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Generating Pancreatic Duct Organoids and Whole- Mount Immunostaining of Intact Organoids

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Abstract

Traditionally, studies on cells and tissues have been performed on isolated primary cells or immortalized cell lines by culturing them in 2D culture dishes or flasks. However, a caveat of 2D culture is that the cells poorly recapitulate their *in vivo* counterparts mainly due to the lack of 3D cell-cell and cell-extracellular matrix interactions. In recent years, the development of *in vitro* organoids as a 3D culture has gained substantial attention as a model to study different tissues. In adults, pancreatic ductal cells have been considered as a source of stem or progenitor cells, thus developing new methods to study ductal cells would be useful. Here, we explain a protocol to isolate mouse pancreatic ductal cells and a cost-effective protocol to generate 3D organoid structures from such ductal cells. Finally, we have devised a protocol for immunostaining of intact whole-organoids without sectioning.

Keywords

organoids; pancreatic ductal cells; whole-mount immunostaining

INTRODUCTION

The pancreas consisting of both endocrine and exocrine components has an important role in physiology and metabolism. Developing new approaches to study different pancreatic cell types could provide valuable information and help to find treatments for pancreas related diseases such as diabetes (Grapin-Botton, 2016). Generally, *in vitro* studies have been done on isolated primary cells isolated freshly from tissues or immortalized cell lines in 2D culture flasks or dishes. While such studies have provided much important data, 2D culture has the limitation that the cells do not maintain their primary characteristics and often rapidly lose their properties (Corritore et al., 2014). This loss is likely to be due to the lack of cell-cell and cell-matrix interactions.

To overcome these obstacle, in recent years, the development of *in vitro* organoids as a 3D culture system has gained attention as a model to study different tissues (Dorrell et al., 2014; Grapin-Botton, 2016; Hattori, 2014; Hindley and Cordero-Espinoza, 2016; Hohwieler et al., 2017; Jackson and Lu, 2016; Jin et al., 2013; Lancaster and Knoblich, 2014; Li et al., 2014;

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Loomans et al., 2018; Oshima et al., 2017; Sato et al., 2009; Shamir and Ewald, 2014; Wills and Drenth, 2017; Xia et al., 2014; Xu et al., 2016). With this method isolated cells are suspended in suitable matrices that provide extracellular interactions, allowing the cells to grow in a 3D format. Culturing isolated cells in a defined 3D environment has been an informative approach to dissect the complex nature of cells in a simple model (Boj et al., 2015; Dorrell et al., 2014; Loomans et al., 2018; Shamir and Ewald, 2014).

In this unit (Figure 1), we describe 4 main protocols in ductal organoid studies: 1) Mouse pancreatic exocrine tissue preparation 2) Isolating mouse pancreatic ductal cells 3) culturing the isolated ductal cells in Matrigel followed by growing organoids and 4) immunostaining the intact whole organoids without sectioning.

Protocol 1:Mouse pancreatic exocrine tissue preparation

Pancreas is composed of both endocrine (islets of Langerhans) and exocrine parts. The exocrine is mainly acinar cells that secretes digestive enzymes which then collected and transported to small intestine by a complex network of ductal cells. To prepare the exocrine portion of the pancreas, you need to follow the islet isolation protocol and at the end of the procedure, instead of collecting islets from gradient upper layers, you collect the pelleted cells that are mainly acinar and ductal cells (**protocol 1**). After isolating exocrine portion, one needs to isolate ductal cells using CD24 as specific mouse pancreatic ductal surface antigen (**protocol 2**).

Animal—C57BL/B6J mice (Jackson Laboratory)

Materials—Ketamine (90–100 mg/kg, AnaSed Akorn Animal Health)/Xylazine (10 mg/kg, KetaVed, Vedco) combined solution.

Collagenase (Vitacyte, CIzyme RI 005–1030)

RPMI 1640 media

Newborn calf serum (NCS)

2 pairs of iris scissors

27g-needle

5 ml syringe

Two pairs of forceps

Bulldog clamp

50 ml conical tube (Corning)

425 μm diameter wire mesh (Cole Parmer cat # SI59987–16)

Ice

Histopaque 1077 (Sigma cat # H 1077 or LSM from Fisher Cat # 25–072-CV)

Serologic pipette

Equipment—Water bath

Centrifuge

Before you begin: Turn on and set water bath to 37°C.

Collagenase solution preparation: Dissolve one aliquot in 30–35ml of RPMI 1640 **without** Serum. You will need 2–3ml of collagenase solution per mouse.

Alternatively, you can dissolve collagenase in either M199 or HBSS.

Prepare additional RPMI 1640 media with 10% newborn calf serum (NCS).

Note: All surgery should be completed within an hour otherwise the first pancreases removed will start digesting.

Note: throughout the protocol unit, RT stands for room temperature which ranges between 22 to 24 °C.

Procedure:

1. Anesthetize the animal with injecting 200 µl of the Ketamine/Xylazine solution/ 100g mouse body weight intraperitoneally.
2. Prepare a 3 ml syringe with 2–3 ml of collagenase solution with a 27 g-needle.

   You can bend the needle at a 90-degree angle to facilitate the injection into the bile duct (see below).
3. Open up the abdomen and expose the pancreas as much as possible by making a V cut from the lower abdomen. Clamp off, with a small bulldog clamp, the pancreatic duct at its duodenal insertion.

   Do not injure the surrounding pancreatic tissue.
4. Isolate the bile duct at the proximal end, being careful not to be above the branch off to the liver.

   If there is a lot of fat, clean it off, make sure not to puncture the portal vein.
5. Cut the duct with the fine iris scissors one third of the way across and insert the needle into the duct as proximal to the liver as possible and rapidly inject the collagenase (2–3 ml per mouse).

   The whole pancreas should distend. Sacrifice the mouse by cutting the diaphragm and heart or aorta. Use tissue or gauze to soak the blood to avoid contaminating the field.

   Note: some may choose to use the dissecting scope.

6. After the collagenase infiltration, carefully dissect the pancreas. Place the pancreas in a 50 ml conical tube. You can put up to two pancreases into one tube.

   Start by removing pancreas from the intestines, moving to along the stomach and then the spleen. When the pancreas is only attached by the bile duct, cut it out of the mouse.

7. Put the tube containing the pancreases in the water bath at 37°C for 17 min.

   For each mouse strain, you should adjust the digestion timing.

8. At the end of the incubation, add 25 ml of RPMI 1640 media supplemented with 10% NCS to each tube to halt collagenase digestion.

   Note: from this step on, the isolation should be done on ice.

9. Hand shake the tubes vigorously for 5–10 seconds to break up the tissue.

10. Wash the islets to remove the collagenase using a clinical centrifuge at approx. 180g for 1.5 min, RT.

11. Pour off the supernatant and then add 25 ml of media and vortex gently. Then centrifuge 1.5 min at 180 g, RT. Repeat this step so there are 3 washes total.

12. Resuspend the tissue in 25 ml media

13. Filter the suspension through wire mesh to remove the remaining undigested tissue, fat and lymph.

    Add 5–10 ml more to the tube to wash any remaining tissue off and filter through the mesh.

14. Centrifuge 1.5 min at 180 g, RT.

15. Remove the supernatant leaving as little excess media as possible.

    This can be done by turning the tubes upside-down on a clean paper towel.

16. Make the gradient as follows: re suspend the pellet in 10–15 ml Histopaque 1077 and vortex until the suspension is homogeneous. Overlay with 5–10 ml media (with or without NCS) being careful to maintain the sharp interface between the Histopaque and the media.

    Add the media by pipetting slowly down the side of the tube.

17. Centrifuge 20 min at 1750 g, 4°C.

    Set the centrifuge to a very slow acceleration and **no braking**.

18. Take out the tubes very gently. You need the pelleted cells at the bottom of the tube which are mainly ductal cells and acinar cells.

Optional: collect the islet layer from the interface with a disposable 10ml serologic pipette.

Protocol 2: Isolating mouse pancreatic ductal cells using immunomagnetic beads

Here, we describe how to isolate mouse pancreatic ductal cells from pancreatic tissue remaining after islet isolation. We used cell surface marker CD24 expressed on mouse ductal cells to isolate pancreatic ductal cells from other pancreatic cells. Briefly, we first washed and digested the remaining pancreatic exocrine tissue to single cells, which then were incubated with anti-CD24 antibody, followed by incubation with magnetic microbeads to which secondary antibody is conjugated. As the last step, the ductal cells labeled with magnetic microbeads on their surface are separated from the rest of the cells by passing them through a strong magnetic field. This approach resulted in a pure ductal cell population that was confirmed by immunostaining and flow cytometry for ductal markers (SOX9, HNF1β and CD24) expression.

NOTE: Proper aseptic procedure should be followed by the operator including wearing sterile gloves and lab coat.

NOTE: The following procedures, except immunofluorescence staining, is performed in a Class II biological hazard flow hood.

NOTE: Working conditions for pancreatic ductal cells isolation and growing organoids must ensure the highest degree of sterility. All solutions must be sterile.

Materials—PBS Buffer Solution (see recipe and Table 1)

Complete RPMI 1640 media (see recipe and Table 2)

Phosphate-buffered saline (PBS, Mg/Ca free) (PBS-CMS)

Ice

Trypsin-EDTA (%0.25) (Thermo Fisher)

15 and 50 ml conical centrifuge tubes (Corning)

10 μl, 100 μl and 1 ml pipet tips

Fisherbrand™ Cell Strainers - 40μm

Rat anti- mouse CD24 antibody (eBioscience)

Goat anti-rat IgG1 microbeads (Miltenyi Biotec, 130–048-501)

LS Columns (Miltenyi Biotec, 130–042-401)

Pipet-aid

5- and 10-ml disposable pipets

Trypan blue (Life Technologies)

37°C CO2 incubator with humidity and gas control

Equipment—Incubating shaker or alternatively shaking water bath, 37°C

Centrifuge

Hemocytometer

NOTE. If the end result is to extract RNA from isolated duct cells, to improve RNA integrity, add additional compounds to the solutions as follows:

RNasin® Plus RNase Inhibitor (Promega) (250 μl/250 ml) to the PBS buffer

EZSolution Y27632 ROCK Inhibitor,10 mM (Y-27632, BioVision) to the RPMI 1640 media

Procedure:

Before you begin,

- prepare fresh PBS buffer and complete RPMI 1640 media according to the recipes.
- Turn on the incubating shaker and set to 37°C.

1. Collect the pancreatic exocrine portion (pelleted cells at the end of the previous protocol) of each mouse in one 50 ml tube. Add 30 ml of complete RPMI 1640 to each tube, vortex briefly. Pipette up and down to resuspend the cells.
2. Let cells settle by gravity for 5 min.

   Do not centrifuge since this would include the dead cells, which otherwise will float.
3. Aspirate off the supernatant as much as possible.
4. Wash cells in 5 ml cold PBS buffer.
5. Centrifuge 3 min at 100 g, RT.
6. To disperse the tissue, prepare the trypsin solution. For each tube make 2 ml trypsin diluted in 10 ml PBS-CMS (not PBS buffer).
7. Add 11 ml of diluted trypsin to each tube and vortex for 10 sec and place in incubating shaker for 10 min at 180 RPM, 37°C.
8. Halt trypsin reaction by adding 10 ml complete RPMI 1640 media and mix well by vortexing for 20 seconds.
9. Pass samples through 40 μm cell strainers to remove big clumps.

   If the digestion is done well, you should not see cell clumps.
10. Centrifuge 3 min at 100 g, RT.
11. Resuspend the cells in 2 ml media and mix thoroughly by pipetting. Then, add 8 ml more of the media to each tube.

Take 100 μl sample of the cells and check under microscope. You should see most of the cells as single cells. If there are small or big clumps of cells, you need to increase the digestion time or gently increase the number of pipetting up and down to obtain homogenous single cell suspension. It is critical to check them at this stage, otherwise you will not get a pure population.

If there are undigested tissue or clumps of cells, you can put them in trypsin solution for few more minutes and check the singularity again. Also, keep in mind that trypsin is a sensitive enzyme and make sure you use an active trypsin.

Moreover, you can check the cells' viability using trypan blue vital dye.

12. Resuspend pellet with primary antibody rat anti- mouse CD24 (1:100) in PBS buffer.

For making 4 ml of the solution per tube: 40 μl antibody+ 4 ml PBS buffer

13. Add 2 ml of primary antibody to each 15 ml tube and incubate for 45 min on slow speed shaker, 4 °C (we put the shaker in cold room).

Set the shaker at a very low speed to avoid shear force on the cells. A constant slow movement to avoid sedimentation of the cells is enough.

14. To remove unbound antibodies, add 10ml PBS buffer.

15. Centrifuge 3 min at 100 g, RT.

16. Dilute microbeads in PBS buffer. Beads are conjugated with goat anti-rat IgG1 antibody.

For each tube prepare 960 μl of beads and 3840 μl of PBS buffer.

17. Incubate 30 min on slow shaker, 20 RPM, 4°C.

Set the shaker at a very low speed to avoid shear force on the cells. A constant slow movement to avoid sedimentation of the cells is enough.

18. Add 10 ml PBS buffer to remove unbound microbeads.

19. Centrifuge 3 min at 100 g, RT.

20. Resuspend pellet in 20 ml PBS buffer and mix thoroughly.

21. Run cells through 40 μm cell strainers.

You should not see any cell clumps at this step and the cell suspension must easily pass through the strainer. If there are any small clumps, it could clog the separation column in the following steps and lead to a poor yield and unpurified ductal population.

22. Place a LS column in magnet attached to stand.

23. Run 3 ml PBS buffer through the column to calibrate the column and collect flow-through in 50 ml tube.

24. Run cell suspension through the LS column (one column for each tube).

    You should have 20 ml in each tube but can add only 4 ml to the column at a time.

25. Collect flow through in a 50 ml tube and label as negative (i.e., **not** ductal cells).

26. Wash the column with 5 ml PBS buffer, repeat 3 times.

27. Detach the column from the magnet and put it in a new sterile 50 ml tube (labelled as ductal cells).

28. Wash the cells from the LS column by adding 4–5 ml of PBS buffer to the column and firmly push out positive ductal cells (which are trapped in the column) using the plunger which is provided by the column. Repeat this step 3 times to release all the cells in the column.

    Removing the LS column from the magnet would let the trapped ductal cells be released with the stream of the PBS buffer.

29. Collect all the positive cells into one tube.

30. Centrifuge 3 min at 100 g, RT.

31. Resuspend the cells in 10 ml complete RPMI 1640 media.

32. Count the cells by hemocytometer and assess the cells viability using Trypan Blue dye.

    If needed, it is better to isolate RNA immediately after collecting live cells to minimize RNA degradation caused by freeze and thaw.

    You can assess the purity of the isolated ductal cells by either FACS or immunostaining for ductal markers.

Using this protocol, we could easily isolate approximately 2 million ductal cells from two C57BL/B6J mice (n=10).

Protocol 3: Growing organoids from freshly isolated pancreatic mouse ductal cells

After isolating primary pancreatic mouse ductal cells, next we resuspend the isolated cells in Matrigel to provide an extracellular matrix for embedded cells to form 3D organoids.

Materials—Advanced DMEM/F12 (Life Technologies, Cat# 12634028)

L-WRN cell line conditioned media

Organogenesis media (table 3)

Penicillin/Streptomycin

Fetal Bovine Serum (FBS)

Glutamax (Life Technologies)

Phosphate-buffered saline (PBS, Mg/Ca free)

FGF10 (Peprotech, cat# 100–26)

EGF (Sigma Aldrich, cat# E9644)

Nicotinamide

BD Matrigel Matrix Growth Factor-Reduced, Phenol Red-Free (Corning, cat#356231)

15 ml and 50 ml canonical tube

Trypsin (0.25%)

Ice

70% ethanol

24- well plate

Equipment—Cell culture incubator

Hemocytometer

Prepare in advance: The organogenesis media was prepared according to Table 3. The main component of this media is 50% L-WRN conditioned media, supernatant media collected from L-WRN cell line. This engineered cell line secretes Wnt, R-spondin and noggin proteins which are essential to grow organoids. Prepare this conditioned media according to Miyoshi et al. 2013 (Miyoshi and Stappenbeck, 2013). Preparing the conditioned media takes 7–16 days. After preparing the conditioned media, add the compounds based on Table 3 and then aliquot into 50 ml tubes and store at −20°C freezer. They are generally active for 3–4 months.

One day prior to the experiment prepare the following:

1. Thaw aliquoted Matrigel. Take 1 ml of aliquoted Matrigel out from −20 freezer, put the vial in ice and then put in refrigerator overnight. It helps the Matrigel to thaw gradually and give rises to a homogenous Matrigel solution.

   To avoid freeze and thaw of the Matrigel, after receiving the main batch, thaw the whole bottle (10 ml) as described and aliquot in 0.5–1 ml volumes and store in −20°C. If not thawed according to the instruction, it hardens faster and sometimes forms small clumps which lead to difficulty.

Procedure (1h)—On the day of beginning procedure, thaw one 50 ml tube of organogenesis media. Avoid multiple freeze and thaw. Keep the thawed media in fridge and use it within one week.

1. After ductal cell isolation, count 10×10^3 isolated cells and centrifuge in a 1 ml vial.

2. Aspirate off the RPMI 1640 media.

3. Add 25 µl of organogenesis media to the cell pellet.

4. Add 50 µl of cool thawed Matrigel. Pipet up and down few times.

   Do not make bubbles. Do not warm the tube (Matrigel solidifies at 37°C quickly). Try to handle the Matrigel while is on the ice. If needed spray the ice bucket with 70% ethanol and place it under hood.

5. Put as droplet of cells/Matrigel mixture (25 µl of organogenesis media plus 50 µl of the Matrigel (1:2) that is used to resuspend 10×10^3 cells) at the center of a well in a 24 well plate. Then using the tip of the pipet, spread out the droplet a little bit.

   The droplet diameter should be approximately 6 mm (well diameter in a 24 well plate is 15.6 mm).

6. Put the plate in incubator upside down for 20 min.

   All culture incubations are performed in a humidified 37°C, 5% CO2 incubator unless otherwise specified. This step solidifies the Matrigel.

3. Take out the plate and under the hood add 500 µl of organogenesis media to each well and replace in the incubator.

4. Refresh the media every 3 days.

Note: we achieved the same results when used 1:1 ratio of Matrigel and organogenesis media.

Alternative media: Instead of the organogenesis media containing 50% L-NRW cell line conditioned media, you can make the organogenesis media according to Table 4. We switched to conditioned media because the R-spondin protein is very expensive and limits the use of this approach.

Using this method, we were able to grow organoids from mouse isolated pancreatic ductal cells with a high efficiency (Fig 2).

Protocol 4: Organoids passaging

You can passage the organoids to expand them and have enough organoid for subsequent experiments. Moreover, you may want to cryopreserve organoids for later. Based on our results, we could passage organoids with a high efficiency. We even cryopreserved organoids (in 10% DMSO+ 90% organogenesis media) for over a year and were able to thaw and grow them again successfully.

Materials—PBS (Mg/Ca free)

Cell Recovery Solution (Corning)

Trypsin

RPMI 1640 supplemented with 10% FBS

Ice

Thawed Matrigel (according to instructions)

15 ml canonical tube

24-well plate

Cell culture incubator

Procedure (1h)

1. Aspirate off the media from each well.
2. Wash each well with 250 µl PBS (Mg/Ca free).
3. Add 250 µl of cold Cell Recovery Solution to each well.
4. Disperse Matrigel in each well with pipette tip and transfer to a 15 ml tube.
5. Wash each well with 200 µl extra cold Cell Recovery Solution.
6. Break down the organoid structures to small pieces by pipetting up and down for 50 times with a tip-bent pipet.

   To make bent-tip pipet, attach a 1 ml tip to pipettor and with a 45- degree angle, press the tip against a sterile surface. Alternatively, you can bend the pipettor tip using forceps. The nearer to the end of a pipettor tip the bend is, the smaller the size of broken organoid pieces would be.
7. Leave the 15 ml tube on ice for 20–30 min.

   If you leave it on ice for enough time, the Matrigel would become liquid.
8. Centrifuge 2 min at 100 g, 4°C.
9. Aspirate off the recovery solution.

   Optional: if you need more singular cells, add 1 ml trypsin (0.25%) to each tube and incubate for 3 min at 37°C. You can adjust the digestion timing according to how digested you want the organoids to be. Normally 3 minutes digestion results in many single and small clumps of cells.
11. Add 1 ml RPMI 1640 medium supplemented with FBS to neutralize the trypsin.
12. Centrifuge 2 min at 100 g, RT.
13. Aspirate off the media. Add 2 ml of RPMI 1640 media to wash the remaining trypsin.
14. Centrifuge 2 min at 100 g, RT.
15. Aspirate off the media.

16. Resuspend small pieces of organoids in an appropriate volume of Matrigel plus organogenesis media (2:1) and re-culture at 1:3 ratio.

    You can passage at different ratios depending on your experiments and density you want the organoid culture to be. We generally re-culture each well of a 24 well plate into 3 new wells.

Protocol 5: Growing organoids in 8- well chamber slide for immunostaining

Materials—Hemocytometer

8-chamber polystyrene chamber slides (Corning, Cat# 08–774-26)

Ice

Organogenesis media (see recipe)

BD Matrigel Matrix Growth Factor-Reduced, Phenol Red-Free (Corning, cat#356231)

Cell culture incubator

Equipment—Cell culture incubator

Hemocytometer

Inverted microscope

Procedure

1. Count 20,000 isolated duct cells and mix with 40 μl cold Matrigel and 20 μl of organogenesis media.

   Alternatively, you can put small pieces of organoids (after passaging) in each chamber.

2. Put 10 μl of the cell-Matrigel mixture in each chamber of the chambered cell culture slides.

   Critical step: the thickness of the duct cells and Matrigel suspension in each chamber has to be thin enough to allow antibodies and reagents to easily penetrate and reach the organoids. In our hands, when we put only 10 μl of the mixture we were able to stain the organoids successfully.

3. Put the chambered cell culture slides in cell culture incubator for 20 min to solidify.

4. Add 200 μl of organogenesis media to each chamber.

5. Replace in the cell culture incubator 37°C and 5% CO2.

6. Refresh the media every 3 days.

7. Check the organoid culture every other day by examining with inverted microscope.

Generally, the organoids are grown enough after 6 days to stain. Depends on your aim, you can stain them earlier or later.

Protocol 6: Immunostaining of whole intact organoids

Materials—Formalin 10%

Permeabilization buffer (see recipe)

Washing Buffer (see recipe)

Avidin/Biotin Blocking Kit (Vector Laboratories)

NDS: Normal Donkey Serum

Antibodies (Supplementary Table 1)

VECTASHIELD Antifade Mounting Medium with DAPI (Vectorlabs)

Equipment: Fluorescence microscope

Slow- speed shaker

Notes: Do all the washes between steps on slow- speed shaker

Day 1.

1. Aspirate off the media from each chamber.
2. Fix the organoids by adding 200µl of 10% formalin to each chamber for 30 min, RT.
3. Wash each chamber with 300µl of PBS for 5 min, repeat 2 times.
4. Add 200 µl of permeabilization buffer (Saponin 0.1%) for 15 min, RT, to permeabilize the organoid structures.
5. Wash each chamber with washing buffer for 15 min, repeat 3 times.
6. Add one droplet of Avidin blocking solution to each chamber and leave for 30 min, RT.
7. Wash each chamber with wash buffer for 15 min, repeat 3 times.
8. Add one droplet of Biotin blocking solution to each chamber and leave for 30 min, RT.
9. Wash each chamber with 200 µl of washing buffer for 15 min, repeat 3 times.
10. Add 200 µl of NDS at 1:50 dilution for 1 hour, RT to block non-specific bindings.

    We used NDS because our secondary antibody was raised in donkey. If you use antibodies raised in other hosts, change accordingly.

11. Aspirate off the blocking buffer.

    Do not wash the chambers. Leave NDS on “only secondary antibody” chambers (negative controls).

12. Add primary antibodies to each well and leave overnight at 4°C (Table S1)

Day 2.

13. Take out the chamber slides from 4°C and allow about 1hr to reach RT.
14. Wash the chambers with washing buffer for 15 min, repeat 3 times.
15. Incubate organoids with biotin-conjugated donkey anti-rabbit immunoglobulin G (IgG)
16. Wash the chambers with washing buffer for 15 min, repeat 3 times.
17. Incubate organoids with SA-Alexaflour 594 (red) at 1:200 dilution for 2 hr.

    From this step on, you should keep the samples at dark as much as possible especially during the incubations.

18. Wash the chambers with washing buffer for 15 min, repeat 3 times.
19. Incubate organoids with mouse anti-Ki67 antibody at 1:200 dilution for 2 hr in RT.
20. Wash the chambers with Washing Buffer for 15 min, repeat 3 times.
21. Incubate spheroids with fluorescein isothiocyanate- conjugated (FITC, green) anti-mouse IgG at 1:200 for 2 hr.
22. Wash the chambers with washing buffer for 15 min, repeat 3 times.
23. Add one droplet of antifade mounting medium with DAPI (Vector Laboratories).
24. Keep slide in fridge until visualization.

It is better to visualize and take pictures as soon as possible. But if you want to archive the samples, you can remove the upper part of the chamber slides according to the manufacturer instructions, then add one droplet of VECTASHIELD Antifade Mounting Medium with DAPI on top of each chamber; VERY gently put a coverslip on top of the slide. Do not push the cover slip down since that could destroy the organoid structures. Leave it overnight in dark and next day you can seal off around the coverslip by nail polish and store the slides for few months in 4°C.

Using this protocol, we had successful staining for SOX9, HNF1β, E-CADHERIN, PDX1, HNF6 and Ki67 (Fig 3). You can use confocal microscope to take Z-stack pictures from organoids and construct 3D picture of intact organoids.

REAGENTS AND SOLUTIONS

PBS-CMF buffer

To make PBS-CMS buffer, add 2.5 g of BSA powder to 500 ml of PBS (Ca/Mg free) and leave in 37°C bath for 30 min to dissolve, then add 375 mg of EDTA. Shake it to dissolve completely, adjust the PH=7.4, and then filter sterilize the solution using 0.2 μm filter, keep in 4°C.

Complete RPMI 1640 media

Add 50 ml of FBS to 500 ml of RPMI 1640 media. Then add 5 ml of Pen/Strep (100X). Filter sterilize the solution using 2 μm filter, store at 4°C.

Organogeneseis media

Permeabilization buffer for immunostaining—Make enough (200 μl/chamber) of 0.1% saponin solution. Weigh the calculated Saponin and add to PBS. Stir using magnet until completely dissolved. You can store it at RT. Before use make sure it is not contaminated.

Washing Buffer for immunostaining—PBS + 2% donkey serum + 0.3% Triton X-100

COMMENTARY

Background Information

Organoid structures have versatile applications such as studying cells in an environment that recapitulate their natural environment more closely, also for understanding normal and disease development, to screen for drug testing and these could finally cut down the use of animals in studies. Devising 3D organoid structures has gained significant attention in recent years mainly because of its advantages compare with conventional 2D cell culture systems. With this *in vitro* culture system, one can study cells or tissues in an environment more closely to their *in vivo* condition. Considering all these advantages of organoids technology, developing and optimizing procedures and protocols for growing organoids has become very important. We recently showed that ductal cells culture in 3D organoids maintain their ductal characteristics (Rezanejad et al., 2018) whereas they go through EMT transition when grow in 2D culture (Corritore et al., 2014). We also used this technique to study distinct subpopulations of pancreatic ductal cells. We showed that when mouse pancreatic ductal cells are FACS sorted according to their CD24 expression (low vs. high CD24), only CD24 high population could form organoid structures. It shows that ductal cells have different subpopulations and each subpopulation possesses distinct characteristics.

Critical Parameters

For pancreatic duct isolation: checking the singularity of dispersed exocrine tissue before incubation with CD24 antibody is important. The digestion time and concentration of trypsin are critical. Over digestion leads to loss of the surface marker, CD24, and diminished viability so consequently lower yield, whereas under digestion leaves small clumps of tissue

that later can clog the LS column which result in low separation yield and unpurified ductal cell population.

For suspending cells in Matrigel: it is critical to thaw the Matrigel according to the instructions. We observed that when thawing Matrigel by ways other than instructed in this protocol, it did not result in a homogenous solution. Moreover, the ductal cell density greatly affects the number of organoids formed, so you may want to vary the number of cells per µl of Matrigel. Make sure you use the prepared organogenesis media within 3–4 months.

For immunostaining: we found that the most critical factor was the thickness of the Matrigel in 8 well chamber slides. We tested 20 µl of Matrigel and got considerable background and low staining efficiency, which we believe was due to the insufficient penetration of antibodies and solutions to the organoids. Moreover, we found that long washes (15 min) on a slow-speed shaker led to clearer staining with a low background.

Troubleshooting

Procedure	Problem	Potential Solution
Ductal cell isolation	Low separation efficiency	Magnetic beads have a fairly short shelf-life (check the expiration)
		Make sure the digestion is sufficient. Any remaining cell clumps can clog the separation column.
		It is possible to saturate the column. Check the maximum capacity of the column. Increase the number of aliquots or columns per pancreas.
	Reduced cell viability	Reduce the digestion time.
		Try to keep the procedure as timely as possible.
	Unpurified ductal cells	Check the singularity of cells at the end of dispersion step
Organoid growing	Low number of formed organoids	Organogenesis media may be too old
		Check the Matrigel expiration.
	Matrigel hardens quickly	Thaw the Matrigel according to the instruction. Keep the Matrigel always on ice.
Immunostaining	High background	Increase the washing time. Always washes on slow- speed shaker.
		Use the wash buffer to wash, not PBS alone.
		Adjust the antibody concentration.
	Faint or no staining	Pay attention to the thickness of the Matrigel.
		Use 1:1 ratio of Matrigel: organogenesis media.

TIME CONSIDERATIONS

The pancreatic exocrine tissue preparation (protocol 1) takes approximately 3 hours. Isolating mouse pancreatic ductal cells using immunomagnetic beads (protocol 2) can be done in 3–4 hours. To culture ductal cells for growing organoids from freshly isolated pancreatic mouse ductal cells (protocol 3) takes about 1 hour. Generally, it takes 1 hour to passage the organoids (protocol 4). Protocol 5 can be done in 1 hour. Then it takes 9–15 days for organoids to form and grow. Immunostaining of whole intact organoids (protocol 6) greatly depends on the antibody incubation times. For our used antibodies it took 2 days.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance of the protocol

In vivo studying of cells and tissues is a challenging task owing to their inaccessibility. *In vitro* studying of cell lines and primary cells in traditional 2D flasks poorly represents the *in vivo* counterparts, whereas, 3D organoid cultures can provide an environment that mimic the natural condition more accurately. Here, we describe a protocol to grow organoids from mouse pancreatic ductal cells. Ductal cells have been considered as a source of pancreatic stem/progenitor cells and are important in pancreatic cancer. 3D organogenesis can used as a unique tool to study these cells. Moreover, one of the limitations in studying organoids is the lack of a standard staining protocol to stain organoids in an intact- whole form. Here, we have detailed a method to stain intact organoids without sectioning.

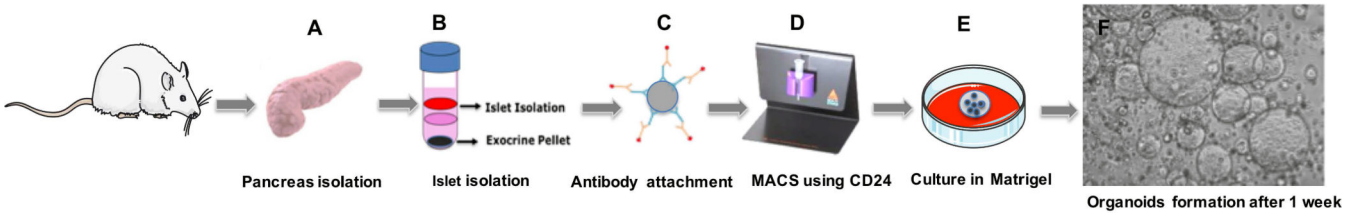

Fig 1. Schematic representation of growing organoid from mouse pancreatic ductal cells.

A. Taking out the pancreas from mouse by surgery **B.** Then, digesting the pancreas and separate the exocrine portion (pelleted cells). **C.** The digested exocrine portion is incubated with CD24 primary antibody. Then, CD24 antibody is labelled with secondary antibody attached to micromagnetic beads. **D.** The exocrine part is passed through a column located in a strong magnetic field. The labeled ductal cells, $CD24^{+}$ cells, are trapped in the magnetic field and unlabeled cells, i.e acinar cells, pass through the column. Afterwards, the column removed from the magnetic field and trapped cells pushed out by strong liquid stream. **E.** After purifying ductal cells, they are mixed with Matrigel, put as droplets in a Petri dish and overlaid by organogenesis media. **F.** After one week growing in incubator, significant number of grown ductal organoids can be observed under microscope.

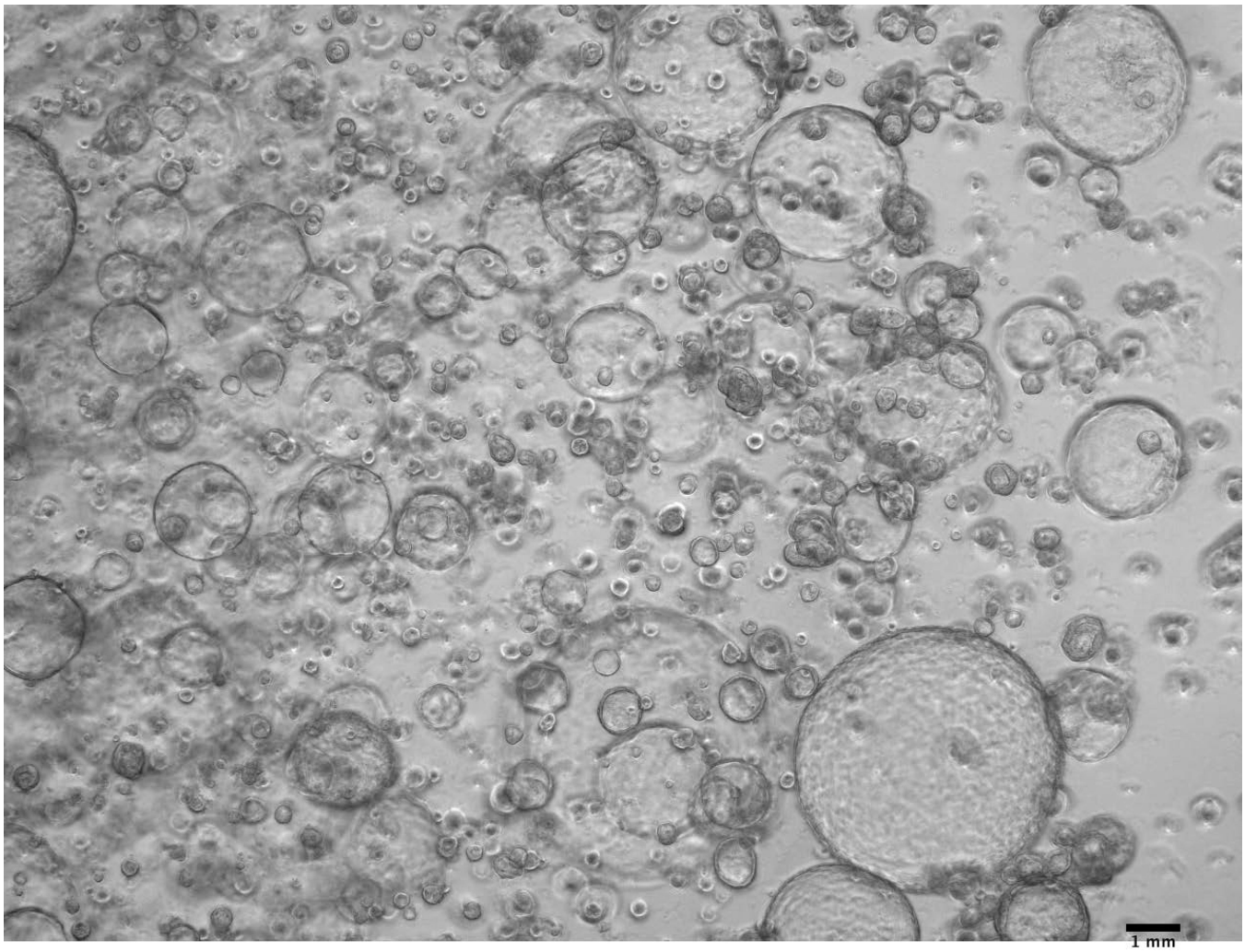

Fig 2.

Isolated ductal cells robustly formed organoids after 10– 14 days in Matrigel. n= 10 experiments

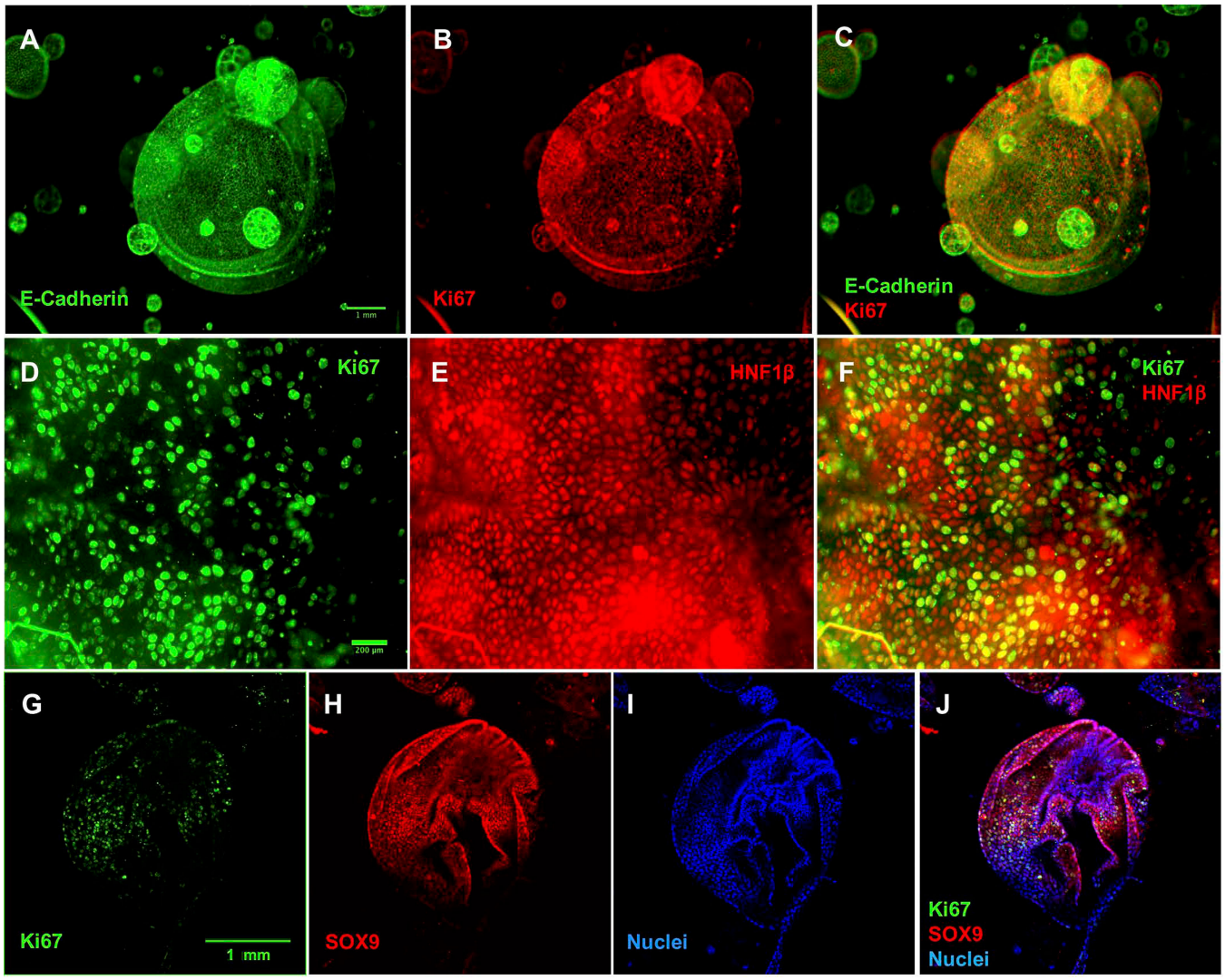

Fig 3. Intact whole- immunostaining of organoids after growing for 14 days.

Immunostaining of whole organoids for **A.** E-Cadherin (green) as a membrane marker **B.** Ki67 (red) as a proliferation marker, which is expressed in the nuclei and **C.** merged picture. It shows the efficiency of our protocol to stain both membrane and nuclear antigens. Organoid stained for Ki67 (green, **D**) and ductal marker HNF1β (red,**E**) and merged channels (**F**). Another organoid stained for Ki67 (**G**), SOX9 (another ductal marker, **H**), DAPI (**I**, to mark the nuclei) and merged channels (**J**).

Procedure	Problem	Potential Solution
Ductal cell isolation	Low separation efficiency	Magnetic beads have a fairly short shelf-life (check the expiration)
		Make sure the digestion is sufficient. Any remaining cell clumps can clog the separation column.
		It is possible to saturate the column. Check the maximum capacity of the column. Increase the number of aliquots or columns per pancreas.
	Reduced cell viability	Reduce the digestion time.
		Try to keep the procedure as timely as possible.
	Unpurified ductal cells	Check the singularity of cells at the end of dispersion step
Organoid growing	Low number of formed organoids	Organogenesis media may be too old
		Check the Matrigel expiration.
	Matrigel hardens quickly	Thaw the Matrigel according to the instruction. Keep the Matrigel always on ice.
Immunostaining	High background	Increase the washing time. Always washes on slow- speed shaker.
		Use the wash buffer to wash, not PBS alone.
		Adjust the antibody concentration.
	Faint or no staining	Pay attention to the thickness of the Matrigel.
		Use 1:1 ratio of Matrigel: organogenesis media.

Table 1.

PBS buffer used to isolate pancreatic primary ductal cells.

PBS Buffer	PBS CMF	500 ml
	EDTA	375 mg
	BSA	2.5 g

Table 2.

PBS buffer used to isolate pancreatic primary ductal cells.

Culture Media	RPMI 1640	500 ml
	FBS	10%
	P/S	1x

Table 3.

Optimized organogenesis media for growing mouse pancreatic duct cell-derived organoids.

Compound	Conc.	Compound	Conc.
Advanced DMEM-F12	50%	FGF10	50 ng/ml
L-WRN condition media	50%	EGF	50 ng/ml
FBS (final concentration)	10%	Nicotinamide	10 mM
Glutamax	1X	Pen/Strep	1X

Table 4.

Alternative organogenesis media to grow organoids from mouse pancreatic ductal cells.

Compound	Conc.	Compound	Conc.
Advanced DMEM-F12	90%	R-spondin	20 μg/ml
FBS (final concentration)	10%	Noggin	20 μg/ml
Glutamax	1X	FGF10	25 μg/ml
N-acetyl cysteine	1.25 μM	EGF	50 ng/ml