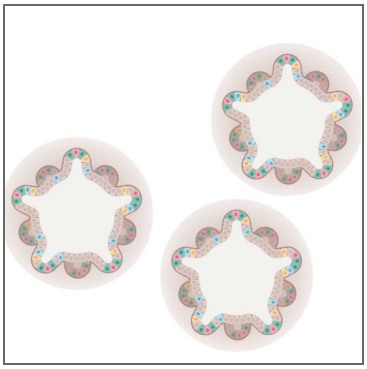

JAN 15, 2024

DOI:
dx.doi.org/10.17504/protocols.io.ewov1qmqkgr2/v1

Protocol Citation: arpine.sokratian, Ian Williamson, Ramos Chavez Katherine, Liddle Roger 2024. Preparation and culture of mouse intestinal organoids. **protocols.io** https://dx.doi.org/10.17504/protocols.io.ewov1qmqkgr2/v1

MANUSCRIPT CITATION:
10.1172/jci.insight.172192

Preparation and culture of mouse intestinal organoids

Ramos Chavez arpine.sokratian[1], Ian Williamson[1], Katherine[1], Liddle Roger[1]

[1]Duke University

ASAP Collaborative Research Network

andrew.west

ABSTRACT

This protocol details an approach for the dissociation of colon epithelial cells from the murine samples. The key goal is to yield intact crypt samples suitable for culture and further dissociation into organoids. The protocols outline specific tools and reagents required for each step ensuring the isolation of crypt units with minimal contamination from other cell types.

ATTACHMENTS

IntestinalOrganoidSOPs 12.3.2023.docx

MATERIALS

SB202190 **LC Laboratories Catalog #S-1700**

A8301 **Merck MilliporeSigma (Sigma-Aldrich)**

Prostaglandin E2 (PGE2) **Cayman Chemical Company Catalog #14010**

Gastrin **Anaspec Catalog #AS-64149**

Nicotinamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #N0636-100G**

Recombinant Murine EGF **Funakoshi Catalog #315-09-1MG**

N-acetyl cysteine **MP Biomedicals Catalog #194603**

B-27™ Supplement (50X) minus vitamin A **Thermo Fisher Scientific Catalog #12587010**

Protocol status: Working
We use this protocol and it's working

Created: Dec 03, 2023

Last Modified: Jan 15, 2024

PROTOCOL integer ID: 91745

Keywords: ASAPCRN

Funders Acknowledgement:
ASAP
Grant ID: 020527

- Primocin **InvivoGen Catalog #ant-pm-1**
- HEPES Buffer **Thermo Fisher Scientific Catalog #15630-080**
- Glutamax (100x) **Gibco - Thermo Fischer Catalog #35050-061**
- Advanced DMEM/F-12 **Thermo Fisher Scientific Catalog #12634-010**
- Disodium phosphate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S7907**
- JAG-1 peptide **Anaspec Catalog #AS-61298**
- Y27632 **ApexBio Technology Catalog #A3008-200**
- UltraPure 0.5M EDTA, pH 8.0 **Thermo Fisher Scientific Catalog #15575-038**
- d-sorbitol **Fisher Scientific Catalog #BP439-500**
- sucrose **Fisher Scientific Catalog #BP220-1**
- KCl **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P5405**
- NaCl **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5886**
- KH2PO4 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P5655**
- Collagenase IV **Fisher Scientific Catalog #NC9919937**
- DPBS no calcium no magnesium **Gibco - Thermo Fischer Catalog #14190250**
- TrypLE™ Express Enzyme (1X), phenol red **Thermo Fisher Catalog #12605036**
- HEPES (1 M) **Thermo Fisher Catalog #15630080**
- 10x Phosphate Buffered Saline **Gibco, ThermoFisher Catalog #70011044**
- Dispase **Corning Catalog #354235**
- DNAse **Merck MilliporeSigma (Sigma-Aldrich) Catalog #DN25-100MG**
- Roche DTT 14-Dithiothreitol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #10197777001**

Dissect Mouse Intestine

1

Note

Up to 3 mice may be processed in parallel. If more than 3 mice are necessary, keep intestines on ice at the end of this procedure before moving to next steps.

Tools and Consumables

☐ IACUC approved isofluorane container for euthanasia (*ex: bell jar*)
☐ Chemical hood
☐ Dissection tools
o Large forceps
o Large scissors
o Small forceps
o Small scissors
☐ Dissection surface (*ex: lid of extra Styrofoam box*)
☐ 4 needles to pin mouse for dissection. (*18-25 gauge work well*)
☐ 10 cm ruler
☐ (Optional) 10 mL syringe fitted with P200 tip trimmed from wide end to fit syringe
☐ 10 cm petri dish (1 per mouse)
☐ Conical tube (1 per mouse)

Reagents

☐ Isofluorane
☐ Ice-cold Epithelial Buffer (~50 mL per mouse) (*PBS may be substituted*)
☐ 70% EtOH in spray bottle

2 Euthanize mouse in chemical hood with isoflurane followed by cervical dislocation in accordance with IACUC-approved protocol.

3 Dissect intestine

Note

1. Mount euthanized animal on dissection plate and secure in supine position with needles.
2. Spray abdomen with 70% EtOH to wet and sterilize fur.
3. Using large scissors and large forceps, make an incision into abdominal skin ~ 2 cm above the anus.
4. Insert scissors below skin and open the blades to separate skin from muscle.
5. Cutting in a V-pattern, free the skin, lifting up and to the side to expose the abdominal muscle. Spray scissors with 70% EtOH to remove any fur that may be stuck on the blades.
6. Perform laparotomy to open abdominal cavity
7. Using small scissors and forceps to shift visceral organs, locate the stomach underneath the liver.
8. Holding the stomach with forceps at pyloric antrum, cut through the stomach immediately proximal to the forceps.
9. Still holding the stomach, gently pull upward and to the left to remove intact intestine from duodenum to rectum.
IMPORTANT: While pulling, use small scissors or a second pair of forceps to release pancreatic adhesions and mesenteric plexus.
10. Transfer to glass plate and stretch intestine to full length.

4 Collect tissue from desired intestinal regions.

Note

1. Trim small intestine by cutting immediately distal to stomach and proximal to cecum.
2. Duodenum: 1st 2-5 cm distal to pyloric sphincter
3. Jejunum: Middle third of the intestine
4. (NOTE: Most small intestinal preps are made from jejunum)
5. Ileum: distal 8-10 cm intestine
6. Trim large intestine by cutting distal to cecum and 10 mm proximal to anus. Remove any remaining non-intestinal tissue.
7. (NOTE: avoid collecting rectum and anus by omitting the distal ~0.5 cm from collection)
8. Proximal colon: Cecum to end of wide curvature
9. Distal colon: end of curvature to ~0.5 cm proximal to the anus

5 Remove luminal debris

Note

1. (optional) Flush intestinal contents with ice-cold EB using a 10 mL syringe fitted with a trimmed P200 tip.
2. Fillet open the intestine by passing scissors through the lumen, starting at the proximal end of the tissue. The intestine may be handled directly or with forceps.
3. Rinse briefly in 10 cm petri dish by agitating filleted intestine in 5-10 mL ice-cold EB.
4. Transfer to conical tube with 5-25 mL of cold EB.
5. Shake to remove any remaining debris.
6. Keep tube on ice until proceeding

Murine Small Intestine Epithelial Dissociation

6

Note

The final prep will be intact crypt/villus units. Crypts may separate from villi, which is desirable if planning to enrich for crypts. Expected contaminating cell types including circulating cells with little contamination from mesenchymal cell sources. Crypt/villi units isolated with this protocol can be fixed, cultured, or further dissociated into single cells.

IMPORTANT: If crypts will be plated in Matrigel, begin thawing Matrigel several hours before starting dissociation, as per SOP: Plate in Matrigel.

Tools and Consumables

☐ Small forceps
☐ Small scissors
☐ 15mL/ 50mL Conical tubes (4 per mouse)
☐ MACSmix™

Tube Rotator

☐ Bacterial plate

Reagents

☐ Intestinal Dissociation Solution: Ice-cold
o 2 tubes per sample containing 5mL per small intestinal segment 10mL sufficient for the entire small intestine
☐ Crypt Shake Solution:
o 2 tubes per sample Containing 5mL per small intestinal segment 10mL sufficient for the entire small intestine

7 Loosen epithelium

Note

1. Use forceps to transfer the intestine from ice-cold EB to the first tube of Intestinal Dissociation Solution.
2. Secure tube in MACSmix™ Tube Rotator rocker and rotate on short intervals in a cold room at 80 rpm for 15 minutes
3. Use sterile forceps to transfer the intestine from the first tube of Intestinal Dissociation Solution to the first tube of Crypt Shake Solution

8 Deplete damaged epithelium

Note

1. Use sterile forceps to transfer the intestine from the first tube of Intestinal Dissociation Solution to the first tube of Crypt Shake Solution
2. Shake sample at 2.5 cycles per second for 2 minutes
3. Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy
3a. The solution should contain villi fragments with very few crypt units
3b. Keep conical for comparison to final preparation
4. Use sterile forceps to transfer the intestine to the second tube of Intestinal Dissociation Solution
5. Secure tube in MACSmix™ Tube Rotator rocker and rotate on short intervals in a cold room at 80 rpm for 30 mins
5a. Crypt yield from distal small intestinal section may be improved by extending to 45 mins

9 Isolate epithelial units

1. Use sterile forceps to transfer the intestine from the second tube of Intestinal Dissociation Solution to the second tube of Crypt Shake Solution
2. Shake sample at 2.5 cycles per second for 2 minutes
2a. Can be extended for large samples containing multiple regions or pooled animal samples
2aa. Tissue fragments float when depleted of epithelium
2aaa. Adipose attached to the intestine can cause premature floating
3. Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy
3a. The solution should contain intact villus/crypt units as well as intact villi and intact crypts
3aa. The solution can be compared to the contents of the first shake tube
3b. Use forceps to transfer a piece of digested intestine to the bacterial plate and

examine with a tissue culture microscope. The remaining crypts appear as dense circles in the epithelium.

3c. NOTE: If crypt yield is sub-optimal and many crypts are visible in the remaining tissue, repeat the shaking step using reserved tissue. To avoid damaging crypts isolated from the first round of shaking, transfer tissue to a new tube with Epithelium Shake. Pool crypts before proceeding to the next step.

10 **Remove digested tissue** using forceps for a sterile cotton swab.

1. Using sterile forceps, transfer digested intestine to a storage tube.

1a. *Optional: Reserve on ice in the event of catastrophic failure at downstream steps*.

2. *Optional: Process intestinal fragment for histology to examine dissociation efficiency.*

Embedding Cypts in Matrigel for Organoid Culture

11

Note

This
protocol is adapted from the Matrigel product sheet and associated literature.
Read the Matrigel product sheet for more detailed handling instructions.

Tools and Consumables

☐ Clinical centrifuge in a cold room
☐ (Optional) cold pipette tips
☐ Conical centrifuge tube
☐ Tissue culture plate
☐ (Optional) 100 µm cell strainer
☐ (Optional) 70 µm cell strainer

Reagents

☐ Growth factor-reduced Matrigel
☐ Organoid Growth Media (several options)
o **Expansion media** (recommended)
o *Note: Many different media combinations*
have been used to grow organoids from crypts. The original media formulations
required expensive recombinant growth factors.
Refer to the Media Formulation sheet for additional recipes. Current
protocols replace Noggin, R-spondin, and/or Wnt3 growth factors with
conditioned media made from L-cells that produce and secrete these factors into
culture media.
§ IntestiCult™ Organoid Growth Medium (Mouse)
- Stem Cell Technologies cat: 06005
- Add supplement A and supplement B

§ 50% L-WRN conditioned media
- Produced according to https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3969856/

§ Antibiotic supplement (several options)
- Pen/Strep, Gibco: 15140-122
- Primocin, InvivoGen: ant-pm-1

☐

(Optional) Crypt Shake Solution:
o 1mL intubes to collect filter flow through

12 **1. (Optional) Separate Crypts from isolated whole epithelial units**

1. Filter isolated epithelial unit solution produced by SOP: Small Intestine Epithelial Dissociation through a 100 µm cell strainer

Gently tap the tube to speed up filtration. Two strainers may be necessary if the whole small intestine is used for crypt isolation.

Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy

The solution should be enriched for crypts but may contain villi fragments that are similar to crypt size

2. Filter the flow-through using a 70 µm cell strainer

Gently tap the tube to speed up filtration. Two strainers may be necessary if the whole small intestine is used for crypt isolation.

Remove a 25 µL aliquot of the Crypt Shake Solution and examine by phase microscopy

The solution should be enriched for crypts but may contain villi fragments that are similar to crypt size

13 **1. Pellet crypts for plating**

1. Remove 3 separate 5 µL aliquots of the filtered crypt solution and examine by phase microscopy
2. Quantify the average crypt contents of the aliquots and extrapolate to determine the total crypt yield
3. Mix the filtered crypt solution by flicking and transfer an adequate volume to a microcentifuge tube
4. Volume is determined by the amount of Matrigel being utilized
5. Transfer 5 crypts/1 µL Matrigel for plating
6. well then aliquot enough crypts for 5 crypts/1 ul Matrigel to 1.5 mL centrifuge tube or up to 5000 single cells per well
7. Centrifuge on benchtop centrifuge in a cold room at 2000g for 5 minutes
8. Remove supernatant and examine an aliquot by phase microscopy to ensure crypts are pelleted efficiently

NOTE: >80% of intact crypts will form organoids. Contaminating single cells that include stem cells can also form organoids.

14 **1. Embed in Matrigel**

1. Centrifuge Matrigel at max speed for 15-30 sec in benchtop centrifuge in a cold room. This will pellet any insoluble collagens that may be in the Matrigel.
2. The amount of insoluble material varies by Matrigel lot.
*3. R*e-suspend pellet directly into the appropriate volume of Matrigel. Mix very well by pipetting ~25-50 times.

**Note: Cells and crypts may be plated in up to a 1:1 dilution in Matrigel.*

Use pre-chilled tubes and tips.

**Optional: Perform pipetting steps on ice.*

4. When pipetting Matrigel from aliquot, pipet up and down one time to coat the inside of the tip. Or, push the pipette plunger slightly past the stop so that no bubbles will be added to the Matrigel when the depressing plunger

DO NOT introduce bubbles into the Matrigel.

5. Plate by adding Matrigel as a hemispheric ‘dome’ to the center of each well in a pre-warmed plate

For 48 well plate, use 25-50 uL Matrigel

For 96 well plate, use 8-10 µL Matrigel

**Note: Place pipette tip on the bottom of the middle of the well, lift slightly, then depress to first stop to plate Matrigel in hemispheric droplet*

6. Carefully transfer the plate to a 37°C incubator and allow the Matrigel to polymerize for 30 minutes

15 **1. Overlay Media**

1. Remove the plate from the incubator
2. Add 10-20x Matrigel volume of desired Culture Media
3. Continue to change culture media on an appropriate schedule
Typically, every 2-3 days
4. Transfer into a standard tissue culture incubator

Organoids are ready for passage after 6-12 days or when the lumens begin to burst and expel contents into the Matrigel

Passage Organoids Grown in Matrigel

16

Note

This protocol is adapted from Matrigel product sheet and associated literature. Read the Matrigel product sheet for more detailed handling instructions.

Tools and Consumables

☐ Clinical centrifuge (in cold room)
☐ (Optional) cold pipette tips
☐ Conical centrifuge tube
☐ Tissue culture plate

Reagents

☐ Growth factor-reduced Matrigel
☐ Organoid Growth Media (several options)
o **Expansion media** (recommended)
o *Note: Many different media combinations have been used to grow organoids from crypts. The original media formulations required expensive recombinant growth factors.*
Refer to the Media Formulation sheet for additional recipes. Current protocols replace Noggin, R-spondin, and/or Wnt3 growth factors with conditioned media made from L-cells that produce and secrete these factors into culture media.
§ IntestiCult™ Organoid Growth Medium (Mouse)
- Stem Cell Technologies cat: 06005
- Add supplement A and supplement B

§ 50% L-WRN conditioned media
- Produced according to https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3969856/

§ Antibiotic supplement (several options)
- Pen/Strep, Gibco: 15140-122
- Primocin, InvivoGen: ant-pm-1

☐ **(Optional) Crypt Shake Solution**:
o 1mL in tubes to collect filter flow through
☐ TrypLE express +10μM Y27632
o 4x matrigel volume being passaged
o Contained in a conical tube of appropriate volume
☐ FBS
o Cold
o 10% of TrypLE volume employed

17 **1. Digest the matrigel and fragment organoid units**

1. Remove culture media from wells being passaged
2. Wash wells in DPBS containing matrigel cultures to remove residual media
3. Add 1.5x culture media volume removed in step 1
4. Remove DPBS and discard

5. Add a small volume of TrypLE +y27 to each well being passaged
6. Add 2x the volume of matrigel in TrypLE +y27
7. Transfer organoids in matrigel for digestion
8. Fragment the matrigel domes in each well using a pipette tip
9. Transfer the fragmented matrigel in the conical tube containing the full volume of TrypLE +y27 being employed
10. Digest the matrigel fragments at 37^{o}C for 5 mins
11. Place the tube upright in a water bath
12. Quench the TrypLE digestion with cold BS
13. Add 10% of the TrypLE solution volume and mix by inverting.

18 **1. Embed organoid fragments in Matrigel**

1.Centrifuge matrigel at max speed for 15-30 sec in benchtop centrifuge in a cold room.
2. This will pellet any insoluble collagens that may be in the Matrigel.
3. The amount of insoluble material varies by Matrigel lot.
4. Pellet organoid fragments for plating
5. Centrifuge digestion solution containing organoid fragments at 2000gs for 5 mins
6. Discard supernatant
7. Re-suspend organoid fragments in the appropriate volume of Matrigel.
Typically 4x the original matrigel volume is used
Mix very well by pipetting (~40x) without adding bubbles to the system
8. Plate organoid fragments in hemispheric matrigel 'domes' in the center of each culture well
Plate matrigel domes in the center of wells in a new tissue culture plate
For 48 well plate, use 25-50 uL Matrigel
For 96 well plate, use 8-10 µL Matrigel
**Note: Place pipette tip on the bottom of the middle of the well, lift slightly, then depress to first stop to plate Matrigel in hemispheric droplet*
9. Transfer plate into tissue culture incubator
10. Incubate at 37^{o}C for 45 mins to polymerize matrigel

19 **1. Overlay Growth Media on Matrigel Domes**

1. Remove the plate from the incubator
2. Add 10-20x Matrigel volume of desired Culture Media
3. Continue to change culture media on an appropriate schedule

Typically, every 2-3 days

4. Transfer into a standard tissue culture incubator