Tuveson Laboratory Murine and Human Organoid Protocols

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Based on methods described in:

Huch M et al. 2013 Unlimited in vitro expansion of adult bi-potent pancreas progenitors through the Lgr5/R-spondin axis. EMBO J. 32: 2708-21.

Boj S et al. 2015 Organoid models of human and mouse ductal pancreatic cancer. Cell 160: 324-38.

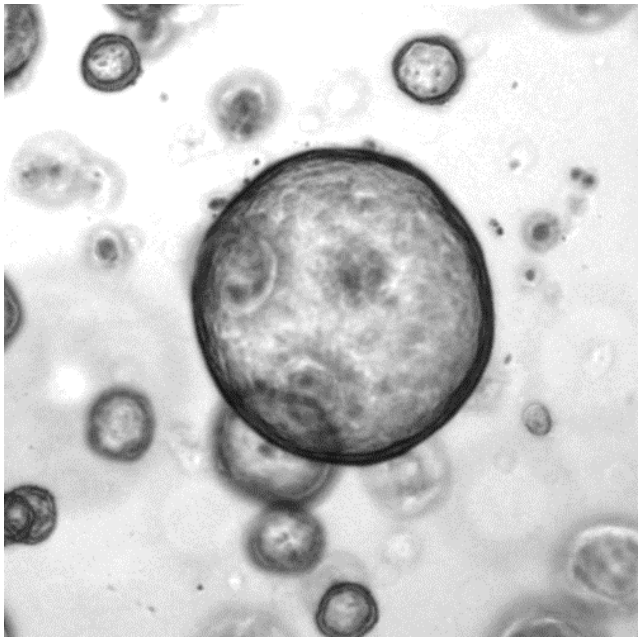

Table of Contents

General Considerations for Working With Pancreatic Organoid Cultures

- We culture organoids in a dome of Matrigel bathed in a nutrient- and growth factor-rich liquid medium. Because organoids are suspended in the Matrigel, they never touch the plastic of a tissue culture dish like a 2-dimensional culture would.
- Matrigel, or Basement-Membrane-Extract is a protein-rich substance secreted by EHS mouse sarcoma cells which contains hundreds of proteins. Because of this, there is some variation between Matrigel content, protein content, and stiffness from lot to lot. Individual lots need to be tested for organoid culture. In practice, we use lots with protein content between 9.4-9.9 mg/mL. We have noticed that lots with lower protein concentrations fail to make solid domes. When the protein concentration is too high, it is difficult to separate the organoids from the Matrigel.
- Matrigel is frozen at -20^{o}C, liquid at 0^{o}C, and begins to harden above 0^{o}C. Always keep Matrigel aliquots on ice when working with it. Matrigel can be purchased from BD Biosciences in several formulations. For our organoid protocols, we use Growth Factor Reduced Matrigel that is Phenol-Red free.

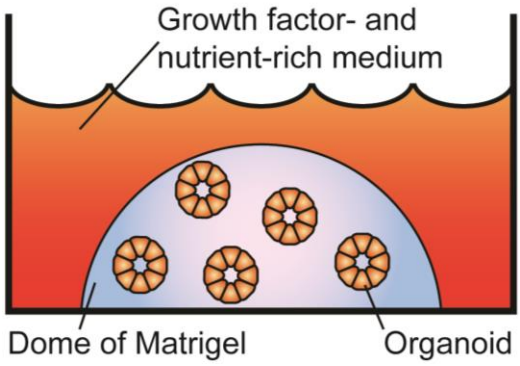

Figure 1. Cross-sectional diagram of an organoid culture.

Protocol for Isolation of Organoids from Normal or PanIN-Containing Murine Pancreas

(edits by LB 2013 and 2017; based on MSS/BC 5.16.2013 N/P Protocol and CH/CC/YP 9.10.2013 T Protocol)

Notes:

- This protocol is for generating normal organoids from a healthy murine pancreas or PanIN organoids from a PanIN-containing pancreas (such as the pancreas of a mouse from the *Kras$^{LSL\text{-}G12D}$; Pdx-Cre* genetic background). To make organoids from tumor-bearing mice, see the "Protocol for Isolation of T and M Organoids from Mouse Pancreatic Tumors and Metastases" on page 10.
- If possible, schedule at least 2 people to be involved:
    - Person 1 will handle the pancreas dissection, will conduct the necropsy if needed, will clean the necropsy area, and then will pick ducts in the microscope.
    - Person 2 will prepare the digestion materials and the tissue culture hood, will set up the digestion, and will eventually assist in duct picking and plating.
- Person 1 should start prepping the Mouse Wash and Digestion Media and the tissue culture hood about 1 hour before the mouse is to be sacrificed.
- Overall protocol time (including preparation and plating) is about 4 hours.
- Organoids grow under same conditions as 2D cell lines (37ºC, 20% O_2, 5% CO_2)

A. Isolation of Murine Pancreas

Reagents and Equipment (have ready at necropsy area):

Mouse	70% Ethanol in spray bottle
Sterile Petri dish containing 2 mL cold Mouse Wash Medium	Blue pad
Paper Towels	Necropsy pins or needles
Strong scissors for decapitation	2 Pairs Dissection Scissors
2-3 Dissection Forceps	Dissection board
CO_2 chamber with CO_2 hookup and regulator	Marker
Hot bead sterilizer for dissection tools	Digital scale
<u>If PanIN mouse, you will also need:</u>	
10% Neutral Buffered Formalin (NBF) for histology	Histology cassettes
Pencil to mark histology cassettes	Histology worksheet
1.5 mL tube for mouse tail	

Procedure:

1. Sterilize dissection tools in hot bead sterilizer.
2. Prepare the necropsy area:
    a. Put down a clean blue pad.
    b. Tack a clean paper towel to your dissection board
    c. Set out paper towels for dissection tools and blotting mouse blood.
    d. Separate dissection tools and scissors into three sets, (1) scissors for the mouse decapitation, (2) dissection tools for cutting through the mouse skin and peritoneum, and (3) dissection tools for removing the pancreas.
    e. Place a paper towel in CO_2 chamber.
3. If PanIN mouse, label cassettes for histology with pencil and begin filling in histology worksheet. (Typically, we don't do histology on wild-type mice.)
4. Tare the digital scale with a sterile Petri Dish containing 2 mL cold Mouse Wash Medium.
5. Record information about mouse (strain, name, date of birth).
6. Sacrifice mouse in CO_2 chamber:
    a. Turn the CO_2 on very slowly to keep the mouse as comfortable as possible.

b. Watch the mouse the entire time.
c. The mouse should go from active, to inactive but breathing normally, to making quick gasps, to making heaving gasps/spasms with longer pauses in between.
d. After 2 or 3 heaving gasps remove mouse from chamber.
e. It is important to work quickly, because the viability of the pancreas will decrease with time. After opening up the mouse you will notice that with time, the pancreas turns from healthy pink to unhealthy gray.

7. While the mouse's heart is still beating, position strong scissors between head and neck, and cut off the head.
8. Blot the mouse's neck onto paper towels until blood flow slows.
9. Spray mouse with ethanol.
10. Skin the mouse.
    a. Locate the mouse's sternum (where the chest bone sticks out).
    b. Pull the fur up, and make a sizeable cut through the skin (but not through peritoneum).
    c. Cut skin towards back side of mouse.
    d. With two fingers of each hand, pinch the skin on both sides of the opening and pull the skin towards the head and tail until entire midsection is exposed.
11. Cut open the peritoneum near the genitals, and cut to expose the intestines and pancreas.
12. Switch to clean dissection tools.
13. Remove the pancreas, while being careful not to rupture the intestines.
    a. To locate the pancreas, either locate the spleen and start there while working down toward the intestines, or carefully pull out the intestines, working your way up from the cecum to the stomach.
    b. If intestines are ruptured, your organoids may be contaminated! If possible, discard this pancreas and start over with a new mouse.
14. Place pancreas in the sterile, tared Petri dish containing 5 mL Mouse Wash Medium.
    a. A Petri dish is preferred over a Tissue Culture dish for this step, since pancreatic tissue will stick to the latter.
15. Place the Petri dish on the digital scale, and record the weight of the pancreas.
16. If mouse had PanIN, cut a piece of pancreas, place in a labeled histology cassette, and place cassette in a container of 10% NBF to fix for histology.
17. Place the the Petri dish containing the pancreas on ice and bring to the Tissue Culture Room to start digestion procedure described in Part B of this protocol.
    a. If you are working alone, go immediately to a tissue culture hood and begin mincing the pancreas as described in Part B of this protocol. Once the pancreas is incubating in Mouse Digestion Medium, return to the necropsy room to finish necropsy and clean room as described below.
    b. Waiting too long between harvesting the pancreas and getting it into digestion can cause the pancreas to over-digest itself and organoid isolation to fail.
18. If mouse had PanIN, continue with full necropsy and fill cassettes to send to histology.
19. Cut tail off of mouse and save in the 1.5 mL tube for confirmation genotyping.
20. Clean up the necropsy area.
    a. At Cold Spring Harbor Laboratory, used mouse cages should be covered with trash bag or hair nets and returned to back room of Animal Facility.
    b. Mouse carcass should be wrapped in a glove and placed in the bucket at the bottom of the chest freezer in the back room of the Animal Facility.

B. Pancreas Digestion and Duct Picking

Reagents and Equipment (have ready in Tissue Culture):

DMEM + glucose, L-glutamine, sodium pyruvate	100% FBS
100X Penicillin/Streptomycin	10 mg/mL DNase I
Dispase II	Mouse Complete Feeding Medium
Collagenase Crude Type XI (from *Clostridium histolyticam)*	10.5 mM (1000X) Rho Kinase Inhibitor (Y-27632)
Growth Factor Reduced Matrigel, Phenol Red Free on ice	Sterile glass bottle
Sterile aspirator pipettes	#10 Scalpels
Ice bucket with ice	Petri Dishes (ones with grids if possible)
Sterile serological pipettes (5, 10 and 25 mL)	Hot water flask (pre-warmed to 37ºC)
15 and 50 mL conical tubes	Marker
Sterile P2, P20, P200, P1000 Pipetmen and tips	Timer
Extra Styrofoam tube holders or boxes (for armrests for pickers)	48-well tissue culture plate (pre-warmed to 37ºC)
Thermomixer or incubated rocker/rotator set to 37ºC	37ºC water bath
Microscope for each duct picker	Pipet-Aid/Pipette controller
Refrigerated 15/50 mL tube centrifuge	Tissue culture incubator
Tissue culture aspirator	Tissue culture hood

Procedure:

21. At least 6 hours before organoid isolation, place a 48-well plate and a hot water flask in a 37ºC tissue culture incubator to warm up.
22. Before starting the organoid preparation, place Mouse Complete Feeding Medium in the 37ºC water bath to ensure it comes to at least room temperature by the time the organoids are ready to be fed.
23. Prepare Mouse Wash Medium and keep on ice:
    500 mL DMEM + glucose, L-glutamine, sodium pyruvate
    5 mL 100X Penicillin/Streptomycin
    5 mL 100% FBS
24. Prepare Mouse Digestion Medium and allow it to warm to room temperature:
    a. Weigh 25 mg Collagenase Crude Type XI and 25 mg Dispase II into a 50 mL conical tube (We typically do this outside of Tissue Culture hood, so these reagents are not technically sterile, but we have not had any contamination problems.)
    b. Inside Tissue Culture Hood: add 50 mL Mouse Wash Medium to 50 mL tube containing enzymes, mix and pour into sterile glass bottle.
    c. Repeat 3 more times with 50 mL Mouse Wash Medium to bring final volume to 200 mL.
25. Thaw aliquots of 10 mg/mL DNase I and Matrigel on ice.
26. Prepare a 15 mL conical tube with 5 mL Mouse Wash Medium, and place tube on ice.
27. Mince pancreas in the Mouse Wash Medium in Petri dish with #10 scalpels, keeping the dish on ice while mincing. Be careful not to over-mince! Just get pancreas broken up into ~1-2 mm size pieces.
28. Transfer minced pancreas to 15 mL conical tube containing Mouse Wash Medium.
29. Use 5 mL Mouse Wash Medium to rinse Petri dish and get all pancreas pieces into conical tube.
30. Let tube stand at room temperature for 1-2 minutes. If present, fat should rise to top and float on surface, while pancreas material should sink.
31. Aspirate off fat and most of Mouse Wash Medium, leaving ~1 mL Mouse Wash Medium with pancreas pieces in bottom of tube. Be careful not to aspirate pancreas pieces.

32. Add 10 mL Mouse Digestion Medium to pancreas.
33. Incubate at 37°C for 20 minutes, with gentle agitation.
    If you are working alone, continue with mouse necropsy during this incubation.
34. Prepare a 15 mL conical tube with 3-5 mL Mouse Wash Medium for each duct picker.
35. After 20 minutes, remove tube with pancreas digest from 37°C.
36. Pipet up and down with 10 mL pipette to break up chunks.
37. Let tube stand ~1 minute to allow pancreas pieces settle by gravity.
38. Transfer 8.5 mL of supernatant to a clean Petri dish that contains 10 mL Mouse Wash Medium. This is "Wash 1."
39. Give Petri dish containing wash to picker to check in microscope for the presence of ducts and if present, pick ducts.
    a. If you are working alone, proceed immediately to step 40, and then pick ducts during 10 minute incubations.
    b. To pick ducts, look under the microscope, using the 4X or 10X objective. With the phase contrast on, in the 10X objective, ducts appear like small chains of cells, are smaller than blood vessels, and often have lariat or cross shapes (see Figure 2).
    c. Use a P10 or P20 set to 8-10 µL. Push Pipetman plunger to first stop, hover pipette tip near a duct. Release Pipetman plunger slightly to suck up duct. You should see the duct go into the pipette tip. Keep finding and sucking up ducts until you have released the plunger all the way. Then dispel ducts into 15 mL conical tube with Mouse Wash Medium.
    d. Most people prefer to use their dominant hand for picking, and to position a box or Styrofoam tube rack under their elbow to help stabilize it.
    e. Avoid picking acinar cells, because in large numbers, these will prevent organoid formation. Avoid picking blood vessels, which are similar to ducts, but are a bit larger in size (thicker diameter) with a visible, hollow lumen. However, **when in doubt, pick it** – small numbers of acinar or blood vessels will still permit organoid formation.
    f. For wild-type mice, ducts usually appear at the 3rd-4th wash. For PanIN and tumor mice, ducts may take longer to appear, and may start showing up as late as the 7th wash.
    g. Try to get as many ducts as possible – at least 100 or more is ideal.
    h. For PanIN mice, pick both normal and abnormal ducts.
    i. If there are too many cells around ducts for clean picks, it may help to dilute washes 1:2 with additional Mouse Wash Medium and split into 2 Petri dishes.
    j. The entire picking process happens outside the tissue culture hood with open tubes and open Petri dishes. For us, there have not been contamination issues.
40. Add 8.5 mL more Mouse Digestion Medium to conical tube with pancreas and incubate at 37°C for 10 minutes with gentle agitation.
41. After 10 minutes, remove tube from 37°C and repeat steps 36-40 to generate "Wash 2." Repeat to generate "Wash 3," "Wash 4," etc.
    a. Starting at the 4th wash (or earlier if cells start to burst and digestion looks viscous before the 4th wash) add 10 µL 10 mg/mL DNase to the Mouse Digestion Medium at each new incubation.
    b. Continue making new washes until no ducts are visible in the washes anymore (usually at least 8 washes, for PanIN could be up to 12)
42. (Optional) Transfer 1-2 mL of a wash that contained a lot of ducts to a 15 mL tube to be able to plate an "unpicked fraction."
    a. These can serve as a backup if you are not confident in your duct picking.
43. Centrifuge 15 mL tubes containing picked ducts and "unpicked fraction" at 850 rpm (145 RCF) for 5 minutes at 4°C.
    a. Pellet from picked ducts will be very small, or may not be visible.

44. If you have more than 1x 15 mL conical tube with ducts from the same mouse, aspirate off most of the medium, use Wash Medium to pool material into 1x 15 mL tube, and centrifuge again.
45. Remove as much medium as possible: aspirate down to ~200 µL and use Pipetman to carefully remove the rest. (Residual medium will dilute the Matrigel and may prevent Matrigel domes from solidifying, so try to remove as much medium as possible without sucking up your pellet.)
46. Resuspend the pellet of picked ducts in Matrigel and plate 25 µL Matrigel domes in a pre-warmed 48-well tissue culture plate on top of a pre-warmed hot water flask.
    a. Typically, we resuspend the pellet in 125 µL Matrigel and make 5 x 25 µL wells in a 48 well plate, but the volume may vary depending on pellet size.
    b. More information about plating Matrigel domes can be found in the "Protocol for Passaging Mouse Organoids" on page 13.
47. (Optional) Resuspend the pellet of the unpicked fraction in Matrigel and plate multiple dilutions.
    a. Resuspend in 200 uL Matrigel and plate 2 x 25 uL domes. Then add 105 uL Matrigel to the remaining cells and plate 2 x 25 uL domes. Repeat for 3-4 dilutions.
    b. If you are trying to culture organoids without enriching for ducts, it is critical to find a dilution that keeps the ducts as dense as possible, while not having too many acinar cells present, which will destroy the culture. Plating multiple dilutions helps ensure that you find the right balance.
48. While keeping plate on hot water flask, carefully move plate and bottle into 37ºC incubator. Keep at 37ºC 5-15 minutes so Matrigel can harden.
49. Add Rho Kinase Inhibitor Y-27632 to the Mouse Complete Feeding Medium. You will need 250 µL medium per well plated. The 10.5 mM stock of Y-27632 is a 1000X stock.
50. After 5-15 minutes, remove 48-well tissue culture plate from incubator, and add 250 µL of warm/room temperature Mouse Complete Feeding Medium with Rho Kinase Inhibitor to each well.
51. Check plate in microscope – ducts should be visible in the Matrigel.
52. Return plate to incubator.

C. Post-Isolation

53. Within 1-2 days, organoids should start to bud out of ducts, as shown in Figure 3.
54. If mouse has PanIN, make sure to finish preparing cassettes for histology.
    In the Tuveson Laboratory, we incdubate histology cassettes overnight in 10% NBF. After 16-24 hours, carefully pour the 10% NBF off of the cassettes and add 70% ethanol to cover the cassettes. Cassettes should be brought to histology within 1-2 days of placing them in 70% ethanol. P and T organoids must have PanIN or Tumor histology confirmed on tissue section.
55. Change medium after 3-4 days, depending on how fast organoids grow. Rho Kinase Inhibitor is not needed in medium after the initial isolation.
56. Let organoids grow for ~ 1 week before passaging. For first passage after isolation:
    a. Do not scrape bottom of plate under Matrigel; leave some Matrigel on bottom to avoid contaminating next passage with fibroblasts that have attached to the plate.
    b. Harvest all wells, pool, and freeze down the equivalent of one well into 2 cryovials as passage 0.5 Expand the rest of the organoids 1:4 or 1:8 (depending on density) in unused wells of the same plate or a new plate.
    c. See the "Protocol for Passaging Mouse Organoids" on page 13 and the "Protocol for Freezing Mouse Organoids" on page 15 for information on passaging and freezing.
    d. The Tuveson Lab convention is that organoids initially isolated from a mouse are passage 0, and that when organoids are frozen they are given a passage number halfway between the passage that they came from and the passage that they will be upon thawing.

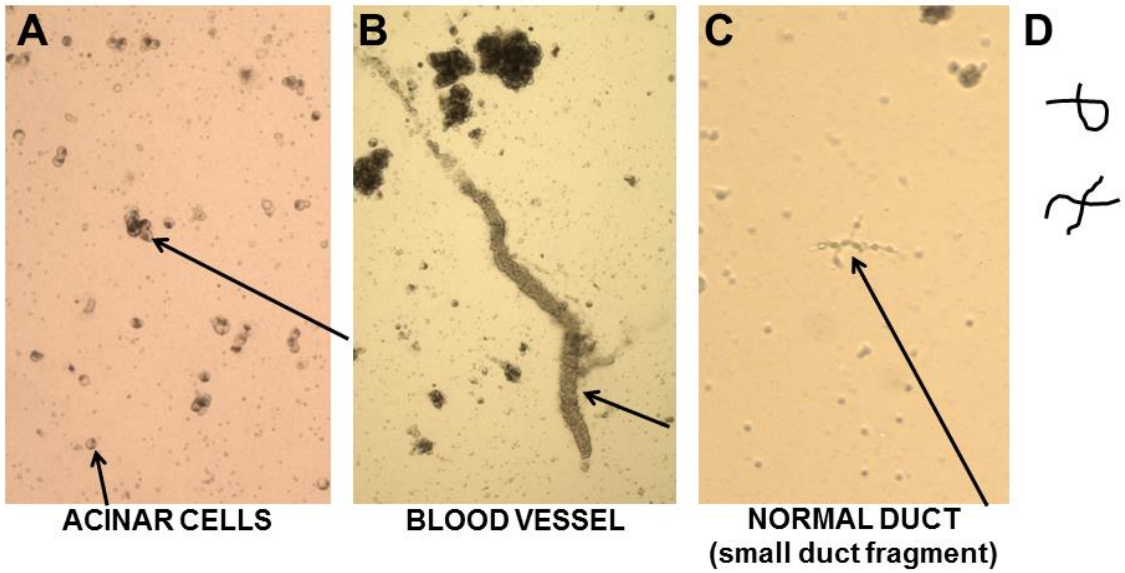

Figure 2. High magnification images of cell types found in pancreas digestion viewed with phase contrast microscope.
A. Arrows denote acinar cells, which are large cells, may come in clumps, and are often more refractile. **B.** Arrow indicates a blood vessel, which is generally larger than most ducts. Unlike most ducts, the lumen of the blood vessel is often visible, and red blood cells may be visible inside the vessel. **C.** Arrow indicates a typical small pancreatic duct fragment. Note that these images were taken with a higher magnification objective, and ducts will look smaller in the 4x objective. **D.** Small duct fragments often have lariat or cross structures, as diagramed here.

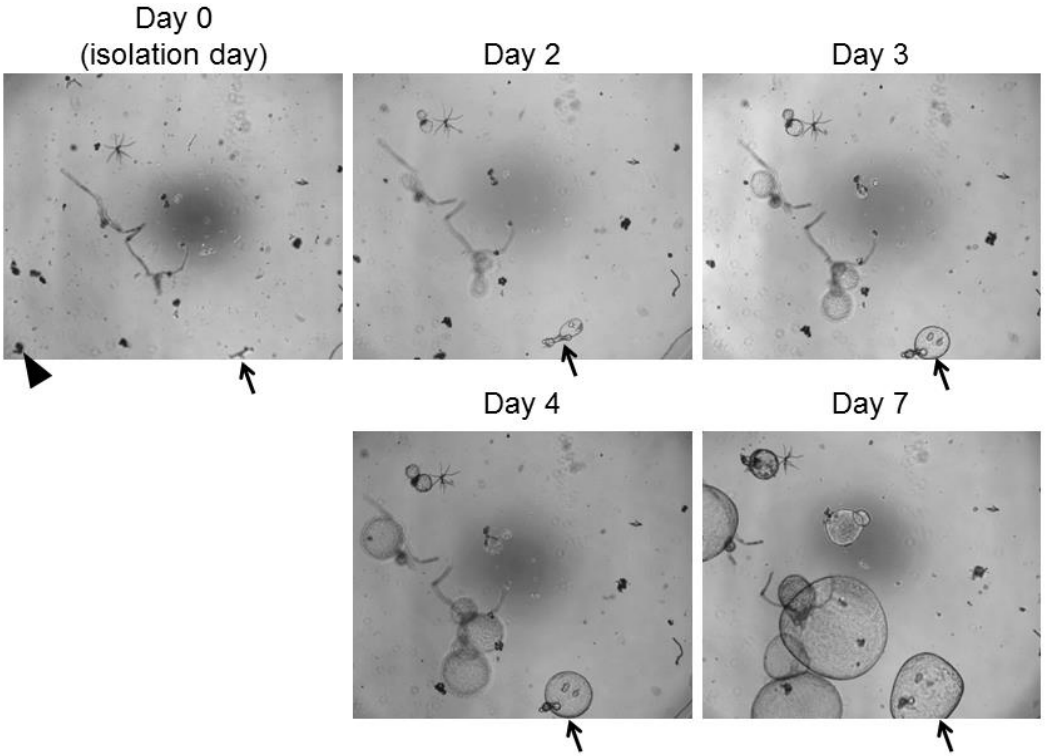

Figure 3. Murine normal organoid formation following duct picking.
Images of a single well immediately following duct isolation and plating, as well as 2, 3, 4, and 7 days post-isolation. Arrowhead indicates an acinar cell, which looks dark in the Matrigel. Arrow indicates a typical small duct, with branched structure, and the organoid that grows out from it.

Protocol for Isolation of T and M Organoids from Mouse Pancreatic Tumors and Metastases

A. Isolation of Murine Tumors and Metastases

Reagents and Equipment (have ready in the necropsy room):

Mouse bearing pancreatic tumor	70% Ethanol in spray bottle
15 mL conical tubes with 10 mL Mouse Wash Medium on ice	10% Neutral Buffered Formalin (NBF) for histology
Blue pad	Paper towels
Dissection board	Marker
Strong scissors for decapitation	#10 Scalpels
2 Pairs dissection scissors	3 Sets dissection forceps
1.5 mL tube for mouse tail	Petri dish (not tissue culture plate)
Pencil to mark histology cassettes	Histology cassettes
(for blood collection) Heparin tube	(for blood collection) Animal lancet
Histology worksheet	Ice bucket with ice
CO_2 chamber with CO_2 hookup and regulator	Digital scale
Hot bead sterilizer for dissection tools	(for blood collection) Refrigerated microfuge

Procedure:

1. Before you isolate the tumors and metastasis, prepare the tissue culture hood, and the Mouse Wash and Digestion Media as described in part B of this protocol.
2. Prepare necropsy area and histology cassettes as described in the “Protocol for Isolation of Organoids from Normal or PanIN-Containing Murine Pancreas,” on page 4.
3. Optional: before sacrificing mouse, using an animal lancet, perform a submandibular bleed on mouse. Collect blood in a tube containing heparin. Spin tube for 2 minutes at 10,000 rpm at 4^{o}C in a microfuge, and pipette supernatant (plasma) into new tube. Flash freeze plasma and store at -80^{o}C.
4. Sacrifice mouse in CO_2 chamber:
    a. Turn the CO_2 on very slowly to keep the mouse as comfortable as possible.
    b. Watch the mouse the entire time.
    c. The mouse should go from active, to inactive but breathing normally, to making quick gasps, to making heaving gasps/spasms with longer pauses in between.
    d. After 2 or 3 heaving gasps remove mouse from chamber.
5. Spray mouse with ethanol.
6. Skin the mouse.
    a. Locate the mouse’s sternum (where the chest bone sticks out).
    b. Pull the fur up, and make a sizeable cut through the skin (but not through peritoneum).
    c. Cut skin towards back side of mouse.
    d. With two fingers of each hand, pinch the skin on both sides of the opening and pull the skin towards the head and tail until entire midsection is exposed.
7. Cut open the peritoneum near the genitals, and cut to expose the intestines and pancreas.
8. Switch to clean dissection tools.
9. Dissect out tumor and place on a Petri dish on ice.
10. In the Petri dish, cut a central piece of the tumor and place in a labeled cassette for histology. Place cassette in a container containing 10% NBF.

11. Mince the tumor in a Petri dish on ice, and move to a 15 mL conical tube containing Mouse Wash Medium.
12. Prepare the rest of the mouse organs for histology. Check carefully for metastases (mets), and if a met is located, send a portion of it for histology, and use a portion of it to make an “M” organoid (see step 21 in part B of this protocol).
13. Cut off a piece of the mouse’s tail and save in a 1.5 mL tube to confirm genotype.
14. Bring the tubes of tumor (and mets) on ice to the tissue culture hood.

B. Digestion of Tumors and Metastases

Reagents and Equipment:

DMEM + glucose, L-glutamine, sodium pyruvate
100X Penicillin/Streptomycin
Collagenase Crude Type XI (from *Clostridium histolyticam)*
TrypLE Express
RhoK Inhibitor (Y-27632)
(for generating 2D line) 2D Cell Culture Medium
Ice bucket with ice
Sterile serological pipettes (5, 10, and 25 mL)
Sterile P20, P200, P1000 Pipetmen with tips
48-well tissue culture plate
Marker
Thermomixer or incubated rocker/rotator set to 37ºC
Pipet-Aid/Pipette controller
37ºC water bath
Tissue culture hood
100% FBS
Dispase II
10 mg/mL DNase I
Mouse Complete Feeding Medium
Mouse Splitting Medium (+++)
Growth Factor Reduced Matrigel, Phenol Red Free on ice
Sterile glass bottle
Sterile aspirator pipettes
15 and 50 mL conical tubes
Hot water flask pre-warmed to 37ºC
Timer
Refrigerated 15/50 mL tube centrifuge
Tissue culture aspirator
Tissue culture incubator

Procedure:

15. At least 6 hours before organoid isolation, place a 48-well plate and a hot water flask in a 37ºC tissue culture incubator to warm up.
16. Prepare Mouse Wash Medium
    500 mL DMEM + glucose, L-glutamine, sodium pyruvate
    5 mL 100X Penicillin/Streptomycin
    5 mL 100% FBS
17. Prepare Mouse Digestion Medium and allow it to warm to room temperature.
    a. Weigh 25 mg Collagenase Crude Type XI and 25 mg Dispase II into a 50 mL conical tube (We typically do this outside of Tissue Culture hood, so these reagents are not technically sterile, but we have not had any contamination problems.)
    b. Inside Tissue Culture Hood: add 50 mL Mouse Wash Medium to 50 mL tube containing enzymes, mix and pour into sterile glass bottle.
    c. Repeat 3 more times with 50 mL Mouse Wash Medium to bring final volume to 200 mL.
18. Thaw aliquots of 10 mg/mL DNase I and Matrigel on ice.
19. Place Mouse Complete Feeding Medium in 37ºC water bath to warm.
20. Allow the tumor pieces to settle, and aspirate the fat as described in Part B of the “Protocol for Isolation of Organoids from Normal or PanIN-Containing Murine Pancreas” on page 6.
21. Add 10 mL Mouse Digestion Medium and incubate with gentle agitation/rocking/rotating at 37ºC for 2-4 hours (for tumors and large mets) and 1 hours (for small mets).

22. Remove digested tumor from 37ºC.
23. Pipet up and down with 10 mL pipette to break up chunks.
24. Let pancreas settle by gravity.
25. Transfer 8.5 mL of supernatant to new tube.
26. Centrifuge this tube at 1000 rpm (200 RCF) for 5 minutes at 4ºC.
27. Aspirate the supernatant.
28. Resuspend the pellet in 1 mL TrypLE Express and 1 µL 10 mg/mL DNase I.
29. Incubate at 37ºC in with gentle agitation for 10 min.
30. Add 10 mL Mouse Splitting Medium (+++) to the digested pancreas.
31. To make a 2D cell line, take 1 mL of pancreas digest and add to 10 cm tissue culture dish containing 9 mL 2D Cell Culture Medium.
    d. Once 2D cell line is established, 2D Cell Culture Medium with 5% FBS can be used to slow growth.
32. Spin remaining pancreas digest at 1000 rpm (200 RCF) for 5 min at 4ºC.
33. Aspirate medium, resuspend pellet in Matrigel, and plate cells as described in steps 17-25 of the "Protocol for Passaging Mouse Organoids" on page 14. Be sure to include Rho Kinase Inhibitor in the Mouse Complete Feeding Medium.

C. Post-Isolation

34. Over the next 2-3 days, small spheres should begin to emerge among the cells plated (Figure 3).
35. At the first passage, there may be fibroblasts at the bottom of the wells. When passaging the first 1-2 times, avoid scraping all the way to the bottom of the well with your pipette tip, to avoid passaging the fibroblasts.

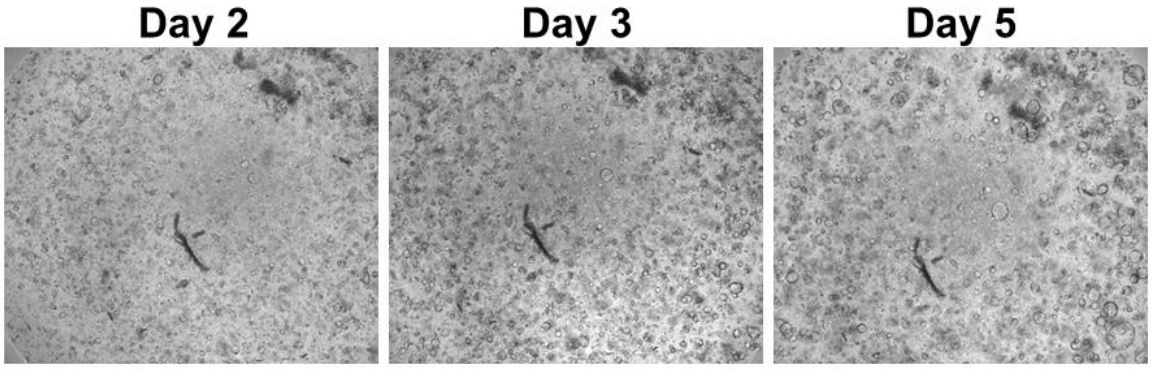

Figure 4. Formation of murine tumor organoids following isolation.
Images of a single well 2, 3, and 5 days following tumor digestion and plating. Small spheres are visible as soon as 2 days following plating.

Protocol for Passaging Mouse Organoids

Notes:

Typical split ratios are between 1:4 and 1:8, depending on organoid confluency. Organoids are typically ready to passage again 3-8 days after passaging. Organoids should be split if any of the following are true:

1. Organoids are very dense in Matrigel
2. Organoids have grown very large (>20% the diameter of a 50 µL Matrigel dome)
3. Matrigel has become very “squishy” and no longer appears firm.

If organoids are kept too long in culture without passaging, they will begin to die in culture, and portions of organoid will appear dark (especially when viewed with phase-contrast). While this is not ideal, some organoids can usually be rescued by passaging such wells.

The traditional passaging protocol uses fire-polished pipettes to disrupt organoids. However, there are several alternatives to using fire-polished pipettes. First, organoids can be broken up during the initial step to disrupt the Matrigel domes: the P1000 tip is angled against the bottom of the well to create a smaller opening through which organoids, Matrigel, and medium are pipetted. Alternatively organoids can be pipetted through a filter-free P10 tip lodged onto the end of a P1000 tip or pipetted rapidly through a P200 tip. Finally, a 22- or 23-gauge needle attached to a 5 mL syringe can be used.

Reagents and Equipment:

- Mouse Splitting Medium (+++) on ice
- Matrigel on ice
- Ice bucket with ice
- 24- or 48- well tissue culture plate
- Sterile serological pipettes (5, 10, and 25 mL)
- Fire-polished glass pipette
- 37ºC tissue culture incubator
- Refrigerated 15/50 mL tube centrifuge
- Mouse Complete Feeding Medium at 37ºC
- Sterile aspirator pipettes
- P1000 and P200 Pipetmen and sterile tips
- 15 mL conical tube on ice
- Hot water flask pre-warmed to 37ºC
- Timer
- Pipet-Aid/Pipette controller
- Tissue culture aspirator

Procedure:

1. Up to 2 weeks before you begin, make Mouse Complete Feeding Medium. Up to 4 weeks in advance, make Mouse Splitting Medium (+++).
2. At least 6 hours in advance, place a hot water flask and a 24- or 48-well tissue culture plate in the tissue culture incubator to warm up.
3. Thaw Matrigel on ice. (A 1000 µL aliquot takes ~1 hour 30 minutes to thaw. A complete Matrigel vial takes ~10-16 hours to thaw.)
4. Just before starting, place Mouse Complete Feeding Medium in 37ºC water bath to warm up. Mouse Complete Feeding Medium must be at least room temperature when you add it to Matrigel domes, or it will cause the Matrigel to melt.
5. If using fire-polished pipettes to break up organoids, make these ahead of time.
6. Prepare a 15 mL conical tube with 8 mL Mouse Splitting Medium (+++) to receive organoids and place on ice.
7. Aspirate medium from organoid wells to be split.
8. Add 500 µL (for 24-well plate) or 250 µL (for 48-well plate) ice-cold Mouse Splitting Medium (+++) to each well to be split, and pipette up and down and scrape the bottom of the well to dislodge the Matrigel and break it up.

9. Transfer the medium containing the broken-up Matrigel and organoids to an ice-cold 15 mL conical tube containing 8 mL ice-cold Mouse Splitting Medium (+++).
10. Use additional ice-cold Mouse Splitting Medium (+++) to wash organoid wells and to fill conical tube containing old Matrigel/organoids to 10 mL.
11. Spin conical tube containing old Matrigel/organoids at 850-1500 rpm (145-250 RCF) for 5 min at 4ºC.
12. Aspirate the supernatant, keeping the tip of the aspirator at the top of the liquid, until approximately 1.5 mL of liquid remain.
13. Attach fire-polished pipette to tissue culture Pipet-Aid, and pipette organoids up and down through pipette 7-12 times to break them up. Initially, you should see large particles coming up into pipette which should get smaller as you pipette them up and down.
14. Fill 15 mL conical tube to 10 mL with ice-cold Mouse Splitting Medium (+++).
15. If you do not plan to use all organoids for split, discard appropriate amount of organoids (or move to another tube for freezing), and refill tube to 10 mL with ice-cold Mouse Splitting Medium (+++).
    a. For example, if you are splitting 1 well of a 24 well plate 1:8, and plan to make 4 new wells in a 24-well plate, discard 5 mL / 10 mL of organoids at this step.
16. Centrifuge organoids at 850-1000 rpm (145-200 RCF) for 5 minutes at 4ºC.
17. Carefully aspirate off as much medium as possible, removing the final ~200 µL with a P200 Pipetman.
18. Resuspend organoid pellet in Matrigel.
    a. Use 50 µL Matrigel per new well of 24-well plate or 25 µL Matrigel per new well of 48-well plate.
    b. Keep Matrigel tube and organoid tube on ice the entire time.
    c. Be very careful not to produce bubbles when pipetting Matrigel.
19. Remove new 24- or 48-well plate, and place on top of a pre-warmed hot water flask in the tissue culture hood.
20. While keeping the cell/Matrigel mixture on ice, gently mix the suspension with a Pipetman and take 50 µL (for one well of 24-well plate) or 25 µL (for one well of a 48-well plate) of the mixture into the tip. Take care to keep the tube containing the Matrigel/cell suspension on ice, or the cells and Matrigel may start to solidify before you plate them.
21. Touch the pipette tip to the center of a well of the tissue culture plate, and push down pipette plunger while slowly pulling Pipetman up. The Matrigel and organoids should form a dome in the center of the well. To avoid forming bubbles, do not push Pipetman plunger to second stop.
22. Carefully bring 24- or 48-well plate on top of hot water flask back to 37ºC tissue culture incubator, and incubate at 37ºC for 5-15 minutes to allow Matrigel to harden.
23. Bring plate back to tissue culture hood and add 500 µL (for wells in a 24-well plate) or 250 µL (for wells in a 48-well plate) of pre-warmed Mouse Complete Feeding Medium to each well.
24. Check wells in phase-contrast microscope. You should see small pieces of organoids distributed throughout the Matrigel.
25. Return the plate containing organoids to 37ºC tissue culture incubator.
26. After 2-4 days, organoids will begin to grow quickly, and medium will change color. Change medium at this point.

Protocol for Freezing Mouse Organoids

Notes:
Typically, we split 1 well of a 24-well plate into 2-4 cryovials, depending on density.

Reagents and Equipment:

- Mouse Splitting Medium (+++) on ice
- Fire-polished pipettes
- P1000 and P200 Pipetmen and sterile tips
- Ice bucket with ice
- Sterile serological pipettes (5 and 10 mL)
- Cell freezing chamber or 2 styrofoam tube racks
- Refrigerated 15/50 mL tube centrifuge
- -80^{o}C freezer
- Recovery Cell Culture Freezing Medium
- 15 mL conical tubes on ice
- Cryovials
- Sterile aspirator pipettes
- Tissue culture aspirator
- Tissue culture hood
- Cryofreezer

Procedure:

1. Follow the procedure for harvesting organoids and mechanical dissociation detailed in steps 5-14 of the “Protocol for Passaging Mouse Organoids” on page 13.
2. If planning to passage and freeze organoids, divide resuspended organoids into separate 15 mL conical tubes, one containing organoids to be split and one containing organoids to be frozen. Set aside organoids to be passaged on ice.
3. Centrifuge organoids to be frozen at 850-1000 rpm (145-200 RCF) for 5 minutes at 4^{o}C.
4. Carefully aspirate off as much medium as possible, removing the final ~200 µL with a P200 Pipetman.
5. Resuspend organoids in 500 µL Recovery Cell Culture Freezing Medium per intended cryovial, and transfer organoids and Freezing Medium to cryovials.
6. Move cryovials into isopropanol cell freezing chamber, and screw lid onto top. (Don’t screw lid on too far or chamber will be difficult to open after freezing!) Alternatively, move cryovials into a 15 mL conical tube Styrofoam rack, cover with a second Styrofoam tube rack, and tape Styrofoam racks together.
7. Incubate freezing chamber with cells at -80^{o}C for 24 hours.
8. If a portion of organoids was set aside for splitting, proceed with step 16 of the “Protocol for Passaging Mouse Organoids” on page 14.
9. The next day, transfer cryovials to liquid nitrogen cryofreezer.

Protocol for Thawing Mouse Organoids

Notes:
It is important to have 10.5 µM (3.38 µg/mL) Rho Kinase Inhibitor in the Mouse Complete Feeding Media when organoids are thawed to help cells cope with the stress of thawing.

Reagents and Equipment:

- Cryovial of frozen organoids
- Growth Factor-Reduced Matrigel, Phenol Red Free on ice
- Mouse Complete Feeding Media
- 15 mL conical tube on ice
- Glass aspirator pipettes
- Sterile serological pipettes (10 mL)
- 37ºC water bath
- Refrigerated 15/50 mL tube centrifuge
- Tissue culture hood
- Tissue culture incubator
- Mouse Splitting Medium (+++) on ice
- 10.5 mM (1000X) Rho Kinase Inhibitor
- 24- or 48- well tissue culture plate
- P1000 and P200 Pipetman and tips
- Hot water flask pre-warmed to 37ºC
- Ice bucket with ice
- Glass aspirator pipettes
- Timer
- Pipet-Aid/Pipette controller
- Tissue culture aspirator

Procedure:

1. At least 6 hours before thawing, place a tissue culture plate and a hot water flask in the tissue culture incubator to warm.
2. Thaw Matrigel on ice. (A 1000 µL aliquot takes ~1 hour 30 minutes to thaw. A complete Matrigel vial takes ~10-16 hours to thaw.)
3. Just before starting, place Mouse Complete Feeding Medium in 37ºC water bath to warm up. Mouse Complete Feeding Medium must be at least room temperature when you add it to Matrigel domes, or it will cause the Matrigel to melt.
4. Prepare a 15 mL conical tube with 10 mL Mouse Splitting Medium (+++) and place on ice.
5. Remove cryovial containing organoids from liquid nitrogen freezer and thaw quickly in a 37ºC water bath.
6. Pipette thawed cells into 15 mL conical tube with Mouse Splitting Medium (+++).
7. Centrifuge organoids at 850-1000 rpm (145-200 RCF) for 5 minutes at 4ºC.
8. Carefully aspirate off as much medium as possible, removing the final ~200 µL with a P200 Pipetman.
9. Resuspend organoid pellet in Matrigel and plate and feed as described in steps 18-25 of the “Protocol for Passaging Mouse Organoids” on page 14.
    a. Depending on size of pellet, make 1-3 wells of organoids. When in doubt, make 1 well. If organoids are too sparse, they will have difficulty recovering from thaw.
    b. Be sure to add Rho Kinase Inhibitor to the Mouse Complete Feeding Medium
10. Depending on how quickly the organoids grow, change media 2-4 days after thawing. Once organoids have formed and begun to grow, Rho Kinase Inhibitor is no longer needed in the Mouse Complete Feeding Medium.

Media and Solutions for Murine Organoid Protocols

For more information on organoid culture reagents, please see page 50. Please see page 70 for suppliers and catalog numbers.

Mouse Wash Medium

Volume	Stock Conc.	Medium Component	Final Conc.
500 mL		DMEM + glucose, L-glutamine, sodium pyruvate	
5 mL	100 X	Penicillin/Streptomycin	1 X
5 mL	100 %	Fetal Bovine Serum (FBS)	1 %

Used for organoid isolation protocols.
Need ~400 mL for N or P organoid isolation (200 for Mouse Digestion Medium + 200 for washes).
Need ~200 mL for T or M organoid isolation (for Mouse Digestion Medium).
Can be prepared in advance and stored at 4ºC for up to 1 month.
Unused Mouse Wash Medium can be stored at 4ºC and used for next isolation.

Mouse Digestion Medium

Volume or Mass	Medium Component	Final Conc.
200 mL	Mouse Wash Medium	
25 mg	Collagenase Crude Type XI	0.125 mg/mL
25 mg	Dispase	0.125 mg/mL

Used for digesting murine pancreas or tumor.
Make this fresh before use.
Need 100 mL per mouse for N or P organoid, and 10 mL for T or M organoid, but it is difficult to make less than 200 mL at a time.

Mouse Splitting Medium (+++)

Volume	Stock Conc.	Medium Component	Final Conc.
485 mL		Advanced DMEM/F-12	
5 mL	1 M	HEPES pH 7.2-7.5	10 mM
5 mL	100 X	Penicillin/Streptomycin	1 X
5 mL	100 X	GlutaMAX Supplement	1 X

Used for passaging murine organoids.
Can be made in advance and stored at 4ºC for up to 2 months.

Mouse 2D Cell Culture Medium

Volume	Stock Conc.	Medium Component	Final Conc.
445 mL		DMEM + glucose, L-glutamine, sodium pyruvate	
50 mL	100 %	Fetal Bovine Serum (FBS)	10 %
5 mL	100 X	Penicillin/Streptomycin	1 X

Can make in advance and store at 4ºC.
Once cell lines are established, may want to switch to 2D medium with 5% FBS instead of 10% to slow cell growth.

Mouse Complete Feeding Medium with Rho Kinase Inhibitor

Volume	Stock Conc.	Stock Conc.	Medium Component	Final Conc.
18 mL			Mouse Splitting Medium (+++)	
20 µL	1000X	0.5 mM (0.21 mg/mL)*	A 83-01*	0.5 µM (0.21 µg/mL)
20 µL	1000X	50 µg/mL*	mEGF*	0.05 µg/mL
20 µL	1000X	0.1 mg/mL*	hFGF-10*	0.1 µg/mL
20 µL	1000X	10 µM (0.021 mg/mL)*	hGastrin I*	0.01 µM (0.021 µg/mL)
20 µL	1000X	100 µg/mL	mNoggin	0.1 µg/mL
50 µL	400X	500 mM (81.5 mg/mL)	N-acetylcysteine	1.25 mM (0.2 mg/mL)
200 µL	100X	1M (122 mg/mL)	Nicotinamide	10 mM (1.22 mg/mL)
2 mL	10X		R-Spondin1-Conditioned Medium	1X
400 µL	50X		B27 Supplement	1X

* 1000X working stocks of A 83-01, mEGF, hFGF-10, and hGastrin I are prepared by diluting 10,000X frozen stocks – see “More Information on Organoid Culture Reagents” on page 50 for more information.

** 20 µL of 1000X Y-27632 stock solution (10.5 mM or 3.4 mg/mL stock concentration) should be added to the medium (final concentration 10.5 µM or 3.4 µg/mL) when organoids are first isolated, when organoids are thawed, or when organoids are dissociated to single cells.

Make up to 2 weeks in advance and store at 4^{o}C.

Protocol for Establishing Human Normal Pancreatic Organoid Cultures from Digested Pancreas Tissue

Notes:

This protocol describes methods to establish cultures of normal organoids from a suspension of exocrine cell aggregates that are leftovers after purification of human islets from an Islet Cell Transplant Center. All human experiments are approved by the IRB of Cold Spring Harbor Laboratory, and written informed consent from the donors for research use of tissue is obtained prior to acquisition of the specimen. The cell suspension is usually shipped on ice in a solution of University of Wisconsin solution (UW solution). All steps should be performed on ice, unless otherwise specified.

Reagents and Equipment:

- Human exocrine pancreas suspension
- Human Wash Medium with 0.1% BSA (h+++B)
- Prostaglandin E2 (PGE2) Stock (10 mM)
- Rho kinase Inhibitor Stock (Y-27632, 10.5 mM)
- Cryovials
- Sterile 5, 10, and 25 mL serological pipettes
- Sterile aspirator pipettes
- Mr. Frosty freezing container
- Hot water flask pre-warmed to 37ºC
- Refrigerated 15/50 mL tube centrifuge
- -80ºC freezer
- Tissue culture hood with aspirator
- Human Complete Feeding Medium
- Growth Factor Reduced Matrigel, Phenol Red Free on ice
- Recovery Cell Culture Freezing Medium
- Ice bucket with ice
- Pipetmen (p20, p200, p1000) and sterile tips
- 15 mL and 50 mL conical tubes
- Pipet-Aid/Pipette controller
- 5 Pre-warmed 24 well culture plates
- 37ºC water bath
- Liquid nitrogen cryofreezer
- 37ºC tissue culture incubator

Procedure:

1. At least 6 hours before organoid isolation, place a 24-well plate in a 37ºC tissue culture incubator to warm up.
2. Upon delivery of the specimen, place the tube containing the cell suspension on ice.
3. Record all information about the specimen, including, but not limited to, date of procedure, providing institution, and institution ID.
4. Place Human Complete Feeding Medium at 37ºC to warm.
5. Divide the cell suspension into two 50 mL conical tubes.
6. Centrifuge tubes at 200 RCF for 5 minutes at 4ºC.
7. Carefully remove the supernatants without disturbing the pellets.
8. Resuspend both pellets in a total of 40 mL Human Wash Medium with 0.1% BSA (h+++B).
9. Divide the resuspended material equally among four 15 mL conical tubes.
10. Centrifuge tubes at 200 RCF for 5 minutes at 4ºC.
11. Wash the pellets twice more using 10 mL Human Wash Medium with 0.1% BSA (h+++B), making sure to break up the pellets by multiple triturations (approximately 15 times).
12. Following the second wash and centrifugation, examine the pellets, choose the tube with the least amount of specimen, and aspirate as much medium as possible.
13. Resuspend this pellet in 6 mL Matrigel, while avoiding making bubbles.
14. Plate cell suspension as 50 µL domes on pre-warmed 24 well plates. To aid in dome solidification, plates can be placed on top of a hot water flask, while you are pipetting the domes.

15. Place the plate into a 37°C tissue culture incubator until Matrigel solidifies (~10 minutes).
16. While the Matrigel domes are solidifying, aspirate the supernatant from the remaining three 15 mL tubes.
17. Resuspend the 3 pellets in Recovery Cell Freezing Medium, combining all the pellets into a single conical tube and a total volume of 25 mL Freezing Medium.
18. Aliquot 1 mL of the cell suspension into each of 25 cryovials.
19. Place cryovials in a Mr. Frosty freezing container and freeze cells overnight at -80°C.
    The cryovials should be labeled with the organoid ID, "p0" (indicating that they contain primary tissue), the date, and your initials.
    After 24 hours, frozen cells can be moved to a liquid nitrogen freezer for long-term storage.
20. Add Rho Kinase Inhibitor (Y-27632) and PGE2 (to final concentrations of 10 µM and 1 µM, respectively) to pre-warmed Human Complete Feeding Medium as described in "Media and Solutions for Human Organoid Protocols" on page 33. You will need 500 µL of medium per well plated.
21. Add 500 µL of pre-warmed Human Complete Feeding Medium supplemented with Y-27632 and PGE2 to each well.
22. Return organoids to 37°C tissue culture incubator.

Protocol for Establishing Human Pancreatic Organoid Cultures from a Resected Tumor Specimen or an Adjacent Normal Specimen

Notes:

This protocol describes methods to establish tumor organoid cultures from human pancreatic cancer specimens. These specimens are obtained from surgical resections of pancreatic neoplasms and are usually delivered in a 5 mL LoBind tube containing Human Wash Medium (h+++ with or without BSA) or RPMI supplemented with GlutaMAX, HEPES, and Primocin. The specimens should be shipped on wet ice (not dry ice) and can be shipped overnight (to arrive in the lab the day after surgery). The specimens should be processed as soon as possible to prevent autolysis or degradation. Except for digestions, all procedures should be performed on ice. The protocol is written for generating organoids from tumor specimens, but the same procedure can be followed to generate organoids from adjacent normal tissue as long as the Human Complete Feeding Medium is supplemented with 1 µM PGE2.

All human experiments are approved by the IRB of Cold Spring Harbor Laboratory, and written informed consent from the donors for research use of tissue is obtained prior to acquisition of the specimen. All the procedures should be performed on ice (unless otherwise specified) under sterile conditions by using sterile tools and by operating in a biological hood.

Reagents and Equipment:

- Human tumor (or adjacent normal) specimen
- 10.5 mM Rho Kinase Inhibitor (Y-27632) Stock
- Human Wash Medium with 0.1% BSA (h+++B)
- Human Digestion Medium
- Tissue-Tek® O.C.T. Compound
- Ice bucket with dry ice
- Sterile scalpels
- Sterile forceps
- P2, P20, P200, P1000 pipettes and sterile tips
- (For CAF isolation) 6 well culture dish
- 5 mL Protein LoBind Tubes
- Sterile serological pipettes (5, 10, and 25 mL)
- Timer
- Mr. Frosty freezing container
- 37ºC water bath
- Tissue culture hood with aspirator
- Hot water flask pre-warmed to 37ºC
- 37ºC tissue culture incubator
- Recovery Cell Culture Freezing Medium
- Human Complete Feeding Medium
- 10 mg/mL DNAse I stock
- Growth Factor Reduced Matrigel, Phenol Red Free on ice
- ACK (Ammonium-Chloride-Potassium) Lysing Buffer
- (For CAF isolationn) Human CAF Medium
- Ice bucket with ice
- Sterile 10 cm tissue culture dish
- Sterile aspirator pipettes
- Cryovials
- 15 and 50 mL conical tubes
- Pre-warmed 24 well culture plates
- Truncated Embedding Mold, size 12x12mm (for O.C.T)
- Small dewar with liquid nitrogen
- Pipet-Aid/Pipette controller
- -80ºC freezer
- Refrigerated 15/50 mL tube centrifuge
- Incubated rocker/rotator set to 37ºC
- Liquid nitrogen cryofreezer

Procedure:

1. At least 6 hours before organoid isolation, place a 24-well plate in a 37ºC tissue culture incubator.
2. Thaw an aliquot of the Human Digestion Medium in the 37ºC water bath and bring temperature to 25-37ºC. You will need 9 mL Digestion Medium per specimen.
3. Add 10 µL of 10 mg/mL DNAse and 10 µL 10.5 mM Y-27632 to the 10 mL aliquot of Human Digestion Medium (to final concentrations of 10 µg/mL and 10.5 µM, respectively).
4. Aliquot 3 mL of pre-warmed Human Digestion Medium supplemented with DNAse and Y-27632 to a 5 mL protein LoBind tube.
   The DNAse stock sometimes comes out of solution, so the tube should be mixed just before adding to the Digestion Medium.

5. Record all the information available for the specimen, including: nosography, hospital, sample identifier, date, and the names of the people processing the sample.
6. Using sterile forceps, transfer the specimen to a sterile tissue culture dish.
7. Examine the tissue macroscopically, and record the size, shape and gross morphological characteristics.
   Since the handle of the scalpel contains centimeter markings, this can be used to measure the specimen. Typically, we keep the specimen inside of the tissue culture dish with the lid on, and hold the scalpel handle up to the outside of the dish for comparison.
8. If adjacent normal tissue is visible on tumor specimen, carefully dissect this tissue away from the tumor tissue.
9. If the specimen is large enough, take a central slice and embed in O.C.T. fluid. Place embedded tissue on dry ice to freeze, and store at -80ºC for subsequent cryosectioning.
10. Mince the remaining specimen into small fragments (1 mm^3 or less) using sterile scalpels.
    a. If there is fat present, try to dissect it away from the specimen.
    b. Note that pancreatic tumors are highly fibrotic, and therefore, the tissue is usually hard.
11. Take a few pieces and place into a cryovial, labelled with the organoid ID, “p0” (indicating that vial contains primary tissue), the date, and your initials. Snap freeze cryovial in liquid nitrogen.
12. Transfer remaining tissue to 5 mL protein LoBind tube containing the 3 mL Human Digestion Medium supplemented with DNAse and Y-27632.
13. Place the tube in rotating incubator set to 37ºC with rapid rotation (35 rpm) for an initial digestion of 15 minutes.
14. Using a P1000 pipette set to 1000 uL, prewet the tip by pipetting Human Wash Medium with 0.1% BSA (h+++B) a few times, and then triturate the digested tumor 10 times.
15. Allow the tube to sit for 1 minute so the larger tissue pieces can settle to the bottom.
16. Transfer the supernatant in a separate LoBind tube, labeled “Fraction 1.”
17. Add Human Wash Medium with 0.1% BSA (h+++B) to Fraction 1 to fill tube to 5 mL total volume.
18. Centrifuge Fraction 1 at 200 RCF for 5 minutes at 4ºC.
19. Carefully remove the supernatant from Fraction 1 without disturbing the cell pellet.
20. Resuspend the Fraction 1 pellet in 2 mL Human Wash Medium with 0.1% BSA (h+++B) and store on ice.
21. Add 3 mL Human Digestion Medium supplemented with DNAse and Y-27632 to the remaining undigested tissue pieces leftover from the first digest.
22. Repeat steps 13-21 to acquire “Fraction 2,” and (if there is sufficient undigested material to continue) “Fraction 3,” but at step 20, resuspend the pellets from each of these fractions in 1 mL Human Wash Medium with 0.1% BSA (h+++B).
23. Combine all fractions together in one 5 mL protein LoBind tube, and fill tube to 5 mL with Human Wash Medium with 0.1% BSA (h+++B).
24. Add 5 mL Human Wash Medium with 0.1% BSA (h+++B) to the remaining, undigested tissue fragments.
25. Centrifuge the tube of combined fractions and the tube of undigested tissue fragments at 200 RCF for 5 minutes at 4ºC.
26. Place Human Complete Feeding Medium at 37ºC to warm.
27. Carefully remove the supernatants from each tube without disturbing the pellets.

28. In very rare cases, large numbers of red blood cells might be seen in the pellet of combined fractions, coloring entire pellet red. If this is the case, perform ACK Lysis of red blood cells as follows. If no or minimal numbers of red blood cells are present, proceed with the next step.
    a. Add 4 mL of room temperature ACK lysing buffer and let sit at room temperature for 1 to 2 minutes.
    b. Invert and flick tube to resuspend the pellet and mix.
    Do not pipette up and down or cells will stick to the pipette.
    c. Centrifuge the tube at 200 RCF for 5 min at 4ºC.
    d. Carefully aspirate the supernatant without disturbing the pellet.
    e. Resuspend cells in 1 mL Human Wash Medium with 0.1% BSA (h+++B) and then fill tube to 5 mL with additional 4 mL Human Wash Medium with 0.1% BSA (h+++B) to wash.
    f. Centrifuge the tube at 200 RCF for 5 min at 4ºC.
    g. Carefully aspirate the supernatant without disturbing the pellet.
29. Resuspend the pellet of combined fractions in Matrigel. The volume of Matrigel to use will depend on the size of the pellet. It is best to err on the side of resuspending the pellet in less Matrigel to keep the cells concentrated, rather than diluting the material too much. As a ballpark estimate, a tumor pellet around 25 µL in volume would be resuspended in 800 µL Matrigel.
30. Plate 50 µL Matrigel domes into a pre-warmed 24 well plates. To aid in dome solidification, plates can be placed on top of a hot water flask, while you are pipetting the domes.
31. Place the plate into a 37ºC tissue culture incubator until Matrigel solidifies (~10 minutes).
32. Resuspend the pelleted undigested tissue fragments in Recovery Cell Freezing Medium. (The volume to resuspend in depends on the volume of the pellet.)
33. Aliquot the tissue fragments to cryovials.
34. Place cryovials in a Mr. Frosty freezing container and freeze cells overnight at -80ºC.
    After 24 hours, frozen cells can be moved to a liquid nitrogen freezer for long-term storage.
35. Add 10.5 mM Rho Kinase Inhibitor (Y-27632) Stock (to final concentrations of 10.5 µM) to the Human Complete Feeding Medium. You will need 500 µL of medium per well plated.
    If generating adjacent normal organoids, the Human Complete Feeding Medium should also be supplemented with PGE2 (to final concentration of 1 µM).
36. Add 500 µL of pre-warmed Human Complete Feeding Medium supplemented with Rho Kinase Inhibitor (Y-27632) to each well of organoids.
37. Return plates of organoids to tissue culture incubator.

<u>Optional: To isolate cancer associated fibroblasts:</u>

38. Separate a portion of digest to a new 15 mL conical tube.
39. Centrifuge at 200 RCF for 5 min at 4ºC.
40. Carefully remove the supernatant.
41. Resuspend pellet in Human CAF Medium.
42. Transfer to 1 well of a 6 well culture plate.
43. Place in 37ºC tissue culture incubator.
    Note: At future passages, very short trypsin incubations will help dissociate fibroblasts while keeping cancer cells attached. This method may be used to enrich for fibroblasts.

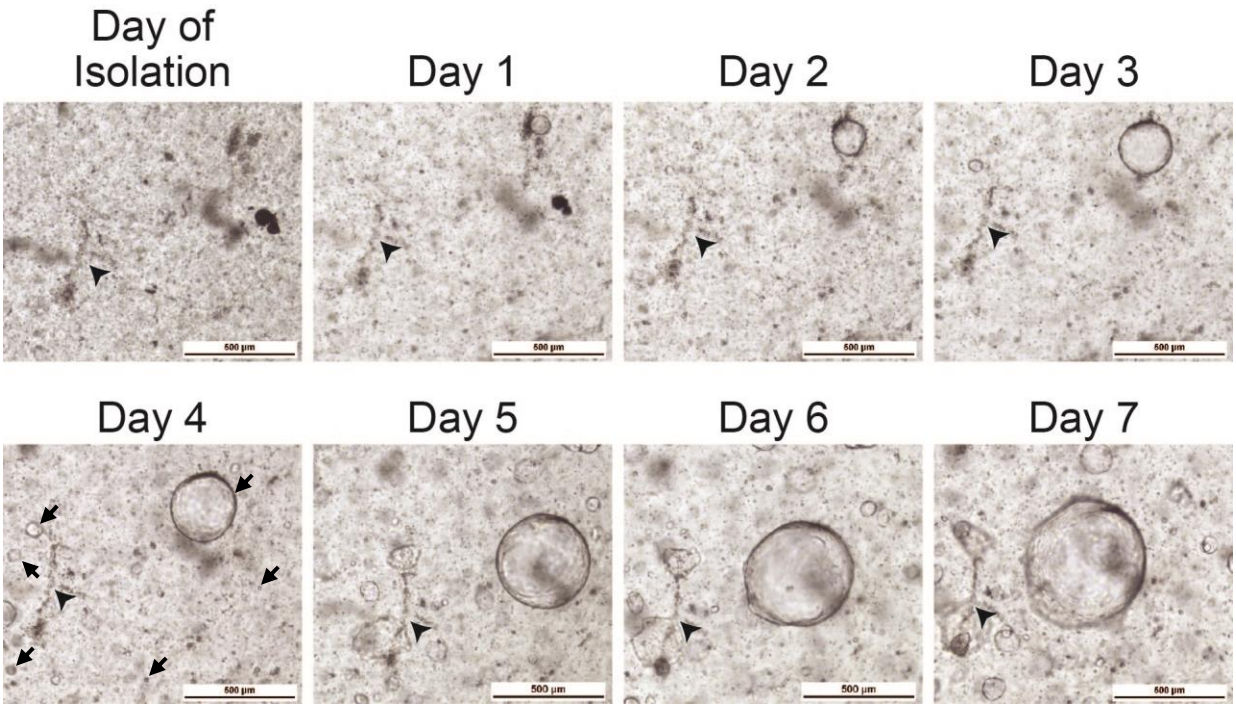

Figure 5. Time-course showing formation of human PDA organoids following isolation from a resected PDA tumor specimen.
The same well was imaged immediately following isolation and each day thereafter for 7 days. By day 4, multiple organoids of varying sizes are visible in the well (denoted by arrows). Arrows demark some of the organoids present in the day 4 image. Arrowhead points to the same chain of cells in each image. Scale bars, 500 µM.
Images courtesy of Candice Megan Young.

Protocol for Establishing Organoid Cultures from Human Fine Needle Aspirates and Cores

Notes:

Similar to resected tissue, Fine Needle Aspirates (FNAs) and cores are usually delivered in a 5 mL Protein LoBind Eppendorf tube containing either Human Wash Medium with or without 0.1% BSA (h+++ or h+++B), or RPMI supplemented with GlutaMAX, HEPES, and antibiotics. The specimen should be shipped on ice and processed as soon as possible to avoid tissue autolysis/degradation. Except for digestions, all procedures should be performed on ice.

Reagents and Equipment:

Human fine needle aspirate or biopsy	Human Complete Feeding Medium
10.5 mM Rho Kinase Inhibitor (Y-27632) Stock	10 mg/mL DNAse I stock
Human Wash Medium with 0.1% BSA (h+++B)	Growth Factor Reduced Matrigel, Phenol Red Free on ice
Human Digestion Medium	ACK (Ammonium-Chloride-Potassium) Lysing Buffer
P2, P20, P200, P1000 pipettes and sterile tips	Ice bucket with ice
5 mL Protein LoBind Tubes	Sterile aspirator pipettes
Sterile serological pipettes (5, 10, and 25 mL)	Pre-warmed 24 well culture plates
Timer	Pipet-Aid/Pipette controller
Hot water flask pre-warmed to 37ºC	Refrigerated 15/50 mL tube centrifuge
37ºC water bath	Incubated rocker/rotator set to 37ºC
Tissue culture hood with aspirator	37ºC tissue culture incubator

Procedure:

1. At least 6 hours before organoid isolation, place a 24-well plate in a 37ºC tissue culture incubator to warm up.
2. Record on a sheet all the information already available including: nosography, hospital, sample identifier, date and names of the people processing the sample.
3. Centrifuge sample at 200 RCF for 5 minutes at 4ºC.
4. Aspirate the supernatant, making sure not to disturb the cell pellet.
5. If the pellet is mostly white and little blood is present, proceed to the next step. If the cell pellet is mostly red (indicating a lot of blood in the sample), perform red blood cell lysis using ACK lysis buffer as follows:
    a. Add 4 mL of ACK lysing buffer.
    b. Invert a few times to dislodge the pellet.
    c. Centrifuge the tube at 200 RCF for 5 min at 4ºC.
    d. Carefully aspirate the supernatant without disturbing the pellet.
    e. If the sample is still bloody, repeat steps a-d until most of the blood is gone.
6. Resuspend the cell pellet in 1 mL Human Wash Medium with 0.1% BSA (h+++B). Triturate 10-20 times to break apart the tissue.
    a. If the tissue falls apart, all tissue will be processed as a single fraction. Add 3 mL of Human Wash Medium with 0.1% BSA (h+++B), and proceed to step 21.
    b. If the tissue still remains largely intact, then proceed to the next step.

7. Let the larger pieces of tissue settle to the bottom of the tube for 1 minute.
8. Transfer the medium above the larger tissue pieces to a new protein LoBind tube. This is "Fraction 1."
9. Centrifuge Fraction 1 at 200 RCF for 5 minutes at 4ºC.
10. Aspirate the supernatant from Fraction 1, making sure not to disturb the cell pellet.
11. Resuspend Fraction 1 in 4 mL Human Wash Medium with 0.1% BSA (h+++B), and set on ice.
12. Thaw an aliquot of Human Digestion Medium in the 37ºC water bath, and add 2 µL 10 mg/mL DNAse I stock and 2 µL 10.5 mM Rho Kinase Inhibitor (Y-27632) stock to 2 mL Human Digestion Medium.
    The DNAse stock sometimes comes out of solution, so the tube should be mixed just before adding to the Digestion Medium.
13. Add 2 mL Human Digestion Medium supplemented DNAse I and Y-27632 to the larger tissue pieces remaining in the original LoBind tube to begin the digestion of Fraction 2.
14. Place the tube containing Fraction 2 in rotating incubator set to 37ºC and 35 rpm for 5 minutes.
15. Centrifuge the tube containing Fraction 2 at 200 RCF for 5 minutes at 4ºC.
16. Remove the supernatant from Fraction 2 and resuspend the pellet in 1 mL of Human Wash Medium with 0.1% BSA (h+++B), triturating at least 10 times to shear the tissue.
17. Add an additional 3 mL of Human Wash Medium with 0.1% BSA (h+++B) to Fraction 2 to bring the volume to 4 mL.
18. Centrifuge Fractions 1 and 2 at 200 RCF for 5 minutes at 4ºC.
19. Remove the supernatants from both fractions.
20. Resuspend each pellet in 2 mL Human Wash Medium with 0.1% BSA (h+++B) and combine into a single tube.
    If there are still large pieces of the biopsy that weren't digested, skip this step to proceed to step 23, resuspending each pellet in Matrigel and plating each separately. In this case, it is better to keep the pellets separate, since these larger fragments tend to tear the Matrigel, and may negatively impact culture formation.
21. Centrifuge the combined fractions at 200 RCF for 5 minutes at 4ºC.
22. Remove the supernatant.
23. Resuspend the cell pellet in Matrigel.
    The amount of Matrigel depends on pellet size. For reference, we typically resuspend in 600 µL Matrigel for a 20 µL pellet.
24. Plate 50 µL domes of the cell/Matrigel suspension into wells of a pre-warmed 24 well plate.
    Plating can be done on top of a pre-warmed hot water flask to aid dome solidification.
25. Place the plate into a 37ºC tissue culture incubator for 10 minutes until Matrigel solidifies.
26. Add 10.5 mM Rho Kinase Inhibitor (Y-27632) Stock (to final concentrations of 10.5 µM) to the Human Complete Feeding Medium. You will need 500 µL of medium per well plated.
27. Add 500 µL of pre-warmed Human Complete Feeding Medium supplemented with Y-27632.
28. Return organoids to 37ºC tissue culture incubator.

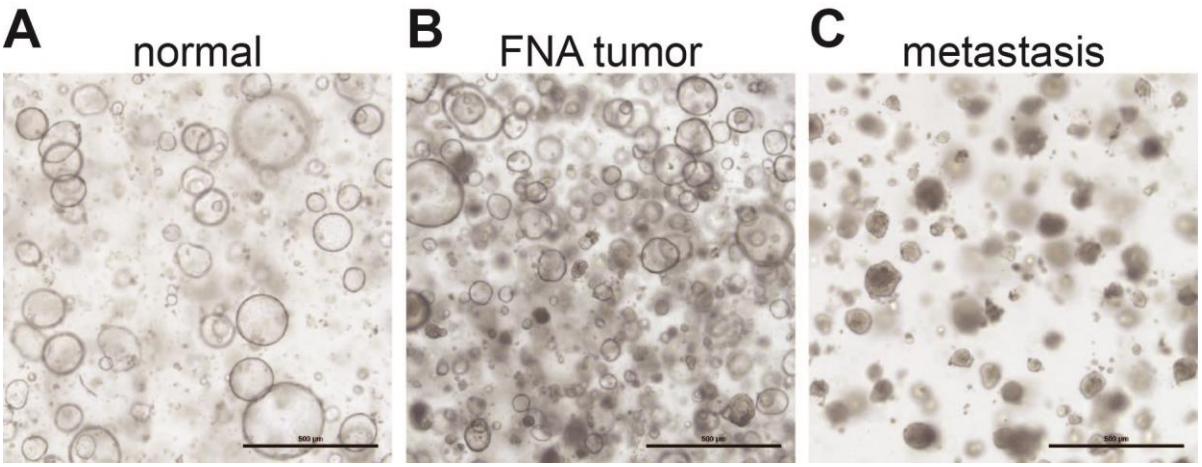

Figure 6. Human normal, tumor, and metastatic organoid cultures.
Human normal organoids appear as spherical cysts, similar to murine organoids. Human tumor and metastatic organoids can take a variety of morphologies, including filled spheres.

Protocol for Passaging Human Tumor Organoid Cultures

Notes:

There are special considerations to keep in mind when working with human organoid cultures. Due to the heterogeneity observed in human cancers, many organoid morphologies can be observed. If the human organoids appear to be cystic, either mechanical or enzymatic dissociation can be used. If the human organoids appear to be filled with cells ("solid"), enzymatic digestion is necessary. Since human organoids do not grow as fast as mouse organoids, and cultures will be healthier at higher densities, the splitting ratios should typically range from 1:1 to 1:3. While human tumor organoids do not appear to have a passaging limit, human normal organoids will stop propagating after 15 passages. All steps should be done on ice unless otherwise specified.

*** Human normal organoids are sensitive to Cell Recovery Solution. To passage human normal organoids, follow to the "Protocol for Passaging Mouse Organoids" on page 13, but use Human Wash Medium with 0.1 % BSA (h+++B) instead of Mouse Splitting Medium.

Reagents and Equipment:

- Confluent wells of organoids
- Prostaglandin E2 (PGE2) Stock (if normal organoids)
- Human Wash Medium with 0.1% BSA (h+++B)
- 10.5 mM Rho Kinase Inhibitor (Y-27632) Stock
- 10 mg/mL DNAse I stock
- Pre-warmed 24 well culture plates
- P2, P20, P200, P1000 pipettes and sterile tips
- 5 mL Protein LoBind Tube or 15 mL conical tube
- Hot water flask pre-warmed to 37ºC
- Tissue culture hood with aspirator
- 37ºC tissue culture incubator
- Human Complete Feeding Medium
- Cell Recovery Solution
- TrypLE Express
- Growth Factor Reduced Matrigel, Phenol Red Free on ice
- Ice bucket with ice
- Sterile aspirator pipettes
- Sterile serological pipettes (5, 10, and 25 mL)
- Pipet-Aid/Pipette controller
- 37ºC water bath
- Incubated rocker/rotator set to 37ºC
- Refrigerated 15/50 mL tube centrifuge

Procedure:

1. At least 6 hours before passaging, place a 24-well plate in a 37ºC tissue culture incubator to warm up.
2. Place Human Complete Feeding Medium at 37ºC to warm.
3. Aspirate medium from each well.
4. Add 1 mL of ice cold Cell Recovery Solution (CRS) to every other well.
5. Starting with a well that had CRS added to it, pipette the liquid up and down until the Matrigel falls apart and the solution is homogeneous.
6. Take the mix of organoids, Matrigel, and CRS, and pipette it onto a Matrigel dome that does not have CRS added to it.
7. Pipette up and down again, until the solution is homogeneous and the 2 domes are combined in 1 mL of CRS.
8. Move the suspension to a 5 mL protein LoBind tube (if harvesting 8 or fewer wells) or a 15 mL conical tube (if harvesting more than 8 wells) on ice.
9. Repeat this procedure for the remaining wells. Harvest up to 24 Matrigel domes per 15 mL conical tube.
10. Incubate on ice for 30 minutes, inverting the tube every 10 minutes.
11. Centrifuge the tube at 200 RCF for 5 minutes at 4ºC.
12. Aspirate the supernatant, being careful not to disturb the pellet.
13. Resuspend the pellet in 1 mL Human Wash Medium with 1% BSA (h+++B), and if the organoids

are in a 15 mL conical tube, transfer them to a 5 mL protein LoBind tube.

14. If organoids are cystic organoids, go directly to step 21 for the mechanical dissociation. If organoids are solid, proceed with the next step.
15. Add 3 mL of TrypLE Express supplemented with 4 µL 10 mg/mL DNAse I stock and 4 µL 10.5 mM Rho Kinase Inhibitor (Y-27632) stock to the resuspended organoids.
16. Incubate tube at 37ºC with 35 rpm rotation for an initial 5 minutes.
17. Examine the contents of the tube under an inverted microscope, checking to see if the organoids have dissociated into cell clumps or single cells.
    a. If most of the organoids are still intact, continue the dissociation, checking every 3 to 5 minutes.
    b. If organoids have dissociated into cell clumps, continue to the next step.
18. Centrifuge the tube at 200 RCF for 5 minutes at 4ºC.
19. Aspirate the supernatant, being careful not to disturb the pellet.
20. Add 1 mL of ice cold Human Wash Medium with 1% BSA (h+++B) to the pellet.
21. Using the P1000 and P1000 tip, triturate the organoid solution at least 20 times, making sure to hit the bottom of the conical tube with the pipette tip to shear the organoids.
22. Add additional Human Wash Medium with 1% BSA (h+++B) to bring the volume to 5 mL total, and invert the tube a couple of times to mix.
23. Centrifuge the tube at 200 RCF for 5 minutes at 4ºC.
24. Aspirate the supernatant, being careful not to disturb the pellet.
25. Resuspend the pellet in Matrigel.
    The volume of Matrigel should be 50 µL multiplied by the number of new wells you plan to plate.
26. Plate the suspension as 50 µL domes into a pre-warmed 24 well plates.
    Plating can be done on top of a pre-warmed hot water flask to aid in dome solidification.
27. Place the plate into a 37ºC tissue culture incubator for 10 minutes until Matrigel solidifies.
28. Add 500 µL of pre-warmed Human Complete Feeding Medium (with the addition of PGE2 if splitting human normal organoids).
29. Return organoids to 37ºC tissue culture incubator.

Protocol for Freezing Human Organoid Cultures

Notes:
It is recommended to freeze two to four confluent wells of human organoids into 1 cryovial for optimal recovery. For filled organoids, it is better to freeze when smaller in size. Organoids should not be passaged enzymatically before cryopreservation.

- Confluent wells of organoids to be frozen
- Human Wash Medium with 0.1% BSA (h+++B)
- P2, P20, P200, P1000 pipettes and sterile tips
- Cryovials
- Ice bucket with ice
- Refrigerated 15/50 mL tube centrifuge
- Tissue culture hood with aspirator
- Liquid nitrogen cryofreezer
- Recovery Cell Culture Freezing Medium
- 5 mL Protein LoBind Tube or 15 mL conical tube
- Sterile serological pipettes (5, 10, and 25 mL)
- Mr. Frosty freezing container
- Sterile aspirator pipettes
- Pipet-Aid/Pipette controller
- -80ºC freezer

Procedure:

1. Label cryovials with the organoid ID, passage number, date, and your initials, and chill on ice.
2. Aspirate medium from each well.
3. Add 1 mL of ice cold Cell Recovery Solution (CRS) to every other well.
4. Starting with a well that had CRS added to it, pipette the liquid up and down until the Matrigel falls apart and the solution is homogeneous.
5. Take the mix of organoids, Matrigel, and CRS, and pipette it onto a Matrigel dome that does not have CRS added to it.
6. Pipette up and down again, until the solution is homogeneous and the 2 domes are combined in 1 mL of CRS.
7. Move the suspension to a 5 mL protein LoBind tube (if harvesting 8 or fewer wells) or a 15 mL conical tube (if harvesting more 8-24 wells) on ice.
8. Repeat this procedure for the remaining wells.
9. Incubate on ice for 30 minutes, inverting the tube every 10 minutes.
10. Centrifuge the tube at 200 RCF for 5 minutes at 4ºC.
11. Aspirate the supernatant, being careful not to disturb the pellet.
12. Add 1 mL of ice cold Human Wash Medium with 1% BSA (h+++B) to the pellet.
13. Triturate at least 20 times, making sure to hit the bottom of the conical tube with the pipette tip to shear the organoids.
14. Add additional Human Wash Medium with 1% BSA (h+++B) to bring the volume to 5 or 10 mL total (depending on tube size), and invert the tube a couple of times to mix.
15. Centrifuge the tube at 200 RCF for 5 minutes at 4ºC.
16. Aspirate the supernatant, being careful not to disturb the pellet.
17. Resuspend the pellet in Cell Recovery Freezing Medium.
    *The volume of Freezing Medium will be 500 µL * the number of cryovials you plan to freeze.*
18. Aliquot 500 µL of the cell suspension to each cryovial.
19. Move cryovials to a Mr. Frosty Freezing Container, and incubate for 24 hours at -80ºC.
20. Transfer cryovials to liquid nitrogen storage for long term cryopreservation.

Protocol for Thawing Human Organoid Cultures

Notes:

Since human organoids are very fragile, this procedure should be done as quick as possible. It is highly recommended that all items needed should be in the biosafety cabinet before starting.

Reagents and Equipment:

- Frozen vial of cryopreserved organoids
- 10.5 mM Rho Kinase Inhibitor (Y-27632) stock
- Prostaglandin E2 (PGE2) Stock (10 mM) (if normals)
- Ice bucket with ice
- Sterile aspirator pipettes
- 5 mL Protein LoBind tube
- 37ºC water bath
- Hot water flask pre-warmed to 37ºC
- Tissue culture hood with aspirator
- Human Complete Feeding Medium
- Growth Factor Reduced Matrigel, Phenol Red Free on ice
- Human Wash Medium with 0.1% BSA (h+++B)
- Pipetmen (p20, p200, p1000) and sterile tips
- Sterile 5, 10, and 25 mL serological pipettes
- Pre-warmed 24 well culture plates
- Pipet-Aid/Pipette controller
- Refrigerated 15/50 mL tube centrifuge
- 37ºC tissue culture incubator

Procedure:

1. At least 6 hours before organoid thawing, place a 24-well plate in a 37ºC tissue culture incubator to warm up.
2. Place Human Complete Feeding Medium at 37ºC to warm.
3. Thaw a vial of frozen organoids quickly in a 37ºC water bath.
4. Once a small bit of ice remains in the vial, bring vial into the biosafety hood.
5. Prewet a P1000 pipette tip with Human Wash Medium with 0.1% BSA (h+++B), and pipette the cell suspension into a 5 mL Protein LoBind tube containing 3 mL of cool (but not ice cold) Human Wash Medium with 0.1% BSA (h+++B).
6. Wash the cryovial with 1 mL of room temperature Human Wash Medium with 0.1% BSA (h+++B), and add to the LoBind tube containing the rest of the organoids.
7. Centrifuge tube at 200 RCF for 5 minutes at room temperature.
8. Resuspend the pellet in Matrigel.
   The resuspension volume will depend on the pellet size, but it is best to err on the side of having the organoids too dense rather than not dense enough. For organoids frozen at a density of 2-4 confluent wells per cryovial, we typically plate 1 thawed vial in 4 wells.
9. Plate the suspension to 50 µL domes on a pre-warmed 24 well plate.
   Plating can be done on top of a pre-warmed hot water flask to aid in dome solidification.
10. Place the plate into a 37ºC tissue culture incubator for 10 minutes until Matrigel solidifies.
11. Add 10.5 mM Rho Kinase Inhibitor (Y-27632) stock to the Human Complete Feeding Medium to a final concentration of 10.5 µM. If you are thawing human normal organoids, add the 10 mM PGE2 stock to final concentration of 1 µM.
    You will need 500 µL of feeding medium per well plated.
12. Add 500 µL of pre-warmed Human Complete Feeding Medium supplemented with Y-27632 (and, if normal organoids, PGE2) to each well.
13. Return organoids to 37ºC tissue culture incubator.

Protocol for Generating a 2D Cell Line from a Human Organoid Culture

Notes:

- The 2D cell line is developed by allowing the outgrowth of organoids that naturally attach to the plastic at the bottom of a well of organoids in Matrigel.
- Not all human organoid cultures form 2D cell lines.
- A couple of passages after you initiate an organoid culture (once you have lost fibroblast contamination), check your plate to see if some cells have attached to the plastic. You will generate your 2D cell line by removing the organoids and Matrigel from the well, and letting these cells outgrow in the same well.
- The more wells with cancer cells stuck to the bottom that you can start with, the easier it is to establish a 2D cell line.
- We culture our 2D lines in the same conditions as the organoids (37ºC, 20% O_2, 5% CO_2).

Reagents and Equipment:

Wells of organoids with cancer cells attached to bottom of wells	Human Wash Medium with 0.1% BSA (h+++B)
Human 2D Medium (pre-warmed to 37ºC)	Cell Recovery Solution
Ice bucket with ice	Pipetmen (p20, p200, p1000) and sterile tips
Sterile aspirator pipettes	Sterile 5, 10, and 25 mL serological pipettes
5 mL Protein LoBind tube	Pre-warmed 24 well culture plates
37ºC water bath	Pipet-Aid/Pipette controller
Hot water flask pre-warmed to 37ºC	Refrigerated 15/50 mL tube centrifuge
Tissue culture hood with aspirator	37ºC tissue culture incubator

Procedure:

1. Use a glass aspirator pipette to remove the medium from each well to be harvested
2. Pre-wet a P1000 tip with ice-cold Human Wash Medium with 0.1% BSA (h+++B), then pipette 500 µL cold Human Wash Medium with 0.1% BSA (h+++B) onto each Matrigel dome to be harvested.
3. Pipette up and down gently to break up the dome and solubilize the organoids. Do not pipette too harshly to ensure cancer cells attached to the plastic stay attached.
4. Transfer the medium with the Matrigel and organoids to a 5 mL Protein LoBind tube.
5. Use 500 µL cold Human Wash Medium with 0.1% BSA (h+++B) to gently rinse each well.
6. Add 500 µL warm Human 2D Medium to each well.
7. Place plate back in tissue culture incubator.
8. If desired, complete the passaging of the organoids you isolated in the conical tube, by centrifuging the cells at 200 RCF for 5 min at 4ºC, and resuspending in Cell Recovery Solution.
9. Monitor the growth of your 2D cancer cells. You should see the cells forming colonies within the well of the 24- or 48-well plate, and those colonies growing in size over the next 1-2 weeks. However, not all human organoid lines are capable of proliferating in 2D culture conditions.
10. Once colonies of cancer cells are covering the majority of the well(s), passage the cells: use PBS and trypsin, as for any other 2D cell line, and combine like wells into one larger dish. *Trypsinization time should be determined empirically for each line. Many pancreas cancer cell*

lines require longer trypsinization times than fibroblast cells.

Media and Solutions for Human Organoid Protocols

All the solutions are prepared before sample processing. The Human Wash Medium (h+++) can be prepared in advance and stored for up to 1 month before use. Human Complete Medium (hCPLT) is used to feed organoids and is prepared no more than 2 weeks prior to use, due to the instability of some of its components. The amount of Digestion Medium is prepared in large batches (up to 200 mL) and frozen into 10 mL aliquots.

For more information on organoid media components, please see “More Information on Organoid Culture Reagents” on page 50. Catalog numbers can be found on page 70.

Human Wash Medium with or without BSA (h+++ and h+++B)

Volume	Stock Concentration	Reagent	Final Concentration
500 mL		Advanced DMEM/F-12	
5 mL	1 M	HEPES pH 7.2-7.5	10 mM
5 mL	100 X	GlutaMAX Supplement	1 X
1 mL	50 mg/mL	Primocin	100 µg/mL
1.67 mL	30 %	BSA*	0.1 %

h+++ is the base for hCPLT, and this can be stored at 4ºC for up to 1 month.

*BSA is typically added to h+++ (generating h+++B) when generating organoids or when passaging. This helps prevent organoids from sticking to plastic surfaces.

Human Complete Feeding Medium (hCPLT)

Volume	Stock Concentration	Reagent	Final Concentration
36.415 mL		Human Wash Medium	
50 mL	2 X	Wnt3a-Conditioned Medium*	1 X
10 mL	10 X	R-spondin1-Conditioned Medium	1 X
2 mL	50 X	B27 Supplement	1 X
1 mL	1 M	Nicotinamide	10 mM
250 µL	500 mM	N-acetylcysteine	1.25 mM
200 µL	50 mg/mL	Primocin	100 µg/mL
100 µL	100 µg/mL	mNoggin	100 ng/mL
10 µL	500 µg/mL	hEGF	50 ng/mL
10 µL	1000 µg/mL	hFGF	100 ng/mL
10 µL	100 µM	hGastrin I	10 nM
2 µL	25 mM	A 83-01	500 nM
100 µL	10.5 mM	Y-27632**	10.5 µM
10 µL	10 mM	PGE2***	1 µM

hCPLT should be prepared no more than 2 weeks prior to use due to the instability of some of its components. All components of this medium should be prepared according to the provided certificate of analysis or product specification sheet from the manufacturer.

* If available, Afamin/Wnt3a conditioned medium can be substituted for Wnt3a conditioned medium, and should also make up 50% of the total volume of the hCPLT medium.

** Y-27632 (Rho Kinase Inhibitor) is necessary when organoids are first generated, when organoids are thawed, or when organoids are enzymatically dissociated. It should be added just prior to feeding.

***Prostaglandin E2 (PGE2) is necessary for normal pancreas organoids. It should be added to the hCPLT to a final concentration of 1 µM just prior to feeding.

Human Digestion Medium

Volume/Mass	Stock Concentration	Reagent	Final Concentration
100 mL		Human Complete Feeding Medium	
500 mg		Collagenase XI	5 mg/mL
100 µL	10.5 mM	Y-27632*	10.5 µM
100 µL	10 mg/mL	DNAse I*	10 µg/mL

Digestion medium is ideally made with hCPLT (with Wnt3a-conditioned medium and therefore, with FBS present) to provide additional nutritional supplement while the primary culture is being digested. If no extra hCPLT is available, prepare hCPLT without mNoggin, hEGF, hFGF, hGastrin, A 83-01, and use this for the base of the digestion medium.

*Prepare digestion medium without the DNAse and Y-27632 in large batches (up to 200 mL), sterile filter, and store in 10 mL aliquots at -20ºC. Thaw in a 37ºC water bath and bring temperature to 25-37ºC. Add the DNAse and Y-27632 just before use.

In previous versions of this protocol, Collagenase II was used for digestions for human pancreatic tumor organoids. However, we decided to switch to Collagenase XI, since this fraction contains less trypsin and is the enzyme used in the Islet Cell Transplant digestions.

Human 2D Medium = Human CAF Medium

Reagent	Stock Concentration	Volume	Final Concentration
RPMI 1640 Medium with L-Glutamine		450 mL	
FBS	100%	50	10%
Penicillin/Streptomycin	100X	5 mL	1X

Protocol for Harvesting DNA from Organoids

Notes:
A minimum of 2 confluent wells of organoids should be harvested to generate DNA.

Reagents and Equipment:

- Wells of organoids to be harvested
- PBS with 0.1% BSA
- 1.5 mL tubes
- Aspirator pipettes
- Aspirator
- Nanodrop Spectrophotometer
- -20^{o}C freezer
- Cell Recovery Solution
- QIAGEN DNeasy Blood and Tissue Kit
- P2, P20, P200, P1000 pipettes and tips
- Ice bucket with ice
- Timer
- Refrigerated microcentrifuge

Procedure:

1. Label a 1.5 mL tube with the organoid ID and the passage number.
2. Aspirate medium from wells to be harvested.
3. Add 1 mL Cell Recovery Solution to one well, and pipette up and down until the Matrigel falls apart and the solution is homogeneous.
4. Pipette the mixture onto the next well to be harvested, and pipette up and down until the Matrigel falls apart and the solution is homogeneous.
5. Repeat until all wells are harvested, and transfer the mixture to the labeled, 1.5 mL tube.
6. Incubate tube on ice for 45-60 minutes.
7. Centrifuge tube at 6000 RCF for 3 minutes at 4^{o}C.
8. Remove the supernatant.
9. Resuspend the pellet in 200 µL ice-cold PBS with 0.1% BSA. Using a P200 pipette, triturate vigorously 20 times to get organoids into solution.
10. Add 1 mL ice-cold PBS with 0.1% BSA.
11. Centrifuge tube at 6000 RCF for 3 minutes at 4^{o}C.
12. Repeat steps 8-11 for a second wash.
13. Use the QIAGEN DNeasy Blood and Tissue Kit per manufacturer’s instructions, but elute in 30 µL of the recommended buffer.
14. Measure and record the DNA concentration and the A260/280 and A260/280 ratios using the NanoDrop Spectrophotometer.
15. Label the tube containing the isolated DNA with the organoid ID, passage number, and DNA concentration.
16. Store the DNA at -20^{o}C.

Protocol for Performing *KRAS* Genotyping on Human Organoids

Notes:

In this protocol, PCR is used to amplify exons on the human *KRAS* gene that contain the G12/13 or Q61 codons, and purified PCR products are sent for Sanger Sequencing.

Reagents and Equipment:

- Genomic DNA isolated from organoids
- UltraPure DNAse/RNAse-free dH_2O
- QIAGEN QIAprep PCR Purification Kit
- 1.5 mL tubes
- P2, P20, P200, P1000 pipettes with tips
- Thermal cycler
- -20°C freezer
- NEBNext High-Fidelity 2X PCR Master Mix
- Sequencing primers (see below for sequencing information)
- 0.5 mL PCR tubes
- Marker
- Ice bucket with ice
- Nanodrop spectrophotometer

PCR Primers for Sequencing:

KRAS G12/13 Forward: 5'- CTG GTG GAG TAT TTG ATA GTG -3'
KRAS G12/13 Reverse: 5'- CTG TAT CAA AGA ATG GTC CTG -3'

KRAS Q61 Forward: 5'- CCA GAC TGT GTT TCT CCC TT -3'
KRAS Q61 Reverse: 5'- CAC AAA GAA AGC CCT CCC CA -3'

Order primers with IDT as oligos with standard desalting. Resuspend to 100 µM in UltraPure DNAse/RNAse-free dH_2O, and make 10 µM dilutions. Store at -20°C.

Procedure:

1. Dilute organoid genomic DNA to 100 ng/uL
2. Prepare master mix on ice following the recipe below. Prepare enough to have 1 reaction per sample plus 2 extra reactions. Mix well but gently. (DO NOT VORTEX.)

Components	Volume for 1 reaction
High Fidelity 2x PCR Master Mix (NEB)	25 µL
10 µM Forward Primer	5 µL
10 µM Reverse Primer	5 µL
UltraPure DNAse/RNAse-free dH_2O	9 µL
TOTAL	49 uL

3. Label 0.5 mL PCR tubes with the human organoid ID, then place on ice.
4. Aliquot 49 uL of the prepared master mix to each PCR tube, then add 1 µL of organoid genomic DNA at 100 ng/uL to the appropriate tubes.
5. Seal tubes and flick to mix.

6. Subject tubes to the following thermal cycle program:

Step	Temperature	Time (min:sec)	Cycles
Initial Denaturation	94ºC	02:00	1
Denaturation	94ºC	00:30	3
Annealing	64ºC	00:30	
Extension	72ºC	00:30	
Denaturation	94ºC	00:30	3
Annealing	61ºC	00:30	
Extension	72ºC	00:30	
Denaturation	94ºC	00:30	3
Annealing	58ºC	00:30	
Extension	72ºC	00:30	
Denaturation	94ºC	00:30	3
Annealing	57ºC	00:30	
Extension	72ºC	00:30	
Final Extension	72ºC	05:00	1
Hold	4ºC	∞	1

7. Purify PCR products using the QIAGEN QIAprep PCR Purification Kit, following manufacturer's instructions.
8. Measure the DNA concentration and the A260/280 and A260/280 ratios using the Nanodrop Spectrophotometer.
9. Record the DNA concentration on the side of the tube and store at -20ºC until ready to send samples for sequencing.

Preparing Purified PCR Products for Sequencing (based on requirements of the CSHL Sequencing Core):

10. Label tubes to send for sequencing.
11. Dilute DNA to be sequenced to 1-5 ng/µL.
12. Dilute primers to use for sequencing to 5 µM.
13. Send diluted DNA and primers for Sanger Sequencing, using the *KRAS* G12/13 Reverse or *KRAS* Q61 Forward primer for sequencing.

Interpreting Sequencing Results

14. Align sequencing results to wild-type *KRAS* gene sequence to look for mutations.
15. For *KRAS* G12/13, search sequence for GTA GTT GGA GCT. The next 2 codons code for amino acids 12 and 13, which are commonly mutated in pancreatic cancer. If a mutation is present, use the codon table to determine what amino acids codon encode.

    Wildtype human *KRAS* sequence: GTA GTT GGA GCT GGT GGC GTA GGC AAG
    Val8 Val9 Gly10 Ala11 Gly12 Gly13 Val14 Gly15 Lys16
16. For *KRAS* Q61, search sequence for GAC ACA GCA GGT. The next codon codes for amino acid 61, which is sometimes mutated in pancreatic cancer. If a mutation is present, use the codon table to determine what amino acids the codon encodes.
    Wildtype human *KRAS* sequence: GAC ACA GCA GGT CAA GAG GAG TAC AGT
    Asp57 Thr58 Ala59 Gly60 Gln61 Glu62 Glu63 Tyr64 Ser65

DNA Codon Table for Reference

Amino acids biochemical properties | nonpolar | polar | basic | acidic

Termination: stop codon

Standard genetic code

1st base	2nd base								3rd base
	T		C		A		G		
T	TTT	(Phe/F) Phenylalanine	TCT	(Ser/S) Serine	TAT	(Tyr/Y) Tyrosine	TGT	(Cys/C) Cysteine	T
	TTC		TCC		TAC		TGC		C
	TTA	(Leu/L) Leucine	TCA		TAA[B]	Stop (*Ochre*)	TGA[B]	Stop (*Opal*)	A
	TTG		TCG		TAG[B]	Stop (*Amber*)	TGG	(Trp/W) Tryptophan	G
C	CTT		CCT	(Pro/P) Proline	CAT	(His/H) Histidine	CGT	(Arg/R) Arginine	T
	CTC		CCC		CAC		CGC		C
	CTA		CCA		CAA	(Gln/Q) Glutamine	CGA		A
	CTG		CCG		CAG		CGG		G
A	ATT	(Ile/I) Isoleucine	ACT	(Thr/T) Threonine	AAT	(Asn/N) Asparagine	AGT	(Ser/S) Serine	T
	ATC		ACC		AAC		AGC		C
	ATA		ACA		AAA	(Lys/K) Lysine	AGA	(Arg/R) Arginine	A
	ATG[A]	(Met/M) Methionine	ACG		AAG		AGG		G
G	GTT	(Val/V) Valine	GCT	(Ala/A) Alanine	GAT	(Asp/D) Aspartic acid	GGT	(Gly/G) Glycine	T
	GTC		GCC		GAC		GGC		C
	GTA		GCA		GAA	(Glu/E) Glutamic acid	GGA		A
	GTG		GCG		GAG		GGG		G

Protocol for Harvesting RNA from Organoid Cultures

Notes:
Typically, 1 confluent well of murine organoids in a 24 well plate yields ~3-5 µg total RNA. For most experiments, pooling 2-4 wells of organoids from a 24 well plate, will yield sufficient RNA.

Reagents and Equipment:

Confluent wells of organoids to be harvested	TRIzol Reagent
(for TRIzol protocol) Chloroform	(for TRIzol protocol) Isopropanol
(for TRIzol protocol) Ethanol	(for TRIzol protocol) Glycogen
(for highly pure RNA) Pure Link RNA Mini Kit	Aspirator pipettes
Aspirator	P1000 and P200 Pipetmen and tips
Ice bucket with ice	Eppendorf tubes
(optional) Liquid nitrogen	(optional) -80^{o}C freezer

Procedure:

1. Place Eppendorf tubes to receive RNA on ice to chill.
2. For the wells of organoids to be harvested, carefully aspirate medium away from Matrigel domes.
3. Pipette 1 mL TRIzol Reagent on top of first Matrigel dome to be harvested.
4. Pipette up and down approximately 10 times, until Matrigel begins to dissolve into TRIzol, and bits of Matrigel dome are no longer visible clinging to the plate.
5. Pipette mixture of TRIzol, cells, and Matrigel onto next well of organoids to be harvested.
6. Repeat steps 4 and 5 until all wells of organoids to be harvested together have been combined into the TRIzol mixture.
7. Transfer the mixture of TRIzol, cells, and Matrigel into cold Eppendorf tube.
8. (Optional) Flash freeze Eppendorf tubes in liquid nitrogen and store at -80^{o}C until ready to prepare RNA.
9. RNA can now be prepared using your favorite protocol:
    a. If using the TRIzol protocol, we suggest the following modifications:
        i. Make sure to measure the amount of aqueous phase and add an equal volume of isopropanol - Often the aqueous phase volume is higher than the 0.5 mL the TRIzol protocol assumes, and a 1:1 ratio of aqueous phase:isopropanol is necessary for RNA to pellet.
        ii. We also recommend using glycogen as recommended in the TRIzol protocol to help pellet the RNA.
    b. If highly pure RNA is needed for RNA-sequencing or other sensitive downstream applications, we recommend purifying RNA using the “TRIzol Plus” protocol which comes with the Pure Link RNA Mini Kit (Life Technologies 12183018A). Briefly, thaw RNA, add chloroform, and spin all as described in the TRIzol protocol. Remove aqueous phase to a new tube, and add 1 volume 70% ethanol as described in the Pure Link RNA Mini Kit protocol. Follow the Binding, Washing, and Elution steps described in the Pure Link RNA Mini Kit protocol.

Protocol for Harvesting Protein from Organoid Cultures

Reagents and Equipment:

Wells of confluent organoids to be harvested	DPBS
PhosSTOP Tablet	Protease Inhibitor Tablet
Lysis Buffer (TNET, RIPA, or SDS)	15 mL conical tubes
Protein LoBind 1.5 mL Tubes	Liquid nitrogen
Ice bucket with ice	Pipettman (P1000, P200) and tips
Aspirator pipettes	Gel loading tips
Aspirator	Refrigerated 15/50 mL tube centrifuge and microfuge
Bioruptor sonicator or high-gauge needle and syringe	-80^{o}C freezer
Refrigerated microfuge	

Lysis Buffer Recipes:

TNET LYSIS BUFFER

50	mM	Tris-HCl pH 7.5
150	mM	NaCl
5	mM	EDTA
1	%	Triton X 100

Recipe to make 100 mL:

5	mL	1M	Tris-HCl pH 7.5
3	mL	5M	NaCl
1	mL	0.5M	EDTA
1	mL	100%	Triton X-100

SDS LYSIS BUFFER

10	mM	Tris-HCl pH 7.5
1	%	SDS

Recipe to make 100 mL:

1	mL	1M	Tris-HCl pH 7.5
10	mL	10%	SDS
89	mL		milliQ H_2O

RIPA LYSIS BUFFER

50	mM	Tris-HCl pH 7.5
150	mM	NaCl
0.1	%	SDS
2	mM	EDTA
0.5	%	Sodium deoxycholate
1	%	Triton X-100

Recipe to make 100 mL:

5	mL	1M	Tris-HCl pH 7.5
3	mL	5M	NaCl
0.5	mL	20%	SDS
0.4	mL	0.5M	EDTA
0.5	g		Sodium deoxycholate
1	mL	100%	Triton X-100
89.6	mL		milliQ H_2O

Procedure:

1. Ensure that organoid wells are at least 80%-90% confluent before beginning, since this is crucial to getting a good cell pellet.
2. Add protease inhibitor tablet and PhosSTOP tablet to DPBS Solution to generate DPBS-PPI Solution. Keep this on ice.
   If you do not require large amounts of buffer, dissolve each tablet to 1 mL DBPS, and use these solutions as a 10X stocks. 10X stocks should be aliquoted and stored at -20^{o}C for a couple of months.
3. Add protease inhibitor tablet and PhosSTOP tablet to Lysis Buffer to generate LYSIS-PPI. If using TNET or RIPA Lysis Buffer, place this solution on ice.
   If you do not require large amounts of buffer, dissolve each tablet to 1 mL Lysis Buffer, and use these solutions as a 10X stocks. 10X stocks should be aliquoted and stored at -20^{o}C for a couple of months.
4. Aspirate medium from the organoid domes.

5. Add 500 µL of ice-cold DPBS-PPI to each dome and pipette up and down to manually disrupt the Matrigel.
6. Pool 2 organoid mounds from a 24 well plate (1 mL total volume) into 1 Protein Lo-Bind 1.5 mL tube, or transfer up to 8 wells of a 24 well plate into a 15 mL tube, in 10 mL total volume of cold DPBS-PPI.
7. Centrifuge cells:
    a. For 1.5 mL tubes: 3000 RCF for 3 minutes in a chilled microfuge.
    b. For 15 mL tubes: max speed for 5 minutes in a chilled 15/50 mL tube centrifuge.
8. If organoids were harvested at 80-90% confluency, the organoids should sediment cleanly below the Matrigel layer and should appear as a white pellet below the gel.
9. Using a gel loading tip, carefully aspirate off the DPBS solution and the Matrigel, leaving behind the cell pellet. To get a clean pellet with minimal Matrigel, you must aspirate on the upper edge of the cell pellet and sacrifice a little bit of the cell pellet.
10. Wash the pellet 2x in ice-cold DPBS-PPI Solution.
    If cells are in a 15 mL conical tube, use 2nd wash to transfer to a 1.5 mL LoBind tube.
11. For TNET or RIPA Lysis:
    a. Resuspend the pellet in 100 µL of LYSIS-PPI and if not already in a 1.5 mL tube, transfer to a 1.5 mL tube.
    b. (Optional) Flash freeze in liquid nitrogen and store at -80^{o}C.
    c. Incubate on ice for 20 minutes.
    d. Shear the chromatin by passing the lysate three times through a 26 Gauge or insulin needle or by sonication (Use Medium setting on Bioruptor, sonicate with 30s on/30s off for 3 minutes, incubate on ice for 3 minutes, and sonicate with 30s on/30s off for 3 minutes).
    e. Centrifuge the lysate 10 minutes at max speed in a microfuge at 4^{o}C.
    f. Transfer supernatant to a new 1.5 mL tube.
12. For SDS Lysis:
    a. Heat LYSIS-PPI Buffer to 100^{o}C.
    b. Add 100 µL boiling LYSIS-PPI Buffer to the pellet of cells.
    c. Incubate cells at 100^{o}C for 5 min and allow to cool to room temperature.
    d. Pass the lysate three times through a 26 Gauge or insulin needle with a 1 mL syringe to shear chromatin.
13. Protein concentration can be determined by Lowry Method.
14. Flash freeze samples in liquid nitrogen and store samples at -80^{o}C until needed.

Alternative Protocols

- Corning Cell Recovery Solution can be used to isolate cells from the Matrigel. Cells can then be washed in DPBS and resuspended in Lysis Buffer.
- A solution of 2 mg/mL Dispase in Mouse Splitting Medium (+++) can also be used to separate organoids from Matrigel. Remove media from domes and add 500 uL Dispase Solution to each dome. Incubate at 37^{o}C for 10 minutes, triturate a few times to disrupt dome, and incubate at 37^{o}C for 5 minutes. Transfer cells to a LoBind tube, wash in PBS with 1 mM EDTA. Cells can then be resuspended in Lysis Buffer.

Protocol for Generating Single Cells from Organoid Cultures

Notes:
This protocol works well for both mouse and human organoids

Reagents and Equipment:

[For single cell prep]

- Wells of confluent organoids
- Splitting Medium (+++)
- TrypLE Express
- Rho kinase inhibitor (Y-27632, 10.5 mM stock)
- Ice bucket with ice
- Serological pipettes (5, 10, 25 mL)
- Aspirator pipettes
- Digital scale
- Refrigerated microfuge
- Tissue culture hood with aspirator
- (optional) Cell counting chambers and solution
- Dispase
- DNAse solution (10 mg/mL stock)
- Human or Mouse Complete Feeding Medium
- Protein LoBind 1.5 mL and 5 mL Tubes
- Pipettman (P2, P20, P200, 1000) and sterile tips
- 50 mL conical tube
- Timer
- 37°C rocking/rotating incubator
- Refrigerated 15/50 mL tube centrifuge and microfuge
- Microscope

[For culturing]

- Growth Factor Reduced Matrigel, Phenol Red Free on ice
- 37°C tissue culture incubator
- Pre-warmed 24 well culture plate
- Hot water flask pre-warmed to 37°C

Procedure:

1. Measure out Dispase in a 50 mL conical tube
    a. You will need approximately 2 mg per well of organoid you are harvesting
    b. Measure out at least 20 mg.
2. Bring tube of Dispase to the tissue culture hood.
3. Add cold or room temperature Splitting Medium (+++) to the Dispase to bring it to a concentration of 2 mg/mL. Invert tube to make sure Dispase is resuspended in the solution.
    a. Technically, the Dispase Solution is not sterile, because the Dispase was measured outside the tissue culture hood. We have not had problems with sterility. However, if you experience problems, you could try sterile filtering the Dispase solution. However, the concentration of Dispase may need to be adjusted if it is filtered to account for Dispase that may stick to the filter.
4. Remove medium from organoid wells to be harvested.
5. Add 500 µL Dispase Solution to each well to be harvested.
6. Pipette up and down with a P1000 to break up each Matrigel dome.
7. Incubate plate in tissue culture incubator (37°C, 5% CO_2, 20% O_2) for 10 minutes.
8. Use pipette to move the Dispase/Organoid mixture to a Protein LoBind 1.5 mL or 5 mL tube
    a. Depending on the number of wells to be harvested.
    b. Up to 2 wells per 1.5 mL tube or up to 8 wells per 5 mL tube.
9. Use 0.5 mL (for the 1.5 mL tube) or 1 mL (for the 5 mL tube) of Dispase Solution to wash the wells and combine with the Dispase/Organoid mixture in the LoBind tube.
10. Add 1 – 5 µL 10 mg/mL DNase I stock (to final concentration of 10 µL/mL) to the Dispase/Organoid mixture.
    a. The purpose of the DNase solution is to prevent DNA that is released from dying cells from causing cells to clump.

11. Incubate at 37ºC with rocking/rotation for 10 minutes.
12. Centrifuge tube at 200-400 RCF for 3 minutes at 4ºC.
13. Remove most of the supernatant, but leave a bit to ensure that you don't lose the pellet.
14. Wash the cells once in 1 mL TrypLE Express and spin again.
15. Remove most of the supernatant, but leave a bit to ensure that you don't lose the pellet.
16. Depending on pellet size, add 1-4 mL TrypLE Express and 2-8 µL 10 mg/mL DNase I stock (to final concentration 10 µg/mL).
17. Pipette up and down gently 20 times to resuspend the cells.
18. Incubate at 37ºC with rocking/rotation for 5 minutes.
19. Gently pipette up and down 10-20 times to help dissociate cells.
20. If stringy clumps can be seen by the naked eye, add 2-8 µL 10 mg/mL DNase I stock (to final concentration 10 µg/mL), and pipette up and down gently 20 times to resuspend the cells.
21. Look at tube in inverted microscope to see if cells are dissociated.
22. If cells are not dissociated, repeat steps 18-21 until cells are dissociated.
    a. This can take up to 35 minutes for some human tumor and mouse metastatic organoids.
23. Centrifuge tube at 200-400 RCF for 3 minutes.
24. Remove most of the supernatant, but leave a bit to ensure that you don't lose the pellet.
25. Wash cells 2 times in 5 mL Splitting Medium (+++).
26. Option #1: To Count the Cells
    a. Wash cells 1 time in 1-5 mL Complete Feeding Media (supplemented with Rho Kinase Inhibitor).
    b. Resuspend the cells in Complete Feeding Medium (supplemented with Rho Kinase Inhibitor) and use a cell counter to count cells
        i. A typical, relatively confluent organoid well in a 24 well plate has 100,000-300,000 cells, so for counting, I usually resuspend the cells in 50-100 µL per well harvested.
    c. Based on cell counts, set aside a set number of cells for replating
        i. Typically, cells are replated at a concentration of 25,000 – 50,000 cells per well of a 24 well plate.
    d. Spin tube with cells to be plated 200-400 RCF for 5 minutes.
27. Option #2: To Virally Transduce Cells
    a. Wash cells once more in 5 mL Splitting Media (+++)
    b. Single cells can be transduced by Lentivirus or Retrovirus.
    c. Aliquot 20,000 – 200,000 cells per transduction.
    d. Refer to the Lentiviral Infection protocol on page 49, step 25.
28. Option #3: To Use Cells for Flow Cytometry
    a. Wash cells once in your flow buffer.
    b. Resuspend cells in flow buffer, and cells are ready for flow cytometry or further staining.
29. Option #4: To Replate Cells

a. Spin cells to be plated 200-400 RCF for 5 minutes.

b. Carefully remove all of the supernatant (use a manual pipetteman for the final 100 µL).

c. Resuspend the cells in 50 µL Matrigel per well to plate.

d. Spot Matrigel domes onto a pre-warmed 24 well plate.

e. Let the domes set by keeping the plate in the tissue culture incubator 15 minutes.

f. While domes set, add Rho Kinase Inhibitor to your organoid Medium. You will need 0.5 mL per plated well.

g. Add 500 µL prewarmed Complete Feeding Medium (supplemented with Rho Kinase Inhibitor) to each well.

30. Option #5: To set up a therapeutics experiment in 96 well plate

a. Make Matrigel bed on white, opaque 96 well plate
Notes: The white, opaque plate is required if Cell Titer Glo will be your readout. The Matrigel bed is optional for murine normal, PanIN, and tumor organoids, which are not likely to attach to the bottom of the dish. It is important for murine metastatic organoids which are likely to attach. Human lines should be evaluated on a case-by-case basis.

b. Pre-chill white plate and regular, 96 well flat bottom plate in freezer

c. Combine equal volumes of Matrigel and cold DPBS. Pipette gently to mix.
Note: You will need 30 µL per well, or 2300 µL for a full plate of organoids.

d. Place plate on ice.

e. Pipette 30 µL of Matrigel mix into each well of the plate, but avoid plating organoids on the edge wells.

f. Centrifuge the plate at 200 RCF for 1 minute at 4^{o}C.

g. Optional: Repeat steps d-f with a clear, flat bottomed, 96 well plate, if you would like to be able to visualize some of the organoids you have plated.

h. Place plates in 37^{o}C tissue culture incubator for at least 20 minutes.

i. Make cell suspension, by combining the following on ice, Complete Feeding Medium (supplemented with Rho Kinase Inhibitor), Matrigel (to a final concentration of 10%), and cells (to a final concentration of 10-20 cells/µL for mouse organoids). Mix by gently pipetting up and down.

j. Plate 100 µL cell suspension (1000-2000 cells) per well on top of the Matrigel bed.

k. Return plate to 37^{o}C tissue culture incubator.

Protocols for Preparing Human Organoids for Therapeutics

Notes:

Before performing the therapeutics, ensure that the organoid cultures are growing well enough that they can be passaged at least once every 10 days. This is to ensure optimal recovery after plating into 384-well format. The average number of human organoid cells that can be isolated from one well of a 24 well plate varies, but is typically around 150,000 cells.

Reagents and Equipment:

- Wells of confluent organoids
- 10.5 mM Rho Kinase Inhibitor (Y-27632) stock
- TrypLE Express
- 10 mg/mL DNAse I stock
- Sterile PBS
- 15 mL conical tube
- Ice bucket with ice
- Sterile aspirator pipettes
- White, ultra low attachment, 384 well plate
- (Optional) Pre-warmed 24 well culture plate
- Multichannel pipette and sterile tips
- Timer
- Incubated rocker/rotator set to 37ºC
- 37ºC tissue culture incubator
- Human Complete Feeding Medium
- Growth Factor Reduced Matrigel, Phenol Red Free on ice
- Prostaglandin E2 (PGE2) Stock (1 mM=1000X) (if normal organoids)
- Cell Recovery Solution
- Human Wash Medium with 0.1% BSA (h+++B)
- Sterile basin
- P2, P20, P200, P1000 pipettes and sterile tips
- Sterile serological pipettes (5, 10, and 25 mL)
- Clear, ultra low attachment, 384 well plate
- Polypropylene tubes with 3 µM filter caps
- Pipet-Aid/Pipette controller
- Tissue culture hood with aspirator
- Refrigerated 15/50 mL tube centrifuge with plate inserts

Procedure:

1. Aspirate medium from each well.
2. Add 1 mL of ice cold Cell Recovery Solution (CRS) to every other well.
3. Starting with a well that had CRS added to it, pipette the liquid up and down until the Matrigel falls apart and the solution is homogeneous.
4. Take the mix of organoids, Matrigel, and CRS, and pipette it onto a Matrigel dome that does not have CRS added to it.
5. Pipette up and down again, until the solution is homogeneous and the 2 domes are combined in 1 mL of CRS.
6. Move the suspension to a 5 mL protein LoBind tube (if harvesting 8 or fewer wells) or a 15 mL conical tube (if harvesting more than 8 wells) on ice.
7. Repeat this procedure for the remaining wells. Harvest up to 24 Matrigel domes per 15 mL conical tube.
8. Incubate on ice for 30 minutes, inverting the tube every 10 minutes.
9. Centrifuge the tube at 200 RCF for 5 minutes at 4ºC.
10. Aspirate the supernatant, being careful not to disturb the pellet.
11. Resuspend the pellet in 1 mL Human Wash Medium with 1% BSA (h+++B), and if the organoids are in a 15 mL conical tube, transfer them to a 5 mL protein LoBind tube.
12. Add 3 mL of TrypLE Express supplemented with 4 µL 10 mg/mL DNAse I stock and 4 µL 10.5 mM Rho Kinase Inhibitor (Y-27632) stock to the resuspended organoids.
13. Incubate tube at 37ºC with 35 rpm rotation for an initial 5 minutes.
14. Examine the contents of the tube under an inverted microscope, checking to see if the organoids have dissociated into cell clumps or single cells.
    a. If most organoids are intact, continue the dissociation, checking every 3 to 5 minutes.

b. If organoids have dissociated into cell clumps, continue to the next step.

15. Centrifuge the tube at 200 RCF for 5 minutes at 4ºC.
16. Aspirate the supernatant, being careful not to disturb the pellet.
17. Add 1 mL of ice cold Human Wash Medium with 1% BSA (h+++B) to the pellet.
18. Using the P1000 and P1000 tip, triturate the organoid solution at least 20 times, making sure to hit the bottom of the conical tube with the pipette tip to shear the organoids.
19. Add additional Human Wash Medium with 1% BSA (h+++B) to bring the volume to 5 mL total, and invert the tube a couple of times to mix.
20. Centrifuge tube at 200 RCF for 5 minutes at 4ºC.
21. Aspirate the supernatant, then resuspend the pellet in Human Wash Medium with 0.1% BSA (h+++B) up to 5 mL final volume.
22. Pass the cell suspension through 35 µM filter.
    Typically we use the 5 mL polystyrene test tubes with cell strainer caps for this step.
23. Count the number of cells to prepare for plating into a 384-well plate.
    a. For the human organoids, plate 500 cells per well in a 20 µL volume. Plate at least 3 wells per condition.
       We have used up to 1000 to 2000 cells per well in up to 30 µL volume, but we have found that the 20 µL volume saves on reagents, while still giving repeatable results.
    b. Each well will receive 20 µL of cell suspension, so for a complete plate, 8 mL of a cell suspension will be enough.
    c. Cells are plated in 10% Matrigel/90% Human Complete Medium supplemented with Rho Kinase Inhibitor (Y-27632).
       If human normal organoids are being used in the experiment, the medium should also be supplemented with PGE2.
    d. As an example, this cell line was counted and the live cell count was $1.0x10^6$ cells/mL. Make the cell mixture as follows: below for 500 cells/well:

Live count: Cells / mL	Volume of Medium	Volume of Matrigel	Volume of Cells	Total Volume	# Cells / µL	# Cells / well = # Cells / 20 µL
1.0E+06	7.0 mL	0.8 mL	0.2 mL	8 mL	25	500 cells

24. Make your cell suspension in a 50 mL conical tube in the following order: add ice-cold Complete Human Medium, Rho Kinase Inhibitor, Matrigel, and then the cells. Mix thoroughly by pipetting.
25. Transfer the cell suspension to a sterile basin on ice.
26. Use a 12-channel pipette to add 20 µL to each well of a white opaque-bottom ultra-low attachment 384-well plate excluding the wells edges.
    Cells settle quickly out of solution. Triturate to mix the cell suspension after every couple of rows.
27. Pipette the some of the remaining cell suspension into a clear ultra-low attachment 384-well plate to allow for observation under the microscope.
28. Centrifuge the plates at 300 RCF at 4ºC, stopping the centrifuge as soon as it gets up to speed.
29. Add 80 µL sterile PBS to wells at the edges of the plate (to prevent evaporation).
30. Incubate at 37ºC until drug dosing.
31. The remaining cell suspension can be pelleted at 200 RCF for 5 minutes at 4ºC, resuspended in Matrigel, and replated as one or more Matrigel domes on a 24 well plate. These organoids should be fed with medium containing Rho Kinase Inhibitor.

Transfection to Make Lentivirus / Lentiviral Infection of Organoids

Courtesy of Daniel Öhlund

Notes:

For this protocol, your lentiviral vector must be compatible with the second generation packaging system (psPAX1 and pMD2.G). Examples of vectors compatible with the second generation system are pLKO.1 and pLVX.

Check with your university on the rules and regulations surrounding lentiviral production.

Reagents and Equipment:

[For transfection and virus production]

Lentiviral Vector (uses 2nd generation packaging)	psPAX1 plasmid
pMD2.G plasmid	DMEM + glucose, L-glutamine, sodium pyruvate
X-tremeGene9 Transfection Reagent	Pipettman (P20, P200, 1000) and sterile tips
Sterile 1.5 mL tubes	293T cells
DMEM, 10% FBS, 1% Penicillin/Streptomycin	PBS
Trypsin	Cell counting chambers and dye
6 cm tissue culture dish	37ºC tissue culture incubator
Glass pipettes and aspirator	5, 10, 25 mL pipettes
Refrigerated 15/50 mL centrifuge	Splitting Medium (+++)
LentiX Concentrator	-80ºC Freezer

[For infection of organoids]

24- or 48-well plate of organoids to be infected	Glass pipettes and aspirator
Pipettman (P20, P200, 1000) and sterile tips	5, 10, 25 mL pipettes
Splitting Medium (+++)	Ice bucket with ice
15 mL conical tube	Fire-polished glass pipettes
Refrigerated 15/50 mL tube centrifuge	Timer
37ºC rocking/rotating incubator	TrypLE Express
Lentivirus aliquot	DMEM, 5% FBS, 1% Penicillin/Streptomycin
48 well organoid culture plate	250X Polybrene Stock
37ºC tissue culture incubator	Non-refrigerated 15/50 mL tube centrifuge
Mouse, Human Normal, or Human Tumor Feeding Medium	Rho kinase inhibitor (Y-27632, 10.5 mM stock)
Matrigel	Hot water flask pre-warmed to 37ºC
Pre-warmed 24 well culture plate	(optional) Antibiotic for selection

4 mg/mL (1000X) Polybrene Solution (recipe for 40 mL)

0.16 g Polybrene
H2O to fill to 40 mL

Polybrene is also known as Hexadimethrine bromide
Polybrene is also known as 1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide
Sterile filter through 0.2 µM filter, aliquot, and store at -20ºC.
Freeze thaws are ok.

Procedure:
Transfection of 293T cells to produce virus

1. Prepare transfection mix inside a 1.5 mL tube in a tissue culture hood.
    a. ____ µL DMEM + glucose, L-glutamine, sodium pyruvate (serum free)
    b. 1 µg Target lentiviral vector (*2nd generation packaging compatible)
    c. 0.75 µg psPAX1
    d. 0.25 µg pMD2.G
    e. Total volume = 94 uL
2. Add 6 µL X-tremeGene9 Transfection Reagent, and pipette up and down gently with a P1000 to mix.
3. Incubate for 20 min at room temperature.
4. Trypsinize a plate of 293T cells, and make a suspension of $2.5x10^6$ cells in 5 mL Medium (DMEM + glucose, L-glutamine, sodium pyruvate, 10% FBS, 1% Penicillin/Streptomycin).
5. Mix cells with the transfection mix.
6. Plate cells and transfection mix in a 6 cm tissue culture dish and incubate overnight in 37°C tissue culture incubator.
7. 24 hours after plating, change medium to 5 mL fresh medium.
8. 24 hours after changing the medium, harvest the virus-containing supernatant and move to 15 mL conical tube.
9. Filter virus through 0.45 µM filter into clean 15 mL conical tube.
10. For 2D cells: virus can be used in a 1:5 dilution
11. For Organoids, Concentrate the virus:
    a. Add Lenti-X Concentrator to the viral supernatant. Determine the volume of Lenti-X Concentrator to add by taking the total volume of viral supernatant and dividing by 3.
    b. Incubate virus and concentrator at 4°C overnight.
    c. Centrifuge virus and concentrator at 1500 RCF for 45 min at 4°C.
    d. Aspirate supernatant away from pellet.
    e. Resuspend viral pellet in 1 mL Splitting Medium (+++)
    f. Aliquot into 0.25 mL aliquots.
    g. Store viral aliquots in – 80°C freezer.
    h. 1 x 0.25 mL aliquot should be used for 1 infection.

Infection of Organoids

12. Thaw lentivirus aliquot on ice.
13. Aspirate medium away from Matrigel domes.
14. Add 500 µL ice-cold Splitting Medium (+++) into the well
15. Pipette up and down to break organoids
16. Transfer organoids into 15 mL conical tube with 10 mL ice-cold Splitting Medium (+++).
17. Centrifuge at 850 rpm (145 RCF) for 5 min at 4°C.
18. Remove 8~8.5 mL of medium and leave 1.5~2 mL medium.
19. Pipette up and down with a fire-polished glass pipette 10 times.
20. Add Splitting Medium (+++) up to 10 mL.
21. Centrifuge at 850 rpm (145 RCF) for 5 min at 4°C.

22. Carefully remove supernatant.
23. Add 1 ml TrypLE Express and incubate at 37ºC with gentle agitation for 5 min.
24. Quench TrypLE by adding 9 ml DMEM + glucose, L-glutamine, sodium pyruvate, 5% FBS, 1% Penicillin/Streptomycin.
25. Centrifuge at 150-200 RCF for 5 min at 4ºC.
26. Carefully remove supernatant.
27. Resuspend cell pellet with 250 µL virus with 1 µL 250X Polybrene Stock
    a. Dilute the 1000X Polybrene Stock in DPBS to get a 250X stock.
28. Transfer the cell suspension with virus into a single well of 48 well culture plate
    a. Be sure to use the special plates for culturing organoids.
29. Centrifuge at 600 RCF (~1700 rpm) for 1 hour at room temperature.
30. Incubate the plate at 37ºC in a tissue culture incubator for 1-6 hours.
31. Resuspend cells with 1 mL Splitting Medium (+++) and transfer cells to 15 ml Conical tube.
32. Add 9ml ice-cold Splitting Medium (+++).
33. Centrifuge at 850 rpm (113 RCF) for 5 min at 4ºC.
34. Remove the supernatant.
35. Resuspend with 100~ 200 µL Matrigel and plate into 24 well plate (50 µL / well) on a hot water flask.
36. Incubate 37ºC tissue culture incubator to allow Matrigel to harden 15 min.
37. Add Rho Kinase Inhibitor to Mouse, Human Normal, or Human Tumor Feeding Medium. You will need 500 µL Feeding Medium per well plated.
38. Add 500 µL of Mouse, Human Normal, or Human Tumor Feeding Medium (with Rho Kinase inhibitor added) to each well.
39. Return plate to 37ºC tissue culture incubator.
40. Select with antibiotics 2 days after infection.
    a. Optimal concentration for antibiotic must be determined empirically.

More Information on Organoid Culture Reagents

Organoid culture reagents should be prepared in sterile conditions and with great care. When resuspending the powders, make sure there is no particulate matter left in the vial. Aliquot into single use volumes and store at -20ºC.

Most dry components can be resuspended with just gentle pipetting. If necessary, some components can be heated at 37ºC or vortexed to help them go into solution. Double check the certificate of analysis or product specification sheet for more details on reconstitution and storage.

Growth Factor Reduced Matrigel, Phenol Red Free

Need 125 µL for organoid isolation and 25 µL per well (48-well plate) or 50 µL per well (24-well plate) for passaging
Matrigel should be in -20 freezer or on ice at all times.
Matrigel begins to harden at temperatures above 0ºC.
Allow enough time for your Matrigel aliquot to thaw on ice before you need it: an 800 µL aliquot of frozen Matrigel takes ~ 1 hour and 15 minutes to thaw on ice, and a full vial takes ~8 hours.
To aliquot a full vial, thaw on ice overnight, and aliquot into pre-chilled tubes using pre-chilled tips.

10% Neutral Buffered Formalin (NBF)

The Tuveson Lab buys NBF.
Store at room temperature.
Used for organoid isolation.

TrypLE

TrypLE is more gentle than ordinary trypsin.
Store at room temperature.
Used for T or M organoid isolation, human isolation, and single cell preps.

Fire-polished pipettes

Used for passaging or freezing organoids.
Fire-polished pipettes are glass pipettes whose ends have been constricted by rotating the pipettes in a fire.
To make a fire-polished pipette, set up a Bunsen burner inside of a tissue culture hood. Rotate a glass tissue culture pipette quickly while holding the very end of the pipette in the flame of the Bunsen burner. The goal is to narrow the opening of the glass pipette to approximately half of the diameter of the starting pipette.

Hot-water flask

At all times, we keep a hot water flask in the 37ºC tissue culture incubator. The flask serves as a warming plate to keep 24-well/48-well tissue culture plates at 37ºC while making Matrigel domes of organoids. Since Matrigel hardens at 37ºC, the hot water flask enables the tissue culture plate to stay warm while Matrigel/organoid domes are spotted, ensuring that the domes begin to solidify immediately after they are spotted. This reduces the risk of Matrigel domes collapsing after spotting.
To make:

In a tissue culture hood, fill a T-75 75cm^2 tissue culture flask to top with sterile water.
Add a few drops of a water bath antifungal agent.
Close up flask, and seal cap with parafilm.
Prepare in advance and store in the tissue culture 37ºC incubator.

Advanced DMEM/F-12

Used for Human and Mouse Media
Store at 4ºC.

100X Penicillin/Streptomycin

Used for Mouse Media
Thaw, aliquot and store at -20ºC.

400X Primocin

Used for Human Media
Thaw and store at 4ºC.

100X (1M) HEPES Buffer (Thermo-Fisher)

Used for Human and Mouse Media
Store at 4ºC.

100X GlutaMAX Solution (Thermo-Fisher)

Used for Human and Mouse Media
Store at 4ºC.

50X B27 Supplement

Thaw, generate 400 µL aliquots, and store aliquots at -20ºC
Once thawed, keep working aliquot at 4ºC.

R-Spondin1-Conditioned Medium

Cells engineered to produce R-spodin1 can be obtained from Trevigen (Catalog # 3710-001-K).
See the "Protocol for Production of R-spondin1-Conditioned Medium" on page 55 for more information.
In the Tuveson Lab, we use R-Spondin1-conditioned medium at 10X.
Others have reported success with recombinant R-Spondin1, but in our hands, this did not work.

Wnt3a-Conditioned Medium

Cells engineered to produce Wnt3a can be obtained from ATCC (L Wnt-3A, ATCC #CRL-2647).
See the "Protocol for Production of Wnt-3a-Conditioned Medium" on page 57 for more information.

Recovery Cell Culture Freezing Medium

For freezing organoids
Thaw, make 3 mL aliquots, and store at -20ºC.
Thaw before use and refreeze unused medium.

Albumin Solution from Bovine Serum, 30% in DPBS (30% BSA, sterile)

For dissolving medium components
Make 0.1% BSA in DPBS solution by mixing 30% BSA in DPBS with sterile DPBS
Make 0.1% BSA in Ultrapure dH_2O solution by mixing 30% BSA in DPBS with Ultrapure dH_2O

DNase I

Resuspend 10 mg in 1 mL sterile DPBS, and aliquot and store at -20ºC.

Y-27632 (Rho Kinase Inhibitor)

1000X Y-27632 Working Stock (10.5 mM, 3.38 mg/mL)

Resuspend 5 mg in 1480 µL UltraPure dH_2O
Store 25 µL aliquots at -20ºC.
Once thawed, keep working aliquot at 4ºC.

Nicotinamide

100X Nicotinamide Working Stock (1M, 122 mg/mL)

Resuspend 4.88 g in 40 mL DPBS and filter through 0.2 µM filter.
Store 1 mL aliquots at -20ºC.
Once thawed, keep working aliquot at 4ºC.

N-Acetylcysteine

400X N-Acetylcysteine Working Stock (500 mM, 81.6 mg/mL)

Resuspend 5 g in 61.2 mL UltraPure dH_2O and filter through 0.2 µM filter.
Store 1 mL and 50 µL aliquots at -20ºC.
Once thawed, keep working aliquot at 4ºC.

A 83-01

50,000X A 83-01 Concentrated Stock (25 mM, 10.52 mg/mL)

Resuspend 10 mg in 950 µL sterile DMSO.
Store 20 µL aliquots at -20ºC

1000X A 83-01 Working Stock (0.5 mM, 0.21 mg/mL)

Mix 5 µL 50,000X A 83-01 concentrated stock with 245 µL sterile DMSO.
Store 20 µL aliquots at -20ºC.
Once thawed, keep working aliquot at 4ºC.

Mouse Noggin (mNoggin)

1000X mNoggin Working Stock (100 µg/mL)

Resuspend 100 µg in 1 mL sterile 0.1% BSA in UltraPure dH_2O
Store 20 µL aliquots at -20ºC. Aliquots are good for 3 months.
Once thawed, keep working aliquot at 4ºC.
Note, a previous version of this protocol said to resuspend in 0.1% BSA/ DPBS.
However, mNoggin requires the acidic pH of H_2O to be fully soluble.

Human FGF-10 (hFGF-10)

10,000X hFGF-10 Concentrated Stock (1 mg/mL)

Resuspend 500 µg in 500 µL sterile 0.1% BSA in DPBS.
Store 50 µL aliquots at -20ºC. Aliquots are good for 3 months.

1000X hFGF-10 Working Stock (0.1 mg/mL)

Mix 50 µL 10,000X hFGF-10 concentrated stock with 450 µL 0.1% BSA/DPBS.
Store 20 µL aliquots at -20ºC.
Once thawed keep working aliquot at 4ºC.

Human Gastrin (hGastrin) I

10,000X hGastrin I Concentrated Stock (100 µM, 0.21 mg/mL)

Resuspend 1 mg in 4.8 mL sterile DPBS.
Store 20 µL aliquots at -20ºC.

1000X hGastrin I Working Stock (10 µM, 0.021 mg/mL)

Mix 20 µL 10,000X hGastrin I stock with 180 µL Human or Mouse Splitting Media
Store at 4ºC.

Mouse EGF (mEGF)

10,000X mEGF Concentrated Stock (500 µg/mL)

Resuspend 1 mg in 2 mL sterile 0.1% BSA in DPBS.
Store 200 µL and 5 µL aliquots at -20ºC.

1000X mEGF Working Stock (50 µg/mL)

Mix 5 µL 10,000X mEGF stock with 45 µL Human or Mouse Splitting Media.
Store at 4ºC.

Human EGF (hEGF)

10,000X hEGF Concentrated Stock (500 µg/mL)

Resuspend 1 mg in 2 mL sterile 0.1% BSA in UltraPure dH_2O
Store 200 µL and 5 µL aliquots at -20°C.

1000X hEGF Working Stock (50 µg/mL)

Mix 5 µL 10,000X hEGF Concentrated Stock with 45 µL Human Wash Medium.
Store at 4°C.

PGE2

10,000X (10 mM) Concentrated Stock

Resuspend 10 mg in 2.84 mL DMSO

1000X PGE2 Working Stock (1 mM)

Dilute concentrated stock 1:10 in DMSO

Summary of Reconstitution Conditions:

Reagent	Used for	Weight	Diluent	Volume	Heat?	Vortex?	Filter?
DNAse I (100-1000X stock)	Human and Mouse	10 mg	DPBS	1 mL	No	No	No
Y-27632 (1000X stock)	Human and Mouse	5 mg	UltraPure dH2O	1480 µL	No	No	No
Nicotinamide (100X stock)	Human and Mouse	4.88 g	DPBS	40 mL	Yes	Yes	Yes
N-Acetylcysteine (400X stock)	Human and Mouse	5 g	UltraPure dH2O	61.2 mL	Yes	Yes	Yes
A 83-01 (50,000X stock)	Human and Mouse	10 mg	DMSO	950 µL	Yes	Yes	No
mNoggin (1000X stock)	Human and Mouse	100 µg	UltraPure dH2O + 0.1% BSA	1 mL	No	No	No
hFGF-10 (10,000X stock)	Human and Mouse	500 µg	DPBS + 0.1% BSA	500 µL	No	No	No
hGastrin I (10,000X stock)	Human and Mouse	1 mg	DPBS	4.8 mL	No	Yes	No
mEGF (10,000X stock)	Mouse	1 mg	DPBS + 0.1% BSA	2 mL	No	No	No
hEGF (10,000X stock)	Human	1 mg	UltraPure dH2O + 0.1% BSA	2 mL	No	No	No
PGE2 (10,000X stock)	Human	10 mg	DMSO	2.84 mL	Yes	Yes	No

Other Reagents:

DMEM + glucose, L-glutamine, sodium pyruvate	For Mouse Wash and Digestion Media
100% FBS	For Mouse Wash and Digestion Media
Collagenase Crude Type XI	For Mouse and Human Digestion Media
Dispase II	For Digestion Media, Single Cell Dissociation
DMSO (sterile)	For dissolving medium components
DPBS (no Ca^{+2}, no Mg^{+2}, sterile)	For dissolving medium components
Ultrapure DNase/RNase-free dH_2O	For dissolving medium components

Functions of Organoid Media Components:

MEDIA COMPONENT	INFO ABOUT COMPONENT
Advanced DMEM/F-12	Base for media. BSA present in this medium helps prevent organoids and growth factors from sticking to tips, tubes, and pipettes.
HEPES	Buffering agent to maintain pH of media
GlutaMAX	Glutamine supplement
Penicillin/Streptomycin	Antibiotics
Primocin	Antibiotic, antifungal, and anti-mycoplasma agent
BSA	Molecular carrier protein to keep organoids and growth factors from sticking to tips, tubes, and pipettes
A 83-01	Inhibits ALK, leading to TGF Beta Inhibition
mEGF/hEGF	Growth Factor
hFGF-10	Growth Factor, Activates NOTCH pathway
hGastrin I	Hormone, stimulates pancreatic acinar cells to secrete digestive enzymes, pancreatic growth factor
mNoggin	Inhibits BMP4, chordin, follistatin, leading to TGF Beta Inhibition
N-acetylcysteine	Antioxidant – replenishes glutathione stores
Nicotinamide	B vitamin; inhibits poly(ADP-ribose) polymerases (PARP-1), promotes endocrine lineage
B27 supplement	Contains the hormones insulin and progesterone. Leads to retinoic acid activation; contains steroids and antioxidants. Full formulation: Biotin, DL Alpha Tocopherol Acetate, DL Alpha-Tocopherol, Vitamin A, BSA (Fatty acid free Fraction V), Catalase, Human Recombinant Insulin, Human Transferrin, Superoxide Dismutase, Corticosterone, D-Galactose, Ethanolamine HCl, Glutathione (reduced), L-Carnitine HCl, Linoleic Acid, Linolenic Acid, Progesterone, Putrescine 2HCl, Sodium Selenite, T3 (triodo-I-thyronine)
R-spondin1	Activates Wnt pathway, will drive Myc
Wnt3a	Activates Wnt pathway, will drive Myc
PGE2	Agonist of Prostoglandin E2 receptor. Activates the Wnt pathway
Y-27632	Rho kinase inhibitor; helps cells cope with stress

Protocol for Production of R-spondin1-Conditioned Medium

Notes:

R-spodin1-expressing cells can be obtained from Trevigen (Catalog# 3710-001-K).
Information about these cells can be found at: http://kuolab.stanford.edu/
Cells can be used for 15 passages to harvest batches of conditioned medium.

Reagents and Equipment:

Vial of 293T-HA-Rspo1-Fc Cells	Culture Medium without Zeocin
100 mg/mL Zeocin Selection Reagent	Sterile PBS
Sterile 0.05% trypsin	Sterile 0.2 µM filter
15 and 50 mL conical tubes	P2, P20, P200, P1000 pipettes and sterile tips
Sterile aspirator pipettes	Sterile serological pipettes (5, 10, and 25 mL)
Sterile glass bottles	15 cm tissue culture dishes
Pipet-Aid/Pipette controller	175 cm^2 tissue culture flasks
37°C water bath	15/50 mL tube centrifuge
Tissue culture hood with aspirator	37°C tissue culture incubator

Culture Medium without Zeocin – Used to expand the cells
500 mL DMEM + glucose, L-glutamine, sodium pyruvate or Advanced DMEM/F-12
60 mL 100% FBS
5 mL 100X Penicillin/Streptomycin
*Both Culture Medium recipes work, but we switched to using Advanced DMEM/F-12 for the base of our medium to better match the rest of the organoid media.

100 mg/mL Zeocin Selection Reagent
Use 3 µL per mL Culture Medium (final concentration 300 µg/mL) for selection.

Mouse Splitting Medium (+++) – The Conditioning Medium
500 mL Advanced DMEM/F-12
5 mL 100X Penicillin/Streptomycin
5 mL 1M HEPES
5 mL 100X GlutaMAX Supplement

Protocol:

1. Thaw a frozen vial of 293T-HA-Rspo1-Fc cells, and transfer cells into a 15 mL conical tube with 9.5 mL Culture Medium without Zeocin.
2. Centrifuge cells at 133 RCF for 5 minutes and remove medium.
3. Resuspend cells 1 mL Culture Medium without Zeocin and seed cells in a 175 cm^2 culture flask containing 50 mL Culture Medium and 150 µL 100 mg/mL Zeocin Selection Reagent (final concentration 300 µg/mL).
4. Incubate in tissue culture incubator until cells reach confluency (typically 2-4 days).
5. Passage cells into 6 x 175 cm^2 flasks:
    a. Wash once with 25 mL PBS
    b. Add 2 mL trypsin and incubate 3-5 minutes until cells detach.
    c. Quenching with 18 mL Culture Medium without Zeocin and move to 50 mL tube.
    d. Centrifuge cells at 133 RCF for 5 minutes and remove medium.
    e. While cells are centrifuging, prepare 6 x 175 cm^2 culture flasks each containing 50 mL Culture Medium without Zeocin. Add 150 µL 100 mg/mL Zeocin Selection Reagent (final

concentration 300 µg/mL) to one of the flasks (the “Selection Flask”), while leaving the other 5 flasks (the “Conditioning Flasks”) free of Zeocin.

f. Remove medium from pelleted cells.

g. Resuspend cells in 6 mL Culture Medium without Zeocin and transfer 1 mL of cells to each of the 6 flasks.

h. Incubate in tissue culture incubator until cells reach confluency (typically 2-4 days).

6. When the “Conditioning Flasks” are confluent, change medium to conditioning medium:

   a. Carefully pour off the medium from the cells and wash the cells 2x with 25 mL PBS.

   b. Add 50 mL Mouse Splitting Medium (+++) to each flask.

   c. Incubate cells for one week in the tissue culture incubator.

   d. After one week, decant the medium from the cells into transfer to 50 mL conical tubes. (Cells may detach easily.)

   e. Centrifuge the medium for 5 minutes at 300 RCF to pellet floating cells.

   f. Pour the supernatants into a 0.2 µM sterile filter attached to a sterile bottle and filter the medium.

   g. Aliquot filtered medium and either freeze and store at -20^{o}C or store at 4^{o}C.

7. When the “Selection Flask” is confluent, repeat step 5.

Protocol for Production of Wnt-3a-Conditioned Medium

Notes:

- Wnt3a requires the presence of lipids to be active, and therefore will only be functional if produced in medium containing FBS.
- The Tuveson Laboratory uses a special Wnt3a-expressing line that has a zeocin selectable marker, using the protocol below. A similar Wnt3a-expressing cell line, with a G418-selectable marker can be purchased from ATCC (L Wnt-3A, ATCC CRL-2647).
- If using the ATCC cell line to produce Wnt3a-conditioned medium, follow the protocol below, but substitute the ATCC-recommended concentration of G418 for the zeocin.
- Cells can be used for around 15 passages to harvest conditioned medium.
- Wnt activity in Wnt3a conditioned medium can be tested using the TOPflash assay

Reagents and Equipment:

Vial of L-Wnt3a Cells	Culture Medium without Zeocin
100 mg/mL Zeocin Selection Reagent	Sterile PBS
Sterile 0.05% trypsin	Sterile 0.2 µM filter
15 and 50 mL conical tubes	P2, P20, P200, P1000 pipettes and sterile tips
Sterile aspirator pipettes	Sterile serological pipettes (5, 10, and 25 mL)
Sterile glass bottles	15 cm tissue culture dishes
Pipet-Aid/Pipette controller	175 cm^2 tissue culture flasks
37°C water bath	15/50 mL tube centrifuge
Tissue culture hood with aspirator	37°C tissue culture incubator

Culture Medium without Zeocin

500 mL DMEM, high glucose, GlutaMAX, pyruvate
60 mL 100% FBS
5 mL Penicillin/Streptomycin

100 mg/mL Zeocin Selection Reagent

Use 1.25 µL per mL Culture Medium (final concentration 125 µg/mL) for selection.

Procedure:

1. Thaw a frozen vial of L-Wnt3a cells and transfer cells into a 15 mL conical tube with 9.5 mL Culture Medium.
2. Centrifuge cells at 133 RCF for 5 minutes and remove medium.
3. Resuspend cells 1 mL Culture Medium without Zeocin and seed cells in a 175 cm^2 culture flask containing 50 mL Culture Medium without Zeocin or a 15 cm culture dish containing 19 mL Culture Medium without Zeocin.
4. Incubate in tissue culture incubator until cells reach confluency (typically 2-4 days).
5. Passage cells into 6 x 175 cm^2 flasks:
    a. If cells are in flask, carefully pour off the medium, wash cells once with 25 mL PBS, incubate with 2 mL trypsin, and quench with 18 mL Culture Medium without Zeocin.
    b. If cells are in 15 cm dish, aspirate off the medium, wash once with 20 mL PBS, incubate with 2 mL trypsin, and quench with 18 mL Culture Medium without Zeocin.
    c. Transfer cells to a 50 mL conical tube.
    d. Centrifuge cells at 133 RCF for 5 minutes.

e. While cells are centrifuging, prepare 6 x 175 cm^2 culture flasks each containing 50 mL Culture Medium without Zeocin. Add 62.5 µL Zeocin to only one of the flasks (the "Selection Flask"), while leaving the other 5 flasks (the "Conditioning Flasks") free of Zeocin.

f. Remove medium from pelleted cells.

g. Resuspend cells in 6 mL Culture Medium without Zeocin and transfer 1 mL of cells to each of the 6 flasks.

6. When the "Conditioning Flasks" are confluent, passage cells into conditioning medium:

   a. Carefully pour off the medium from the cells and wash the cells with 25 mL PBS.

   b. Trypsinize the cells using 2 mL trypsin.

   c. Quench the trypsin with 18 mL Culture Medium without Zeocin

   d. Pool all the cells in a 1 L sterile glass bottle. Add additional Culture Medium without Zeocin to bring the total volume to 600 mL.

   e. Plate 20 mL of cells onto 15 cm culture dishes, making a total of 30 dishes.

   f. Incubate cells for one week in the tissue culture incubator.

   g. After one week, use a serological pipette to collect the medium from the cells, and transfer the medium to 50 mL conical tubes.

   h. Centrifuge the medium for 5 minutes at 300 RCF to pellet floating cells.

   i. Carefully pour the supernatants into a 0.2 µM sterile filter attached to a sterile bottle and filter the medium.

   j. Aliquot filtered medium and either freeze and store at -20^{o}C or store at 4^{o}C.

7. When the "Selection Flask" is confluent, repeat step 5.

Protocol for Production of Afamin/Wnt3a-Conditioned Medium

Notes:

- Afamin is a serum glycoprotein that forms a complex with Wnt proteins and helps keep Wnt soluble. While serum must normally be present to solubilize Wnt3a in conditioned medium, co-expression of Wnt3a with afamin bypasses the requirement for serum in the conditioning medium since the presence of afamin alone will keep the Wnt soluble.
- The Afamin/Wnt3a-expressing cell line can be obtained from the laboratory of Dr. Junichi Takagi at Osaka Univeristy, and is described in the following publication:

  Mihara E, Hirai H, Yamamoto H, Tamura-Kawakami K, Matano M, Kikuchi A, Sato T, Takagi J. 2016. Active and water-soluble form of lipidated Wnt protein is maintained by a serum glycoprotein afamin/a-albumin. *eLife* 5:e11621.
- Wnt activity in Wnt3a conditioned media can be tested using the TOPflash assay.

Reagents and Equipment:

Vial of Afm/Wnt3a Cells	Culture Medium without Zeocin/Hygromycin
Mouse Splitting Medium (+++)	100 mg/mL Zeocin Selection Reagent
50 mg/mL Hygromycin B	Sterile PBS
TrypLE Express	Sterile 0.2 µM filter
P2, P20, P200, P1000 pipettes and sterile tips	Sterile serological pipettes (5, 10, and 25 mL)
15 and 50 mL conical tubes	15 cm tissue culture dishes
Sterile aspirator pipettes	Sterile glass bottles
Pipet-Aid/Pipette controller	15/50 mL tube centrifuge
37°C water bath	37°C tissue culture incubator
Tissue culture hood with aspirator	

Culture Medium

500 mL DMEM, high glucose, GlutaMAX, pyruvate
50 mL 100% FBS
5 mL 100X MEM Non-Essential Amino Acids Solution
2.5 mL Penicillin/Streptomycin

Mouse Splitting Medium (+++) – The Conditioning Medium

500 mL Advanced DMEM/F-12
5 mL 100X Penicillin/Streptomycin
5 mL 1M HEPES
5 mL 100X GlutaMAX Supplement

100 mg/mL Zeocin Selection Reagent

Use 5 µL per mL Culture Medium (final concentration 500 µg/mL) for selection.

50 mg/mL Hygromycin B

Use 4 µL per mL Culture Medium (final concentration 200 µg/mL) for selection.

Procedure:

1. Thaw a cryopreserved vial of hAFM/Wnt3a cells quickly in a 37°C water bath.
2. Transfer the cells to a 15 mL conical tube containing 10 mL Culture Medium at room temperature.
3. Centrifuge cells at 200 RCF for 5 minutes at room temperature.

4. Remove the supernatant, and resuspend the cells in 500 µL Culture Medium.
5. Seed cells in a 15 cm tissue culture dish containing 20 mL Culture Medium.
6. Place plate in a 37ºC tissue culture incubator, and culture until confluent.

7. When cells are ready to be passaged, prepare four 15 cm tissue culture dishes containing 20 mL media + 100 µL of Zeocin + 80 µL hygromycin B, and place in the 37ºC incubator to pre-warm.
8. Remove medium from confluent plate of hAFM/Wnt3a cells.
9. Wash cells with 20 mL PBS.
10. Add 3 mL TrypLE Express to plate and incubate at room temperature to detach cells from plate. *Typically it takes ~2 minutes for cells to detach.*
11. Quench the TrypLE by adding with 6 mL Culture Medium, and transfer the cells to a 15 mL conical tube.
12. Centrifuge the cells at 200 RCF for 5 minutes.
13. Remove the supernatant, and resuspend the cells in 1 mL of Culture Medium.
14. Seed 250 µL of the cell suspension in each of the four prepared dishes.
15. Return plates to the 37ºC tissue culture incubator, and culture until confluent (typically up to 4 days).

16. When cells are confluent, prepare two sets of 15 cm dishes:
    a. 4 “Selection Plates” each containing 20 mL Culture Medium + 100 µL of Zeocin + 80 µL hygromycin B, to keep the cells in culture.
    b. 16 “Conditioning Plates” each containing 20 mL Culture Medium without Zeocin or hygromycin B, to generate conditioned medium.
17. Follow steps 8-12 to passage the 4 plates of confluent cells, and resuspend the pellets in a total volume of 5 mL.
18. Seed 250 µL of the cell suspension in each of the 20 prepared dishes.
19. Return plates to the 37ºC tissue culture incubator, and culture for 2 days.

20. After two days, change the medium of the Selection Plates to fresh Culture Medium without Zeocin & hygromycin B.
21. When the Selection Plates are confluent (typically four days after the last passage or two days after changing the medium), split the four Selection Plates into 20 new 15 cm plates as described in steps 16-19 above.
22. On the day that the Selection Plates are passaged, change the medium on the Conditioning Plates to 20 mL Mouse Splitting Medium (+++), and return cells to the 37ºC tissue culture incubator for 7 days.

23. After 7 days, collect the medium from the Conditioning Plates (as batch 1), and replace the medium with 20 mL Mouse Splitting Medium (+++).
    It is best to do this two plates at a time so that the cells don't dry out.
24. Centrifuge the collected conditioned medium at 300 RCF for 6 minutes at 4ºC, and separate the conditioned medium from any pelleted cells.
25. Filter-sterilize the conditioned medium through a 0.2 µm filter, and store at 4ºC.
26. 5 days after the first conditioned medium collection, collect and filter a second batch of conditioned medium as described in steps 23-25, and then throw away the Conditioning Plates.
27. Store the batches of Afm/Wnt3a-Conditioned Medium at 4ºC.
    The Wnt3a activity is comparable between the two batches. However, we have noticed that Wnt3a activity in this medium decreases after freezing, so we recommend not to freeze. We have stored medium up to 2 months at 4ºC, and noticed no decrease in Wnt3a activity.

Protocol for Dual Luciferase Reporter Assay to Measure Wnt Activity in Wnt3a-Conditioned Medium

Notes:

- The protocol is written for measuring Wnt activity in Wnt3a-conditioned medium, but it could be modified to compare the Wnt activity in Wnt3a-conditioned medium vs that in a combination of the Wnt3a-conditioned and RSPO1-conditioned media. The RSPO1-condtioned media should potentiate the Wnt signal.
- This assay uses a TOP plasmid. In this plasmid, TCF/LEF binding sites are upstream of the Firefly luciferase gene. If cells containing this construct are exposed to medium containing Wnt activity, the Wnt drives expression of Firefly lucerifase. As a negative control, this assay uses a FOP plasmid, which is similar to the TOP construct, but with a mutated TCF/LEF binding site. This construct is expected to be unresponsive to Wnt activity. This assay also uses a constitutive renilla luciferase plasmid for normalization.
  Plasmids used for this assay are similar to plasmids available on Addgene or those supplied with the Qiagen "Cignal TCF/LEF Reporter Assay Kit (LUC)" available as product # CCS-018L from SABiosciences Qiagen:
  http://www.sabiosciences.com/reporter_assay_product/HTML/CCS-018L.html
- This protocol is written to perform the assay in a 96 well plate format, with 3 replicate wells per condition for 12 media conditions.
- This protocol is based on that available with the promega dual luciferase reporter assay kit. For more information, see:
  https://www.promega.com/-/media/files/resources/protcards/dual-luciferase-reporter-assay-and-dual-luciferase-reporter-1000-assay-systems-quick-protocol.pdf

Reagents and Equipment:

Confluent 15 cm tissue culture dish of HEK293T cells	Media to be tested
1000 ng/µL FOP plasmid	1000 ng/µL TOP plasmid
DMEM + glucose, L-glutamine, sodium pyruvate	100 ng/µL Renilla plasmid (100 ng/ul)
Sterile PBS	X-tremeGENE9
Promega Dual-Luciferase® Reporter Assay System	TrypLE Express
Sterile 1.5 mL tubes	HEK Culture Medium
15 mL conical tubes	Parafilm
White 96 well plates	P2, P20, P200, P1000 pipettes and sterile tips
Sterile aspirator pipettes	Sterile serological pipettes (5, 10, and 25 mL)
Hemocytometer or cell counter and reagents	Multichannel pipette and tips
Pipet-Aid/Pipette controller	15/50 mL tube centrifuge
Tissue culture hood with aspirator	37ºC water bath
-80ºC freezer	37ºC tissue culture incubator
Rocker	Plate reader with ability to read luciferase signal

HEK Culture Medium

500 mL DMEM + glucose, L-glutamine, sodium pyruvate
50 mL 100% FBS
5 mL Penicillin/Streptomycin

Procedure:

Day 1: Transfect HEK293T Cells With Reporter Constructs

1. Prepare transfection mixes in sterile 1.5 mL tubes:

Transfection Tube 1:	**Volume:**
DMEM + glucose, L-glutamine, sodium pyruvate	37.6 µL
X-tremeGENE9 Transfection Reagent	2.4 µL
TOP plasmid (1 ug/ul)	0.5 µL
Renilla plasmid (100 ng/ul)	0.5 µL

Transfection Tube 2:	**Volume:**
DMEM + glucose, L-glutamine, sodium pyruvate	37.6 µL
X-tremeGENE9 Transfection Reagent	2.4 µL
FOP plasmid (1 ug/ul)	0.5 µL
Renilla plasmid (100 ng/ul)	0.5 µL

2. Gently mix the transfection mixes and incubate at room temperature for at least 20 minutes.
3. Meanwhile, split HEK293T cells (a 10 cm dish usually gives enough cells for this assay).
    a. Aspirate medium from plate.
    b. Wash cells with 3 mL sterile PBS.
    c. Add 2 mL TrypLE Express and incubate at room temperature until cells detach.
    d. Quench with 4 mL HEK Culture Medium, and transfer cells to 15 mL conical tube.
    e. Centrifuge cells at 200 RCF for 5 minutes at room temperature.
    f. Remove supernatant, and resuspend cells in 4 mL of HEK Culture Medium.
4. Count cells using a hemocytometer or cell counter
5. Based on the cell concentration, calculate the volume of cell suspension you will need to make your cell dilution for plating.
    a. For a 96 well plate, you will need to plate 3000 cells per well.
    b. For example, to plate 36 wells at 3000 cells/well, plan to make enough cell suspension for 50 wells.
    3000 cells/well * 50 wells = 150,000 total cells needed.
    The volume of cell suspension needed in mL = $\frac{150{,}000\ cells}{X\ cells/mL\ (from\ cell\ counter)}$
6. Generate two 15 mL conical tubes with cell dilutions. In each tube, combine the volume of cell suspension calculated in the previous step with HEK Culture Medium to a total volume of 5 mL.
7. To one 15 mL tube of diluted cells, add the contents of transfection tube 1, and mix gently by inverting the tube. This is the TOP transfection.
8. Repeat to add the contents of transfection tube 2 to the second 15 mL tube of diluted cells. This is the FOP transfection.
9. Seed 100 µL of the TOP transfection to rows B, D, and F of a clear 96 well plate.
10. Seed 100 µL of the FOP transfection to rows C, E, and G of a clear 96 well plate.
11. Place the 96 well plate in the tissue culture incubator overnight (ideally 24 hours).

Day 2: Treat Cells with Conditioned Medium

12. Prepare the media conditions to be tested.
    a. For each planned condition, prepare 1 mL medium per condition to have more than enough to add 100 µL each to the 3 replicate TOP-transfected, and 3 replicate FOP-transfected wells.
    b. The conditioned media should be at room temperature or at 37ºC before adding to the cells.
    c. Examples of conditions might be:
        i. a negative control (i.e. just the base culture medium used to make the Wnt3a conditioned medium - DMEM with GlutaMAX and pyruvate, 10% FBS, 1% pen/strep)
        ii. undiluted Wnt3a-conditioned medium
        a 50:50 mixture of Wnt3a-conditioned medium and Mouse Splitting Medium (+++)
        iii. a 50:50 mixture of Wnt3a-conditioned medium and Rspo1-conditioned medium
13. Carefully add 100 µL of the prepared media conditions to the appropriate wells of transfected HEK293T cells so that 3 TOP-transfected and 3 FOP-transfected wells receive each treatment.
    a. Since cells were already in 100 µL medium, this becomes 50% HEK Culture Medium, 50 % medium condition to be tested.
    b. Be very careful when adding the treatments since the cells will detach from the plate
    c. Change tips every time to ensure you are pipetting accurately and not mixing wells.
14. Keep in incubator overnight (ideally 24 hours).

Day 3: Lyse Cells

15. Prepare 1x Passive Lysis Buffer by mixing 1.6 mL of ultrapure water with 400 µL 5x Passive Lysis Buffer.
    a. The 1x Passive Lysis Buffer should be used at room temperature.
16. Using a multichannel pipette, carefully remove the media from the HEK293 cells.
    a. Be very careful, as cells will detach off of the plate.
    b. Change tips every time.
17. Add in 5 to 10 µL of Passive Lysis Buffer (depending on confluence of the wells).
18. Swirl / shake the plate sideways to ensure even coverage of passive lysis buffer.
19. Parafilm the plate and keep at -80ºC for a few hours to freeze (can go up to overnight).

Later on Day 3 or Day 4: Read Luciferase

20. When ready to read the plate, thaw the Stop & Glo Buffer and Luciferase Assay Buffer II at room temperature.
21. Thaw the 96 well plate on a rocker with gentle agitation at room temperature for 30 minutes. On the Tuveson Lab rocker, we use dial 4.
22. Make up Luciferase Assay Reagent by adding 10 mL of the Luciferase Assay Buffer II to the lyophilized Luciferase Assay Substrate* powder. Set aside 5 mL of this solution for reading the plate, and store the rest at -80ºC.
    This solution is light sensitive and cannot go through more than 1 freeze-thaw cycle.
23. Make up the Stop & Glo Reagent by adding 100 µL of the Stop & Glo substrate to 5 mL of the Stop & Glo buffer.
24. Transfer 5 µL of the lysed cells to a white 96 well plate.
25. Set up the plate reader to read out the assay:
    a. If using the Softmax Pro Software:
        i. Click on Protocol Manager > Reporter Assays > Dual Glo Assay
        ii. Click the button with gears on it:
            1. For “Plate,” choose “nunc white opaque plate.”
            2. Select the read area (i.e. the wells in which you have cells in).
            3. Make sure these settings are true for both the “Luciferase” plate and the “Renilla” plate
26. Using a multichannel, add 50 µL of Luciferase Assay Reagent to each well, changing tips every time.
27. In the software, select the Luciferase plate at the “Luciferase” tab, and read the plate.
28. When done reading, add 50 µL of Stop & Glo Reagent to each well, changing tips every time.
29. In the software, select the Renilla plate at the “Renilla” tab, and read the plate.
30. Save the plate reader data as an .sda file.
31. Export the raw data as an .xls file for later analysis.

Data Analysis

32. Divide the Luciferase value by the Renilla value
33. This gives you the amount of luminescence normalized to the number of transfected cells
34. Divide the Luciferase/Renilla value for TOP by the Luciferase/Renilla value for FOP.
35. This gives you the luminescence value normalized to the background signal.

Protocol for Orthotopic Transplant of Organoids into Pancreas

Courtesy of Dannielle Engle

Notes:

Please see the following reference for additional guidance and images:

Kim MP, Evans DB, Wang H, Abbruzzese JL, Fleming JB, Gallick GE. Generation of orthotopic and heterotopic human pancreatic cancer xenografts in immunodeficient mice. Nat Protocols. 2009;4(11):1670-80.

Suggestions/Cautions:

- Practice this procedure with your animal facility veterinarian on euthanized mice before attempting this surgery on live animals, especially suturing and wound clipping.
- In the beginning, your procedures will take longer and will benefit from the use of a heated surface. If you can trim down the procedure time to 10 minutes, the heating pad is not necessary.
- Using high cell numbers can increase the risk of cell leakage. If cells leak into the abdominal cavity, a secondary tumor at the wound site on the peritoneum as well as development of malignant ascites is possible and will require the mice to be euthanized earlier.

Reagents and Equipment:

[Cell Preparation]

Ice bucket with ice	Sterile glass pipettes and aspirator
Pipetmen (P20, P200, P1000) and sterile tips	24- or 48-well plate of organoids to harvest
Dipase	Splitting Media (+++)
5 mL LoBind tube	37^{o}C rocking/rotating incubator
Refrigerated 15/50 mL centrifuge	TrypLE
10 mg/mL DNAse	Phenol Red Free Splitting Media (PRF+++)
Cell counting chambers and dye	Matrigel

[Surgery]

Hot bead sterilizer	Surgery cards
Isoflurane/Oxygen mixture	Cells to be transplanted
Shea scissors*	Graefe Forceps*
Dissolvable Sutures, Taper needle (Ethicon Vicryl or PDS II)	0.3-0.5 cc insulin syringes
Wound Clipper (Reflex)	Wound Clips (Reflex 7mm)
Matrigel	Tissue Glue
Analgesic (Ketoprofen)	Eye Gel
Topical Antibiotic	Isoflurane
Betadine	Sterile Sponges
Sterile Cotton Tips	Trypan Blue or GI Spot

* Tool available from Fine Science Tools

Procedure:

1. Prepare cells (always prepare more than needed - 20% excess):
    a. Begin with organoids grown to confluence and dissociate them into single cells.

b. Remove media and harvest Matrigel mounds in 1–4 mg/ml Dispase in Splitting Media (+++) into a 5 mL LoBind tube.
    i. Harvest 6 wells with 1 mL of Dispase in a MW24.
    ii. Dispase concentration depends on the activty of current lot of Dispase. Begin with 1 mg/mL, but if this is insufficient to dissolve Matrigel in 30 min, increase in 1 mg/ml increments.
c. Incubate tube rocking at 37ºC for 15-30 minutes.
d. Spin cells (200 RCF, 5 minutes) and remove sup
    i. Determine whether there is residual Matrigel.
    ii. If there is, add fresh Dispase and dissociate for another 30 minutes as above.
    iii. If not, then proceed.
e. Add TrypLE (2–3 mL) with 10 µL 10 mg/mL DNase.
f. Incubate at 37ºC for 5-30 minutes with occasional vortexing
g. Monitor thereafter every 5 minutes until single cell dissociate looks sufficient
    i. Add more DNAse if see clumping (5 µL / 5 mL).
h. Spin cells as above and remove sup
i. Wash cells in Phenol Red Free Splitting Media (PRF+++), spin, and remove sup
j. Resuspend cells in PRF+++, and count cells.
k. Make working dilution of cells in 50% Matrigel/PRF+++
    i. Some people use the more concentrated form of Matrigel or Matrigel that has not been depleted with growth factors, but growth factor reduced Matrigel also works
l. Keep cells on ice

2. Turn on the hot bead sterilizer and 37ºC Recovery Chamber
3. Begin labeling Surgery Cards
4. Clean and organize work space
5. Perform laparotomy
    a. Sterilize tool tips
    b. Induce the mouse with 4% Isoflurane/Oxygen
    c. Transfer the mouse to nose cone, reduce Isoflurane to 2%
    d. Apply eye gel to mouse
    e. Administer analgesics (5 mg/kg Ketoprofen)
    f. Apply ear code to the mouse
    g. Pluck the hair from the wound site (over the spleen) (~ 3 cm diameter)
        i. Alternatively, remove fur with hair clippers followed by Nair (5 min)
        ii. Make sure to remove all Nair from wound site using water and sponges
    h. Sterilize the area using Betadine + Cotton Tip, three times, beginning in the center and moving outwards
    i. Make a small incision (0.5 – 1.0 cm) in the skin perpendicular to the spleen using Shea scissors
    j. Make a smaller incision through the peritoneum same orientation
    k. Exteriorize the spleen by applying very gentle traction using Graefe forceps
        i. Do not close your forceps all the way, this will cut the spleen in half
    l. The pancreas should be attached to the spleen, avoid handling the pancreas excessively, but if needed pull gently to lay out the pancreas
    m. Do not fully expose the pancreas until ready to proceed so that it stays moist
6. Prepare cell injection
    a. Load an insulin syringe with the appropriate volume of cells (20 – 50 µL)
7. Pancreas injection
    a. If the pancreas appears dry, add a drop of sterile saline
    b. Pull the pancreas up vertically
    c. Make sure to locate the major blood vessels

    d. Insert needle in the tail of the pancreas in between your forceps and push the needle down parallel to the major blood vessel as far as the needle will insert
    e. Pull the needle a very small fraction to either side to insure you are within the parenchyma and not outside the pancreas
    f. Press down the plunger and you should be able to see the formation of a clear bubble
        i. You can add 1 µL of Trypan Blue or GI Spot per injection (sterile filtered) to aid in visualizing injections until you are comfortable
    g. Slowly retract the needle by pulling it up straight
    h. Release the pancreas by gently laying it back down

8. End of procedure
    a. Gently move the spleen and pancreas into the peritoneal cavity
        i. Do not put pressure on the bubble from the injection, use the spleen as a handle for the pancreas
    b. Suture the peritoneum (purse string)
    c. Wound clip the skin
        i. Make sure to tent the skin when wound slipping to prevent snagging the peritoneum
    d. Apply tissue glue

9. Place mouse in the 37^{o}C recovery chamber and allow it to wake up prior to transferring to its cage

10. Annotate the details of each mouse by ear code
    a. Include whether the bubble was intact, or estimate percentage of leakage
    b. Include whether there was bleeding or if you nicked the pancreatic blood vessels, if a hematoma formed
    c. Record whether any mouse took longer than normal to recover and any other notable instances (i.e. if the mouse just ate and its cecum is bloated, it can obscure the pancreas and will require that the cecum be gently exteriorized. This will lengthen procedure time and require a lengthening of the incision).

Orthotopic Transplant Worksheet

Line Name, Passage & Date	Viability	Cell Concentration	Working Concentration	Injection Volume Pancreas

Mouse Ear Code & ID, Cage #	Notes

Product Information

Reagent	Vendor	Catalog #	Quantity
For Organoid Culture:			
Matrigel – Growth Factor Reduced, Phenol Red Free	Corning	356231	10 mL
CELLSTAR Cell Culture Multiwell Plates, Polystyrene, 24 well tissue culture plates	VWR (Greiner BioOne)	82050-894	120 plates per case
CELLSTAR Cell Culture Multiwell Plates, Polystyrene, 48 well tissue culture plates	VWR (Greiner BioOne)	82051-002	120 plates per case
For Organoid Isolation:			
Neutral Buffered Formalin	Fisher Scientific	22-110-869	2.5 Gal
Bard-Parker Sterile Disposable Scalpels, #10 Blade	VWR	89176-380	Pack of 10
SWINGSETTE M516 Biopsy Cassettes	VWR	97000-474	500 per case
Petri dishes	VWR	25384-342	500 per case
Square Petri Dishes for Duct Picking	VWR	60872-310	500 per case
Dissection tools (assorted)	Fine Science Tools		
DMEM + glucose, L-glutamine, sodium pyruvate	VWR (Corning)	45000-304	6 x 500 mL per case
FBS	VWR	97068-085	500 mL
Dispase II	Thermo Fisher	17105041	5 g
Collagenase Crude Type XI	Sigma-Aldrich	C9407-1G	1 g
DNase I (bovine pancreas)	Sigma-Aldrich	D5025-150KU	150 KU
ACK Lysing Buffer	Thermo Fisher	A1049201	100 mL
Primocin	Invivogen	ant-pm-2	1g in 20 mL liquid
TrypLE Express Enzyme (1X), Phenol Red	Thermo Fisher	12605010	100 mL
Bovine Serum Albumin solution 30% in DPBS, sterile	Sigma-Aldrich	A9576-50ML	50 mL
Wide Orifice Pipette Tips (with aerosol filter)	VWR	89049-168	6 x 96 tips
Tissue Culture Dishes, Polystyrene, Sterile, 10 cm	VWR (Corning)	25382-166	200 dishes
Truncated Mold, size 12x12mm (for O.C.T.)	Electron Microscopy Sciences	70181	288 molds
Tissue-Tek® O.C.T. Compound, Sakura® Finetek	VWR (Sakura)	25608-930	4 oz.
For Splitting Media (+++):			
Advanced DMEM/F-12	Thermo Fisher	12634010	500 mL
GlutaMAX 100X	Thermo Fisher	35050061	100 mL
Penicillin/Streptomycin (10,000 U/mL)	Thermo Fisher	15140122	100 mL
HEPES 1 M	Thermo Fisher	15630080	100 mL
For Growth Factor Stock Reconstitution			
DMSO	Sigma-Aldrich	D2650-100ML	100 mL
Dulbecco's PBS (DPBS), no Ca^{+2} or Mg^{+2}	Thermo Fisher	14190144	500 mL
Ultrapure DNase/RNase-free dH2O	Thermo Fisher	10977015	500 mL
For Genotyping Organoids			
DNeasy Blood and Tissue Kit	Qiagen	69054	50 columns
QIAquick PCR Purification Kit	Qiagen	28104	50 columns
NEBNext High Fidelity 2x PCR Master Mix	New England Biolabs	M0541S	50 reactions

Reagent	Vendor	Catalog #	Quantity
For Organoid Complete (Feeding) Media:			
N-Acetylcysteine	Sigma-Aldrich	A9165-5G	5 g
Nicotinamide	Sigma-Aldrich	N0636-100G	100 g
Recombinant Murine Noggin	Peprotech	250-38	100 µg
EGF Recombinant Mouse Protein	Thermo Fisher	PMG8043	1 mg
Animal-Free Recombinant Human EGF	Peprotech	AF-100-15	1 mg
Recombinant Human FGF-10	Peprotech	100-26	500 µg
Gastrin I (human)	TOCRIS	3006	1 mg
A 83-01	TOCRIS	2939	10 mg
B27 supplement 50x (serum-free)	Thermo Fisher	17504044	10 mL
Y-27632 (Rho Kinase inhibitor)	Sigma-Aldrich	Y0503-5mg	5 mg
Prostaglandin E2 (PGE2)	TOCRIS/R&D Systems	2296 / 10	10 mg
Polystyrene Tissue Culture 175 cm^2	VWR	89090-950	case of 50
Tissue Culture Dishes, Polystyrene, 150 mm	VWR (Corning)	25383-103	case of 100
Cultrex® R-spondin1 (Rspo1) Cells	Trevigen	3710-001-K	1 vial
L Wnt-3A Cells	ATCC	CRL-2647	1 vial
Zeocin Selection Reagent	Thermo Fisher	R25001	8 x 1.25 mL
Hygromycin B (50 mg/mL)	Thermo Fisher	10687010	20 mL
DMEM, high glucose, GlutaMAX, pyruvate	Thermo Fisher	10569010	500 mL
MEM Non-Essential Amino Acids Solution	Thermo Fisher	11140050	100 mL
Cignal TCF/LEF Reporter Assay Kit (LUC)	Qiagen	CCS-018L	1 kit
293 [HEK-293] cells	ATCC	CRL-1573	1 vial
Promega Dual-Luciferase® Reporter Assay System	Promega	E1910	100 assays
Multiwell Flat-Bottom Plates, Sterile, 96 well	VWR (Corning/Falcon)	62406-081	50 plates
Nunclon 96-Well Plates, Sterile, White	VWR (NUNC)	43300-430	50 plates
For Working With Organoids			
Recovery cell culture freezing medium	Thermo Fisher	12648010	50 mL
Trizol Reagent	Thermo Fisher	15596018	200 mL
Protein LoBind 5 mL Tubes, PCR clean	Eppendorf	0030108302	2 x 50 tubes
Protein LoBind 1.5 mL Tubes, PCR clean	Eppendorf	022431081	2 x 50 tubes
X-tremeGENE 9 DNA transfection reagent	Sigma-Aldrich	06365779001	400 µL
Cell Recovery Solution	Corning	354253	100 mL
Lenti-X™ Concentrator	Clontech	631231	100 mL
Corning cellgro RPMI 1640 Medium w/ L-Gln	Fisher Scientific	MT10040CV	6 x 500 mL
PhosSTOP	Sigma-Aldrich	4906845001	10 tablets
cOmplete Mini, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	11836170001	25 tablets
Hexadimethrine bromide (Polybrene)	Sigma-Aldrich	H9268-5G	5 g
Mr. Frosty Freezing Container	Thermo Fisher	5100-0001	1 container
Nunc 384-Well Tissue Culture Plates, White	VWR (NUNC)	62409-072	30 plates