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Modeling Intestinal Development and Injury-Repair In Vitro using Mouse Colonic Stem Cell Monolayer Culture under Air-Liquid Interface

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February 4, 2020

Kyle Jewhurst, Ph.D.
Science Editor, JoVE

Dear Dr. Jewhurst,

Thank you for the invitiation to author a protocol for consideration at JoVE. We are please to submit our original research article entitled "**Modeling Intestinal Development and Injury-Repair In Vitro using Mouse Colonic Stem Cell Monolayer Culture under Air-Liquid Interface**" to be considered for publication at JoVE.

Intestinal epithelial stem cells are an important model of stem cell biology and cellular renewal during injury and repair. Existing 3D organoid systems are powerful tools to study intestinal development but are limited in the study of injury-repair because they provide only a snapshot of a specific cellular state. In this protocol we present an approach to generate long-lived, self-organizing 2D monolayer cultures of mouse colonic epithelium that contains all epithelial cell types. We believe that this method will be of broad interest to your readers and accelerate future mechanistic studies of intestinal development, repair, and interactions with microbes and other cell types.

Based on our previously published and widely reproduced methods, we first demonstrate how to collect and expand mouse colonic stem cells. Next, we present a new detailed protocol to collect colonic stem cells and seed them on Transwell membranes as epithelial monolayers. When the monolayer is exposed to an Air-liquid interface, the cells undergo a rapid proliferative burst followed by cytodifferentiation of all colonic cell types including goblet, enteroendocrine, and absorptive cells. The monolayer self-organizes to resemble flattened colonic crypts with foci of proliferative stem cells interspersed amongst differentiated, non-proliferating cells. Importantly, this self-renewing monolayer is culturable for at least four weeks, which enables studies of long-term development as well as host-microbe and epithelial-mesenchymal interactions. Another key advantage of this system is the ability to model injury-repair cycles by resubmerging the monolayer after ALI. This leads to numerous cellular and functional changes so that the monolayer resembles an atrophic crypt, which is the pathological feature of intestinal damage. After re-establishing ALI, the monolayer returns to a homeostatic state, modeling the cycle of colonic injury and repair. We believe that this protocol will be an important tool for mechanistic studies of cellular renewal and translational models of human intestinal disease.

Sincerely,

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TITLE:

Modeling Intestinal Development and Injury-Repair In Vitro Using Mouse Colonic Stem Cell Monolayer Culture Under Air-Liquid Interface

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KEYWORDS:

intestinal stem cells, organoid, air-liquid interface, developmental biology, injury, repair

SUMMARY:

This protocol describes the generation of a long-lived self-renewing monolayer culture system for mouse colonic stem cells that contains all major epithelial cell types. This culture system can be used to study epithelial biology, intestinal wound repair, and host-pathogen interactions.

ABSTRACT:

Intestinal organoid culture is a powerful tool to model stem and epithelial cell biology. Here we present a protocol to generate long-lived two-dimensional monolayers of all major intestinal epithelial cell types using primary mouse colon stem cells grown under air-liquid interface. An advantage of this protocol over conventional 3D organoid culture is that the monolayer is self-renewing for at least four weeks without passaging, allowing long-term studies of intestinal development and response to injury or challenge. Mouse colonic stem cells are first expanded in a conditioned medium containing Wnt, R-spondin, and Noggin. The stem cells are then seeded on a semi-permeable membrane to form a continuous monolayer. After seven days of submerged cell growth, the monolayer is exposed to an Air-liquid interface (ALI) by removing conditioned media from the apical compartment. This results in epithelial differentiation and formation of numerous self-organizing proliferative foci that resemble “flattened” colonic crypts. Stem cells and differentiated lineages co-exist in this monolayer for at least four weeks. We

further demonstrate the ability to model injury-repair cycles by re-submerging the cells under conditioned media, which leads to a loss of differentiated cells while sustaining the regenerative stem cells. The differentiating monolayer can then be re-established by resuming the Air-liquid interface. In this protocol, we additionally present methods for histological analysis including paraffin embedding and whole mount imaging. This monolayer system can be adapted to study many aspects of long-term intestinal development, including stem cell dynamics, host-pathogen interactions, and metabolism.

INTRODUCTION:

The intestinal epithelium is a self-renewing barrier with high regional and cellular diversity. An ideal in vitro model of this barrier would be long-lived, composed of all relevant epithelial cell types, and model cycles of homeostasis perturbation and restoration. In this protocol, we present a method to generate a two-dimensional monolayer culture from mouse colonic stem cell spheroids that allows the study of colonic injury, repair, and infection dynamics.

In vitro 3D intestinal organoid culture methods have been widely and powerfully used to study stem cell biology and intestinal differentiation[1,2]. Multiple groups have adapted protocols to stem cells derived from multiple regions of the intestinal tract as well as embryonic or induced pluripotent stem cells[3,4]. Despite their immense power, there are several drawbacks to existing organoid culture methods. They are relatively short-lived, requiring passage every 5 to 7 days. The culture conditions used to induce differentiation leads to the loss of proliferative stem cells. Finally, organoids embedded in a three- dimensional extracellular matrix require microinjection techniques to introduce, for instance, bacterial pathogens[5,6].

Two-dimensional monolayer cultures of immortalized intestinal cell lines have long been used to model simple epithelial repair and study absorption process[7,8]. However, these transformed cell lines can't fully recapitulate homeostasis and normal cellular differentiation of all epithelial lineages. Primary stem cells under an Air-liquid interface (ALI) have been reported in other tissue types including skin, respiratory tract, and pancreas[9–13]. Some progress has been reported growing intestinal monolayer cultures under ALI, but these models are short-lived and often discontinuous[14–17].

Here we present a protocol that addresses the gap in intestinal culture by generating long-term 2D cultures of mouse colonic stem cells under an ALI. We demonstrate the use of this system to model a proliferative injury-repair cycle by resubmersion of the cells. This protocol is based on our previous reports of development, infection, and repair[18,19]. The two-dimensional culture system will be generally useful for studies of long-term adaptation of the epithelium to environmental factors, such as aerotolerant microbes or oxygen tension. Spheroid stem cells can be grown from other regions of the gut and from other species including humans[20–22], and we have preliminarily been able to generate ALI monolayers from these other sources with minor modifications to the protocol. It will also be an ideal platform to study more complex mixtures of cell types from different tissue compartments.

We have validated this protocol using conditioned media prepared from L-cells expressing Wnt,

R-spondin, and Noggin (ATCC # CRL-3276)[23, 24]. Our laboratory has previously published a detailed protocol describing the generation of this conditioned media[20]. Multiple independent laboratories have used this protocol to generate this media for the growth of intestinal stem cells[22,25,26]. Before establishing colonic spheroid cultures, protocol users should generate a batch of the conditioned media (hereafter referred as 50% L-WRN CM). The 50% L-WRN CM can be frozen at -20 °C for long-term use.

PROTOCOL:

All animal experiments described in the manuscript were approved by the Washington University School of Medicine Animal Studies Committee. All centrifugation steps can be performed at room temperature.

1. Establish and expand 3D colonic spheroid culture according to Miyoshi et al[20].

NOTE: Refer to **Table 1** for media recipes.

1.1. Establish a 3D colonic spheroid culture

1.1.1. Briefly, dissect out a 1 cm segment of colonic tissue from an 8-10-week-old C57/BL6J mouse. Remove any fat or connective tissues with fine scissors.

NOTE: Spheroid lines are typically generated from male mice in this age range, but lines can be established from other genetic backgrounds, ages, or genders with identical procedures.

1.1.2. Flush the lumen with ice cold PBS using a 10 mL syringe fitted with a blunt 19 G needle, then open the colonic segment longitudinally with scissors.

1.1.3. Transfer the tissue to a 90 mm Petri dish containing ice-cold PBS and swirl the tissue to rinse.

1.1.4. Transfer the tissue fragment to a 50 mL conical containing ice-cold PBS. Wash by vigorous shaking.

1.1.5. Move the tissue fragment to a 40 mm Petri dish and transfer into the tissue culture hood. Mince the tissue with sterilized scissors in the Petri dish until the tissue can be easily pipetted with a P1000. The pieces at this point will be <1 mm^2. Add 1 mL of collagenase solution to the minced tissue and mix the tissue homogenate by gentle pipetting.

1.1.6. Transfer the tissue homogenate to a 15 mL conical flask. Digest at 37 °C for 20-40 min. Pipette 20x every 5-10 min until crypts fall out into the solution readily.

NOTE: Colonic tissue typically requires 30-40 min of total digestion. Adequacy of digestion can be assessed using a phase or dissection microscope. Digestion is complete when 50-80% of single epithelial units (crypts or pits) are free from the larger colonic tissue fragments.

1.1.7. Filter the crypt suspension through a 70 µm strainer into a new 15 mL conical. Wash the strainer with 9 mL of Washing Media.

1.1.8. Centrifuge the filtered solution for 5 min at 100 *x g*. Aspirate the supernatant, leaving about 200 µL of solution around the loose cell pellet. Resuspend the cell pellet in 10 mL of washing media and repeat the centrifugation (second wash).

1.1.9. Aspirate all but 200 µL of solution. Resuspend the cell pellet in 1 mL of washing media. Transfer the solution to a 1.5 mL tube. Centrifuge 5 min at 350 *x g*.

1.1.10. Remove as much of the supernatant as possible with a pipette without disturbing the cell pellet.

1.1.11. On ice, re-suspend the crypt pellet with 15 µL of extracellular matrix (see **Table of Materials**) per planned well of a 24 well plate. Typically, there will be enough crypts for 2-6 wells of a 24 well plate. Dispense one 15 µL droplet of the extracellular matrix mix to the center of each well of a 24 well plate.

NOTE: Approximately 1,000 to 3,000 epithelial units can be dispersed to each well. This can be estimated by evaluating a droplet of the solution with a phase or dissection microscope. Empirically, if starting with 1 cm of mouse colon, the user could resuspend the crypt pellet in 60 µL of extracellular matrix to seed 4 wells and adjust future experiments based on yield.

1.1.12. Carefully invert the 24-well plate and incubate at 37 °C for 10 min for the extracellular matrix to solidify.

1.1.13. Return the plate to the tissue culture hood and re-invert. Add 500 µL of 50% L-WRN CM supplemented with 10 µM Y-27632 to each well.

NOTE: Y-27632 is an inhibitor of Rho-associated protein kinase (ROCK). ROCK inhibition helps to prevent anoikis in isolated epithelial cells. For mouse culture, supplement 50% L-WRN CM with Y-27632 when establishing stem cell lines and when spheroids are dissociated to single cell suspension (step 2.12–2.15). Y-27632 is not required for media change or passaging of established murine spheroids (step 1.2) or for media change of ALI monolayers (step 2.16).

1.1.11. Culture the spheroids at 37 °C in a tissue culture incubator with 5% CO_2 supplementation for 3 days.

NOTE: Stem cells from different regions of the intestine or different animals may require additional co-factors. Consult relevant literature if using tissue other than mouse colon[21]. For instance, mouse small intestine stem cell spheroids do not need additional factors while human spheroids require a TGF-beta inhibitor.

1.2. Passage and expand 3D colonic spheroid culture

NOTE: Place an aliquot of 1x Trypsin in a 37 °C water bath to warm before beginning to collect the spheroids. All steps are performed at room temperature unless otherwise noted.

1.2.1. After 3 days of culture, remove the 50% L-WRN CM by aspiration.

1.2.2. Add ~ 0.5 mL of 0.5 mM EDTA (PBS-EDTA) to each well. Scratch the colonic spheroid-containing extracellular matrix bubble in each well with a pipette tip to re-suspend in the PBS-EDTA. Collect the suspension from each well into a 15 mL conical.

1.2.3. Wash the collected spheroids by centrifuging at 350 *x g* for 5 min.

1.2.4. Aspirate the supernatant but don't disturb the loose cell pellet. Add 300 µL of prewarmed 1x Trypsin to the cell pellet and mix by pipetting 1-2x.

1.2.5. Place the tube in the 37 °C water bath and incubate for 1.5 to 2 min. Pipette the mixture ~ 20x to complete the dissociation process.

1.2.6. Add 5 mL of washing medium to the dissociated spheroid fragments to quench the trypsin. Wash by centrifugation at 350 *x g* for 5 min.

1.2.7. Aspirate the washing media, leaving 200 µL of solution. Add 1 mL of washing media, resuspend by pipetting, and transfer the mixture to a 1.5 mL tube. Wash by centrifuging the 1.5 mL tube at 350 *x g* for 5 min.

1.2.8. Remove as much supernatant as possible with a pipette. Determine how many wells of a 24 well plate will be seeded based on the desired passage ratio and resuspend the cell pellet in 15 µL extracellular matrix per well on ice. Thoroughly mix by gentle pipetting but avoid introducing air bubbles.

NOTE: Typical passage ratios for mouse colon are 1:5 to 1:20. For example, if stem cells are collected from 4 wells of a 24 well plate in step 1.2.1 and a 1:10 passage ratio is desired, 600 µL of extracellular matrix would be added (4 wells x 1:10 passage ratio x 15 µL extracellular matrix per well). The ratio needs to be empirically adjusted for each researcher, depending on adequacy of trypsinization, pipetting losses, and other factors. New users should start with a ratio closer to 1:5 and increase dilution as skills increase. See also previous publications on spheroid culture for discussion and details[20,21].

1.2.9. Dispense 15 µL of the extracellular matrix suspension into 24-well plates as described in 1.1.8 and 1.1.9.

1.2.10. After extracellular matrix has hardened, reinvert the plate and add 400 µL of 50% L-WRN CM containing 10 µM Rock inhibitor Y-27632 to each well.

1.2.11. Culture the spheroids for 3 days at 37 °C. Change media on day 2.

NOTE: To seed 12 individual cell culture membrane inserts for 2D ALI culture, spheroids from approximately two 24-well plates are needed. The exact number of wells of spheroids required for seeding one plate depends on the density of spheroids plated and the efficiency of cell recovery from trypsinization described in step 2 and needs to be empirically determined by each individual researcher who performs the experiments. One might need to repeat the passage and expansion steps more than once to generate enough wells.

2. Seed 2D ALI monolayer culture

NOTE: Spheroids of Passage 3 to Passage 20 are typically used for seeding ALI culture.

2.1. To seed one plate (containing 12 x 6.5 mm diameter cell culture membrane inserts), dilute 120 µL extracellular matrix with 1,080 µL PBS to make 10% extracellular matrix solution. Keep the solution on ice until use.

NOTE: If using a membrane insert product other than that listed in the **Table of Materials**, be sure to select a product with transparent or clear membranes if microscopic imaging needs to be performed at later steps.

2.2. Add 100 µL of 10% extracellular matrix solution onto the membrane in each insert on top of the membrane. Dispense solution gently to avoid damaging the membrane. Incubate at 37 °C for 20-30 min to pre-coat. After incubation, aspirate all solution off the membrane.

2.3. Collect spheroids from two 24-well plates containing day 3 colonic spheroids. First, aspirate the 50% L-WRN CM from all wells of the plates.

2.4. Add 500 µL of PBS-EDTA to each well and scratch the extracellular matrix containing spheroids off the well. Transfer to 15 mL tubes. Collect up to 6 wells of extracellular matrix into one 15 mL tube.

2.5. Wash the collected spheroids by centrifugation at 350 *x g* for 5 min.

2.6. Aspirate the supernatant from each conical tube. Add 500 µL of 1x Trypsin (pre-warmed in 37 °C water bath) to each 15 mL tube. Pipette 5x to dislodge the pellet.

2.7. Incubate in 37 °C water bath for 3 min.

2.8. Pipette the contents of each tube vigorously (recommend 70x) to create a single-cell suspension. Filter the cell suspension through a 40 µm strainer and collect the flow through in a clean 50 mL conical. Use one strainer and 50 mL collection tube for up to two 15 mL conical tubes of cellular mixture.

2.9. Add 5 mL of washing media into each original 15 mL tube to rinse any remaining cells, then use this mixture to rinse the strainers. Collect the flow through in the 50 mL conical tube.

2.10. Transfer the filtrate from each 50 mL conical tube into a clean 15 mL tube. Centrifuge at 350 *x g* for 5 min.

2.11. Aspirate the supernatant. Wash the pellet again by adding 10 mL washing media to the pellet, resuspending by several pipettes, and centrifuging at 350 *x g* for 5 min.

NOTE: A solid cell pellet without extracellular matrix should be readily visible in the 15 mL tube after centrifugation. If the pellet appears to be semi-transparent, trypsin digestion was incomplete. The optimal amount of time for trypsinization can vary based on handling conditions and should be determined empirically by individual researchers.

2.12. Dislodge the pellet with 1 mL of 50% L-WRN CM supplemented with 10 µM Y-27632. If there is more than one tube of cell pellets, transfer the suspension from the first tube onto the cell pellet in the second tube and mix by pipetting. Repeat the transfer and suspension until all the cell pellets are resuspended together in ~ 1 mL of 50% L-WRN CM. Mix well by pipetting 10x.

2.13. Determine the number of cells collected using a manual or automated cell counter.

NOTE: On the cell counter, cells should be a mix of single cells or small clusters (less than 10 cells) with minimal cell death. If the cells at this step are not sufficiently dissociated, increase the trypsinization time in step 2.7 by 30 s and or increase the number of pipettes in step 2.8. Conversely, if there is a large amount of cell death, decrease the number of pipettes in step 2.8 by 10 and make sure that all steps are performed as quickly as possible. Trypan blue can be used to determine the extent of cell death. If many cells are dead or large clusters of cells remain, seeding will be inefficient.

2.14. Transfer 2.4 x 10^6 stem cells to a new 15 mL tube and bring the volume up to 1.8 mL by adding 50% L-WRN CM supplemented with 10 µM Y-27632. Mix well by pipetting 5-10x.

NOTE: Typical yields from 2 full 24 well plates of colonic spheroids are 2 to 4 million cells.

2.15. Dispense 150 µL of cell suspension into each insert (2 x 10^5 cells per insert, 12 cell culture membrane inserts per plate). Add 350 µL 50% L-WRN CM supplemented with 10 µM Y-27632 outside of the insert to each well (bottom of well) (**Figure 1**).

2.16. Incubate at 37 °C with 5% CO_2. Change media both inside and outside of the insert every 2-3 days with 50% L-WRN (150 µL inside the insert, 350 µL outside the insert).

NOTE: 10 µM Y-27632 is not required for media changes.

2.17. On day 7 after seeding, carefully remove the 150 µL media from the inside of the insert to create Air-liquid interface (ALI) for the epithelial monolayer. This day is counted as ALI day 0 (**Figure 1**).

2.18. Keep changing media outside of the insert every 2-3 days. The ALI culture can be maintained for up to 1 month. Evenly distribute across the surface the light brown apical mucus layer which will become apparent around ALI day 7.

3. Whole mount staining for Ki67 and UEA1 on ALI monolayer culture

3.1. On ALI day 21, fix the culture by removing the 50% L-WRN media. Apply 4% paraformaldehyde (PFA) both inside (100 µL) and outside (300 µL) of the insert. Incubate at room temperature for 30 min or at 4 °C for overnight.

3.2. Remove PFA and discard according to local regulations. Wash with PBS 3x by applying 100 µL inside and 300 µL outside the insert. Soak the insert with PBS at 4 °C overnight.

NOTE: PBS soaking helps to hydrate and soften the mucus layer on top of the epithelial monolayer. Membrane inserts can be left in PBS at 4 °C for up to two weeks before proceeding to subsequent steps.

3.3. Gently remove the PBS from the inside of the insert by pipetting to lift the mucus layer. Remove the PBS from the bottom of the insert. Wash once more with PBS applied to the inside and outside of the insert, followed by immediate aspiration.

3.4. Remove the insert from the well and place on a cutting board. Carefully cut the membrane out of the plastic insert frame using a #11 surgical blade.

3.5. Transfer each cut membrane (with cell side facing upwards) into a separate well of a 24 well plate containing 200 µL of blocking buffer (PBS with 1% BSA and 0.1% Triton-X). Incubate at room temperature for 1 h to block the cells.

3.6. Dilute the Ki67-FITC antibody 1:200 or UEA1-Rhodamine 1:500 in blocking buffer.

3.7. Remove the blocking buffer and add 200 µL diluted primary antibody solution to each sample. Incubate at 4 °C overnight.

3.8. Wash the membrane with 500 µL of PBS for 5 min with gentle rocking. Repeat this wash two more times.

NOTE: If using un-conjugated primary antibodies, remove the blocking buffer and perform a one-hour incubation with secondary antibody at room temperature, followed by three PBS washes.

3.9. Aspirate PBS and incubate in 200 µL of diluted Hoechst solution (1:5,000 in PBS) at room

temperature for 10 min to stain nuclei. Wash once with PBS.

3.10. Carefully transfer the membrane to a glass slide using fine tweezers with the cell side facing upwards. Add a droplet of mounting media and cover the membrane with a coverslip. Gently press the coverslip and clean any excessive mounting media outside of the coverslip.

3.11. Use an inverted confocal microscope to take whole mount images (**Figure 2**). Place the slide with the cell side facing the lens. Find the focal plane for the cells by using a 10x lens. Then use a 40x or 60x lens (use water or oil as needed) to capture images at the desired fluorescent channels. Z stacks of 20-30 µm is recommended to capture the entire span of cell height.

4. Agar embedding of ALI culture for paraffin blocks

4.1. Perform the fixation, PBS wash and membrane cutting steps as described in 3.1 to 3.4.

4.2. Transfer each cut membrane to an individual well of a 24 well plate. Add 1 mL of 70% ethanol to each sample. Incubate overnight at 4 °C.

NOTE: Samples can be stored in 70% ethanol for up to one week at 4 °C.

4.3. On the day of agar embedding, prepare 2% agar solution by microwaving 1 g of agar powder in 50 mL of distilled water for 1 min or until agar dissolves completely. Keep the agar solution warm in a 60 °C water bath.

4.4. Remove the cut membrane from the ethanol using fine tweezers and lay flat on a colored cutting board with cell side facing up.

4.5. Use a transfer pipette to cover the top of the membrane with warm 2% agar from the solution kept in 60 °C water bath ("Agar 1" in **Figure 3B**). Wait until the agar solidifies.

4.6. Use a razor blade to trim the agar droplet making a square shape with the membrane at the center of the square.

4.7. Cut the agar square with the embedded membrane in the midline to make two halves. Use forceps to make each half stand vertically with the cut / midline edge down on the cutting board surface. Then align the two pieces parallel and next to each other with the apical side of all membrane sections pointing in the same direction (**Figure 3B**).

4.8. Place several drops of 2% agar on top and around the two vertically standing halves and wait until it solidifies. This results in a bigger agar block for sectioning ("Agar 2" in **Figure 3B**).

4.9. Place the agar block within a histology cassette and store in 70% ethanol at 4 °C before subjecting it to standard paraffin processing and sectioning.

5. Model injury and repair with re-submersion and re-ALI

5.1. On ALI day 21, add 150 µL of 50% L-WRN media on the apical side of ALI culture to re-submerge the monolayer.

5.2. Change media inside and outside of membrane insert every 2 days.

5.3. After 7 days of re-submersion, remove media on the apical side of the monolayer to re-create air-liquid interface.

5.4. Incubate for another 14-21 days to re-establish a self-renewal, differentiated monolayer.

NOTE: During the course of re-submersion and re-ALI, histology and whole mount staining analysis can be performed at any time as described in steps 4 and 5.

REPRESENTATIVE RESULTS:

The colonic ALI monolayer culture consists of two distinct phases: the submerged phase and the ALI phase (**Figure 1**). During the submerged phase, 50% L-WRN CM is applied both inside and outside of the membrane insert. Colonic epithelial cells will settle and attach to insert membrane overnight after the initial seeding step. Over the first seven days of the submerged phase, the epithelial cells will form a confluent monolayer in the insert. Upon creation of ALI, monolayer cells undergo a proliferative burst in the first two days. Over the next 2-3 weeks, the monolayer self-organizes into proliferative foci containing stem cells and regions of cellular differentiation including UEA1 expressing goblet cells (**Figure 2**)[19]. At day 0 of ALI culture, a continuous, flat monolayer will be present and cover the membrane. After 3 weeks of ALI culture, cell height dramatically increases compared to ALI day 0 and mucus-producing goblet cells are plainly visible (**Figure 3A**). Between ALI day 7 to day 14, a layer of apical mucus will become visible to the naked eye. Enteroendocrine and absorptive lineages are also present as detailed in our previous work[19]. Upon re-submersion on ALI day 21, the monolayer will revert back to a flat, regenerative epithelium. Re-establishing the ALI following the period of re-submersion will again result in a long-term self-renewing monolayer.

To assess the histology of the monolayer in paraffin sections, an agar-embedding step is performed prior to paraffin processing and embedding. In the agar block, membrane halves are aligned parallel to each other while vertically positioned to the future cutting plane of a paraffin block (**Figure 3B**). This agar pre-embedding step will facilitate the generation of cross sections of the monolayer. Standard immunostaining and H&E staining can then be performed to examine the morphology and differentiation of the monolayer from a side view of the cells over the course of ALI culture. In contrast, whole mount immunostaining provides a top-down view of the monolayer culture and can be used in combination with cross sections to achieve a comprehensive understanding of this monolayer system.

FIGURE AND TABLE LEGENDS:

Figure 1: Illustration of ALI culture. ALI culture is composed of two phases. In the submerged phase (first 7 days), 50% L-WRN media is applied both inside and outside the membrane insert. During the air-liquid interface phase, epithelial monolayer is cultured without media on top of its apical side.

Figure 2: Whole mount staining on ALI Day 21 monolayer. Whole mount images of Ki67 (red) and UEA1 (grey) staining on ALI day 21 monolayer culture as described in step 3. Scale bar: 20 µm.

Figure 3: Histology of ALI monolayer culture on cross sections. (**A**) H&E staining on cross sections of ALI culture on ALI day 0 and ALI day 21. (**B**) Illustrated side view of an agar block containing multiple membrane insert halves as described in step 4. Scale bar: 50 µm.

Table 1: Media recipes.

DISCUSSION:

Because the monolayers are long-lived, it is especially important to practice sterile culture technique to prevent accidental contamination. All work should be performed in an appropriate biosafety cabinet using sterilized and/or single use consumables, where possible. It is also essential to generate a high number of stem cells by spheroid culture to create the initial seeding lawn. If the user does not have experience with stem cell practice, it is advisable to become familiar with basic handling and passaging techniques through repetition with a manageable number of culture wells before expanding to large collection volumes which are more time consuming and expensive.

The presented protocol is for the two-dimensional culture of mouse colonic stem cells. Numerous protocols have been developed in the literature to grow spheroids and organoids from different intestinal regions and different animal hosts. Future directions would include extending these ALI methods to stem cells derived from other intestinal regions or host species, notably human. This would enable patient-specific studies of development and disease. In preliminary studies in our laboratory, we have been able to generate ALI cultures from human rectal stem cells with minor modifications to the protocol. However, further work is needed to determine stem cells from different regions or animal hosts would undergo the same proliferative burst and self-organization as mouse colonic stem cells when exposed to ALI. Similarly, it is possible that there are rare differentiated epithelial cells types in other intestinal regions that would not emerge without additional growth signals.

The intestinal epithelium is a polarized barrier, with distinct apical and basolateral surfaces. A major benefit of a 2D monolayer culture model is that the apical and basolateral surfaces are each readily accessed. For instance, microbes from the intestinal lumen contact the apical surface of the colon. Studies of host-microbe interactions in 3D organoid systems require micro-injection of bacteria or bacterial components to the luminal (interior) surface of the 3D structure[5,6,27,28]. By contrast, viruses and aerotolerant bacteria can be introduced to intestinal monolayers without

special equipment[29,30]. Similarly, several groups have leveraged the ability to separate cell types on opposite sides of a semi-permeable membrane to study the interactions between primary epithelial monolayers and immune cells or enteric neurons[31,32]. An advantage of our protocol is that all epithelial cell types our present simultaneously in a long-lived monolayer organized to resemble flattened crypts. Co-culture and pathogenesis studies in this model might be useful to determine which epithelial cell type or region of the crypt-villous axis is most critical for a particular interaction.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

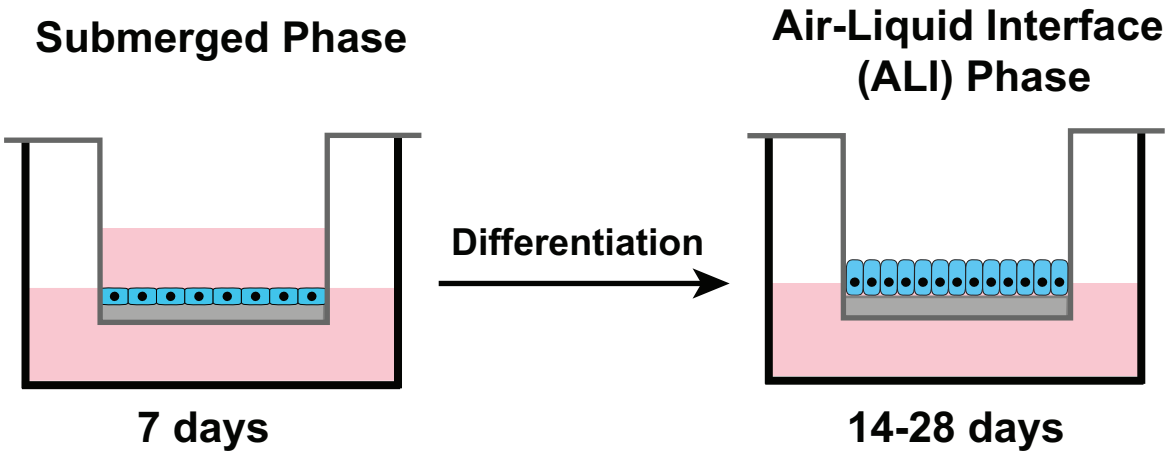


Figure 2

Click here to access/download;Figure;Figure 2.eps

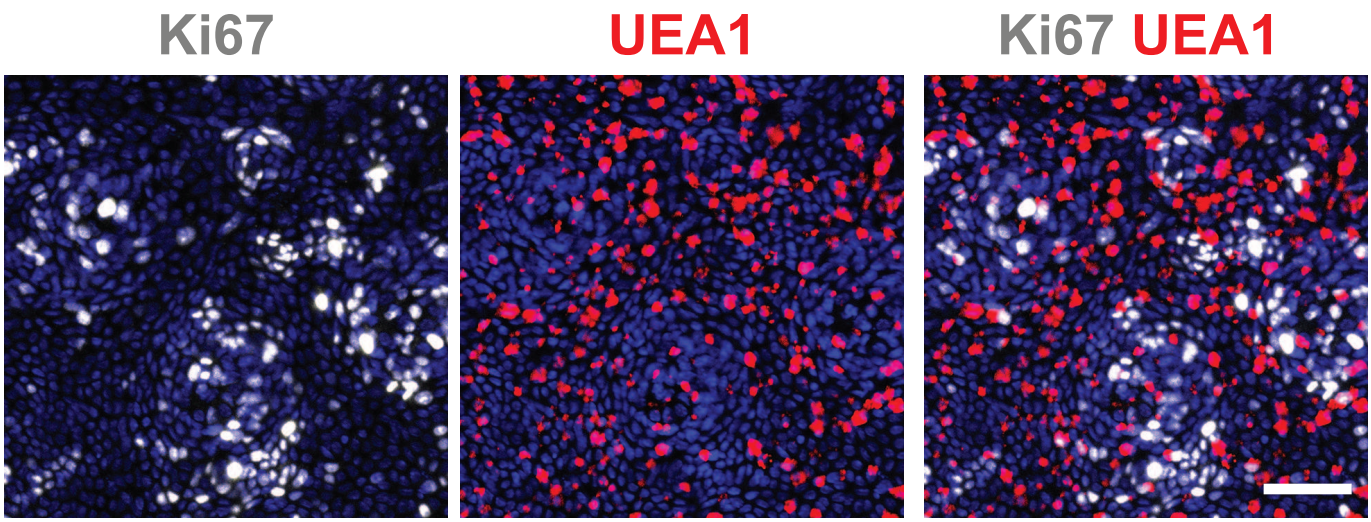
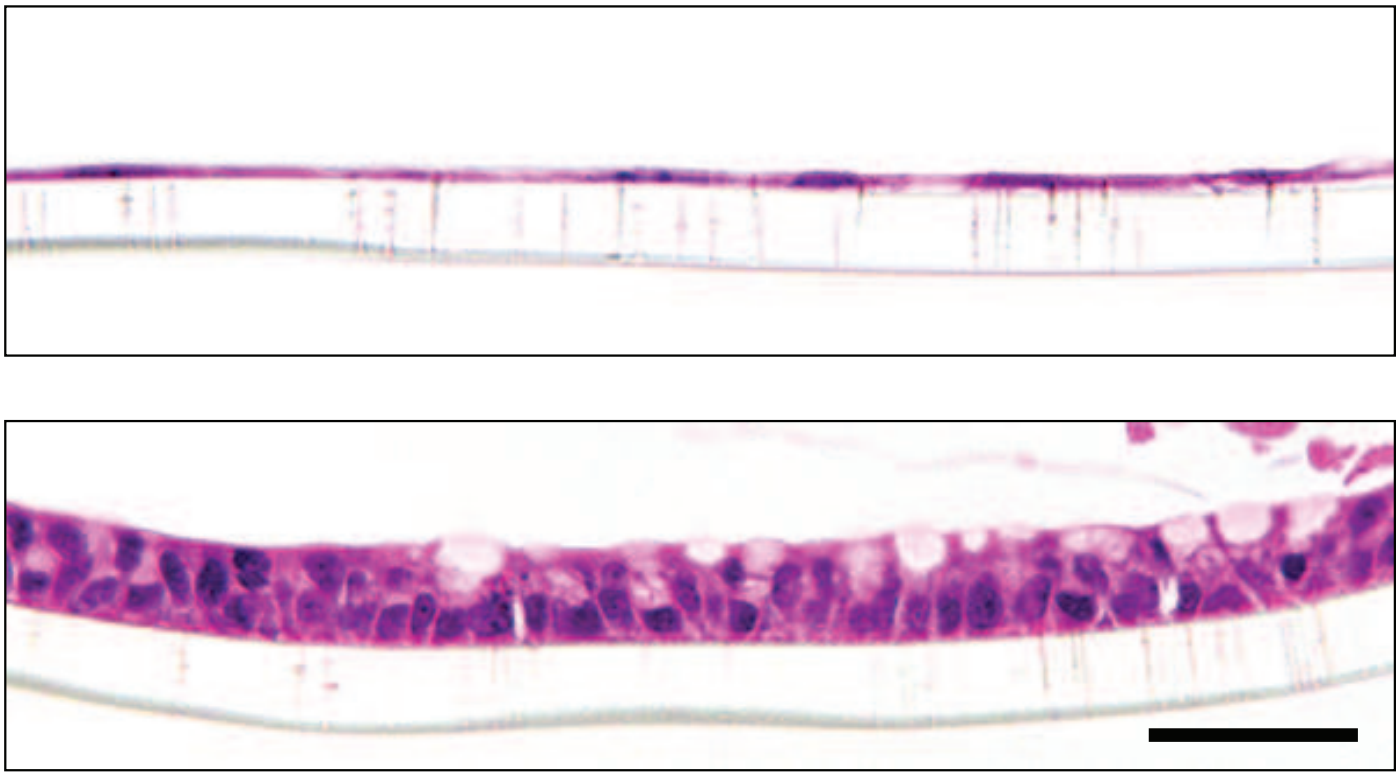
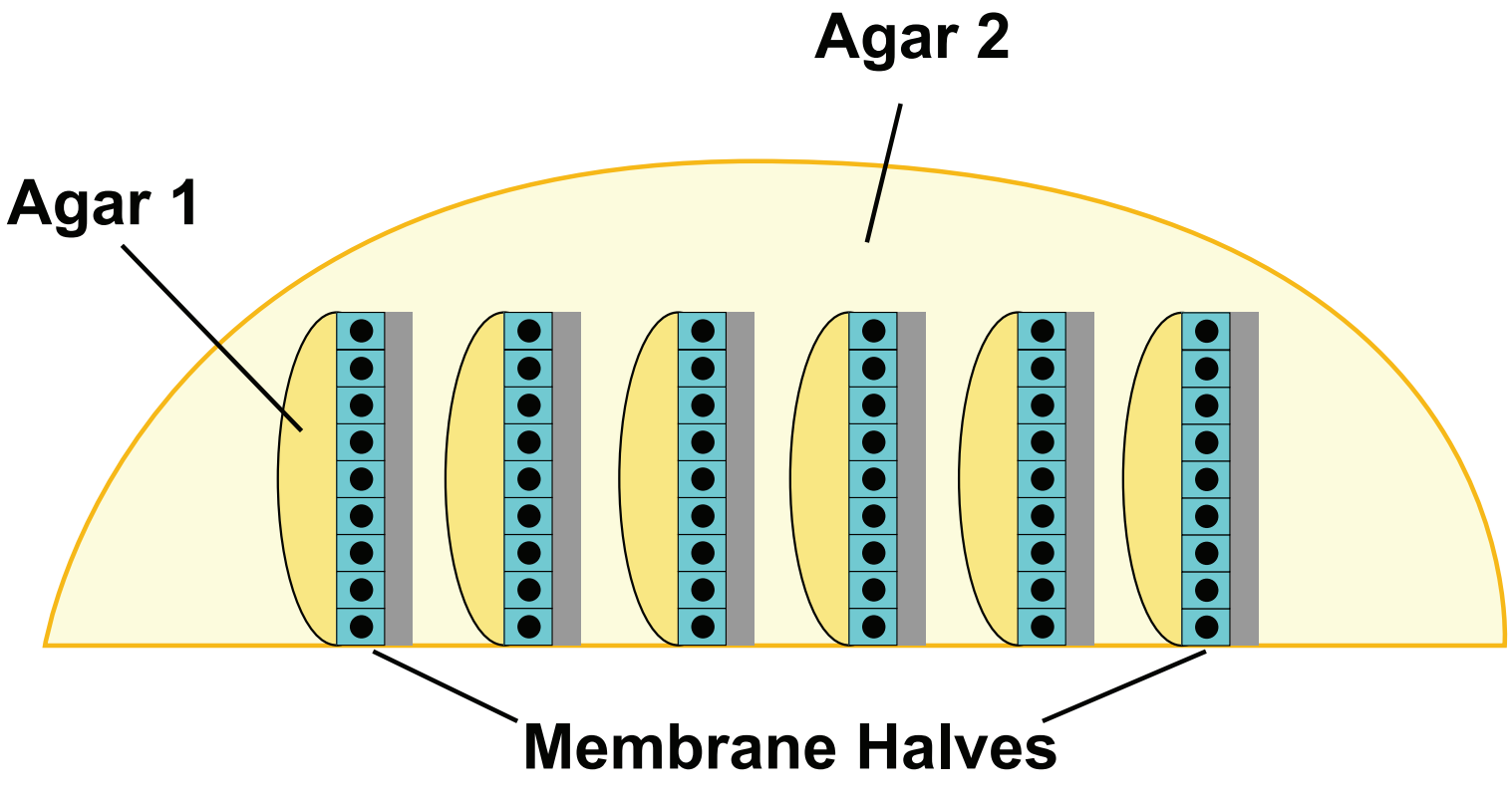


Figure 3

A



B



Table

Media Recipes

Washing Media

DMEM/F12 with HEPES	500 mL
100X L-glutamine	5 mL
100X Penicillin/Streptomycin	5 mL
FBS	50 mL

Collagenase Solution

Washing Media	50 mL
Collagenase, Type 1, powder	100 mg
Gentamicin (SIGMA: G1397)	50 µL

- Filter through a 0.22 µm filter. Store at -20 °C in 1 mL aliquots

10 mM Y-27632 Stock Solution

- Resuspend 10 mg of Y-27632 in 3.04 mL sterile water.
- Store in aliquots of 50-100 uL at -80 °C.
- Thaw and use 1:1000 in 50% LRN-CM for 10 µM final concentration.

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_MarchRevised.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
#11 surgical blade	Henry Schein	1126190	
0.5M EDTA	Thermo Fisher Scientific	15575020	
10x Trypsin	Sigma	T4549	
32% paraformaldehyde	Fisher Scientific	50-980-495	
Advanced DMEM/F12 for primary culture media	Thermo Fisher Scientific	12634010	
Agar	Sigma	A7921-500G	
Collagenase, Type 1, powder	Thermo Fisher Scientific	17100-017	
DMEM for L cell culture media	Sigma	D5796-500ML	
DMEM/F12 with HEPES for washing media	Sigma	D6421-500ML	
FBS	Sigma	F2442-500mL	
G418	Sigma	G8168-10mL	
Gentamicin	Sigma	G1397	
Hoechst 33342	Thermo Fisher Scientific	H3570	
Hygromycin B	InvivoGen	ant-hg-1	
Ki67-FITC	Thermo Fisher Scientific	11-5698-82	
L-Glutamine (100x)	Sigma	G7513-100mL	
L-WRN cell line	ATCC	CRL-3276	
Matrigel	Corning	354234	
Mounting media	Vector Laboratories	H1000	
Pen/Strep (100x)	Sigma	P4333	
Transfer Pipette	Fisher Scientific	13-711-7	
Transwell Permeable Supports, 6.5 mm diameter	Corning	3470	

UEA1-Rhodamine	Vector Laboratories	RL-1062
Y-27632	R&D Systems	1254

Dear Editors,

We thank the reviewers for their time in reviewing our manuscript "Modeling Intestinal Development and Injury-Repair in Vitro using Mouse Colonic Stem Cell Monolayer Culture under Air-Liquid Interface", and for their very helpful suggestions. We have responded to all of the requests for revision or clarification in the attached revised manuscript. We highlight our responses here in red and include significant changes to the revised manuscript in blue.

Reviewers' comments:
Reviewer #1:

"-Line 102: perhaps helpful to point readers to clear resource on preparation of 50% L-WRN CM."
In our submission the reference to the previously published protocol for L-WRN generation was included but not emphasized as the main reference. We have added an explicit sentence to clarify the reference that readers may use to find the published and validated protocol.
(Revised Lines 103-105): "Our laboratory has previously published a detailed protocol describing the generation of this conditioned media.[20] Multiple independent laboratories have used this protocol to generate this media for growth of intestinal stem cells[22, 25, 26]."

"-Line 110: how important is it that cells originate from 8-wk M C57 mice? Assuming not very, but it may be worth including brief discussion on author's experiences in any potential differences between animals, etc…"
We have not encountered significant differences in line generation between animals of different ages or genetic backgrounds but have added a comment of clarification.
(Revised NOTE under Step 1.1.1): "NOTE: We typically generate spheroid lines from male mice in this age range, but lines can be established from other genetic backgrounds, ages, or genders with identical procedures."

"-Line 117: how do you assess whether crypts "fall out into the solution readily"? Visually? Under magnification? What may be indicators of too-short or too-long digestion?"
We have expanded our text to describe the visual inspection of the solution by microscopy to gauge adequacy of digestion.
(Revised NOTE under Step 1.1.6): "NOTE: Colonic tissue typically requires 30-40 min of total digestion. Adequacy of digestion can be assessed using a phase or dissection microscope. Digestion is complete when 50-80% of single epithelial units (crypts or pits) are free from the larger colonic tissue fragments."

"-Line 135: any more quantitative guidance on assessing how many crypts per gel?"
We roughly target 1,000 to 3,000 epithelial units per well of a 24 well plate. Overcrowding is more of a problem than low input - even a very low starting number of epithelial units can be expanded over several passages to generate a robust number of cells. We have added a note clarifying an empiric approach and guidance.
(Revised NOTE under step 1.1.11): "NOTE: We recommend that approximately 1,000 to 3,000 epithelial units be dispersed to each well. This can be estimated by evaluating a droplet of the solution with a phase or dissection microscope. Empirically, if starting with 1 cm of mouse colon, the user could resuspend the crypt pellet in 60 µL of extracellular matrix to seed 4 wells and adjust future experiments based on yield."

"-Line 139: would like to see the technique of inversion demonstrated"
We will demonstrate in the video protocol at step 1.2.9 and 1.2.10.

"-Line 182: guidance on when a passaging ratio of 1:5 versus 1:20 is preferred would be helpful"
Unfortunately this ratio is empiric for each researcher, and depends on adequacy of trypsinization and loss of cells in pipetting steps among other factors. We have expanded the note under step 1.2.8. Also, as the main point of this JoVE protocol is to describe the generation of the ALI culture, and we have previously published other papers explicitly detailed the spheroid culture component, we emphasize that readers should review those prior papers for full discussion and detail.

(Revised NOTE under Step 1.2.8): "NOTE: Typical passage ratios for mouse colon are 1:5 to 1:20. For example, if stem cells are collected from 4 wells of a 24 well plate in step 1.2.1 and a 1:10 passage ratio is desired, 600 µL of extracellular matrix would be added (4 wells x 1:10 passage ratio x 15 µL extracellular matrix per well). The ratio needs to be empirically adjusted for reach researcher, dependent on adequacy of trypsinization, pipetting losses, and other factors. New users should start with a ratio closer to 1:5 and increase dilution as skills increase. See also previous publications on spheroid culture for discussion and details [20, 21]."

"-Line 225: how important is it that 70 pipettes are performed? Perhaps a range or a visual cue would be more instructive."
The count of 70 is somewhat arbitrary but is chosen because it results in nearly complete single-cell suspension. We don't recommend any visual analysis at this point because the single-cell suspensions are prone to cell death and speed is paramount. The visual analysis of suspension adequacy comes in step 2.13. We have expanded the Note under step 2.13 with suggested troubleshooting if the suspension is not sufficiently dispersed to single cells or hampered by excessive cell death.
(REVISED additional text beginning on Line 380): "If the cells at this step are not sufficiently dissociated, increase the trypsinization time in Step 2.7 by 30 seconds and or increase the number of pipettes in step 2.8. Conversely, if there is a large amount of cell death, decrease the number of pipettes in step 2.8 by 10 and make sure that all steps are performed as quickly as possible."

"-Line 271: guidance on what a successful culture looks like would be helpful here"
The membrane makes it hard to assess the cells by microscopy without fixation. We have added text about the emergence of a mucus layer which is a robust indicator of successful culture.
*(Addition to Step 2.18): "*A light brown apical mucus layer will become apparent around ALI day 7 and should be evenly distributed across the surface."

"-Line 422: Authors state in introduction the method can be applied to other GI tissues / organisms (line 87), though from discussion it sounds as though this has not been tested. Clarification here would be helpful."
Thank you for this clarifying comment. In preliminary work from our lab we have been able to generate ALI monolayers from other regions of the mouse gut and from human spheroids with only minor modifications to the protocol. We are working to submit these studies for publication. We have revised the language in the introduction and the discussion to reflect that this is preliminary work so that there is no confusion.

(Revised Introduction, beginning at new line 90) "Spheroid stem cells can be grown from other regions of the gut and from other species including humans [20–22], and we have preliminarily been able to generate ALI monolayers from these other sources with minor modifications to the protocol."

(Revised Discussion, beginning at new line 576) "The presented protocol is for the two-dimensional culture of mouse colonic stem cells. Numerous protocols have been developed in the literature to grow spheroids and organoids from different intestinal regions and different animal hosts. Future directions would include extending these ALI methods to stem cells derived from other intestinal regions or host species, notably human. This would enable patient-specific studies of development and disease. In preliminary studies in our laboratory, we have been able to generate ALI cultures from human rectal stem cells with minor modifications to the protocol."

Reviewer #2:
Manuscript Summary:
"Current manuscript describes a novel 2D-culture model of colon epithelial cells. The manuscript is clearly written and easy to read. Most important points are included appropriately.

My only advice is to add some tips for visualization of whole-mount samples (as shown in Fig. 2), if there is any. Is it possible to acquire high-quality pictures through any kind of trans-well membrane, or is it specific for the one used in the present manuscript?"

We appreciate these clarifying comments. We have added a note after step 2.1 that clear or transparent membranes are recommended for any microscopic imaging. We have expanded the details in step 3.11 to explicitly describe orientation of the cells and detailed steps for whole mount microscopy.

(Revised NOTE after Step 2.1): "NOTE: If using a transwell product other than that listed in the Table of Materials, be sure to select a product with transparent or clear membranes if you will be performing microscopic imaging at later steps."

(Revised text for Step 3.11): "Use an inverted confocal microscope to take whole mount images (**Figure 2**). Place the slide with the cell side facing the lens. Find the focal plane for the cells by using a 10x lens. Then use a 40x or 60x lens (use water or oil as needed) to capture images at the desired fluorescent channels. Z stacks of 20-30 um is recommended to capture the entire span of cell height."

Reviewer #3:

"In steps 1.1.1, 1.1.5, 1.1.6, it may help to suggest carrying out the initial tissue harvest on ice and continue working at 4°C in the centrifugation steps after incubation with the collagenase/dispase solution."
Thank you for this helpful suggestion. We expanded the early steps of mouse tissue collection to make clearer which steps should be performed on ice and which can be performed at room temperature. The expanded steps are found in 1.1.1 to 1.1.5 of the revised protocol.

(Revised steps 1.1.1 to 1.1.5):
"1.1.1. Briefly, dissect out a 1 cm segment of colonic tissue from a 8-10 week-old C57/BL6J mouse. Remove any fat or connective tissues with fine scissors.

NOTE: We typically generate spheroid lines from male mice in this age range, but lines can be established from other genetic backgrounds, ages, or genders with identical procedures.

1.1.2. Flush the lumen with ice cold PBS using a 10 mL syringe fitted with a blunt 19 gauge needle, then open the colonic segment longitudinally with scissors.

1.1.3. Transfer the tissue to a 90 mm petri dish containing ice cold PBS and swirl the tissue to rinse.

1.1.4. Transfer the tissue fragment to a 50 mL conical containing ice cold PBS. Wash by vigorous shaking.

1.1.5. Move the tissue fragment to a 40 mm Petri dish and transfer into the tissue culture hood. Mince the tissue with sterilized scissors in the petri dish until the tissue can be easily pipetted with a P1000. The pieces at this point will be <1 mm^2. Add 1 mL of Collagenase solution to the minced tissue and mix the tissue homogenate by gently pipetting."

We don't perform centrifugation steps at 4°C. Not all labs have a refrigerated centrifuge with 15 mL or 50 mL conical capacity in the tissue culture space. Although we have never compared results between a room-temperature and refrigerated centrifuge, we have not encountered any problems generating spheroid cultures from mouse or human tissue in the room-temperature centrifuge. To make this explicit, we added the following in the revised protocol:
(Addition to Line 110): "All centrifugation steps can be performed at room temperature."

"In step 1.1.2, there is no catalogue number provided for Dispase."
We apologize for the typographic error. The solution is Collagenase Type 1 with no dispase. We have corrected this in the text, now step 1.1.5 as entered above. We have provided explicit directions for making and storing this solution in the Table of Materials.

"In step 1.1.4, is it necessary to coat tubes with BSA?"
We don't coat the tubes with BSA, even when starting from small pieces of tissue. Even a small number of stem cells can be greatly expanded in 50% L-WRN media over one or two passages.

"In step 1.1.9, it is unclear how to proceed with the plate inversion step. Would inversion affect the position of the Matrigel droplet?"
Stem cells and spheroids can sink under the control of gravity and adhere to the plastic in upright plates. If this occurs, there is more heterogeneity in the culture. Inversion while the extracellular matrix is polymerizing keeps the spheroids from attaching to the plastic and preserves three-dimensional structure and proliferation. We demonstrate inversion in the video protocol in step 1.2.9 and 1.2.10.

"In step 1.1.10, there is initial supplementation of 10 µM Y-27632. It may be helpful to describe for how many days the inhibitor should be kept in the medium at this step."
We explicitly wrote which steps require Y-27632 supplementation. All other steps can be performed with 50% L-WRN without Y-27632. To make this clearer we added a NOTE after the first mention of Y-27632 (now 1.1.13)
(Additional NOTE after Step 1.1.13): "NOTE: Y-27632 is an inhibitor of Rho-associated protein kinase (ROCK). ROCK inhibition helps to prevent anoikis in isolated epithelial cells. For mouse culture, we supplement 50% L-WRN CM with Y-27632 when establishing stem cell lines and when spheroids are dissociated to single cell suspension (Step 2.12–2.15). Y-27632 is not required for media change for established murine spheroids on day 2 (Step 1.2.11) or for media change of ALI monolayers (Step 2.16)."

"In step 1.2.2, would it be better to carry the colonic spheroid collection on ice?"
"In steps 1.2.6-1.2.7, again here, it may be useful to recommend centrifugation at 4°C."
As addressed above, we do not perform centrifugation steps at 4 °C. We perform all spheroid passage and expansion steps at room temperature except for the Matrigel suspension (now step 1.2.8). We have expanded the note at the beginning of Step 1.2 to make this clear.
(Revised Note at Line 231): "All steps are performed at room temperature unless otherwise noted."

"In steps 2.2, it may be helpful to advise to be careful about not disrupting the membrane of the Transwell inserts during Matrigel coating."
We have revised step 2.2 to incorporate this helpful suggestion.
(*Revised Step 2.2):* "Dispense solution gently to avoid damaging the membrane."

"In steps 2.5, 2.10 and 2.11, should centrifugation be performed at 4°C.?"
As addressed previously, these centrifugations are performed at room temperature.

"In step 4.5, it is unclear how big the size of the agar droplet should be in the first step of membrane embedding."
Thank you for pointing out this ambiguity. We have expanded the text including more specific reference to the representative Agar droplet in Figure 3B.
(Revised Step 4.5): "Use a transfer pipette to cover the top of the membrane with warm 2% agar from the solution kept in 60 °C water bath ("Agar 1" in **Figure 3B**). Wait until the agar solidifies."

Editorial comments:

Please track the changes within the manuscript to identify all of the edits.
All modifications in the revised manuscript were made with Track Changes.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

We have uploaded all figures files in high resolution .eps format and the table in .xlsx format.

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We have proofread our manuscript and are not aware of any errors.

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We have replaced all occurrences of Matrigel with 'extracellular matrix' and replaced capitalized occurrences of Transwell with 'transwell', 'insert', or 'membrane' as appropriate.

3. 1.1.1: Please describe how to dissect out the colon tissue from the mouse. Alternatively, cite a relevant reference here.
We expanded the mouse tissue dissection in steps 1.1.1 to 1.1.5. These steps are also described in our prior publications on spheroid culture, notable Miyoshi and Stappenbeck 2013.

4. Figure 3B: Please replace Transwell with a generic term.
We replaced the word Transwell with 'Membrane'.

5. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.
We have made the requested modifications to the table.

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Author(s):	Brian D. Muegge, Yi Wang, Thaddeus S. Stappenbeck

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