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Organoid-derived Epithelial Monolayer: A Clinically Relevant In Vitro Model for Intestinal Barrier Function

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Organoid-derived Epithelial Monolayer: A Clinically Relevant In Vitro Model for Intestinal Barrier Function

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epithelial monolayer, human intestinal organoids, membrane insert, barrier function, permeability, transport

SUMMARY:

Here, we describe the preparation of human organoid-derived intestinal epithelial monolayers for studying intestinal barrier function, permeability, and transport. As organoids represent original epithelial tissue response to external stimuli, these models combine the advantages of expandability of cell lines and the relevance and complexity of primary tissue.

ABSTRACT:

In the past, intestinal epithelial model systems were limited to transformed cell lines and primary tissue. These model systems have inherent limitations as the former do not faithfully represent original tissue physiology, and the availability of the latter is limited. Hence, their application hampers fundamental and drug development research. Adult stem-cell-based organoids (henceforth referred to as organoids) are miniatures of normal or diseased epithelial tissue from which they are derived. They can be established very efficiently from different gastrointestinal (GI) tract regions, have long-term expandability, and simulate tissue- and patient-specific responses to treatments in vitro. Here, the establishment of intestinal organoid-derived epithelial monolayers has been demonstrated along with methods to measure epithelial barrier integrity, permeability, transport, viability, as well as antimicrobial protein and cytokine secretion

by histology. Moreover, intestinal organoid-derived monolayers can be enriched with proliferating stem and transit-amplifying cells as well as with key differentiated epithelial cells. Therefore, they represent a model system that can be tailored to study the effects of compounds on target cells and their mode of action. Although organoid cultures are technically more demanding than cell lines, once established, they can reduce failures in the later stages of drug development as they truly represent in vivo epithelium complexity and interpatient heterogeneity.

INTRODUCTION:

The intestinal epithelium acts as a physical barrier between the luminal content of the intestines and the underlying tissue. This barrier comprises a single epithelial layer of mainly absorptive enterocytes that are connected by tight junctions, which establish strong intercellular connections between adjacent cells. These cells form a polarized epithelial lining that separates the apical (lumen) and basolateral sides of the intestine, while simultaneously regulating paracellular transport of digested nutrients and metabolites. In addition to enterocytes, other important epithelial cells such as goblet, Paneth, and enteroendocrine cells also contribute to intestinal homeostasis by producing mucus, antimicrobial peptides, and hormones, respectively. The intestinal epithelium is constantly replenished by dividing leucine-rich repeat-containing G-protein-coupled receptor 5-positive ($LGR5^+$) stem cells in the bottom of intestinal crypts producing transit-amplifying (TA) cells that migrate upwards and differentiate into other cell types[1]. Disruption of intestinal epithelial homeostasis by genetic and environmental factors, such as exposure to food allergens, medicinal compounds, and microbial pathogens, leads to disruption of intestinal barrier function. These conditions cause several intestinal diseases including inflammatory bowel disease (IBD), celiac disease, and drug-induced GI toxicity[2].

Studies on the intestinal epithelium are performed using several in vitro platform systems such as membrane inserts, organs-on-a-chip systems, Ussing chambers, and intestinal rings. These platforms are suitable for establishing polarized epithelial monolayers with access to both apical and basolateral sides of the membrane, using transformed cell lines or primary tissue as models. Although transformed cell lines, such as the colorectal (adeno)carcinoma cell lines Caco-2, T84, and HT-29, are able to differentiate into polarized intestinal enterocytes or mucus-producing cells to some extent, they are not representative of the in vivo epithelium as several cell types are missing, and various receptors and transporters are aberrantly expressed[3]. In addition, as cell lines are derived from a single donor, they do not represent interpatient heterogeneity and suffer from reduced complexity and physiological relevance. Although primary tissues used in Ussing chambers and as intestinal rings are more representative of the in vivo situation, their limited availability, short-term viability, and lack of expandability make them unsuitable as a medium for high-throughput (HT) studies.

Organoids are in vitro epithelial cultures established from different organs such as the intestine, kidney, liver, pancreas, and lung. They are proven to have long-term, stable expandability as well as genetic and phenotypic stability and therefore are representative biological miniatures of the epithelium of the original organ with faithful responses to external stimuli[4–9]. Organoids are efficiently established from either resected or biopsied normal, diseased, inflamed, or cancerous

tissue, representing heterogeneous patient-specific responses[10–16]. This paper demonstrates how to establish intestinal epithelial monolayers derived from organoid cultures. Monolayers have been successfully established from small intestinal as well as colonic and rectal organoid cultures. This model creates an opportunity to study the transport and permeability of the epithelial cells to drugs as well as their toxicological effects on the epithelium. Moreover, the model allows co-culture with immune cells and bacteria to study their interactions with the intestinal epithelium[17–19]. Furthermore, this model can be used to study responses to therapies in a patient-specific manner and initiate screening efforts to look for the next wave of epithelial barrier-focused therapeutics. Such an approach could be extended to the clinic and pave the way toward personalized treatments.

Although the epithelial monolayers in this protocol are prepared from human normal intestinal organoids, the protocol can be applied and optimized for other organoid models. Epithelial organoid monolayers are cultured in intestinal organoid expansion medium containing Wnt to support stem cell proliferation and represent intestinal crypt cellular composition. Intestinal organoids can be enriched to have different intestinal epithelial fates, such as enterocytes, Paneth, goblet, and enteroendocrine cells, by modulating Wnt, Notch, and epidermal growth factor (EGF) pathways. Here, after the establishment of monolayers in expansion medium, they are driven toward more differentiated intestinal epithelial cells, as described previously[20–25]. For screening purposes, depending on the mode of action of the compound of interest, its target cells, and the experimental conditions, the monolayers can be driven toward the cellular composition of choice to measure the effects of the compound with relevant functional readouts.

PROTOCOL:

1. Preparing reagents for culture

NOTE: Perform all steps inside a biosafety cabinet and follow standard guidelines for working with cell cultures. Ultraviolet light is used for 10 min before starting up the biosafety cabinet. Before and after use, the surface of the biosafety cabinet is cleaned with a tissue paper drenched in 70% ethanol. To facilitate the formation of three-dimensional drops of extracellular matrix (ECM), keep a prewarmed stock of 96-, 24-, and 6-well plates ready in the incubator at 37 °C.

1.1. Basal medium preparation

1.1.1. Prepare basal medium (BM) in a 500 mL of Advanced Dulbecco's Modified Eagle Medium with Ham's Nutrient Mixture F-12 (Ad-DF) medium bottle by adding 5 mL of 200 mM glutamine, 5 mL of 1 M 4-(2-hydroxyethil)-1piperazineethanesulfonic acid (HEPES), and 5 mL of penicillin/streptomycin (pen/strep) solutions (10,000 U/mL or 10,000 µg/mL). Store it in the refrigerator at 4 °C for at least 4 weeks.

1.2. Wnt sources

1.2.1. Prepare Wnt3a-conditioned medium (Wnt3aCM) according to the previously described method[26].

NOTE: Recently, a next-generation surrogate Wnt (NGS-Wnt), which also supports expansion of human intestinal organoids, has been generated[27].

1.3. Intestinal organoid base medium preparation

NOTE: Use all growth factors and reagents according to the manufacturer's recommendations. Use small aliquots and avoid freeze-thaw cycles; functional growth factors are essential for successful organoid culture.

1.3.1. Prepare concentrated 2x intestinal organoid base medium (2x IBM) by supplementing BM with 1 µM A83-01, 2.5 mM N-acetylcysteine, 2x B27 supplement, 100 ng/mL human epidermal growth factor (hEGF), 10 nM gastrin, 200 ng/mL hNoggin, and 100 µg/mL of an antimicrobial formulation for primary cells (see the **Table of Materials**).

1.3.2. Aliquot the 2x IBM and freeze at -20 °C for up to 4 months. When needed, thaw an aliquot overnight at 4 °C or for several hours at room temperature (RT).

1.3.3. To prepare intestinal organoid expansion medium (IEM), supplement 2x IBM with either 50% Wnt3aCM or 50% BM and 0.5 nM NGS-Wnt, 250 ng/mL human Rspondin-3 (hRspo3), 10 mM nicotinamide, and 10 µM SB202190.

1.4. Intestinal organoid differentiation medium preparation

1.4.1. Prepare enterocyte differentiation medium (eDM) by supplementing 2x IBM with 50% BM, 250 ng/mL hRspo3, and 1.5 µM Wnt pathway inhibitor (IWP-2). Store eDM at 4 °C for up to 10 days.

1.4.2. Prepare combination differentiation medium (cDM) by supplementing 2x IBM with either 40% BM and 10% Wnt3aCM or 50% BM and 0.1 nM NGS-Wnt, 250 ng/mL hRspo3, 10 µM DAPT and 100 nM PD0325901. Store cDM at 4 °C for up to 10 days.

1.5. Manipulation of extracellular matrix (ECM)

NOTE: Prepare the extracellular matrix (ECM) (see the **Table of Materials**) according to the manufacturer's recommendation.

Thaw ECM overnight on ice; transfer the ECM from the bottle to a 15 mL conical tube using a 5 mL pipette, both pre-cooled at -20 °C. Refreeze aliquots only once at -20 °C. Once thawed, store the ECM in a refrigerator at 4 °C for up to 7 days. Incubate for at least 30 min on ice before use.

NOTE: Mix ECM properly and ensure that it is cold before embedding crypts or organoids.

2. Organoid cultures

2.1. Establishing cultures from frozen organoids

NOTE: Let BM reach RT, and keep a 10 mL aliquot, warmed to 37 °C, ready before starting the procedure of thawing one cryovial containing frozen organoids.

2.1.1. Thaw the organoid cryovial rapidly by agitating in a 37 °C water bath until only a sliver of ice remains. Immediately add 500 µL of warm BM dropwise to the cryovial, and pipet up and down a few times to dilute the freezing medium and mix the contents carefully.

2.1.2. Using a P1000 pipette, transfer the organoids to a 15 mL conical tube, and add another 1 mL of warm BM dropwise while gently mixing the bottom of the tube. Pipet up and down a few times to dilute the freezing medium and mix the contents carefully.

2.1.3. Add up to 12 mL of warm BM dropwise to the 15 mL conical tube containing the organoids, and pipet up and down with a 10 mL sterile pipette to gently resuspend the organoids.

2.1.4. Centrifuge the organoid suspension for 5 min at 85 × *g* and 8 °C. Discard the supernatant carefully without disturbing the pellet, and resuspend the organoids in 30% v/v of IEM supplemented with 10 µM Y27632 or other rho-associated coiled-coil-forming protein serine/threonine kinase inhibitor (ROCK inhibitor). Place the tube on ice.

2.1.5. Add 70% v/v of ECM in the 15 mL conical tube containing the organoids. Mix the organoid suspension keeping the 15 mL conical tube on ice, and seed 5 µL of the suspension to check the density (**Figure 1A**). Continue plating if the density is appropriate; if the density is too high, add more IEM/ECM solution in the same ratio of 30–70% v/v, respectively.

2.1.6. In each well of a prewarmed 24-well plate, seed 50 µL of the organoid suspension by pipetting 5 separate drops of 10 µL (**Figure 1B**). Turn the plate upside down, and leave it in the biosafety cabinet for 5 min. Transfer the plate still upside down to the 37 °C incubator, and leave it for another 30 min.

2.1.7. Add 500 µL of IEM with 10 µM ROCK inhibitor to each well, and transfer the plate to the incubator. Image one drop regularly to monitor the growth, and refresh IEM every 2–3 days by aspirating the old medium and adding 500 µL of fresh IEM.

2.1.8. Passage the organoids once they have recovered properly from thawing and have reached the right size to be processed (**Figure 1C**), as described in section 2.2.

2.2. Passaging of intestinal organoids

NOTE: Chill the ECM on ice for at least 30 min, and keep the IEM at RT for at least 1 h before use.

2.2.1. Use the medium from one culture well to break up the organoid domes using a 1250 µL low-retention filter-tip, and transfer the well contents to a labeled 15 mL conical tube. Wash the well with 1 mL of BM, and transfer it to the same 15 mL conical tube.

2.2.2. Repeat steps 2.2.1 and 2.2.2 with all other wells (a maximum of half a plate or 600 µL of ECM drops can be washed and added to one 15 mL conical tube).

2.2.3. Add BM to fill the tube up to 12 mL, and pipet up and down 10x using a 10 mL pipette. Centrifuge at 85 × *g* for 5 min at 8 °C.

2.2.4. Before removing the BM, check under the microscope to see whether all organoids are pelleted at the bottom of the 15 mL conical tube (**Figure 1D**). If there is no ECM overlaying the organoid pellet or the ECM layer is either clean or contains just debris, single cells, or very few organoids compared with the pelleted organoids, aspirate the supernatant and pipet out the ECM overlaying the organoid pellet very carefully using a P200 pipette.

NOTE: Organoids can get trapped in the ECM and do not sediment as a compact pellet due to low centrifugation force. If the ECM contains organoids, centrifuge the tube again at 450 × *g* for 5 min at 8 °C, and carefully remove the supernatant as described in 2.2.4. If there are multiple 15 mL conical tubes, they can be pooled after step 2.2.4.

2.2.5. Add 1 mL of BM to each pellet (of volume of 50–200 µL, depending on the organoid culture and density), and resuspend carefully. Pipet the organoids up and down at least 5x to shear them, avoiding foam formation. Check under the microscope to see whether the organoids are disrupted (**Figure 2A**). If the organoids are disrupted, proceed to step 2.2.7; if the organoids are not disrupted, pipet them another 5x. This time, touch the wall of the plastic tube with the pipette tip to exert more mechanical force to disrupt the organoids.

NOTE: Mechanical shearing of cystic (**Figure 1C**) and budding (**Figure 1E**) organoids is possible with either a 200 µL or 10 µL plastic pipette tip fitted on a low-retention 1250 µL filter-tip **(Figure 1F)**, depending on the volume required for disrupting the organoids. The use of a narrowed glass pipette **(Figure 1F)** is recommended when more than 200 µL of ECM containing the organoids are processed (one well of a 6-well plate or 4 wells of a 24-well plate).

2.2.6. Check under the microscope again to see whether the organoids are disrupted. If disrupted, proceed with the next step; if not, pipet the organoids up to 20x, checking the organoids under the microscope regularly. If the organoids are still not disrupted, add 25% v/v cell dissociation reagent (see the **Table of Materials**) to the suspension, incubate in the water bath at 37 °C for 2 min, and pipet the organoids up to 20x, checking the organoids under the microscope regularly to make sure they are not digested to single cells.

2.2.7. Add up to 12 mL of BM to the 15 mL conical tube, and wash the organoid pellet by pipetting up and down. Centrifuge at 85 × *g* for 5 min at 8 °C. Discard the supernatant, and adjust the final concentration to 70% v/v ECM by adding IEM and ECM to the organoid pellet.

2.2.8. Start resuspending the organoid pellet with double the volume of IEM/ECM collected for passaging, and seed 5 µL of the suspension to check the density. Continue plating if the density is appropriate (**Figure 2B**); add more IEM/ECM solution if the density is too high. Add 200 µL of the suspension to each well of a prewarmed 6-well plate, making separate drops of 10 µL volume.

2.2.9. Turn the plate upside down, and leave it in the biosafety cabinet for 5 min. Transfer the plate still upside down to the 37 °C incubator, leaving it for another 30 min. Add 2 mL of IEM with 10 µM ROCK inhibitor to each well, and transfer the plate to the incubator.

2.2.10. Image one drop regularly to monitor the growth, and refresh IEM every 2–3 days by aspirating the old medium and adding 2 mL of fresh IEM.

2.3. Passaging of intestinal organoids for epithelial monolayer preparation

2.3.1. Passage organoids 3 days prior to harvest to prepare the monolayers by following the same passaging protocol described in section 2.2 with one exception. In step 2.2.7, resuspend the organoids in 1–1.5x the starting volume of IEM/ECM to have a higher density and expansion potential when they are harvested for monolayer preparation (**Figure 3A**).

3. Epithelial monolayer preparation

3.1. Culture epithelial monolayers on both 24-well and 96-well membrane inserts with a variety of available plate types (**Table 1**). Use high-throughput system (HTS) membrane inserts for both sizes as these contain an integral tray with the membrane inserts and a receiver plate. For the 24-well format, use plates with separate removable membrane inserts.

NOTE: Different membrane types (polyethylene terephthalate (PET) or polycarbonate) and pore sizes (0.4–8.0 µm) are available and can be used depending on experimental needs. Monolayers can only be imaged by brightfield when inserts with PET membranes are used. Light-tight membranes block fluorescent light leakage from the apical to the basolateral compartment and can be considered when dynamic transport or permeability of fluorescently labeled substrates is studied. The current protocol uses 24-well membrane inserts; adaptations for 96-well membrane inserts are described in section 5. Depending on the density, morphology, and size of the organoids (**Figure 3A**)**,** 6 wells of a 6-well plate (as seeded in section 2.3) are enough for seeding a full 24-well plate of membrane inserts.

3.2. Coating membrane inserts with ECM

NOTE: If there are doubts about having enough cells, coat the inserts after counting the cells. This is to prevent unnecessary coating and loss of the expensive membrane inserts.

3.2.1. Place the membrane inserts into the support plate in the biosafety cabinet. Dilute the ECM 40x in ice-cold Dulbecco's phosphate-buffered saline (DPBS) with Ca^{2+} and Mg^{2+}, and pipet 150 µL of the diluted ECM into the apical compartment of each insert. Incubate the plate at 37 °C for at least 1 h.

3.3. Preparation of cells for seeding

3.3.1. Prewarm aliquots of the cell dissociation reagent in the water bath (37 °C). Prepare 2 mL of the reagent for each well of a 6-well plate.

3.3.2. Transfer the culture plate containing the organoids (prepared in section 2.3) from the incubator to the biosafety cabinet. Process the organoids, as described in steps 2.2.1.–2.2.4. Do not pool multiple tubes into one tube.

3.3.3. Fill the tube, containing organoids from a maximum of 3 wells of a 6-well plate, up to 12 mL with DPBS (without Ca^{2+} and Mg^{2+}), and pipet up and down 10x using a 10 mL pipette. Centrifuge at 85 × *g* for 5 min at 8 °C, and aspirate the supernatant without disturbing the organoid pellet.

3.3.4. Add 2 mL of the prewarmed cell dissociation reagent per well of a 6-well plate used as the starting material and resuspend. Incubate the tubes diagonally or horizontally for 5 min in the water bath at 37 °C, to prevent the sinking of the organoids to the bottom of the tube.

3.3.5. Pipet up and down 10x using a 5 mL sterile plastic pipette or a P1000 pipette, depending on the total volume of the cell dissociation reagent. Check the organoid suspension under the microscope to see if a mixture of single cells and some cell clumps consisting of 2–4 cells has formed (**Figure 3B**). If needed, continue the digestion by repeating steps 3.3.4–3.3.5 until the mixture looks similar to **Figure 3B.**

NOTE: Avoid digesting the organoids fully to single cells. It is necessary to have some small groups of cells (i.e., groups of 2–4 cells).

3.3.6. Stop cell dissociation by adding up to 12 mL of BM to the cell suspension. Centrifuge at 450 × *g* for 5 min at 8 °C, and aspirate the supernatant without disturbing the cell pellet. When handling the same organoid culture in several 15 mL conical tubes, pool the cell pellets and resuspend them in 12 mL of BM.

3.3.7. Filter the cell suspension through a 40 µm strainer prewetted with BM, and harvest the flow-through into a 50 mL conical tube. Wash the strainer with 10 mL of BM, and harvest the flow-through into the same 50 mL conical tube.

3.3.8. Transfer the strained cell suspension into two new 15 mL conical tubes. Centrifuge at 450 × *g* for 5 min at 8 °C, and aspirate the supernatant without disturbing the cell pellet. Resuspend

the cells in 4 mL of IEM supplemented with 10 µM ROCK inhibitor per full culture plate used as starting material.

3.3.9. Mix a small amount of cell suspension in a 1:1 ratio with trypan blue for counting. Count the live, not blue, cells (**Figure 3C**), and calculate the total number of live cells. In small clumps, count each individual cell.

3.3.10. Prepare a cell suspension containing 3×10^6 live cells per mL of IEM supplemented with 10 µM ROCK inhibitor.

3.4. Seeding cells on polyester membrane inserts

3.4.1. Carefully aspirate DPBS from the ECM-coated inserts (step 3.2.1), whilst keeping the plate horizontally. Pipet 800 µL of IEM supplemented with ROCK inhibitor into each basolateral compartment. Pipet 150 µL of the cell suspension prepared in step 3.3.10 onto the ECM-coated membrane in the apical compartment dropwise. Per plate, be sure to have at least one "blank" well with BM only.

3.4.2. Once the cells have sedimented onto the membrane, measure transepithelial electrical resistance (TEER), as described in section 4.1, and image the membrane inserts using a microscope. Place the plate in the incubator at 37 °C and 5% CO_2. Measure TEER every day, and acquire images regularly to monitor monolayer formation (**Figure 4A–D**).

3.5. Refreshing monolayers

NOTE: Refresh the medium every 2–3 days, adhering to the following order to maintain a positive hydrostatic pressure above the cells and prevent cells from being pushed off the membrane. While refreshing the medium, make sure the monolayer, which is visible upon aspiration of the medium, is not damaged by the pipette tip.

3.5.1. Remove the medium from the basolateral compartments of the plate containing the membrane inserts. Then, carefully aspirate the medium from the apical compartments of the membrane inserts.

3.5.2. Add 150 µL of fresh IEM dropwise to each apical compartment, and then add 800 µL of fresh IEM to each basolateral compartment.

3.6. Enrichment of the monolayer for desired intestinal epithelial cell types

3.6.1. Allow the monolayer to become confluent in IEM, corresponding to a TEER value of around 100 Ω·cm² (as calculated in step 4.1.1.4). Check under the microscope to determine whether the monolayers have completely formed **(Figure 4D)** and for the absence of holes (as seen in **Figure 4B,C**).

3.6.2. Carefully remove IEM from the basolateral and apical compartments of the membrane inserts, and replace with either eDM or cDM as prepared in section 1.4. Culture the monolayer for another 3–4 days in the specific differentiation medium to get the organoid cells enriched with the desired specific cell type. Refresh the medium every 2–3 days, as described in section 3.4.

3.6.3. Measure TEER daily, and acquire images regularly if desired (**Figure 5A–C**).

NOTE: The TEER value that indicates a fully organized enriched monolayer varies per organoid culture; typically TEER values increase to 600 and can increase up to 1000 Ω·cm² (as calculated in step 4.1.1.4) after 3 days in differentiation media and are stable for 3–5 days.

4. Epithelial monolayer assay readouts

4.1. Measurement of transepithelial electrical resistance (TEER)

NOTE: TEER measurements are widely accepted as a method to analyze tight junction dynamics and barrier function integrity in biological models of physiological barriers, such as epithelial monolayers[28, 29]. Increase in TEER after differentiation because of increased cellular interaction at tight junctions can be measured using a manual TEER meter (for 24-well plates containing membrane inserts) or an automated TEER measurement robot (for HTS 24- and 96-well plates containing membrane inserts).

4.1.1. Measurement of TEER using a manual TEER meter

4.1.1.1. Clean the electrode with 70% ethanol, and let it air-dry inside the biosafety cabinet. Place the electrode in a tube containing BM. Connect the electrode to the manual TEER meter. Turn the **Function** switch to measure in **Ohms (Ω)**. Turn the **Power** switch on.

4.1.1.2. Place the short electrode in the apical compartment of the insert, while the long electrode is positioned in the basolateral compartment **(Figure 6A)**. Avoid touching the monolayer.

4.1.1.3. Measure resistance in the blank well (R_{blank}), and then measure the remaining samples (R_{sample}) in the same way. Wash the electrode with BM between samples with different conditions. Clean the electrode first with demi water and then with 70% ethanol and let it air-dry.

4.1.1.4. Calculate TEER (Ω·cm²): [R_{sample} (Ω) – R_{blank} (Ω)] × membrane area (cm²) **(Table 1** and **Figure 6B**).

4.1.2. Measurement of TEER using an automated TEER measurement robot

4.1.2.1. Perform automated TEER measurements when using HTS systems for 96-well and 24-well HTS plates containing membrane inserts. Use different electrodes for TEER measurement for both types (24- and 96- HTS membrane inserts). To measure TEER using an automated TEER measurement robot, follow the manufacturer's instructions.

4.2. Measurement of epithelial barrier integrity and permeability

NOTE: This protocol introduces Lucifer Yellow permeability from the apical to basolateral compartment as an indication of monolayer integrity. This section describes fluorescence measurement in the basolateral compartment after a 1 h incubation step to evaluate monolayer permeability and thus, barrier integrity. This measurement is an end-point assay and is especially useful when testing compounds for their effect on barrier integrity.

4.2.1. Thaw Lucifer Yellow on ice, and let BM equilibrate to RT. For one 24-well plate of membrane inserts, prepare 5 mL of working solution of 60 µM Lucifer Yellow in BM.

NOTE: Lucifer Yellow is light-sensitive. Prepare dilutions in dark 1.5 mL sterile tubes with the biosafety cabinet light switched off.

4.2.2. Carefully remove the medium from the basolateral and apical compartments of the membrane inserts, as described in step 3.5.1. If desired, scratch one untreated monolayer using a pipette tip as a positive control for Lucifer Yellow leakage through a damaged barrier.

4.2.3. Add 150 µL of BM with 60 µM Lucifer Yellow to each apical compartment, and add 800 µL of BM without Lucifer Yellow to each basolateral compartment. Place the plate on a shaker at 37 °C, 50 rpm for 60 min.

4.2.4. In the meantime, prepare a standard curve of Lucifer Yellow in BM starting with the working solution prepared in step 4.2.1. Dilute 1:3 in each step until a concentration of 3 nM is reached. Include a negative control (BM only).

4.2.5. Transfer 100 µL of each standard in triplicate to a 96-well transparent plate. After 60 min incubation, transfer 100 µL of each basolateral well from the plate containing the membrane inserts (step 4.2.3) in triplicate to the 96-well transparent plate. Measure fluorescence of the plate using a plate reader at an excitation wavelength of 400 nm and an emission wavelength of 590 nm.

4.2.6. After correcting for the negative control value (BM only), use the standard curve values to calculate the Lucifer Yellow concentration in the basolateral compartment (final receiver concentration (µM)).

4.2.7. Calculate the apparent permeability coefficient (P_{app}) according to the following formula (**Figure 6C**):

$$P_{app}\ (cm/s) = \frac{\text{receiver volume (mL)} \times \text{final receiver concentration (µM)}}{\text{initial apical concentration (µM)} \times \text{membrane area (cm}^2\text{)} \times \text{assay time (s)}}$$

4.2.8. For a 24-well plate containing membrane inserts, use the following formula:

$$P_{app}\ (cm/s) = \frac{0.8\ \text{mL} \times \text{final receiver concentration (µM)}}{60\ \mu\text{M} \times 0.33\ \text{cm}^2 \times 3600\ \text{s}}$$

4.3. Fixing monolayers and preparing paraffin blocks for histology

NOTE: Epithelial monolayers can be used for histologic staining for evaluation of their cellular composition, polarity, and expression of different proteins of interest such as junctional proteins, proliferation, or differentiation markers. This section describes paraffin block preparation for histologic staining.

4.3.1. Carefully remove the medium from the basolateral and apical compartments of the membrane inserts, as described in steps 3.5.1.

4.3.2. Wash the monolayers by adding 150 µL of DPBS (without Ca^{2+} and Mg^{2+}) to each apical compartment and 800 µL to each basolateral compartment. Carefully aspirate the DPBS again, first from the basolateral compartment and then from the apical compartment.

NOTE: The basolateral compartment will stay empty from this step on.

4.3.3. In a fume hood, add 150 µL of 4% paraformaldehyde to each apical compartment, and incubate for 30 min at RT.

NOTE: From this step onwards, perform all actions in this section inside a fume hood, as paraformaldehyde is toxic.

4.3.4. Carefully aspirate the fixative from the apical compartments of the membrane inserts, and dispose of it as liquid halogen waste.

NOTE: From this step onwards, dispose of all liquid waste as liquid halogen waste.

4.3.5. Wash the monolayers by adding 200 µL of DPBS (without Ca^{2+} and Mg^{2+}) to each apical compartment, and carefully aspirate the DPBS again. Repeat this step one more time.

4.3.6. Add 200 µL of 25% ethyl alcohol (EtOH) to each apical compartment, and incubate for 15 min at RT. After 15 min, carefully aspirate the 25% EtOH from the apical compartments of the membrane inserts. Repeat with 50% EtOH solution and subsequently with 70% EtOH solution.

4.3.7. Add 200 µL of 70% EtOH to each apical compartment, and wrap the plate with parafilm. Store it at 4 °C until further use.

4.3.8. Carefully aspirate the 70% EtOH, and use a scalpel to carefully cut the monolayer membranes from the inserts. Cut from the basolateral side, around the edge of the insert.

4.3.9. Prepare paraffin blocks following the standard procedure.

4.3.9.1. When the paraffin is still warm, take the monolayer from the paraffin with tweezers, and place it on a precooled surface.

4.3.9.2. Be careful not to damage the monolayer. Cut the monolayer in half using a single edge blade.

4.3.9.3. When the paraffin in the bottom of the cassette starts to solidify, use heated tweezers to place the two monolayer parts in the paraffin, next to each other with the straight side down and in a vertical direction to ensure that the monolayer will be vertical in the coupe.

4.3.10. When paraffin blocks are ready, cut the blocks using a microtome, and make slides of 4 µm thick sections following standard procedure. Make sure that the monolayers end up vertical in the coupe.

4.3.11. Perform histologic stains as described previously[7,9]. Use hematoxylin and eosin (H&E), Ki67, mucin-2 (MUC2), and Alcian Blue to show general morphology, proliferative cells, mucus production and goblet cells, respectively (**Figure 6E**).

NOTE: Additional differentiation markers, such as lysozyme for Paneth cells, can be used as well. This marker is not presented in **Figure 6E** as Paneth cells are present in small intestinal epithelium rather than colon epithelium.

4.4. Secreted protein measurement in medium supernatant

4.4.1. Measure lysozyme levels in the apical supernatant of ileal monolayers (see **Figure 6D**). If desired, measure levels of different cytokines and other proteins of interest.

4.5. Gene expression analysis

4.5.1. Quantify the effects of the differentiation media on the expression of epithelial cell marker genes using quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

4.5.1.1. Lyse the monolayers in 350 µL of RNA lysis buffer followed by RNA isolation according to the manufacturer's instructions. Perform cDNA synthesis and qPCRs, as described earlier[7,9], using the cDNA synthesis kits, master mix, and oligonucleotides listed in the **Table of Materials**.

5. Upscaling to 96-well plates containing membrane inserts

NOTE: Prepare epithelial monolayers for higher throughput drug screenings or multiple medium conditions using HTS 96-well plates containing membrane inserts. TEER measurements will then require an automated TEER measurement robot (**Table of Materials**).

5.1. Adaptations when preparing monolayers in 96-well format

5.1.1. Follow all steps described in this protocol for 24-well plates containing membrane inserts, changing volumes and cell numbers to those described in **Table 1**. For preparing monolayers on 96-well plates with membrane inserts, proceed as described in section 3 with the following differences.

5.1.1.1. NOTE: Approximately 9 wells of a 6-well culture plate with organoid density represented in **Figure 3A** are needed to seed a full 96-well plate with membrane inserts. In step 3.2.1, precoat the membranes with 67 µL of 40x diluted ECM in DPBS (with Ca^{2+} and Mg^{2+}).

5.1.1.2. In section 3.5, first transfer the integral plate of membrane inserts to another 96-well plate to allow medium refreshment of both apical and basolateral compartments.

REPRESENTATIVE RESULTS:

Figure 1A shows a representative brightfield image of intestinal organoids after thawing them from a cryovial. It is important to thaw organoids at a high density to ensure optimal recovery. Organoids are plated in 24- or 6-well plates in ECM domes of approximately 10 µL (**Figure 1B**). Most normal intestinal organoids have a cystic morphology. After recovering from the thawing process, the organoids grow to a bigger size and are ready to be passaged after 3–7 days depending on the organoid culture (**Figure 1C**). After harvesting the organoids and washing the ECM away (**Figure 1D**), organoids can be disrupted to a small size by mechanical shearing. Depending on the morphology of the organoids (cystic **Figure 1C**, budding **Figure 1E**), organoids can be disrupted using plastic or glass pipettes (**Figure 1F**). Organoids are disrupted in a suspension (**Figure 2A**), which should be regularly monitored under the microscope. It is important to avoid making them too small, as groups of cells need to stay together to ensure organoid growth. **Figure 2B** shows the organoids in ECM drops just after passaging. In general, cystic organoids are plated at a relatively high density while budding organoids are plated in a low density; however, this can differ between different organoid cultures.

When passaging organoids for the preparation of monolayers, be sure to plate them at a high density, and let them grow for three days so they are in optimal expansion conditions. Organoids can be harvested for monolayer preparation when they are comparable with **Figure 3A** in size and density, where 6 wells of a 6-well plate, each containing 200 µL of organoid domes, are typically enough for seeding a full 24-well plate of membrane inserts. After the preparation of a single-cell suspension with the cell dissociation reagent, single cells and small clumps of cells should be visible (**Figure 3B**), and live cells can be counted (**Figure 3C**). The arrows indicate dead

cells stained with trypan blue, which should be excluded from counting. The single cells and small clumps are then seeded in the membrane inserts as seen in **Figure 4A**. Monolayer formation is visible after 1–3 days (**Figure 4B,C**), and the monolayers will be generally be confluent after 3–6 days depending on the organoid culture (**Figure 4D**). Monolayers stay in expansion medium until they are confluent, after which they can be enriched with, amongst others, enterocytes or goblet cells using different enrichment media. **Figure 5A** shows a monolayer that was cultured for 8 days in expansion medium (IEM). When enriched with enterocytes (eDM), a structure is seen, as in **Figure 5B**, while monolayers exposed to combination medium (cDM) show a smoother structure (**Figure 5C**).

Monolayer formation can be quantitatively followed by measuring TEER (**Figure 6A**). A completely confluent monolayer has a TEER value of ~100 $\Omega \cdot cm^2$, which increases to ~1000 $\Omega \cdot cm^2$ when exposed to either differentiation medium (**Figure 6B**). Monolayers in all medium conditions are impermeable to Lucifer Yellow (0.45 kDa), while an increase in apparent permeability (P_{app}) can be seen when the monolayers were purposely scratched (**Figure 6C**). Lysozyme secretion by ileal monolayers cultured in IEM was higher than that of monolayers cultured in IEM until confluent and for another 4 days in eDM or cDM (denoted as + subsequent eDM or cDM) (**Figure 6D**). Monolayers cultured in IEM, IEM + subsequent eDM or IEM + subsequent cDM show different morphology, as can be observed with H&E staining (**Figure 6E**). While colon organoid-derived epithelial monolayers in IEM and cDM media have a smooth apical surface, enterocyte-differentiated monolayers present an invaginated apical morphology in the absence of Wnt. Ki67-positive proliferative cells can be detected in expansion conditions only. Alcian Blue and MUC2 stain mucus produced by goblet cells, which is visualized in the monolayers differentiated in eDM and more prominently in cDM when Wnt, Notch, and EGF signaling are inhibited, respectively (**Figure 6E**). Upon differentiation, proliferative cells decrease while goblet cell and enterocyte marker gene expression increases in comparison to that observed under IEM conditions, as shown by LGR5, MUC2, and ALPI gene expression quantification by qRT-PCR, respectively (**Figure 6F**).

FIGURE AND TABLE LEGENDS:

Figure 1: Establishing an intestinal organoid culture from frozen organoids. (**A**) Representative brightfield image of an intestinal organoid culture after thawing. (**B**) ECM domes (50 µL) seeded in each well of a 24-well culture plate. (**C**) Representative image of a normal intestinal organoid culture ready for passaging. (**D**) Representative image on how to check the presence of ECM in a 15-mL tube containing organoids under a light microscope. (**E**) Representative image of a budding intestinal organoid culture ready for passaging. (**F**) A 10 µL plastic pipette tip fitted on a low-retention 1250 µL filter tip (left) and a narrowed glass pipette (right) for mechanical shearing of organoids. Scale bars = 100 µm. Abbreviation: ECM = extracellular matrix.

Figure 2: Processing intestinal organoids for maintenance or preparation of monolayers. (**A**) Representative image of intestinal organoids after mechanical disruption. (**B**) Representative image of an intestinal organoid culture seeded after passaging. Scale bars = 100 µm.

Figure 3: Preparing single cells from intestinal organoids for monolayer preparation. (**A**) Intestinal organoids ready to harvest for monolayer preparation. (**B**) Single cells and small clumps of cells after single cell preparation. (**C**) Visible single cells and small clumps during counting in a grid chamber. Scale bars = 100 µm.

Figure 4: Monolayer formation after seeding single cells on membranes. (**A**) Single cells just after seeding on membranes. On average, (**B**) the monolayer is around 50% confluent 1–3 days after seeding, (**C**) ~90% confluent at day 3–5, and (**D**) the complete monolayer has formed around day 4–7. Scale bars = 100 µm.

Figure 5: Enrichment of specific cell types in the monolayer. (**A**) Monolayer after 8 days in IEM. (**B**) Monolayer enriched with enterocytes after 4 days in IEM and another 4 days in eDM. (**C**) Monolayer enriched with goblet cells and other cell types after 4 days in IEM and another 4 days in cDM. Scale bars = 100 µm. Abbreviations: IEM = intestinal organoid expansion medium; eDM = enterocyte differentiation medium; cDM = combination differentiation medium.

Figure 6: A variety of possible readouts using epithelial organoid monolayers. (**A**) Electrode in the membrane insert to measure TEER. (**B**) TEER values increase in time with a value of ~100 $\Omega \cdot cm^2$ when the monolayer reaches confluence. After enriching monolayers with enterocytes or a combination of different epithelial cells, TEER increases to 1000 $\Omega \cdot cm^2$ or higher. (**C**) Monolayers in all medium conditions (IEM + 4 days IEM/eDM/cDM) are impermeable to Lucifer Yellow. (**D**) Expression of lysozyme is higher in ileum monolayers when grown in expansion medium than in either type of differentiation medium (IEM + 4 days IEM/eDM/cDM). (**E**) Colon monolayers show different morphologies when exposed to different medium conditions (IEM + 4 days IEM/eDM/cDM) as visualized by H&E, Ki67, Alcian Blue, and MUC2 stains. As expected, monolayers cultured in expansion medium are very proliferative, as shown by Ki67 staining. Monolayers differentiated with eDM show a columnar epithelium without proliferative cells. Monolayers exposed to cDM are also not proliferative and develop more goblet cells. Scale bar = 100 µm. (**F**) Stem cell (LGR5), goblet cell (MUC2), and enterocyte (ALPI) marker gene expression in colon monolayers by qRT-PCR. Abbreviations: TEER = transepithelial electrical resistance; IEM = intestinal organoid expansion medium; eDM = enterocyte differentiation medium; cDM = combination differentiation medium; P_{app} = apparent permeability coefficient; LGR5 = leucine-rich repeat-containing G-protein-coupled receptor 5; H&E = hematoxylin and eosin; AB = Alcian Blue; MUC2 = mucin-2; ALPI = intestinal alkaline phosphatase; qRT-PCR = quantitative reverse-transcription polymerase chain reaction .

DISCUSSION:

This protocol describes the general manipulation and maintenance of intestinal organoids as well as the preparation and possible applications of epithelial monolayers derived from these organoids. To date, monolayers have been successfully prepared from the duodenum, ileum, and different regions of colon organoids derived from normal as well as previously and actively inflamed intestinal tissue (unpublished data). The application of patient-derived organoid monolayers facilitates the study of barrier function in a disease- and patient-specific manner as well as the study of patient-specific responses to a variety of drug treatments. Although cell lines

can form differentiated and polarized monolayers containing intestinal enterocytes and goblet-like cells, many different enzymes and transporters are aberrantly expressed in these cell lines, resulting in reduced complexity and physiological relevance[3,30]. Organoid culture is technically more demanding and laborious than cell culture; however, organoids are more representative of the in vivo situation, whereas cell lines have repeatedly failed to represent tissue responses in complex setups.

Although primary tissue-based models can be representative of the in vivo situation, they require the use of test animals or access to human material, which is associated with limited availability and ethical constraints. Additionally, primary tissue has no or limited expandability and is not stable in extended experimental timeframes. Organoid technology requires limited amount of primary tissue to establish genetically and physiologically stable long-term expanding cultures while still representing epithelial tissue complexity and patient heterogeneity. Different cell lines, such as Caco-2, are often used to prepare epithelial monolayers. Caco-2 cells take 21 days to establish a polarized epithelial monolayer that can be used for any experiment[30]. Organoid-derived epithelial monolayers are prepared from organoid single cells as described in the current protocol, and after 3–6 days, form a polarized epithelium that can be further differentiated to enrich them with enterocytes or goblet cells.

Monolayers are a static model lacking a microfluidic flow or mechanic stretch as is seen in organs-on-a-chip. They do, however, offer the opportunity for co-culturing with (autologous) immune cells, as well as bacteria or parasites[17–19, 31]. Organoids are suitable for monolayer preparation when they are in their expansion phase; for intestinal organoids, this is usually 3 days after passaging. Dissociation of organoids to single cells should be performed quickly to avoid long-term exposure to digestive enzymes. To increase the survival of stem cells after the preparation of a single-cell suspension, the Rho-associated protein kinase inhibitor (ROCK inhibitor), Y27632, is added to the cells to prevent anoikis-induced cell death[32]. Furthermore, it is critical to culture the monolayers on a membrane precoated with ECM to ensure that they maintain their organoid characteristics and polarization. For functional screening assays that quantify GI tract epithelial cell responses to external stimuli, it is important that organoid cultures represent the required cellular complexity depending on the type of assay to be developed and eventual readouts.

Intestinal organoids represent different epithelial cell types present in vivo, such as stem, TA, enterocyte, Paneth, goblet, and enteroendocrine cells[4,5], and are prepared and maintained using a defined organoid medium prepared as described in this protocol. Enterocyte differentiation can be promoted by culturing organoids for an additional four days using culture conditions that dually inhibit the Wnt pathway, by removal of Wnt molecules/activators, and the addition of the Porcupine inhibitor, IWP-2 (enterocyte colon differentiation medium, eDM). As mucus-producing goblet cells are essential for barrier function homeostasis as well, a second culture condition aims to produce a more heterogeneous cell population containing stem, enterocyte, and goblet cells, in which Notch and EGF pathways are inhibited while Wnt signaling is kept partially active by reducing Wnt3aCM to 10% (or 0.1 nM for NGS-Wnt) instead of 50% (0.5 nM NGS-Wnt) used in IEM. In contrast to eDM, which enriches for enterocyte differentiation, this second condition

supports the presence of several cell types and is therefore called combination differentiation medium (cDM).

Applications of organoid-derived monolayers include the monitoring of epithelial barrier integrity as well as examining tight junction and transporter expression. Barrier integrity, permeability, and transport can be analyzed using the readouts introduced in this protocol. While TEER measures ionic conductance through tight junctions, the permeability assay measures water flow and thus paracellular permeability through the tight junctions[28,29]. Epithelial barrier integrity, permeability, and transport functionality of the monolayers can be evaluated by measuring the traffic of different fluorescent or radioactive substrates across apical and basolateral compartments of the membrane inserts. TEER measurements allow for the quantification of barrier integrity, showing an increase in values when differentiating towards polarized enterocytes and goblet cells, and a decrease after inducing injury to the monolayers.

The Lucifer Yellow permeability assay can be used for initial barrier integrity assessment as well as the confirmation of reduced integrity after inducing injury to the monolayers. This protocol introduces Lucifer Yellow permeability from the apical to basolateral compartment as an indication of monolayer integrity. Similarly, other fluorescently labeled reagents, such as 4 or 40 kDa dextran, can be employed to evaluate increased paracellular permeability as the result of barrier damage-inducing agents. Fluorescently labeled substrates, such as Rhodamine 123, can be used for measuring the activity of different transporters, such as P-glycoprotein-1. The fluorescence assay described in this protocol allows the measurement of the levels of proteins, such as lysozyme, that are secreted into the apical compartment. Responses to injury induction by pro-inflammatory cytokines can be measured with these readouts, as well as the potential effects of barrier-restoring compounds.

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DISCLOSURES:
The authors declare no conflict of interest.

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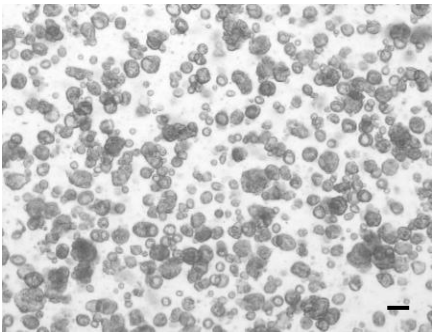
Figure 1

A



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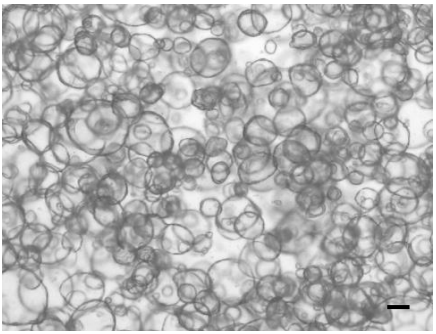

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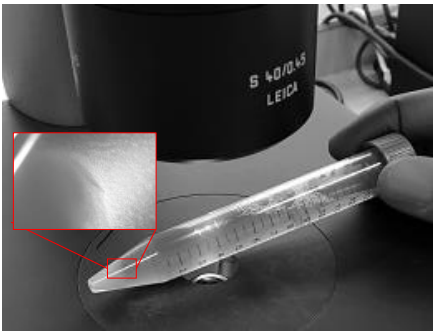

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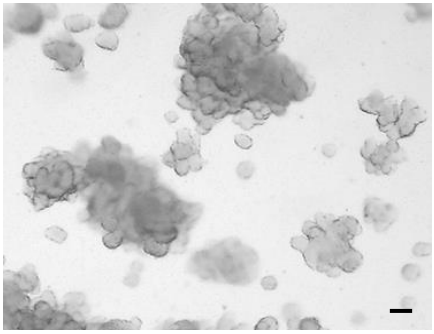

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Figure 2

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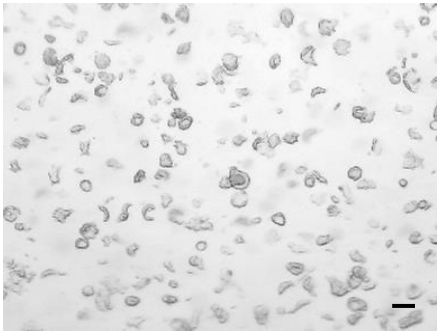

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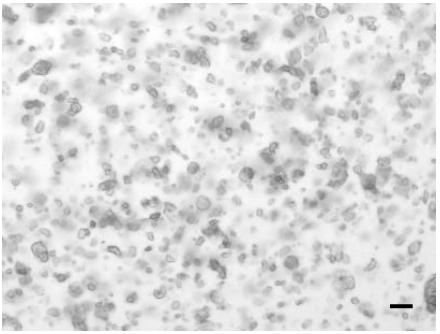

Figure 3

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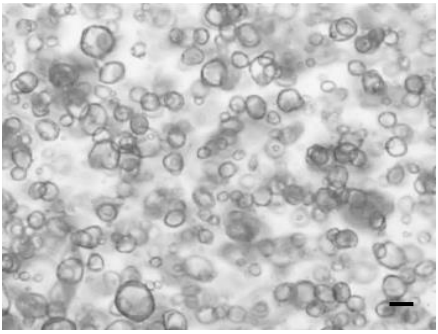

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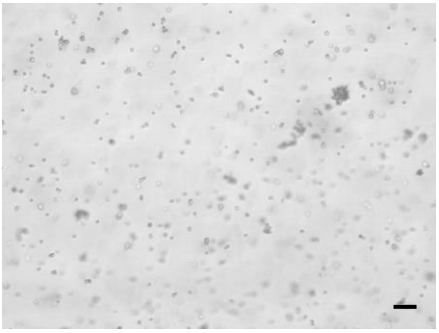

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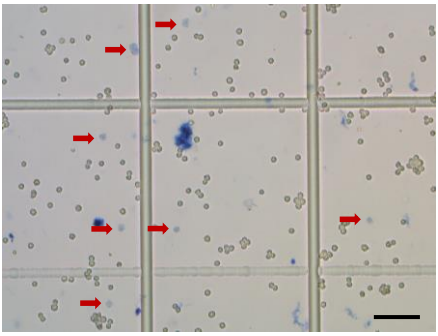

Figure 4

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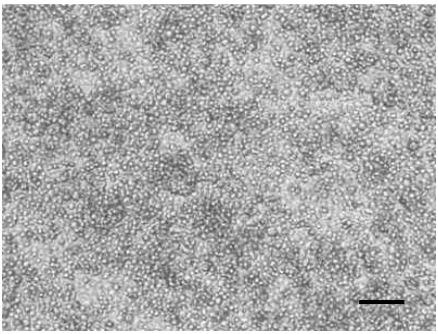

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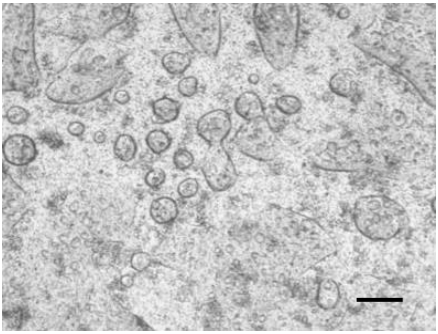

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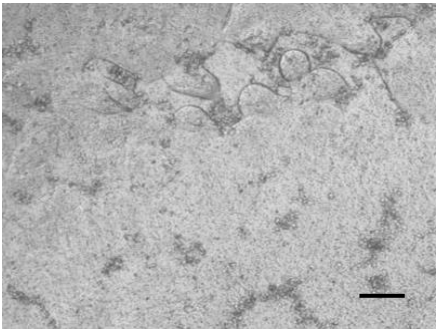

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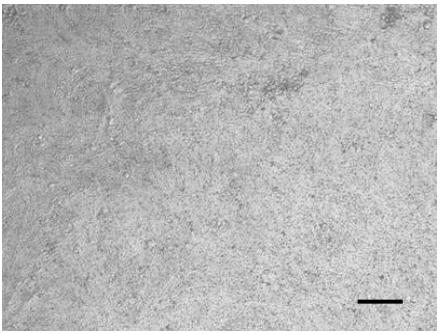

Figure 5

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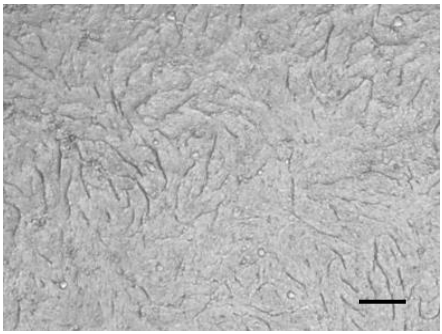

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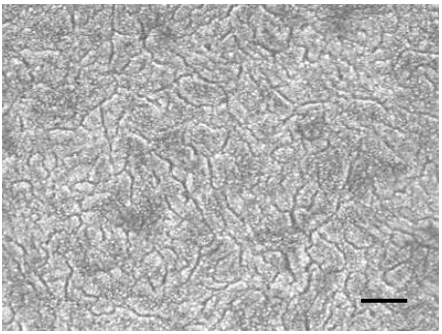

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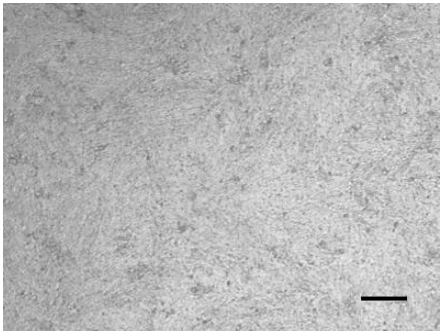

Figure 6

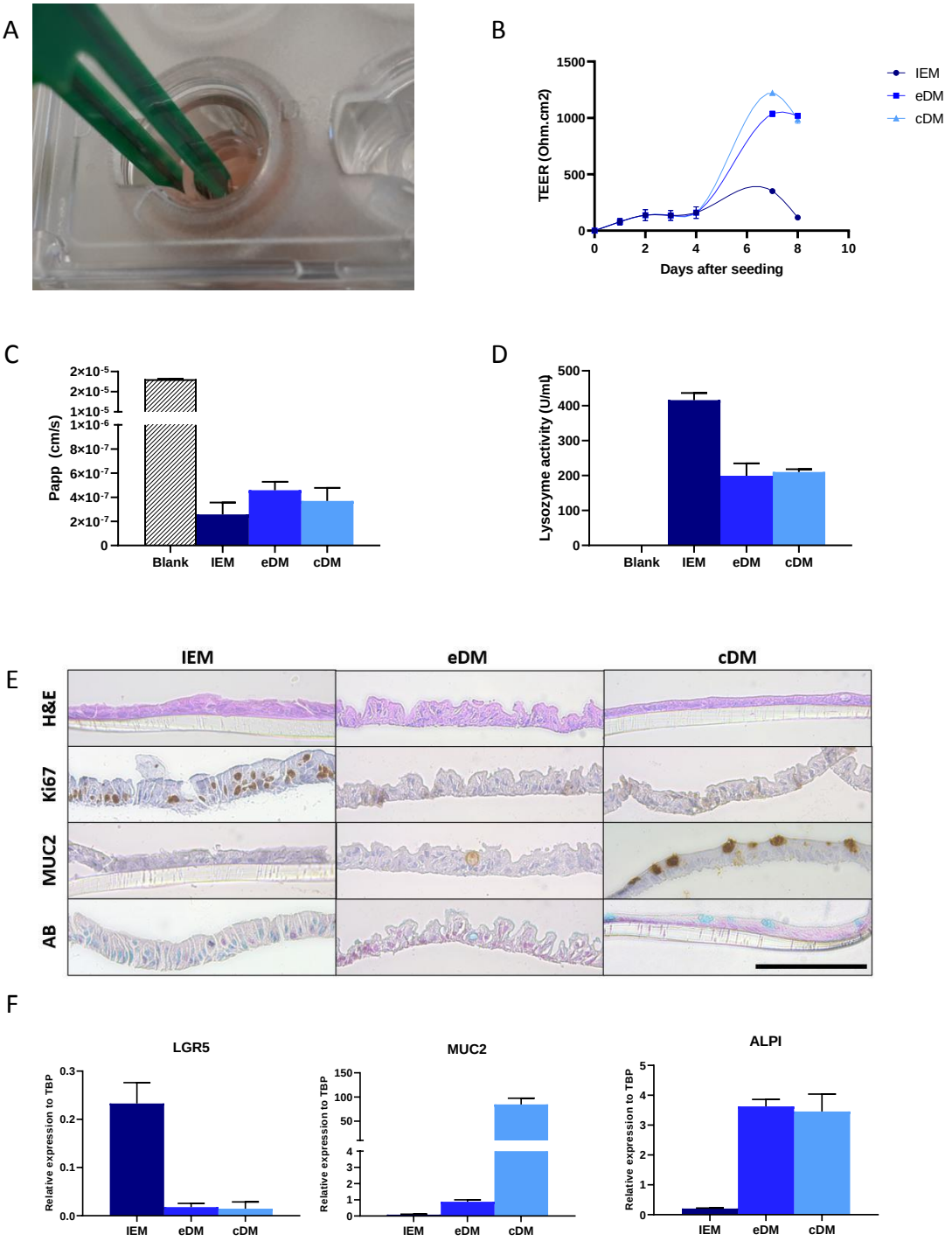

Table 1

	Membrane inserts	
	24-well plates	**96-well plates**
Membrane surface (cm^2)	0.33	0.143
Apical volume (µL)	150	100
Basolateral volume (µL)	800	300
# Cells to seed per well	450,000	200,000
HTS plates with membrane inserts	PET	PET
Plates with separate inserts	PET	NA
Light-tight plates with membrane inserts		
Electrodes are different for the two formats	Refer to the Table of Materials	

Name of Material/ Equipment	Company	Catalog Number	Comments
100% ethanol	Fisher Emergo	10644795	
1250, 300, and 20 µL low-retention filter-tips	Greiner bio-one	732-1432 / 732-1434 / 732-2383	
15 mL conical tubes	Greiner bio-one	188271	
24-well cell culture plates	Greiner bio-one	662160	
24-well HTS Fluoroblok Transwell plate (light-tight)	Corning	351156	
24-well HTS Transwell plates (Table 1)	Corning	3378	Plates require REMS AutoSampler for TEER measurements
24-well plate with Transwell inserts	Corning	3470	membrane inserts
40 µm cell strainer	PluriSelect	43-50040-01	
50 mL conical tubes	Greiner bio-one	227261	
6-well cell culture plates	Greiner bio-one	657160	
96-well black plate transparent bottom	Greiner bio-one	655090	
96-well fast thermal cycling plates	Life Technologies Europe BV	4346907	
96-well HTS Fluoroblok Transwell plate	Corning	351162	
96-well HTS Transwell plates (Table 1)	Corning	7369	
96-well transparent culture plate	Greiner bio-one	655180	
A83-01	Bio-Techne Ltd	2939	
Accutase Cell Dissociation Reagent	Life Technologies Europe BV	A11105-01	
Advanced DMEM/F-12	Life Technologies Europe BV	12634028	
B27 supplement	Life Technologies Europe BV	17504001	
Cell culture microscope (light / optical microscope)	Leica		
CellTiter-Glo	Promega	G9683	
Centrifuge	Eppendorf		
CO_2 incubator	PHCBI		
DAPT	Sigma-Aldrich	D5942	

DEPC treated H_2O	Life Technologies Europe BV	750024	
Dulbecco's phosphate-buffered saline (DPBS) with Ca^{2+} and Mg^{2+}	Life Technologies Europe BV	14040091	
DPBS, powder, no calcium, no magnesium	Life Technologies Europe BV	21600069	
EnzChek Lysozyme Assay Kit	Life Technologies Europe BV	E22013	
EVOM2 meter with STX electrode	WTI		
Gastrin	Bio-Techne Ltd	3006	
Glass pipettes	Volac		
GlutaMAX	Life Technologies Europe BV	35050038	
hEGF	Peprotech	AF-100-15	
HEPES	Life Technologies Europe BV	15630056	
Human Noggin	Peprotech	120-10C	
Human Rspo3	Bio-Techne Ltd	3500-RS/CF	
IWP-2	Miltenyi Biotec	130-105-335	
Ki67 primary antibody	Sanbio	BSH-7302-100	
Ki67 secondary antibody	Agilent	K400111-2	
Kova International Glasstic Slide with Counting grids	Fisher Emergo	10298483	
Laminar flow hood	Thermo scientific		
Lucifer Yellow CH dilithium salt	Sigma-Aldrich	L0259	
Matrigel, Growth Factor Reduced (GFR)	Corning	356231	extracellular matrix (ECM)
MicroAmp Fast 8-Tube Strip, 0.1 mL	Life Technologies Europe BV	4358293	
MicroAmp Optical 8-Cap Strips	Life Technologies Europe BV	4323032	
Microcentrifuge tubes	Eppendorf	0030 120 086	
Micropipettes (1000, 200, and 20 µL)	Gilson		
Microtome	Leica		
MUC2 primary antibody	Santa Cruz Biotechnology	sc-15334	
MUC2 secondary antibody	VWR	VWRKS/DPVR-HRP	

Multichannel pipette (200 µL)	Gilson		
N-acetylcysteine	Sigma-Aldrich	A9165	
NGS Wnt	U-Protein Express	N001-0.5mg	
Nicotinamide	Sigma-Aldrich	N0636	
Oligonucleotide ALPI1/Forward	Custom-made	GGAGTTATCCTGCTCCCCAC	
Oligonucleotide ALPI1/Reverse	Custom-made	CTAGGAGGTGAAGGTCCAACG	
Oligonucleotide LGR5/Forward	Custom-made	ACACGTACCCACAGAAGCTC	
Oligonucleotide LGR5/Reverse	Custom-made	GGAATGCAGGCCACTGAAAC	
Oligonucleotide MUC2/Forward	Custom-made	AGGATCTGAAGAAGTGTGTCACTG	
Oligonucleotide MUC2/Reverse	Custom-made	TAATGGAACAGATGTTGAAGTGCT	
Oligonucleotide TBP/Forward	Custom-made	ACGCCGAATATAATCCCAAGCG	
Oligonucleotide TBP/Reverse	Custom-made	AAATCAGTGCCGTGGTTCGTG	
Optical adhesive covers	Life Technologies Europe BV	4311971	
PD0325901	Stemcell Technologies	72184	
Penicillin/streptomycin	Life Technologies Europe BV	15140122	
Plate shaker	Panasonic		
PowerUp SYBR Green Master Mix	Fisher Emergo	A25776	
Primocin	InvivoGen	ANT-PM-2	antimicrobial formulation for primary cells
Qubit RNA HS Assay Kit	Life Technologies Europe BV	Q32852	
Reagent reservoir for multichannel pipet	Sigma-Aldrich	CLS4870	
REMS AutoSampler with 24-probe or 96C-probe	WTI		
Richard-Allan Scientific Alcian Blue/PAS Special Stain Kit	Thermo scientific	87023	
RNase-Free DNase Set	Qiagen	79254	
RNeasy Mini Kit	Qiagen	74106	
SB202190	Sigma-Aldrich	S7076	
Serological pipettes	Greiner bio-one	606180 / 607180 / 760180	
Serological pipettor (Pipet-Aid)	Drummond		

Single edge razor blade	GEM Scientific		
Superscript 1st strand system for RT-PCR	Life Technologies Europe BV	11904018	
Tecan Spark 10M plate reader	Tecan		
Trypan Blue Solution, 0.4%	Life Technologies Europe BV	15250-061	
TrypLE Express Enzyme (1x)	Life Technologies Europe BV	12605-010	Cell dissociation reagent
Water bath	Grant		
Y27632 (ROCK inhibitor)	AbMole	M1817	

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Dr. Vineeta Bajaj
Review Editor JoVE
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Dear Dr. Bajaj,

After our revisions, we gladly resubmit our manuscript entitled: "Organoid-derived epithelial monolayer: a patient-relevant in vitro model for intestinal barrier function." (reference JoVE62074).

We wish to thank the editor and reviewers for the critical review of our manuscript and providing their valuable suggestions, which have improved our manuscript considerably. We would also like to thank the editor for providing us with the opportunity to resubmit a revised version of our manuscript. The manuscript has been revised according to the comments and suggestions put forward by the reviewers.
Unfortunately, due to the conflict of interest from our industrial partner, we had to remove data that was presented in the first submission under Figure 7. We believe the manuscript can stand as a method paper without this data, since the feasibility of barrier function experiments has been elaborated in the discussion section.
In the rebuttal letter we have included our detailed reply, in which we have provided point by point responses.

We hope our revisions will make the manuscript suitable for publication in your journal.

Yours sincerely, on behalf of all authors

Wies van Dooremalen
Sylvia Boj, PhD
Farzin Pourfarzad, PhD

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 have reviewed the manuscript and all spelling errors are corrected. Abbreviations are also defined in their first use.

2. 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: Mimetas OrganPlates; FluoroBlok; Accutase; REMS AutoSampler; EnzChek Lysozyme Assay Kit; CellTiter-glo 3D etc

All the trademarks, registered symbols and company names are removed and referred to in the Table of Materials.

3. 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 have revised the manuscript and imperative tense has being used along the text. We have also included sentences about the use of biosafety cabinets (line 115-116).

4. 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 have included more details in some of the procedures to ensure proper understanding of the protocol.

5. 2.1.1: Please clarify what "a little frozen material" means to help readers replicate this step.

We have replaced the sentence "a little frozen material" by "until only a sliver of ice remains".

6. Line 521: please remember to use period as decimal, not comma.

We have replaced all comma's with periods in the decimals described in the manuscript.

7. 4.3.12: for staining procedures, please cite references even if you do not provide details for these techniques in this protocol.

We have included several references in step 4.3.12.

8. As we are a methods journal, please also include limitations of your method in the Discussion section.

We have addressed limitation points of our method in the Discussion section.

9. Please rename Table 2 as Table 1 and Table of Materials as Table of Materials.

We have renamed the Tables as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This is a very thorough protocol describing the thawing, passage, and establishing of monolayers from organoids as well as various assays that can be performed with these monolayers. It will be very helpful for beginners in the field.

Dear reviewer 1,
Thank you very much for your positive comments. We are very glad to hear that our manuscript will be helpful in the field. Below we will respond to your comments.

Major Concerns:
none

Minor Concerns:
Minor clarity issues in the protocol:
Protocol: EGF, hRspo3 ,iWP: please spell out the full names of these compounds at first mention to avoid confusion.

All compounds have now been spelled out completely at first mention.

Basement membrane matrix: There is many different kinds and manufacturers of BMM. The material section mentions Matrigel. Does the protocol work with all of them? If yes, please indicate. If not, please specify also in the text.

In the manuscript all experiments were performed with Matrigel, which is now referred to as Extracellular Matrix (ECM) in the text. The Matrigel specification is referred to in the Table of Materials. We have tested other ECMs such as Cultrex® Basement Membrane Extract (BME) from Trevigen, but human healthy intestinal organoids did not grow neither behaved the same way as when using Matrigel, so we recommend users to use Matrigel as ECM.

Section 2.2.6.+7 "Before removing the BM, check under the microscope whether all organoids are pelleted at the bottom of the 15 mL conical tube (Figure 1D). If there is no BMM or the BMM is either clean or contains just debris, single cells or very few organoids compared with the organoids pelleted, aspirate supernatant and pipet out the BMM overlaying the organoid pellet very carefully using a P200 pipette.
If BMM contains organoids, spin the tube again at 450 x g for 5 min at 8 °C."

This part of the instructions is very confusing for someone that didn't work with organoids before. Please attempt to explain that due to the low g spin in 2.2.5., organoids can get stuck in the matrix

Thank you for your critical read and reflection. This is now clarified in the text.

Section 3.2.19. Count the live, not blue, cells (Figure 3C) and calculate the total number of live cells."
Please specify whether the cell clumps mentioned in 3.2.9. are being counted as one or individual cells

We have specified how to count clumps of cells in step 3.2.19.

Line 617: the abbreviation MG here probably means Matrigel? Specify

The reviewer is correct, and it has been replaced by ECM.

4.3.3.4% paraformaldehyde and 4% formalin is not equivalent. I assume you mean 10% formalin. Maybe also point out that fixative is toxic and needs to go into special waste.

Thank you for the comment. The correction has been made and additional sentences for toxicity have been added.

Results and legends: I think the representative results section could possibly be integrated into the figure legends (especially as the figure legends don't contain descriptions of all panels.)

We would like to avoid describing figure results in the figure legend and therefore leave representative result section as it is. We have added descriptions to all figure panels in the figure legends.

Discussion: Why is Y27632 mentioned for the first time in the discussion and never in the methods? I assume in the rest of the text this is what is meant with RhoKi? Please unify.

Thank you for pointing out the discrepancy. This compound was used in step 2.1.7. but identified with another name. We have included Y27632 in this step.

Reviewer #2:

Manuscript Summary:

This manuscript describes a protocol to establish intestinal organoid epithelial monolayers. They also described some methods to characterize the epithelial functions such as epithelial barrier integrity, permeability and secretion, as well as a method to perform histology. Organoid cultures are actually a very powerful tool and easily overpass regular cell lines. This method is certainly of interest as one of the challenges is indeed to use these cells in a 2D setup.

The authors have done a very good job with the manuscript. Well written, with good image which are well annotated. I recommend only small modification.

Dear reviewer 2,
Many thanks for your interest, positive feedback, and comments. Below we have addressed your comments point by point.

Major Concerns:

—Sections 4.4 and 4.5: These sections are not novel or of sufficient interest to be included in this protocol. Indeed, these sections only summarizes the protocol of commercial kits as we would do for any cell types. Only a small note suggesting the use of this kit may be included.

This point has been well taken. Unnecessary text has been removed and methods are referred to kit manufacturer instructions. Section 4.5 cell viability has been completely removed since Figure 7 had to be removed in the final version of the manuscript due to restrictions from our industrial partner.

Minor Concerns:

—Line 86: (Normal) should be removed

Instead of removing this word, we have chosen to remove the term 'healthy' from the text and replaced this with the term 'normal' everywhere. This choice was made because the normal tissue is taken from patients as the counterpart of tumor tissue and often, it is taken in close proximity from the tumor. From sequencing data we know that this tissue might contain some genomic alterations as well. The term normal is therefore more suitable than the term healthy.

—Page 3: Not sure about the rationale to put a capital letter to organoids. It is also sometimes written without a capital letter.

We have changed "Organoids" to "organoids" in all instances.

—Line 231: Should we read this sentence : "If there is no BMM layer at the bottom or the BMM layer is either clean or contains..."? If not please add more precision.

Thank you for the comment. We have clarified the sentence.

—Lines 230-231: Should BM or BMM replace by another abbreviation? It is confusing at some moment. I suggest the use of the word "matrix" throughout the protocol.

We agree with the reviewers point. BMM has been changed to ECM (extracellular matrix) in all instances.

—Line 444: Should it be repeated that the electrode should be kept in the hood for air dry and then in sterile BM?

We have added the extra instruction to step 4.1.1.1.

—Line 724: (Normal) should be removed

As explained in the previous similar comment, we have changed all terms 'healthy' into 'normal'.

Reviewer #3:

Manuscript Summary:

This manuscript provides very useful protocols for intestinal barrier function assessment using organoids grown as monolayers on transwells. Both TEER and permeability assay using fluorescent probes are described, including protocol for barrier disruption. Figures 1-5 are great for reference/visual aid, particularly for crucial organoid culture steps and transwell work. Some aspects of this manuscript however should be improved.

Dear reviewer 3,

We would like to thank you for your positive feedback, comments, and critical read, which have helped us to improve the quality of our manuscript. Below we have responded to your comments point by point.

Major Concerns:

1. Important information is missing, please include details on organoid media, supplements and antibodies used (manufacturer and product codes). Specify that BMM used is Matrigel (perhaps in line 157?)

Information about the reagents used has been carefully checked and reflected in the Table of Materials. BMM has been changed to ECM (extracellular matrix) in all instances and we have specified the use of Matrigel in the manuscript (line 165).

2. Dynamic models with microfluidic flow and mechanic stretch that mimics peristalsis are a better approximation of in vivo situation than the static 2D model on transwells (although they all lack other components- for example immune cells). Please provide evidence to support the claim "organoid cultures are truly representing in vivo situation... and tissue complexity" in last sentence in abstract and in text later on.

We recognize the comment of the reviewer and references from previously published literature have been added to reflect these points. Additionally, the advantages of the microfluidic system have been added to the Discussion. In the abstract, we have specified that the statement about organoids representing the in vivo situation is regarding the epithelial compartment and when compared with the use of conventional 2D cell lines for monolayer preparation.

3. Figure 6D should be improved:

-all three conditions (IEM, eDM, cDM) have big differences in epithelium thickness and morphology within their group,

-this model should demonstrate the presence of differentiated epithelial cells, hence a marker of differentiated enterocytes should be included as well as enteroendocrine (and Paneth) cells

-MUC2 and AB staining is limited to goblet cells in cDM group, whereas in eDM group it looks like the positive staining is limited to mucus layer caught in the invaginations, the staining is not clear

-images would be greatly improved if there was also a magnified region of interest shown
-include scale bar

To improve Figure 6, some alternative images have been included for better representation of histological changes.
We agree with the point of the reviewer, however, we thought that H&E stain showing a simple columnar epithelium in eDM condition compared with the simple cuboidal epithelium shown in IEM condition, would already indicate the enrichment for enterocytes in eDM condition. Due to the impossibility to perform an enterocyte-specific stain in monolayers, we have included a new panel in updated version of Figure 6 (Figure 6F) that shows RT-qPCR data for the enterocyte marker ALPI.
Regarding the presence of specific cell types, such as Paneth cells, the images from panel D (panel E in new version of Figure 6) were generated from monolayers established from colon organoids, and as such Paneth cells are not present. In case of goblet cells, their differentiation requires inhibition of Notch and Wnt signalling. In the eDM condition only Wnt signalling is inhibited. Since cDM condition contains the Notch inhibitor DAPT, MUC2 expression was detected in this specific condition. We have included RT-qPCR data (Figure 6 F) to support this observation.
The images shown in Figure 6E are made using the highest magnification that our microscope with image software allowed. Therefore, it was not possible to show a magnified region of interest in the images.
A scale bar has also been included.

Minor Concerns:
line 87 references quote cancer tissue derived organoids, please provide references to organoids derived from inflamed tissue

Two extra references (15. and 16.) have been included.

line 137 primocin at 100 ug/ml is recommended for working concentration (hence this reagent is not at 2x as the line 135 suggests). Media that is used to dilute the 2xIBM doesn't contain 1x primocin (line 144, 149/150,..), is this correct?

We appreciate your accuracy in reading the text. This is correct, we use half of the recommended concentration of Primocin for organoids.

line 638 it would be very helpful to include the approximate numbers of BMM organoid domes needed to seed one 24 well transwell.

We have indicated the volume of organoid domes approximately needed for a full 24-well Transwell plate in line 636.

line 654 include the size/molecular weight of lucifer yellow

We have indicated the molecular weight of Lucifer Yellow (0.45 KDa) in the requested section (line 653)

figure 7A this figure would be improved with a wider range of colours to make the distinction between results easier

Due to the conflict of interest from our industrial partner, we had to remove Figure 7 from the manuscript. Although the data in Figure 7 in the first submission would have added value to the manuscript, we believe the manuscript still contains relevant information for a method paper without this data. Nevertheless, feasibility of barrier function experiments has been elaborated in the discussion section.

Reviewer #4:

Manuscript Summary:
The authors nicely describe techniques to generate and analyze primary organoid-derived Transwell-like models. This approach is of high interest in preclinical research and development e.g. in drug testings.

Dear reviewer 4,
Thank you very much for your positive feedback. Below we have addressed your concerns.

Major Concerns:
Please mention in the method description and/or figure legend, why we see histomorphological differences in the epithelium in figure 6D. The H&E stainings mostly show a flat epithelium, wheras in the IHC stainings the epithelium appears with invagination (crypt-villus structures?), especially under eDM conditions. Please also provide some quantitative data, how the proportion of differentiated cells (i.e. Ki67+ and goblet cells, EEC?) change between the different medium conditions (eDM and cDM vs control).

We thank the reviewer for this comment since it is indeed very important to explain the observed differences. This point has been addressed in the representative results section (lines 659-664). Quantitative data has been provided in the form of RT-qPCR data: stem cell (LGR5), goblet cell (MUC2) and enterocyte (ALPI) marker gene expression in different medium conditions are added in Figure 6F.

Minor Concerns:
Please include the term BMM (matrigel?) in the materials table. Include the differentiation time point when the readouts in figure 6C-E were generated (4 days?).

The term ECM (Extracellular Matrix) has been used to replace the term BMM, and the specification for Matrigel has been included in the Table of Materials.
Differentiation time points have been indicated in the legend of Figure 6.