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TITLE:

Generation of Murine Primary Colon Epithelial Monolayers from Intestinal Crypts

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primary culture, 2D-enteroids, 2D-colonoids, epithelial cells, epithelial monolayers, intestinal crypts

SUMMARY:

In this protocol, we describe how to generate murine primary epithelial colon monolayers directly from intestinal crypts. We provide experimental approaches to generate confluent monolayers on permeable filters, confluent monolayers for scratch wound healing and biochemical studies, and sparse and confluent monolayers for immunofluorescence analysis.

ABSTRACT:

The intestinal epithelium is comprised of a single layer of cells that act as a barrier between the gut lumen and the interior of the body. Disruption in the continuity of this barrier can result in inflammatory disorders such as inflammatory bowel disease. One of the limitations in the study of intestinal epithelial biology has been the lack of primary cell culture models, which has obliged researchers to use model cell lines derived from carcinomas. The advent of three dimensional (3D) enteroids has given epithelial biologists a powerful tool to generate primary cell cultures, nevertheless, these structures are embedded in extracellular matrix and lack the maturity characteristic of differentiated intestinal epithelial cells. Several techniques to generate intestinal epithelial monolayers have been published, but most are derived from established 3D enteroids making the process laborious and expensive. Here we describe a protocol to generate primary epithelial colon monolayers directly from murine intestinal crypts. We also detail experimental approaches that can be used with this model such as the generation of confluent cultures on permeable filters, confluent monolayer for scratch wound healing studies and sparse and confluent monolayers for immunofluorescence analysis.

INTRODUCTION:

Epithelial cells (IEC) line the intestines forming a selectively permeable barrier that allows nutrient and water absorption while prevent microorganisms and toxins to enter the body [1]. The intestinal mucosa is composed of luminal projections called villi (only present in small intestine) and invaginations named crypts. Villi and the surface of colon crypts are covered by differentiated epithelial cells while the base of the crypts is comprised of stem cells that make the rapid renewal of the intestinal epithelia, which has a turnover from 3 to 7 days. Intestinal stem cells (ISC) are not only important for maintaining gut homeostasis but for adequate repair of damaged epithelia[2].

Study of the intestinal epithelia biology was limited by the lack of primary cell cultures and transformed cell lines were the only tool available. Intestinal epithelial model cell lines are not capable to accurately replicate the physiology of the normal intestinal epithelium. The development of 3D cultures derived from ISC provided intestinal mucosal biologists with in vitro models that resemble in vivo gut mucosal conditions[3]. Crypts can be easily isolated from murine samples, embedded in a basement membrane matrix medium (e.g., Matrigel) and grown in conditioned media containing Wnt3a, R-spondin, and Noggin, generating 3D structures known as enteroids (small intestine) or colonoids (large intestine)[4]. Enteroids and colonoids are polarized spheroidal structures where the apical domain is facing an internal lumen and the basolateral region is in direct contact with the extracellular matrix. Enteroids and colonoids contain all major differentiated intestinal epithelial sub-types such as Enterocytes/Colonocytes, Paneth, Enteroendocrine and Goblet cells and they appear in relatively the same proportions as they do in the section of the gut where they were isolated from[5]. Even though 3D enteroids and colonoids represent a major advance in the study of intestinal development and physiology, these models present certain drawbacks such as limited access to the apical surface of the epithelial cells (lumen) and the ability to scale cultures up or down to achieve high-throughput screening of molecules of interest. To overcome these limitations, protocols to obtain primary 2D cultures of IEC derived from 3D enteroids/colonoids were generated. 2D enteroids/colonoids grow as sheet of cells just as model cell lines do and are ideal to study intestinal wound repair, host-pathogen interactions, and regenerative medicine amongst others. Several published papers describe how to generate 2D monolayers from 3D structures or directly from intestinal crypts, (see[6-11]) but these methods tend to be labor intensive and hard to reproduce. A fast, simple, and reproducible method to obtain monolayers directly from freshly isolated mouse intestinal crypts is outlined in this protocol.

Here we explain in detail the process for crypt extraction with minimal generation of debris, extracellular matrix choice and different surfaces and applications for this technique. This experimental approach was optimized for colon crypts, but similar results are obtained when applied for small intestine.

PROTOCOL

All procedures described below have been approved and conducted in accordance with the guidelines set by University of Michigan Institutional Animal Care and Use Committee.

1. Preparation of reagents for crypt isolation and culture (prepare in tissue culture hood)

1.1. 50 mM ethylenediamine tetraacetic acid (EDTA): Add 50 mL 0.5 M stock to 450 mL of phosphate buffered saline, without calcium (Ca^{2+}) and magnesium (Mg^{2+}) to prepare 500 mL. In this protocol PBS will refer to PBS without calcium and magnesium unless otherwise stated.

1.2. Shake buffer: Dissolve 7.4 g sucrose (43.3 mM) and 5 g sorbitol (54.9 mM) in PBS to prepare 500 mL.

1.3. L-WRN (L-Wnt-3A, R-spondin and Noggin) media: Supplement Advanced Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12) (780 mL) with 20% Fetal Bovine Serum (FBS) (200 mL), 1x commercially available glutamine supplement (10 mL), 100 U/mL penicillin and, 100 g/mL streptomycin (10 mL), and filter sterilize with 0.22 µm filter.

1.3.1. Get L-WRN cells through ATTC, grow in T175 flasks, and select using Geneticin and Hygromycin. Media is changed and collected for 12 days.

NOTE: Every batch of media is tested for Wnt activity using a TOPflash Wnt Reporter assay. In this case the Michigan Medicine Translational Tissue Modeling Laboratory protocols (https://www.umichttml.org/protocols) were followed. The TOPflash HEK 293 cell line is grown to confluency in a T75 flask, trypsinized and plated onto a 96-well plate. The following day different dilutions of the collected media is added to cells and incubated in a 5% CO_2 incubator at 37 ˚C overnight. Next day, the cells are lysed, and Firefly Luciferase assay is performed according to manufacturer's instructions. The assay is normalized using recombinant Wnt-3A. The media is divided into 25 mL aliquots in 50 mL conical tubes and store at -80 ˚C.

1.4. Base media: For 500 mL, supplement Advanced DMEM/F12 (448 mL) with 2x commercially available glutamine supplement (10 mL), 20 mM HEPES (10 mL), 100 U/mL penicillin and, 100 g/mL streptomycin (10 mL), 2 mM N-acetyl-L-cysteine (2 mL), N2 supplement (10 mL) and B27 supplement (20 mL), filter sterilize with 0.22 µm filter. Divide the media into 25 mL aliquots in 50 mL conical tube and store at -80 ˚C.

1.5. LWRN complete media: Combine 25 mL LWRN media with 25 mL of base media and supplement with 200 ng/mL Epidermal growth factor (EGF) (20µL) and 2x antibiotic-antimycotic solution (1 mL). Store the complete media at 4 ˚C.

1.6. Collagen and Laminin: Dissolve 5 mg powder in 5 mL of filter sterilized 100 mM acetic acid (add 60 µL of acetic acid stock to 9.94 mL of molecular grade water) to produce a stock concentration of 1 mg/mL. Rotate at 4 ˚C for 4 h and make 100 µL aliquots in 0.2 mL tubes. Freeze at -20 ˚C. Laminin is purchased at a stock concentration of 100 µg/mL.

1.7. Complete media without growth factors (CMGF-): Supplement Advanced DMEM/F12 (500 mL) with 1x commercially available glutamine supplement (5 mL), 10 mM HEPES (5 mL), 100 U/mL penicillin and, 100 g/mL streptomycin (5 mL).

1.8. Differentiation media: To 9.2 mL of CMGF- media, add 200 µL of B27 supplement, 100 µL of N2 supplement, 20 µL of N-acetyl-L-cysteine, 500 µL of Noggin media[12] (made from Noggin-producing cells) and 2 µL of EGF to make 10 mL of differentiation media.

2. Preparation of plates, chamber slides and cell culture membrane inserts

2.1. Coating 48-well plate and chamber slides for plating 2D monolayer: Use the coating solution which constitutes laminin (1:40 dilution, see **Table of Materials**) and collagen (1:30 dilution) in cold Dulbecco's Phosphate buffered saline, with Ca^{2+} and Mg^{2+} (DPBS). Add 200 µL of coating solution to each well and pre-incubate the plate/chamber slide in a 5% CO_2 incubator at 37 ˚C for at least 2 h.

2.2. Coating 0.4 µm cell culture membrane inserts: Make 1:30 dilution of collagen in molecular grade water and add 200 µL to each insert. Keep the plate containing the membrane insert on ice at 4 ˚C for 30 min. Following 30 min incubation, keep the plate in a 5% CO_2 incubator at 37 ˚C for 1.5-2 h. Polyester and polycarbonate membrane inserts yield comparable results.

NOTE: Any tissue culture plate can be seeded (up and downscaling can be performed) using this protocol by adjusting the collagen/laminin volume to obtain full coverage of the plating surface.

3. Crypt isolation

NOTE: Before starting the dissection, prepare collagen and/or laminin coated plate/membrane inserts/chamber slide and leave them in a 5% CO_2 incubator at 37 ˚C. Prepare a clean work bench and sterile surgical instruments appropriate for surgery, and a biological safety cabinet for culturing 2D monolayers. Confirm all other standard equipment for 2D monolayer culturing such as humidified CO_2 incubator, tabletop centrifuges (maintained at 4 ˚C), microscope and pipettes (including serological pipettes) are ready.

3.1. Use C57Bl/6 mice, 8-12 weeks old. Euthanize mice using an approved method of euthanasia.

3.2. Spray the mice carcasses with 70% ethanol (EtOH) solution to clean the dissection area and remove excess EtOH using paper tissue.

NOTE: Ensure that the crypt isolation reagents (such as PBS, 50 mM EDTA, shake buffer) crypts are kept cold. These reagents can be prepared one-day prior and are good to use for at least 3 months when stored at 4 ˚C. Also, make sure that the LWRN complete media is kept in a bead/water bath maintained at 37 ˚C until use.

3.3. Using clean dissection scissors and forceps, dissect out the colon from the rectum to the cecum. Hold the end of the colon using forceps and very gently flush off the feces using ice cold PBS in a 10 mL syringe, fitted with a 20 G feeding tube (**Figure 1A**). Make sure not to rupture the

colon.

3.3.1. Remove the proximal colon. This will be the portion of the colon closest to the ileocecal junction.

3.4. With forceps, slide the distal colon gently onto the 20 G feeding tube, tying off the colon at the end of the tube by the tip with 4-0 silk suture thread (**Figure 1B**).

3.5. Invert the colon inside out using one's fingers over the end tied off and tie the other end with 4-0 silk suture thread. Using surgical scissors, cut colon below the tip of the feeding tube (**Figure 1C,D,E**).

3.6. Using the plunger of a 1.25 mL repeat syringe, gently open the untied end of the inverted colon onto the tip of a 1.25 mL repeat syringe. Slide the inverted colon onto the end of the syringe and tie tightly with 4-0 silk suture thread (**Figure 1F,G**).

3.7. Insert the plunger into the syringe and inflate the colon to form a sausage. Inflate until the colon sausage looks turgent with no visible wrinkles (**Figure 1H**).

3.8. Place syringe/colon in 15 mL tube with 5 mL of Cell Recovery Solution on ice for 20 min, inflate and deflate the colon once every 5 min (**Figure 1I**). The sausage must remain inflated during the incubation.

3.9. Tie off using 4-0 silk suture thread below the tip of the repeat syringe with the colon inflated. Cut the sausage off the repeat syringe and place in 15 mL tube containing 10 mL of 50 mM (2mM for small intestine) EDTA for 40 min and rotate at 4 °C (**Figure 1J,K**).

3.10. Decant off the EDTA solution and replace with 5 mL of shake buffer. Shake the sausage manually in vertical position (vigorously) for 2 min.

3.11. Decant the shaking solution into a new 15 mL tube and repeat shaking step for a total of 10 mL of crypts in shaking buffer.

3.12. Take 20 µL of the crypt suspension in a Petri dish and count the number of crypts under a microscope. Calculate the crypt concentration in crypts/µL. Depending on the concentration, dilute the samples to obtain 5 crypts/ µL at the moment of plating (1000 crypts/cm^2).

3.13. Spin the tube with isolated crypts using tabletop centrifuge at 400 x *g* for 10 min at 4 °C.

3.14. In the meantime, remove 48-well plate/insert/chamber slide from the incubator and place it in biosafety cabinet. Aspirate the coating solution using P200 and leave the plate with the lid slightly offset until cells are ready to be plated.

4. Culturing 2D monolayer

NOTE: For a detailed protocol on how to generate intestinal epithelial monolayers from 3D colonoids check protocols by Estes and Kovbasnjuk labs ([7,11]).

4.1. Remove the shaking buffer using 10 mL serological pipette. Make sure that the pellet is intact and may using P1000 to remove any liquid left. Re-suspend the pellet in 3 mL of LWRN complete media and pipette up and down with P1000. Add 200 µL of crypts to each well of a pre-coated 48-well plate/chamber slide and incubate in a 5% CO_2 incubator at 37 ˚C.

4.2. The following day aspirate the media using P200 and add fresh media. The cells become confluent in 24-48 h.

4.3. For cell culture membrane inserts, add 200 µL crypts (5 crypts/µL) to top of the inserts and 600 µL of complete L-WRN media to the bottom part. The following day aspirate the media using P200 and add fresh media only to the top chamber. Incubate the plate in a 5% CO_2 incubator at 37 ˚C. Transepithelial Electrical Resistance (TEER) is measured every day using Epithelial Volt/Ohm meter (EVOM).

NOTE: If the culture has a TEER reading greater than 300 $\Omega.cm^2$, they are meant to be confluent. Confluency is achieved in 3-4 days. Change media every 2 days.

REPRESENTATIVE RESULTS:

To illustrate the reliability of the primary epithelial colon monolayer cultures, a summary of the crypt isolation and representative images derived from the protocol is shown. The user must have in mind that the isolated crypts are cultured in sterile conditions, so a correct dissection and cleaning of the colon is a priority. **Figure 1** features key steps during crypt isolation. Isolated crypts (**Figure 2A**) are now counted and concentrated (**Figure 2B**) to obtain a concentration of 5 crypts/µL. After a concentrated preparation of crypts, the cells will be plated in the desired format (culture dish, membrane inserts or chamber slides) and incubated with the appropriate media depending on experimental needs. **Figure 3** shows culture progression after 24 and 48 h of culture in 48-well plates. The cells are incubated until the desired confluency is reached. To exemplify possible applications of this method, we allowed 48-well plate wells to reach confluency and proceeded to do a scratch-wound assay. **Figure 4** depicts a freshly created scratch (**Figure 4A**) in a 2D colonoid monolayer and the same wound 24 h after (**Figure 4B**). It is clear that the culture is still healthy and viable and there is wound repair. To generate differentiated monolayers, media is changed from LWRN to differentiation media. Differentiation is achieved by showing high TEER values (**Figure 5A**), decrease of ISC markers ((Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) and Achaete-scute like 2 (Ascl2)) and increase of differentiation markers ((Alanyl aminopeptidase (Anpep), Mucin 2 (Muc2), lysozyme 1 (Lyz1), Sucrose iso-maltase (Sis) and Chrmogranin A (Chga)) (**Figure 5B**,**C**) by PCR. Other markers as CDX2 and KRT20 can also be included in this panel. In addition to mRNA expression levels, the appearance of sub-types of differentiated epithelial cells in 2D colonoids grown in differentiation conditions is also shown by immunofluorescence (Muc2 and Chga; **Figure 5D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Sample preparation for healthy monolayers generation. Sample preparation is crucial for generation of healthy monolayers. Key steps of the isolation process are depicted in this figure to make it easier to the reader. The colon is excised from the mouse, making sure there is no fur leftovers. Carefully remove the feces making sure you do not perforate the colon; this is of vital importance as the colon needs to be able to hold air and be inflated and deflated.

Figure 2: Crypt isolation count and concentration. (**A**) Colonic crypts after shaking the colon sausages. The image depicts a field of a 20 µL drop. (**B**) Crypt concentration in order to obtain 5 crypts/µL. Scale bar: 1 mm.

Figure 3: Primary IEC Monolayer growth. (**A**) 2D IEC monolayers after 24 h of plating and removal of cell debris. (**B**) Confluent 2D IEC monolayers 48 h after plating. Scale bar: 10 µm.

Figure 4: **Scratch wound assays using murine primary IEC.** Images showing wound healing after a scratch was made in epithelial colon monolayer. Scale bar: 5 µm.

Figure 5: Differentiation of epithelial colon monolayers. (**A**) Differentiation media creates tight epithelial barriers as demonstrated by TEER. Differentiation media causes a drop in mRNA expression of (**B**) stem cells markers and upregulation of (**C**) differentiation markers. (**D**) Markers of specialized differentiated epithelial cells such as Muc2 and Chromogranin-A can also be detected via immunofluorescence of direct 2D colonoids. Scale bar: 5 µm.

DISCUSSION:

Our protocol provides a fast, reproducible, and reliable method to generate direct primary 2D IEC monolayers. One of the main differences in our protocol compared to previously published protocols to generate colon epithelial monolayers is that we do not cut the colon in small pieces to liberate the crypts. Instead, we adapted a protocol to separate intestinal epithelium from mesenchyme[13] by a combination of chemical and mechanical forces to release crypts in an extremely clean preparation, (**Figure 2**), providing the researcher with ideal material to generate primary cultures. Our isolation method can be also used to generate 3D enteroids and colonoids. It is important to do a crypt count every time an experiment is performed to normalize the number of crypts that are being plated. Variables such as colon sausage turgor, speed of isolation, user expertise can affect the number of crypts isolated. The suggested plating crypt concentration mentioned in the protocol is a starting point, but every user to account for user driven variables must optimize it. We have used this technique with male and female WT mice ranging from 8 to 20 weeks and we have not seen major differences in cell survival, in theory crypts isolated from younger mice have better chances to survive. Crypts are plated in excess as only a small percentage of them attach to the surface and survive. A balance where there are enough crypts to have a 50% confluency one day after plating but not too many crypts where the dying crypts will have a cytotoxic effect is the goal. LWRN media must be carefully removed 24 hours after plating to eliminate dead crypts and debris, this must be done carefully to avoid detaching cells that are already growing as a monolayer.

After initial removal of LWRN media, the user must decide if the experimental conditions require primary IEC monolayers that remain closer to stem cells and add fresh LWRN media or if differentiation of the monolayer is desired, replace with differentiation media. The single most important factor in this protocol is to assure the integrity of the colon during the isolation process. If a rupture occurs, the sausage can be shortened to eliminate the damaged area. Before putting the sausage in EDTA be sure that the knots are as tight as possible. If the sausage is deflated after the EDTA incubation the protocol can be continued with little to no effect in the overall crypt yield. If the repeat syringe is not available, a regular micropipette tip attached to a regular syringe can also be used for the process of inflation and deflation. Also, if no cell recovery solution is available, the inflation and deflation steps can be done in EDTA (2mM small intestine, 50 mM colon), but this substitution is not recommended. If confluency is not required, crypts can grow in plates coated only with collagen and even in uncoated plates. Only use uncoated plates cases there is no other option, but the cells will not grow healthy as they would in collagen-coated plates. When it comes to culture health and stability, monolayers plated in plastic are healthy for 4 to 5 days while monolayers plated in transwells can be carried for up to 8 days.

One of the main limitations of this method is the cell media required to grow the epithelial colon monolayers. LWRN cells are available at ATCC, but LWRN conditioned media generation is labor intensive and requires access to a fluorescent spectrometer to determine Wnt activity. Differentiation media requires a number of reagents that are added fresh before use, which makes it a tedious process. Finally, most of these reagents are costly and is easy to burn the reagents in a fast pace. If a laboratory desires to establish this technique without previous intestinal primary cell culture, it is highly recommended to find a collaborator/college with experience and train one of their members.

Maintenance of 3D culture could be expensive due to cost of basement membrane matrix medium and high volumes of conditioned media needed for organoid cultures, but it has the advantage of using a reduced number of mice and the generated structures can be passaged many times. Enteroids (derived from small intestine) are relatively easy to isolate and maintain while colonoids are more delicate, grow in a slower pace and have a more limited passage capacity. Monolayer generation from 3D colonoids require a disproportionate amount of 3D structures, that make these kinds of experiments time consuming and costly. On the contrary, direct epithelial colon monolayer prep is fast and is a quick way to obtain the results. One colon prep can generate a confluent area of 75 cm^2 (10 to 15 mL of conditioned media, replaces once) 2 to 3 days after plating (this area would require 144 wells of 3D colonoids, which means almost 6mL of Matrigel and more than 250 mL of conditioned media). The lower consumption of media, low-cost maintenance of the cell culture and ability to perform functional tests-easy and fast downstream processing are big advantages of epithelial colon monolayers.

This protocol is a valuable tool in the study of intestinal epithelial cell biology in areas such as cell-adhesion, polarity, and differentiation. It gives the advantage to generate primary cell cultures from genetically modified mice (knock-out, overexpressing, reporters). The primary intestinal epithelial monolayers allow easy access to the apical and basolateral surfaces (when

plated on transwells) allowing the study of permeability, barrier and transepithelial migration of different cells types. Finally, this model can be useful in different fields like host-pathogens interactions, epithelial damage and repair and drug discovery.

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DISCLOSURES:

The authors do not have any conflicts of interest.

REFERENCES

1. Quiros, M., Nusrat, A. Contribution of wound-associated cells and mediators in orchestrating gastrointestinal mucosal wound repair. *Annual Reviews in Physiology.* **81**, 189-209 (2019).
2. Blutt, S. E. et al. Use of organoids to study regenerative responses to intestinal damage. *American Journal of Physiology-Gastrointestinal and Liver Physiology.* **317** (6), G845-G852 (2019).
3. Zhang, M., Liu, Y., Chen, Y. G. Generation of 3D human gastrointestinal organoids: principle and applications. *Cell Regeneration.* **9** (1), 6 (2020).
4. Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature.* **459** (7244), 262-265 (2009).
5. Zachos, N. C. et al. Human enteroids/colonoids and intestinal organoids functionally recapitulate normal intestinal physiology and pathophysiology. *Journal of Biological Chemistry.* **291** (8), 3759-3766 (2016).
6. Kozuka, K. et al. Development and characterization of a human and mouse intestinal epithelial cell monolayer platform. *Stem Cell Reports.* **9** (6), 1976-1990 (2017).
7. In, J. G., Foulke-Abel, J., Clarke, E., Kovbasnjuk, O. Human colonoid monolayers to study interactions between pathogens, commensals, and host intestinal epithelium. *Journal of Visualized Experiments.* (146), e59357 (2019).
8. Cardenas, D. et al. Two- and three-dimensional bioengineered human intestinal tissue models for cryptosporidium. *Methods in Molecular Biology.* **2052**, 373-402 (2020).
9. Moon, C., VanDussen, K. L., Miyoshi, H., Stappenbeck, T. S. Development of a primary mouse intestinal epithelial cell monolayer culture system to evaluate factors that modulate IgA transcytosis. *Mucosal Immunology.* **7** (4), 818-828 (2014).
10. VanDussen, K. L. et al. Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays. *Gut.* **64** (6), 911-920 (2015).
11. Zou, W. Y. et al. Human intestinal enteroids: New models to study gastrointestinal virus infections. *Methods in Molecular Biology.* **1576**, 229-247 (2019).
12. Heijmans, J. et al. ER stress causes rapid loss of intestinal epithelial stemness through activation of the unfolded protein response. *Cell Reports.* **3** (4), 1128-1139 (2013).
13. Nik, A. M., Carlsson, P. Separation of intact intestinal epithelium from mesenchyme. *Biotechniques.* **55** (1), 42-44 (2013).

Figure1

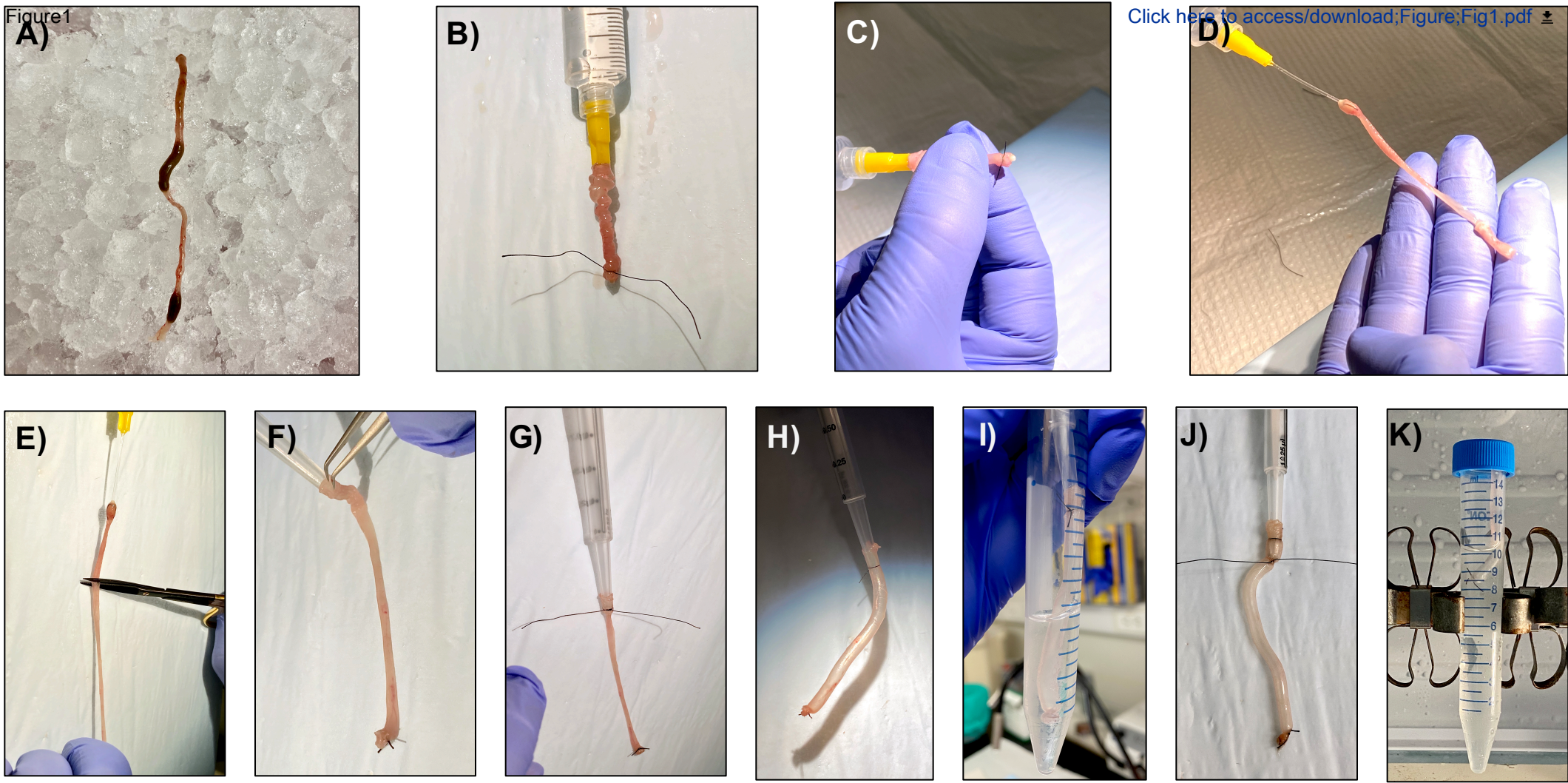
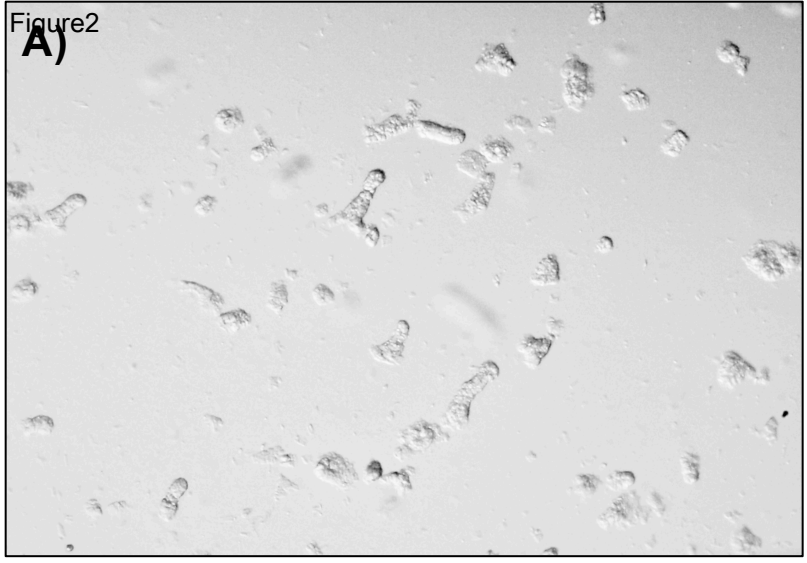


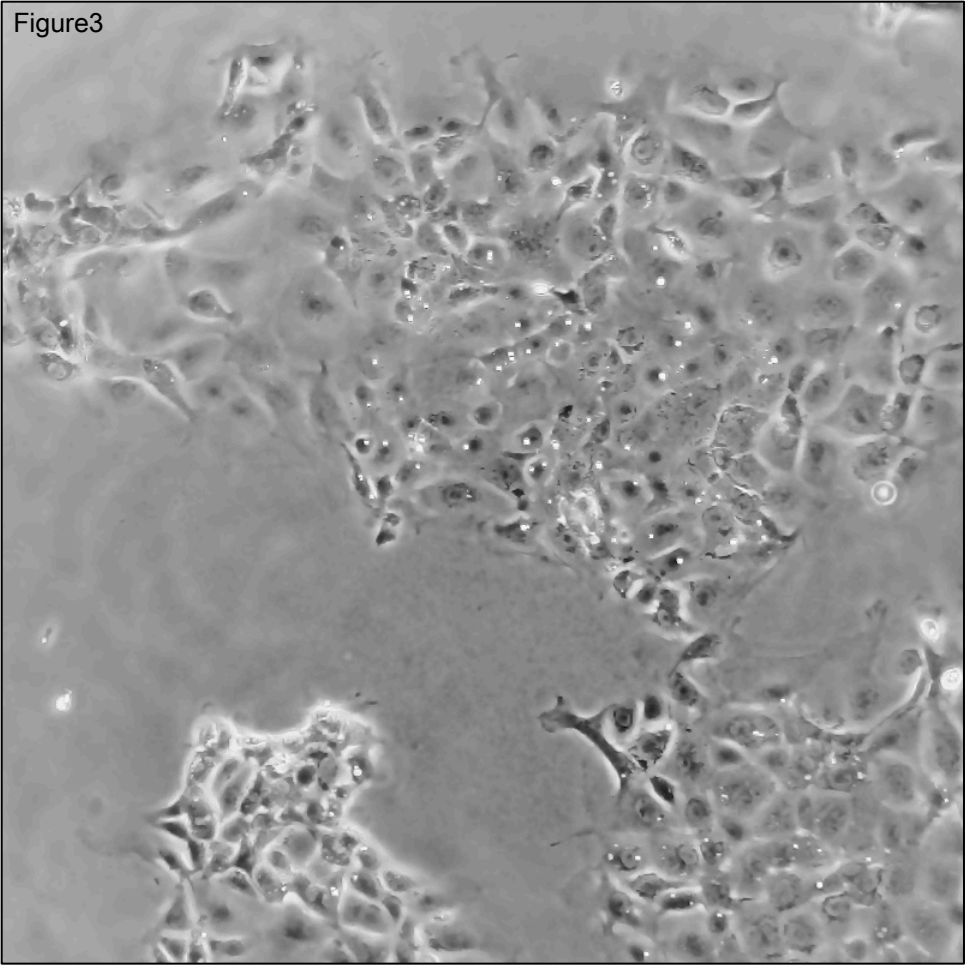
Figure2

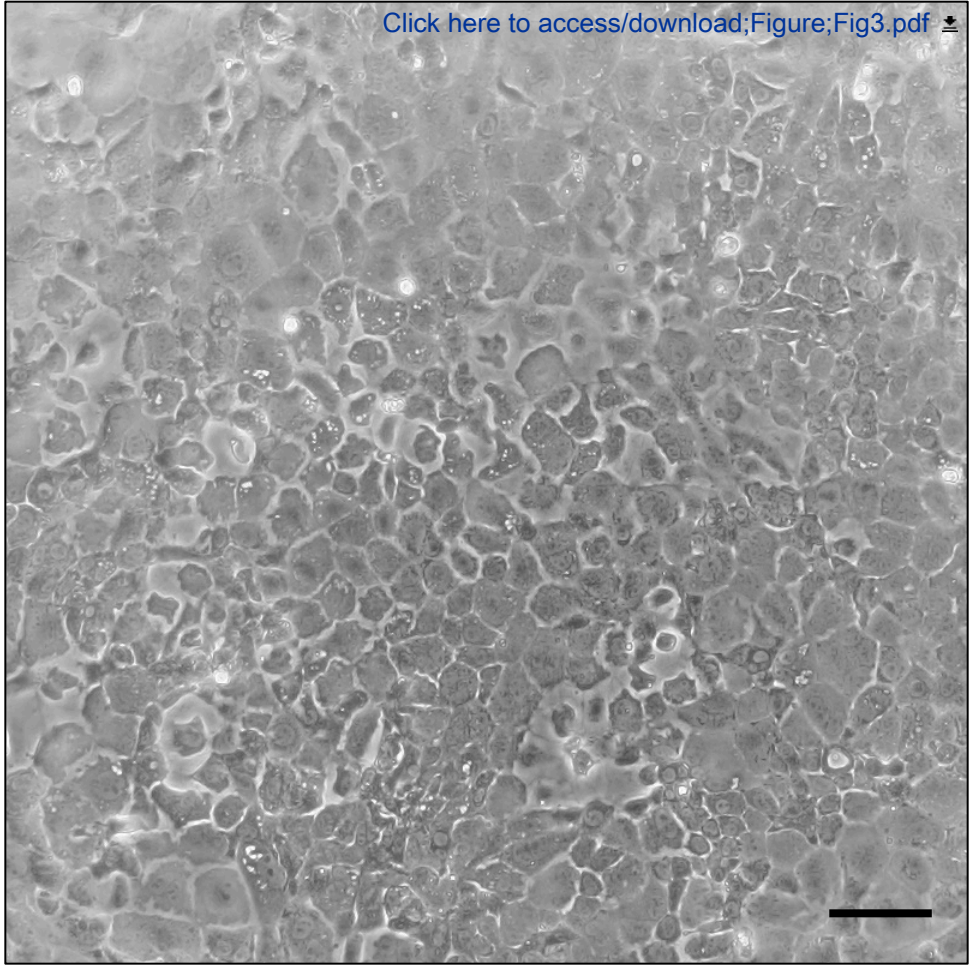


Initial preparation

After concentration

Figure3





24h after plating

48h after plating

Figure4

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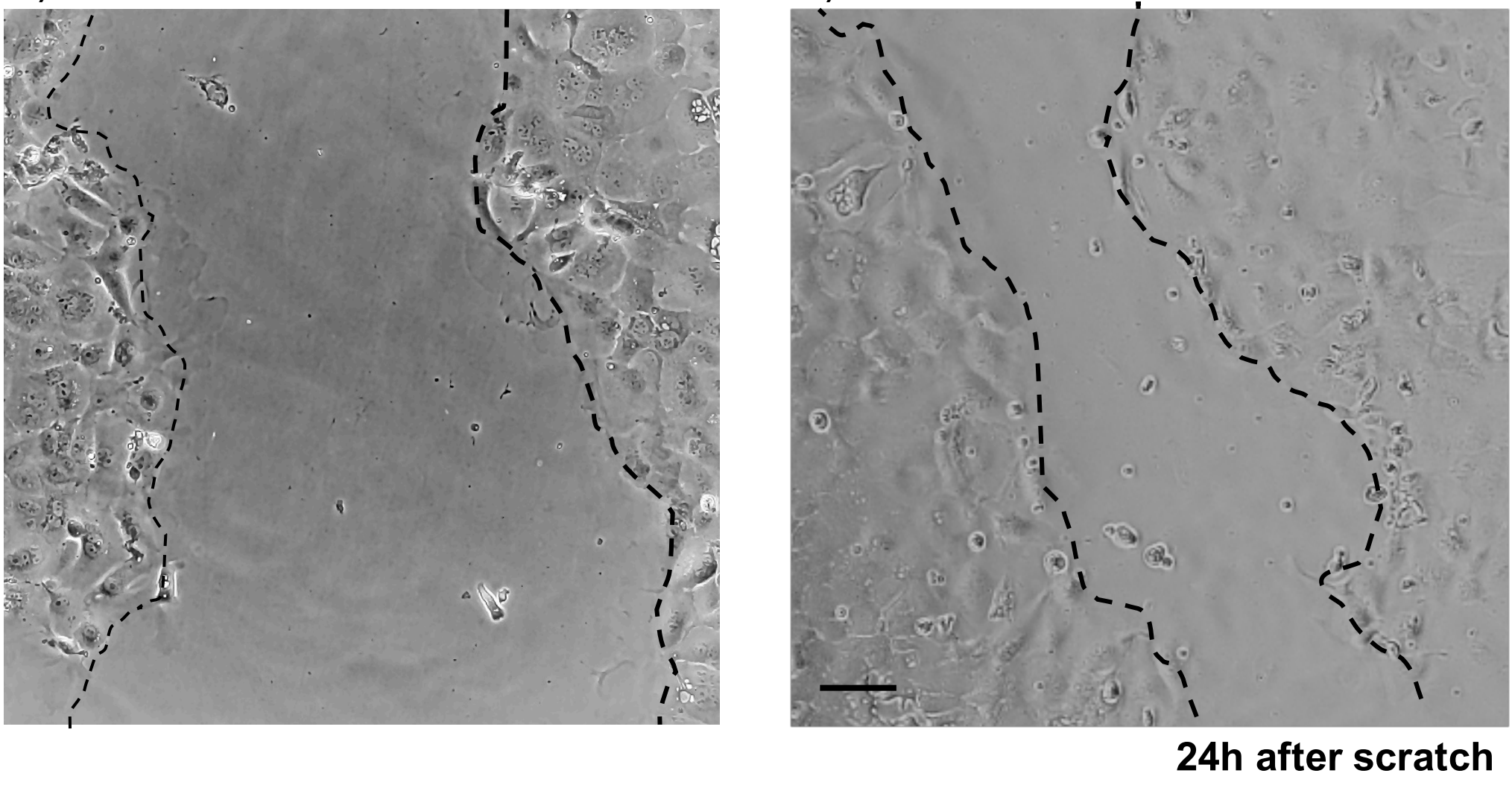
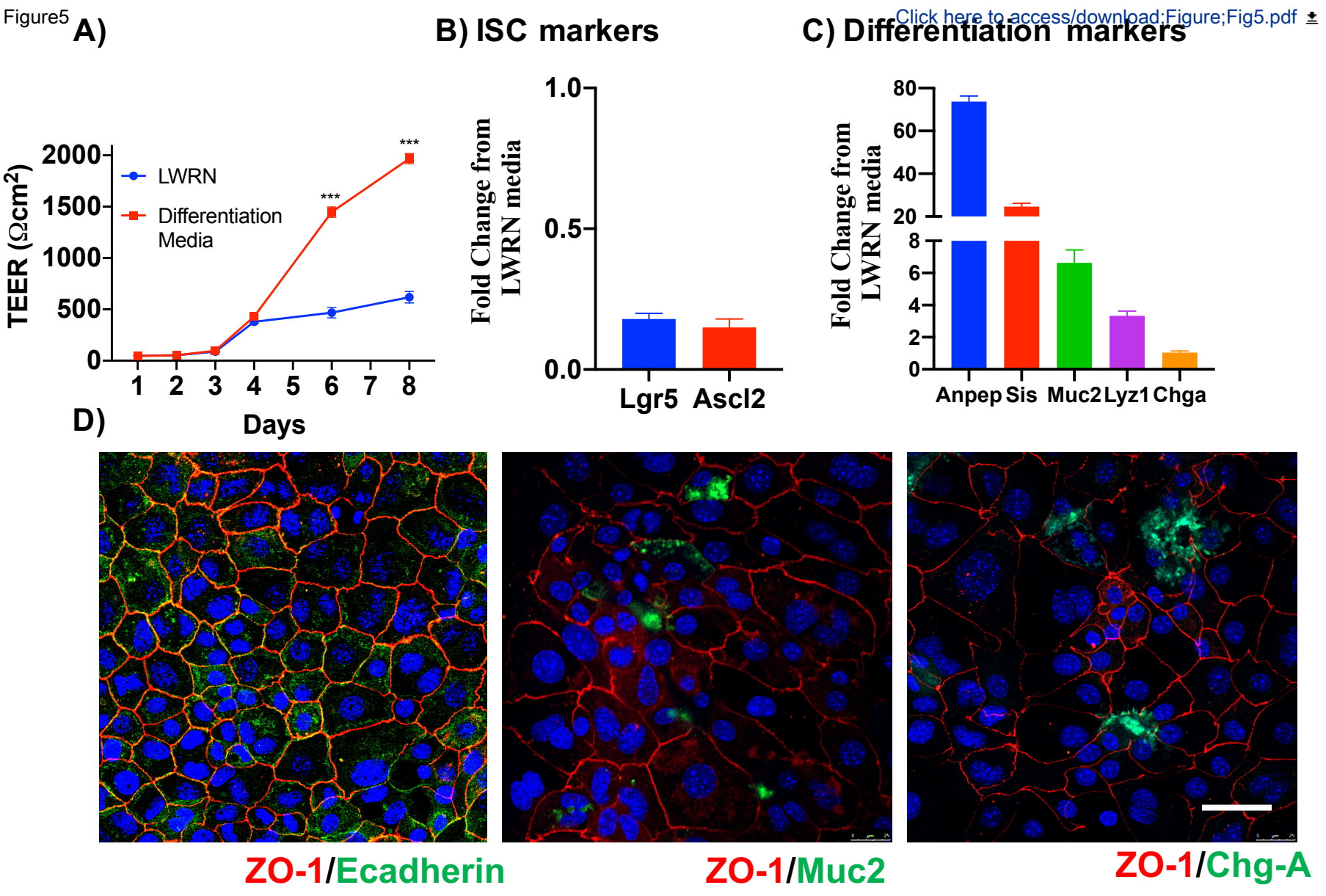


Figure5



Name of Material/ Equipment	Company	Catalog Number
Advanced DMEM/F12	Gibco	12634-010
Antibiotic Antimycotic solution	Corning	30-004CI
B27 supplement (50X)	Gibco	12587-010
Cell Recovery Solution	Corning	354253
Collagen from human placenta (type IV)	Sigma-Aldrich	C5533
D-Sorbitol	Sigma	85529-250G
D-Sucrose	Fisher Scientific	BP220-1
Dulbecco's phosphate buffered saline, with Ca^{2+} and Mg^{2+} (DPBS)	Corning	21-030-CV
Epithelial Volt/Ohm meter	World Precision Instruments	0-10KΩ with STX2
Ethylenediamine tetraacetic acid (EDTA)	Lonza	51201
Fetal Bovine Serum (FBS)	Corning	35-016-CV
Firefly Luciferase assay	Biotium	30085-2
Geneticin	Gibco	10131-035
GlutaMAX (100X)	Gibco	35050-061
HEPES (1M)	Corning	25060CI
Human recombinant EGF	R&D systems	236-EG
Human recombinant Wnt-3A	R&D systems	W3a-H-005
Hygromycin B	Invitrogen	10687010
LWRN cells	ATCC	CRL-3276
Molecular grade water	Corning	46-000-CV
N2 supplement (100X)	Gibco	17502-048
N-acetyl-L-cysteine	Sigma-Aldrich	A9165-5G
Noggin	Conditioned media	-
Nunc Lab-Tek Chamber slide system	Sigma-Aldrich	C7182-1PAK
Pencillin-Streptomycin (10,000U/mL)	Corning	30002CI
Phospahte buffered saline, Ca^{2+} and Mg^{2+} free (PBS)	Corning	21-040-CV
Plastic 20G feeding tube	Fisher Scientific	50-810-46
rh-laminin-521	Gibco	A29248
Roboz Surgical 4-0 Silk Black Braided 100YD	Fisher Scientific	NC9452680
TOPflash HEK293 cells	ATCC	CRL-3249
Transwell Permeable supports (0.4µm)	Corning	3470

Comments/Description

Stock Concentration: 500µg/mL

Stock Concentration: 500mM

Stock concentration: 100µg/mL

Reviewer Response

We would like to thank the reviewers for their insightful and constructive comments, and the editor for allowing us to address the concerns in this revised manuscript. A detailed point-by-point response to the referees' criticisms follows below.

All changes in the revised manuscript are highlighted in **blue color**. We hope that the revisions satisfactorily address the reviewers' concerns, and that the manuscript is now acceptable for publication in *Journal of Visualized Experiments*.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We carefully read the article to minimize potential spelling and grammar issues. All abbreviations are defined.

2. Please revise the title to "Generation of Murine Enteroid and Colonoid Monolayers from Intestinal Crypts".

To reflect the editors' advice and better describe the end goal of our protocol we decided to change the title to: "Generation of Murine Primary Colon Epithelial Monolayers from Intestinal Crypts".

3. Please provide an email address for each author.

An email address for each author is now provided.

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We removed all personal pronouns.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and

Reagents. For example: Matrigel; ATCC; Glutamax; Supplement Advanced DMEM/F12; permanox slide; cell recovery solution.

We added all the commercial products to the table of materials and reagents.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We revised the manuscript and changed the text accordingly.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We believe that we have written the protocol in enough detail to supplement the actions in the video and make it easy to de viewer/reader to replicate

8. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We highlighted 3 pages identifying the essential steps of the protocol for the video.

9. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Any limitations of the technique

b) The significance with respect to existing methods

Limitations and significance of the protocol are part of the discussion

10. Please do not abbreviate journal names in the reference list.

The reference list is automatically generated by the EndNote using the JoVE output style.

11. Please add labels A, B, C etc to the figure panels wherever needed.

We labeled figures accordingly.

12. Please sort the Materials Table alphabetically by the name of the material.

We did.

Reviewers' comments:

Reviewer #1:

"Title: Generation of Murine Enteroid and Colonoid Monolayers directly from Intestinal Crypts"

Manuscript summary:

This protocol for generating 2D enteroids/colonoids monolayers from Muraleedharan et al., is both timely and likely to have a significant impact on the field of gut/epithelial biology. Several reports in the literature claim to have created 2D cultures but fail to provide adequate experimental details to enable others to replicate their findings. The Quiros group herein provide a detailed protocol to establish 2D monolayers of primary mouse enteroids/colonoids that will provide a platform for many laboratories studying gut epithelial biology to benefit. They elegantly demonstrate the monolayer integrity and the more biologically relevant heterogeneous cellular composition of their 2D monolayers. Futhermore, they demonstrate that commonly used assays such as TEER and wound-healing assays can be used.

We appreciate the reviewer's thoughtful comments about our manuscript.

Major concerns:

-The clear advantage of this protocol is to rapidly establish 2D monolayers from fresh tissue, bypassing the need to culture in 3D prior to plating. However, many labs do not have access to live mice and obtain their colonoids/enteroids or receive transgenic colonoids/enteroids from collaborators. If possible, a short optional section for harvesting 3D cultures from Matrigel prior to plating would greatly enhance the impact of this protocol.

We appreciate this comment. We included and highlighted a reference to a protocol to generate 2D enteroids from 3D structures.

Minor concerns:

-Line 98. Is the PBS used here also without Ca2+/Mg2+?

Yes, throughout the protocol we use PBS without $Ca2^{+}/Mg^{2+}$, we have made this clear in the protocol (line 212)

-Line 100. I believe the "L" in L-WRN refers to the mouse fibroblast LMTK-cell line, originally used to create L-Wnt3a and later L-WRN, and not LGR5.

The reviewer is correct, we apologize about this mistake and we now changed to L-Wnt3A in the protocol (line 104)

-Line 106. No details or reference provided on TOPflash assay, cell line(s) transfected etc.

To provide more clarity details about the TOPflash assay were are added in the protocol line 110 and the catalog number for the kit was added to the table of reagents.

-Line 125. Does "500uL Noggin media" refer to L-WRN media, or recombinant Noggin, or conditioned media from a Noggin secreting cell line?

We apologize for the lack of clarity in this section. In this case is conditioned media from a Noggin secreting cell line. We clarified this in the text and provided a reference. (line 138)

-Line 130 indicates a 1:40 dilution of stock laminin but does not indicate an initial stock concentration of laminin. (Furthermore, line 118 includes instructions for preparation of Collagen stock, however, Laminin stock preparation is not included).

We use as stock concentration of laminin the concentration of the purchased product. We now clarify this the protocol. We also added the stock concentration to the table of reagents (line 129).

-Line 134 indicates pore size of the transwell. I assume these are 6.5mm sized inserts? Does the membrane type (polycarbonate/polyester etc.) affect the protocol?

Yes, the permeable supports used in this protocol are 6.5mm transwell with 0.4 µm pore. We have used both polyester and polycarbonate membranes inserts, and we see no difference in cell behavior. We included a note to this in the protocol discussion (line150).

-Line 157. How much inflation pressure is applied? This seems a bit vague. I assume the inflation helps to release crypts from the ECM?

We inflate the intestine until a turgent sausage is formed, we will add a line in the protocol describing this. Indeed, the inflation helps the crypts to be release from the ECM. (line 189)

-Line 158/159. During the 20-minute incubation, does the colon remain inflated twice for 5 minutes and remain deflated twice for 5 minutes, or is the colon deflated/reinflated once every 5 minutes and remain inflated during the 5-minute incubation? This is not clear.

The colon sausage is inflated and deflated once every 5 minutes and it remains inflated during the incubation. Details were added to the protocol (line191).

-Line 163. Is this achieved with manual/mechanical shaking? If mechanical, what type of machine is used and what setting? Is it performed vertically/horizontally/vortex adapter etc?

To release the crypts from the ECM, a manual shaking is performed vertically. We made sure this is clear in the protocol (line 198).

-Line 167. Using a hemocytometer?

A hemocytometer could be used but crypts are big enough to count them in a 20 ul drop of the solution containing the individual crypts.

-Line 172. Should the coated transwells/plates be allowed to dry out prior to use or does that affect adhesion?

The coated transwells are not completely dried out but, it is to be noted that the coating solution is aspirated from the plate or transwell while the cells are being centrifuged. Therefore, there is approximately 10 minutes of “drying” time.

-Table of materials: The LWRN cell line should be listed as available from ATCC (cat#: CRL-3276?) and if the Noggin is secreted by a cell line, the identity/source of this line should also be identified.

The details of the L-WRN cell line are now included in the table of reagents and the reference for the preparation of noggin media is cited in the protocol.

Reviewer #2:

The paper titled Generation of Murine Enteroid and Colonoid Monolayers directly from Intestinal Crypts by Miguel Quiros and the colleagues touches upon very important model of mouse gut primary epithelial monolayer that could be applicable in numerous experimental setting.

We value the recognition that this technique could be applicable in numerous experimental settings

Major:

Unfortunately, the technique is not novel. Similar techniques were described in great detail for both human and mouse intestinal epithelial tissues. Please, see below several representative references that were not acknowledge by the authors.

Kozuka K, He Y, Koo-McCoy S, Kumaraswamy P, Nie B, Shaw K, Chan P, Leadbetter M, He L, Lewis JG, Zhong Z, Charmot D, Balaa M, King AJ, Caldwell JS, Siegel M. Development and Characterization of a Human and Mouse Intestinal Epithelial Cell Monolayer Platform. Stem Cell Reports. 2017;9:1976-1990.

In J, Foulke-Abel J, Kovbasnjuk O. "Human Colonoid Monolayers to Study Interactions between Pathogens, Commensals, and Host Intestinal Epithelium". 2019 January. JoVE59357R1.

We apologize for not properly acknowledge previous protocols that describe generation of intestinal epithelial monolayers. Although we acknowledged this in the manuscript, we failed to include the references. We have now included a number of valuable manuscripts that describe different ways to generate primary intestinal epithelial cultures (line 76).

The novelty of our protocol is in the approach, we modified a crypt isolation technique that has not been published as a tool to generate a clean crypt preparation and described a combination of extracellular matrix proteins that permit the generation of confluent monolayers. Our protocol is easy to follow and reproduce and to our knowledge there is no other identical protocol published.

The authors pointed out that the major difference between their approach and published techniques that they use the mouse crypts directly instead creating the 3D cultures of mouse epithelium that usually are used to expand the organoid culture that could be propagated indefinitely. They stated that crypt-to-monolayer approach in less expensive compared to 3D approach. This statement is questionable. For the technique the author proposed, many mice

have to be sacrificed for each experiment. In contrast, establishment of 3D primary organoid cultures that can be cultured for years prevents unnecessary animal sacrifice. 3D cultures are in line with animal rights activists and are greatly supported by federally mandated Institutional Animal Care and Use Committee (IACUC) across the globe. The technology proposed by the authors leads to the unnecessary sacrifices of numerous animals.

We understand and support the reviewer concern about animal rights. Our protocol does not replace generation of 3D colonids, it is just a viable option for labs that do not work with organoids routinely or that need a couple of experiments in a primary cell model. Researchers could plan experiments in a way that one mouse is used for more than one experimental purpose, avoiding unnecessary sacrifices of numerous animals. Finally, establishment of 3D colonoids is more complicated than small intestine as the survival rate is low and passaging is limited compared to enteroids. Having this in consideration, plus the fact that a large number of 3D structures is necessary to generate a small area monolayer, we believe our technique does not require an excess of mice sacrifice when it comes to generate primary epithelial colon monolayers. This is not the case for small intestine so we will make this clear in our discussion (line 320).

Protocol lacks several crucial details:

1. Line 100: The qualities and quantities of growth factors are crucial for establishment of any primary cell cultures. What is the standard ration between Wnt3A, R-sponidn-1 and Noggin in L-WRN media that is the standard for the procedure? How many passages of L cells support this crucial ratio? How users should test for the quantity/quality of R-spondin and/or Noggin in the L cell produced media?

We use a commercially available cell line of L-fibroblasts transfected with a Wnt3A expressing vector and a R-spondin 3 and noggin co-expressing vector (ATCC CRL-3276). ATTC writes this about the cell line in their website: “The L-WRN cells were derived by transfecting L-Wnt3A (ATCC® CRL-2647) with an R-spondin 3 and noggin co-expressing vector and stable clones were selected in medium containing G418 and Hygromycin B. The L-WRN cells secrete the factors Wnt3A, R-spondin 3 and noggin into the medium. Wnt3A binds the frizzled receptor family and activate β-catenin-dependent transcription. Members of the r-spondin protein family are potent co-activators of canonical Wnt signaling in the intestine and are essential for isolation of small intestinal stem cells. Noggin, a bone morphogenetic protein (BMP) signaling inhibitor, enables the maintenance and passage of small intestinal organoids in vitro. Although these three factors are commercially available, it is costly to maintain the large-

scale cultures that are required for standard assays currently with immortalized cell lines. Using the conditioned medium from CRL-3276 provides relatively intact and high titer proteins compared to medium made with reconstituted proteins and is a cost-effective alternative. Note: Since the conditioned medium contains other factors besides Wnt3A, R-spondin 3 and noggin proteins, it is necessary to control any experiments involving the Wnt3A conditioned medium with control conditioned medium from the grand-parental cell line (ATCC® CRL-2648)". In order to determine the effectiveness of the conditioned media, we measured Wnt activity as described in the protocol. We follow the Michigan Medicine Translational Tissue Modeling Laboratory protocols (https://www.umichttml.org/protocols)

2. Line 116: The authors propose to use 2X antibiotic antimycotic solution.
What is this antibiotic? Is it different for this used for 3D human and mouse cultures?
If it is different, what is the advantage?

It is a common practice when culturing primary cells to use 2X antibiotic solutions, especially when working with a tissue rich in microbiota such as the colon. This practice decreases the chance of having problems of contamination.

3. Line 6: Collagen: What collagen? This is crucial information, because not each type of collagen supports the confluency of mouse and/or human monolayers.

This information is included in the table of materials and reagents.

4. Line 125: 500 µl Noggin media… How Noggin media is obtained? What is Noggin concentration in this media? What is a quality control?

The reference for the preparation of noggin media is cited in the protocol. This Noggin conditioned medium contains an equivalent of 1 µg/ml of Noggin (line 138).

5. Differences in coating between plates and traswells - does it affect the physiology?

Because transwells provide a matrix that is less stiff than plastic tissue culture plates, cells usually attach easier to transwells. We include laminin in our plastic tissue culture wells to facilitate cell-matrix adhesion.

6. This method of crypt isolation is not novel - please, include the original references.

Our crypt isolation method is indeed novel. We have modified a protocol to separate intestinal epithelium from mesenchyme ([1]) and adjusted it in a way that we can obtain very clean

and pure crypt preps. The original paper that we adapted was cited from the beginning.

7. How many monolayers could be obtained from one gut segment preparation (48 well plate/ confluency at 24-48h) vs. same Transwell (confluency at 3-4 days)?

One mouse colon can yield a confluent area of 75cm^2 2 to 3 days after plating, this is independent of plastic or transwells. We included this in our discussion (line 328).

8. Figure 5A: it would be helpful to show the TER separately for confluent undifferentiated monolayers vs. confluent differentiated.

We are now showing graphed TEER values of undifferentiated (LWRN) vs differentiated (differentiation media).

9. For how long the monolayers are stable in undifferentiated vs. differentiated state?

The stability of the monolayers depends more on the surface they are plated. Monolayers plated in plastic will be healthy for 4 to 5 days while monolayers plated in transwells can be carried up to 8 days. We added a line explaining this in the discussion (line 309).

Minor:

Line 251: "must" instead of "most"

We corrected this typo.

Reviewer #3:

Manuscript Summary:

In this manuscript, Muraleedharan and colleagues present a new, detailed method to create monolayer small intestine or colon primary cell cultures. The technique is broadly applicable and will be of interest to the field which has yet to settle on a single methodology for intestinal organoid monolayers.

We appreciate the reviewer positive comment

Major Concerns:

None

Minor Concerns:

1. Step 7 - nomenclature is confusing, as the media is not growth factor free since one will be spiking in Noggin and EGF and only L-WRN conditioned media is excluded.

We apologize for any confusion in the way this step was written. This paragraph describes complete media without growth factors and differentiation media, potentially creating a confusion about growth factors being present in the complete media without growth factors. To avoid this confusion, we created a new section for complete media without growth factors before differentiation media (line 134).

2. Lines 76-77 "Several papers describe how to generate 2D monolayers..." Please cite these papers so readers can compare and contrast those protocols with this approach.

We now cite different protocols to generate 2D monolayers so the reader can contrast and compare (line 76).

3. Can the authors explain why their chelation/distension method is cleaner than opening & chelating intestine (e.g. the protocol standardization published here: Am J Physiol Gastrointest Liver Physiol 305: G542-G551, 2013)? Seems like less tissue handling as compared to the eversion, tying, and inflating sausage approach.

This method is superior because it yields a clean prep. Classical methods require cutting of tissue and their shaking step needed to be in a very specific speed and force to avoid debris. Even being very careful, crypts derived from these methods always contained undesirable cells and debris that affected the quality of the culture. As shown in figure 2, our protocol generates crypt preps with little to no contamination of other cells or debris.

4. Can the authors explain the different concentrations of EDTA for small intestine versus colon?

The reviewer raises an interesting point that we questioned in the past, and it's the fact that colon requires way higher concentrations of EDTA in order to release individual crypts. Unfortunately, we do not have a definitive answer. We hypothesize that absorptive capacity changes between the small and large intestine create differences in cell-cell and cell-matrix adhesions that might be responsible for disparities in their sensitivity to EDTA.

Reviewer #4:

Manuscript Summary:

In this paper, Muraleedharan et al. described how to generate murine enteroids and colonoid monolayers (2D) directly from intestinal crypts. This paper has immense importance in the gut physiology field. But the paper has some drawbacks that need to be addressed.

We appreciate and value the reviewer's comments.

Major Concerns:

1) Why is their method superior to the methods where a 2D layer is prepared from 3D-organoids? They just stated the method is laborious but still, the culture of 3D organoids and the preparation of 2D layer is cost-effective and the same sample can be used for repeat experiments.

The establishment of 3D colonoids is more complicated than small intestine as the survival rate is low and passaging is limited compared to enteroids. Monolayer generation from 3D colonoids require a disproportionate amount of 3D structures, that make these kinds of experiments time consuming and costly. On the contrary, direct epithelial colon monolayer prep is fast and is a quick way to obtain the results. One colon prep can generate a confluent area of 75cm^2 (10 to 15 mL of conditioned media, replaces once) 2 to 3 days after plating (this area would require 144 wells of 3D colonoids, which means almost 6mL of Matrigel and more than 250 mL of conditioned media). The lower consumption of media, low-cost maintenance of the cell culture and ability to perform functional tests-easy and fast downstream processing are big advantages of epithelial colon monolayers. We included this information as part of the discussion (line 321).

2) In Fig 5, they have measured the TEER values, but they need to continue the measurement to see how many days it takes to reach the plateau and when the values drop off

This is a good observation, but it goes beyond the scope of our paper. Our representative results show the feasibility of the technique and then the readers and viewers can create their own experimental settings.

3) Is there any difference between the age and gender of the mice in the TEER and the quality of the 2D monolayer

We did not notice any gender difference in monolayer quality. When it comes to age, we have used mice up to 20 weeks old and have always been able to generate healthy

monolayers. We cannot assure older mice would be as successful. We included a comment about this important point in our discussion (line 287).

4) In Fig 5, what's the status of the differentiation marker CDX2 and KRT20? Why there is no error bar in the qRT-PCR data?

This is a very good question. Unfortunately, we did not use these markers in our panel, but I will add this to the protocol as options for the users and will keep it in mind for the future. We apologize for our mistake in the expression graft, it has now been corrected.

Minor Concerns:

5) In Fig 1, the steps and the details need to be further elaborated. For example, how to remove dead cells generated in the procedures. What precautions need to be taken.

We appreciate this comment, we will keep this in mind for the video part of the protocol.

6) They need to compare the cell death probably using the LDH-assay

It is not clear in which step is the reviewer suggesting measuring cell death and to what should we compare.

References

1 Nik, A. M. & Carlsson, P. Separation of intact intestinal epithelium from mesenchyme. *Biotechniques.* **55** (1), 42-44, doi:10.2144/000114055, (2013).