Protocol for Organ-Chips:

Basolateral Recruitment of Peripheral Blood Mononuclear Cells (PBMCs) on the Colon Intestine-Chip

EP215 Rev A

Goals:	Key Steps:	Associated Emulate Documentation:
• Inflammation-specific migration of PBMCs from the vascular (bottom) channel to the epithelial (top) channel • Release of pro-inflammatory mediators by recruited PBMCs and disruption of epithelial barrier • Abrogation of recruitment and associated downstream inflammatory cascades using α4 integrin-targeting therapeutic molecule AJM300 as a pharmacological modulator	1. Inflammatory activation of endothelial cells with TNFα 2. Chemoattractant addition to the epithelial channel 3. Vascular delivery of PBMCs 4. Daily permeability measurements 5. Imaging and effluent collection endpoints	• **EP203:** Colon Intestine-Chip Culture Protocol (link) • **EP187:** Barrier Function Analysis (link) • **EP216:** Immune Cell Recruitment 3D Analysis User Guide (provided upon request)

Basolateral Administration of PBMCs Protocol

Overview

Introduction

This protocol describes how to induce the migration of peripheral blood mononuclear cells (PBMCs) from the vascular endothelial (bottom) channel to the epithelial (top) channel in the Emulate Colon Intestine-Chip.

In this protocol

This protocol contains the parts listed below.

Part I. Introduction

Protocol Description

This protocol describes how to induce the migration of peripheral blood mononuclear cells (PBMCs) from the vascular endothelial (bottom) channel to the epithelial (top) channel in the Emulate Colon Intestine-Chip. This includes steps for:

- Inflammatory treatment of the vascular channel, addition of chemoattractants to the epithelial channel, preparation of buoyancy media, and administration of PBMCs through the vascular channel
- Measuring key permeability readouts, imaging recruited PBMCs in the epithelial channel, and carrying out Meso Scale Discovery (MSD) analyses of cytokines in the effluent collected from the epithelial channel
- Using AJM300, which selectively binds α4β1 and α4β7 integrins, to exert an anti-inflammatory effect by inhibiting lymphocytes from excessively invading sites of inflammation

Part II. Experimental Timeline

Overview

Introduction

This part discusses the experimental timeline

Contents

Experimental Timeline

Total Timeline

The total timeline for this protocol is six to nine days from seeding (Day 0) the Colon Intestine-Chips, depending on the timing of epithelial tissue maturation (Figure 1, 2, and 3) and desired timeline following PBMC administration (e.g., 48 to 72 h; Figure 3).

Days	Description
3	• Inflammatory activation of the chip with tumor necrosis factor alpha (TNFα) is initiated once an epithelial monolayer with acceptable barrier function is established, as defined in Figure 2 (this typically occurs by Day 3 but may extend to Day 4 or 5) • TNFα is then administered alongside a cocktail of chemoattractants (defined in Experimental Readouts). The former is kept in the chip for a total of 24 h, while the latter is continually dosed until the end of the protocol
4	• Following completion of TNFα administration, PBMCs are mixed with buoyancy media and introduced into the vascular channel (this typically takes place on Day 4, but it may occur on Day 5 or 6; i.e., depending on the timing of barrier maturation, as described above) • The PBMCs are introduced to the vascular channel as a bolus at a high flow rate of 1000 µL / h for 4 h and then flushed with media at the same flow rate for 30 min • The flow rate is then adjusted to 30 µL / h until the end of the protocol as a control, PBMCs can be pretreated and administered together with AJM300 to selectively inhibit PBMC attachment and migration
3-5	• A chip treated with interferon gamma (IFNγ) can act as a parallel, stand-alone (i.e., not combined with PBMC administration) positive permeability control • This treatment can be started in a separate chip at the same time as TNFα/chemoattractant administration (typically Day 3 but may be Day 4 or 5 depending on barrier maturation) and continued to the end of the protocol (Figure 3)

Exemplary phase contrast micrographs of epithelial tissue and functional barrier measurement are depicted in Figure 1 and Figure 2, respectively, and a schematic timeline of the protocol is depicted in Figure 3 below.

Figure 1

Phase contrast images of colonic epithelial tissue in the chip. **A)** Immature epithelial tissue that is not ready for TNFα treatment. **B)** Mature epithelial tissue that is ready for TNFα treatment. The areas between fragments exhibit a monolayer structure with defined cell borders (indicated with white arrows). **C)** "Old" epithelial tissue in culture for multiple days past optimal window for TNFα treatment. **D)** Injured colonic epithelium (i.e., post-72 h IFNγ treatment), where cell border definition is lost, and cell debris is prevalent (indicated with white arrows). Scale bar: 100 µm.

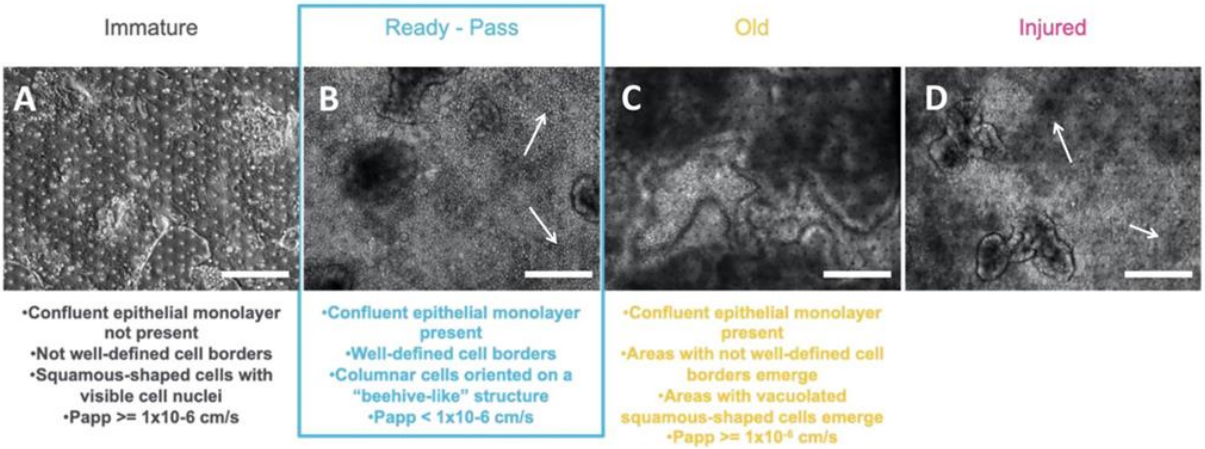


Figure 2

Representative time for barrier maturation on a Colon Intestine-Chip, characterized by apparent permeability to 3kDa dextran. Note that on Day 4 P_{app} has reached a threshold of 1 x 10^{-6} cm / s. N=2 chips per timepoint, error bars represent SEM.

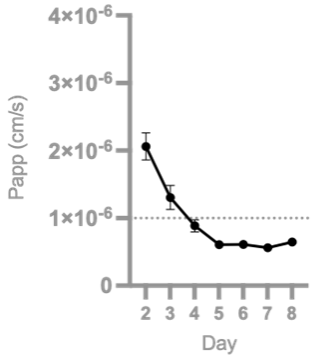
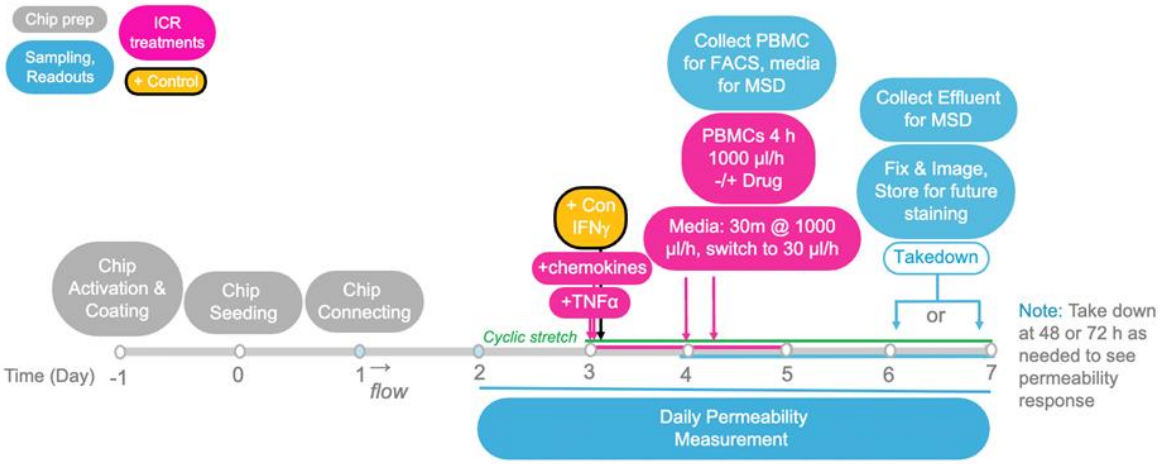


Figure 3

Schematic representation of this protocol's key steps following a typical timeline. Note that cytokine administration may occur on Day 3 (as shown) 4, or 5, depending on the epithelial cells' level of coverage and differentiation. The IFNγ group is a stand-alone positive control for barrier disruption should not be combined with immune cell recruitment treatments.



Part III. Experimental Readouts

Experimental Readouts

- Barrier function by apparent permeability (P_{app}; EP203)
- PBMC localization by fixed microscopy (and associated analysis)
- Cytokine release by MSD U-Plex assay (Meso Scale Diagnostic)
  - Cytokines: IFNγ, IL-22
  - Performed according to manufacturer instructions

Part IV. Colon Intestine-Chip Protocol

Overview

Introduction

Protocol to establish the Colon Intestine-Chip.

Contents

Timeline

Required Material

Emulate Colon Intestine Bio-Kit – 24-pack (Emulate, Cat. BIO-CH1-24)

Timeline

To establish the Colon Intestine-Chip, please follow the instructions in EP203: Colon Intestine-Chip Culture Protocol (link)

Day	Description
-1	• Chip activation and extracellular matrix coating is done on the day before seeding
0	• Organ-Chips are seeded first with colonic human intestinal microvascular endothelial cells (cHIMECs) and then with primary colon organoid-derived fragments
1	• The seeded Colon Intestine-Chips are connected to Pod® Portable Modules and media flow is started by connecting to Zoë® Culture Module
3	• Stretch is introduced to the chips (2% stretch at 0.15 Hz)
4	• Stretch is increased further on the chip (10% stretch at 0.15 Hz) • The basolateral PBMC recruitment protocol begins by introducing inflammatory cytokines

Effluent Collection for Analysis (Day 2 through Takedown)

Protocol

Step	Action
1	Using a P200 multichannel pipette, collect 200 µL from the Sampling/Aspirating zone of the Pod inlets and outlets – Follow EP124: Effluent Sampling Protocol for detailed instructions (link)
2	Collect into 96-well V-bottomed plates and repeat for all samples *Optional: centrifuge effluent collection plate at 300 x g for 5 min to pellet any residual cell debris*

Barrier Function Dosing, Collection, and Analysis

Required Materials

- Cell culture grade water
- Dextran, Cascade Blue™, 3000 MW, Anionic, Lysine Fixable (ThermoFisher Scientific, Cat. D7132)
- Endothelial Cell Growth Medium (EGM) MV 2 (PromoCell, Cat. 22121)
- IntestiCult™ Organoid Growth Medium (StemCell Technologies, Cat. 06010)
- Serological pipettes
- Pipettes including multichannel and filter tips
- 37°C water or bead bath
- 70% ethanol
- 96-well flat-bottom plates

Preparation of Cascade Blue Stock Solution (from EP203: Basic Colon Intestine-Chip Protocol)

Step	Action
1	• In a biosafety cabinet (BSC), resuspend 10 mg of Dextran, Cascade Blue™, 3000 MW, Anionic, Lysine Fixable in 1 mL of cell culture grade water to obtain 3 kDa Dextran Cascade Blue working solution at a 10 mg / mL concentration • One vial containing 10 mg of 3 kDa Dextran Cascade Blue is sufficient for 200 mL of complete IntestiCult media
2	• Any remaining working solution can be stored up to 1 week at 4°C

Preparation of 3 kDa Cascade Blue Dosing Media

Step	Action
1	Warm sufficient volume of complete IntestiCult per day, including 200 µL additional volume per chip for effluent collection
2	Add Cascade Blue Stock Solution to make 50 µg/mL concentration in media, or 1:200 dilution

Continuation of Flow after Effluent Collection

Step	Action
1	Aspirate Pod top and bottom outlets
2	Add media to Pod inlets to ensure enough volume per chip for 30 µL/h flow
3	Add Cascade Blue-dosed IntestiCult media to the epithelial channel Pod inlets – Add equivalent volume of EGM to bottom vascular Pod inlet (without Cascade Blue tracer)
4	Return the Pods to Zoë and continue flow for the epithelial and vascular channels
5	Repeat the collection procedure daily until the experiment takedown to evaluate the barrier function of the epithelium over the course of the experiment

Barrier Function Collection (Day 2 through Takedown)

If the barrier function is included as a readout, follow the instructions in the Emulate protocols below:

Protocol	Document Number
Barrier Function Analysis	EP187
Colon Intestine-Chip Culture Protocol	EP203

Step	Action
1	Pipette 50 µL of sample from the effluent collection plate. Dilute the sample if necessary – For the Cascade Blue tracer molecule at 50 µg / mL, dilute samples to 1:3 in the PBS
2	Follow Emulate protocol EP187, Protocol for Emulate Organ-Chips: Barrier Function Analysis' for standard barrier function protocol – For the Cascade Blue tracer molecule, measure the signal with the plate reader at an excitation/emission of 400/420

Effluent Collection for MSD Analysis

Protocol

Step	Action
1	Transfer 100 µL of sample from the effluent collection plates to the MSD analysis plates (96-well black-walled plates)
2	Cover the wells with plate sealer film or seal the plate with parafilm. Store plate at -20°C for up to 1 month, or -80°C for longer-term storage before measuring – It is recommended to use the MSD cytokines IFNγ and IL-22 for this step

Part V. Day 3-5: TNFα and Chemoattractant Treatment

Overview

Introduction

Protocol for TNFα and chemoattractant treatment.

Contents

Day 3-5: TNFα and Chemoattractant Treatment

Goals

- To induce endothelial cell activation with 24 h TNFα treatment in vascular channel, with simultaneous treatment of epithelial channel with gut-specific chemoattractant cocktail to create appropriate inflammatory conditions for PBMC recruitment
- In parallel, use an optional stand-alone IFNγ treatment (i.e., not to be introduced with PBMC or with any other treatment) as a positive control condition for inflammation-driven barrier disruption in order to compare with any effects of PBMC recruitment
- Note that TNFα treatment should not be started until complete barrier maturation is achieved (i.e., $P_{app} \leq 1x10^{-6}$ cm/s)

Required Materials

Material Name	Model Information
0.45 µm PVDF sterile filters	--
Bovine serum albumin	BSA, MilliporeSigma, Cat. A7030
Cell culture grade water	--
Dulbecco's Phosphate Buffered Saline 1X	Millipore Sigma, Cat. D8537
EGM MV-2 complete media	PromoCell, Cat. 22121
Primocin	VWR, MSPP-ANTPM1
TNFα	R&D Systems, Cat. 210-TA-020
Recombinant human IP-10	CXCL10, PeproTech, Cat. 300-12
Recombinant human MIP-3α	CCL20, PeproTech, Cat. 300-29A
Recombinant human MEC	CCL28, PeproTech, Cat. 300-57
Recombinant human GPR15L	PeproTech, Cat. 300-71
Recombinant human TECK	CCL25, PeproTech, Cat. 300-45
Recombinant human MCP-1	CCL2, PeproTech, Cat. 300-04
Recombinant human SDF-1α	CXCL12, PeproTech, Cat. 300-28A
Recombinant human MIP-3β	CCL19, PeproTech, Cat. 300-29B
Dextran, Cascade Blue™, 3000 MW, Anionic, Lysine Fixable	ThermoFisher Scientific, Cat. D7132
Additional Required Materials (if including IFNγ treatment control)	
Recombinant human IFNγ	PeproTech, Cat. 300-02

Cytokine Treatment with TNFα (24 h)

Preparation of TNFα Stock Solution (as below, or according to vendor instructions)

Step	Action
1	Prepare 0.1% (w/v) BSA solution: – Weigh 50 mg BSA into 50 mL conical tube – Add cell culture grade water up to 50 mL – Put solution on shaker on low speed to mix until dissolved – Sterile filter solution through 0.2 µm filter
2	Centrifuge TNFα vial for a few seconds to pellet lyophilized contents
3	Reconstitute TNFα using BSA solution to 1 mg / mL. Pipette to mix – Reconstituted stock can be stored at -20°C for up to 3 months – Aliquot solution to avoid multiple freeze-thaw cycles

Preparation of TNFα Dosing Media

Step	Action
1	Prepare EGM MV2 following manufacturer instructions – Thaw supplement pack and add to Basal Media MV2 component – Add 500 µL Primocin (1:1000) – Sterile filter complete media through 0.45 µm PVDF filter
2	Warm sufficient volume of complete EGM MV2 media to 37°C – Account for at least 24 h of 30 µL / h flow, plus 200 µL per chip for effluent readouts (recommended at least 1.2 mL total per chip)
3	Add 1 mg/mL TNFα stock solution to the EGM MV2 to a concentration of 50 ng/mL – If the necessary volume of TNFα is exceedingly small, dilute the stock 1:10 in 0.1% BSA solution to allow for larger pipetting volumes

Chemokine Treatment with Cocktail (24 h)

Preparation of Chemokine Stock Solutions (as below, or according to vendor instructions)

Step	Action
1	Centrifuge each chemokine vial (8 vials) to pellet lyophilized contents
2	Reconstitute each chemokine individually to 1 mg / mL concentration in PBS. Pipette to mix – Aliquot stock solutions (5 µL / aliquot) and store at -20°C to avoid multiple freeze-thaw cycles

Preparation of Dosing Media with Chemokine Cocktail

Step	Action
1	Prepare IntestiCult media following manufacturer instructions
2	Warm sufficient volume of complete IntestiCult media to 37°C – Account for at least 24 h of 30 µL / h flow, plus 200µL per chip for effluent barrier analysis (recommended at least 1.2 mL total per chip)
3	Add 1 mg / mL chemokine stock solution to IntestiCult media to achieve a concentration of 100 ng / mL. Repeat for each of 8 chemokines – If the necessary volume is exceedingly small, dilute the stock 1:10 in PBS to allow for larger pipetting volumes
4	Add 10 mg / mL 3 kDa Dextran Cascade Blue working solution to a final concentration of 50 µg / mL (1:200 dilution) for barrier function analysis if necessary (See Barrier Function Dosing, Collection, and Analysis)

Note that by day 3, IntestiCult media should no longer include Rock inhibitor or CHIRR99021.

Administration of Dosing Media to Chip

Step	Action
1	Aspirate all four Pod reservoirs
2	Add the bottom dosing media (1.2 mL of EGM MV2 + TNFα) to the bottom inlet reservoir of appropriate chips
3	Add the top dosing media (1.2 mL of IntestiCult + chemokine cocktail + 50 µg / mL Cascade Blue Dextran) to the top inlet reservoir of appropriate chips
4	Return chips to Zoë and resume flow for 24 h

Note on TNFα Treatment

TNFα treatment must ensue for a full 24 hours to ensure sufficient inflammation of the endothelium. Thus, for logistical reasons, it is recommended to start TNFα treatment in the morning, to allow a reasonably early start of PBMC administration the next day.

Cytokine treatment with IFNγ for control (96 h, optional)

Preparation of IFNγ Stock Solution (as below, or according to vendor instructions)

Step	Action
1	Prepare 0.1% (w/v) BSA solution: – Weigh 50 mg BSA into 50 mL conical tube – Add cell culture grade water up to 50 mL – Put solution on shaker on low speed to mix until dissolved. – Sterile filter solution through 0.2 µm filter
2	Centrifuge IFNγ vial to pellet lyophilized contents
3	Reconstitute IFNγ using BSA solution to 1 mg / mL. Pipette to mix – Reconstituted stock can be stored at 4°C for up to 1 week. Use a fresh vial for each experiment

Preparation of IFNγ Dosing Media

Step	Action
1	Prepare EGM MV2 following manufacturer instructions: – Thaw supplement pack and add to Basal Media MV2 component – Add 500 µL Primocin (1:1000)
2	Warm sufficient volume of complete EGM MV2 media to 37°C – Account for 96 h of 30 µL / h flow, plus 200 µL per chip for daily effluent barrier analysis (recommended 4 mL per chip)
3	Add 1 mg/mL IFNγ stock solution to the EGM MV2 to a concentration of 50 ng / mL – If the necessary volume of IFNγ is exceedingly small, dilute the stock 1:10 in 0.1% BSA solution to allow for larger pipetting volumes

Administration of IFNγ Dosing Media to Chip

Step	Action
1	Aspirate all four Pod reservoirs
2	Add the bottom dosing media (4 mL of EGM MV2 + IFNɣ) to the bottom inlet reservoir of appropriate chips
3	Add epithelial channel media with tracer (IntestiCult complete media + 50 µg / mL 3 kDa Dextran Cascade Blue)
4	Return chips to Zoë and resume flow until end of experiment

Part VI. Day 4: PBMC Administration

Overview

Introduction

Protocol for PBMC administration.

Contents

Day 4: PBMC Administration

Goals

- Administer a bolus of PBMCs to vascular channel using buoyancy media with continuous chemokine administration in epithelial channel to induce PBMC recruitment into the epithelial channel
- Validate the dependence of recruitment on α4β7 integrin adhesion receptor by pretreatment (and co-administration) of α4 integrin small molecule inhibitor AJM300 as a control

Key Steps

- Prepare 2X RPMI and Gelzan reagents
- Incubate PBMCs with Cell Tracker stain
- Pretreatment and co-administration of PBMC with α4 integrin small molecule inhibitor AJM300
- Prepare concentrated buoyancy solution of Percoll with 1.6% v/v Gelzan
- Create concentrated cell suspension in 2X RPMI media
- Add buoyancy solution to cell suspension
- Replenish chemokine cocktail to media
- Administer PBMCs bolus and flush to remove non-adherent PBMCs

Required Materials

- Mature Colon Intestine-Chips with Pods
- α4 integrin small molecule inhibitor (AJM300, carotegrast methyl; MedChemExpress, Cat. HY-124290)
- Human Peripheral Blood Mononuclear Cells, Frozen (PBMCs) (StemCell, Cat. 70025)
  - *Note: Suggested donor selection criteria include inclusion of healthy donors and avoidance of donors with underlying pro-inflammatory conditions (e.g., smoking, hypertension, or obesity)*
- Cell Tracker stain (Deep Red, ThermoFisher Scientific, Cat. C34565)
- DMSO
- RPMI 1640 Media, powder, HEPES (ThermoFisher Scientific, Cat. 23400021)
- Cell culture grade water
- Sodium Bicarbonate (NaHCO3, Millipore Sigma, Cat. S5761)
- Fetal bovine serum (FBS, Millipore Sigma, Cat. F4135)
- Pen-Strep (Millipore Sigma, Cat. P4333)
- Percoll® (Millipore Sigma, Cat. P4937)

- GelzanTM CM (low acyl gellan gum; MilliporeSigma, Cat. G1910, Cas# 71010-52-1, EC#:2740117-5, MLD#: MFCD00131909)
- P200 multichannel pipette
- 96-well V-bottom plates
- 50-mL conical tubes
- Aspirator and sterile tips
- Trypan Blue
- Hemocytometer
- Dextran, Cascade Blue™, 3000 MW, Anionic, Lysine Fixable (ThermoFisher Scientific, Cat. D7132)

Preparation of Materials

Preparation of 2X RPMI 1640 Media

Follow modified instructions (below) from manufacturer for RPMI 1640 Media, Powder, HEPES (Cat. 23400021, ThermoFisher Scientific)

Step	Action
1	To a mixing container that is as close to the final volume as possible, add 390 mL of cell culture grade water
2	Add powdered media to room temperature (15°C to 30°C) cell culture grade water with gentle stirring – Do not heat the water
3	Rinse inside of package to remove all traces of powder
4	Add sodium bicarbonate (NaHCO3) to media to concentration of 2 g / L (w/v)
5	Add 100 mL FBS to a final concentration of 20% (v/v)
6	Add 10 mL Pen/Strep to a final concentration of 2% (v/v)
7	Adjust the pH between 6.8 to 7.1 by slowly adding, with stirring, 1 N NaOH or 1 N HCl to achieve the desired final working pH of 7.0 - 7.4 – The pH may rise 0.1 to 0.3 units upon filtration
8	Process the media immediately into sterile containers by membrane filtration with a 0.2-µm filter using a positive-pressure system

Solutions

2X RPMI Media Component	Weight/Volume	% (v/v) of Final Solution
Cell culture grade water	390 mL	78%
RPMI 1640 Media, powder, HEPES (Cat. # 23400021, ThermoFisher Scientific)	Total material in package	N/A
Sodium Bicarbonate ($NaHCO_3$, Millipore Sigma, Cat. S5761)	2 g / L (w/v)	N/A
Fetal Bovine Serum (FBS, Millipore Sigma, Cat. F4135)	100 mL	20%

Pen-Strep (Millipore Sigma, Cat. P4333)	10 mL	2%

Preparation of Gelzan 1% w/v Stock Solution

Step	Action
1	Make 1% w/v GelzanTM (Millipore Sigma, Cat. G1910; below) in cell culture grade water (e.g., 1 g/100 mL)
2	Dissolve by stirring at 90°C with magnetic stir bar
3	Sterilize solution at 121°C for 20 min in autoclave
4	Cool to room temperature
5	Store at room temperature or use immediately in buoyancy media – Gelzan stock solution is stable for at least 3 months when stored at room temperature in aseptic conditions – Emulate recommends mixing fresh buoyancy reagent beyond this time

Preparation of 2X (25µg/mL; 44 µM) Stock Solution of α4 Integrin Small Molecule Inhibitor (AJM300)

Note: *The following steps are required to ensure AJM300 solubility*

Step	Action
1	Pre-warm bath sonicator to 37°C
2	AJM300 (carotegrast methyl; MedChemExpress, Cat. HY-124290) is supplied as lyophilized powder – Reconstitute 5 mg of AJM300 into 400 µL DMSO (12.5 mg / mL)
3	Sonicate in bath sonicator for 5 min
4	Add 1,200 µL warm 2X RPMI for a 3.125 mg / mL AJM300 stock solution
5	Sonicate in bath sonicator for 5 min
6	Vortex solution immediately before step 7
7	Dilute 3.125 mg/mL stock solution 1:125 in desired amount of warm 2X RPMI (i.e., 400 µL / 50 mL media) for a final 2X stock concentration of 25 µg / mL AJM300 and 0.02% DMSO in 2X RPMI

PBMC Thawing and Resting

PBMC Thawing and Resting

Vials of commercially available freshly frozen PBMC cells (StemCell, Cat. 70025) are allowed to rest for at least 3 to 4 h in culture media at 37°C after thawing. If fresh PBMC are to be used, they can be placed in culture media and used immediately or kept in culture for no longer than 4 hours.

Markers

Users can consider an initial immunophenotyping of PBMCs each time a new donor is introduced, through standard immunofluorescence staining and flow cytometric analysis. The goal is to demonstrate normal ranges of immune cell subsets and activation states and to capture donor-to-donor variability as an additional layer of quality control. We would recommend the following as a basic panel of markers to satisfy this goal:

- CD3
- CD4
- CD8
- CD14
- CD19
- CD45RO
- CD56,
- CD69
- beta-7 Integrin

Required Materials

- RPMI complete media
  - RPMI 1640 media (ThermoFisher Scientific, Cat. 11875093)
  - 10% Fetal bovine serum (FBS) (Millipore Sigma, Cat. F4135)
  - 1% Pen-Strep (Millipore Sigma, Cat. P4333)
- 37°C water bath
- 70% ethanol
- Pipettes and filtered tips
- 50 mL conical tubes
- Aspirator and sterile tips
- Trypan Blue
- Hemocytometer
- 75 or 150 cm^2 flasks
- Sonicator bath

Cell Number and Volume Calculations

Below are considerations for the total number of PBMCs required

Step	Action
1	For each hour of dosing to Pod vascular channel inlets, 1 mL of PBMC cell suspension (recommended $2x10^6$ cells / mL) is needed – The maximum volume of the Pod reservoir is 4 mL (i.e., 4 h of PBMC dosing). Thus, $8x10^6$ cells in 4 mL buoyancy media is recommended per chip – *Note: $2x10^6$ cells/mL is physiological*
2	Multiply the total number of chips (recommended at least three chips per condition) by the volume per chip to be added (i.e., recommended 4 mL cell suspension per chip) to determine the total volume needed
3	Multiply by the volume needed by the recommended $2x10^6$ cells / mL to determine the minimum number of PBMCs required
4	Increase the total numbers by ~20% to ensure all chips get adequate volume

Thaw PBMCs

Step	Action
1	Warm RPMI complete media to 37°C in water bath
2	Thaw cryopreserved PBMCs in 37°C water bath
3	Transfer PBMCs to an empty 50 mL conical tube with very slow, gentle, pipetting to resuspend before transfer
4	Add 10 mL of warm complete RPMI media drop wise (i.e., one drop per second) while gently mixing the cells
5	Use 1 mL of media to rinse out the vials and collect in the 50 mL tube
6	Count cells and determine viability – Invert the tube gently to homogenize the cell suspension prior to pipetting out 30 µL – Perform the counting at 3-fold dilution (i.e., 30 µL cell suspension + 30 µL PBS + 30 µL Trypan Blue) – Count the cells with a hemocytometer

7	Spin the cells: 300 x g for 8 min at room temperature – Remove supernatant by tube inversion or careful aspiration
8	Gently disturb the cell pellet and resuspend the 1 mL RPMI complete media, using a P1000 pipet, then add media to adjust the volume to achieve a cell concentration of 2-6 x10^6 cells/mL (for AJM300 treatment studies, replace this step with steps 1-5, immediately below)
9	For untreated PBMCs, move cells to 75 or 150 cm^2 flasks depending on working volume of cell suspension (10-15 mL per T75 or 20-3 0mL per T175)
10	Incubate for 4 h at 37°C

PBMC Pre-Incubation with α4 integrin small molecule inhibitor (AJM300)

Protocol

Step	Action
1	Separate desired volume of freshly thawed PBMC suspension (prepared in steps 1-7 of preceding section) for AJM300 treatment (at least $8x10^6$ cells per treated chip, i.e., 4 mL cell suspension per chip) in conical tubes
2	Centrifuge at 300 x g for 8 min
3	Dilute required volume of 2X AJM300 stock solution (see above) with equal volume of cell culture grade water (1:1) *Note: final concentration of AJM300 on cells is 12.5 µg / mL*
4	Resuspend cells in AJM300 solution at $2x10^6$ cells / mL
5	Transfer PBMC suspension to 75 or 150 cm^2 and incubate for 4h at 37°C

PBMC Staining with Cell Tracker

Protocol

Step	Action
1	Transfer the resting PBMCs from flasks to 50 mL conical tubes – Collect PBMCs using a 10 mL serological pipette. Pipette multiple times while washing the bottom flask surface. – Add an additional 10 mL of PBS to the flask and wash 3-4 times – Collect in 50 mL conical tube, check flask under microscope for complete collection of cells – It is expected that some monocytes will adhere during this time
2	If excessive cell clumping is visible, pour cell suspension through a 100 µm filter
3	Count cells: – Invert the tube gently to homogenize the cell suspension prior to pipetting out 30 µL – Perform the counting at 3-fold dilution (i.e., 30 µL cell suspension + 30 µL PBS + 30 µL Trypan Blue) – Count the cells with a hemocytometer – Calculate volume needed to resuspend cells at $2x10^6$ cells / mL – Add appropriate volume of RPMI complete media
4	Centrifuge at 300x g for 8 min
5	Prepare Cell Tracker staining solution in PBS: – For Cell Tracker Deep Red ▪ Prepare a working solution by adding 20 µL of DMSO to one 15 µg vial ▪ Dilute working solution in serum-free media to1 µM final concentration (e.g., add 10 µL dye to 10 mL of PBS) – For all other Cell Tracker products (ThermoFisher Scientific) ▪ Prepare a 10 mM working solution by adding 10.75 µL of DMSO to one 50 µg vial

	▪ Dilute staining solution in serum-free media to 5 µM final concentration
6	Remove supernatant and gently loosen the PBMC pellet by tapping the tube
7	Resuspend PBMCs in 1-2mL of staining solution per 100 M cells using a P1000 pipette
8	Incubate for 30 min at 37°C
9	After incubation, add 20 mL of complete RPMI media to dilute stain and wash
10	Centrifuge at 300 x g for 8 min
11	User can move directly to PBMC Administration steps, or reconstitute PBMCs at $2x10^6$ cells / mL in complete RPMI and store temporarily at 37°C

PBMC Administration

Preparation of Buoyancy Media and PBMC Dosing Solution

Note: The Buoyancy Media described herein is required to overcome the effects of gravity and ensure that the PBMCs remain evenly distributed within the bottom inlet reservoir and vascular channel during perfusion. This is necessary to allow the PBMCs to interact with the endothelial cells on the top surface of the vascular channel that faces the epithelial compartment and is meant to mimic the distribution of PBMCs in blood.

Note: *Prepare at least 4 mL of cell suspension in buoyancy media per chip.*

Step	Action
1	Warm 2 mL of Percoll per chip at 37°C (volume dependent on number of samples)
2	Prepare Percoll and Gelzan buoyancy media components (use a 50 mL conical tube): – Calculate volume of Gelzan stock solution (see above) to prepare a 1.6% v/v solution in Percoll (e.g., 1.6 mL Gelzan + 98.4 mL Percoll) – Pipette Gelzan into Percoll suspension while swirling tube to ensure complete mixing ▪ Additionally, mix with serological pipette to ensure homogeneity – Warm the conical tubes with media at 37°C in a water or bead bath for at least 30 minutes – Immediately connect the 50 mL tube containing each warmed buoyancy media to a Steriflip unit – Do not flip the tube – Attach the unit as-is (buoyancy media in the bottom tube) and apply vacuum for 15 minutes to remove dissolved gas without filtering the solution
3	Collect PBMCs from resting into 50 mL conical tubes: – If PBMCs are already in 50 mL conical tubes, proceed to next substep – If PBMCs are in flask: ▪ Collect the suspension from the flask using a serological pipette, rinsing the flask while collecting

	▪ Once the flask is empty, perform an additional rinse with PBS (half the original suspension volume) in order to collect any remaining PBMC adhered – Centrifuge PBMCs at 300 x g for 8 min, remove supernatant
4	Working one tube at a time, loosen pellet by gently tapping tube, then resuspend at $4x10^6$ cells / mL density based on prior cell counts in 2x RPMI media or 2X AJM300 stock solution – Resuspend the first 1000 uL of the 2X media using a P1000 pipette in order to homogenously incorporate the pellet – *Note: Immediately proceed to next steps to minimize PBMC exposure to 2X concentrated RPMI media*
5	Add buoyancy media to the PBMC suspension at a 1:1 (v/v) ratio: – Final solution: 2 $x10^6$ cells/mL PBMC in 49.2% (v/v) Percoll and 0.8% (v/v) Gelzan with 50% (v/v) 2X RPMI. This makes a 1X RPMI buoyancy media – Mix gently and thoroughly with serological pipette to homogenize cells
6	Repeat for any remaining conical tubes of PBMC
7	For any control groups not including PBMC, prepare complete buoyancy media for dosing: – Mix buoyancy media (Percoll with 0.016% Gelzan) at a 1:1 (v/v) ratio with 2X RPMI media – Keep at 37°C until administration

PBMC Administration to the Colon Intestine-Chip

Step	Action
1	Warm required volume of IntestiCult (4 mL per chip) in 37°C water bath: – Add 100 µg / mL of Cascade Blue dye – Add 100 ng / mL of each chemokine, if being used
2	Add 4 mL of IntestiCult media (treated or not) to the inlet of the epithelial channel of each Pod
3	Mix PBMC dosing solutions (cell suspensions in buoyancy media) with serological pipettes

4	Add 4 mL of PBMC dosing solution to the inlet of the vascular channel of each Pod – For controls not receiving PBMCs, add complete buoyancy media prepared previously without cells
5	Set Zoë flow rates to 1,000 µL / h for epithelial and vascular channels
6	Return Pods to Zoës and run flow for up to 4 h *Optional: Repeat steps 4-6 immediately one or more times to increase PBMC recruitment*

Media Flow Post-Administration

Protocol

Step	Action
1	After PBMC administration (4 h), perform a media flush to remove non-adhered PBMCs: – Aspirate the Pod inlets and outlets – Determine the volume of media needed per chip (roughly 1.2 mL per day) to flow until takedown (see note below), and then account for 750 µL extra per chip – Add complete EGM MV2 to Pod bottom inlet – Add epithelial channel media (IntestiCult + Cascade Blue + chemokines where used) to Pod top inlet – Flow both channels at 1,000 µL / h for 30 min
2	After media flush, set floww to 30 µL / h with 10% stretch (0.15 Hz) and continue flow until end of experiment

Note: *Current protocol recommends maintaining media flow for 48 to 72 h to observe barrier disruption. However, the user can reduce or extend this duration for desired endpoints.*

Part VII. Takedown

Overview

Introduction

Protocol for takedown.

Contents

Takedown

Required Material

- P200 multichannel pipette
- 4% Paraformaldehyde
- 1x DPBS
- *Optional: PBS + 0.05% Sodium Azide (Teknova, Cat. P0202)*

PFA Chip Fixation (ON-Zoë) (Recommended)

Step	Action
1	Stop flow of Zoës and aspirate Pod inlets and outlets
2	Add 300 µL of 4% Paraformaldehyde (PFA) to Pod inlets
3	Flush Pods in Zoë for epithelial and vascular channel at 1000 µL / h for 5 min to perfuse the PFA into the channels
4	Stop flow and let Pods sit for 15 min at 37°C for fixation
5	Aspirate Pod inlets and outlets
6	Add 300 µL of DPBS to Pod inlets
7	Flush Pods in Zoë for epithelial and vascular channel at 1000 µL/h for 20 min to remove PFA
8	Disconnect chips from Pods and store chips in DPBS at 4°C. Protect from light – Store chips in PBS + 0.05% sodium azide solution for long-term storage at 4°C

PFA Chip Fixation (OFF-Zoë) (Not Recommended)

Step	Action
1	Stop flow of Zoës and disconnect all chips. Place (labeled) chips in square cell culture plate in biosafety cabinet
2	Perfuse 100 µL of 4% Paraformaldehyde (PFA) slowly into both channels using P200 pipette, aspirating outflow
3	Incubate for 20 min at room temperature
4	Wash channels three times with 100 µL DPBS using P200 pipette
5	Store chips in PBS at 4°C. Protect from light – Store chips in PBS + 0.05% sodium azide solution for long-term storage at 4°C

Note: *OFF-Zoë fixation through manual pipetting provides a source of variability as differential levels of shear forces can differentially dislodge attached PBMCs. The above ON-Zoë fixation avoids this issue.*

Experimental Design Recommendations

Introduction

Guidelines on experimental design are provided below. In a first example a complete set of controls is provided to support in house validation of application ('Validation Mode'). A second example lists the minimal controls needed to screen larger number of drugs in parallel ('Screening Mode').

Experimental Design 1 - Validation Mode

The experimental design outlined in the table below provides a complete set of controls to properly validate the application in house in a 21 chip, 2 Zoë experiment.

- Groups 1 and 2 do not include PBMC administration in order to provide validation that the inflammatory priming regime itself does not induce barrier alterations. They also capture any downstream cytokine production (by the epithelial and endothelial cells) that might be induced by priming. This is necessary to determine the degree to which cytokines detected in PBMC containing groups can or cannot be directly attributed to the PBMC (i.e., versus priming).
- Group 3 also does not include PBMC administration and is a validated stand-alone positive control for barrier dysfunction.
- Groups 4 and 5 determine the primary signal, >4-fold selective PBMC recruitment on primed (Group 5) versus resting (Group 4).
- Groups 6 and 7 add a validated inhibitory drug and recruitment, cytokine, and barrier dysfunction responses compared to Groups 4 and 5.

Group	Description	Endothelial Treatment (bottom channel)	Chemokines (top channel)	Drug Treatment	PBMC	# of Chips
1	Resting control	-	-	-	-	3
2	Primed control	TNFα	+	-	-	3
3	Positive leak control	IFNγ	-	-	-	3
4	Resting + PBMC	-	-	-	+	3
5	Primed + PBMC	TNFα	+	-	+	3
6	Resting + PBMC + Drug	-	-	AJM	+	3
7	Primed + PBMC + Drug	TNFα	+	AJM	+	3
Total						**21**

Experimental Design 2 – Screening Mode

Once the application has been validated in house (above), a modified experimental design can be implemented with much fewer controls to increase throughput for drug screening, as indicated below. In this example, a 24-chip, 2-Zoë experiment includes assessment of PBMC recruitment in resting and primed chips in the absence of drug (Groups 1 and 2). The recruitment on the primed chips set the primary response signal (Group 2). Subsequent Groups 3-8 recapitulate Group 2 with the additional of 6 different drugs or (drug concentrations) to assess inhibition responses.

It is recommended that AJM300 be included as a validated inhibitor of PBMC recruitment (positive control drug), cytokine secretion, and barrier disruption.

Group	Description	Endothelial Treatment (bottom channel)	Chemokines (top channel)	Drug Treatment	PBMC	# of Chips
1	Resting + PBMC	-	-	-	+	3
2	Primed + PBMC	TNFα	+	-	+	3
3	Primed + PBMC + Drug 1	TNFα	+	AJM	+	3
4	Primed + PBMC + Drug 2	TNFα	+	Drug 2	+	3
5	Primed + PBMC + Drug 3	TNFα	+	Drug 3	+	3
6	Primed + PBMC + Drug 4	TNFα	+	Drug 4	+	3
7	Primed + PBMC + Drug 5	TNF	+	Drug 5	+	3
8	Primed + PBMC + Drug 6	TNF	+	Drug 6	+	3
Total						**24**

Troubleshooting

Common Issues:

Procedure	Possible Issue	Troubleshooting Recommendations
Colon Intestine-Chip Culture	Epithelium has not reached acceptable criteria for barrier function by Day 3 of protocol (TNFα and Cytokine Treatment)	Delay TNFα and Cytokine Treatment for another 24 hours, continuing culture under flow to allow for increased barrier function. Perform barrier function analysis the next day and assess. Repeat for another 24 hours if necessary. If barrier function does not improve, this is indicative of poor epithelial coverage (confirm with brightfield imaging), and the experiment will have to be repeated.
Colon Intestine-Chip Culture / Media Flow Post-Administration	Low effluent in Pod outlet reservoirs after overnight flow	An air bubble or debris may be present in the chip, obstructing flow. Check for bubbles in Pod via or in co-culture channel. After effluent collection, perform a media flush at 1,000µL/h on Zoë for 5 minutes according to the Colon Intestine-Chip Culture protocol. If no media remains in the outlet after flush, perform Regulate™ Cycle on Zoë to remove any lodged air in Pod.
PBMC Culture	Low yield of PBMC collected from culture flask	Collect as much suspension as possible using serological pipette. Add PBS at half the working volume of the flask. Incubate at 37°C for 10 minutes. Gently tap the side of the flask and collect, washing the flask during collection. Check the flask under a brightfield microscope to ensure that nearly all the PBMCs have been collected (up to 10% adhesion expected)
PBMC Culture	Excessive clumping of PBMCs in RPMI media after thawing	Clumping of PBMC can happen due to environmental stresses during cryopreservation and thawing. Before incorporating into buoyancy media, add 100 µg / mL of DNase I enzyme following protocol from StemCell (https://www.stemcell.com/how-to-reduce-cell-clumping-in-single-cell-suspensions-with-dnase.html). Incubate

		for 15 minutes at room temperature and wash with RPMI complete media. If large clumps persist, filter the PBMC suspension with a 100 µm filter until clumps are removed.
PBMC Administration	PBMC count is lower than needed for 4 hours of flow at 2 x 10^6 cells / mL	The Pod bottom inlet reservoir can be dosed with as little as 3.8 mL of cell suspension per chip (i.e., as little as 7.6 x 10^6 cells per chip) to flow for 4 hours, due to the higher viscosity of the administration suspension with buoyancy media.
Post-Administration Media Wash	Flow issues/No flow	In the case of flow issues or no flow after PBMC administration, Pods may need to be re-Primed. After effluent collection, proceed to disconnect the Chip with flow issues from its Pod. Add 300 µL of Intesticult media (without Dextran Cascade Blue) directly onto the top outlet via of the Pod. Add 300 µL of EGM-MV2 media directly onto the bottom outlet via of the Pod. Make sure all inlets in the Pod have their respective media. Run the Prime cycle twice in the Zoë. Add a droplet of appropriate media to the top of each Chip via. Reconnect Chips to Pods. Run the Regulate Cycle on all Zoës.

References

Buoyancy Media

The Buoyancy Media consists of cell culture media together with Percoll and Gellan Gum.

Percoll

Percoll affords increased density to the media that prevents gravity-dependent sinking of PBMCs to the bottom of the Pod reservoir and Organ-Chip channel.

Gellan Gum provides a degree of cross-linking that stabilizes the solution and promotes an even distribution of PBMCs in both the reservoir and channel.

Figure 4

Schema Illustrating the functional rationale for the Buoyancy Media formulation (Media + Percoll + Gellan Gum).