Protocol for Isolation of Organoids from Normal or PanIN Mouse Pancreas

(edits by LB 12.04.2013; based on MSS/BC 5.16.2013 N/P Protocol and CH/CC/YP 9.10.2013 T Protocol)

Notes

This protocol is for making normal organoids or organoids from mice containing PanINs (e.g. KC mice).
To make organoids from tumor-bearing mice, see the "Protocol for Isolation of T and M Organoids from Mouse Pancreatic Tumors and Metastases" below.
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 room, 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 digestion solution and 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 (37^{o}C, 20% O_2, 5% CO_2)

Part 1: Isolation of Mouse Pancreas

You will need the following (have ready in the necropsy room):

Petri dishes (not tissue culture plates)	Digital scale
Dissection tools	Strong scissors for decapitation
Dissection board	Paper towels and blue pads
Marker	CO_2 chamber with CO_2 hookup and regulator
Hot bead sterilizer for dissection tools	Mouse
Eppendorf tube for mouse tail	
If PanIN/Tumor mouse, you will also need:	
10% Neutral Buffered Formalin (NBF) for histology	Histology cassettes
Pencil to mark histology cassettes	Histology worksheet

Procedure:

1. Sterilize dissection tools in hot bead sterilizer.
2. Prepare your work area: put down a clean blue pad, tack a clean paper towel to your dissection board, and set out paper towels for dissection tools and blotting mouse blood. Separate dissection tools and scissors into three sets, (1) scissors for the mouse decapitation, (2) dissection tools for cutting through the mouse skin, and (3) dissection tools for removing the pancreas. Place a paper towel in CO_2 chamber.
3. If PanIN/tumor mouse, label cassettes for histology with pencil and begin filling in histology worksheet.
4. Label a clean Petri dish, and tare the digital scale with it.
5. Record information about mouse (strain, name, date of birth).
6. Sacrifice mouse in CO_2 chamber.
   Turn the CO_2 on very slowly to keep the mouse as comfortable as possible.
   Watch the mouse the entire time. 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. After 2 or 3 heaving gasps remove mouse from chamber. (Mouse should still be alive but unconscious when you decapitate.)
   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. Alcohol off the mouse.
10. Skin the mouse.
    Locate the mouse's sternum (where the chest bone sticks out). Pull the fur up here, and make a sizeable cut. Cut skin towards back side of mouse. 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. Switch to clean dissection tools!
12. Cut open the peritoneum near the genitals, and cut to expose the intestines and pancreas.
13. Remove the pancreas, while being careful not to rupture the intestines.
    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.
    If intestines are ruptured, your organoids will be contaminated! Discard this pancreas and start over with a new mouse.
14. Place pancreas in the clean, tared Petri dish.
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. Pass the Petri dish containing the pancreas to the person who will digest pancreas. The pancreas should be brought immediately to the Tissue Culture Room to start digestion procedure – do not wait to long to get pancreas into solution.
    If you are working alone, go immediately to a tissue culture hood and begin mincing the pancreas as described in Part 2 of this protocol. Once the pancreas is incubating in Digestion Media, return to the necropsy room to finish necropsy and clean room as described below.
18. If mouse had PanIN or tumor, continue with full necropsy and fill cassettes to send to histology.
19. Cut tail off of mouse and save for confirmation genotyping.
20. Clean up room.
    At Cold Spring Harbor Laboratory, used mouse cages should be covered with trash bag or hair nets and returned to back room of mouse facility. 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 mouse facility.

Part 2: Pancreas Digestion and Duct Picking

You will need the following (have ready in tissue culture):

- Ice bucket with ice
- Digestion Media at room temp
- Timer
- Thermomixer set to 37°C (or rocker in 37°C incubator)
- 15 mL Falcon Tubes
- #10 Scalpels
- Petri Dishes
- Arm rest for each duct picker
- Microscope for each duct picker
- Matrigel on ice
- 48-well plate
- 37°C water bath
- Wash Media on ice
- 10 mg/mL DNase I
- Sterile glass bottle
- 10 and 25 mL pipettes
- Glass pipettes for tissue culture aspirator
- P20, P200, P1000 Pipetmen with tips in TC hood
- P10 or P20 with tips for each duct picker
- Feeding Media with RhoK Inhibitor
- Hot water bottle
- Marker
- 37°C tissue culture incubator

Procedure:

1. Make 2 mL Feeding Media with Rho Kinase Inhibitor before the organoid preparation. (Media can be made up to 3 days in advance.) Before starting the organoid preparation, place Feeding Media with Rho Kinase Inhibitor in the Tissue Culture hood to ensure it comes to room temperature by the time the organoids are ready to be fed. (Feeding Media can also be warmed in a 37°C water bath.)
2. Prepare Wash Media and keep on ice:
   Add 5 mL 100% FBS and 5 mL 100X Penicillin/Streptomycin to 500 mL DMEM
   Wash Media can be stored for 1 month at 4°C, and used for additional isolations.
3. Prepare Digestion Media in a sterile glass bottle and allow it to warm to room temperature.
   Add 12.5 mg Collagenase Crude Type IX and 12.5 mg Dispase II to 100 mL Wash Media.
4. Place a 48-well tissue culture plate on top of a pre-warmed hot water bottle in a 37°C tissue culture incubator.
5. Thaw aliquots of 10 mg/mL DNase I and Matrigel on ice.
6. Prepare a 15 mL Falcon tube with 5 mL Wash Media to receive the minced pancreas, and place tube on ice.
7. Mince pancreas in Petri dish with 2x #10 scalpels. Be careful not to over-mince! Just get pancreas broken up into ~1-2 mm size pieces.
8. Transfer minced pancreas to 15 mL Falcon Tube containing Wash Media.
9. Use 5 mL Wash Media to rinse Petri dish and get all pancreas pieces into Falcon Tube.
10. 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.
11. Aspirate off fat and most of Wash Media, leaving ~1 mL Wash Media with pancreas pieces in bottom of tube. Be careful not to aspirate pancreas pieces.
12. Add 10 mL Digestion Media and incubate at 37°C in Thermomixer at 450 rpm (inverting tube every 5 minutes). Alternatively, a rocker in a 37°C incubator or a water bath (inverting tube every 5 minutes) can be used.
    If you are working alone, continue with mouse necropsy during this incubation.
13. Prepare a 15 mL Falcon Tube with 10 mL Wash Media for each duct picker.
14. After 20 minutes, remove tube with pancreas digest from 37°C.
15. Pipet up and down with 10 mL pipette to break up chunks.

16. Let tube stand ~1 minute to allow pancreas pieces settle by gravity.
17. Transfer 8.5 mL of supernatant to a clean Petri dish that contains 10 mL Wash Media. This is "Wash 1."
18. 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 19, and 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.
    c. Use a P10 or P20 set to 8-10 µL. Push Pipetman plunger all the way down, 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 Falcon tube with Wash Media.
    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 these may prevent organoids from growing. Avoid picking blood vessels, which are similar to ducts, but are a bit larger in size (thicker diameter) with a visible, hollow lumen.
    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 Wash Media 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.
19. Add 8.5 mL more Digestion Media to Falcon tube with pancreas and incubate at 37^{o}C for 10 minutes as in step 12.
20. After 10 minutes, remove tube from 37^{o}C and repeat steps 15-19 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 Digestion Media 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)
21. Once ducts are picked into 15 mL Falcon Tube(s), spin tube(s) in the clinical centrifuge at 850 rpm (145 RCF) for 5 minutes at 4^{o}C.
    Pellet will be very small, or may not be quite visible.
22. If you have more than 1x 15 mL Falcon tube with ducts from the same mouse, aspirate off most of the media and use Wash Media to pool all ducts into 1x 15 mL Falcon tube. Then spin again at 850 rpm (145 RCF) for 5 minutes at 4^{o}C.
23. Remove as much media as possible: aspirate down to ~200 µL and use Pipetman to carefully remove the rest. (Residual media will dilute the Matrigel and may prevent Matrigel domes from solidifying, so try to remove as much media as possible without sucking up your pellet.)
24. Resuspend pellet in Matrigel and plate 25 µL Matrigel domes in a pre-warmed 48-well plate on top of a pre-warmed hot water bottle.

Typically, we resuspend in 125 µL Matrigel and make 5 x 25 µL wells in a 48 well plate, but may vary depending on pellet size.
More information about plating Matrigel domes can be found in Part 1 of the "Protocols for Culturing, Passaging, Freezing, and Thawing Mouse Organoids."

25. Keeping the 48-well plate on hot water, carefully place plate and water bottle into 37^{o}C incubator for 15 minutes so Matrigel can harden.
26. After 15 minutes, remove 48-well plate on hot water bottle, and carefully add 250 µL of warm/room temperature Feeding Media with Rho Kinase Inhibitor to each well.
Once organoids are fed, plate no longer needs to sit on hot water bottle.
27. Check plate in microscope – ducts should be visible in the Matrigel.
28. Return plate and hot water bottle to incubator.

Part 3: Post-Isolation:

1. Within 1-2 days, organoids should start to bud out of ducts.
2. If mouse has PanIN or tumor, make sure to finish preparing cassettes for histology.
In the Tuveson Laboratory, we incubate 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.
3. Change media after 2-4 days, depending on how fast organoids grow. Rho Kinase Inhibitor is not needed in media after the initial isolation.
4. 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 Part 1 of the "Protocols for Culturing, Passaging, Freezing, and Thawing Mouse Organoids" for more information on how to passage and freeze organoids.
    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.

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

1. The procedure is very similar to the procedure described above in the "Protocol for Isolation of Organoids from Normal or PanIN Mouse Pancreas", with the changes described below.
2. In addition to the reagents outlined in the "Protocol for Isolation of Organoids from Normal or PanIN Mouse Pancreas," you will need TrypLE, Splitting Media, and 2D Cell Culture Media. For blood collection, you will also need an animal lancet, a heparin tube, and a refrigerated microfuge.
3. Prepare necropsy area and histology cassettes as described in the "Protocol for Isolation of Organoids from Normal or PanIN Mouse Pancreas."
4. 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.
5. Rather than dissecting out whole pancreas, dissect out tumor.
6. In the Petri Dish, be sure to cut a piece of the tumor and place in a labeled cassette for histology. Place cassette in a container containing 10% NBF.
7. 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 23 below).
8. Mince the tumor in a Petri dish, move to a 15 mL Falcon tube containing Wash Media, and aspirate the fat as described in steps 7-11 in Part 2 of the "Protocol for Isolation of Organoids from Normal or PanIN Mouse Pancreas" above.
9. Add 10 mL Digestion Media and incubate on a rocker at 37^{o}C for 16-24 hours.
10. Remove digested tumor from 37^{o}C.
11. Pipet up and down with 10 mL pipette to break up chunks.
12. Let pancreas settle by gravity.
13. Transfer 8.5 mL of supernatant to new tube.
14. Spin this tube at 850 rpm (145 RCF) for 5 minutes at 4^{o}C.
15. Aspirate the supernatant.
16. Resuspend the pellet in 1 mL TrypLE.
17. Add 1 µL 10 mg/mL DNase I.
18. Incubate at 37^{o}C in Thermomixer for 10 minutes at 450 rpm or on a rocker in a 37^{o}C incubator.
19. Add 10 mL Splitting Media to TrypLE-digested pancreas.
20. 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 Media.
    Once 2D cell line is established, 2D Cell Culture Media with 5% FBS can be used to slow growth.
21. Spin remaining pancreas digest at 800 rpm (128 RCF) for 5 min at 4^{o}C.
22. Aspirate media, resuspend organoids in Matrigel and plate as described in steps 23–28 in Part 2 of the "Protocol for Isolation of Organoids from Normal or PanIN Mouse Pancreas" above.
23. For small mets, incubate for 1 hour in Digestion Media at 37^{o}C with 450 rpm shaking or rocking, and follow steps 8-22 above.

Protocols for Culturing, Passaging, Freezing, and Thawing Mouse Organoids

Part 1. Passaging Organoids

Notes:
We culture organoids in a dome of Matrigel bathed in a liquid media. Because organoids are suspended in the Matrigel, they never touch the plastic of a tissue culture dish like a 2-dimensional culture would. Matrigel is a gelatinous protein-rich substance secreted by EHS mouse sarcoma cells, and sold by BD Biosciences. 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 8-9 mg/mL.
Matrigel is frozen at -20°C, liquid at 0°C, and begins to harden above 0°C. Always keep Matrigel aliquots on ice when working with it.

You will need:

24- or 48- well tissue culture plate	Hot-water bottle
37°C tissue culture incubator	Feeding Media at room temp or 37°C
Splitting Media on ice	Ice bucket with ice
15 mL Falcon tube on ice	Matrigel aliquot on ice
P1000 and P200 Pipetmen and tips	Glass aspirator pipettes
Fire-polished glass pipette or needle w/ syringe	

Procedure:

1. Up to 3 days before you begin, make Feeding Media.
2. Just before starting, place Feeding Media at room temp or in 37°C water bath to warm up. Feeding Media must be at least room temperature when you add it to Matrigel domes, or it will cause the Matrigel to melt.
3. Remove Matrigel aliquot and place on ice to thaw.
   An 800 µL aliquot takes ~1 hour 15 minutes to thaw. A complete Matrigel vial takes ~8 hours to thaw.
4. Place a 24- or 48-well tissue culture plate on top of a pre-warmed hot water bottle in a 37°C tissue culture incubator.
5. If using fire-polished pipettes to break up organoids, make these ahead of time.
6. Place Splitting Media on ice. Prepare a 15 mL Falcon tube with 8 mL Splitting Media to receive organoids and place on ice.
7. Aspirate media from organoid wells to be split.
8. Add 500 µL (for 24-well plate) or 250 µL (for 48-well plate) ice-cold Splitting Media 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 Splitting Media containing the broken-up Matrigel to an ice-cold 15 mL Falcon Tube containing 8 mL ice-cold Splitting Media.
10. Use additional ice-cold Splitting Media to wash organoid wells, and fill Falcon Tube containing old Matrigel/organoids with Splitting Media to 10 mL.
11. Spin Falcon Tube containing old Matrigel/organoids at 750 rpm (113 RCF) for 5 min at 4°C.
12. Aspirate off media until approximately 1.8 mL media remain.
    Aspirate carefully, keeping the tip of the aspirator pipet at the top of the liquid.

13. Use fire-polished pipette to break up organoids. Alternatively, use a 22- or 23-gauge needle attached to a 5 mL syringe.
    a. If using a fire-polished pipette, attach pipette to pipette gun and pipet organoids p and down through pipette 7-12 times. Initially, you should see large particles coming up into pipette which should get smaller as you pipette them up and down.
    b. If using a needle and syringe, carefully assemble needle and syringe, remove syringe cap, and lower needle and syringe into 15 mL Falcon tube. Slowly bring up syringe plunger to take in as much media as possible. (With a 1 inch needle and 5 mL syringe, you won't be able to take in all the media, but this is ok.) While holding needle at bottom of tube, push media back out through needle. This will dislodge organoid pellet from bottle of tube. Repeat pulling media and organoids into syringe and pushing out again 6-8 times.
14. Fill 15 mL Falcon tube to 10 mL with ice-cold Splitting Media.
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 Splitting Media.
    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. Spin organoids at 750 rpm (113 RCF) for 5 minutes at 4^{o}C.
17. Carefully aspirate off as much media as possible.
    Get as much media as possible with aspirator and then carefully remove the rest 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 on top of hot water bottle from 37^{o}C tissue culture incubator and spot 50 or 25 µL Matrigel dome for each new well of organoids.
    a. To spot Matrigel dome, keeping 15 mL Falcon tube with Matrigel and organoids on ice, draw Matrigel and organoids into Pipetman, touch tip to the center of a well on a 24-/48-well plate on top of water bottle, and push down pipette plunger while slowly pulling Pipetman up. The Matrigel and organoids should form a dome in the center of the well.
    b. Do not push Pipetman plunger to second stop. This will form bubbles.
    c. Keep tube containing organoids in Matrigel on ice throughout this procedure. This will keep Matrigel liquid and prevent it from hardening too early or inconsistently.
20. Carefully bring 24- or 48-well plate on top of hot water bottle back to 37^{o}C tissue culture incubator, and incubate at 37^{o}C for 15 minutes to allow Matrigel to harden.
21. Bring plate and hot water bottle back to tissue culture hood and add 250 µL of Feeding Media (for wells in a 48-well plate) or 500 µL (for wells in a 24-well plate) of Feeding Media to each well.
22. Check wells in phase-contrast microscope. You should see small pieces of organoids distributed throughout the Matrigel.
23. Return 24- or 48-well plate containing organoids to 37^{o}C tissue culture incubator. The plate no longer needs to be on top of hot water bottle. Return hot water bottle to incubator until next passage.

24. After 2-4 days, organoids will begin to grow quickly, and media will change color. Change media at this point.
25. Organoids are ready to be split again, after 3-7 days, if any of the following are true:
    a. Organoids are very dense in Matrigel
    b. Organoids have grown very large (>20% the diameter of a 50 µL Matrigel dome)
    c. Organoids have been in the same Matrigel for 7 days

    If organoids are not split when one of these is true, they will begin to die in culture, and portions of organoid will appear dark (especially when viewed with phase-contrast).

Part 2. Freezing Organoids

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

You will need:

- Ice bucket with ice
- 15 mL Falcon tube on ice
- P1000 and P200 pipettman and tips
- Fire-polished pipette or needle w/ syringe
- Splitting Media on ice
- Matrigel aliquot on ice
- Glass aspirator pipettes
- Recovery Cell Culture Freezing Medium
- Cell freezing chamber or 2 Styrofoam tube racks for cell freezing

Procedure:

1. Follow the steps for harvesting organoids and breaking them up detailed in steps 1 - 14 in the "Passaging Organoids" Protocol.
2. If planning to passage and freeze organoids, divide resuspended organoids into separate 15 mL Falcon tubes, one containing organoids to be split and one containing organoids to be frozen.
3. Spin organoids at 750 rpm (113 RCF) for 5 minutes at 4^{o}C.
4. Carefully aspirate off as much media as possible.
   Get as much media as possible with aspirator and then carefully remove the rest with a P200 Pipetman.
5. Resuspend organoids in 500 uL Recovery Cell Culture Freezing Medium per intended cryovial, and pipette organoids into 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 Falcon 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 at least 24 hours before transferring cryovials to liquid nitrogen storage.

Part 3. Thawing Organoids

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

You will need:

- 24- or 48- well tissue culture plate
- 37°C water bath
- 37°C tissue culture incubator
- Ice bucket with ice
- 15 mL Falcon tube on ice
- P1000 and P200 Pipetman and tips
- Hot-water bottle
- Splitting Media on ice
- Matrigel aliquot on ice
- Glass aspirator pipettes
- Feeding Media with Rho Kinase Inhibitor

Procedure:

1. Place an aliquot of Matrigel on ice.
2. Place Feeding Media at room temperature or in a 37°C water bath.
3. Prepare a 15 mL Falcon Tube with 10 mL Splitting Media and place on ice.
4. Remove cryovial containing organoids from liquid nitrogen freezer and thaw quickly in a 37°C water bath.
5. Pour thawed cells into 15 mL Falcon Tube with Splitting Media.
6. Spin organoids at 750 rpm (113 RCF) for 5 minutes at 4°C.
7. Carefully aspirate off as much media as possible.
   Get as much media as possible with aspirator and then carefully remove the rest with a P200 Pipetman.
8. Resuspend organoid pellet in Matrigel and plate and feed as described in steps 18 - 25 of Part 1 of the “Protocols for Culturing, Passaging, Freezing, and Thawing Mouse Organoids.”
9. 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 Feeding Media.

Part 4. Making Organoid Feeding Media

Notes:
Because some of the components of the Organoid Feeding Media may not be stable in solution, we try to make the media no more than 3 days before using it.
The base for the Organoid Feeding Media is Organoid Splitting Media (called "+++" in the Tuveson Lab). This can be made in advance and stored at 4^{o}C, and is good for at least 1 month.

Organoid Splitting Media Recipe

VOLUME	STOCK CONCENTRATION	STOCK
500 mL		DMEM-F12 Advanced
5 mL	100X (1M)	Invitrogen HEPES Buffer
5 mL	100X	Invitrogen Penicillin/Streptomycin
5 mL	100X	Invitrogen GlutaMax

Organoid Feeding Media Recipe

VOLUME	STOCK CONCENTRATION	STOCK CONCENTRATION	STOCK	FINAL CONCENTRATION
20 mL			Organoid Splitting Media	
20 uL	1000X	0.5 mM (0.21 mg/mL)*	A83-01*	0.5 uM (0.21 ug/mL)
20 uL	1000X	50 ug/mL*	mEGF*	0.05 ug/mL
20 uL	1000X	0.1 mg/mL*	FGF-10*	0.1 ug/mL
20 uL	1000X	10 uM (0.021 mg/mL)*	Gastrin I*	0.01 uM (0.021 ug/mL)
20 uL	1000X	100 ug/mL	mNoggin	0.1 ug/mL
50 uL	400X	500 mM (81.5 mg/mL)	N-acetylcysteine	1.25 mM (0.2 mg/mL)
200 uL	100X	1M (122 mg/mL)	Nicotinamide	10 mM (1.22 mg/mL)
200 uL	100X	100 ug/mL	Recombinant R-Spondin I**	1 ug/mL
400 uL	50X		B27 supplement (50x)	1X
20 uL	1000X***	10.5 mM (3.4 mg/mL)	Y-27632***	10.5 uM (3.38 ug/mL)

* 1000X working stocks of A83-01, mEGF, FGF-10, and Gastrin I are prepared by diluting more concentrated frozen stocks.
** As an alternative to Recombinant R-Spondin I, conditioned media from R-Spondin-producing cells can be used. The Tuveson Lab typically uses conditioned media at 10X.
*** Y-27632 (Rho Kinase Inhibitor) is only necessary when organoids are first prepared, when organoids are thawed, or when organoids are dissociated to single cells.

Solutions and Key Reagents

Note – See Organoid Reagent List for Suppliers and Catalog Numbers

MEDIA RECIPES

Wash Media

500 mL	DMEM
5 mL	100X Invitrogen Penicillin/Streptomycin (f.c. 1x)
5 mL	100% FBS (f.c. 1%)

Used for mincing pancreas, fat aspiration, diluting washes for picking, and for tubes that receive picked ducts.
Need ~250 mL per mouse for N or P organoid (100 for Digestion Media + ~250 for washes).
Need 10 mL for T or M organoid isolation.
Can be prepared in advance and stored at 4°C for up to 1 month.
Unused DM can be stored at 4°C and used for next isolation.
The Tuveson Lab also calls this "Predissociation Media" or "Dissociation Media"

Digestion Media

100 mL	Wash Media
12.5 mg	Collagenase Crude Type XI (f.c. 0.125 mg/mL)
12.5 mg	Dispase (f.c. 0.125 mg/mL)

Used for digesting pancreas or tumor.
Make this fresh before use.
Need 100 mL per mouse for N or P organoid.
Need 10 mL for T or M organoid, but difficult to make less than 100 mL at a time.
Typically, we make as follows:

a. Take Collagenase out of -20°C freezer and Dispase out of fridge, and allow containers to come to room temperature.
b. Weigh Collagenase and Dispase carefully and pour each into 50 mL Falcon Tube. (Technically, this step is not done in sterile conditions, but seems to work ok.)
c. In Tissue Culture Hood, add 50 mL of DM to to Falcon Tube with enzymes and invert to get enzymes into solution
d. Pour DM with enzymes into a sterile glass bottle
e. Add 50 mL more DM to 50 mL Falcon Tube to rinse tube, and pour this into sterile bottle.

Splitting Media

500 mL	DMEM-F12 Advanced
5 mL	100X Invitrogen HEPES Buffer
5 mL	100X Invitrogen Penicillin/Streptomycin
5 mL	100X Invitrogen GlutaMax

Can be made in advance and stored at 4°C for up to 2 months.
The Tuveson Lab calls Splitting Media "+++"

2D Cell Culture Media

500 mL	DMEM
50 mL	FBS (f.c. 10%)
5 mL	100X Penicillin/Streptomycin

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

Feeding Media with Rho Kinase Inhibitor

	DMEM-F12 Advanced
1X	Invitrogen HEPES (comes as 100X stock)
1X	Invitrogen Penicillin/Streptomycin (comes as 100X stock)
1X	Invitrogen GlutaMax (comes as 100X stock)
0.5 µM (0.21 µg/mL)	A83-01 (TGF Beta Inhibitor)
0.05 µg/mL	mEGF
0.1 µg/mL	FGF-10
0.01 µM (0.021 µg/mL)	Gastrin I
0.1 µg/mL	mNoggin
1.25 mM (0.2 mg/mL)	N-acetylcysteine
10 mM (1.22 mg/mL)	Nicotinamide
1X	B27 supplement
10%	R-spondin-conditioned media
10.5 µM (3.38 µg/mL)	Y-27632 (Rho Kinase Inhibitor)

Make up to 3 days in advance and store at 4^{o}C.
See "Protocol for Culturing Organoids" for further information about the Organoid Media and components.
The Tuveson Lab calls Feeding Media " "Complete +++"
The Y-27632/Rho Kinase Inhibitor helps cells cope with extreme stress, and is only added to the Feeding Media when organoids are isolated for the first time, when organoids are thawed, or when organoids are dissociated down to single cells.

KEY SOLUTIONS AND REAGENTS

Matrigel

Need 125 uL for organoid isolation and 25 uL per small (48-well plate) well or 50 uL per large (24-well plate) well when passaging
Matrigel should be in -20 freezer or on ice at all times.
Matrigel begins to harden at temperatures above 0^{o}C.
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 mg/mL DNase I

Prepare in advance at 10 mg/mL in sterile DPBS, and aliquot and store at -20^{o}C.
Used for organoid isolation

10% Neutral Buffered Formalin (NBF)

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

Invitrogen TrypLE

TrypLE is more gentle than ordinary trypsin.
Store at room temperature.
Used for T or M organoid isolation.

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 bottle

At all times, we keep a hot water bottle in the 37°C tissue culture incubator. The bottle serves as a warming plate to keep 24-well/48-well tissue culture plates at 37°C while making Matrigel domes of organoids. Since Matrigel hardens at 37°C, the hot water bottle 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 37°C incubator.

FEEDING MEDIA FACTORS

DMEM-F12 Advanced

Used for Splitting Media, Feeding Media
Store at 4°C.

Invitrogen 100X Penicillin/Streptomycin

Used for Wash Media, Digestion Media, Splitting Media, Feeding Media
Aliquot and store at -20°C.

Invitrogen 100X (1M) HEPES Buffer

Used for Splitting Media, Feeding Media
Store at 4°C.

Invitrogen 100X GlutaMax Solution

Used for Splitting Media, Feeding Media
Store at 4°C.

A83-01

50,000X A83-01 Concentrated Stock (25 mM, 10.52 mg/mL)

Resuspend 10 mg in 950 uL DMSO.
Store 20 uL aliquots at -20°C

1000X A83-01 Working Stock (0.5 mM, 0.21 mg/mL)

Mix 5 uL 50,000X A83-01 Concentrated Stock with 245 uL DMSO.
Store 20 uL aliquots at -20°C.
Once thawed, keep working aliquot at 4°C.

mEGF

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

Resuspend 1 mg in 2 mL 0.1% BSA/DPBS.
Store 200 uL and 5 uL aliquots at -20°C.

1000X mEGF Working Stock (50 ug/mL)

Mix 5 uL 10,000X mEGF Concentrated Stock with 45 uL Splitting Media.
Store at 4°C.

FGF-10

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

Resuspend 250 ug in 250 uL 0.1% BSA/DPBS.
Store 50 uL aliquots at -20°C. Aliquots are good for 3 months.

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

Mix 50 uL 10,000X FGF-10 Concentrated Stock with 450 uL 0.1% BSA/DPBS.
Store 20 uL aliquots at -20°C.
Once thawed keep working aliquot at 4°C.

Gastrin I

10,000X Gastrin I Concentrated Stock (100 uM, 0.21 mg/mL)

Resuspend 0.1 mg in 480 uL DPBS.
Store 20 uL aliquots at -20°C.

1000X Gastrin I Working Stock (10 uM, 0.021 mg/mL)

Mix 20 uL 10,000X Gastrin I Concentrated Stock with 180 uL Splitting Media.
Store at 4°C.

mNoggin

1000X mNoggin Working Stock (100 ug/mL)

Resuspend 20 ug in 200 uL 0.1% BSA/DPBS.
Store 20 uL aliquots at -20°C. Aliquots are good for 3 months.
Once thawed, keep working aliquot at 4°C.

N-Acetylcysteine

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

Resuspend 5 g in 61.2 mL sterile H2O and filter through 0.22 uM filter.
Store 1 mL and 50 uL aliquots at -20°C.
Once thawed, keep working aliquot at 4°C.

Nicotinamide

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

Resuspend 1.22 g in 10 mL DPBS.
Store 500 uL aliquots at -20°C.
Once thawed, keep working aliquot at 4°C.

50X Invitrogen B27 Supplement

Purchase and store 400 uL aliquots at -20°C
Once thawed, keep working aliquot at 4°C.

Recombinant R-Spondin1

100X Human Recombinant RSPO1 Working Stock (100 ug/mL)

Resuspend 100 ug in 1 mL sterile DPBS
Filter through 0.22 uM filter
Store 100 uL aliquots at -20°C.
Once thawed, keep working aliquot at 4°C.

Alternatively, use conditioned media from a cell line that produces RSPO1.
In the Tuveson Lab, we use RSPO1-conditioned media at 10X.

Y-27632 (Rho Kinase Inhibitor)

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

Resuspend 5 mg in 1480 uL sterile H2O.
Store 25 uL aliquots at -20°C.
Once thawed, keep working aliquot at 4°C.

OTHER SOLUTIONS

DMEM
For Wash Media, Digestion Media

100% FBS
For Wash Media, Digestion Media

Collagenase Crude Type XI
For Digestion Media

Dispase II
For Digestion Media

DMSO (sterile)
For dissolving media components

DPBS (no Ca^{+2}, no Mg^{+2}, sterile)
For dissolving media components

Albumin Solution from Bovine Serum, 30% in DPBS (30% BSA, sterile)
For dissolving media components (make 0.1% BSA/PBS solution using 30% BSA and DPBS)

Sterile H2O
For dissolving media components

Recovery Cell Culture Freezing Medium
For freezing organoids
Store 3 mL aliquots at -20°C.
Thaw before use and refreeze unused media.

MORE INFORMATION ABOUT MEDIA COMPONENTS

MEDIA COMPONENT	INFO ABOUT CHEMICAL
A83-01	TGF Beta Inhibitor
mEGF	Growth Factor
FGF-10	Growth Factor, Activates NOTCH pathway
Gastrin 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
Nicotinamide	B vitamin; inhibits poly(ADP-ribose) polymerases (PARP-1), promotes endocrine lineage
B27 supplement	Leads to retinoic acid activation; contains steroids and antioxidants
R-spondin	Activates Wnt pathway, will drive Myc
Y-27632	Rho kinase inhibitor; helps cells cope with stress of being separated or thawing