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Generation of a canine enteroid-derived monolayer model to study gut barrier structure and function

By

VERONICA MARTINA LOPEZ CERVANTES

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

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DAVIS

Approved:

Amir Kol, Chair

Natalia Vapniarsky Arzi

Sina Marsilio

Committee in Charge

2022

Dedication

To Hope.

To my dear husband, Omar.

To my parents, José Luis and María Elena.

Acknowledgments

First, I thank God for guiding me every day of my life.

For his love, support, and encouragement to Omar, my lovely husband, only we know all we have been through to get to this point.

To my parents, José Luis and Maria Elena, for their support and love during this journey. To my siblings, Natalia and Alejandro. And of course, my tia Chabela and Lore and Carlita. Also, to my tios Susana and Pedro, my family in California. And my madrina Soledad for always encouraging me to be better.

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Veronica M. Lopez Cervantes

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Integrative Pathobiology

Generation of a canine enteroid-derived monolayer model to study gut barrier structure and function

Abstract

Conventional in-vitro models of intestinal epithelium such as human intestinal/colonic cancer cell lines lack essential features of the canine intestine, limiting their use to study canine intestinal barrier function, cellular metabolism, or host-microbe interactions. Intestinal organoids, also known as enteroids, represent a recent primary cell culture technology that has revolutionized the field of intestinal disease and physiology modeling. Enteroids are 3D structures that are composed of a single layer epithelium arranged in crypt- and villi-like domains with its apical and basal membranes facing the luminal and external sides of the enteroid, respectively. The conversion of the 3D organization of enteroids into a 2D monolayer enables accessibility to intestinal cells' apical membrane for experimental infections or drug treatments. This study aimed to develop a canine enteroid-derived monolayer model to study gut barrier structure and function. Jejunal segments were obtained from three donor dogs during a resection and anastomosis surgery and used to isolate the intestinal crypts by EDTA-based tissue dissociation. Then, the intestinal crypts were resuspended in growth factor reduced Matrigel and supplemented with the commercial IntestiCult™ Human Organoid Growth Medium to generate 3D enteroids. Canine enteroids were mechanically and enzymatically disrupted into single cells and cultured on Matrigel-coated porous membranes of transwell culture systems, reaching 100% confluency around day four. We performed immunofluorescence, histochemistry, and

transmission electron microscopy (TEM) to study intestinal epithelium morphology and structure. Intestinal epithelium barrier function was analyzed by Fluorescein Isothiocyanate-Dextran 4kD (FITC-D4) intestinal permeability Assay and Trans-Epithelial Electrical Resistance (TEER) measurement. Canine enteroids and 2D monolayers showed positive staining for ZO-1, Ki67, and Periodic Acid of Schiff (PAS), indicating epithelial cell heterogeneity and polarization. A functional intestinal barrier was confirmed by FITC-D4 assay and TEER measurement. This protocol provides a canine-specific, complex, and long-term ex-vivo model to study intestinal epithelial function.

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1. Introduction

In dogs, the intestinal epithelium is commonly involved in several diseases and disorders that affect its normal function and structure (Zachary, 2017). The lack of canine-specific and physiologically relevant intestinal models results in canine-specific research that is conducted in human cancer cells lines or in vivo rodent models that often have limited translational value. Standard *In vivo* and *in vitro* models of the intestinal epithelium have both advantages and disadvantages that are often opposite.

For instance, laboratory animal models such as mice or dogs are more physiologically relevant. But they are costly and often unethical or impractical to develop. On the other hand, most traditional *in vitro* models are human intestinal/colonic cancer cell lines that are easily cultured and genetically manipulated. Nevertheless, these traditional cell lines, such as Caco2 cells, are not derived from dogs and they further lack essential intestinal epithelium features, including cell diversity, functional, and structural properties (Kar *et a.l*, 2021).

Enteroids are 3-Dimensional (3D) *in vitro* structures derived from intestinal crypts that can be expanded ex-vivo and maintained long-term in culture. They are composed of a single layer of epithelium and an enclosed lumen. Intestinal crypts contain the adult intestinal stem cells (ISCs) that can proliferate, self-renew, and differentiate into all of the fully differentiated intestinal cell types. Enteroids recapitulate essential functions and structure of the intestinal mucosa such as crypt and villi domains. Thus, enteroids are extremely valuable as disease and developmental models of the intestinal epithelium (Sato *et al*., 2011, Sato and Clevers, 2013).

The epithelium of the enteroids is arranged in crypt- and villi-like structures with its apical and basal sides facing the luminal and external sides of the enteroid, respectively. This arrangement of the enteroids mimics the in vivo organization of the intestinal epithelium. However, it hinders experimental access to the apical side of the epithelium for experimental infections or drug treatments. This

limitation can be addressed by transforming the 3D-structural organization of the enteroid into a 2D monolayer and culturing them in transwell systems, where both the basal and apical components of the epithelium are accessible (Ambrosini et al., 2020).

Enteroid-derived 2D monolayers (EDMs) are a novel tool to investigate pathogen-host interactions, cell-to-cell interactions, intestinal development, barrier function, and explore novel therapeutics. Optimal culture conditions of this 2D model rely on enteroid media that provides appropriate biochemical and physical cues to mimic the stem cell niche of the native intestine and allow the rapid and efficient intestinal epithelium renewal that guarantees the intestinal epithelium function.

When establishing enteroids and EDMs, as with any in-vitro model, different enteroid lines and passage levels should replicate similar growth patterns such as enteroid morphology and size, cell viability, and proliferation rates. EDMs have been previously reported in dogs (Ambrosini et al., 2020) using an in-house media formulation that successfully supports this system. However, we obtained depleted enteroid growth patterns when replicating their formulation. In particular, our enteroids had different morphology and reduced cell viability within and between enteroid lines. To address this inter-laboratory variability, we opted to use a commercially available enteroid media.

Here, we develop a protocol to establish a 2D monolayer model derived from 3D canine enteroids using commercially available enteroid media. Our protocol reliably supports the formation of a differentiated epithelium with mucus-producing cells and a functional intestinal barrier supported by tight junctional proteins.

This model is a novel tool for studying canine disorders involving interaction with the intestinal epithelium. For instance, the access to the apical and basal components of the canine EDMs epithelium can facilitate the study of the mechanisms of host-pathogen interaction of parasitic infections (i.e., Giardia lamblia), viral infections (i.e., Canine Distemper virus, Canine Parvovirus-2, Canine Coronavirus, etc.) and numerous bacterial agents. Also, EDMs are a convenient model to investigate endocrine

conditions (i.e., Diabetes Mellitus) or inflammatory bowel diseases that affect intestinal barrier function. In addition, the culture of canine EDMs in a transwell system is ideal for drug testing representing a potential tool to be used in preclinical phases of drug studies. These features make canine EDMs an approachable and valuable instrument for exploring biological and pathogenic mechanisms in research.

2. Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
ZO-1 Monoclonal Antibody	Thermo Fisher Scientific	Cat# 33-9100
Ki-67 Recombinant Rabbit Monoclonal Antibody	Thermo Fisher Scientific	Cat# MA5-14520
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	Thermo Fisher Scientific	Cat# A-21203
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-21206
Biological samples		
Canine intestinal biopsies	Soft Tissue Surgery Service, Veterinary Medical Teaching Hospital, UC Davis	N/A
Chemicals, peptides, and recombinant proteins		
Fetal bovine serum	R&D Systems	Cat# S11150
CHIR 99021 10 mg	Tocris (R&D)	Cat# 495310
Y27632 10 mg	Tocris (R&D)	Cat# 1254
Gibco™ TrypLE™ Express Enzyme (1X), no phenol red	Fisher Scientific	Cat# 12-604-013
Advanced DMEM/F12	Thermo Fisher Scientific	Cat# 12634028
Corning Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free, 10 ml	Corning	Cat# 356231
IntestiCult™ Human Organoid Growth Medium	StemCell Technologies	Cat# 06010
Gentamicin (50 mg/ml)	Thermo Fisher Scientific	Cat# 15750060
Lonza™ AccuGENE™ 0.5 M EDTA Solution	Fisher Scientific	Cat# BMA51201
B-27 Supplement (50x)	Gibco	Cat# 12587-010
Fluorescein isothiocyanate–dextran KDa	Sigma Aldrich	Cat# 46944-100MG-F
Recombinant Canine TNF-alpha Protein	R&D Systems	Cat# 1507-CT-025
CanineIFN-gamma Recombinant Protein	Thermo Fisher Scientific	Cat# 781-CG-050
DMSO	Sigma Aldrich	Cat# D2650

Richard-Allan Scientific™ HistoGel™	Thermo Fisher Scientific	HG-4000-012
ProLong™ Gold Antifade Mountant	Thermo Fisher Scientific	Cat# P36934
Periodic Acid	Thermo Fisher Scientific	Cat # 453175000
Schiff's Reagent, Electron Microscopy Sciences	Fisher Scientific	Cat# 50-301-27
Triton™ X-100 Detergent	Sigma Aldrich	Cat# 9036-19-5
Tween 20	Sigma Aldrich	Cat# 9005-64-5
Bovine Serum Albumin, Fraction V	Fisher Scientific	Cat# BP1605-100
Fisher Chemical™ Gill Method Hematoxylin Stains	Fisher Scientific	Cat# CS400-1D
Formaldehyde, 37% by Weight (Histological), Fisher Chemical™	Fisher Scientific	Cat# F75P-20
Software and algorithms		
Fiji (ImageJ)	National Institute of Health	https://imagej.nih.gov/ij/index.html
TightJunctionAnalysis.ijm	Grosheva et al. 2020	https://github.com/WIS-MICC-CellObservatory/Intestinal_Barrier_Function
Other		
Fisherbrand™ Sterile Cell Strainers 70 µm	Fisher Scientific	Cat# 22-363-548
Fisherbrand™ Sterile Cell Strainers 40 µm	Fisher Scientific	Cat# 22-363-547
24-Well TC-treated Multi Well Plates	Genesee Scientific	Cat # 25-107MP
Fisherbrand™ Petri Dishes with Clear Lid, 100 mm	Fisher Scientific	Cat # FB0875713
Corning™ Transwell™ Multiple Well Plate with Permeable Polyester Membrane Inserts	Fisher Scientific	Cat # 07-200-154
BD Disposable Syringes with Luer-Lok™ Tips	Fisher Scientific	Cat# 14-823-30
EasyTouch® Hypodermic 25G - 1.5" Needles	Thomas Scientific	Cat # 1145G65
Corning® Cryogenic Vials with Orange Caps, 2 mL	StemCell technologies	Cat# 38053
Microplate, 384 well, ps, f-bottom, fluotrac, med. binding, black.	Greiner BIO-ONE	Cat# 781076
FilterMax F3 Multi-Mode Microplate Reader	Molecular Devices	Cat# F3
Epithelial Volt/Ohm (TEER) Meter 3	World Precision Instruments	Cat# EVOM3
Mr. Frosty™ Freezing Container	ThermoFisher	Cat # 5100-0001

CRITICAL: Make aliquots of Corning Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix (Matrigel), Phenol Red-Free, following the manufacturer's handling instructions website (https://www.corning.com/worldwide/en/products/life-sciences/products/surfaces/matrigel-matrix.html)

Note: Matrigel is solid at -20°C, gel at 37°C and, liquid at 4°C. Keeping Matrigel in its liquid form is crucial during this protocol.

3. Materials

IntestiCult™ Human Organoid Growth Medium (commercially available IntestiCult™ Human Organoid Growth Medium complete kit)

Reagent	**Final concentration**	**Amount**
Basal Medium/Component A (provided by the kit)	N/A	50 ml
Organoid Supplement/Component B (provided by the kit)	N/A	50 ml
Penicillin-Streptomycin (10,000 U/ml)	100 U/ml	1 ml
Y-27632 (Rock inhibitor) 10 mM	10 µM	100 µl
CHIR 99021 (GSK3 inhibitor) 2.5 mM	2.5 µM	100 µl

Note: The feeding media for canine enteroids and EDMs used in this protocol is the commercially available IntestiCult™ Human Organoid Growth Medium (Intesticult).

To store the Intesticult media, make aliquots of each of the two components, A and B. The volumes of the aliquots will vary according to the number of experiments, number of lines ongoing, etc. Store these aliquots at - 20°C for up to 3 months.

To prepare the Intesticult media, mix aliquots of equal volumes of each component and add 1X of Penicillin-Streptomycin (10,000 U/ml). Use immediately or store at 4°C for up to 1 week.

To feed the cells, use the media at 22-24 °C.

CRITICAL: The first feeding media of any passage of enteroids and EDMs should be supplemented with Y-27632 (10 µM final concentration) and CHIR 99021 (2.5 µM final concentration).

Note: Make aliquots of the supplements and store them at -20°C. Avoid repetitive freeze and thaw.

- Y-27621 stocks: To prepare a 10 mM solution, dilute 10 mg of Y-27621 (MW: 320.26 g/mol) in 3.12 ml of water and then filter using a 0.22 µm syringe filter according to the manufacture

instructions at the website (https://www.tocris.com/products/y-27632-dihydrochloride_1254).

- CHIR 99021: To prepare a 2.5 mM solution, dilute 10 mg of CHIR 99021 (MW: 574.72 g/mol) in 6.96 ml water and then filter using a 0.22 µm syringe filter according to the manufacture instructions at the website (https://www.tocris.com/products/chir-99021-trihydrochloride_4953).

Organoid collection media (OCM)

Reagent	**Final concentration**	**Amount**
Advanced DMEM/F-12	N/A	44.25 ml
B-27 Supplement (50X), minus Vit A	1x	5 ml
Penicillin-Streptomycin (10,000 U/ml)	100 U/ml	500 µL
Gentamicin 50 µg/ml	12.5 µg/ml	250 µL

Note: Make 1 ml aliquots of a B-27 supplement. Thaw these aliquots no more than 2 additional freeze/thaw cycles.

CRITICAL: Add Gentamicin (12.5 µg/ml final concentration) only to the OCM media used for crypt isolation : OCM + G.

PBS with antibiotics (PBS+A)

Reagent	**Final concentration**	**Amount**
PBS (-Ca,-Mg)	N/A	989.5 ml
Penicillin-Streptomycin (10,000 U/ml)	100 U/ml	10 ml
Gentamicin 50 µg/ml	12.5 µ/ml	500 µL

Note: Store at 4°C and keep on the ice during the workflow of the crypt isolation protocol.

20 mM EDTA solution

Reagent	Final concentration	Amount
PBS (-Ca,-Mg)		23.625 ml
Penicillin-Streptomycin (10,000 U/ml)	100 U/ml	250 µL
Gentamicin 25 microgram/ml	12.5 µ/ml	125 µL
Lonza™ AccuGENE™ 0.5 M EDTA Solution	20 mM	1 ml

Note: Store at 4°C and keep on the ice during the workflow of the crypt isolation protocol.

Media to dilute Tryple express enzyme™

Reagent	Final concentration	Amount
Advanced DMEM/F-12	N/A	44.5 ml
Fetal Bovine Serum (FBS)	10%	5 ml
Penicillin-Streptomycin (10,000 U/ml)	100 U/ml	500 µL

Note: Store at 4°C.

Freezing media

Reagent	Final concentration	Amount
IntestiCult™ Human Organoid Growth Basal Medium	N/A	500 µl
Penicillin-Streptomycin (10,000 U/ml)	100 U/ml	10 µL
Y-27632 (Rock inhibitor) 10 mM	10 µM	1 µL
DMSO	10%	100 µl
Fetal Bovine Serum (FBS)	40%	400 µl

Note: Store at 4°C.

1mg/ml FITC-D4

Reagent	Final concentration	Amount
IntestiCult™ Human Organoid Growth Medium (warm)	N/A	332 µl
Fluorescein isothiocyanate–dextran KDa 50 mg/ml	1 mg/ml	8 µL

Note: Use immediately after preparation.

To prepare the Fluorescein isothiocyanate–dextran 4 KDa (FITC-D4) 50 mg/ml stock solution, mix cell culture water with 100 mg of Fluorescein isothiocyanate–dextran 4000 Da to obtain a 2 ml final volume solution. Store at 4°C light-protected.

IFN- γ /TNF-α solution

Reagent	Final concentration	Amount
IntestiCult™ Human Organoid Growth Medium (warm)	N/A	540 µl
IFN-γ 5 µg/ml	500 ng/ml	20 µL
TNF-alpha 5 µg/ml	500 ng/ml	20 µL

Note: Use immediately after preparation.

Dilute the 50 µg of IFN-γ protein in PBS 1%BSA to obtain a 10 ml final volume solution with a concentration of 5 µg/ml. Store up to 3 months at -20 to -70 °C. Avoid repeated freeze-thaw cycles.

To prepare the TNF-alpha 5 µg/ ml stock solution, mix 25 µg of TNF-alpha protein with sterile 0.1% BSA/PBS to obtain a 5 ml final volume solution. Store up to 3 months at -20 to -70 °C.

4% Formaldehyde

Reagent	Final concentration	Amount
PBS (-Ca,-Mg)	N/A	3.516 ml
Formaldehyde, 37% by Weight (Histological), Fisher Chemical™)	4%	484 µL

Note: Store at 22-24°C.

Washing buffer

Reagent	Final concentration	Amount
PBS (-Ca,-Mg)	N/A	450 ml
Triton™ X-100 Detergent	N/A	4.84 ml
Teen 20	N/A	225 µL

Note: Store at 22-24°C.

Blocking solution

Reagent	Final concentration	Amount
Washing buffer	N/A	20 ml
Bovine Serum Albumin	N/A	0.2 g

Note: Store at 4°C for up to 1 week.

Permeabilization solution

Reagent	Final concentration	Amount
Washing buffer	N/A	20 ml
Bovine Serum Albumin	N/A	0.2 g

Note: Store at 22-24°C

Periodic acid 0.5%

Reagent	Final concentration	Amount
Distilled water	N/A	500 ml
Periodic acid	N/A	2.5 g

Note: Store at 4°C.

4. Prologue

Here we provide a detailed protocol to generate functional EDMs from intestinal surgical tissue samples. This protocol has been validated in three different canine intestinal enteroid cell lines supporting long-term maintenance, multiple serial passages, and viable passages after cryopreservation.

To ensure the effectiveness of this protocol, solid skills and knowledge in cell culture techniques such as sample handling and process, passaging, cryopreservation, and freezing are required.

It is essential to plan a Mycoplasma-control program starting from the crypt isolation and applying it onwards.

The success of this protocol relies on the isolation of intestinal crypts that contain the ISCs. To isolate intestinal crypts, use surgical fragments or fresh cadaveric samples. We recommendsampling cylindrical segments of 5-10 cm in length of ileal, jejunal, or colonic sections and processing them within 2 hours of its collection. Older and smaller samples have been processed, but the success rate decreases within time, given the rapid autolysis of the intestinal tissue.

5. Harvesting of intestinal segments from fresh cadavers

Timing: [30 min]Required equipment and reagents:

- Scissors
- Tweezers
- Styrofoam cooler
- Ice
- 50 ml conical tubes
- PBS + Antibiotics

Procedure:

1. Using aseptic technique, scissors, and tweezers, make an incision in the ventral midline of the abdomen from the xiphoid process through the pubis.
2. Open the abdominal cavity to expose the intestine.
3. Identify the intestinal tract's anatomical segments and define which section(s) will be harvested.
4. Remove the intestinal content of the section of interest by gently squeezing the intestinal tube by hand.
5. CRITICAL: Remove as much intestinal content as possible without damaging the tissue. This step is crucial to avoid contamination and facilitate the crypt isolation process.
6. Using scissors, resect the intestinal segment and place it in a 50 ml conical tube containing 25 ml of cold PBS + Antibiotics (PBS + A).

Note: The composition of PBS+Antibiotics solution is detailed in Materials. Prepare and label one tube with PBS+A per intestinal section.

7. Place the tube or tubes in a container with ice.
8. Process the samples within 2 hours from its collection; crypt viability decreases over time.

6. Method details

6.1 Isolation of intestinal crypts and enteroid culture

Timing: [6-8 hours]

Required equipment and reagents:

- Biosafety cabinet
- Incubator (37°C/5%CO2)

- Refrigerated Laboratory Centrifuge
- Water Bath
- Microscope
- Scissors
- Tweezers
- Spoons
- Ice
- Styrofoam cooler
- 10 and 25 ml serological pipettes
- 50 ml conical tubes
- 100 mm Petri dishes
- 100 ml beaker
- 250 ml Erlenmeyer flask.
- Microscopic glass slides
- 70 µm cell strainers
- 24-Well TC-treated Multi Well Plate
- 70% ethanol
- Sterile PBS+A
- 10% bleach solution container
- Corning Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix aliquot
- 20mM EDTA solution
- Organoid Collection Media with Gentamicin

- IntestiCult™ Human Organoid Growth (Intesticult) media supplemented with ROCK inhibitor Y-27632 and GSK3 inhibitor CHIR 99021.

Procedure:

Part 1: Non-sterile process

CRITICAL: The day before, thaw an aliquot of Corning Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free (Matrigel) at 4 °C overnight.

CRITICAL: Place a 24-Well TC-treated Multi Well Plate (24 WP) in an incubator at 37 °C for 1 hr.

Note: All the reagents and calculations for this protocol have been done for 24 WP. Adjust solutions' volumes and cell seeding densities according to the type of the Multi-Well plate used.

CRITICAL: Since the samples may have intestinal content, the first part of this workflow will be performed on the bench.

CRITICAL: The time to process fresh cadaveric samples will be longer than biopsies.

CRITICAL: Process samples in separate tubes.

1. Prepare the reagents and instruments.
    a. Clean and spray with 70% ethanol the bench area where the samples will be processed. Repeat this step as needed during the procedure.
    b. Organize the bench area with the reagents and instruments.
    c. Add 70% ethanol into the 100 ml beaker.
    d. Prepare 20mM EDTA solution following details in Materials.
2. Spray with 70% ethanol the 50 ml conical tube(s) containing the sample(s)/PBS + A
3. Firmly hold the sample and place it into a 100 mm Petri dish using a tweezer.

CRITICAL: From this step onwards, place the instruments in the beaker containing 70% ethanol every time after its use, and dry them before their use.

4. Gently, hold the sample from one of its open ends using a tweezer.
5. Place the tip of a 10 ml pipette into the open end of the sample and carefully flush with PBS+A to clean the intestinal segment. Discard the used PBS+A. Repeat this step until no luminal content is visible (5-10 times).

Note: The number of repetitions of this step depends on the size and cleanness of the sample and the intestinal segment. Usually, colon segments take longer to clean.

6. Using a surgical scissor, cut the intestinal segment longitudinally.
7. Place the sample in the Petri dish with the intestinal lumen facing up.
8. Wash with PBS+A to remove any intestinal content leftover as needed.

Note: From this step, moisturize the intestinal section with PBS+A continuously. Do not allow the sample to dry.

9. Using one of the shortest ends of a microscopic glass slide, apply gentle pressure and remove the mucus by scrapping off the luminal side of the tissue.
10. Wash the sample with PBS+A.
11. Discard the removed mucus and the used PBS+A.
12. Transfer the sample to a clean 100 mm Petri dish with the luminal side facing up.

Note: Remember to moisturize the sample continuously.

13. Using a microscopic glass slide, apply firm pressure and scrape off the luminal side of the tissue to remove the entire mucosal layer (Fig. 1).

CRITICAL: This step is crucial because the mucosal layer carries the intestinal crypts.

14. Keep intestinal mucosa and discard the tissue's leftovers into the 10% bleach container.
15. Using a surgical scissor, mince the mucosal layer into approximately 5 mm2 pieces.

Note: Alternatively, use a disposable scalpel to mince the mucosal layer.

16. Place the minced intestinal mucosa into a clean 50 ml conical tube containing 25 ml of cold PBS+A and vortex at high speed.
17. Let the intestinal pieces settle by gravity for 2-4 min.
18. Using a 25 ml serological pipette, discard the supernatant without disturbing the sample and leave approximately 2 ml of PBS+A covering the intestinal pieces. Add about 25 ml of cold PBS+A and vortex. Repeat this step until the appearance of the supernatant is clear (10-20 times).
19. Note: The number of repetitions of this step depends on the size and cleanness of the sample and the intestinal segment. Usually, colon segments take longer to clean.
20. Centrifuge at 300 g for 2 min at 4°C.
21. Discard the supernatant without disturbing the pellet.
22. Resuspend the intestinal mucosal pieces in 25 ml of cold 20 mM EDTA solution.

Note: The composition of the 20mM EDTA solution is detailed in Materials.

EDTA is a calcium chelator that facilitates the isolation of the intestinal crypts by interrupting calcium dependent-attachment of the intestinal epithelium to the extracellular matrix.

23. Incubate for 30-40 minutes with intermittent swirling at 4°C.

Note: The tube should be placed horizontally to ensure the homogenous EDTA action. Check the samples every 15 min to confirm sample dissociation.

24. After incubation, centrifuge at 300 g for 5 min at 4°C.

Part 2: Sterile process

CRITICAL: This part of the workflow should be performed in the biosafety cabinet (BSC).

25. Thoroughly clean and disinfect with 70% ethanol the 50 ml conical tube(s) containing the sample and transfer it to the BSC.
26. Discard the supernatant without disturbing the pellet.

27. Resuspend the intestinal pieces in 10 ml of cold, sterile PBS+A. Pipette up and down for 3-4 times.
28. Note: Pre-wet the 10 ml serological pipette with PBS+A to avoid the sample sticking to the walls of the pipette.
29. Let the intestinal pieces settle by gravity for 2-4 min.
30. Place a 70 µm cell strainer into the top of a new 50 ml conical tube.

Note: This step enriches for intestinal crypt cells and removes the remaining large tissue and matrix fragments from the intestinal mucosal layer.

31. Using a 10 ml serological pipette, recover the supernatant and pass it through the 70 µm cell strainer into the 50 ml conical tube.
32. Repeat steps 26-29 using the same 50 ml conical tube.

Note: Replace the cell strainer if it appears clogged.

33. Filter the supernatant and the intestinal pieces through the 70 µm cell strainer into the 50 ml conical tube.
34. Centrifuge at 150 g for 5 min at 4°C.
35. Discard the supernatant without disturbing the pellet.
36. Resuspend the isolated intestinal crypts in 5-10 ml cold Organoid Collection Media with Gentamicin (OCM+G).

CRITICAL: Add an appropriate volume of OCM+G according to the size of the intestinal crypt pellet. The number of crypts obtained per intestinal section varies with the sample and the processor. As a guide, add 10 ml of OCM+G when the intestinal segment is fresh and 10 cm in length.

Note: The composition of the OCM+G media is detailed in Materials.

37. Transfer 20 µl of the crypt suspension into a microscopic glass slide and cover it with a cover slide.
38. Use an inverted light microscope at 4x of lens magnification to count all the crypts in the 20 µl sample manually.
39. Plate 300 crypts per well in a 24WP. Determine the appropriate volume of suspension containing the desired number of crypts according to the number of wells to be plated.
40. Place the final volume into a 15 ml tube.
41. Centrifuge at 150 g for 5 min at 4°C.
42. Discard the supernatant and resuspend the pellet in 20 µL per well of OCM+G.
43. Bring an aliquot of Matrigel and keep it on ice.
44. Add 30 µl per well of fresh Matrigel and slowly pipette up and down to homogenize the crypt solution avoiding air bubble formation.
45. Using a P200 tip, slowly aspirate 50 µl of the crypt solution and pipette it into the center of a well of a pre-warmed 24 WP.

Note: Repeat this step according to the desired number of wells to be plated. We recommend plating at least 3 wells of crypts to generate an enteroid line.

46. Place the plate into an incubator at 37°C, 5% CO2 for 10-15 min to allow Matrigel polymerization. After this incubation, the Matrigel should form a crypt-containing dome.
47. Add 700 µl of warm IntestiCult™ Human Organoid Growth (Intesticult) media supplemented with ROCK inhibitor Y-27632 and GSK3 inhibitor CHIR 99021 by slowly discharging the media by the sidewall of the well.

CRITICAL: Do not add the Intesticult media directly to the dome.

Note: Follow the instructions on Materials to prepare Intesticult media.

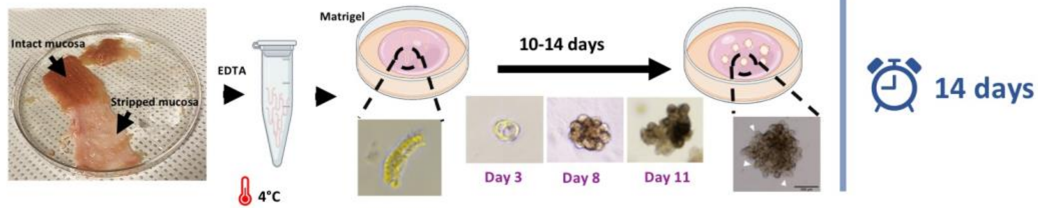
48. Change the media every two days.

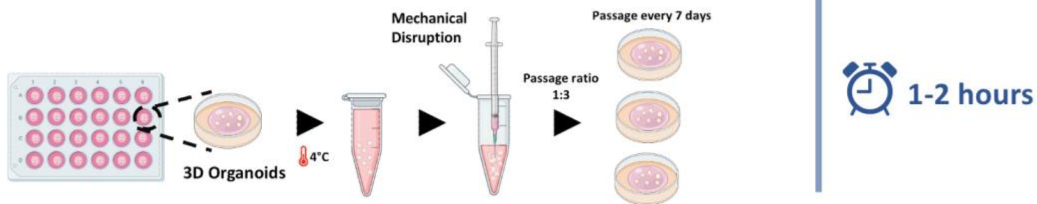
49. Monitor daily growing and morphological characteristics (Fig. 1).

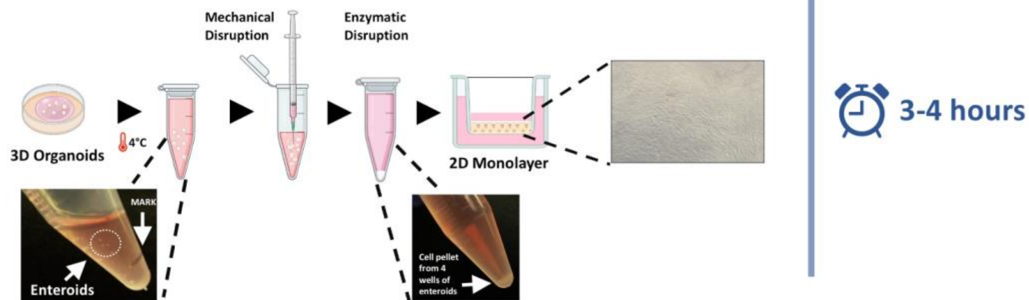
50. Passage the primary culture (passage 0) of enteroids at days 10-14.

CRITICAL: During the first 2-3 passages, keep the splitting ratio between 1:1-1:2 to avoid low enteroid confluency and potential death of the enteroid line. For older passages, use a splitting ratio between 1:3-1:4.

CRITICAL: After finishing the crypt isolation process, disinfect the BSC thoroughly.







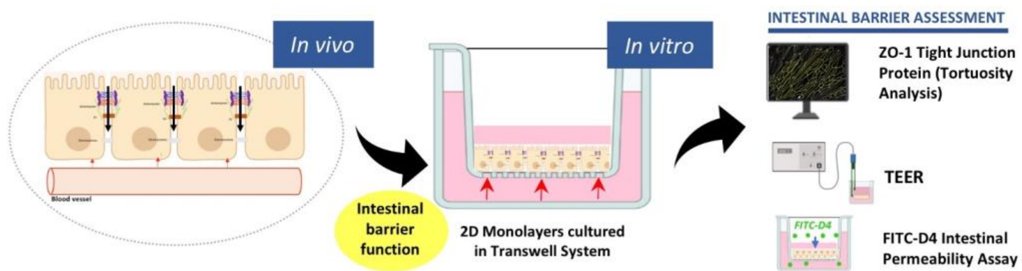


Figure 1. Workflow to generate canine enteroid-derived 2D monolayers.

6.2. Passaging of enteroids

Timing: [1-2 hours]

Required equipment and reagents:

- Biosafety cabinet
- Incubator (37°C/5%CO2)
- Refrigerated Laboratory Centrifuge
- Water Bath
- Microscope
- Alcohol Resistant Cryogenic Permanent Marker
- Ice
- Styrofoam cooler
- 24-Well TC-treated Multi Well Plate
- 1.5 ml microcentrifuge
- 5 ml microcentrifuge
- BD Disposable Syringes with Luer-Lok™ Tips (1ml)
- Sterile EasyTouch® Hypodermic 25G - 1.5" needle
- 70% ethanol
- PBS
- 10% bleach solution container
- Corning Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix aliquot
- Organoid Collection Media
- IntestiCult™ Human Organoid Growth (Intesticult) media supplemented with ROCK inhibitor Y-27632 and GSK3 inhibitor CHIR 99021.

Procedure:

1. Prepare the reagents and instruments.

    a. The day before, thaw an aliquot of Matrigel at 4 °C overnight.

Note: Allot 30 µl per well of a 24WP to be plated.

    b. Place a 24 WP in an incubator at 37°C, 5% CO_2 for a minimum of 1 h before starting the procedure.

    c. Prepare Intesticult and OCM media following the instructions on Materials.

    d. Keep OCM media on the ice during all the procedure.

CRITICAL: Keeping OCM cold is essential to avoid Matrigel polymerization during the process.

2. Label a 1.5 ml microcentrifuge sterile tube and add 20 µl of PBS per well to be plated.

Note: For example: For passaging one well of enteroids under a 1:3 splitting ratio, add 60 µL of PBS. This volume of PBS is added to draw a mark to determine the final volume of OCM required to form the OCM-Matrigel-enteroid solution on step 17.

Alternatively, use a 5 ml macrocentrifuge tube when passaging more than 3 wells of enteroids of the same line and passage. This will allow adding more volume of cold OCM and prevent Matrigel polymerization.

3. Draw a mark on the tube at the PBS top-level using Alcohol Resistant Cryogenic Permanent Marker

4. Discard the PBS.

5. Keep the tube on ice.

6. Remove the 24 WP containing the enteroids from the incubator and place it in the BSC.

7. Using a P1000 tip, aspirate the total volume of old Intesticult media from the culture well by tilting the plate and putting the tip against the sidewall of the well.

Note: Avoid touching the Matrigel dome when aspirating the old Intesticult media.

8. Using a P1000 tip, add 500 µl of cold OCM directly onto the enteroid-containing Matrigel dome.

Note: Adding cold OCM dilutes Matrigel and keeps it liquid.

CRITICAL: When passaging more than one well from the same line and passage, use the same 500 µl of OCM to recover the Matrigel domes with enteroids. Combine up to 3 wells of enteroids in the same collection tube to avoid Matrigel polymerization. When passaging more than 3 wells of enteroids, prepare more tubes on step 2 or use 5 ml macrocentrifuge tubes.

9. Scrape the enteroid-containing Matrigel dome using the end of the P1000 tip.
10. Pipette up and down to dissociate the domes. Repeat this step 10-15 times, avoiding air bubble formation.
11. Transfer the complete volume of OCM containing the enteroids into the tube from step 2.

Note: Minimize manipulation of the tube and keep it on ice until its use.

12. Using a P1000 tip, add up to 450 µl of cold OCM to wash the well(s). Transfer the complete volume to the microcentrifuge tube from the previous step.

Note: The final volume in the tube should be 1 ml maximum. Consider the volume of the Matrigel domes when adding the cold OCM.

For example: When passaging 3 wells, add a maximum of 350 µl of OCM on step 12. Consider the initial 500 µl of OCM on step 8, plus the 150 µl of the Matrigel domes from the 3 wells.

13. Use a sterile BD Disposable Syringes with Luer-Lok™ Tips (1ml syringe) and a sterile EasyTouch® Hypodermic 25G - 1.5" needle to mechanically break down the enteroids.
14. Hold the syringe like a pencil, with the needle pointed down. Put the needle tip into the tube containing the enteroids. Slowly, pull back the plunger and aspirate the entire volume avoiding air bubble formation. Place the needle tip into the bottom of the tube and firmly force the entire volume out of the syringe. Repeat this step 1-2 times to dissociate the enteroids.

CRITICAL: Avoid over-dissociation of the enteroids.

15. Add cold OCM media into the tube to dilute the Matrigel and avoid its polymerization. Keep the tube on ice until its use.

Note: The OCM volume depends on the number of wells with enteroids combined in the tube. For example: Add 1 ml of media if the tube contains enteroids from 4 wells of a 24WP; it may not be necessary to add additional OCM when passaging only 1 well of enteroids.

16. Centrifuge the tube at 150 g for 5 min at 4°C.
17. Using a P1000 tip, discard the supernatant until the mark level from step 3 without disturbing the enteroid pellet.
18. Bring an aliquot of Matrigel and keep it on ice.
19. Using a P200 tip, add 30 µl of Matrigel per well to be plated to the tube containing the enteroid pellet.

Note: For example: For passaging one well of enteroids under a 1:3 splitting ratio, add 90 µL of Matrigel to the tube containing 60 µL of OCM and the enteroids pellet.

20. Slowly pipette up and down to resuspend the enteroid pellet.
21. Using a P200 tip, slowly aspirate 50 µL of the Matrigel-OCM-enteroid solution and discharge it into the center of well of a 24 WP. Repeat this step until plating the desired number of wells.
22. Place the 24 WP into an incubator at 37°C for 10-15 min.
23. Carefully add 700 µL of warm Intesticult media supplemented with ROCK inhibitor Y-27632 and GSK3 inhibitor CHIR 99021 by the sidewall of the well.
24. Change the media every two days.
25. Passage the organoids every 7 days from passage 1.

CRITICAL: Enteroid lines will vary on their morphology, size, and confluence; therefore, it is essential to monitor the enteroids at each passage to determine the appropriate splitting ratio. In our experience,

a splitting ratio between 1:3 to 1:4 maintains optimal growth and survival. Over or under splitting ratios lead to unhealthy enteroid cultures.

6.3. Validation of 3D enteroid morphology

In the matrigel domes, the isolated intestinal crypts have their typical cylindrical shape that turns into spherical within the first 2-3 days. These spheroids are composed of proliferating intestinal stem cells that will form a single layer of cells enclosed, creating a lumen. Around day 8, the spheroids grow and develop small budding structures. In the following 3-5 days, most enteroids have numerous villi- and crypt-like structures (Fig. 2A).

At passages 2-3, the proliferative profile of the enteroid line stabilizes, allowing a passage frequency every 7 days. Canine enteroids are very complex structures that reach an approximate diameter of 300 µm at day 7 (Fig. 2B). They contain brush border and tight junctions, relevant elements of the intestinal epithelium function (Fig. 2C). The enteroid epithelium comprises a single polarized layer of cells along villus- and crypt-like units that show positive staining for ZO-1 and Periodic Acid of Schiff (PAS) (Fig. 2D, E, F).

Morphological and proliferative features are essential to determine how healthy are the enteroids in each passage. A healthy culture has the majority of enteroids of similar sizes and shapes. Usually, they are ready to passage on day 7, when they are very complex and have several protruding crypt-like structures with a small lumen (Fig. 3A).

On the contrary, an unhealthy culture will present enteroids of variable sizes and shapes with a very dark lumen due to the higher number of cells dying (Fig. 3B).

The splitting ratio is another critical factor when determining an enteroid line's healthy because it indicates proliferation and survival rates. Around passages 2-3, the splitting ratio stabilizes at 1:3-1:4,

and it should remain the same during passages. Higher or lower splitting ratios will produce cultures with too scarce (Fig. 3C) or crowded enteroids (Fig. 3D), respectively; in either case, proliferation and survival features are diminished.

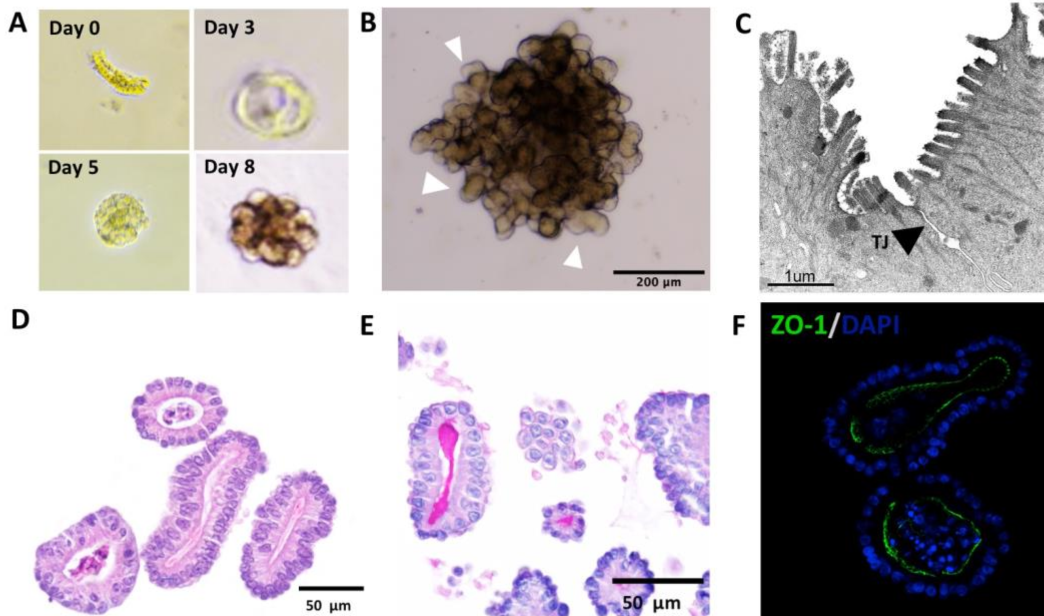


Figure 2. Characterization of canine enteroids. (A) Representative images of primary culture of Matrigel-embedded crypts/enteroids at days 0,3, 5, and 8. (B) Matrigel-embedded 7-day-old canine enteroid at passage 2, with protruding crypt-like structures (white arrowheads). (C) Ultrastructural image of the apical surface demonstrating microvilli and tight junction (TJ, black arrowhead) in canine enteroids. (D) Cross-section of canine enteroids stained with H&E. (E) Positive PAS staining within the enteroid lumen indicating the presence of carbohydrate (i.e., mucus). (F) Confocal immunofluorescence of ZO-1 (green) and nuclei (DAPI, blue) demonstrate an apical lattice-like pattern of the key tight junction protein ZO-1.

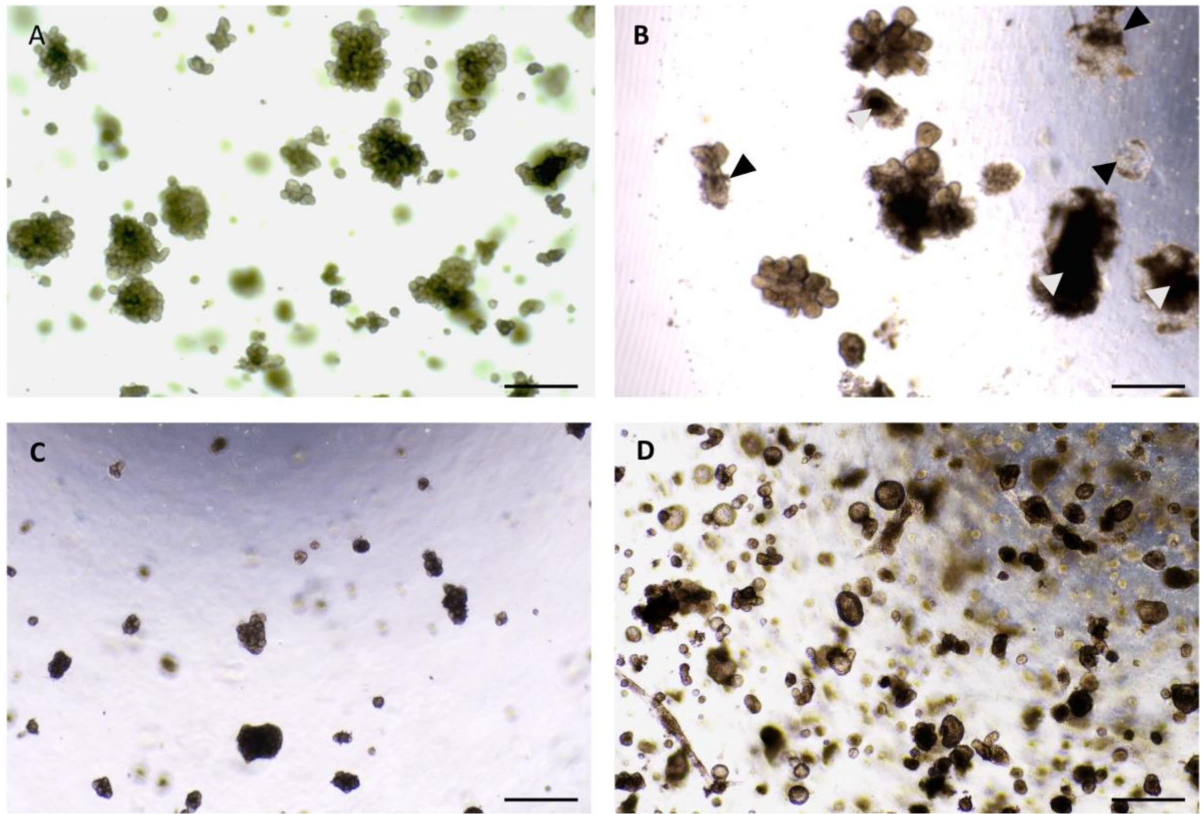


Figure 3. Matrigel-embedded canine enteroids. (A) Representative images of good quality 7-day-old enteroids with similar sizes and complex morphology with numerous branching structures and good confluence. (B) Representative images of poor quality 7-day-old enteroids with a dark lumen and non-continuous border evidencing low viability. Representative image of low (C) and over-confluent (D) 7-day-old enteroids showing different morphology and small size. Scale bar= 400 µm.

6.4. Freezing of enteroids

Timing: [30 minutes]

Required equipment and reagents:

- Biosafety cabinet
- Incubator (37°C/5%CO2)
- Refrigerated Laboratory Centrifuge
- Water Bath
- Microscope
- Liquid nitrogen storage tank
- Cryogenic Gloves
- Alcohol Resistant Cryogenic Permanent Marker
- Ice
- Styrofoam cooler
- Mr. Frosty™ Freezing Container.
- 1.5 ml microcentrifuge
- 5 ml microcentrifuge
- 2 ml cryogenic vials
- BD Disposable Syringes with Luer-Lok™ Tips (1ml)
- Sterile EasyTouch® Hypodermic 25G - 1.5" needle
- 70% ethanol
- PBS
- 10% bleach solution container
- Organoid Collection Media

- Freezing media
- IntestiCult™ Human Organoid Growth (Intesticult) media supplemented with ROCK inhibitor Y-27632

Procedure:

1. Prepare Freezing and OCM media following the instructions on Materials and keep both on ice.
2. Label a sterile cryovial noting the date, passage number, cell line, and the number of wells.
3. Label a sterile 1.5 ml microcentrifuge tube.
4. Remove the 24 WP containing the enteroids from the incubator and place it in the BSC.
5. Using a P1000 tip, aspirate the total volume of old Intesticult media from the culture well by tilting the plate and putting the tip against the sidewall of the well.

Note: Avoid touching the Matrigel dome when aspirating the old Intesticult media.

6. Using a P1000 tip, add 500 µl of cold OCM directly onto the enteroid-containing Matrigel dome.

Note: Adding cold OCM dilutes Matrigel and keeps it liquid.

CRITICAL: When freezing more than one well from the same line and passage, the same 500 µl of OCM can be used to recover the Matrigel domes with enteroids. Freeze a maximum of 3 wells of enteroids per cryovial to ensure enteroid survival.

7. Scrape the enteroid-containing Matrigel dome using the end of the P1000 tip.
8. Pipette up and down to dissociate the domes. Repeat this step 10-15 times, avoiding air bubble formation.
9. Transfer the complete volume of OCM containing the enteroids into the tube from step 2.

Note: Minimize manipulation of the tube and keep it on ice until its use.

10. Using a P1000 tip, add up to 450 µl of cold OCM to wash the well(s). Transfer the complete volume to the microcentrifuge tube from the previous step.

Note: The final volume in the tube should be 1 ml maximum. Consider the volume of the Matrigel domes when adding the cold OCM.

For example: When freezing 3 wells, add a maximum of 350 µl of OCM in step 10. Consider the initial volume of 500 µl of OCM in step 6, plus the 150 µl of the Matrigel domes from the 3 wells.

11. Use a sterile BD Disposable Syringes with Luer-Lok™ Tips (1ml syringe) and a sterile EasyTouch® Hypodermic 25G - 1.5" needle to mechanically break down the enteroids.
12. Hold the syringe like a pencil, with the needle pointed down. Put the needle tip into the tube containing the enteroids. Slowly, pull back the plunger and aspirate the entire volume avoiding air bubble formation. Place the needle tip into the bottom of the tube and firmly force the entire volume out of the syringe. Repeat this step 1-2 times to dissociate the enteroids.
13. Add up to 500 µl of cold OCM media into the tube to better dilute the Matrigel and avoid its polymerization. Keep the tube on ice until its use.

Note: The OCM volume depends on the number of wells with enteroids combined in the tube. For example: Add 500 µl of media if the tube contains enteroids from 3 wells of a 24WP.

14. Centrifuge at 150 g for 5min at 4°C.
15. Using a P1000 tip, discard the entire supernatant.
16. Resuspend in 1 ml of freezing media and store at -80°C for 24 hours using a Mr. Frosty™ Freezing Container.
17. Transfer to liquid nitrogen for long-term storage.

6.5. Thawing of enteroids

Timing: [30 minutes]

Required equipment and reagents:

- Biosafety cabinet
- Incubator (37°C/5%CO2)
- Refrigerated Laboratory Centrifuge
- Water Bath
- Microscope
- Cryogenic gloves
- Alcohol Resistant Cryogenic Permanent Marker
- Ice
- Styrofoam cooler
- 24-Well TC-treated Multi Well Plate
- 15 ml sterile conical tube
- 70% ethanol
- 10% bleach solution container
- Corning Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix aliquot
- Organoid Collection Media
- IntestiCult™ Human Organoid Growth (Intesticult) media supplemented with ROCK inhibitor Y-27632 and GSK3 inhibitor CHIR 99021.

Procedure:

1. Prepare the reagents and instruments.

e. The day before, thaw an aliquot of Matrigel at 4 °C overnight.

Note: Allot 30 µl per well of a 24WP to be plated.

f. Place a 24 WP in an incubator at 37°C, 5% CO2 for a minimum of 1 h before starting the procedure.

g. Prepare Intesticult and OCM media following the instructions on Materials.

2. Label a 15 ml sterile conical tube and add 6 ml of OCM media. Let sit at 23°C for 15 min.
3. Remove a cryovial containing the frozen enteroids from the liquid nitrogen storage.
4. Place the cryovial in a 37°C water bath for approximately 2 min until only a small piece of ice is left.
5. Remove the cryovial from the water bath.
6. Dry and spray the cryovial with ethanol 70%.
7. Using a 5 ml pipette, slowly aspirate the entire volume of the cryovial and transfer it into the 15 ml conical tube containing OCM media.
8. Using a P1000 tip, add 1000 ul of OCM media to wash the cryovial. Transfer the entire volume into the 15 ml tube.
9. Centrifuge at 150 g for 5min at 4°C.
10. Discard the entire supernatant.
11. Add 20 µl of OCM media per well to be plated.

Note: Plate the thawed enteroids using a splitting ratio of 1:1-3:2.

12. Bring an aliquot of Matrigel and keep it on ice.
13. Using a P200 tip, add 30 µl of Matrigel per well to be plated to the tube containing the enteroid pellet.
14. Slowly pipette up and down to resuspend the enteroid pellet.

15. Using a P200 tip, slowly aspirate 50 µL of the Matrigel-OCM-enteroid solution and discharge it into the center of well of a 24 WP. Repeat this step until plating the desired number of wells.
16. Place the 24 WP into an incubator at 37°C for 10-15 min.
17. Carefully add 700 µL of warm Intesticult media supplemented with ROCK inhibitor Y-27632 and GSK3 inhibitor CHIR 99021 by the sidewall of the well.
18. Change the media every two days.
19. Monitor daily growing and morphological characteristics.

Note: Usually, it takes two passages for the enteroids to recover their optimal growth characteristics after a thawing process.

6.6. Processing of enteroids for staining

Timing: [2 hours]

Required equipment and reagents:

- Biosafety cabinet
- Incubator (37°C/5%CO2)
- Refrigerated Laboratory Centrifuge
- Benchmark Digital Hotplate
- Microscope
- Alcohol Resistant Cryogenic Permanent Marker
- Ice
- Styrofoam cooler
- 15 ml conical tube
- 100 mm Petri dishes
- 100 ml beaker

- 250 ml Erlenmeyer flask.
- Plastic Pasteur pipette
- Disposable scalpel
- Cell lifter
- Histology cassette
- 70% ethanol
- PBS
- Di water
- 4% Formaldehyde
- 10% bleach solution container
- Organoid Collection Media
- Richard-Allan Scientific Histogel

Procedure:

1. Liquefy Richard-Allan Scientific Histogel (histogel) by warming it at 60°C for 1 hour.
    a. Add 150 ml of Di water into a 250 ml Erlenmeyer flask
    b. Warm the Di water to 60°C using a benchmark digital hotplate
    c. Place the histogel tube into the Erlenmeyer flask and keep the temperature at 60°C

Note: Alternatively, make 2 ml aliquots of histogel to reduce the warming time.

Note: Use a minimum of 5 wells of enteroids for this procedure. Few enteroids will generate paraffin blocks with sparse enteroids per cut section.

2. Add 3 ml of cold OCM into the 15 ml conical tube and keep on ice.
3. Remove the 24 WP containing the enteroids from the incubator and place it in the BSC.

4. Using a P1000 tip, aspirate the total volume of old Intesticult media from the culture wells by tilting the plate and putting the tip against the sidewall of the wells.

Note: Avoid touching the Matrigel dome when aspirating the old Intesticult media.

5. Using a P1000, select a volume of 400 µl.
6. Set the tip and cut approximately ¼ of the total length of the tip from its fine tapered end.

Note: This tip will be used to absorb the entire Matrigel dome.

7. Load 300 µl of cold OCM.
8. Place the tip in the center of the Matrigel dome.
9. Release some OCM (approx. 50 µl) to push the Matrigel dome gently and immediately absorb the entire dome and transfer it into the 15 ml conical tube containing cold OCM.

Note: Repeat steps 5-9 for each well of enteroids.

CRITICAL: The enteroids will remain in their 3D organization by absorbing the entire Matrigel domes.

Note: Minimize manipulation of the tube and keep it on ice until its use.

10. Centrifuge the tube at 150 g for 5 min at 4°C.
11. Discard the supernatant.

Note: If some Matrigel remains within the enteroid pellet, do not remove it.

12. Add 1.5 ml of 4% Formaldehyde into the 15 ml conical tube containing the enteroids.

Note: Use an approximate volume of 1.5-2 ml of 4% Formaldehyde every 5 wells of enteroids.

13. Set a tip in a P1000. Cut approximately ¼ of the total length of the tip from its fine tapered end. Use this tip to resuspend the pellet gently.
14. Incubate for 10 min.
15. Centrifuge the tube at 150 g for 5 min at 4°C.
16. Discard supernatant in the appropriate Formaldehyde discard container.

Note: Move to the bench to continue the procedure.

17. Add 1 ml of prewarmed histogel into the 15 ml conical tube and vortex at low speed.
18. Allow the histogel to solidify by incubating the tube on ice or at room temperature.
19. Use a cell lifter to remove the entire solidified histogel containing the enteroids and place it in a petri dish.
20. Cut the gel to fit it into a histology cassette using a disposable scalpel.

Note: Discard the parts of the gel that do not contain enteroids.

21. Transfer the gel containing the enteroids into the histology cassette and label it.
22. Place the histology cassette in a plastic container.
23. Add 70% ethanol, covering the histology cassette completely.
24. Embed the gel containing enteroids into paraffin wax.
25. Follow routine immunofluorescence and histochemical protocols for formalin-fixed paraffin-embedded tissues.

6.7. Dissociation of canine enteroids to plate EDMs into transwell inserts

Timing: [3-4 hours]

Required equipment and reagents:

- Biosafety cabinet
- Incubator (37°C/5%CO_2)
- Refrigerated Laboratory Centrifuge
- Guava® Muse® Cell Analyzer or hemocytometer
- Epithelial Volt/Ohm Meter 3
- Water Bath
- Microscope

- Alcohol Resistant Cryogenic Permanent Marker
- Ice
- Styrofoam cooler
- 40 µm cell strainers
- 24-Well TC-treated Multi Well Plate
- Corning™ Transwell™ Multiple Well Plates
- 1.5 ml microcentrifuge
- 5 ml microcentrifuge
- BD Disposable Syringes with Luer-Lok™ Tips (1ml)
- Sterile EasyTouch® Hypodermic 25G - 1.5" needle
- 70% ethanol
- PBS (-Ca -Mg)
- 10% bleach solution container
- Tryple Express Enzyme.
- Corning Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix aliquot
- DMEMF12 with FBS 10% media
- Organoid Collection Media
- IntestiCult™ Human Organoid Growth (Intesticult) media supplemented with ROCK inhibitor Y-27632 and GSK3 inhibitor CHIR 99021.

Procedure:

1. Prepare the reagents and instruments.
    a. The day before, thaw an aliquot of Matrigel at 4 °C overnight.

b. Prepare Intesticult and OCM media following the instructions on Materials.

c. Keep OCM media on the ice during all the procedure.

CRITICAL: Keeping OCM cold is essential to avoid Matrigel polymerization during the process.

2. Prepare Matrigel-coated inserts of a Corning™ Transwell™ Multiple Well Plate (Transwell)

Note: The transwell used in this protocol corresponds to 24 WP with inserts of a culture area of 0.33 cm^2. When utilizing another size of transwells, adjust the volumes and cell seeding densities accordingly.

a. Bring the aliquot of thawed Matrigel and keep it on ice.

b. Dilute Matrigel in PBS using a 1:40 dilution factor.

c. Add the PBS (-Ca -Mg) at room temperature (22-24 °C) into a sterile 1.5 microcentrifuge tube.

d. Using a P200 tip, add the corresponding volume of Matrigel

e. Carefully mix the solution by pipetting up and down several times without forming air bubbles.

Note: Prepare 100 µl of this solution per insert of EDM expected to plate.

f. Using a P200 tip, add 100 µl of the Matrigel diluted solution into a transwell insert without touching the membrane.

g. Incubate for 1 h at room temperature without disturbing.

h. After 1 h, use a P200 tip to remove the Matrigel solution carefully.

Note: Handle the insert by its upper section without touching the membrane.

CRITICAL: When removing any liquid from the insert: Incline the insert horizontally and carefully remove the Matrigel solution without touching the membrane.

i. Wash the insert using a P200 tip by adding 150 µl of PBS (-Ca -Mg).

j. Using a P200 tip, carefully remove the PBS (-Ca -Mg).

k. Again, add 100 µl of PBS (-Ca -Mg).

l. Keep the transwell plate with the Matrigel-coated insert at room temperature until use.

3. Label a 1.5 ml microcentrifuge sterile tube.
4. Remove the 24 WP containing the enteroids from the incubator and place it in the BSC.
5. Using a P1000 tip, aspirate the total volume of old Intesticult media from the culture well by tilting the plate and putting the tip against the sidewall of the well.

Note: Avoid touching the Matrigel dome when aspirating the old Intesticult media.

6. Using a P1000 tip, add 500 µl of cold OCM directly onto the enteroid-containing Matrigel dome.

Note: Adding cold OCM dilutes Matrigel and keeps it liquid.

CRITICAL: When using more than 1 well of enteroids of the same line and passage to generate EDMs, use the same 500 µl of OCM to recover the Matrigel domes with enteroids. Combine up to 3 wells of enteroids in the same collection tube to avoid Matrigel polymerization or use a 5 ml macrocentrifuge tube.

7. Scrape the enteroid-containing Matrigel dome using the end of the P1000 tip.
8. Pipette up and down to dissociate the domes. Repeat this step 10-15 times, avoiding air bubble formation.
9. Transfer the complete volume of OCM containing the enteroids into the tube from step 3.

Note: Minimize manipulation of the tube and keep it on ice until its use.

10. Using a P1000 tip, add up to 450 µl of cold OCM to wash the well(s). Transfer the complete volume to the microcentrifuge tube from the previous step.

Note: The final volume in the tube should be 1 ml maximum. Consider the volume of the Matrigel domes when adding the cold OCM.

For example: When using 3 wells of enteroids to generate EDMs, add a maximum of 350 µl of OCM on step 10. Consider the initial 500 µl of OCM on step 6, plus the 150 µl of the Matrigel domes from the 3 wells.

11. Use a sterile BD Disposable Syringes with Luer-Lok™ Tips (1ml syringe) and a sterile EasyTouch® Hypodermic 25G - 1.5" needle to mechanically break down the enteroids.
12. Hold the syringe like a pencil, with the needle pointed down. Put the needle tip into the tube containing the enteroids. Slowly, pull back the plunger and aspirate the entire volume avoiding air bubble formation. Place the needle tip into the bottom of the tube and firmly force the entire volume out of the syringe. Repeat this step 5-6 times to dissociate the enteroids further.
13. Add cold OCM media into the tube to dilute the Matrigel and avoid its polymerization. Keep the tube on ice until its use.

Note: The OCM volume depends on the number of wells with enteroids combined in the tube. For example: Add 1 ml of media if the tube contains enteroids from 4 wells of a 24WP; it may not be necessary to add additional OCM when using only 1 well of enteroids.

14. Centrifuge the tube at 150 g for 5 min at 4°C.
15. Using a P1000 tip, discard the entire supernatant without disturbing the pellet.
16. Add 300-400 µl of Tryple Express Enzyme per well of enteroids, resuspend the pellet and transfer the entire solution into a 15 ml conical tube.
17. Using a P1000 tip, vigorously pipette up and down for 4 to 5 min.
18. Place the tube in a 37°C water bath for 2-3 min. Gently shake the tube to avoid the enteroids to sediment.
19. Bring the tube back into the BSC.
20. Inactivate the Tryple express enzyme by diluting in DMEMF12 with FBS 10% media. Add 1 ml of this media every 300-400 µl of Tryple Express Enzyme.

Note: The composition of the DMEMF12 with FBS 10% media is detailed in Materials.

21. Place a 40 µm cell strainer into the top of a sterile 50 ml conical tube.
22. Using a 10 ml serological pipette, filter the solution through the 40 µm cell strainer.

Note: This step removes the remaining large enteroids that did not dissociate.

23. Using a P1000 tip, wash the cell strainer with 1 ml of DMEMF12 FBS 10% media.
24. Centrifuge at 200 g for 5min at 4°C.
25. Discard the entire supernatant.
26. Resuspend the pellet in warm Intesticult media supplemented with ROCK inhibitor Y-27632 and GSK3 inhibitor CHIR 99021.

Note: Add an appropriate volume of Intesticult media according to the size of the pellet. The number of cells obtained per well of enteroids varies with the enteroid line and the processor. As a guide, add 100 µl per insert of EDM expected to plate. This volume can be adjusted after counting the cells.

The single-cell solution may have small clumps of cells; they won't affect the development of the EDM.

27. Count the number of cells.

Note: Use either a Guava® Muse® Cell Analyzer or a hemocytometer to count cells.

28. Adjust the volume of the single-cell solution to obtain a concentration of 30 x 10^3 cells per 100 µl of media.

Note: Dilute the solution when too concentrated or centrifuge at 200 g for 5 min at 4°C when a higher concentration is needed.

29. Take out the Matrigel-coated insert from its well and place it into an empty well.
30. Using a P1000, add 600 µl of warm Intesticult media supplemented with ROCK inhibitor Y-27632 and GSK3 inhibitor CHIR 99021.
31. Using a P200 tip, discard the PBS (Ca- Mg-) from the Matrigel-coated insert without touching the membrane.

32. Place the insert in the well with Intesticult media.
33. Using a P200 tip, plate 100 µl of Intesticult media containing 2.5-3 x 10^4 cells.
34. Gently place the plate in the incubator at 37°C with 5% CO_2.
35. Change the media of the insert every other day and the media of the well every two days during the first 7-10 days. Then the media change may be done more often depending on the number of dead cells.

CRITICAL: Visualization of monolayers is complex under a light microscope when cultured in transwells; therefore, daily monitory is crucial to determine confluency.

36. Monitor daily growing and morphological characteristics.
37. Measure Trans-Epithelial Electrical Resistance (TEER) daily using the Epithelial Volt/Ohm Meter 3 (EVOM3).
    a. Prepare a Matrigel-coated insert with no EDM (blank insert) when plating EDMs. Add and change the media of this blank well at the same frequency as with the wells with EDMs.
    b. Incubate transwell plate containing the EDMs at room temperature for 30 min. If necessary, change the media before the incubation time starts.
    c. Add 1.4 ml of warm fresh Intesticult media into a 1.5 ml microcentrifuge tube and keep it at room temperature for 30 min.
    d. Add 1.5 ml of 70% Ethanol into a 1.5 ml microcentrifuge tube.
    e. Sterilize the EVOM3 system with 70% Ethanol.
    f. After incubation, carefully place the electrodes into the 1.5 ml microcentrifuge tube with 70% Ethanol for 1 min.
    g. Move the electrodes into the 1.5 ml microcentrifuge tube with fresh media for 1 min.
    h. Place the electrode into the basal well and record the TEER.

i. Measure the TEER in the wells with EDMs.

j. Remove the electrode set and carefully wash the electrodes.

Note: Follow the manufacturer's EVOM3 manual for more detailed information about the operation, data acquisition, and maintenance: https://www.wpiinc.com/media/wysiwyg/pdf/EVOM3_IM.pdf.

6.8. Validation of EDMs morphology

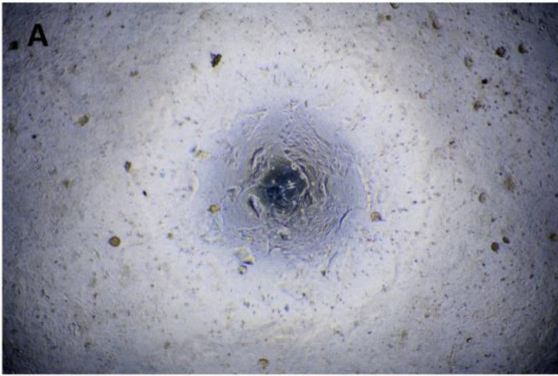
When EDMs are plated under a cell density of 2.5-3 x 10^4 cells/0.33 cm^2 cells, they double their number at day 2 after seeding and are 100% confluent by day 4. Around day 10, EDMs become densely cellular, and the number of dead cells increases. EDMs epithelial cells are polygonal and given that the intestinal epithelium has a high cell turnover rate, it is expectable that the number of cells in the EDMs increases within the days; this causes the size of the cells to reduce as the EDMs get older (Fig. 4 A, B, C).

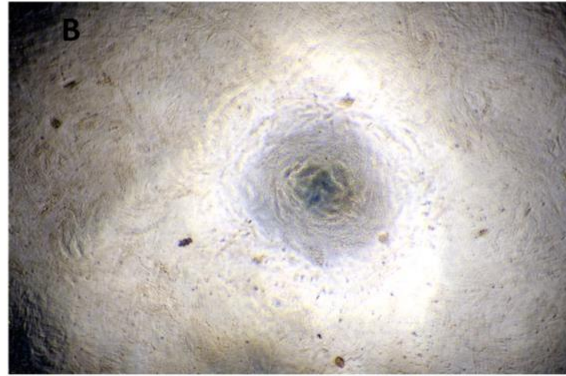
We have culture 100% confluent, healthy and proliferative EDMs until day 18. PAS staining showed that EDMs have mucus-producing cells that increase from day 8 to day 18 (Fig. 4 E, F). EDMs also showed positive staining for ZO-1 and Ki67 (Fig. 4 G, H).

EDMs displayed increase-decrease cyclic TEER values, showing values of 219 $\Omega*cm^2$ at day 5 and 4637 $\Omega*cm^2$ at day 5 with decreasing values the following four days. On day10, the TEER values increased to 4324 $\Omega*cm^2$ with another decreasing 4-day-cycle. TEER values stabilized from day 14 to day 18 with measurements higher than 5000 $\Omega*cm^2$ (Fig. 4 D).

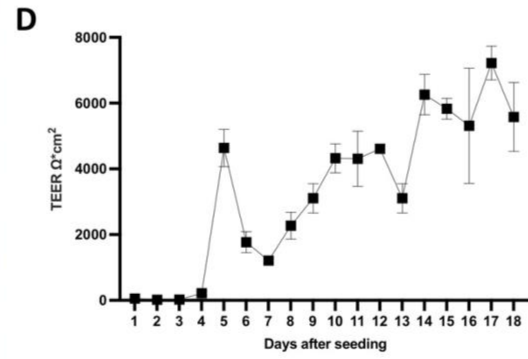
All this data validates the hypothesis that canine EDMs are unique in vitro

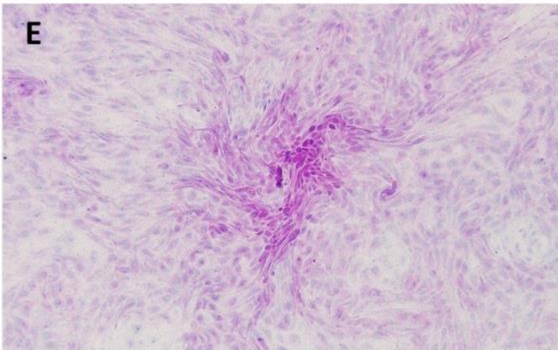
models of canine intestinal epithelium because they show cell heterogeneity and barrier function.

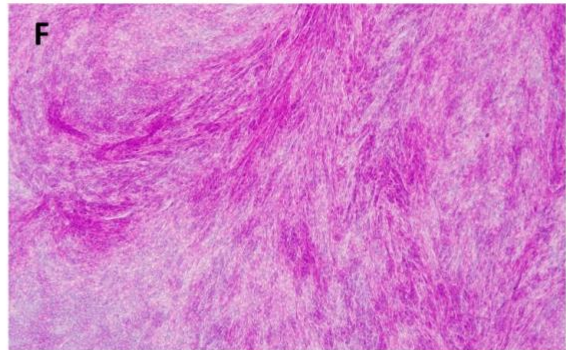

A

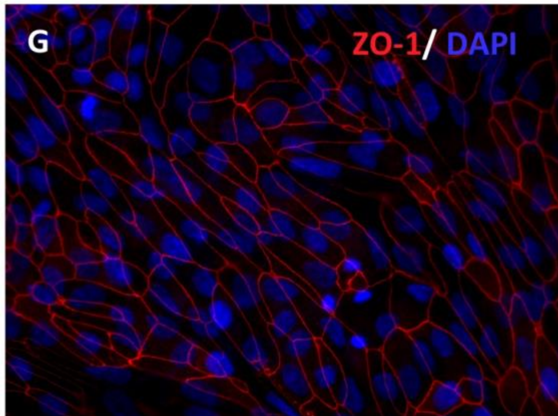

B

C


D
8000
6000
4000
2000
0
TEER Ω*cm²
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Days after seeding


E


F


G
ZO-1/ DAPI

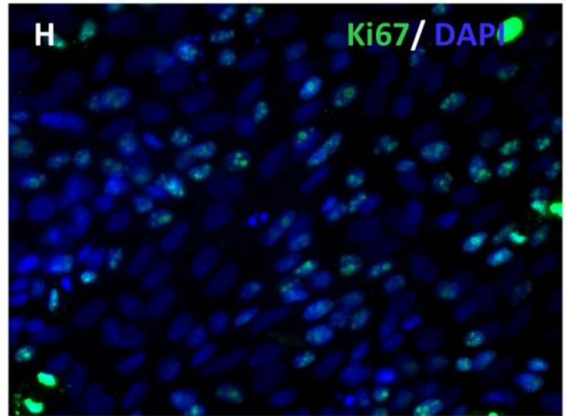

H
Ki67/ DAPI

Figure 4. Characterization of canine enteroid-derived EDMs. Representative images of EDMs at days 2(A),4(B), and 7(C), Bright-field images 4X. (D) Trans-Epithelial Electrical Resistance (TEER) dynamic values over 18 days of EDM culture, each square shows mean TER values (n = 3). (E, F) PAS staining of EDMs at days 8 (E) and 18 (F) demonstrating accumulating mucus, 10X. (E) Staining for tight junction protein ZO-1 at day 8, 40X. (F) Staining for the cell proliferation marker Ki67 at day 8, 40X.

6.9. Fluorescein isothiocyanate-dextran 4 intestinal permeability assay

Timing: [2 hours)

Required equipment and reagents:

- Biosafety cabinet
- Incubator (37°C/5%CO2)
- FilterMax F3 Multi-Mode Microplate Reader
- Water Bath
- Alcohol Resistant Cryogenic Permanent Marker
- 24-Well TC-treated Multi Well Plate
- Corning™ Transwell™ Multiple Well Plates
- Greiner BIO-ONE Microplate, 384 well, black.
- 1.5 ml microcentrifuge
- 1.5 ml black microcentrifuge
- 70% ethanol
- PBS (-Ca -Mg)
- 10% bleach solution container

- Fluorescein Isothiocyanate-Dextran 4 (1mg/ml)
- 4% Formaldehyde
- IntestiCult™ Human Organoid Growth (Intesticult) media supplemented with ROCK inhibitor Y-27632 and GSK3 inhibitor CHIR 99021.

Procedure:

Note: The controls we used for the Fluorescein Isothiocyanate-Dextran 4 (FITC-D4) intestinal permeability assay are (Fig. 5) :

- No cell control: Matrigel-coated insert with no EDM.
- Epithelium disruption control: EDM is treated with high concentrations of the cytokines IFN-γ and TNF-α, well-known intestinal barrier disrupters. Detailed instructions to prepare IFN-γ/TNF-α solution are on Materials
- Background fluorescence control: PBS (step 16).

1. Prepare the reagents and instruments.
    a. Prepare 1mg/ml FITC-D4 solution and 4% Formaldehyde following instructions on Materials.

Note: Prepare 100 µl of 1mg/ml FITC-D4 solution per insert.

    b. Label sterile 1.5 ml black microcentrifuge tubes (one tube per insert).
    c. Add 500 µl PBS (Ca- Mg-) into the wells of a 24 WP (one well per insert). This plate will be used to wash the inserts after FITC-D4 incubation.
    d. Label the transwell inserts before removing them from the wells (Fig 3A).
2. Remove the old Intesticult media from the inserts and wells.
3. Add PBS (Ca- Mg-) into the inserts (100 µl) and wells (600 µl). Discard the PBS.
4. Remove inserts from transwells.

5. Add PBS into the wells (600 µl). Label each well using the lid of the transwell.

Note: It is important to add the exact volume to each "receiver" well.

6. Put the inserts in the corresponding receiver wells.
7. Add 100 µl of 1mg/ml FITC-D4 solution to each insert.
8. Cover the transwell plate with Aluminum foil.
9. Incubate for 1 h at 37°C, 5% CO2

Note: The rationale of this Assay is that the more permeable the monolayer, the more molecules of FITC-D4 will cross from the insert towards the "receiver" well during this incubation.

10. Remove the inserts containing 1mg/ml FITC-D4 solution and put them in the 24 WP from step 1c.
11. Cover the receiver wells with aluminum foil.
12. Fix the EDMs for further Microscopic Analysis:

Note: FITC-D4 absorbs light maximally around 492 nm and fluoresces with a peak around 520 nm. When using the EDMs for Immunofluorescence staining, use an Alexa Fluor 594 secondary antibody.

a. Remove the 1mg/ml FITC-D4 solution from the inserts and discard.
b. Add 100 µl of PBS (Ca- Mg-) into the insert and discard. Repeat this step twice.
c. Add 100 µl of 4% Formaldehyde into the insert and incubate for 20 min.

Note: During this 20 min of incubation, continue to steps 13-17.

d. Discard the 4% Formaldehyde in the appropriate Formaldehyde discard container.
e. Add PBS (Ca- Mg-) into the inserts (100 µl) and wells (600 µl). Incubate at room temperature for 5 min with gentle continual shaking. Discard the PBS. Repeat this step twice.
f. Add PBS (Ca- Mg-) into the inserts (100 µl) and wells (600 µl). Seal the 24 WP with Paraffin Wax film and storage at 4°C.

13. Using a P1000 tip, collect the entire volume (600 ul) of the receiver wells and transfer it into the corresponding 1.5 ml black microcentrifuge tubes from step 1b.
14. Vortex the 1.5ml black microcentrifuge tubes at medium speed.
15. Add 50 µl of each sample in triplicates in wells of a Greiner BIO-ONE Microplate (Greiner microplate), 384 well, black.
16. Add 50 µl of PBS in triplicates into wells of the Greiner microplate.
17. Cover the Greiner microplate with aluminum foil.
18. Read the plate using a FilterMax F3 Multi-Mode Microplate Reader.

Note: Follow instructions in the user guide on the manufacturer's website: https://www.moleculardevices.com/sites/default/files/en/assets/user-guide/br/softmax-pro-data-acquisition-and-analysis-software.pdf

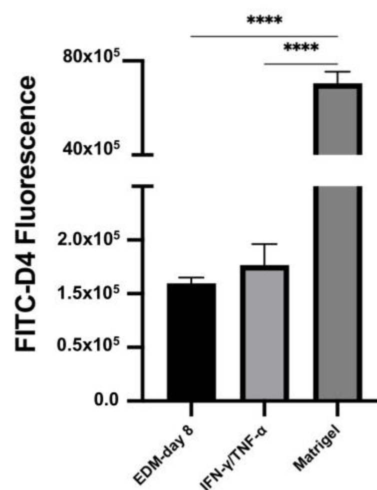


Figure 5. EDMs Intestinal permeability function. Monolayer-free Matrigel-coated membranes show marked increased intensity compared to membranes containing EDMs treated with IFN-γ/TNF-α or not treated, indicating proper intestinal barrier function of EDMs Each bar indicates the mean FITC-D4 intensity in the lower transwell chamber (n = 3).

6.10. Histochemical staining of EDMs

Timing: [3 hours)

Required equipment and reagents:

- Tweezers
- Alcohol Resistant Cryogenic Permanent Marker
- 24-Well TC-treated Multi Well Plate
- Disposable scalpels
- Wash bottles
- Microscopic glass slides
- Coverslips
- Clear nail polish
- Small lab tray
- 70% ethanol
- PBS (-Ca -Mg)
- Distilled water
- 4% Formaldehyde
- Bluing reagent
- Periodic acid 0.5%
- Schiff reagent
- Hematoxylin 1
- ProLong™ Gold Antifade Mountant

Procedure:

CRITICAL: Label the insert before removing them from the well.

1. Prepare 4% Formaldehyde and Periodic acid 0.5% following instructions on Materials.
2. Remove the old Intesticult media from the inserts and wells.
3. Add 100 µl of PBS (Ca- Mg-) into the inserts and discard. Repeat this step twice.
4. Add 100 µl of 4% Formaldehyde into the insert and incubate for 20 min.
5. Discard 4% Formaldehyde in the appropriate Formaldehyde discard container.
6. Add PBS (Ca- Mg-) into the inserts (100 µl) and wells (600 µl). Incubate at 22-24°C for 5 min with gentle continual shaking. Discard the PBS. Repeat this step twice.
7. Add Distilled water into the inserts (100 µl) and wells (600 µl) and discard.
8. Add 50 µl of 0.5 % Periodic Acid solution into the insert. Incubate at 22-24°C for 5 min.
9. Using a P200 tip, discard 0.5 % Periodic Acid solution.
10. Place the insert into a small lab tray.
11. Use a Wash bottle with Distilled water to gently rinse the insert.
12. Discard Distilled water.
13. Place the transwell inserts in a well of a 24 WP.
14. Add 50 µl of Schiff reagent into the transwell insert. Incubate at 22-24°C for 20 min.
15. Place the insert into a small lab tray.
16. Use a Wash bottle with warm tap water to gently rinse the insert for 8-10 min.
17. Discard running tap water.
18. Place the transwell inserts in a well of a 24 WP.
19. Add 50 µl of Hematoxylin 1 for 10 sec.
20. Place the insert into a small lab tray.

21. Use a Wash bottle with Distilled water to gently rinse the insert.
22. Discard Distilled water.
23. Place the transwell inserts in a well of a 24 WP.
24. Add 50 µl of the Bluing reagent. Incubate at 22-24°C for 15 sec.
25. Place the insert into a small lab tray.
26. Use a Wash bottle with Distilled water to gently rinse the insert.
27. Discard Distilled water.
28. Using a disposable scalpel, carefully cut the insert membrane (Fig. 3B).
29. Using tweezers, gently hold the membrane from the border without damaging the EDM.
30. Place the membrane into a microscopic glass slide with the 2D EDM facing up.
31. Using a P20 tip, add 10- 15 µl of ProLong™ Gold Antifade Mountant directly in the membrane.
32. Cover the membrane with a squared coverslip without forming air bubbles.
33. Let dry the mounting media for 24 h at 22-24°C.
34. Seal coverslips with nail polish.
35. Store EDMs at 22-24°C.

6.11. Immunostaining and preparation of EDMs for microscopic examination

Timing: [2 days)

Required equipment and reagents:

- Laboratory Shaker
- Tweezers
- Alcohol Resistant Cryogenic Permanent Marker
- 24-Well TC-treated Multi Well Plate

- Disposable scalpels
- Microscopic glass slides
- Coverslips
- Clear nail polish
- Hydrophobic barrier pencil
- 70% ethanol
- PBS (-Ca -Mg)
- 4% Formaldehyde
- Blocking solution
- Permeabilization Solution
- Washing buffer
- ZO-1 Monoclonal Antibody
- Ki-67 Recombinant Rabbit Monoclonal Antibody
- Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488
- Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594
- 1µ/ ml DAPI solution
- ProLong™ Gold Antifade Mountant

Procedure:

CRITICAL: Label the transwell inserts before removing them from the wells (Fig. 6A).

Part 1:

1. Prepare 4% Formaldehyde, Washing buffer, Blocking solution, and Permeabilization Solution following instructions on Materials.

2. Remove the old Intesticult media from the inserts and wells.
3. Add 100 µl of PBS (Ca- Mg-) into the inserts and discard. Repeat this step twice.
4. Add 100 µl of 4% Formaldehyde into the insert and incubate for 20 min.
5. Discard the 4% Formaldehyde in the appropriate Formaldehyde discard container.
6. Add PBS (Ca- Mg-) into the inserts (100 µl) and wells (600 µl). Incubate at 22-24°C for 5 min with gentle continual shaking. Discard the PBS. Repeat this step twice.
7. Add Washing Buffer solution into the inserts (100 µl) and wells (600 µl). Incubate at 22-24°C for 5 min with gentle continual shaking.
8. Discard the Washing Buffer solution.
9. Add Permeabilization solution into the inserts (100 µl) and wells (600 µl). Incubate at 22-24°C for 20 min with gentle continual shaking.
10. Discard Permeabilization solution.
11. Add Washing Buffer solution into the inserts (100 µl) and wells (600 µl). Incubate at 22-24°C for 5 min with gentle continual shaking. Discard the Washing Buffer solution. Repeat this step twice.
12. Add Blocking solution into the inserts (100 µl) and wells (600 µl). Incubate at 22-24°C for 30 min with gentle continual shaking.

Note: Prepare the primary antibody solution by adding the primary antibodies to the Blocking solution according to the dilution factor of each antibody. Under our laboratory conditions, the dilution factors for ZO-1 Monoclonal Antibody and Ki-67 Recombinant Rabbit Monoclonal Antibody were 1:75 and 1:150, respectively.

13. Discard Blocking solution.

14. Add Washing Buffer solution into the inserts (100 µl) and wells (600 µl). Incubate at 22-24°C for 5 min with gentle continual shaking. Discard the Washing Buffer solution. Repeat this step twice.
15. For the antibody incubation, draw a circle in the bottom of a well of a 24WP using a hydrophobic barrier pencil. The circle area should be similar to the bottom of the transwell insert.
16. Place the insert into the well from the previous step.
17. Add 100 µl of the primary antibody solution in the insert.
18. Add 600 µl of PBS to the surrounding wells.
19. Seal the 24 WP with Paraffin Wax film and incubate overnight at 4°C with continual light shaking.

Part 2:

20. Remove the primary antibody solution from the insert.
21. Locate the insert into a clean well of a 24 WP.
22. Add Washing Buffer solution into the inserts (100 µl) and wells (600 µl). Incubate at 22-24°C for 5 min with gentle continual shaking. Discard the Washing Buffer solution. Repeat this step twice.

Note: Prepare the secondary antibody solution by adding the secondary antibodies to the Blocking solution according to the dilution factor of each antibody. Under our laboratory conditions, the dilution factor for secondary antibodies is 1:1000.

23. For the antibody incubation, draw a circle in the bottom of a well of a 24WP using a hydrophobic barrier pencil. The circle area should be similar in size to the area of the bottom of the transwell insert.

Note: Alternatively, use the lid of a 24 WP for antibody incubation (Fig. 6A)

24. Locate the insert into the well/lid from the previous step.

25. Add 100 µl of the secondary antibody solution and incubate at 22-24°C for 1 h with gentle continual shaking and light-protected.

Note: From this step, protect EDMs from light.

26. Remove the secondary antibody from the insert.
27. Locate the insert into a clean well of a 24 WP.
28. Add Washing Buffer solution into the inserts (100 µl) and wells (600 µl). Incubate at 22-24°C for 5 min with gentle continual shaking. Discard the Washing Buffer solution. Repeat this step twice.

Note: Prepare a 1µ/ ml DAPI solution by diluting the DAPI stock solution in the Washing buffer.

29. Add 100 µl of DAPI solution and incubate at 22-24°C for 10 min with gentle continual shaking and light-protected.
30. Remove the DAPI solution and discard it in an appropriate container.
31. Locate the insert into a clean well of a 24 WP.
32. Add Washing Buffer solution into the inserts (100 µl) and wells (600 µl). Incubate at 22-24°C for 5 min with gentle continual shaking. Discard the Washing Buffer solution. Repeat this step twice.
33. Discard Washing buffer.
34. Using a disposable scalpel, carefully cut the insert membrane (Fig. 6B).
35. Using tweezers, gently hold the membrane from the border without damaging the EDM.
36. Place the membrane into a microscopic glass slide with the EDM facing up.
37. Using a P20 tip, add 10- 15 µl of ProLong™ Gold Antifade Mountant directly in the membrane.
38. Cover the membrane with a squared coverslip without forming air bubbles.
39. Let dry the mounting media for 24 h at 22-24°C.
40. Seal coverslips with nail polish.

41. Store EDMs at 4°C and protect them from light.

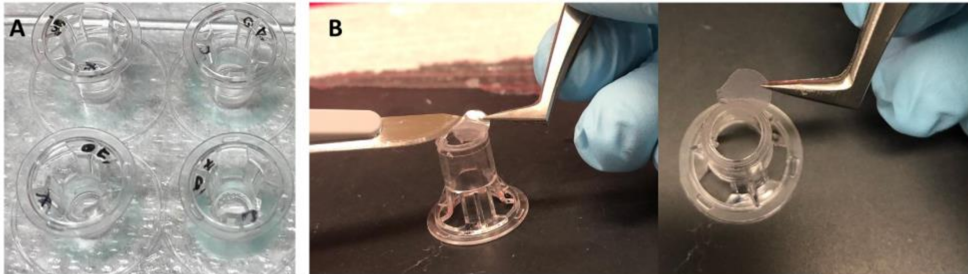


Figure 6. Handling of Transwell inserts for Staining techniques. (A) Incubation of EDMs with secondary antibody using a lid of a 24 WP. (B) Removal of the EDM-containing membrane using a disposable scalpel and tweezers.

6.12. ZO-1 tight junction protein morphological analysis

Tight junction proteins are determinants to maintain the intestinal barrier function. Grosheva *et al*. (2020) developed a Fiji macro to analyze the structural disposition of this ZO-1 tight junction protein to assess the intestinal barrier. This Fiji macro is available online with detailed instructions. Here we provide the steps and settings we applied to analyze our EDMs with this Fiji Macro.

1. Perform Immunostaining of EDMs for ZO-1.
2. Use an Immunofluorescence microscope to acquire the images
3. When imaging, adjust the intensity at high values. For the EVOS-inverted microscope, we used 80-90% intensity values and an exposure time of 1.5 s.
4. Save the images in TIFF format.
5. Open Fiji
6. Open the image
7. Select Image>color>split channels

8. Three images from the three channels will appear; keep only the channel used when acquiring the image.
9. Select Process> subtract background-Rolling ball radius: 10.0 pixels.

Note: Adjust the number of pixels looking for the optimal background subtraction.

10. Save the image in TIFF format. Generate a folder to save all the images for further analysis.
11. Download the TightJunctionAnalysis.ijm Fiji macro at this website https://github.com/WIS-MICC-CellObservatory/Intestinal_Barrier_Function
12. Open the Fiji macro
13. Adjust the morphological segmentation parameters as needed.

Note: Some parameters may need to be adjusted with different experimental conditions.

For terms of our experimental conditions and acquired images, we adjusted the following parameters:

- Tolerance: 10
- MinCellSize: 100
- MaxCellSize: 1500000

14. In the Fiji Macro, select Run>Whole folder> open the folder.
15. Depending on the total number of images, the Macro will take seconds or minutes to complete the analysis.
16. After the analysis is complete, the Fiji macro will generate the "Results" folder within the initial folder.
17. One of the critical information generated is the Tortuosity values that represent how regular is the structural disposition of the ZO-1 protein in the cell.
18. To find these data open the excel files that end in _JTResults.xls (name_of_the_image_JTResults.xls).

Note: There would be one of these files per analyzed image.

19. The last column of these excel files is under the name Straightness. To obtain the tortuosity data, divide one by the Straightness values (Tortuosity=1/Straightnes).

7. Limitations

In this protocol, EDMs have been validated using microscopic analysis. Unfortunately, there is not a specific antibody to recognize canine ISCs. Therefore, investigating their function is limited using this protocol.

This limitation could be addressed using RNA hybridization, but this will increase the cost of laboratory supplies.

Although the commercially available cell culture media used in this protocol has the advantage that it is ready to use, the formulation of this media is proprietary, and the concentrations of the components are unknown.

8. Troubleshooting

Problem 1: Matrigel leftovers when generating the EDMs

Potential solution:

When recovering enteroids from the Matrigel domes, the latter can get polymerized if the sample is unintentionally heated up. This could be due to over manipulation of the tube or failure in maintaining a cold environment. To remove Matrigel leftovers, follow the following steps:

1. If the enteroids are in a 1.5 ml microcentrifuge tube, transfer the entire solution into a 5 ml microcentrifuge tube.
2. Place the tube on ice for 5 min.

3. Add 2 times the sample volume of cold OCM media into the tube.
4. Using a P1000 tip, slowly pipette up and down 3 times.
5. Centrifuge at 150 g for 5 min at 4°C.

After this, the Matrigel leftover should be diluted on the cold OCM media.

Problem 2: Low number of healthy enteroids in the Matrigel dome

Potential solution:

When nearly 1/3 of the total enteroids of a dome are healthy, they can be rescued following the next steps during the passaging process:

1. Using an inverted light microscope, note the section of the Matrigel dome with the healthy enteroids.
2. Using a P1000 tip, remove the old Intesticult media in the BSC.
3. Using a P200 tip, cut the dome isolating the area of interest (Fig. 7).
4. Using a P200, load 100 µl of cold OCM media and pipette up the section of Matrigel dome with the healthy enteroids.
5. Place the recovered enteroids into a sterile 1.5 ml microcentrifuge and add 200 µl of cold OCM.
6. Continue with step 13 of Passaging enteroids protocol.

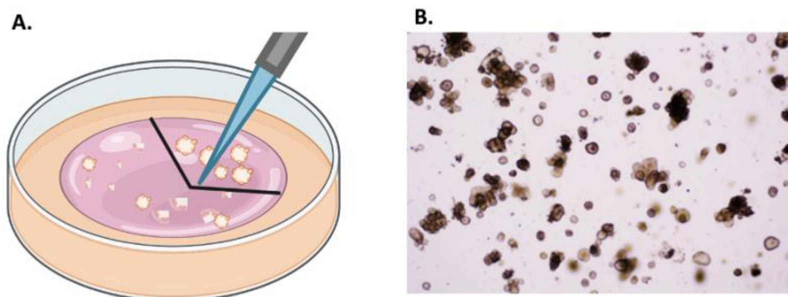


Figure 7. (A) Section of the Matrigel dome to rescue healthy enteroids. (B) Appearance of enteroids derived from an enteroid line with unhealthy morphology and size.

9. References

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