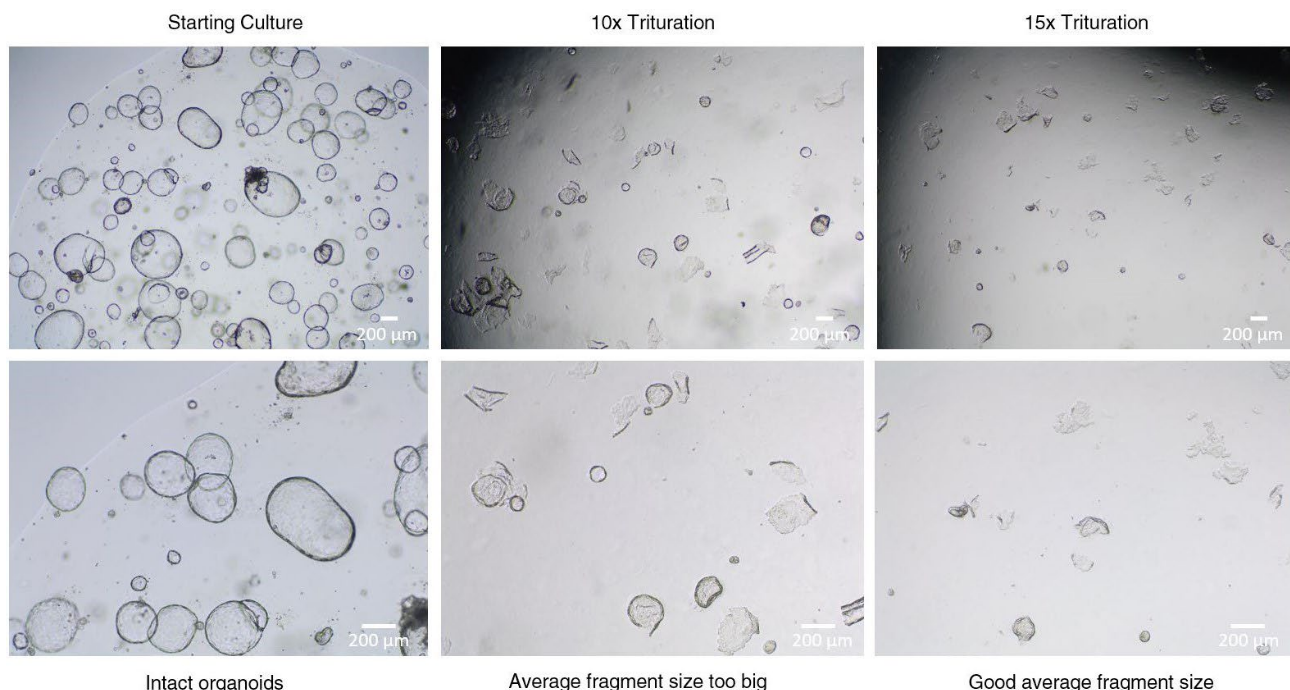

Figure 6. Representative Images of Organoids and Fragment Sizes Before and After Trituration

Fragment sizes of 50 - 200 µm are recommended. Cystic organoids can be broken into appropriately sized fragments with 10 - 20 trituration steps.

7. Once organoids are dispersed, transfer fragment suspension into a 15 mL conical tube on ice (if appropriate for analysis, replicates of the same condition can be pooled).
8. Add 0.5 mL of cold Wash Medium to each well to rinse off remaining fragments, and transfer to the corresponding 15 mL conical tube.

   Note: Keep fragment suspension on ice to support viability and keep Matrigel® from gelling. Keeping the volume of Wash Medium during harvest consistent is important when determining total fragment counts per well or condition.
9. Repeat steps 3 - 8 for all wells/conditions.
10. To determine fragment number in suspension, refer to the fragment counting protocol in section 8.0.
11. Re-seed organoid domes as described in section 6.0, steps 11 - 22, using PancreaCult™ OGM + PGE2 (Y-27632 is not required for fragment passaging). Refer to Figure 7 for examples of good seeding and harvesting densities. Optimal seeding density might require adjustment according to growth characteristics of individual donors and passage number of culture.

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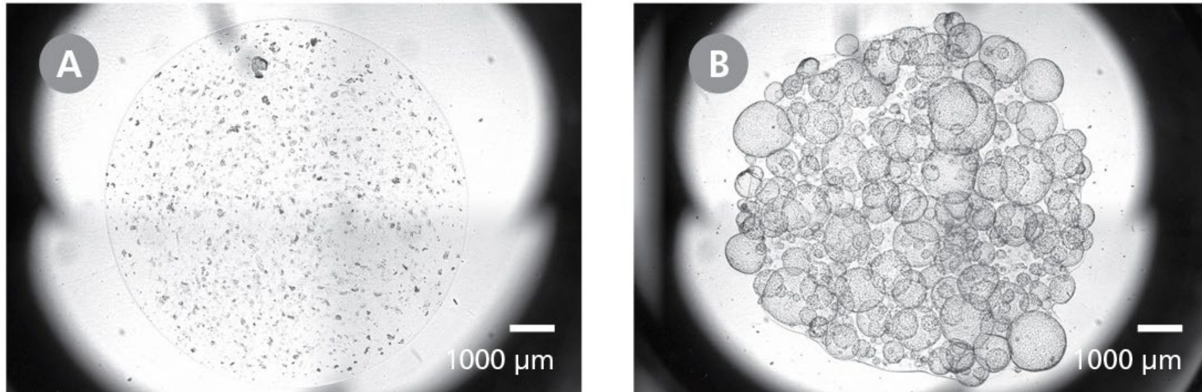

Figure 7. Representative Images of (A) Normal Organoid Fragments After Seeding and (B) Organoids on Day 7, Seeded at 800 Fragments/Dome

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8.0 Fragment Counting

1. Prepare a 96-well flat-bottom plate (e.g. Catalog #38022) for easier counting by drawing a cross under each well with a fine-tipped marker to separate the well into four quadrants. Prepare triplicate wells per sample, and add 40 µL D-PBS per well.
2. Record total volume of fragment suspension in the 15 mL conical tube and (if multiple wells were pooled) the number of domes harvested per vial.
3. Vortex tube briefly to resuspend settled fragments, then immediately transfer 10 µL of the suspension to the first well containing 40 µL D-PBS.

   Note: Resuspending fragments by hand may be insufficient to fully dislodge and evenly distribute fragments in the pellet; ensure that fragments are fully resuspended. As an alternative to vortexing, fragments can also be resuspended using a 1 mL pipettor.

   Note: If 96-well flat-bottom plates are not available, 3 x 10 µL can be transferred into one well of a dry 6-well plate to create three independent drops for fragment counting.
4. Repeat vortex and transfer (step 3) twice to create triplicates.

   Note: When working with non-sterile counting plates, switch tips between sampling steps to prevent contamination of the fragment suspension.
5. Place the tube containing the suspension on ice and repeat steps 3 - 4 for all conditions.
6. Using a brightfield microscope, count and record all fragments that are 50 - 200 µm for each triplicate.

   Note: We recommend using a brightfield microscope with an integrated scale in the ocular, or digital imaging with scale, to estimate fragment sizes. If fragments are too dense to count, dilute the fragment suspension further with a defined volume and repeat count. Count fragments in all four quadrants to determine total fragments per well. Quadrants only serve to help guide the eye during counting.
7. Calculate the average number of fragments per µL suspension in sample tube.

   Example: (53 + 48 + 65) ÷ 3 = 55.3 fragments/10 µL = 5.5 fragments/µL
8. Calculate total fragments per sample tube by multiplying fragment count per µL with the total suspension volume. If multiple domes were harvested, divide by number of domes to determine average fragments per dome.

   Example 1: 5.5 fragments/µL x 1500 µL suspension (1 dome) = 8250 total fragments

   Example 2: (5.5 fragments/µL x 3000 µL suspension) ÷ 2 domes = 8250 fragments/dome
9. Aliquot desired fragment numbers and seed domes as described in section 6.0.

   Note: This method will not yield precise fragment numbers in the final dome. Some variability is introduced due to the counting of small volumes and some material loss during pipetting or uneven suspension in Matrigel®. If precise numbers of fragments per dome are required for evaluation, we recommend counting fragments in the seeded dome.

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9.0 Organoid Cryopreservation

1. Label and cool cryovials.

   Note: We recommend tracking Donor ID, tissue type, total passage number, number of fragments or confluent domes per vial, and date of banking.

2. Harvest organoids as described in section 7.0.
3. Centrifuge 15 mL conical tube(s) containing organoid fragments at 300 x *g* for 5 minutes at 2 - 8°C. Aspirate supernatant and place the tube on ice.
4. If defined fragment numbers per cryovial are to be banked, divide the total number of fragments per pellet by the number of desired fragments per vial; this will give the number of cryovials that can be banked and the volume (mL) of CryoStor® CS10 in which to resuspend the pellet (1 mL per vial). Resuspend the pellet in the appropriate volume of CryoStor® CS10. Immediately transfer 1 mL of suspension into a pre-cooled cryovial and transfer into a freezing container. Repeat for remaining cryovials. Immediately transfer freezing container to -80°C.

   Example: The pellet from one dome contains 8250 fragments. To bank vials with 4000 fragments each, divide 8250 fragments by 4000 to determine the number of vials that can be banked. Resuspend pellet in 1 mL CryoStor® CS10 per vial.

   8250 fragments ÷ 4000 fragments/vial = 2.1 vials x 1 mL CryoStor® CS10/vial = 2.1 mL CryoStor® CS10

   Note: When banking without fragment counts, we recommend freezing fragments from at least one densely grown dome per vial, which can be re-seeded at a split ratio of 1:2 or 1:4, depending on optimal seeding densities.

5. After 24 hours, transfer vials from -80°C into the gas phase of a liquid nitrogen tank for long-term storage. Fragments can be stored for at least 9 months. Do not store fragments for extended times at -80°C.

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10.0 Harvesting Organoids for Immunocytochemistry (ICC) Staining

10.1 Materials Required

- Corning® Cell Recovery Solution (0.5 mL per well) (Corning Catalog #354253)
- **Optional:** Anti-Adherence Rinsing Solution (Catalog #07010)
- Wide-bore or cut-off 1 mL pipette tips
- Pre-cooled and labeled 15 mL conical tubes
- D-PBS (Without Ca++ or Mg++), ice-cold
- 4% Paraformaldehyde (PFA)
- Serological pipettes
- Shaker
- Ice box

10.2 Protocol

It is recommended to harvest pancreatic organoids when they are < 200 µm. Large organoids can collapse or tear more easily during harvest, and the organoids will be more difficult to image.

1. Remove plate containing pancreatic organoids from incubator.
2. Check organoids for health and domes for integrity, and record images if required.
3. Tip plate and aspirate medium from all wells as described in step 3 of section 7.0.
4. Use a 1 mL pipettor to add 0.5 mL of ice-cold Cell Recovery Solution directly on top of each dome, to partially disrupt the Matrigel®.

   Note: To maintain intact organoids, do not disrupt Matrigel® with additional pipetting at this stage.
5. Place plate on ice and shake on rocking platform at medium speed for 1.5 hours to release organoids from Matrigel®.
6. Using a brightfield microscope, check to ensure that Matrigel® has mostly dissolved and organoids have been released.

   Note: Some organoids might remain attached at the well bottom.
7. Gently collect organoids with wide bore tips, cut-off 1 mL pipette tips, or a serological pipette, and transfer into pre-cooled 15 mL conical tubes. Organoids from replicates of the same condition can be pooled in the same tube.

   Note: Avoid disrupting organoids by rapid or repeated pipetting or narrowing of the pipette tip in the well edge. Organoids can adhere to plastic not previously exposed to Matrigel® or BSA. To reduce this risk, tubes and pipettes can be rinsed once with Anti-Adherence Rinsing Solution followed by a rinse with D-PBS. When using multiple tubes, Rinsing Solution can be sterilely transferred from one tube to the next.
8. Rinse each well once with 1 mL ice-cold D-PBS and add to the tube containing organoids.
9. Leave organoids on ice for 5 - 10 minutes to settle.

   Note: Centrifuging organoids at 200 x g for 3 minutes can speed up settling, but can result in the deformation, collapse, and/or aggregation of organoids.

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10. Carefully aspirate supernatant without aspirating organoids. Pellets can be difficult to see; thus, we recommend leaving a small volume (~ 50 µL) at the bottom at the tube.

    Note: If multiple domes are pooled in the same tube, a cushion of partially dissolved Matrigel® can form at the bottom, which can prevent organoids from fully settling. If this is the case, remove as much supernatant as possible, then add 5 mL ice-cold D-PBS to the tube without trituration to further dilute Matrigel® and let organoids resettle on ice before aspiration.

Note: Once Matrigel® has been removed, organoids can become very sticky and will easily adhere to plastic. To avoid losing material, do not pipette the washed organoids with tips or serological pipettes that have not been rinsed with Anti-Adherence Rinsing Solution or Wash Medium containing BSA.

11. Add 1 mL 4% PFA to organoids and gently swirl or flick tube to resuspend pelleted organoids. Do not triturate.

    Note: If working with unrinsed tubes, avoid tipping or inverting the tube to prevent organoids getting stuck in the upper parts of the tube that have not been exposed to the dissolved Matrigel®.

12. Fix organoids at room temperature for 1 hour or overnight at 2 - 8°C in the dark.
13. Slowly remove tube from holder, without stirring up pelleted organoids. Use a 1 mL pipettor to remove as much PFA supernatant as possible.
14. Resuspend organoids in 2 mL D-PBS and store the tube upright at 2 - 8°C until required for staining.

    Note: When handling fixed organoids, use pipettes and staining vessels rinsed in medium or D-PBS with BSA or serum, or use Anti-Adherence Rinsing Solution.

15. For guidance on whole-mount immunofluorescent staining of organoids, refer to the online protocol: Performing Immunocytochemical Staining of Epithelial Organoids, available at www.stemcell.com.

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11.0 Harvesting Organoids for RNA

11.1 Materials Required

- RNeasy Mini Kit (Qiagen 74106)
- **Optional:** QIAshredder homogenizers (Qiagen 79656)
- β-Mercaptoethanol (e.g. Sigma 516732)
- DMEM/F-12 with 15 mM HEPES (1.5 mL per dome)
- 15 mL conical tubes
- Nuclease-free water (e.g. Catalog #79001)

11.2 Protocol

1. Label RNA collection tubes and place on ice.
2. Perform the following step in a fume hood: For each RNA sample, add 3.5 µL β-mercaptoethanol (β-ME) to 350 µL of Buffer RLT (component of RNeasy Mini Kit). Mix thoroughly.
3. Remove organoids from incubator and check dome integrity and organoid health under the microscope.
4. If domes are intact, aspirate medium and proceed to step 5. If domes are loose or damaged, top volume up with cold DMEM/F-12 with 15 mM HEPES as described in section 7.0 step 3, then proceed to step 6.
5. Add 1 mL of cold DMEM/F-12 with 15 mM HEPES to the well.
6. Triturate 3 - 5 times with a 1 mL pipettor to break up and detach the Matrigel® domes.
7. Transfer replicates of each condition into a 15 mL conical tube. Rinse wells with 0.5 mL DMEM/F-12 with 15 mM HEPES and transfer to the tube.
8. Centrifuge at 300 x *g* for 5 minutes.
9. Aspirate the supernatant. Resuspend each pooled condition in 350 µL Buffer RLT + β-ME (prepared in step 2). Triturate the cell lysate until all visible organoid fragments are dissolved and the mixture first acquires a viscous consistency, then becomes slightly less viscous.
10. **Optional:** If organoid fragments remain, centrifuge lysate though a QIAshredder homogenizer to fully break up tissue.
11. Transfer lysate into labeled collection tubes on ice.
12. Immediately store the sample at -80°C or proceed directly to the Qiagen RNeasy RNA purification protocol.

    Note: Four densely grown domes should yield 6 - 12 µg RNA.

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12.0 Troubleshooting

PROBLEM	POSSIBLE CAUSE	SOLUTION
Domes dissolve during organoid establishment	Enzyme secretion from acinar cells in primary tissue sample	• Use PancreaCult™ OIM + PGE2 + Y-27632 for organoid establishment • Re-seed dissociated material into fresh dome. Even dissociated cultures often robustly form organoids after re-seeding. • Reduce amount of tissue seeded per dome when working with large amounts of starting tissue such as islet-depleted fractions. We recommend testing different dilutions of each tissue donor to determine the optimal density (see TIP in section 5.3).
Domes are shearing/ breaking apart	• Medium addition is too forceful or domes are damaged through contact with tips • Medium is too cold • Cultures are maintained too long without passage and gel is losing integrity	• Ensure to add medium warmed to room temperature slowly along the well wall • Keep pipette tip away from dome
Uneven fragment distribution in domes	Resting tip on well bottom during dome dispensing	Lift tip slowly while dispensing the dome into the well
Uneven fragment distribution between domes when seeding multiple domes from the same vial	• Insufficient resuspension of fragments in Matrigel® prior to seeding • Resting the pipette tip on the tube bottom during aspiration of fragment/Matrigel® mix can prevent larger fragments from being aspirated • Fragment loss during supernatant aspiration	• When seeding only small numbers of domes, prepare extra volumes of Matrigel®/fragment suspension for ease of resuspension and aspiration • Matrigel® is viscous; carefully resuspend fragment pellet in Matrigel®. If necessary, add additional trituration steps while moving tip through the gel. • Use a 200 µL pipettor instead of an aspirator, and do not completely remove supernatant (to prevent accidental aspiration of pellet)
2D outgrowths attach to well bottom	• No Y-27632 added during first feed of establishment or after thaw • At p0/p1, presence of non-epithelial cells from primary tissue and exposure to PancreaCult™ OIM + PGE2 + Y-27632 in p0 can result in some 2D outgrowth • Organoid fragments are too large and/or Matrigel® is too diluted, causing fragments to sink to the bottom of the well • Organoid attachment can indicate declining organoid growth performance at higher passage number	• Early passage outgrowths should disappear after 1 - 2 passages and switching to PancreaCult™ OGM • Adjust number of trituration steps to create recommended fragment size • Confirm that Matrigel® protein concentration is ≥ 8 mg/mL • Remove as much medium supernatant from pellet as possible without disturbing pellet prior to resuspension in Matrigel® • Restart culture with cryopreserved sample from lower passage number

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PROBLEM	POSSIBLE CAUSE	SOLUTION
Organoid fragments are not pelleting during centrifugation or are overlaid with Matrigel® cushion	• Wash Medium not cold enough • Too many domes pooled in one vial	• Keep Wash Medium on ice during organoid harvest • Avoid letting Wash Medium sit in plate after addition to domes or for rinse. Process 1 - 2 wells at a time and immediately transfer fragment suspension into tube on ice. Rinse well with cold Wash Medium and immediately add volume with suspension on ice. • Limit the number of pooled domes/samples per 15 mL tube to four
Cultures at end of p0 contain debris that makes fragment counting difficult	Debris from nonviable cells accumulated in dome	If fragments cannot be counted reliably, split domes into p1 at a ratio between 1:1 and 1:3, depending on organoid density and size
Organoids are not establishing in p0 or after thaw	• Low viability of starting material • No Y-27632 added during first feed of organoid establishment or after thawing • Dome dissociation due to enzyme release from primary tissue • Slow recovery/growth	• Optimize tissue dissociation and/or shipping conditions. Optimally, test for cell number and viability. • Add freshly thawed aliquot of Y-27632 during first medium addition after dome seeding in p0 or after thawing of cryopreserved organoids • Collect all material, centrifuge, and re-seed into fresh dome. If organoids do not form by the end of p1, perform titrations with the cryopreserved normal tissue to determine optimal seeding density. • Extend passage duration to up to 2 weeks post seeding with recommended feeding schedule, and regularly check culture for growth

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13.0 References

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2. Tuveson Lab Organoid Protocols. Available at http://tuvesonlab.labsites.cshl.edu/protocolsreagents.

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TECHNICAL MANUAL

Generation of Human Pancreatic Organoids Using PancreaCult™ Organoid Medium Kits

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