Redefining Cell Culture Using a 3D Flipwell Co-culture System: A Mimetic for Gut Architecture and Dynamics In Vitro

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Gut mucosae are composed of stratified layers of microbes, a selectively permeable mucus, an epithelial lining, and connective tissue homing immune cells. Studying cellular and chemical interactions between the gut mucosal components has been limited without a good model system. We have engineered a three-dimensional (3D) multi-cellular co-culture system we coined "3D Flipwell system" using cell culture inserts stacked against each other. This system allows an assessment of the impact of a gut mucosal environmental change on interactions between gut bacteria, epithelia, and immune cells. As such, this system can be utilized in examining the effects of exogenous stimuli, such as dietary nutrients, bacterial infection, and drugs, on the gut mucosa that could predetermine how these stimuli might influence the rest of body. Here, we describe the methods of construction and application of the new 3D Flipwell system we utilized previously in assessing the crosstalk between the gut mucosa and macrophage polarization. We demonstrate the physiological responses of different components of the co-cultures to Sepiapterin (SEP), the precursor of the nitric oxide synthase cofactor tetrahydrobiopterin (BH_4). We reported previously that SEP induces a pro-immunogenic shift of macrophages having acquired an immune suppressive phenotype. We also showed that SEP induces a defense mechanism of commensal gut bacteria. The protocol describing the assembly and use of the 3D Flipwell co-culture system herein would grant its utility in evaluating the concurrent effects of pharmacologic and microbiologic stimuli on gut mucosal components. © 2025 The Author(s). Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: 3D Flipwell construction, assembly, and collagen coating
Basic Protocol 2: Flipwell cell seeding and cell culture
Basic Protocol 3: Addition of bacterial culture to the Flipwell system
Basic Protocol 4: Flipwell disassembly for scanning electron microscopy (SEM) studies
Basic Protocol 5: Immunofluorescence antibody staining for confocal microscopy

Keywords: 3D co-culture • Caco-2 • gut • immune cells • macrophages • microbiome

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INTRODUCTION

Gut microbe–immune cell interactions play critical roles in prevention/treatment of various diseases, in particular cancer. Nevertheless, microbe–immune cell interactions are largely impacted by host metabolism, diet, diseases, and drug treatment, potentially leading to dysbiosis, i.e., an imbalance in the composition of the microflora (Goldsmith & Sartor, 2014; Xuan et al., 2014; Yoo et al., 2020). Screening for specific stimuli impacting microbe–immune cell interactions would be greatly assisted by a robust model system. The gut-on-chip system, developed by Donald Ingber's group, consists of a microfluidic device enabling the reconstruction of the mechanical and physiological properties of the gut microenvironment (Kim et al., 2016). This system has been utilized to establish the three-dimensional (3D) architecture of colon epithelium and to co-culture colon epithelium with bacteria and immune cells (Donkers et al., 2021; Kim et al., 2016). However, fabrication of this system is complex and labor-intensive (Donkers et al., 2021). Also, the chip is made of adsorbent materials that alter the concentrations of analytes impairing its pharmacological utility (Donkers et al., 2021). Another co-culture system developed by Noel et al. (2017) is relatively easy to fabricate and effectively models the gut mucosal microenvironment. This system utilizes a single culture insert to seed colon epithelium on one side of the membrane and macrophages on the other side (Noel et al., 2017). Despite the great advantage of this new co-culture system, their system requires pre-differentiation and chemical dissociation of macrophages from another vessel, potentially damaging their integrity.

In this article, we describe our recently reported co-culture system based on a dual insert stacked against each other (Beamer et al., 2023). This co-culture consists of stratified layers of commensal bacteria, mucus, intestinal epithelium, and immune cells. This system enables seeding of colon epithelial cells on one side and seeding of undifferentiated non-adherent monocytes on the other side of the membrane. This new co-culture system allows for the synchronous progression of colon epithelial polarization, mucus formation, and monocyte-macrophage differentiation/polarization within a single co-culture, without the need to detach and replate pre-differentiated macrophages. Furthermore, fabrication of our system is easy with no need for special materials. This co-culture model could be utilized for high throughput analyses. We tested the utility of this system by analyzing the different responses of co-culture components to Sepiapterin (SEP), i.e., the endogenous precursor of tetrahydrobiopterin (BH_4), a cofactor of nitric oxide synthase (NOS). We previously showed that SEP exerts pro-immunogenic activities on breast tumor microenvironment by reprogramming tumor associated macrophages to a pro-immunogenic type (Fernando et al., 2023; Ren et al., 2019; Zheng et al., 2020). We show here that SEP induced biofilm formation by gut bacteria, mucus production by colon epithelium, and pro-immunogenic polarization of macrophages in co-cultures, indicating activation of a certain mucosal defense mechanism. These observations support the utility of this new co-culture system to recapitulate the gut mucosal microenvironment.

In Basic Protocol 1, we describe construction and collagen coating of the 3D Flipwell. In Basic Protocol 2, we detail gut epithelial and goblet cell co-culture, THP-1 cells

seeding, and SEP treatments. In Basic Protocol 3, we detail bacterial culture preparation and addition to the co-culture. In Basic Protocol 4, we describe Flipwell disassembly for the downstream scanning electron microscopy (SEM) imaging. In Basic Protocol 5, we detail immunofluorescence (IF) staining for confocal microscopy.

STRATEGIC PLANNING

The 3D Flipwells must be assembled in a sterile environment and UV sterilized several days in advance of the cell culture seeding. We used a BSL2 cabinet with a functional UV light for the sterilization steps. We used sterile instruments and ethanol for the initial sterilization steps as described. For the Flipwell construction, we found good reproducibility after several initial attempts of laying a consistent bead of the silicone adhesive. Some initial practice may be required, and extra Flipwells may be needed. We found that a gavage needle works best for the air bubble removal after the Flipwell is flipped. We tested and determined the best shape of the needle for this application. The needle must be prepared in advance of the second cell seeding and sterilized. Each cell type should be grown and maintained independently prior to the co-culture. Bacterial insert must be prepared and fit tested in advance of the bacterial addition to the Flipwell. *Bacillus subtilis* bacterial culture should be grown overnight prior to experimental addition to the co-culture system. Sepiapterin (SEP) treatments of the bacterial culture can be done as pre-treatments for several days prior to the experiment termination.

CAUTION: Bacillus subtilis, Caco-2, and HT-29-MTX cells are BSL-1 pathogens. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms.

CAUTION: Construction of Flipwells requires some basic mechanical skills and coordination for safe handling of basic tools, and specifically the scalpel blade. The authors carry no liability for unsafe practices and use of such tools resulting in any injuries sustained by the reader during their attempts at recreating these protocols. Additional PPE can be used by the reader, e.g., Kevlar or other cut-resistant gloves can be used under the nitrile gloves to increase personal safety level.

CAUTION: When attempting to use other types of bacteria for co-culture, consult biosafety level and guidelines for the use and handling of those pathogenic microorganisms. The authors bear no liability for unsafe and improper handling of other higher level BSL microorganisms resulting in any injuries sustained by the reader during their attempts at recreating these protocols.

3D FLIPWELL CONSTRUCTION, ASSEMBLY, AND COLLAGEN COATING

BASIC PROTOCOL 1

In this protocol we describe 3D Flipwell construction and assembly steps (Fig. 1). We used commercially available inserts to create a cell culture system that can be used to culture multiple cell types concurrently. This protocol is scalable to obtain a desired number of Flipwells depending on the treatment conditions and downstream analysis workflows.

Materials

Sterile phosphate-buffered saline (PBS) without calcium or magnesium (Fisher Scientific, cat. no. BSS-PBS-1X6)
H_2O, deionized, sterile
100 µg/ml PureCol working collagenase solution (see recipe)

PPE, i.e., nitrile powder-free latex free gloves (MidSci Aurelia Amazing, cat. no. 92885, or Top Quality Manufacturing BioGrip Pink, cat. no. P2810), and optional Kevlar or other cut-resistant gloves

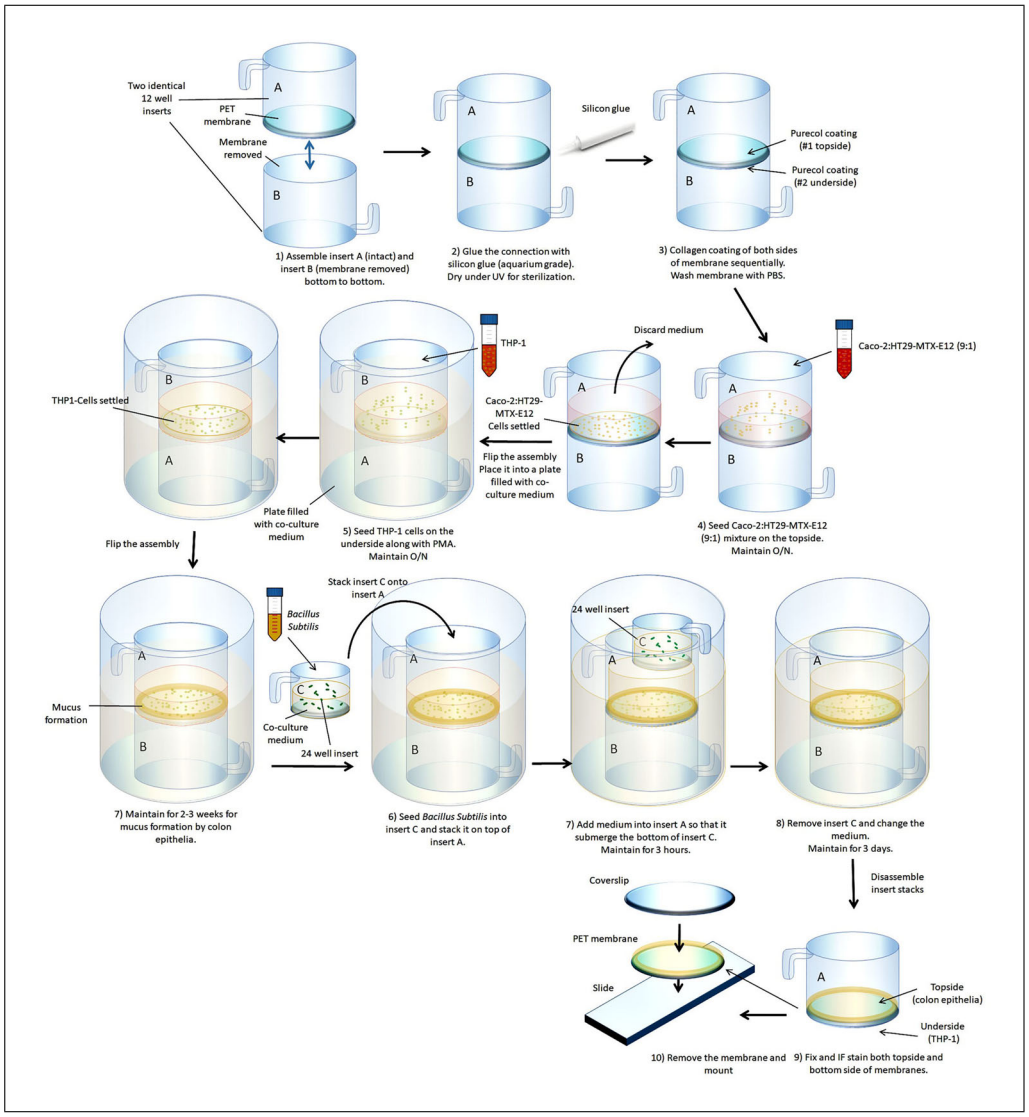

Figure 1 Schematic of the 3D Flipwell assembly and co-culture method. Originally published in Beamer et al., 2023.

Laminar flow hood with UV light (The Baker Company SterilGARD, Thermo Fisher Scientific 1300 Series A2 BSC Class II)

Scalpel blades, sterile, disposable (Mopec, cat. no. AJ136, or Feather #10, cat. no. 2975#10)

Petri dishes, sterile, 100 × 15–mm (Corning Falcon, cat. no. 351029)

Culture inserts for 12-well plate, PET, 0.4-µm, sterile (BrandTech Scientific, cat. no. 782730)

Forceps, stainless steel, sterile autoclaved, 2 sets (VWR Specimen Forceps, cat. no. 82027-438)

Silicone adhesive (Loctite clear silicone IDH, cat. no. 1745658, or 3M marine grade silicone sealant clear with a nose tip, cat. no. PN08019)

Wipes (Kimwipes Kimberly-Clark Professional with LintGuard Anti-Stat Polyshield, cat. no. 34120)

200-µl single channel pipet, 200 (Eppendorf Research Plus)

200-µl tips (USA Scientific, cat. no. 1111-1700)

Deep Petri dish bottoms, 100 × 25–mm deep polystyrene stackable Petri dish, sterile (USA Scientific, cat. no. 8609-0625)

Petri dish lids, sterile 100 × 15–mm style (Corning Falcon, cat. no. 351029)

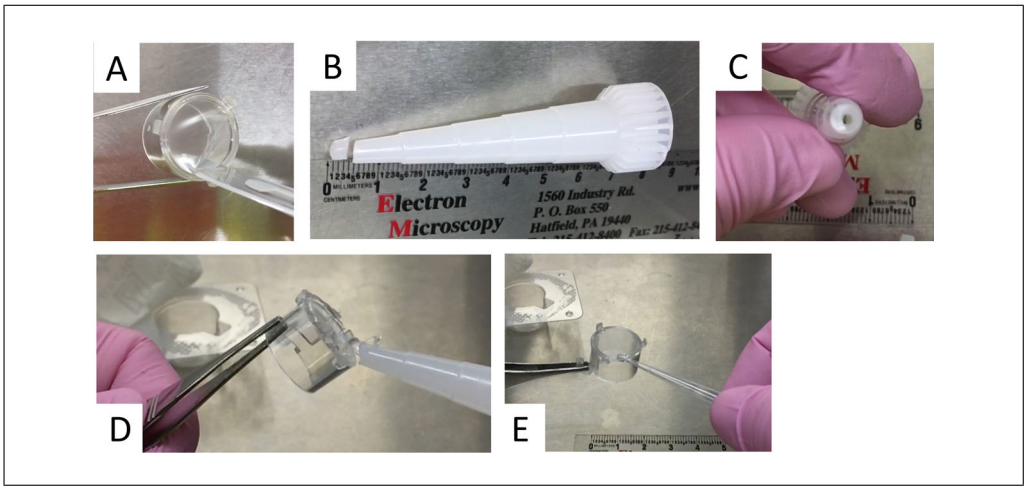

Figure 2 3D Flipwell construction: Membrane removal and glue application methods. (**A**) Membrane is cut out in a clock-wise direction with a sterile scalpel blade. (**B**) Using a sterile scalpel blade, carefully cut off the tip end from the plastic nozzle provided with the silicone adhesive tube to create an opening 3-mm wide. (**C**) 3-mm opening in the plastic nozzle. (**D**) Using sterile forceps, pick up one of the inserts and apply a consistent bead of silicone 2-3 mm in thickness to the bottom of the insert following the round contour of the plastic rim at the bottom of the insert. (**E**) Alternative to the dispensing nozzle method. Use a sterile 200-µl pipet tip to spread a thin 2-3 mm bead of silicone glue around the insert's bottom edge.

Flipwell construction

1. Assemble and place all required materials inside a laminar flow hood. All work should be done inside a cell culture cabinet to keep the inserts as sterile as possible.
2. Prepare the scalpel by opening its protective cover and resting it on the rim of a Petri dish bottom.
3. Open the sterile packaging of the first culture insert.
4. Using sterile forceps, remove the insert out of its sterile packaging and hold it up with the bottom facing you.
5. Pick up the scalpel blade.
6. With a circular clockwise motion (if right-handed) carefully cut out PET membrane (Fig. 2A) with the sterile scalpel. The PET membrane is not needed for the downstream steps and can be discarded.
7. Scrape off any loose material from the rim of the insert.
8. Place the insert back into its protective sterile packaging.
9. Prepare the silicone adhesive's dispensing nozzle provided with the tube. Using a sterile scalpel blade, carefully cut off the tip end from the plastic nozzle provided with the tube to create a 3-mm wide opening (Fig. 2B and 2C).

   The opening needs to be wide enough to make the bead ~2 to ~3 mm wide. Practice squeezing a consistent bead on a Kimwipe before going forward with the assembly. Alternatively, a sterile 200-µl pipet tip with a cut off tip end can be used to lay down and spread a thin layer of silicone around the rim. Cut off ~3 to ~4 mm of the pipet tip. Exercise safe handling of the scalpel blade while cutting (see Strategic Planning).

10. Open a second culture insert and let it rest inside its sterile protective packaging.
11. Using sterile forceps, pick up the second insert (with its membrane still intact) and apply a consistent bead of silicone 2 to 3 mm in thickness to the bottom of the insert following the round contour of the plastic rim at the bottom of the insert (Fig. 2D).

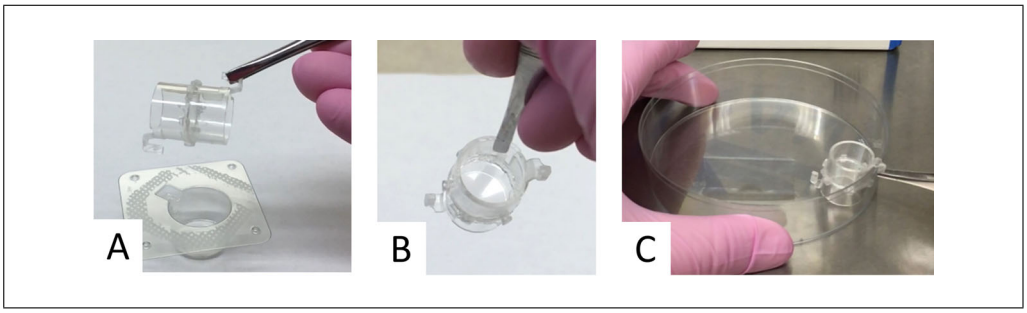

Figure 3 3D Flipwell assembly and silicone glue drying method. (**A**) Two Transwells are glued bottom-to-bottom. (**B**) The final assembly with a single PET membrane and L-shaped handles at 180° from each other. (**C**) Place the Flipwell inside a deep Petri dish, cover, and allow the Flipwell to dry and cure 24-72 hr.

12. Using a second pair of sterile forceps, pick up the first insert without the membrane (from steps 3 to 8 above). Affix the first insert (lacking the membrane) bottom-to-bottom to the second insert (with intact membrane) and the glue.

    The final assembly should only have a single PET membrane with the L-shaped handles at the opposite ends positioned at 180° from each other (Fig. 3A-C).

13. Place the Flipwell inside one of the protective insert covers, or inside a deep Petri dish, cover with a Petri dish lid, and allow the Flipwell to dry and cure 24 to 72 hr (Fig. 3C).

    We found that an overnight drying is sufficient to proceed to the sterilization with UV light and coating steps, although final cure is achieved after 72 hr.

14. Once the silicone adhesive is dry, place the Flipwell inside the deep Petri dish bottom. If already in a deep Petri dish, remove the lid before UV sterilization.

    The deep Petri dish is deep enough to allow for the Flipwell to hang from the rim (Fig. 3C).

15. Using the BSC's UV light, expose the Flipwell to the UV light for 30 to 60 min for each side to sterilize both sides of the Flipwell.

    The safety cabinet sash must be closed. The user should not be near the UV light during this step.

16. Turn off the UV light after use.

17. Cover the deep dish and the hanging Flipwell with the Petri dish lid and set aside.

18. Optional. The Flipwells can be tested for leakage prior to collagen coating with either sterile PBS or sterile deionized water (Fig. 4B; we used DMEM for images to better illustrate this step).

    Add 500 µl of either sterile PBS or sterile deionized water to the top of the Flipwell. Cover and let rest overnight inside the deep Petri dish. Check the Flipwell the next morning for leakage. If the Flipwell has lost the majority of the fluid, discard the Flipwell and start over. Otherwise, proceed to the collagen coating steps or store uncoated inside the deep Petri dish covered with the Petri dish lid inside the BSC.

Collagen coating of the membrane

19. To pre-coat the Flipwell membrane for adherent cell seeding, add 200 µl PureCol solution to one side of the Flipwell membrane for 1 hr.

20. Aspirate and add 200 µl sterile PBS.

21. Aspirate and allow to dry 1 hr inside the BSC.

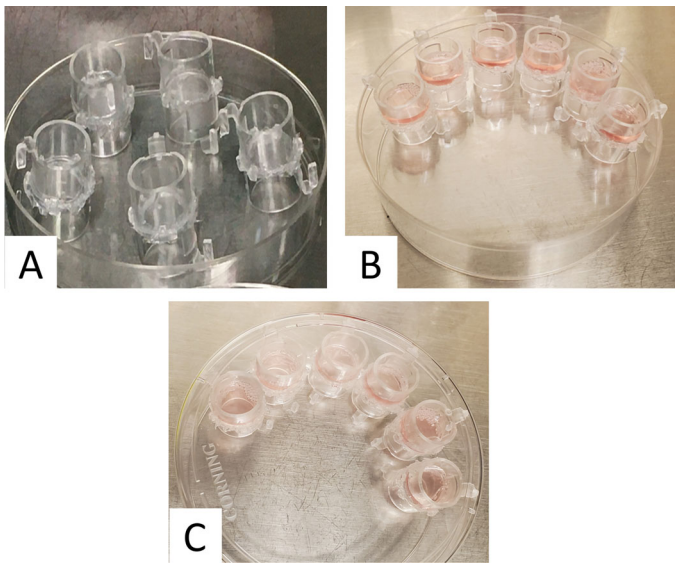

Figure 4 3D Flipwell UV sterilization and leakage test. (**A**) Flipwells are allowed to dry and each side of the Flipwell is UV sterilized prior to collagen coating. (**B**) Flipwells are hung over the deep Petri dish rim, and 200 µl of sterile medium or sterile PBS is added. We used DMEM for better illustration in this case, but we used PBS in our studies; both liquids serve the same purpose. (**C**) Flipwells are covered with a lid and left inside a BSC overnight to test for leakage.

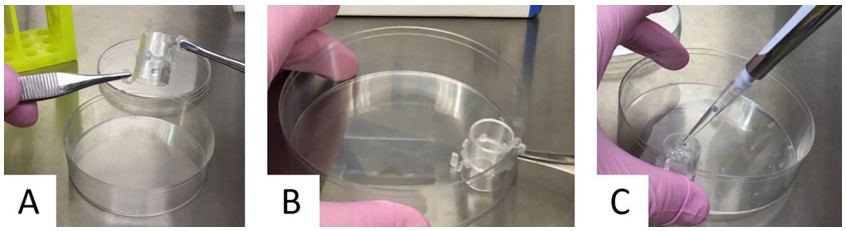

Figure 5 Collagen coating of the 3D Flipwell. (**A**) Flipwell is rotated for collagen coating of each side of the membrane. (**B**) Flipwell is hung over the rim before, during, and after collagen coating. (**C**) Collagen coating with 200 µl PureCol solution. Flipwell is left inside a deep Petri dish, covered (not shown), and allowed to dry and cure 24-72 hr. The same process is repeated for the other side of the membrane.

22. Using forceps, flip and hang the Flipwell on the rim (Fig. 5A-B).
23. Repeat collagen coating and a PBS wash (steps 19 to 21) on the opposite side of the Flipwell membrane (Fig. 5C).
24. Cover the Petri dish and keep inside the cabinet.
25. Proceed to cell seeding or allow to dry overnight inside the BSC and plate the next day.

The Flipwells are now sterile and pre-coated with collagen. We did not store precoated Flipwells for longer than 24 hr for our workflows.

FLIPWELL CELL SEEDING AND CELL CULTURE

BASIC PROTOCOL 2

This protocol describes cell seeding of the apical and basal layers. Gut epithelial Caco-2 and goblet HT29-MTX-E12 cells are grown separately and then plated at a 9:1 ratio and cultured for 7 to 10 days to start mucosal layer development and gut epithelial polarization. The Flipwell is then flipped over, any air bubbles are removed with a gavage needle,

and THP-1 cells are seeded with a PMA treatment to ensure monocyte adhesion and macrophage differentiation. After THP-1 cells attach overnight, the Flipwell is flipped back, air bubbles are removed with a gavage needle, and both cell layers in the apical and basolateral compartments are maintained until a mucosal layer is established (additional 7 to 9 days after Caco-2:HT29-MTX cells are plated). The media are changed every other day until mucus is formed over colon epithelial cells. The Flipwells are further subjected to bacterial co-culture and confocal and electron microscopy analysis.

Materials

Caco-2 cells (ATCC, cat. no. HTB-37)
Medium for Caco-2 cells (DMEM high glucose, see recipe)
HT-29-MTX-E12 cells (Millipore Sigma, cat. no. 12040401)
Medium for HT-29 cells (DMEM high glucose, see recipe)
PBS without calcium or magnesium (Fisher Scientific, cat. no. BSS-PBS-1X6)
0.25% trypsin-EDTA (Thermo Fisher Scientific, cat. no. 25200-056)
Trypan blue solution (VWR, cat. no. 97063-702)
Collagen pre-coated 3D Flipwells (see Basic Protocol 1)
70% to 95% ethanol (Decon Labs)
THP-1 cells (ATCC, cat. no. TIB-202)
Medium for THP-1 cells (RPMI, see recipe)
50 µg/ml phorbol myristate acetate (PMA) stock solution (see recipe)
50 mM Sepiapterin (SEP) stock solution (see recipe)

Deep Petri dish bottoms, 100 × 25–mm deep polystyrene stackable Petri dish, sterile (USA Scientific, cat. no. 8609-0625)
Petri dish lids, sterile 100 × 15–mm style (Corning Falcon, cat. no. 351029)
Cell culture incubator, 37°C, 5% CO_2 (Eppendorf CellXpert)
15-ml conical centrifuge tubes (SpectraTube, Alkali Scientific cat. no. C2715)
Centrifuge (Eppendorf, Centrifuge 5804/5804 R or equivalent)
Hemacytometer (Neubauer with cover slips)
Laminar flow hood with UV light (The Baker Company SterilGARD, Thermo Fisher Scientific 1300 Series A2 BSC Class II)
Needle nose pliers, 2 sets
Ruler
Animal feeding needles, 20-G, 1.5'' length, single use (Fisher Scientific, cat. no. 01-208-87)
3- or 5-ml syringe with Luer-Lok tip (BD, cat. no. 309585 or 309603)
50-ml conical tubes (Greiner Bio-One, cat. no. 227261)
Forceps, stainless steel, sterile autoclaved, 2 sets (VWR Specimen Forceps, cat. no. 82027-438)
Additional lab equipment, including:
  2-, 10-, 200-, and 1000-µl single channel pipettes
  10-, 200-, and 1000-µl pipet tips (Alkali Scientific PurePoint or equivalent)
  Transfer pipets, sterile (Fisherbrand, cat. no. 13-711-20)
  Microcentrifuge tubes (Thermo Fisher, cat. no. 3451)
  10-ml serological pipette (Corning, cat. no. 4488)
  5-ml serological pipette (Corning, cat. no. 4487)
  Brightfield microscope (Nikon or equivalent)

Caco-2 and HT-29 culturing and seeding in the Flipwell

1. Culture Caco-2 and HT-29 cells in their respective growth medium separately in 100-mm dishes. When Caco-2 and HT-29 cells are ready to passage, remove culture media, and wash once with 10 ml PBS for a 100-mm dish.

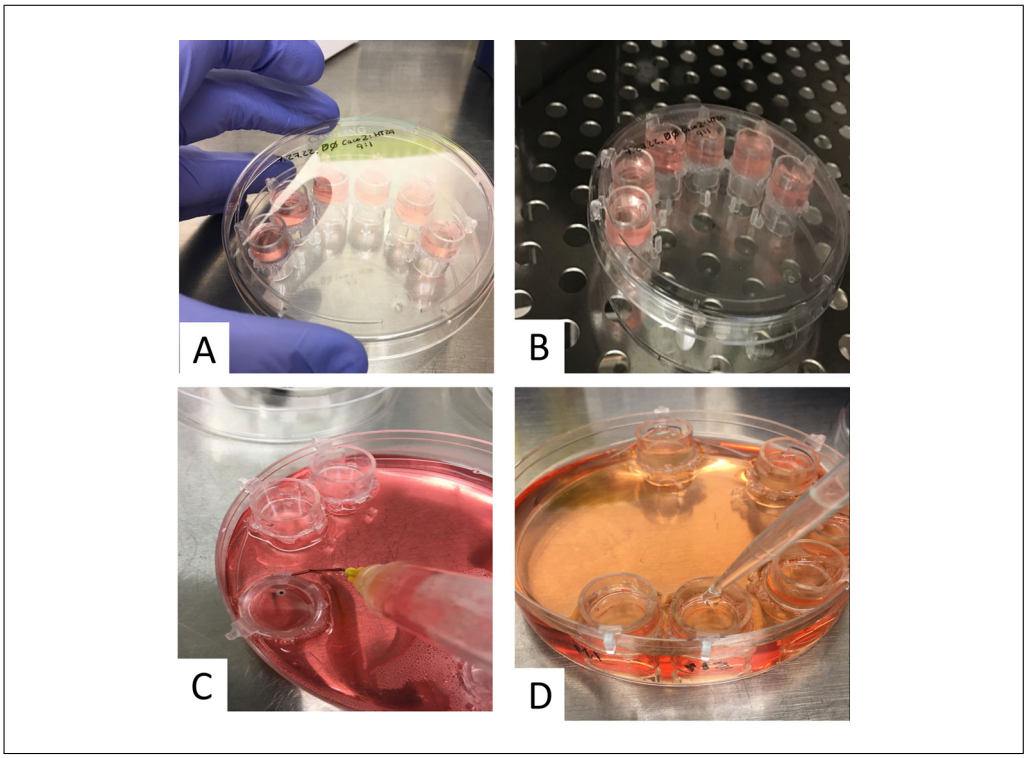

Figure 6 3D Flipwell seeding and culturing. (**A**) Flipwells seeded with epithelial cells. (**B**) Flipwells are left overnight in an incubator for the epithelial cell attachment. (**C**) Flipwells are flipped, and large air bubble aspirated with a needle from underside of each membrane. (**D**) Flipwells are seeded with THP-1 cells on top with Caco-2:HT29 cells at the bottom. Two different media are used.

2. Add 2 ml of 0.25% trypsin-EDTA to the dishes and incubate 5 to 10 min at 37°C with 5% CO_2.
3. Once cells dissociate, add 6 ml of cell-line specific growth medium to each plate and transfer dissociated cells into a sterile 15-ml centrifuge tube.
4. Centrifuge 5 min at 500 × *g*, room temperature.
5. Remove supernatants without disturbing the pellets and resuspend each pellet in 1 ml pre-warmed DMEM-based HT-29 medium.
6. Count the cells with your method of choice, e.g., using trypan blue and a hemacytometer.
7. Combine Caco-2 and HT-29 cells in a ratio of 9:1.
8. Place collagen pre-coated Flipwells (see Basic Protocol 1) in a separate deep Petri dish bottom and hang the Flipwell from the rim of the dish.
9. Plate 500 µl of 7.5 × 10^4 cells/cm^2 (2.6 × 10^5 cells/12-well insert) per Flipwell's apical side (Fig. 6A). Scale up based on the number of Flipwells required.
10. Cover with Petri dish lid and incubate at 37°C with 5% CO_2 overnight until the cells attached begin to produce mucus for 7 to 10 days (Fig. 6B).

Needle construction (see Fig. 7A and 7B)

11. Work inside the laminar flow hood for all steps below.
12. Using a pair of needle nose pliers and a ruler, measure proper distance for the location of each bend and bend the needle (as pictured in Fig. 7A).

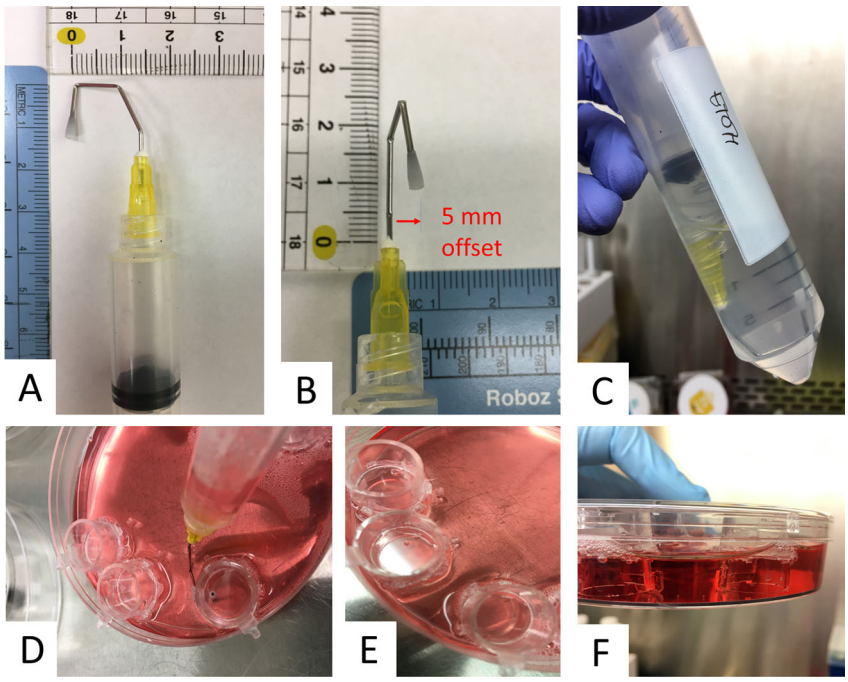

Figure 7 Gavage needle construction, sterilization, and use. (**A**) Gavage needle is bent using pliers. (**B**) 5-mm offset to one side is used to ensure the needle will be at the outer edge of the membrane when inserted under the Flipwell. (**C**) Syringe and constructed needle are sterilized in 70%-95% ethanol for 5-15 min and dried well before use. (**D**) Pre-bent needle is inserted under the Flipwell membrane and is aimed at the outer edge of the membrane. Caco-2:HT-29 cells are now at the bottom. The Flipwell is hung over the rim and is ready for seeding THP-1 cells on top. (**E**) 2-mm air bubble is left behind after the air bubble aspiration due to the gavage needle's cone-like tip. No other air bubbles are present. (**F**) An example of the adequate medium level. The level should reach to the bottom of the membrane but not allow for the Flipwell to rise above the deep Petri dish rim. Shallow U-shaped/concave meniscus is formed between the Flipwell's L-shaped arms as expected.

13. Create a 4- to 5-mm twist in the needle bottom.

    This step is key; 4 to 5 mm from the axis is ideal (Fig. 7B).

14. Connect a sterile 3- or 5-ml syringe to the needle. Insert the needle and syringe assembly inside a 50-ml conical tube filled with 20 ml of 70% to 95% ethanol. Pick up some of the ethanol through the needle to sterilize the needle internally (Fig. 7C). This can be done in advance.

15. Dry the needle and syringe by purging excess ethanol through the syringe and needle and leaving to air dry for ~3 min.

THP-1 cell culture

THP-1 cells should already be growing and maintained in culture. Culture THP-1 cells in its specific RPMI-based medium (see recipe). Start culturing several days in advance and scale up depending on the number of Flipwells needed. After 7 to 10 days of Caco-2:HT-29 culture in the apical side of the Flipwell, the gut epithelial cells begin to produce mucus. Prepare to plate THP-1 cells.

16. Pellet THP-1 cells 5 min at 94 × *g*, room temperature.

17. Resuspend and count THP-1 cells with your preferred counting method.

18. Prepare the cell suspension at 0.5×10^6 cells per 500 µl THP-1 medium.

Flipwell flip and THP-1 cell seeding

19. Aspirate the medium from the apical side of the Flipwell.
20. Using sterile forceps, pick up the Flipwell by the arm and rotate upside-down.
21. Using the second pair of sterile forceps, pick up the Flipwell by the arm on the opposite side and hang over the deep-well Petri dish.
22. Add 500 µl of THP-1 cell suspension to the apical side of the Flipwell (Fig. 6D).
23. Add 1 µl PMA stock solution to each well (i.e., a 1:500 dilution for a final PMA concentration of 100 ng/ml) to allow THP-1 cell differentiation and polarization in the well.
24. Add enough HT-29 medium to the deep-well Petri dish to raise the medium level to the underside of the Flipwell membrane.
25. Remove the air bubble with a gavage needle to stabilize the Flipwell (Fig. 6C).

Air bubble removal

The Flipwell may float up and tilt due to the presence of a large air bubble. We found that the Flipwell resides best with the air-bubble removed. We created a specially constructed needle that was tested to give beautiful results by nearly eliminating the air bubble and allowing the Flipwell to sit firmly in place without tilting (Fig. 7D-E). We learned that the soft cone shaped tip of the gavage needle will retain some small air space when most of the air bubble is aspirated from under the membrane and is visually gone when observing from the top of the Flipwell. The gavage needle's cone-shaped tip will leave a very tiny air bubble (3 mm in diameter) that may remain when most of the air is removed. We discovered that a complete air bubble elimination may result in some backflow of the medium from the other side of the membrane. We also tested other needles; however, the soft tip of the gavage needle gave the best results compared to the non-gavage needle designs that resulted in occasional membrane puncture during air aspiration.

26. To remove the air bubble with the ethanol pre-sterilized gavage needle and syringe assembly, hold the Flipwell with the forceps while the Flipwell is hanging over the rim of the deep-well Petri dish and tilt the Flipwell to one side such that the air bubble will come to the outer edge of the membrane.
27. Gently and slowly place the gavage needle under the Flipwell membrane and position the soft tip of the needle just inside the air bubble but to the outer edge of the membrane. (Fig. 7D)
28. Gently pull the syringe plunger up and aspirate the air slowly until most of the air bubble is gone.

    Do not create a lot of suction as that will force the medium from the apical side to enter through the membrane. We found this process to be easy once the technique is mastered. The key element is to get the air bubble to the outer edge of the membrane and allow the small air bubble from the gavage needle cone-shaped tip to remain behind when aspiration step is completed. Another key element is that the gavage needle must be pre-bent to be as close to the shape pictured and have a twist to either right or left depending on the user's preference. (Fig. 7B). This 4- to 5-mm twist will allow the needle to go to the outer edge of the membrane during needle placement. We learned that this technique did not affect our data when imaging cellular morphology across the entire membrane. Both media need to be present across the membrane for the air bubble removal.

Flipwell culture maintenance

29. Cover the plate and incubate in the cell culture incubator at 37°C overnight with 5% CO_2 and 100% humidity.

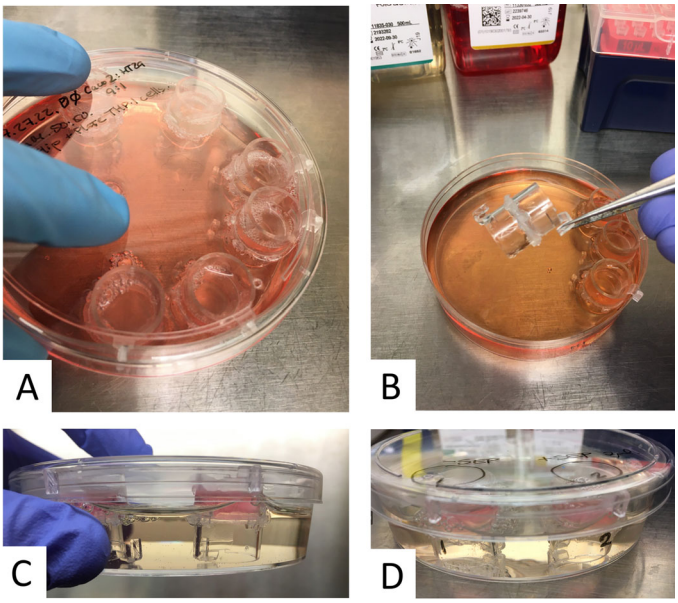

Figure 8 3D Flipwell treatments with SEP. (**A**) Epithelial (bottom) and THP-1 cells (top) are cultured until THP-1 cells attach. (**B**) The Flipwell is transferred into a new sterile deep Petri dish and flipped back to have the epithelial cells on top. (**C**) Flipwells are flipped back to have the epithelial cell on top for differentiation and SEP treatments. Air removed with the syringe and gavage needle as previously shown (see Fig. 7). Two different media are used for each cell type. (**D**) The upper (epithelial) layer is treated with SEP. Labeling is done on the side and top of Petri dish and on the Flipwells.

30. Culture Caco-2 and HT-29-MTX epithelial cells overnight until THP-1 cells attach (Fig. 8A).

Flipwell flip

The next morning, prepare to flip the Flipwell.

31. Using sterile forceps, flip the Flipwell into a fresh sterile deep-well Petri dish (Fig. 8B).

32. Add 500 µl HT-29 medium to the apical side of the Flipwell (Fig. 8C).

33. Add enough THP-1 medium to the deep-well dish to raise the medium level to the underside of the Flipwell membrane (Fig. 8C). This will be ~40 to ~55 ml but will differ with extra Flipwells in the deep Petri dish (see Fig. 7F).

34. Aspirate the air with a gavage needle.

35. Cover the plate and culture for additional 7 to 9 days to allow for the goblet cell differentiation and mucus production in the apical compartment.

36. Partially refresh the medium every 48 hr.

    Do not allow the medium in the basolateral (bottom) compartment to drop below the Flipwell membrane underside and maintain HT-29 medium to prevent air infiltration. The best method for changing medium is to refresh only half of the medium for the basolateral side and leave a small volume of medium in the apical side to prevent air infiltration.

37. Add 1 µl SEP stock solution to 500 µl medium in each well (100 µM final SEP concentration) at this time if bacterial culture is not used (Fig. 8D).

    See Reagents and Solutions for SEP reconstitution and use. If bacterial culture and insert are used, SEP may be added to the bacterial culture. See Basic Protocol 3.

ADDITION OF BACTERIAL CULTURE TO THE FLIPWELL SYSTEM

This protocol describes bacterial insert construction, bacterial culture, SEP treatment, and addition of the co-culture insert into the apical compartment of the Flipwell.

Materials

Miller LB medium (see recipe)
Bacillus subtilis wild-type (3A1T) strains in glycerol stock (*Bacillus* Genetic Stock Center: *https:// bgsc. org/*)
50 mM Sepiapterin (SEP) stock solution (see recipe)
3D Flipwells seeded with gut epithelial and THP-1 cells (see Basic Protocol 2)
PBS without calcium or magnesium (Fisher Scientific, cat. no. BSS-PBS-1X6)

10-ml serological pipet
Drummond Pipet Aid XP
50-ml conical tubes (Greiner Bio-One, cat. no. 227261)
200- and 1000-µl single channel pipettes
200- and 1000-µl pipet tips (Alkali Scientific PurePoint or equivalent)
Shaking incubator set at 220 rpm, 37°C
Culture inserts for 24-well plate, PET, 0.4-µm, sterile 24-well inserts (BrandTech Scientific, cat. no. 782710)
Spectrophotometer
Biosafety cabinet (BSC)
Forceps, stainless steel, sterile autoclaved, 2 sets (VWR Specimen Forceps, cat. no. 82027-438)
Petri dishes, sterile, 100 × 15–mm (Corning Falcon, cat. no. 351029)

NOTE: We performed 3 treatments with SEP: day 1, a.m.; day 1, p.m.; and day 2, a.m.

Bacterial culture and SEP treatments

1. The day prior to bacterial culture, prepare Miller LB and cool to room temperature.
2. The day prior to adding bacterial culture insert, start growing *B. subtilis*. Early morning, add 10 ml Miller LB using a sterile 10-ml serological pipet into a sterile 50-ml conical tube.
3. To initiate a starter culture of *B. subtilis* from a frozen glycerol stock, remove the glycerol stock from –80°C freezer. Do not defrost the glycerol stock.
4. Use a single channel pipet and a sterile 200-µl tip to pick a small amount of bacterial glycerol stock.
5. Dispense the tip into the 50-ml conical tube containing the 10 ml LB.
6. Add 20 µl SEP stock into 10 ml bacterial culture (final SEP concentration of 100 µM) in the 50-ml conical tube.
7. Shake the culture all day in a shaking incubator set at 220 rpm, 37°C.
8. Later that day, add the second treatment of 20 µl SEP stock into 10 ml LB with *Bacillus*.
9. Shake the culture overnight in a shaking incubator set at 220 rpm, 37°C.
10. Early next morning, add the third treatment of 20 µl SEP into 10 ml LB with *Bacillus*.
11. Shake the culture until you are ready to add it to the 24-well bacterial inserts.

Additional considerations could be taken when culturing other bacteria. Doubling time and OD at 600 nm measurements using a spectrophotometer can be used to establish

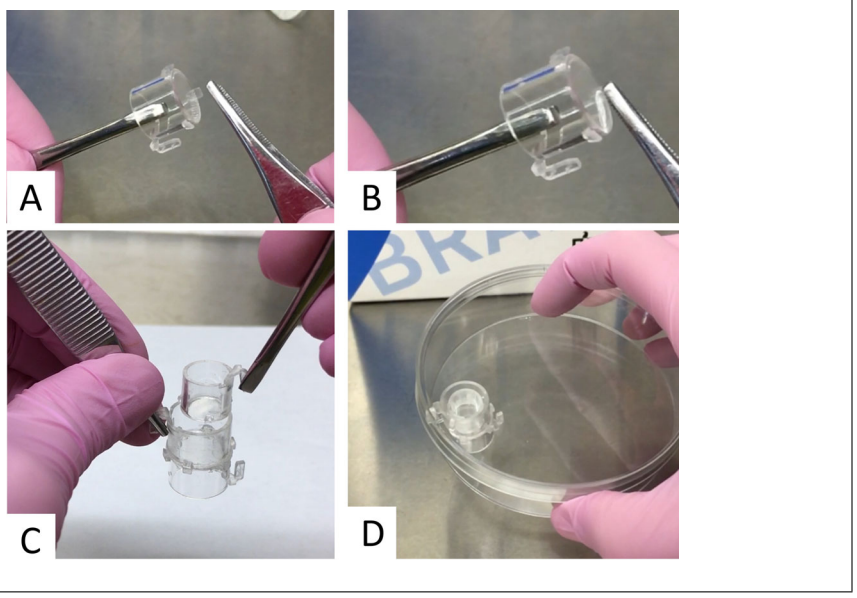

Figure 9 Bacterial Insert preparation and fit test. (**A**) Bacterial insert is prepared by breaking off three small "feet" using sterile tweezers, (**B**) Break off and discard insert's "feet." (**C**) Test to see how well the bacterial insert can fit into the Flipwell, (**D**) Place the Flipwell inside a deep Petri dish and cover. The assembly is complete.

optimal growth conditions for your desired bacterial culture when it is necessary to maintain bacterial culture for several days under various treatment conditions. To maintain bacteria in exponential growth phase, it may be necessary to remove a small volume of bacterial culture and add it to fresh LB each day during cell culture and treatments.

Bacterial insert preparation and addition of the bacterial insert into the Flipwell

Work inside a BSC. Split the work area in half and maintain bacterial and cell cultures apart. Do not return cell cultures back to the incubator. The BSC will need to be sterilized and exposed to UV for 24 hr after this procedure.

12. Using 2 pairs of sterile forceps, remove the sterile 24-well insert from its packaging and break off the small plastic feet at the bottom of the insert (Fig. 9A-B). This insert will be able to fit inside the Flipwell (Fig. 9C). Optional: use ethanol and UV to sterilize the insert.
13. Rest the 24-well insert inside a sterile Petri dish, cover with a lid, and set aside (Fig. 9D).
14. Remove the Flipwells from the incubator and set inside the BSC on the opposite side of the cabinet, being careful not to cross contaminate the cultures.
15. Remove 300 µl HT-29 medium from the Flipwell's apical compartment leaving only ~200 µl in the well. Cover the dish and set aside.
16. Carefully add 50 to 100 µl of *B. subtilis* culture to the 24-well bacterial insert being careful not to spill and contaminate the insert's outer walls.

    Bacterial culture density may vary but the bacteria should be in the exponential growth phase.

17. Using sterile forceps, place the bacterial insert inside the apical compartment of the Flipwell (Fig. 10C).
18. Hang bacterial insert from the rim of the Flipwell and, for stability, position away from the Flipwell hook (Fig. 10A-B).

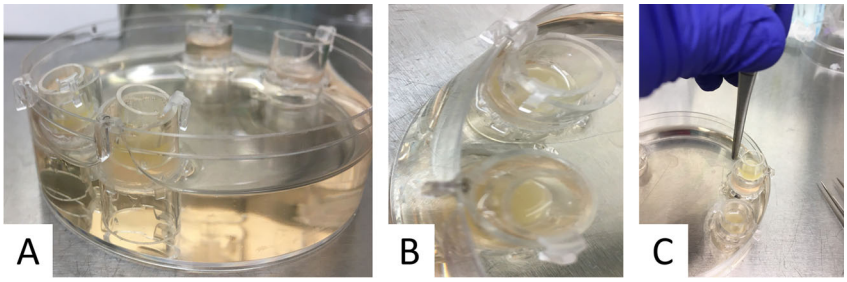

Figure 10 Bacterial inserts with *B. subtilis* are added to the Flipwells. (**A**) 200 µl *B. subtilis* in LB is added for 3 hr in a separate 24-well "bacterial" insert. The Petri dish is covered. Remove 100 µl DMEM to have ≤400 µl when adding the bacterial insert to prevent DMEM overflow. (**B**) Bacterial insert is placed inside the Flipwell and hung over the Flipwell rim (with epithelial cells on top and THP-1 at the bottom) for 3 hr and then removed. (**C**) Bacterial insert is carefully removed after 3 hr.

19. Cover the dish and let sit for 3 hr inside the BSC.
20. Using sterile forceps remove bacterial insert from the Flipwell (Fig. 10C).
21. Discard the bacterial insert following proper safety guidelines for the culture.
22. Aspirate the medium from the apical side of the Flipwell and wash with 500 µl PBS.
23. Remove the Flipwell from the Petri dish into a new sterile deep Petri dish, flip and hang over rim.
24. Wash each side with 500 µl PBS. The membrane is now ready for downstream processing (immunofluorescent or confocal microscopy).

FLIPWELL DISASSEMBLY FOR SCANNING ELECTRON MICROSCOPY (SEM) STUDIES

BASIC PROTOCOL 4

To assess macrophage repolarization across the mucous barrier and gut epithelial layer after Sepiapterin (SEP) treatments, the Flipwell membrane can be processed and prepared for scanning electron microscopy (SEM). We used SEM to evaluate morphological changes in epithelial cells and macrophages after SEP treatment.

NOTE: It is best to fix cells prior to disassembling the Flipwell. Determine which microscopy technique will be used, as it will dictate which fixation buffer will be used. Consult your confocal and SEM facilities for guidelines on fixation, buffer type [glutaraldehyde or paraformaldehyde (PFA)], and buffer concentrations for the initial fixation and subsequent storage duration prior to the dehydration steps.

Materials

Flipwells from Basic Protocol 1
4% PFA (see recipe)
0.4% PFA (see recipe)
Blocking buffer (see recipe)
PBS without calcium or magnesium (Fisher Scientific, cat. no. BSS-PBS-1X6)

Biosafety safety cabinet (BSC) (optional)
Markers, solvent proof (VWR Lab Marker, cat. nos. 52877-310 or 95042-566)
50-ml conical tubes (Greiner Bio-One, cat. no. 227261)
Microcentrifuge tubes or small glass bottles with lids
Scalpel blade, sterile, disposable (Mopec, cat. no. AJ136, or Feather #10, cat. no. 2975#10)

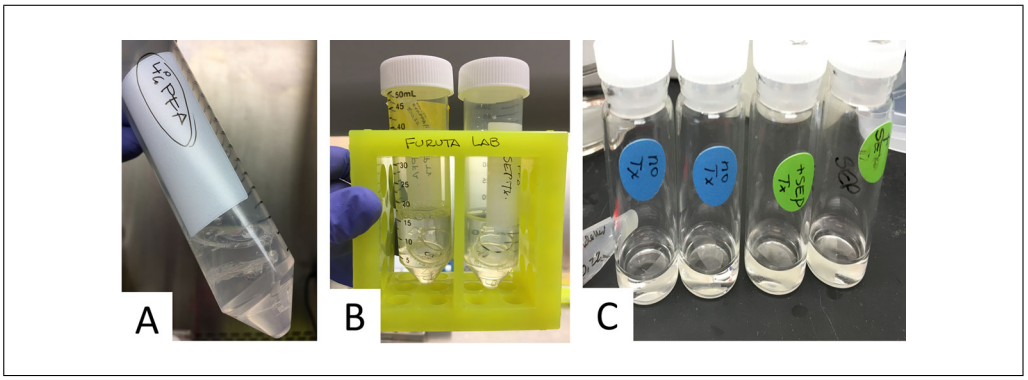

Figure 11 Flipwells are fixed in 4% PFA, transferred into 0.4% PFA, and later cut in half for SEM. (**A**) Flipwells in 4% PFA for the initial fixation overnight. (**B**) 0.4% PFA is used for longer storage until processing for SEM imaging can be done. (**C**) Membranes are cut out, cut in half, and left in 0.4% PFA for the downstream imaging of both sides of the membrane with SEM.

Forceps, stainless steel, sterile autoclaved, 2 sets (VWR Specimen Forceps, cat. no. 82027-438)

Beckman Coulter Allegra 6 centrifuge with a swing bucket rotor for 50-ml conical tubes

Flipwell disassembly for SEM

1. Perform these steps either inside or outside of the BSC.
2. Label each side of the Flipwell walls (top/bottom or cell type) with a marker.
3. Optional. Label the membrane with a dot or an "x" to code the side and cell type.
4. Fix without disassembly inside a 50-ml conical tube (Fig. 11A) or cut out and place in a microcentrifuge tube or a small glass bottle (4% PFA initially then 0.4% PFA for longer storage).
5. To disassemble, twist off each glued part of the Flipwell.
6. Flip the insert with the membrane pointing upward (membrane side up).
7. Using a scalpel blade and forceps, cut out the membrane carefully while supporting the membrane with forceps.
8. Cut the membrane in half if you want to scan each side of the membrane. Place each half into a separate 0.4% PFA solution (0.5 to 1 ml) inside a microcentrifuge tube or small glass bottle (Fig. 11C).
9. Samples are ready for dehydration steps, processing, and imaging.

Scanning electron microscopy (SEM)

After the Flipwells (seeded with Caco2:HT29-MTX cells on one side and THP-1 cells on the other side) were treated with SEP and bacterial metabolites (inside the insert), the membranes were fixed in 4% paraformaldehyde, washed twice with 10× PBS and then DI water. Chemical drying was performed using increasing concentrations of ethanol in water (25%, 50%, 70%, and 100%), followed by washing each sample with a 1:1 hexamethyldisilazane (HMDS):ethanol solution, and then with 100% HMDS. Each membrane was removed from the wells with forceps, placed onto a piece of weighing paper, and cut in half with a scalpel. One side of the membrane was bound with carbon tape to the aluminum SEM sample stub with the top facing up. The remaining side was flipped over and bound to the stub with the bottom facing up. All samples were sputter coated with gold for 15 s using a Denton Vacuum Desk II Cold Sputter unit. The coated samples were imaged in the JEOL 7500F Scanning Electron Microscope at an acceleration voltage of

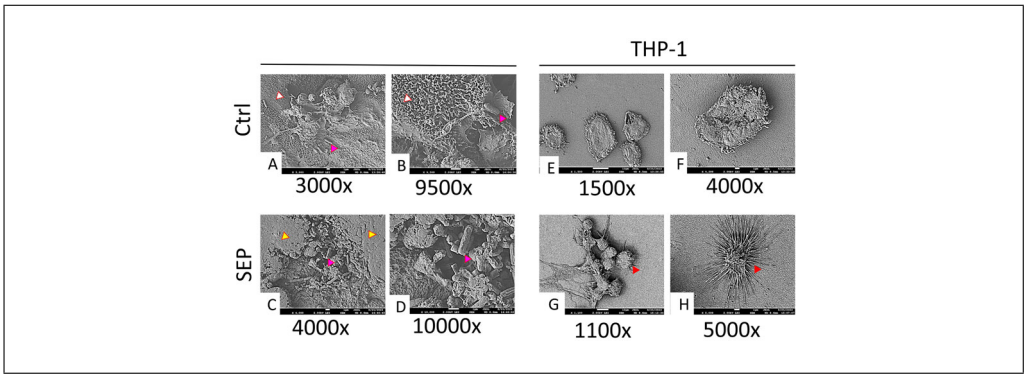

Figure 12 Scanning electron microscopy (SEM) images of Caco2-HT29 epithelial layer and THP1 cells cocultured on the opposite side of the 3D Flipwells membrane. (**A**-**B**) Caco-2/HT29 epithelial layer: Untreated control. Images captured at the magnifications of 3000× (**A**) and 9500× (**B**). Solid pink arrows indicate *B. subtilis*, while solid white arrows point to microvilli. (**C**-**D**) Caco-2/HT29 treated with SEP: Images captured at the magnifications of 4000× (**A**) and 10000× (**B**). Solid pink arrows indicate *B. subtilis*, while solid yellow arrows point to vast and thick mucosal secretions not present in the untreated control (see **A**-**B** above). Microvilli are submerged in mucus and not easily seen. Here, bacteria are present in aggregates encapsulated in thick mucus unlike the untreated control (see **A**-**B** above). (**E**-**F**) THP-1 cells treated with PMA (M0) for attachment to the membrane and cultured on the opposite side of the 3D Flipwell: untreated control. Images captured at the magnifications of 1500× (**E**) and 4000× (**F**). (**G**-**H**) THP-1 cells [initially treated with PMA (M0) for attachment as in **E**-**F**)] treated with SEP appear to have gone through a morphological change. Images captured at the magnifications of 1100× (**G**) and 5000× (**H**). Solid red arrows indicate elongated pseudopodia typical of the M1-like phenotype. Note that *B. subtilis* was cultured in LB and added to the epithelial side of the Flipwell in the smaller Transwell insert for co-culture. It was added to the epithelial cells for imaging and visualization of bacterial clustering in mucus validating presence of thick bacterium trapping mucus.

2 kV using the LEI detector. Using this technique, variation in mucous and changes in bacterial morphology in SEP-treated (Fig. 12C-D) vs SEP-untreated Caco-2:HT29-MTX cells with *Bacillus* (Fig. 12A-B) were observed, as well as macrophage polarization toward M1 phenotype in SEP-treated samples (Fig. 12G-H) were observed compared to the untreated controls (Fig. 12E-F).

IMMUNOFLUORESCENCE ANTIBODY STAINING FOR CONFOCAL MICROSCOPY

BASIC PROTOCOL 5

To assess microphage repolarization across the mucous barrier and gut epithelial layer after Sepiapterin (SEP) treatments, the Flipwell membrane can be processed and prepared for immunofluorescent (IF) staining and confocal microscopy. We used fluorescently-labeled antibodies targeting mucosal, epithelial, and macrophage polarization markers for confocal microscopy to image both sides of the membrane with a confocal microscope. We did not disassemble the Flipwell for the fixation, initial wash, and blocking steps. We fixed, washed, and blocked the Flipwells inside a 50-ml conical tube by submerging the Flipwell and gently rotating the conical tube on a rotator (Fig. 11A).

Large droplet and 12-well plate incubation methods

For the subsequent steps of permeabilization and staining, we washed and stained both sides of the membrane at the same time. We used a "droplet method" for the bench top room temperature steps and the "12-well plate method" for the overnight 4°C incubation (Fig. 13B).

To label each side of the membrane, we placed the insert on top of a large droplet, either inside a 12-well plate for overnight incubation at 4°C, or on a plastic wrap section taped to the bench top (Fig. 13B-C) for room temperature incubation steps. For the wash steps,

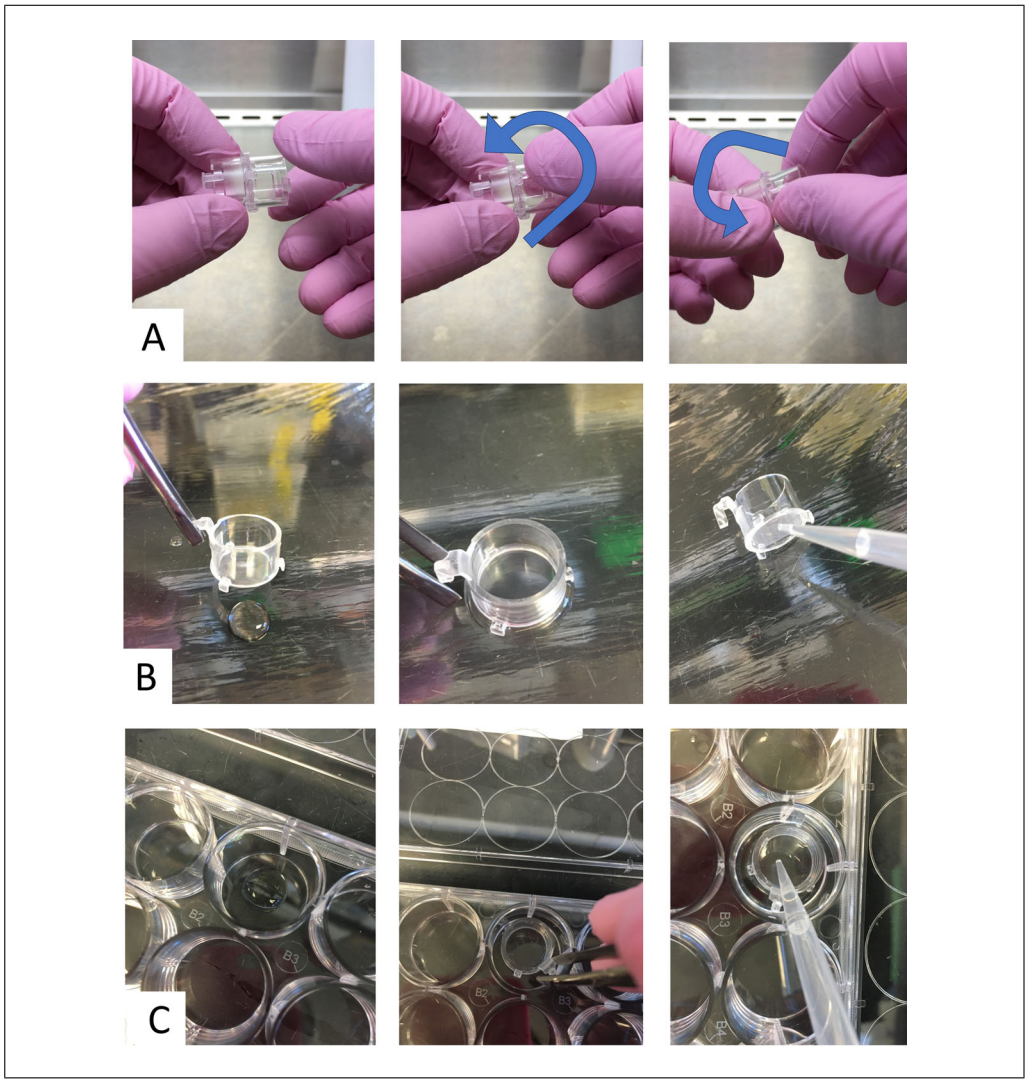

Figure 13 Flipwell disassembly for IF staining and confocal microscopy. (**A**) Disassembly of the Flipwell with a twist-off action. (**B**) Wash, permeabilization, fixation, and antibody incubation steps on the plastic wrap. (**C**) Alternative method using a 12-well plate instead of the plastic wrap. This is better for the overnight antibody incubation step.

we made a large droplet of wash buffer and placed the insert on top of the droplet (Fig. 13B). We also used a series of gentle washes with the tip done drop-by-drop (Fig. 13C). For permeabilization, we used a large "droplet method" for the bottom of insert (Fig. 13B). For the overnight antibody incubation step, we used the "12-well plate method" (Fig. 13C). For the benchtop "droplet method" we used a 200-µl volume of either the permeabilization and wash buffers, or antibody in blocking buffer for the apical (top) of the insert, and a 200-µl droplet at the bottom for the plastic wrap setup.

Materials

Flipwells from Basic Protocol 1
PBS without calcium or magnesium (Fisher Scientific, cat. no. BSS-PBS-1X6)
4% PFA (see recipe)
0.4% PFA (see recipe)
Triton X-100 (Sigma-Aldrich, cat no. X100)
Blocking buffer (see recipe)
Antibody dilution buffer (see recipe)
Primary antibodies:

Anti-human MUC2 (996/1) (Thermo Fisher, cat. no. MA5-12345)
Anti-human CK20 (Life Technologies, cat. no. PA5-82875)
Anti-human CD80 (Cell Signaling Technologies, E3Q9V, cat. no. 15416)
Anti-human CD163 (Abcam, cat. no. 156769) or anti-human CD68 (Novus Biologicals, cat. no. NB100-683)
Secondary antibodies:
Alexa Fluor 488 goat anti-rabbit IgG (Thermo Fisher, cat. no. A11008) for CD80 and CK20
Alexa Fluor 594 goat anti-mouse IgG (Thermo Fisher, cat. no. A11005) for MUC2 and CD68 or CD163
DAPI, VectaShield mounting medium (Vector Laboratories, cat. no. H-1000)

50-ml conical tubes (SpectraTube, Alkali Scientific cat. no. C2750)
Plastic wrap
Lab tape or shipping tape
Tweezers
12-well tissue culture plate (Corning Costar, cat. no. 3513)
6-well dish
Aluminum foil
Scalpel
Round cover slips, #1, 1 oz (Corning Float Glass, cat. no. 50143822, or Fisherbrand cat. no. 12-546-2 25CIR-2)
Laboratory marker (VWR, cat. no 95042-566)
Attofluor cell chamber coverslip holder (Thermo Fisher Scientific, cat. no. A7816)
Confocal microscope and software [e.g., Leica Microsystems TCS SP5, multi-photon laser scanning, and Suite Advanced Fluorescence (LAS AF) software]
Deep Petri dish bottoms, 100 × 25–mm deep polystyrene stackable Petri dish, sterile (USA Scientific, cat. no. 8609-0625)
10-, 200-, and 1000-µl pipet tips (Alkali Scientific PurePoint or equivalent)
Beckman Coulter Allegra 6 centrifuge with a swing bucket rotor for 50-ml conical tubes
Optional: ImmunoPen (Fisher Scientific, cat. no. 4021761EA)

Wash, permeabilization, disassembly, and blocking steps

1. Wash the Flipwell in 15 ml PBS inside a 50-ml conical tube.
2. Discard PBS and add 15 ml of 4% PFA for 10 min (Fig. 11A) inside a 50-ml conical tube.
3. Discard 4% PFA and wash with 15 ml PBS to process for immunostaining immediately or leave in 15 ml of 0.4% PFA in PBS overnight to stain the next day.
4. Tape plastic wrap (a square piece 12 × 12–in.) to the bench top with lab tape or shipping tape.
5. Make a large droplet with 200 µl PBS (Fig. 13B).
6. To separate the Flipwell inserts, twist the top and bottom in the opposite direction (Fig. 13A).

   When disassembling the Flipwell, it is important to label which cell type is located inside the insert and know the cell type on the bottom of the insert. This will dictate which antibody needs to be used and the orientation of the insert during IF incubation.

7. Remove the silicone glue gasket ring with tweezers (not shown).

8. Place the bottom of the insert with the membrane (with THP-1 cells) on top of the 200 µl PBS droplet.
9. To permeabilize the gut epithelial side (top) of the membrane, add 200 µl of 0.1% (v/v) Triton-X100 in PBS for 10 min, aspirate, and add 200 µl PBS. Repeat PBS wash twice.
10. Create a large 250 µl droplet of blocking buffer.
11. Place the insert on top of this droplet and block for 30 min at room temperature.
12. Add 200 µl blocking buffer to the apical side of the insert (top) and block for 30 min at room temperature.

Primary and secondary antibody incubation

Apply primary antibodies at a 1:100 ratio in antibody dilution buffer overnight at 4°C. We used a 12-well plate method for overnight incubation steps and larger buffer volumes. We used 300 µl for the droplet and 300 µl for the apical (top) side of the insert for overnight incubations, but only 200 µl for room temperature incubations and droplets placed on top of the plastic wrap. We used CK-20 and MUC2 primary antibodies for the gut epithelial side of the Flipwell, and CD80 and CD68 or CD163 primary antibodies for the microphage side of the Flipwell. For the secondary antibodies, we used Alexa Fluor 488 for CK20 and CD80 and Alexa Fluor 594 for MUC2 and CD68 or CD163 for both sides of the Flipwell membrane. We therefore were able to apply primary and secondary antibodies to both sides of the Flipwell membrane. This is a new method and allows for dual staining of the cell types across the membrane.

13. Prepare primary antibody dilutions in antibody dilution buffer (i.e., antibody diluted in the 1% BSA in PBS mixture).
14. Add 200 to 300 µl of primary antibody to the insert (200 µl for the room temperature incubation and 300 µl for overnight at 4°C).

    For example, if the gut epithelial cells are on the insert side, use anti-human CK20 rabbit IgG (you will use goat anti-rabbit Alexa Fluor 488 secondary in step 12) and anti-human MUC2 mouse IgG (you will use goat anti-mouse Alexa Fluor 594 in step 24).
15. Place a large droplet of the second primary antibody (200 µl for the plastic wrap method or 300 µl inside a 12-well tissue culture plate) (Fig. 13B and 13C).

    For example, if the macrophage cells are on the underside of the insert, use CD80 and CD68 or CD163 primary antibodies (you will use CD80 primary with Alexa Fluor 488 and CD68 or CD163 with Alexa Fluor 594 in step 24).
16. Place the insert bottom on top of the droplet (Fig. 13B).
17. Cover the dish.
18. Incubate 2 hr at room temperature or overnight at 4°C.
19. If using a fluorochrome-conjugated primary antibody, then skip to step 25.
20. Gently rinse three times in 1× PBS for 5 min each. Use the large droplet method for the bottom of the insert and/or apply PBS gently drop-by-drop (Fig. 13B).
21. Prepare secondary antibodies in the antibody dilution buffer. Dilute 1:1000 or more per manufacturer's recommendations.
22. Add 200 to 300 µl of the secondary antibody inside the insert.

23. Place a large 300 µl droplet of the second secondary antibody diluted per manufacturer's recommendations inside a well of a 6-well dish (Fig. 13C).
24. Incubate with secondary antibodies 1 to 2 hr at room temperature protected from light. Cover with aluminum foil or store in a bench drawer.
25. Wash with PBS 3 times. Use the large 300 µl droplet method for the bottom of the insert and add 300 µl to the inside of the insert.
26. Using a sterile scalpel blade and tweezers for support, cut out the membrane and hold it with tweezers. Note the orientation. You can place a small dot or small "x" on one side of the membrane to denote orientation and cell type.
27. Place 1 to 3 droplets of DAPI on a round cover slip (DAPI diluted 1:100 in PBS).
28. Place the membrane on a round cover slip.
29. Add 1 to 2 droplets of DAPI to the top of the membrane (DAPI diluted 1:100 in PBS).
30. Cover with the second round-shaped cover slip.
31. Label each side of cover slip with cell type or make a mark and note cell type.
32. Place the cover slip-membrane "sandwich" into a stainless-steel holder for confocal microscope imaging.

    This may be microscope-specific. Consult your confocal microscopy facility for the holder that would work best with their confocal microscope.
33. Wrap the sandwich in aluminum foil or keep in the dark.
34. Scan each side of the membrane with appropriate wavelength depending on the fluorophores used.
35. For long term storage, store at 4°C protected from light.

Imaging

By utilizing confocal microscope's Z-stack feature, we were able to image both sides of the membrane and all cell types in the same session. We used the two metal rings of the Attofluor cell chamber and two round cover slips per membrane to "sandwich" and hold the Flipwell membrane. We imaged both sides of the membrane by imaging one side first and then flipping the sandwich over to image the other side of the membrane. We used Leica Microsystems TCS SP5 multi-photon laser scanning confocal microscope using Suite Advanced Fluorescence (LAS AF) software.

Imaging is done the same way as any rectangular microscope slide would be imaged. In this case, the only difference is that we had to use Attofluor cell chamber rings to hold the glass specimen because the cover slip we used is round in shape. We used two round cover slips to hold the membrane for imaging. We had to flip the "sandwich" to image each side of the membrane.

REAGENTS AND SOLUTIONS

Antibody dilution buffer

0.1 g bovine serum albumin (BSA) (Sigma-Aldrich, cat. no. 05470), 1% (w/v) final concentration
10 ml of 1× PBS (Fisher Scientific, cat. no. BSS-PBS-1X6)
Stir until dissolved

Add 30 µl Triton-X 100 (Sigma-Aldrich, cat. no. X100), 0.3% (v/v) final concentration
Stir until dissolved
Use fresh or store up to 2 days at 4°C

Blocking Buffer

250 µl serum from the same species as the secondary antibody, 5% (v/v) final concentration
5 ml PBS (Fisher Scientific, cat. no. BSS-PBS-1X6)
15 µl Triton-X100 (Sigma-Aldrich cat no X100), 0.3% (v/v) final concentration
Stir until dissolved
Use fresh or store up to 2 days at 4°C

Caco-2 medium

440 ml Dulbecco's modified Eagle medium (DMEM) with high glucose (4.5 g/L) and 1% (v/v) GlutaMax (Thermo Fisher Scientific, cat. no. 35050061)
50 ml fetal bovine serum (FBS), heat-inactivated (Sigma, F2242-500ML), 10% (v/v) final concentration
5 ml Pen-Strep (Thermo Fisher Scientific, cat. no. 15140122), 1% (v/v) final concentration
5 ml non-essential amino acids (Thermo Fisher Scientific, cat. no. 11140076), 1% (v/v) final concentration
Store up to 1 month at 4°C

HT-29-MTX medium

440 ml (DMEM) with high glucose (4.5 g/L) and 1% (v/v) GlutaMax (Thermo Fisher Scientific, cat. no. 35050061)
50 ml FBS, heat-inactivated (Sigma, F2242-500ML), 10% (v/v) final concentration
5 ml Pen-Strep (Thermo Fisher Scientific, cat. no. 15140122), 1% (v/v) final concentration
5 ml HEPES (Thermo Fisher Scientific, cat. no. 15630080), 10 mM final concentration
Store up to 1 month at 4°C

Miller LB Medium

To a 500-ml glass flask, add:
6.25 g Miller LB (Sigma-Aldrich, cat no. L3522)
250 ml H_2O, deionized
Autoclave for 15 min at 121°C, cool to room temperature
Store up to 1 month at room temperature

PFA, 0.4%, methanol-free

1 L of 1× PBS (Fisher Scientific, cat. no. BSS-PBS-1X6)
4 g paraformaldehyde (Sigma-Aldrich, cat. no. 441244)
Store up to 6 months at 4°C

Alternatively, dilute 4% PFA (see recipe) 1:10 with 1× PBS (Fisher Scientific, cat. no. BSS-PBS-1X6).

PFA, 4%, methanol-free

1 L of 1× PBS (Fisher Scientific, cat. no. BSS-PBS-1X6)
40 g paraformaldehyde (Sigma-Aldrich, cat. no. 441244)
Store up to 6 months at 4°C

Phorbol myristate acetate (PMA) stock solution, 50 µg/ml)

Add 5 mg PMA (InvivoGen, cat. no. Tlrl-pma) to 1 ml DMSO to make a concentrated 5 mg/ml solution. Vortex to mix. Prepare 20-µl aliquots and store up to 1 month at –20°C. Prepare a PMA stock solution (50 µg/ml) by diluting 1 µl of concentrated 5 mg/ml PMA solution in 99 µl (1:100) complete growth medium. In the Flipwell, use 1 µl of working stock added to 500 µl medium.

PureCol working collagenase solution (100 µg/ml)

Prepare a 1:30 dilution of the commercial stock solution to obtain a 100 µg/ml concentration as follows. Add 33 µl PureCol (Advanced BioMatrix, PureCol solution 3 mg/ml, cat. no. 5005-100ML) to 1 ml sterile deionized water in a sterile microcentrifuge tube. Vortex to mix. Prepare fresh. Do not reuse.

L-Sepiapterin stock solution, 50 mM

100 mg L-Sepiapterin (SEP) (Career Henan Chemical Co., CAS#: 17094-01-8)
8.43 ml DMSO
Vortex to mix
Make 100-µl aliquots
Store up to 6 months at –20°C

Use at a final concentration of 100 µM (e.g., add 1 µl SEP stock into 500 µl medium per Flipwell, or 20 µl SEP stock into 10 ml Miller LB when treating bacterial culture).

THP-1 Medium

420 ml RPMI-1640 medium (Thermo Fisher Scientific, cat. no. 11875119)
50 ml FBS, heat-inactivated (Sigma, F2242-500ML), 10% (v/v) final
5 ml GlutaMax, 1% (v/v) (Thermo Fisher Scientific, cat. no. 35050061), 2 mM final concentration
5 ml HEPES (Thermo Fisher Scientific, cat. no. 15630080), 10 mM final concentration
11 ml glucose (Thermo Fisher Scientific, cat. no. A249001), 4.5 g/L final concentration
5 ml sodium pyruvate (Thermo Fisher Scientific, cat. no. 11360070), 1 mM final concentration
5 ml Pen-Strep (Thermo Fisher Scientific, cat. no. 15140122), 1% (v/v) final concentration
Store up to 1 month at 4°C

COMMENTARY

Background Information

Evaluation of the macrophage polarization across the gut epithelial and mucosal layers in a three-dimensional (3D co-culture) format has been challenging due to the lack of a robust multi-cellular co-culture system. By modifying a previously described system (Noel et al., 2017), we have engineered and tested a new multi-cellular, multi-layer co-culture system that we termed 3D Flipwell co-culture system (Fig. 1). This 3D Flipwell co-culture system allows for the use of the bacterial culture and pharmacologic agents to assess their effects on the gut mucosa and macrophage polarization. The new 3D Flipwell design utilizes commercially available transwell inserts that are stacked bottom-to-bottom with a single layer of PET membrane. By testing various pharmacological and microbiological agents, further investigation of the gut mucosal and immune cell responses can be achieved effectively in a 3D multi-cellular multi-layer system mimicking the gut epithelial environment.

Critical Parameters

The most critical parameter is the cell seeding ratios, cell counts at plating, and growth timing to produce and maintain gut epithelial monolayer and macrophage adherence. Initial experimentation should include fewer than suggested cell numbers to establish good

Table 1 Troubleshooting Flipwell Assembly and Set Up

Problem	Possible cause	Solution
PBS test of the Flipwell assembly results in leakage	Insufficient adhesive used; bead is not applied uniformly around the Transwell bottom rim, and the bead is not of adequate thickness; bottom-to-bottom alignment of the two inserts is altered, the Transwell assembly moved during the curing process	Improve uniformity of the adhesive bead and increase thickness of the adhesive bead; avoid twisting the Transwell inserts during the drying process; remake the Flipwell
Cells do not adhere	Collagen solution is compromised	Recoat new Flipwells; can increase collagen concentration and thickness; dry well
Contamination	Compromised sterile technique and sterility of reagents and process	Use fresh Pen-Strep, assess sterile technique processes
Flipwell capsizes during the flip	Insufficient hold and grip	Obtain forceps with serrated tips; improve holding technique and grip
Large air bubble is still present after the flip	It is expected to have an air bubble after the flip, please see solution	Use needle and syringe as describe to remove large air bubble and stabilize the Flipwell
Culture medium crossed into the opposite side of the membrane	Cells kept in culture too long, phenol dye migrated; Flipwells leak, compromised adhesion; leak test not performed	Decrease culture duration; check newly made Flipwells for leakage prior to collagen coating
Culture medium not touching cells on the basolateral side	Evaporation during incubation; insufficient medium	Add more medium to deep well Petri dish; check incubator humidity pan; check plate every other day and add medium as needed

monolayer under users' media formulations and incubator settings. Phenol red presence in our media was beneficial but not necessary. Bacterial cell counts, toxicological effects of the bacterial secretome, and pharmacological effects of the desired agents on cells and bacteria may be analyzed prior to the experimental setup.

Troubleshooting

For possible problems and solutions for Flipwell assembly and set up, please see Table 1.

Understanding Results

Here we report our new co-culture system that aims to recapitulate the gut mucosal environment. This article is a procedural guide to the publication by Beamer et al. (2023). To test the usability of this system, we tested the effects of SEP, an endogenous precursor of BH_4, a cofactor of NOS (Feng et al., 2021) on different components of the co-culture, and monitored differential responses. We previously showed that application of SEP to breast tumors effectively reprograms tumor associated macrophages from the immuno-suppressive M2-type to immuno-stimulatory M1-type, while also suppressing tumor cell growth (Fernando et al., 2023; Ren et al., 2019; Zheng et al., 2020). Here, we tested the effects of SEP on colon epithelial Caco2 and mucus-producing HT-29/MTX cells, THP-1 macrophages, and Gram-positive commensal *B. subtilis* (Beamer et al., 2023). SEP could activate both mammalian and bacterial NOS isoforms for NO production (Chen et al., 2022). We made comparisons between monocultures and our co-cultures.

Macrophages in monocultures showed clear morphological differences among different subtypes. As previously reported by Kainulainen et al. (2022), M1-type showed elongated, spindle-like structures with prominent pseudopods, whereas M0 and M2 types showed spherical and smoother structures. After SEP treatment, however, both M0 and M2 types exhibited spindle-like structures comparable to M1 type. Such morphological shift of M0 and M2 types by SEP supported our previous finding that SEP caused their functional reprogramming to M1 type (Zheng et al., 2020). SEM imaging of control *B. subtilis* in monocultures showed smooth, rod-shaped structures with single polar flagella. However, after SEP treatment, these bacteria

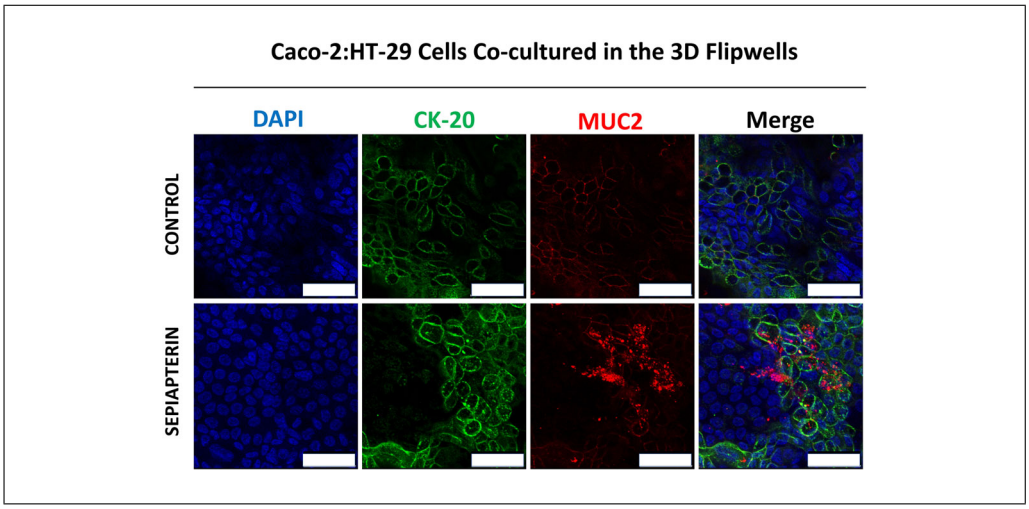

Figure 14 Sepiapterin promotes MUC2 and Cytokeratin-20 expression in Caco-2:HT-29 epithelial layer of the 3D Flipwell system co-cultured with THP-1 cell layer. Confocal Images of Caco-2:HT-29 cells co-cultured with THP-1 cells in the 3D Flipwells and stained for CK20 (green) and MUC2 (red). Cytokeratin rearrangement is very pronounced surrounding massive mucus production as indicated by Muc2. Scale bar = 50 µm.

exhibited a wrinkled, rougher surface, indicating a morphological change possibly due to biofilm formation (Trejo et al., 2013). Biofilm partly accounts for the roles of these bacteria in disease prevention of the host organisms (Goya et al., 2020; Gusarov et al., 2013). In fact, increased NO production in *B. subtilis* is reported to induce biofilm formation (Arora et al., 2015; Chen et al., 2022). In our co-cultures, control colon epithelia (consisting of Caco2 and HT-29/MTX cells) showed prominent microvilli on the surface (Fig. 12B). Conversely, after SEP treatment these cells showed a dense, mucus production assisting in bacterial clustering and entrapment (Fig. 12D), as reported for a mucosal defense mechanism (Schroeder, 2019; Wallace, 2019). Also, in our co-cultures, control M0 (naïve) macrophages showed spherical, smooth morphology similar to mono-cultured M0 cells (Fig. 12E-F). After SEP treatment, however, M0 macrophages acquired M1-type phenotype (Fig. 12G-H) characterized by spindle-like structures with numerous pseudopods (Kainulainen et al., 2022). These observations were consistent with previous reports that certain probiotics, such as *B. subtilis*, could induce M1 polarization of macrophages (Ji et al., 2013; Tobita & Meguro, 2022). These results suggest that our co-culture system could reproduce the synchronous responses of gut bacteria, mucus, colon epithelia and immune cells to an exogenous stimulus and crosstalk among them. Furthermore, to quantitatively analyze phenotypic changes of co-cultured cells in response to an exogenous stimulus, we measured the expression of specific markers by confocal imaging (Fig. 14). We found that SEP treatment caused a large increase in the mucus protein, MUC2, and epithelial marker, CK20, in mucus/colon epithelial layers. Furthermore, SEP treatment of M0 macrophages caused a large increase of a M1 marker CD80 (Fig. 15A), but neither a M2 marker CD163 nor a pan-macrophage marker CD68, indicating their dedicated M1 polarization (Fig. 15B). These observations altogether attest to the utility of our new co-culture system in studying different responses of individual components of the gut mucosal microenvironment to an exogenous stimulus as well as their crosstalk.

Time Considerations

The 3D Flipwells must be assembled in a sterile environment and UV sterilized 1 to 2 days in advance of the cell culture seeding. The gavage needle must be prepared in advance of the second cell seeding and sterilized. Each cell type should be grown and maintained independently for 1 to 2 weeks prior to the co-culture. It takes 7 to 9 days to differentiate a 1:9 mixture of MTX:Caco-2 cells to develop a mucosal layer. Bacterial insert must be prepared and fit tested 1 to 2 days in advance of the bacterial addition to the Flipwell. *B. subtilis* bacterial culture should be grown overnight prior to experimental addition to the co-culture system. Sepiapterin (SEP) treatments of the bacterial culture can be done as pre-treatments for 3 days prior to the experiment termination.

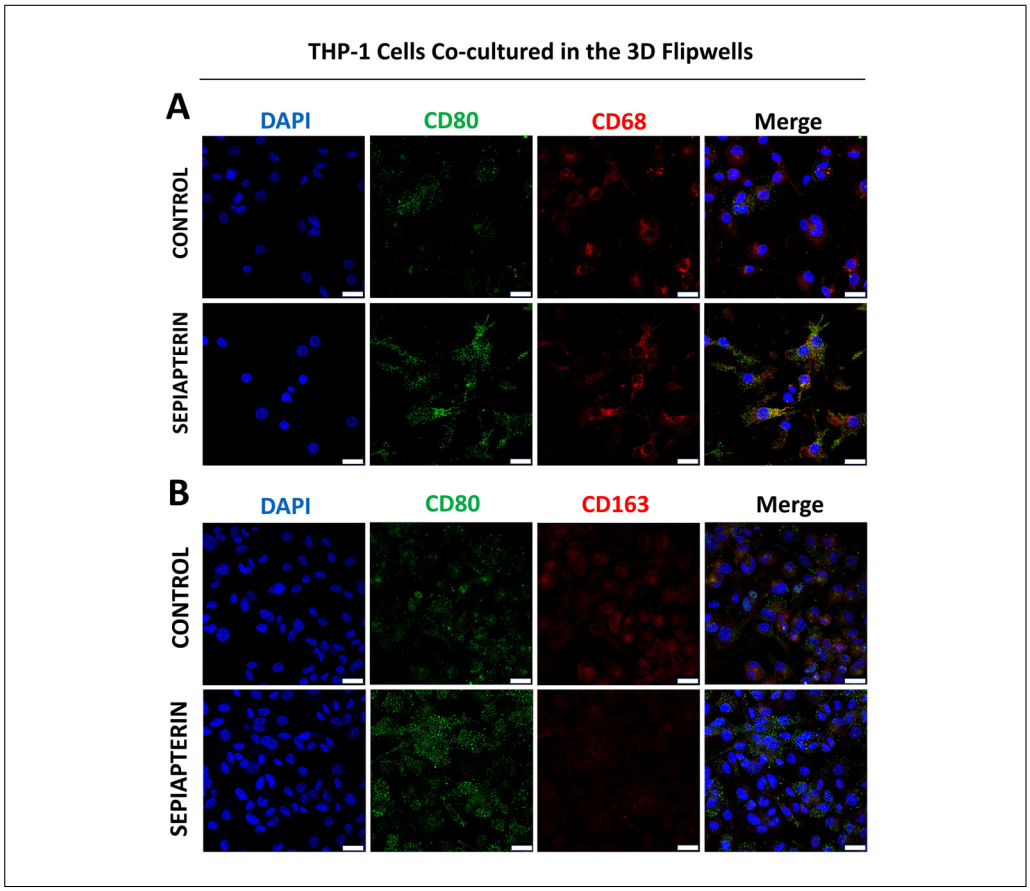

Figure 15 THP-1 dramatic polarization toward an M1-like phenotype with Sepiapterin treatment in the 3D Flipwell coculture system. (**A**) Confocal images of THP-1 cells cocultured with Caco-2:HT-29 in the 3D Flipwells and stained for pan macrophage marker CD68 (red) and CD80 (green). Pan macrophage CD68 marker is present in both the SEP treated sample and the untreated control. CD80 marker, typical of the M1 polarization phenotype, is more prominently present in the SEP treated sample with long pseudopodia extending outward typical of the M1 phenotype. (**B**) Confocal images of THP-1 cells cocultured with Caco-2:HT-29 in the 3D Flipwell and stained for pan macrophage marker CD68 and CD163 to validate phenotypic polarization toward M1 phenotype and away from M2 phenotype. Scale bar = 25 µm.

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Author Contributions

Maria A. Beamer: conceptualization; methodology; formal analysis; investigation; data curation; writing—original draft; writing—review and editing. **Saori Furuta:** conceptualization; methodology; formal analysis; investigation; data curation; funding acquisition; project administration; resources; supervision; writing—original draft; writing—review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

No underlying data are available for this article, since no datasets were generated or analyzed during this study.

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