emulate

Colon Intestine-Chip Culture Protocol

March 19, 2021

EP203 v1.0

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Introduction to the Human Emulation System

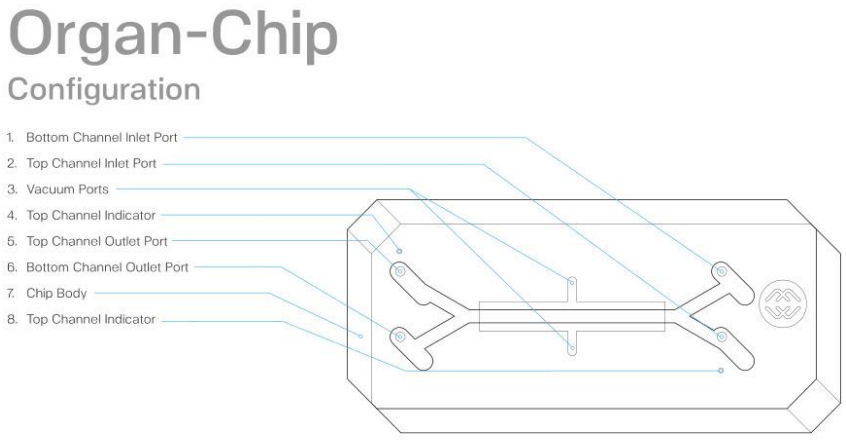
This section provides an overview of key components of the Human Emulation System®, including the Chip-S1® Stretchable Chip, the Pod® Portable Module, the Zoë® Culture Module, and the Orb™ Hub Module.

An Organ-Chip is a living, micro-engineered environment that recreates the natural physiology and mechanical forces that cells experience within the human body. The Pod provides the media to the Organ-Chip that is needed to promote cell growth; the Pod also acts as the interface between the Organ-Chip and the Zoë Culture Module. Zoë provides the flow of nutrient-rich media at a rate determined by the user and provides the mechanical forces that emulate the physical forces experienced *in vivo* by tissue. The Orb delivers a mix of 5% CO_2, vacuum stretch, and power for up to four Zoës.

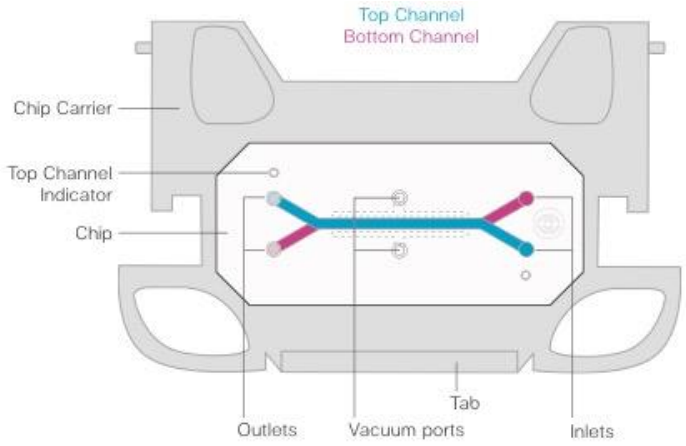
Organ-Chip (Chip-S1)

Our Chip-S1 can be configured to emulate multiple different organ tissues including lung, liver, intestine, kidney, and brain. The chip, when connected to the Pod and Zoë, recreates the body's dynamic cellular microenvironment, including tissue-to-tissue interfaces, blood flow, and mechanical forces. This protocol describes the methodology for the Colon Intestine-Chip.

The microenvironment created within each Chip-S1 includes epithelial cells in the top channel and endothelial cells in the bottom channel. Top and bottom channels are separated by a porous membrane that allows for cell-cell interaction like those that are seen *in vivo*. These two channels are fluidically independent. Cells in each channel are seeded with organ- and cell-specific extracellular matrix proteins (ECM), and can be maintained in static culture for up to four days, depending on cell type, before being connected to Zoë, which provides continuous flow of cell culture media.



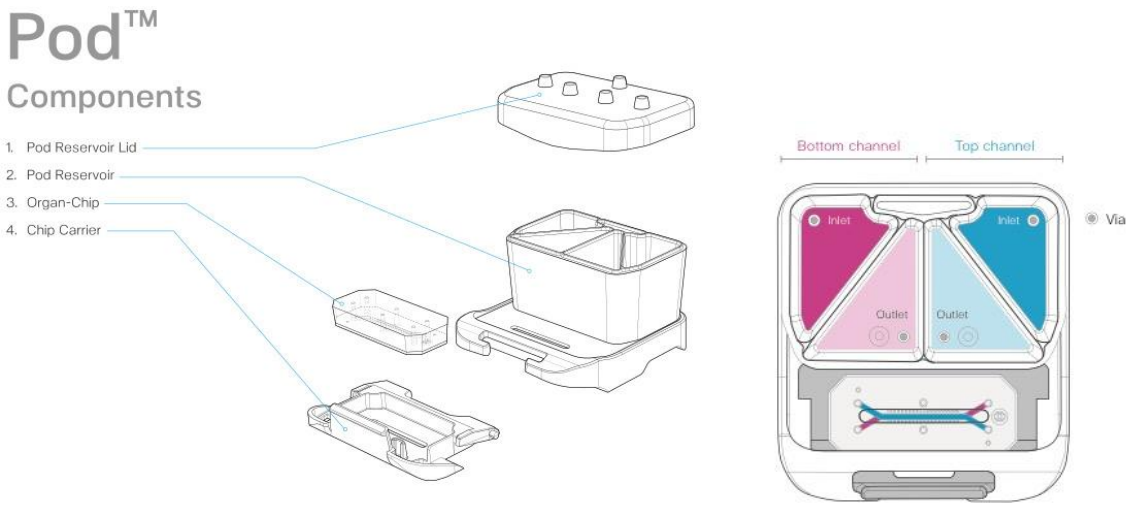
Chips are supplied pre-packaged in the chip carrier and should be maintained in the chip carrier at all times. The chip carrier is designed to hold chips in place, eliminating the need to disturb or distort the chip during preparation. In addition, the chip carrier connects securely to the Pod, which acts as the interface between Zoë and the chip. Use sterile tweezers when handling chips in chip carriers. Removal of the chip from the chip carrier prior to or during an experiment could result in chip failure. (The only appropriate time to remove the chip from the carrier is at end of the life of the chip in the experiment, when it is no longer used for live culture of the cells.)



Pod

The Pod houses the chip, supplies media, and enables compatibility with microscopes and other analysis equipment. The Pod, which has a height of 41.5 mm, is designed to allow Organ-Chips to be easily transported and placed on standard microscopes for imaging. It also allows simple portability of Organ-Chips between our hardware and other analysis equipment.

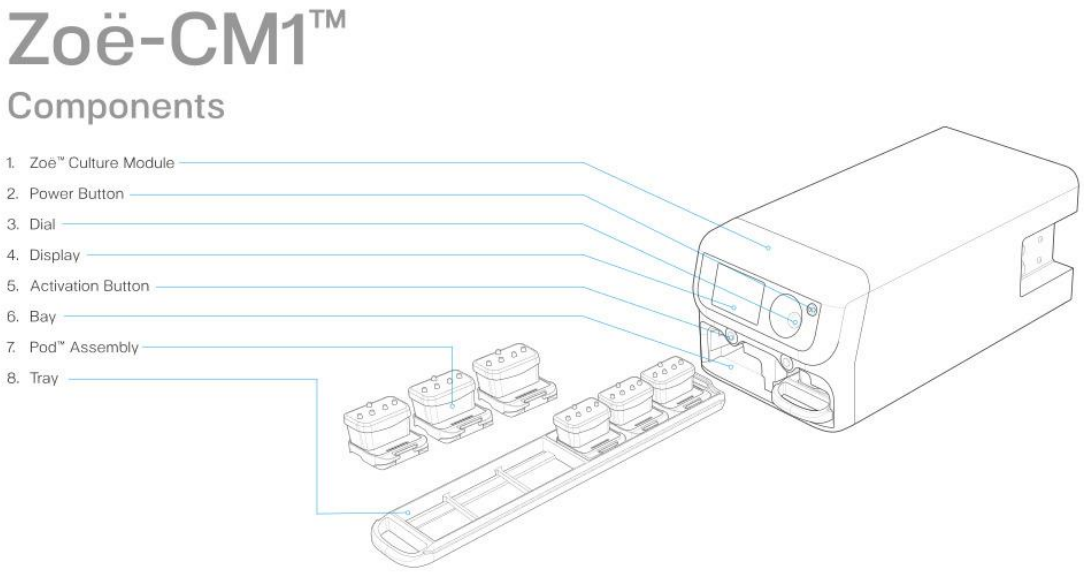
The Pod's reservoirs allow users to introduce nutrient media, precisely control dosing to test drugs or other inputs, and sample chip effluent. Each Pod reservoir holds up to 4 mL



Zoë Culture Module

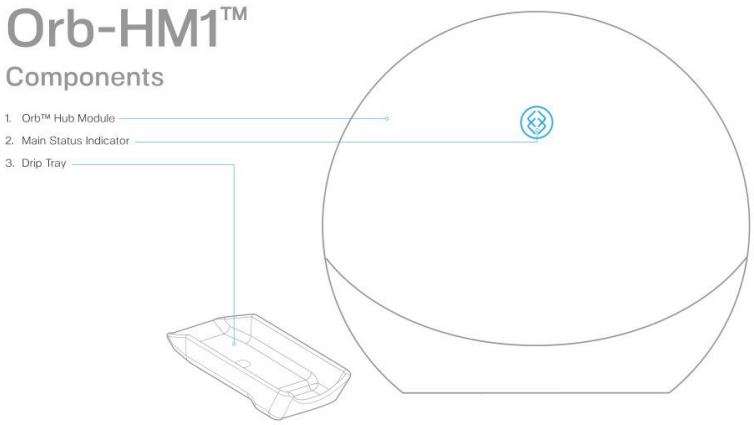
The Zoë Culture Module is designed to sustain the life of cells within Emulate's Organ-Chips. It provides the dynamic flow of media and the mechanical forces that help recreate the microenvironment cells experience *in vivo*. The instrument automates the precise conditions needed for simultaneous cell culture of up to 12 Organ-Chips.

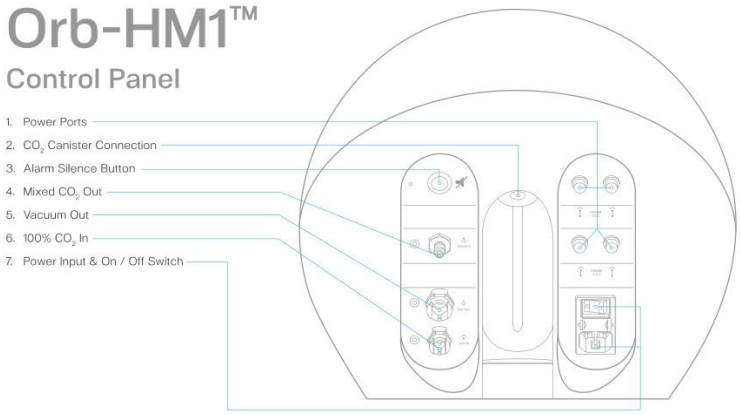
Zoë enables independent control of the flow rate of media through the top and bottom channels of the Organ-Chips, as well as stretch parameters — including frequency and amplitude — of the Organ-Chip. Additionally, Zoë has automated algorithms to prime the fluidic channels of Pods with media, and programming to maintain the culture microenvironment for optimal cell performance.



Orb Hub Module

The Orb is a central hub that connects to up to four Zoë Culture Modules. It provides a simple solution for installing and operating the Human Emulation System within the lab environment. The Orb delivers a mix of 5% CO_2, vacuum stretch, and power from standard lab connections. It generates a 5% CO_2 supply gas to Zoë by combining air with an external 100% carbon dioxide supply (or from a portable CO_2 canister for increased flexibility) in a controlled mixture. Orb also generates the vacuum required to apply Organ-Chip stretching (-70 kPa), and contains four individual power ports.





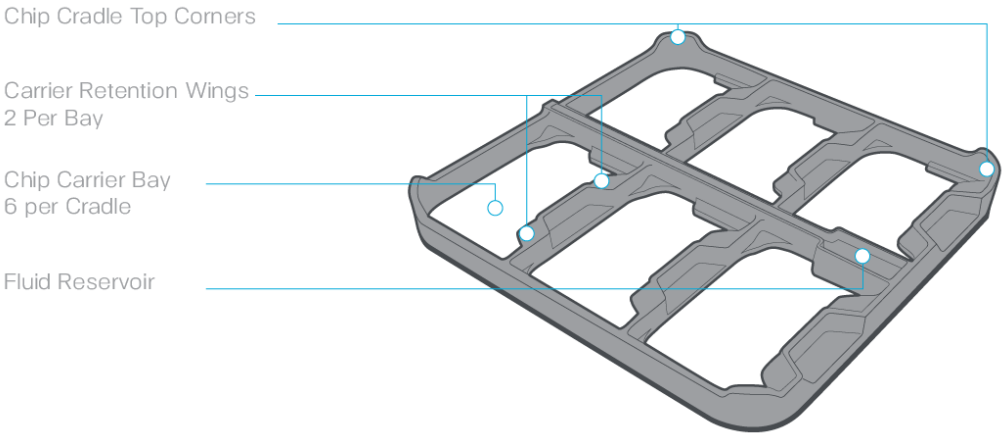
Chip Cradle

The Chip Cradle is an accessory that holds and organizes up to six chips. It offers several benefits, including:

- It allows inversion of the chips during seeding.
- It has a DPBS reservoir that keeps the chips humidified.
- It is made from an autoclavable plastic.
- It contains slots with numbered labels to help keep chips organized.

Chip Cradle

Configuration



Cells

The Primary Colon Intestine Bio-Kit includes pre-qualified primary human parenchymal epithelial cells (from human biopsy-derived organoids) and human microvascular endothelial cells (from large intestine). Vials are shipped in a cryogenic storage vacuum flask and must be stored in liquid nitrogen until use.

The Colon Intestine Bio-Kit includes the following cells:

Parenchymal epithelial cells (top channel):

- Human biopsy-derived organoids

Endothelial cells (bottom channel):

- Human colonic microvascular endothelial cells

Media and Gas Equilibration

Proper gas equilibration of media is essential for successful Organ-Chip culture. As the system is sensitive to bubbles, all media must be equilibrated to 37°C and excess gas must be removed prior to use in the chip and in Zoë. To do this, 37°C medium is placed under vacuum using the 0.45 µm PVDF filter of a Steriflip® conical filter unit. Failure to carry out this step could lead to failure of the chips in the experiment.

It is also important to look for bubbles throughout the experiment. Observed bubbles can often be flushed out of the system to avoid a disruption in medium flow and / or improper surface coverage. However, instances of chips with visible bubbles or blocked flow should be recorded and considered when analyzing experimental data.

Experimental Overview

Workflow Overview

Day -4: Reagent Preparation

- Aliquot reagents (media supplements, ECM, Matrigel, etc.)

Day -3: Thaw HIMEC

- Prepare HIMEC culture medium and flask
- Thaw and plate HIMEC

Day -1: Chip Preparation

- Prepare chips
- Prepare ER-1 solution
- Introduce ER-1 solution to channels
- Activate chips
- Prepare ECM solution
- Coat chips with ECM

Day 0: Cells to Chips

HIMEC to Chips

- Prepare HIMEC Culture Medium
- Prepare Chips
- Prepare Cell Counting Solution
- Harvest HIMECs
- Cell Counting and Viability Assessment
- Seed HIMECs to bottom channel
- Wash chips

Organoids to Chips

- Prepare Organoid Growth Medium
- Prepare Dissociation Solution
- Recover organoids from Matrigel
- Adjust cell density
- Seed colonic organoids to the top channel

Day 1: Chips to Pods and Pods to Zoë

- Gas equilibration of media
- Prime Pods
- Wash chips
- Chips to Pods

- Pods to Zoë and Regulate

Day 2: Replenish Medium

- Via wash
- Medium replenish without CHIR99021 and Y27632
- Observe Cell Morphology
- Effluent Sampling

Day 3: Initiating Stretch

- Stretch introduction
- Effluent sampling

Day 4+: Maintenance and Sampling, Increasing Stretch to 10%

- Maintenance and Sampling of Chips
- Increasing stretch to 10%

Required Materials

Ensure all equipment, materials, and reagents are accessible prior to beginning each experiment. Exact catalog numbers are provided for specific materials required for successful experiments.

Equipment & Materials

Equipment	Description	Supplier	Catalog Number
Human Primary Colon Intestine Bio-Kit	Co-Culture (12- or 24-pack)	Emulate	-
Zoë CM-1™ Culture Module	1 per 12 chips	Emulate	-
Orb-HM1™ Hub Module	1 per 4 Zoës	Emulate	-
Chip-S1™ Stretchable Chip	12 per Zoë	Emulate	-
Pod™ Portable Modules	1 per Chip-S1	Emulate	-
UV Light Box	1 per Zoë	Emulate	-
Chip Cradle	Autoclaved, 1 per 6 chips	Emulate	-
Steriflip®-HV Filters	0.45 µm PVDF filter sterile	EMD Millipore	SE1M003M00
Square Cell Culture Dish (120 x 120 mm)	Sterile, 1 per 6 chips	VWR	82051-068
Handheld vacuum aspirator	-	Corning	4930
Aspirating pipettes	2 mL, polystyrene, individually wrapped	Corning / Falcon	357558
Aspirating tips	Sterile (autoclaved)	-	-
Serological pipettes	2 mL, 5 mL, 10 mL, and 25 mL low endotoxin, sterile	-	-
Pipette	P20, P200, P1000 and standard multichannel	-	-
Pipette tips	P20, P200, and P1000. Sterile, low-adhesion	-	-
Conical tubes (Protein LoBind® Tubes)	15 mL and 50 mL polypropylene, sterile	Eppendorf	15 mL – 0030122216 or 50 mL - 0030122240
Eppendorf Tubes® lo-bind	1.5 mL, sterile	Eppendorf	022431081
96 wells black walled plate	For permeability assessment	-	-
Microscope (with camera)	For bright-field imaging	-	-
Water bath (or beads)	Set to 37°C	-	-

Vacuum set-up	Minimum pressure: -70 kPa	-	-
T75 flasks	-	BD Falcon	353136
Ice bucket	-	-	-
70% ethanol and wipes	For surface sterilization	-	-
96 well black walled	For permeability assay	-	-

Reagents, Media & Supplements

Reagent	Description	Supplier	Catalog Number
ER-1	5 mg powder	Emulate	-
ER-2	25 mL bottle	Emulate	-
Dulbecco's PBS (DPBS - / -) (without Ca^{2+}, Mg^{2+})	1X	Corning	21-031-CV
Trypan blue	0.4% solution	Sigma	93595
TryplE Express	Organoids dissociation and endothelium cells detachment	ThermoFisher Scientific	12604013
Advanced DMEM/F12	Medium	ThermoFisher Scientific	12634028
IntestiCult™ Human Organoid Growth Medium	Component A and Component B	Stem Cell technologies	06010
Endothelial Cell Growth Medium MV 2	Basal Medium and Supplements	Promocell	C-22121
Fetal bovine serum (FBS)	Sterile heat-inactivated	Sigma	F4135
Primocin™	50 mg / mL	InvivoGen	ANT-PM-1
Attachment Factor™	1X	Cell Systems	4Z0-210
Matrigel - Growth Factor Reduced	Phenol Red Free	Corning	356231
Collagen IV	1 mg / mL	Sigma	C5533
Fibronectin	1 mg / mL	Corning	356008
Y-27632	ROCK inhibitor 10 mM	Stem Cell technologies	72304
CHIR99021	GSK3 enzyme inhibitor 5 mM	Reprocell	04-0004-10
Cell Recovery Solution	Recovery solution	Corning	354253
BSA	30%, Sterile	Sigma	A9576
Cell Culture Grade Water	Sterile, Water	Corning	MT25055CV
DMSO	Sterile	Sigma	D2650
3KDa Dextran Cascade Blue	10 mg powder	Invitrogen	D7132
3KDa Dextran Texas Red	10 mg powder (optional for permeability assay)	Invitrogen	D3329
Cascade Blue	10 mg powder (optional for permeability assay)	Invitrogen	C687
Lucifer Yellow	25mg power (optional for permeability assay)	Invitrogen	D3329

Notes for ER-1 and ER-2

- Upon arrival, store ER-1 (powder) unopened in the metalized pouch at -20°C, protected from light and humidity.
- Upon arrival, store ER-2 solution at 4°C.

Aseptic Technique

- Prepare and sanitize the work surface of the biosafety cabinet (BSC) prior to beginning. Ensure that the work space within the BSC is organized and free from clutter. Arrange tips, media, and other necessary materials in the sterile field, easily within reach, without blocking the path of airflow.
- Sanitize all reagents and materials entering the BSC with 70% ethanol. Spray and wipe thoroughly.
- Always avoid touching the chip directly.
- Handle the chip carrier only by the sides or by the tab with gloves.
- Never remove the chip from the chip carrier prior to during an experiment.

Cell Storage

Cryopreserved cells must be stored in liquid nitrogen. Severe damage to cell membrane and cytoskeletal components can result from chronic temperature fluctuations. Never store in dry ice or -80^{o}C freezer.

Chip Handling Techniques

Always work with chips within the chip carrier in a sterile environment, such as the BSC.

When pipetting to fill each channel, 35 µL volume is generally used for the top channel, and 15 µL is used for the bottom channel. These volumes allow for simple pipetting and a slight overfill to avoid bubbles or dry channels. All wash steps, unless otherwise stated, are performed using 200 µL of the specific wash solution.

Top Channel: 30-35 µL

Bottom Channel: 10-15 µL

The specific channel and membrane dimensions are outlined below:

Top Channel

Width x height dimensions	1000 µm x 1000 µm
Area	28.0 mm^2
Volume	28.041 µL
Imaging distance from bottom of chip to top of membrane	850 µm

Bottom Channel

Width x height dimensions	1000 µm x 200 µm
Area	24.5 mm^2
Volume	5.6 µL

Membrane

Pore diameter	7.0 µm
Pore spacing	40 µm (hexagonally packed)
Thickness	50 µm

Co-Culture Region

Area	17.1 mm^2

A P20 or a P200 pipette with a sterile pipette tip is used to add solution directly to the channels of the chip, as when coating, washing, and seeding cells prior to attaching the chip to Zoë. Prior to seeding the cell suspension into the chips, empty the channel in which the cells will be seeded. To introduce solution to the channels, place the pipette tip perpendicular to chip channel inlet, ensuring that the tip is securely in the port, and steadily dispense liquid through the channel. Always introduce liquid to the bottom channel before pipetting into the top channel.

Colon Intestine-Chip Culture Protocol

Day -4: Reagent Preparation

Aliquot Reagents

Aliquot reagents prior to use to avoid multiple freeze-thaw cycles of the stock solutions.

Aliquot reagents including media supplements and ECM prior to use and store at -20°C to avoid multiple freeze-thaw cycles.

1. Y-27632 (ROCK inhibitor)

Reagent	Conc. [Stock]	Amount	Volume	Solvent
Y-27632	10 mM	5 mg	1.56 mL	0.1% BSA in DPBS

- Resuspend 5 mg of Y-27632 compound in 1.56 mL of 0.1% BSA in DPBS according to manufacturer's instructions, yielding a stock concentration of 10 mM
- The final concentration of Y-27632 used in Organoid Growth Medium will be 10 µM
- Aliquot reconstituted Y27632 to single-use volumes and store at -20°C

2. CHIR99021 (GSK-3 inhibitor)

Reagent	Conc. [Stock]	Amount	Volume	Solvent
CHIR99021	5 mM	10 mg	4.29 mL	DMSO

- Resuspend 10 mg of CHIR99021 compound in 4.29 mL of DMSO according to manufacturer's instructions, yielding a stock concentration of 5 mM
- The final concentration of CHIR99021 used in organoid medium will be 5 µM
- Aliquot to single-use volumes and store at -20°C

3. Matrigel – Growth Factor Reduced (GFR)

Reagent	Volume [Stock]
Matrigel - Growth Factor Reduced	10 mL

- The stock bottle of Matrigel must be thawed overnight on slushy ice in a 2°C to 6°C refrigerator (in the back) or cold room and handled on slushy ice at all times. (Maintain Matrigel on ice at all times, as this solution gels rapidly at temperatures above 8°C.)
- After thawing, aliquot Matrigel to suitable single-use volumes based on the specific stock concentration and amount needed in experiment
    - Keep all materials on ice at all times
    - Use pre-chilled pipettes, tips, and tubes at -20°C prior to aliquoting
    - Freeze aliquots immediately at -20°C
- Thaw aliquots on ice just prior to use
- Once aliquots are thawed, do not re-freeze

  Note: Prepare aliquots of 1.4mL for organoids expansion and 100 µL for Chip ECM coating.

4. Collagen IV

Reagent	Conc. [Stock]	Amount	Volume	Solvent
Collagen IV	1 mg / mL	5 mg	5 mL	Cell Culture Grade Water

- Resuspend 5 mg of Collagen IV in 5 mL of sterile cell culture grade water and incubate at 4°C until dissolved.
- Aliquot the next day 300 µL aliquots and store aliquots at -20°C.

5. Fibronectin

Reagent	Conc. [Stock]	Amount	Volume	Solvent
Fibronectin	1 mg / mL	5 mg	5 mL	Cell Culture Grade Water

- Resuspend 5 mg of Fibronectin in 5 mL of sterile cell culture grade water and leave the mix at room temperature for 30 min to dissolve (avoid harsh agitation or vortexing). Swirl gently before aliquoting.
- Store aliquots at -20°C.

6. 3KDa Dextran Cascade Blue

Reagent	Conc. [Stock]	Amount	Volume	Solvent
Dextran, Cascade Blue™, 3000 MW, Anionic, Lysine Fixable	-	10 mg	1 mL	Sterile Water Cell Culture Grade

- In BSC, resuspend 10 mg of **Dextran, Cascade Blue™, 3000 MW, Anionic, Lysine Fixable** in 1 mL of sterile water cell culture grade to obtain 3KDa Dextran Cascade Blue Working Solution at 10mg/mL concentration. The final concentration in medium is 10 ug/mL or 1:100 dilution. One vial of 10mg of 3Kda Dextran Cascade Blue is sufficient for 100 mL
- Any remaining working solution can be freeze it at -20C

Day -3: Thaw HIMEC

Goals:

- Expand HIMEC in flask prior to chip seeding as per established protocols.

Key Steps:

- Prepare HIMEC culture media and flask
- Thaw and plate HIMEC

Required Materials:

- Complete HIMEC Culture Medium (at 37°C)
- 15 mL conical tube
- Attachment Factor
- T-75 flask
- Serological pipettes
- Pipettes and tips
- Aspirator
- Centrifuge
- Chips in Pods
- 70% ethanol
- Microscope

Prepare HIMEC Culture Media and Flask

HIMEC Culture Media

HIMEC Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Endothelial Cell Basal Medium Kit	500 mL	-	-	PromoCell	C-22121
Primocin™	500 µL	50 mg / mL	50 µg / mL	InvivoGen	ANT-PM-1

HIMEC Culture Medium is composed by Endothelial Cell Growth Medium Kit (C-22121). It is supplied as a kit containing Endothelial Cell Basal Medium (C-22221) and Endothelial Cell Growth Medium MV2 Supplement Pack (C-39221). Replace the supplied FCS with heat inactivated FBS from Sigma (cat# F4135).

- Aseptically transfer all components of Endothelial Cell Growth Medium MV2 Supplement Pack to the Endothelial Cell Basal Medium bottle
- Primocin is a non-toxic antimicrobial agent for primary cells
- Store complete media at 4°C for up to 30 days

Prepare Flask

1. Warm sufficient amount of HIMEC Culture Medium to 37°C
2. Label the culture flask with relevant information (e.g., cell type, passage number, date, initials).
3. Pipette Attachment Factor onto the growth surface of flask ensuring full coverage (5 mL of Attachment Factor used per T-75 flask)
4. Ensure that the coating solution covers the culture surface of the flask evenly. Incubate at room temperature for 5 minutes
5. Aspirate excess coating solution after incubation
6. Add 15 mL of HIMEC Culture Medium to the flask, and leave it into 37°C incubator until ready for plating

Thaw and Plate HIMEC

HIMEC require one passage of expansion in culture after thawing before seeding in the chip.

1. Thaw the vial(s) of cells by immersing in a 37°C water bath for approximately 60 to 90 seconds Closely observe the thawing process while gently agitating and remove the vial from the water bath just before the last ice pellet disappears
2. Spray vial(s) with 70% ethanol and wipe dry prior to placing them in the BSC
3. Immediately transfer the contents of the vial using a P1000 pipette into the prepared flask containing warm HIMEC Culture Medium
4. Rinse the vial with 1 mL of HIMEC Culture Medium and add to the flask

5. Incubate the flask in the incubator at 37°C for 6 hours
6. Aspirate medium and carefully add 15 ml of fresh HIMEC Culture Medium
7. Return the flask back to incubator at 37°C and 5% CO_2 overnight
8. Exchange medium in flask with fresh HIMEC Culture Medium every other day until use for seeding in the chip
9. Alternately, add 30 mL of HIMEC Culture Medium to the flask. Leave the flask in the incubator at 37°C and 5% CO_2 overnight, followed by a media replenishment the next day.
10. Exchange medium in flask with fresh HIMEC Culture Medium the following day, and every other day until use for seeding in the chip.

Day -1: Chip Preparation

Goals:

- Activate the inner surface of the chip channels for proper ECM attachment
- Coat top channels with an ECM mixture of collagen IV and Matrigel
- Coat bottom channels with an ECM mixture of Collagen IV and fibronectin

Key Steps:

- Prepare chips
- Prepare ER-1 solution
- Introduce ER-1 solution to channels
- Activate chips
- Prepare ECM solutions
- Coat chips with ECM

Required Materials:

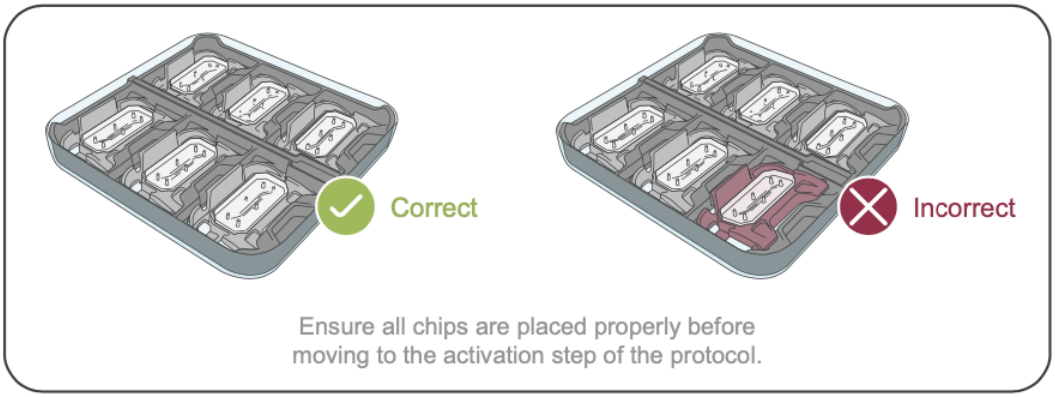
- Chip-S1 (12 chips per Zoë)
- ER-1 reagent
- ER-2 buffer
- 15 mL conical tubes
- DPBS (- / -) at room temperature
- DPBS (- / -) aliquot at 4°C
- Collagen IV
- Matrigel
- Fibronectin
- 70% ethanol
- 150 mm cell culture dish
- Ice and ice bucket
- Pipettes and filtered tips
- Aspirator and sterile tips
- UV light box
- UV safety glasses

Prepare Chips

1. Spray chip packaging, 120 mm square cell culture dish, and Chip Cradle with 70% ethanol and bring into the BSC.
2. Open the Chip Cradle sterile packaging and place the cradle into the 120 mm square dish, making sure the Chip Cradle is oriented properly with the corners facing up.
3. Open the chip packaging carefully and place the first chip into the cradle by sliding the back of the carrier under the tabs on the cradle (Figure 1).

Figure 1. Place chips by sliding under the tab of the Chip Cradle.

4. Repeat as necessary for all of the chips included in the experiment.
5. Label each chip with ID numbers on the chip carrier tab.



Prepare ER-1 Solution

ER-1 is light-sensitive. Prepare ER-1 solution immediately before use and discard any remaining ER-1 solution 1 hour after reconstitution. Failure to protect from light or use of ER-1 solution that has not been freshly prepared will lead to failure of chips. Note: ER-1 is an eye irritant and must be handled in the BSC with proper gloves and eye protection.

1. Turn off the light in BSC and allow ER-1 and ER-2 to equilibrate to room temperature before use (approximately 10 to 15 minutes)
2. Wrap an empty sterile 15 mL conical tube with foil to protect it from light
3. In the BSC, remove the small vial of ER-1 powder from the packet, and briefly tap the vial to concentrate the powder at the bottom
4. Add 1 mL of ER-2 to the vial, and transfer contents directly to the bottom of the 15 mL conical tube. Do not pipette to mix. Note: The color of the solution transferred to the conical tube will be deep red
5. Add an additional 1 mL of ER-2 to the ER-1 vial to collect any remaining material, and transfer the solution directly to the 15 mL conical tube. Note: The color of the transferred ER-1 solution will become lighter with each additional wash of the ER-1 bottle
6. Repeat Step 5 twice more, with an additional 1 mL of ER-2 each time
7. On the last addition of 1 mL ER-2 to the ER-1 bottle, cap the bottle and invert to collect any remaining ER-1 powder from the lid. Transfer the collected solution to the conical tube, bringing the total volume in the tube to 4 mL ER-1 solution.
8. Add 6 mL of ER-2 solution to the 4 mL of ER-1 solution in the 15 mL conical tube (for a final working concentration of 0.5 mg / mL). Pipette gently to mix without creating bubbles. ER-1 should be fully dissolved within the ER-2 solution prior to use

Introduce ER-1 Solution to Channels

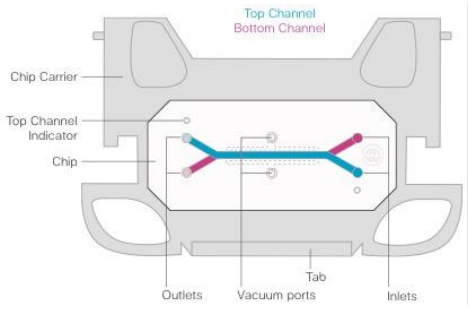


Figure 1: Top view of chip, with labelled ports, in chip carrier

1. Using a P200 pipette and a sterile 200 µL filtered pipette tip, take up 200 µL of ER-1 solution. Note: 200 µL of ER-1 solution will fill approximately 3 chips

2. Carefully introduce approximately 20 µL of ER-1 solution through the bottom channel inlet, pipetting until the solution begins to exit the bottom channel outlet
3. Without releasing the pipetting plunger, take the pipette out from the bottom channel inlet, and move the pipette containing remaining ER-1 solution to the top channel inlet
4. Introduce approximately 50 µL of ER-1 solution to the top channel inlet, pipetting until the solution begins to exit the top channel outlet
5. Remove all excess ER-1 solution from the surface of the chip by gentle aspiration. Be sure only to remove ER-1 solution from the chip surface — do not aspirate ER-1 from the channels. (See Fig. 3 below.)
6. Repeat Steps 1 through 5 for each chip.
7. Inspect the channels for bubbles prior to UV activation. If bubbles are present, dislodge by washing the channel with ER-1 solution until all bubbles have been removed. If bubbles persist, it may be helpful to aspirate the channel dry and slowly re-introduce ER-1 solution

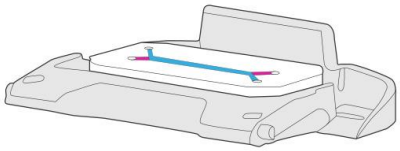


Figure 3: View of chip with no excess ER-1 solution on surface

Activate Chips

1. Bring the 120 mm square dish containing the ER-1-coated chips to the UV light box.
2. Before placing the square dish into the UV light box, make sure to remove the cover from the 120 mm square dish. Note: If the lid is not removed prior to placing the dish in the UV light box, the chips will not activate properly and could result in poor cell attachment.
3. Set the switch at the back of the UV light box to the “Constant” setting. Turn on the “Power,” and press the “On” button to begin UV activation.
4. Allow the chips to activate under UV light for 15 minutes.
5. While the chips are being treated, prepare ECM Solution. (For more information, see the next section: Prepare ECM Solution.)
6. After UV treatment, bring chips back to the BSC. Note: The light may be on in the BSC from this point forward.
7. Fully aspirate the ER-1 solution from both channels.
8. Wash each channel with 200 µL of ER-2 solution.
9. Fully aspirate the ER-2 from the channels.
10. Wash 2x each channel with 200 µL of sterile DPBS.
11. Leave DPBS inside the channels.

Prepare ECM Solutions

The ECM solution is prepared fresh each time by combining the individual ECM components with cold DPBS to the final working concentrations. Separate ECM solutions will be used to coat both the top and bottom channels.

For human Primary Intestine Colon-Chips, the final concentration of proteins in the ECM working is:

Top channel: Collagen IV: 200 µg / mL

Matrigel: 100 µg / mL

Bottom channel: Collagen IV: 200 µg / mL

Fibronectin: 30 µg / mL

1. Bring an ice bucket and ice to the BSC.
2. Thaw one aliquot of fibronectin (1 mg / mL), Collagen IV (1mg/ml) and Matrigel (concentration is dependent on specific lot used) on ice. Maintain all ECM components and mixture on ice at all times.
3. Calculate total volume of ECM solution needed to coat all chips.
    1. Volume required per channel = approximately 50 µL
    2. For every 12 chips to coat, prepare 1.5 mL of each ECM solution (12 chips x 100 µL / chip + extra 300 µL = 1.5 mL of ECM solution). (See calculation example below.)
4. Combine components to prepare ECM working solutions.
5. Keep the ECM solutions on ice until ready to use.

Calculation Example – Top Channel

Collagen IV [stock] = 1 mg / mL (C_1)

Collagen IV [final] = 0.2 mg / mL (C_2)

Matrigel [stock] = 10 mg_/ mL (C_1)

Matrigel [final] = 0.1 mg / mL (C_2)

Total volume of ECM solution = 1.5 mL (V_2)

Collagen IV

$C_1V_1 = C_2V_2$ à solve for V_1

(1 mg / mL) x (X mL) = (0.2 mg / mL) x (1.5 mL)

X = 0.3 mL = 300 µL of collagen IV stock solution

Matrigel

$C_1V_1 = C_2V_2$ à solve for V_1

(10 mg / mL) x (Y mL) = (0.1 mg / mL) x (1.5 mL)

Y = 0.015 mL = 15 µL of Matrigel stock solution

DPBS

DPBS = (total volume of ECM needed) – (volume of Collagen IV) – (volume of Matrigel)

= 1500 µL – 300 µL – 15 µL

= 1,185 µL of DPBS

Calculation Example – Bottom Channel

Collagen IV [stock] = 1 mg / mL (C_1)

Collagen IV [final] = 0.2 mg / mL (C_2)

Fibronectin [stock] = 1 mg / mL (C_1)

Fibronectin [final] = 0.03 mg / mL (C_2)

Total volume of ECM solution: 1.5 mL (V_2)

Collagen IV

$C_1V_1 = C_2V_2$ à solve for V_1

(1 mg / mL) x (X mL) = (0.2 mg / mL) x (1.5 mL)

X = 0.3 mL = 300 µL of Collagen IV stock solution

Fibronectin

$C_1V_1 = C_2V_2$ à solve for V_1

(1 mg / mL) x (Y mL) = (0.03 mg / mL) x (1.5 mL)

Y = 0.045 mL = 45 µL of Fibronectin

DPBS

DPBS = (total volume of ECM needed) – (volume of Collagen IV) – (volume of Fibronectin)
= 1500 µL – 300 µL – 45 µL

= 1,155 µL of DPBS

Coat Chips with ECM

1. Fully aspirate the cold DPBS from both channels.
2. Set a P200 pipette to take up 100 µL of bottom channel ECM solution. (50 µL of ECM solution will be used per chip per channel.)
3. Carefully introduce the bottom channel ECM solution through the bottom channel inlet until a small ECM droplet forms on the bottom channel outlet.
4. Introduce ECM solution through the top channel inlet, leaving small droplets of excess ECM solution on both ports in both channels (Figure 3).

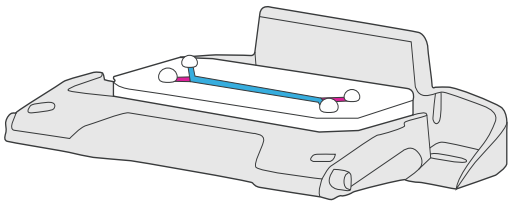


Figure 3. Chip in chip carrier with small droplets of ECM solution at ports

5. Inspect channels to ensure that no bubbles are present. If bubbles are present, dislodge by washing the channel with ECM solution until all bubbles have been removed.
6. Repeat steps 1 through 6 for each chip.
7. To prevent evaporation during incubation, fill central reservoir with 0.75 mL of DPBS (See Figure 4) and place lid onto 120 mm square dish and incubate overnight at 37°C and 5% CO2.

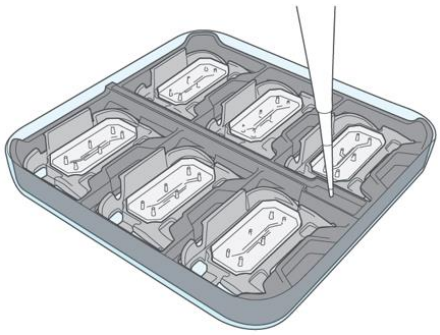


Figure 4. Fill central reservoir of Chip Cradle with 0.75 mL of DPBS

Day 0: Cells to Chips

Goals:

- Seed HIMECs in the bottom channel of chip
- Seed organoids in top channel of chip

Key Steps:

HIMEC to Chips

- Prepare HIMEC Culture Medium
- Prepare Chips
- Prepare Cell Counting Solution
- Harvest HIMECs
- Cell Counting and Viability Assessment
- Seed HIMECs to bottom channel
- Wash chips

Organoids to Chips

- Prepare Organoid Growth Medium
- Prepare Dissociation Solution
- Recover organoids from Matrigel
- Adjust cell density
- Seed colonic organoids to the top channel

Required Materials:

- Square culture dishes (1 dish per 6 chips)
- Autoclaved chip cradle (1 cradle per 6 chips)
- HIMEC culture medium (at 37°C)
- Organoid Growth medium (at 37°C)
- 15 mL and 50 mL conical tube
- Cell Recovery Solution
- Dissociation Solution
- TrypLE
- Y-27632
- CHIR99021
- Advanced DMEM/F12
- Mini Cell Scraper
- 1X DPBS (at room temperature)
- Aspirator and sterile tips
- Trypan blue counting solution
- Hemocytometer

- Installed and qualified Zoë
- Serological pipettes
- Pipettors and filtered tips
- Ice bucket, ice
- 70% ethanol
- 37°C water or bead bath
- Centrifuge

HIMEC to Chips

Prepare HIMEC Culture Medium

Seeding Medium	Volume	Source	Cat. No.
HIMEC Culture Medium	50 mL	Recipe pg. 20	-

- Keep medium at 37°C

Prepare Chips

1. Transfer ECM-coated chips from incubator into the BSC.
2. Flush ECM from both channels with medium, gently washing each channel of the chip with 200 µL of complete HIMEC medium (both channels).
3. Aspirate the medium outflow at the surface of the chips, leaving the medium in the channels.
4. Repeat the wash with an additional 200 µL of Complete HIMEC Culture Medium per channel, leaving the excess medium outflow covering the inlet and outlet ports.
5. Cover the square dish and place the chips in the incubator until the cells are ready for seeding.

Prepare Cell Counting Solution

Trypan Blue Cell Counting Solution

Reagent	Volume	Source	Cat. No.
Cells Suspension	20 µL	-	-
Trypan blue	20 µL	Sigma	93595

- Maintain counting solution at room temperature.
- Prepare in Eppendorf tube fresh for each use.

Harvest HIMECs

HIMECs that have been expanded in culture must be harvested and counted for bottom channel seeding. Typically, HIMECs are adjusted to a density range of 8 x 10^6 cells / mL to achieve a complete monolayer prior to seeding in the bottom channel.

1. Bring the culture flasks containing HIMECs from the incubator into the BSC.
2. Aspirate culture media and add 15 mL of DPBS to wash the culture surface. Aspirate the DPBS wash.
3. Add 3 mL of TrypLE to the flasks. Incubate for 2 to 3 minutes at 37°C.
4. Tap the side of the flask gently, and inspect the culture under the microscope to assess complete detachment of cells from the culture surface.

5. Add 3 mL of warm HIMEC Culture Medium to the flasks and pipette gently to mix, while collecting all cells from the culture surface of the flasks.
6. Transfer the contents of the flasks into a sterile 15 mL conical tube.
7. Transfer 20 µL of the cell suspension to a 1.5 mL tube containing 20ul of trypan blue cell counting.
8. Count cells using a manual hemocytometer (See figure 5.)
9. Transfer the cells suspension in the 15 mL tube to the centrifuge. Set and run at 150 x g for 5 minutes.
10. Carefully aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet. Note: The cell pellet will be very small. Aspirate carefully.
11. Loosen the cell pellet by flicking the tube gently.
12. Using a P1000 pipette, gently resuspend the cells by adding 200 µL of complete HIMEC culture medium.

Cell Counting and Viability Assessment

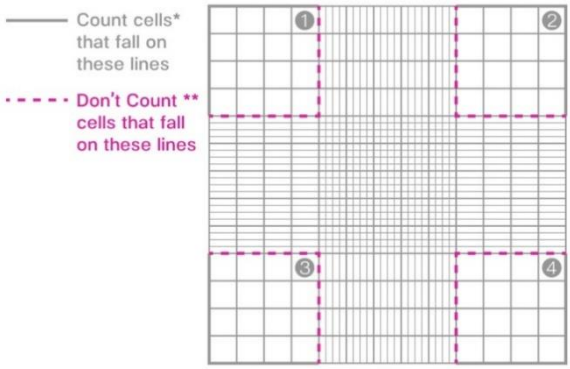


Figure 5: Hemocytometer.

a. Count both viable and non-viable cells in each quadrant of the hemocytometer.
Live Cell Count; Dead Cell Count; Total Cell Count

b. Calculate percent viability of the cell solution.
(Live Cells) ÷ (Total Cells) x 100 = % Viability

c. Calculate viable cell concentration. The dilution factor is 2 when prepared in the trypan blue solution above.
(Live Cell Count x 2 x 10^4) ÷ 4 = Viable Cell Concentration (cells / mL)

d. Calculate viable cell yield.
(Viable Cell Concentration) X (Cell Suspension Volume) = Viable Cell Yield (cells)

1. Re-suspend the HIMECs to 8-10 x 10^6 cells / mL in Complete HIMEC Culture Medium.

Seed HIMECs to Bottom Channel

Work with one chip at a time. After seeding the first chip, invert the chip and assess cell density within the channel through the microscope, adjusting the density of the cell suspension accordingly for the next chips if necessary.

1. Bring the square dish containing the prepared chips to the BSC.
2. Avoiding contact with the ports, carefully aspirate excess medium droplets from the surface of one chip.
3. Gently agitate cell suspension before seeding each chip to ensure a homogeneous cell suspension.
4. Using a pipette, pipet out the medium from the bottom channel, leaving empty.
5. Seed 10 to 15 µL of the HIMEC suspension into the bottom channel of one chip first
6. Cover the dish and transfer to the microscope to check the seeding density within the chip.
7. If seeding density is not optimal, close to 8 M/ml return the chip to the BSC and wash the channel with 200 µL of fresh medium 2 times (See Figure 6.) Do not aspirate the medium from the channel. Adjust cell density accordingly, and repeat steps 3 through 5 until the correct density is achieved within the channel. Note: Correct seeding density is essential for success of the chip cultures.
8. After confirming the correct cell density, seed cells in the remaining chips, invert each chip, and rest the edge of the chip carrier on the chip cradle. Note: Each chip cradle can support up to 6 chips inside a square cell culture dish. (See Figure 7.)
9. Place DPBS at the cradle to provide humidity for the cells. Replace dish lid. Note: Minimize the amount of time the cells are outside the incubator by seeding no more than 12 chips at a time, immediately placing the chips in the incubator at 37°C after seeding each batch of 12.
10. Place the chips still in the dish in the 37°C incubator for approximately 30 minutes - 1 hour, or until cells in the bottom channel have attached.
11. Once HIMECs have attached (approximately 1 hour post-seeding), flip the chips back to an upright position. Note: Remove the chip cradle from the dish, wipe with 70% ethanol to clean, and autoclave for use in next experiment.

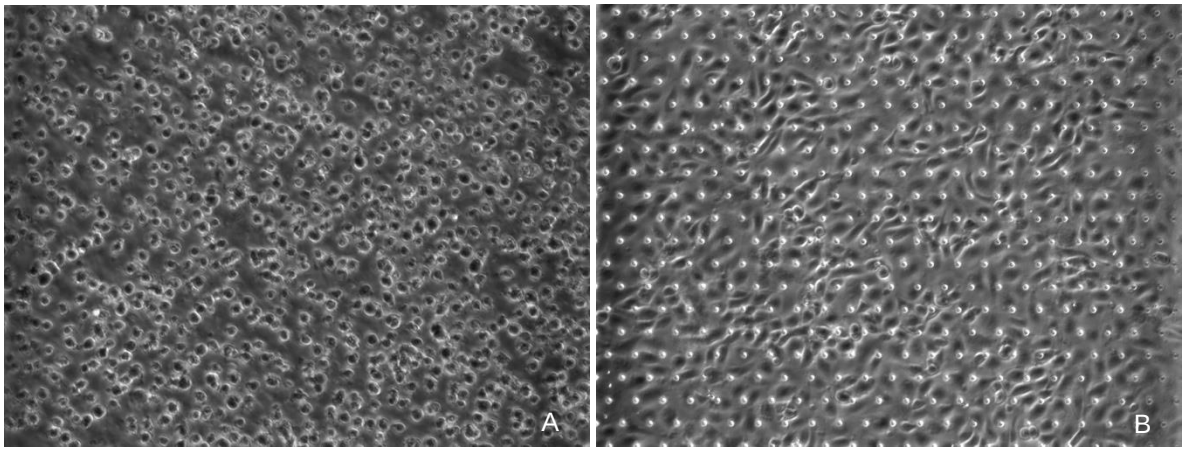


Figure 6: A. HIMEC optimum cell seeding density; B. HIMEC attached on chip 2 hours post-seeding.

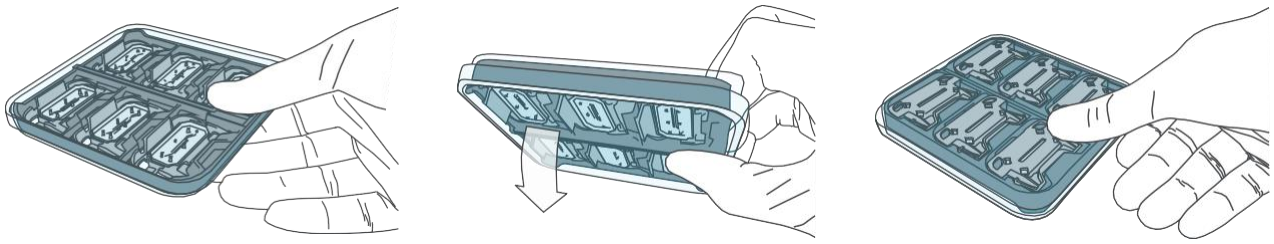


Figure 7: Inverting chips during endothelial attachment

Wash Chips

A wash is performed 30 minutes - 1 hour post-seeding, after the HIMECs in the chip have attached, to ensure that nutrients are replenished, and the channels do not dry out. During the wash the medium should be observed to flow through the channel and outflow from the outlets.

1. With a P200 pipette, gently wash the bottom channel of each chip with warm, equilibrated HIMEC Culture Medium to remove any possible bubbles in the channel.
2. Repeat the step using complete Organoid Growth Medium to wash the top channel

Organoids to Chips

Prepare Organoid Growth Medium

Organoid Growth Medium

Organoid Growth Medium (100 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
IntestiCult™ OGM Component A	50 mL	-	-	Stemcell Technologies	06010 (kit); 06011 (part)
IntestiCult™ OGM Component B	50 mL	-	-	Stemcell Technologies	06010 kit; 06012 (part)
Primocin	200 µL	50 mg / mL	100 µg / mL	InvivoGen	ANT-PM-1
Y-27632	100 µL	10 mM	10 µM	Stemcell Technologies	72302
CHIR99021	100 µL	5 mM	5 µM	Reprocell	04-0004-10

- IntestiCult™ Organoid Growth Medium (Human) (06010) is supplied as a kit containing IntestiCult™ OGM Human Component A (06011) and IntestiCult™ OGM Human Component B (06012).
- Primocin is a non-toxic antimicrobial agent for primary cells.
- Y-27632 is a selective inhibitor ROCK1 and ROCK2 used to enhance survival of cells.
- CHIR99021 is a small molecule inhibitor glycogen synthase kinase 3 β (GSK-3β).
- Store prepared Organoid Growth Medium at 4°C for up to 7 days.

Note: Y-27632 and CHIR99021 are added to the medium only up to day 1 post-seeding, and should be removed from medium on the next day when medium is replenished (day 2).

Prepare Dissociation Solution

Dissociation Solution (2 mL per plate)

Reagent	Volume	Source	Cat. No.
TrypLE	1 mL	GIBCO	12604013
DPBS	1 mL	Sigma	D8537
Y-27632	2 µL	Sigma	72302

- Prepare Dissociation Solution fresh for each use. Calculate the volume based on 2 mL of dissociation solution per 24 well plate.

Note: The volume should be adjusted according to the number of plates used. 2 mL is suggested volume for the dissociation of 50 µL of organoids/Matrigel in 24 well plates.

Recover Organoids from Matrigel

Prior to recovering organoids, ensure all equipment and materials are ready to use, organized, and that all required reagents are prepared and are at the appropriate temperature. Transfer the 24 well plate containing organoids into the BSC.

1. Carefully aspirate medium from each well without disturbing the Matrigel dome.
2. Gently add 500 µL of Cell Recovery Solution to each well.
3. Scrape the Matrigel using a cell scraper.
4. Using a P1000 pipette, collect contents of each well and transfer to a cold 15 mL conical tube.
   Note: It is highly suggested to use Low Protein Binding tubes (Lo Bind Eppendorf) to prevent the organoids from sticking to the tube and to minimize the loss of organoids.
5. Incubate conical tube on ice for 45 minutes, inverting tube every 2 to 5 minutes during this time.
6. While cells are incubating on ice, ensure the centrifuge is cooled down to 4°C.
7. Centrifuge the organoid suspension at 300 x g for 5 minutes at 4°C.
8. After centrifugation, observe the tube to confirm complete disappearance of Matrigel and clear formation of a cell pellet.
   Note: If a thin layer of Matrigel is present, carefully using a P1000 pipette, remove the supernatant without disrupting the pellet, and add 5 mL of new Cell Recovery Solution. Incubate for 5 minutes on ice, and repeat centrifugation in step 8. If no clear pellet is formed, repeat step 8
9. Once a defined cell pellet is observed, aspirate the supernatant, gently flick the tube to disrupt the pellet and add the prepared Dissociation Solution, 2 mL for every 24 well plate.
10. Incubate the conical tube in the water bath at 37°C for 1 to 2 minutes to dissociate the organoids into fragments. NOTE: Incubation time will vary based on the size of organoids, however, do not incubate for longer than 2 minutes, as this may result in dissociation of organoids into single cells, leading to decreased seeding efficiency.
11. Dilute with at least 2 times Advanced DMEM/F12 medium to wash.
12. Centrifuge to pellet the dissociated organoids at 300 x g for 5 minutes at 4°C.
13. Aspirate the supernatant and adjust seeding density by suspending the pellet in Organoid Growth Medium.

Adjust Cell Density

The seeding density depends on the size and density of organoids cultured on the 24 well plate. Use of 2 to 3 wells of organoids per chip has been recommended (See Figure 7.)

To calculate volume for suspension of dissociated organoids:

Chip Seeding Volume = 30 µL

Number of chips = 6

Volume of media required to re-suspend dissociated organoids = (30 µL) x (6 chips) = 180 µL

Dilute the organoids with warm Organoid Growth Medium to the required final cell density (See Figure 7.)

Seed Colonic Organoids to Top Channel

Work with one chip at a time. After seeding the first chip, assess cell density within the channel through the microscope, adjusting the density of the cell suspension accordingly for the subsequent chips if necessary.

1. Bring the square dish containing the prepared and washed ECM-coated chips to the BSC.
2. Pipet out the medium from the top channel, leaving empty.
3. Very gently, agitate the cell suspension before seeding each chip to ensure a homogeneous mixture for even seeding.
4. Quickly and steadily, pipette 30 µL of the cell suspension into the top channel inlet. Avoid direct contact of the aspirator tip with the outlet port.
5. Cover the dish and transfer to the microscope to check the seeding density within the chip. Note: At the optimal seeding density, the organoid fragments will form an even cell layer on the top channel of the chip, covering the whole chip membrane (See figure 8).
6. If seeding density is not optimal, return the chips to the BSC, and wash the channel with 200 µL of fresh medium 2 times. Do not aspirate the medium from the channel. Adjust cell density accordingly, and repeat steps 3 through 5 until the correct density is achieved within the channel.
14. To avoid density gradient between chips, once the cells density is confirmed, transfer ~ 300 µL aliquots of the cells suspension to 1.5 mL Low Protein Binding tubes.
15. Seed cells in the remaining chips. Note: Minimize the amount of time the cells are outside the incubator by seeding no more than 12 chips at a time, immediately placing the chips in the incubator at 37°C after seeding each batch of 12 chips.
16. Place the dish of chips (with the DPBS reservoir) at 37°C and incubate undisturbed overnight.

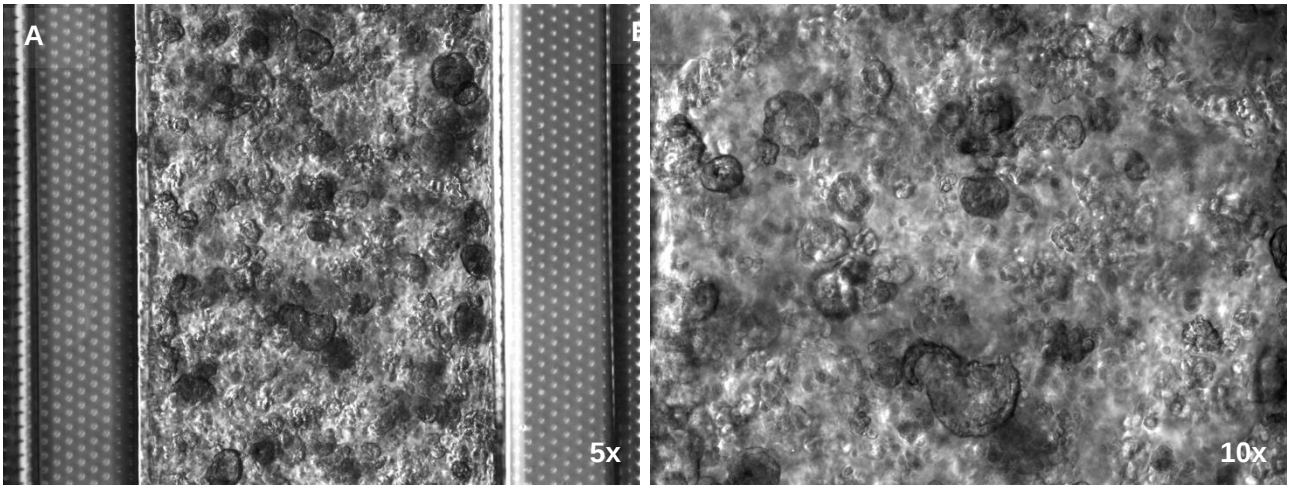


Figure 8: Optimal single and fragments organoids density. A. Low magnification; B. Higher Magnification. Note that the porous membrane is completely covered by the cells.

Day 1: Chips to Pods and Pods to Zoë

Goals:

- Gas equilibration of media
- Connect chips to Pods
- Connect Pods to Zoë

Key Steps:

- Gas equilibration of media
- Prime Pods
- Wash chips
- Chips to Pods
- Pods to Zoë and Regulate

Required Materials:

- HIMEC Culture Medium
- Organoid Growth Medium
- Installed and qualified Zoë
- Prepared chips
- Pods (sterile) — 1 per chip
- Tray — 1 per 6 chips
- Steriflip filtration unit: PVDF filter 0.45 µm (sterile)
- Vacuum source (minimum -70 kPa)
- Serological pipettes
- Pipettors and filtered tips
- 37°C water or bead bath
- 70% ethanol
- 3KDa Dextran Cascade Blue

Gas Equilibration of Media

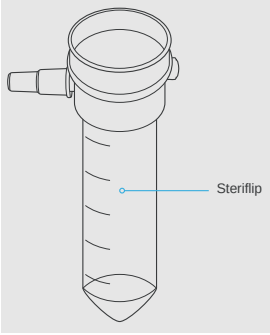
The media equilibration step is critical to the success of Organ-Chip culture. Omission of this step will cause the eventual formation of bubbles in the chip, the Pod, or both, which will in turn cause irregular flow and negatively impact cell viability. Minimize the time medium is outside of a warmed environment (no more than 10 minutes), such as an incubator or bath, as gas equilibrium can become compromised when medium is allowed to cool.

1. Prepare 50 mL of HIMEC Culture Medium per 12 chips in separate 50 mL conical tubes
2. Prepare 50ml of Organoid Growth Medium per 12 chips in separate 50 mL conical tubes with the addition of 10 µM Y-27632, 5µM CHIR99021 and tracer of your choice to check the permeability. The most common one is the 3KDa Dextran Cascade Blue at final concentration in medium of 100 ug/ml. The tracer can be kept in culture medium through the course of experiment to ensure a tighter barrier function. The Permeability Assay Section will provide further instructions.

   Note: Approximately 2 mL of medium will be used per reservoir of the chip. For 12 chips prepare around 30 mL of medium for the top channel and 30 mL of medium for the bottom channel.

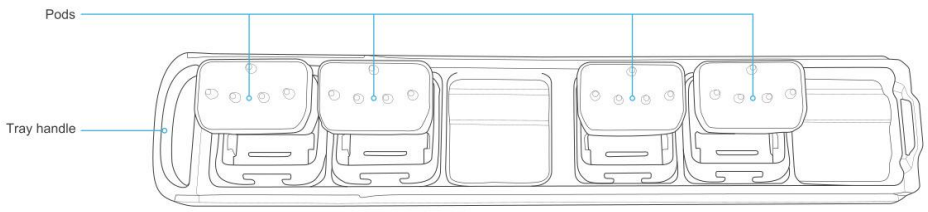
3. Warm the conical tubes with media at 37°C in a water or bead bath for at least 1 hour.
4. Immediately connect the 50 mL tube containing each warmed medium to a Steriflip unit.
   a. Attach each conical tube containing warmed medium to a Steriflip unit.
   b. With the unit "right-side-up" (medium in the bottom conical tube), apply vacuum for 10 seconds.
   c. Invert the Steriflip-connected tubes, and check that the medium begins to pass from the top conical tube to the lower tube. Note: The vacuum source must operate at least -70 kPa. At this correct pressure, it should take about 2 seconds for every 10 mL of medium to flow through the filter. If it takes longer, stop and see the troubleshooting protocol — medium not equilibrated properly.
   d. Leave the filtered medium under vacuum for 5 minutes.
5. Remove the vacuum tubing from the Steriflip units.
6. Separate the conical tubes containing media from the Steriflip unit and immediately place the conical tubes containing media in the incubator with the caps loose.

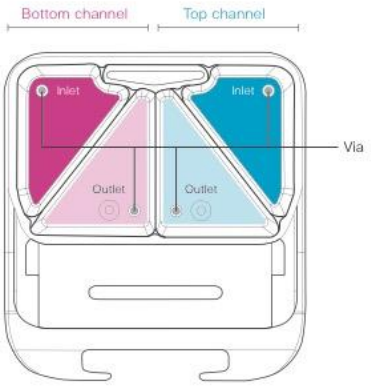
   As noted above, minimize the time media is outside of the incubator during Pod preparation to maintain temperature. This is a critical step to ensure success of the chips.

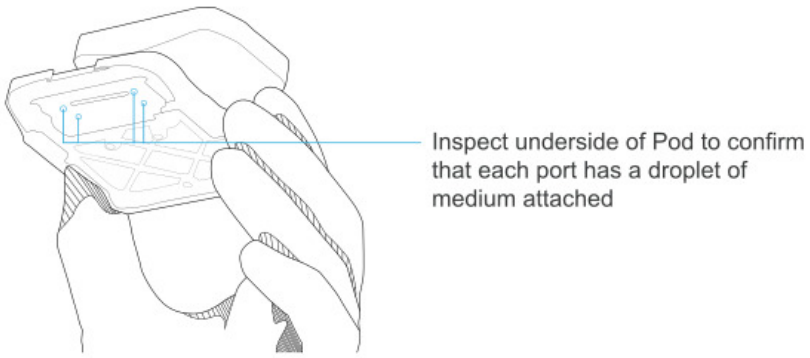


Prime Pods

1. Sanitize the exterior of the Pod packaging and trays with 70% ethanol, wipe, and transfer to the BSC.
2. Open the Pod package and place the Pods into the trays. Orient the Pods with the reservoirs toward the back of the tray.
3. Place 2 mL of pre-equilibrated, warm Organoid Growth Medium to the top and HIMEC Culture Medium to the bottom inlet reservoirs of each Pod.
4. Pipette 300 µL of pre-equilibrated, warm Organoid Growth Medium to the top outlet, and HIMEC Culture Medium to the bottom outlet reservoir of each pod, placing the media directly over each outlet via.
5. Bring trays containing Pods to the incubator and slide completely into Zoë with the tray handle facing outward.
6. Run the Prime cycle on Zoë.
    a. Use the rotary dial to highlight “Prime” on the display.
    b. Press the rotary dial to select “Prime.”
    c. Rotate the dial clockwise to highlight “Start.”
    d. Press the dial again to select “Start” and begin. Note: Once “Start” is selected, there will be an audible sound as Zoë engages the Pods.
7. Close the incubator door, and allow Zoë to prime the Pods; this process takes approximately 1 minute. Note: When the status bar reads “Ready,” the “Prime” cycle is complete.
8. Remove the tray from Zoë, and bring to the BSC.
9. Verify that the Pods were successfully primed and if it is necessary, perform a second prime cycle
    a. Inspect the underside of each Pod — look for the presence of small droplets at all four fluidic ports. Droplets will vary in size from a small meniscus to larger droplets; often droplets on the outlet ports will be larger.
    b. If any Pod does not show droplets, re-run the “Prime” cycle on those Pods.
    c. If any media dripped onto the tray (this may occur more often by the outlet ports), clean tray with a wipe sprayed with 70% ethanol.
10. Once it is confirmed that all Pod ports are wet with droplets, put the tray of Pods to the side in the BSC.







Wash Chips

1. Transfer the seeded chips in a 120 mm square dish from the incubator to the BSC.
2. Wash twice the top channel of each chip with warm, 2x of 200 µL, equilibrated Organoid Growth Medium, aspirating the outflow from the chip surface.
3. Wash the bottom channel of each chip with warm, equilibrated HIMEC Culture Medium, aspirating outflow from the chip surface.
4. Place small droplets of equilibrated Organoid Growth Medium on all inlet and outlet ports of the top channel and HIMEC Culture Medium on the inlet and outlet of the bottom channel.
5. Repeat steps 2 through 4 for each chip.

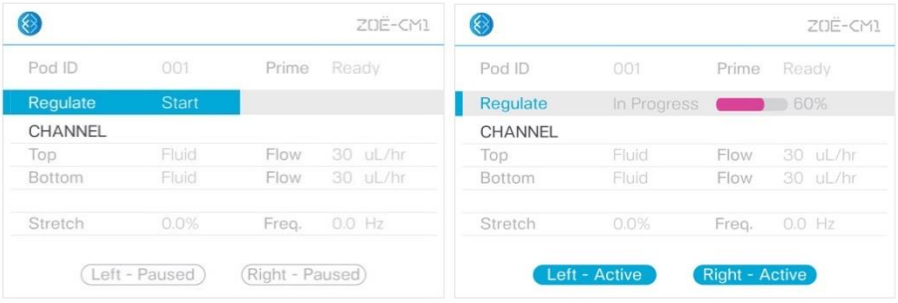
Chips to Pods

1. Holding one chip (while it remains in the chip carrier) in the dominant hand and one Pod firmly in the non-dominant hand, slide the chip carrier into the tracks on the underside of the Pod until the chip carrier has seated fully.
2. Place thumb on the chip carrier tab and gently, but firmly, depress the tab in and up to engage the tab of the chip carrier with the Pod.

3. Aspirate any excess medium on the chip surface from the Pod window.
4. Place the Pod with connected the chip onto the tray.
5. Repeat steps 1 through 4 for each Pod and chip carrier.
6. Confirm that there is sufficient media in each Pod inlet and outlet reservoirs and that the Pod lids are flat and secure.

Pods to Zoë and Regulate

1. Place trays that are holding Pods and chips immediately into Zoë to prevent media from cooling and losing its gas equilibration.
2. Program the appropriate Organ-Chip culture conditions on Zoë. These conditions will start as soon as the Regulate cycle is complete.
   - For Colon Intestine-Chips, set the flow rate to 30 µL / hour for both top and bottom channels.
3. Run Regulate cycle.
   a. Using the rotary dial, highlight the “Regulate” field.
   b. Press the dial to select “Regulate,” and rotate the dial clockwise to “Start.”
   c. Press the dial again to select “Start” and begin the Regulate cycle. Note: Once “Start” is selected, there will be an audible sound as Zoë engages the Pods.
   d. At this point the “Activation” button will glow blue.
   e. The Regulate cycle lasts 2 hours. After the cycle has finished, Zoë will begin flow at the preset Organ-Chip culture conditions. To cancel the Regulate cycle (only if needed), on Zoë, select the “Regulate” field with the dial and press the button to select. Rotate the dial counter-clockwise to select “Cancel.” Press the dial once more and wait 1 minute for the cycle to end, after which the tray can be removed. If cancelled, always rerun a complete Regulate cycle before proceeding.



Day 2: Replenish Medium

Goals:

- Replenish Medium without Y-27632 and CHIR99021
- Cell culture inspection
- Assess barrier function

Key Steps:

- Via wash
- Replenish medium of without CHIR99021 and Y27632
- Chip inspection and Debris Flushing
- Observe cell morphology
- Effluent Sampling

Required Materials:

- HIMEC Culture Medium
- Organoid Growth Medium
- Serological pipettes
- Pipettes, Multichannel and filtered tips
- 37°C water or bead bath
- 70% ethanol
- 3KDa Dextran Cascade Blue
- 96 wells black walled plates
- Microscope

Via Wash

1. The day after connecting chips and Pods to Zoë, which begins the process of Organ-Chip culture, pause the Zoë by pressing the silver “Activation” button located above the tray bays. This stops flow and releases the Pods.
2. Slide the tray out of the bay and transfer to the BSC.
3. Remove the Pod lids. Using a 200 µL pipette, perform a via wash on each Pod inlet and outlet reservoir:
   a. Using media within the Pod reservoir, pipette 200 µL of medium directly over the top of the via to dislodge any bubbles that may be present.
   b. Repeat this wash step for each of the four Pod reservoirs.

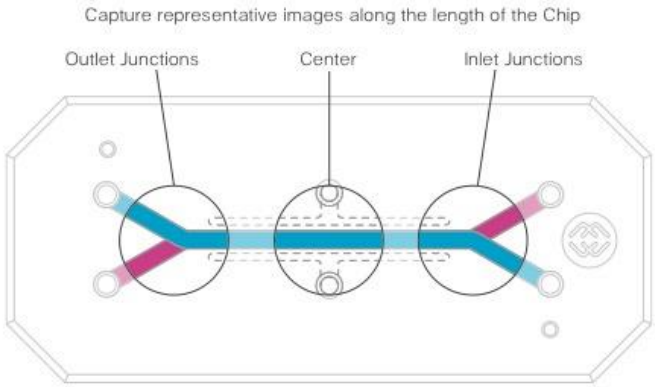
Replenish Medium without CHIR99021 and Y27632

1. Replenish Organoids Growth Medium without the addition of Y-27632 and CHIR99021 inhibitors to the top inlet reservoir.
2. Add HIMEC Culture Medium to the bottom inlet reservoir.
4. Replace Pod lids
5. Inspect the chip for bubbles

Observe Cell Morphology

1. Using a microscope, inspect cells in the chips to assess morphology and viability. Capture representative images at 10X or 20X magnification at the following locations:
   - Inlet junction
   - Center of channel
   - Outlet junction

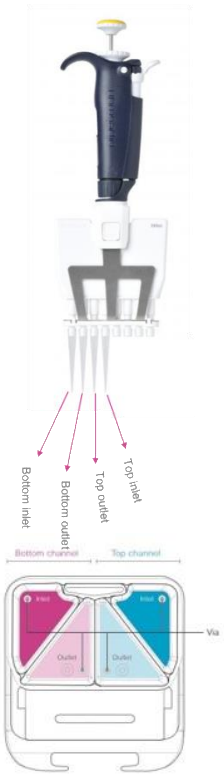
   Note: At day 2 of culture, if a confluent monolayer is not observed, the apparent permeability obtained will be higher than $1x10^{-6}$ cm/s. The optimum permeability will be obtained gradually by day 4-5 upon development of a confluent monolayer.

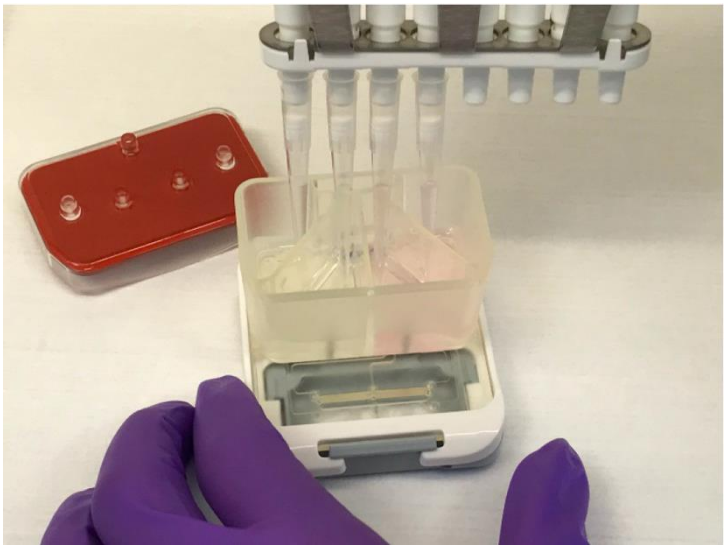


Effluent Sampling

1. Use a standard multichannel pipette; adjust the volume to 50 µL to collect effluent and media from all the four reservoirs simultaneously at once by placing the pipette tips into the Pods such that one tip is in each reservoir as depicted in Figure 9.
2. Dispense the collected effluents in an appropriately labelled 96 wells black walled plate. Change tips between Pods to avoid cross contamination.
3. Cover the Pods and move to the next Pod collecting medium from all your chips.

A





B

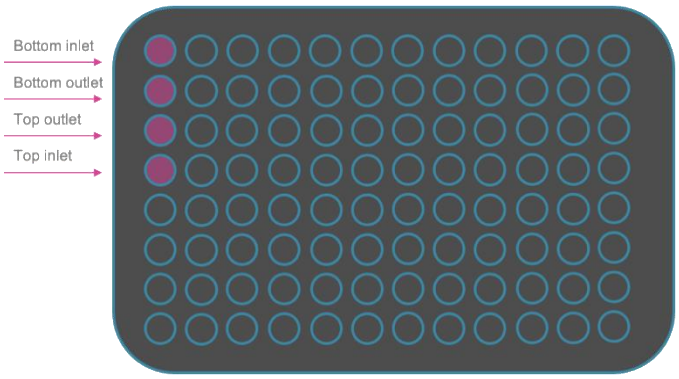
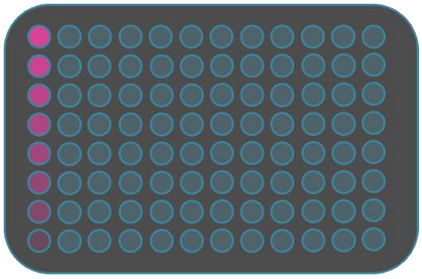


Figure 9: Effluent Sampling. A. Multichannel collecting effluent from the Pod. B. Medium dispensed in the 96 wells black walled plate.

1. Add 100 µL of PBS to the effluent collected on the plate for a final dilution of 1:3
2. To prepare the standard curve, label two 1.5 mL tube as diluent and neat medium.
   a. To prepare the diluent, add 800 µL of DPBS to the tube and collect 400 µL of medium from the bottom inlet reservoir
   b. On the tube labeled neat collect 100 µL of medium from the top inlet reservoir. Add 200 µL of DPBS to the tube. This will be the highest concentration on your standard curve.
3. From the tube labeled diluent, dispense 150 µL of the diluted bottom reservoir medium to wells 1-7 and dispense the diluted medium from tube neat on well 8 on the plate.
4. Perform serial dilution collecting 150 µL from well 8 to 2, leaving only the diluent medium on well the last well (1). Well 1 will be used as your blank.
5. Save the plate wrapped in foil at room temperature to read later or freeze it at -20°C to read at the end of the experiment. Using a Spectrophotometer Microplate Reader, adjust the Ex/Em based on the fluorescent tracer used. (e.g. For Dextran Cascade Blue, set Ex/Em at 375/420, gain 60).
6. Perform assay daily to monitor barrier function and test when it reaches levels below $1x10^{-6}$ cm/s. For more information, refer to emulatebio.com website's Barrier Function Protocol and Permeability and Standard Curve Calculator to download permeability and standard curve.



7. Return trays to Zoë.
8. Using the rotary dial, highlight the “Flow rate” field and set it at 1000 mL/hour. This step provides removal of cell debris.
9. Press the silver “Activation” button and let it flush out any cell debris for 5 minutes.
10. Pause Zoë by pressing the silver “Activation” button and re-set the flow rate to 30 mL/hour.
11. Press the silver “Activation” button to resume pre-set Organ-Chip culture conditions.

Note: The flush cycle is performed at day 4, 6 and 8 for cellular debris removal. It is also used for priming during experimental treatment (e.g., introducing compounds, cytokine challenges.)

- Zoë will engage when the “Activation” button glows blue.

Day 3: Initiating Stretch

Goals:

- Set stretch to 2%, 0.15 Hz

Key Steps:

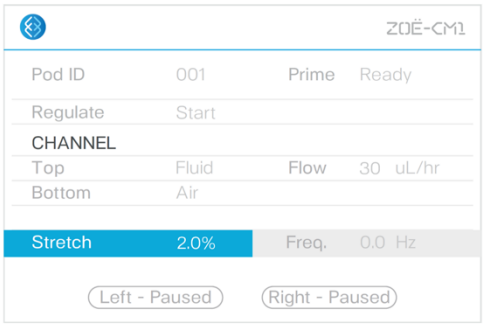
- Stretch introduction
- Effluent sampling

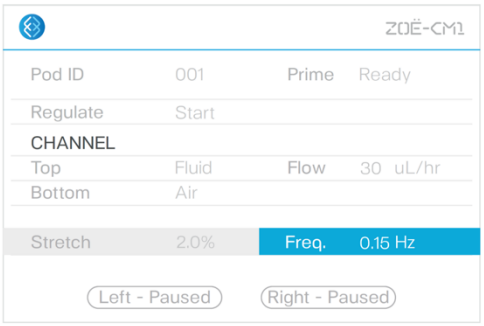
Required Materials:

- Serological pipettes
- Pipettes, Multichannel and filtered tips
- 96 wells black walled plates

Stretch Introduction

1. Pause Zoë by pressing the silver “Activation” button.
2. Using the rotary dial, highlight the “Stretch” field.
3. Press the dial to select “Stretch” and rotate the dial clockwise to increase stretch to “2%”.
4. Press the dial to select “Freq.” and rotate the dial clockwise to increase stretch to “0.15 Hz”.





Effluent Sampling

1. Perform Effluent Sampling as done on day 2 or click on the link for more information on barrier function protocol, permeability and standard curve calculator
2. Press the “Activation” button.

Day 4: Maintenance and Sampling, Increasing Stretch to 10%

Goals:

- Maintenance of Chips in Zoë
- Inspect cells in chips
- Set stretch to 10%, 0.15 Hz

Key Steps:

- Maintenance and Sampling of Chips
- Increasing stretch to 10%

Required Materials:

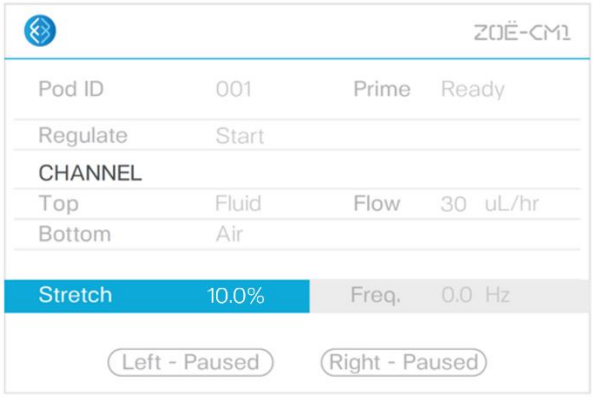
- HIMEC Culture Medium
- Organoid Growth Medium
- Serological pipettes
- Pipettes, multichannel and filtered tips
- 37°C water or bead bath
- 70% ethanol
- 3KDa Dextran Cascade Blue
- 96 wells black walled plate
- Microscope

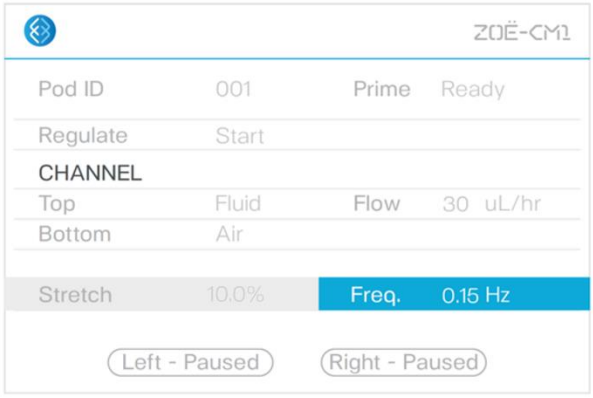
Maintenance and Sampling of Chips

1. Pause Zoë by pressing the silver “Activation” button.
2. Remove the trays and place in the BSC.
3. Inspect each chip for bubbles by eye.
4. Using a microscope, inspect cells in the chips to assess morphology and viability. Capture representative images at 10X or 20X magnification at the following locations:
5. Remove the Pod lids and collect effluent medium from Pod outlet reservoirs for analysis.
   - Collect effluent from the indicated regions, avoiding disturbing the Pod reservoir vias.
   - Check on the links for effluent sampling, barrier function protocol for more information or here to download the permeability or standard curve calculator.
6. Gently aspirate any medium not collected for analysis, ensuring that a thin liquid film still covers the reservoir vias so that no air is introduced into the vias.
7. Refill the Pod media reservoirs with appropriate fresh cell culture medium and perform a via wash by pipetting 200 µL of medium directly over the top of the via to dislodge any bubbles that may be present.
8. Replace the Pod lids and return trays to Zoë.
9. Press the silver “Activation” button to resume pre-set Organ-Chip culture conditions. Zoë will engage when the “Activation” button glows blue.

Increase stretch to 10%

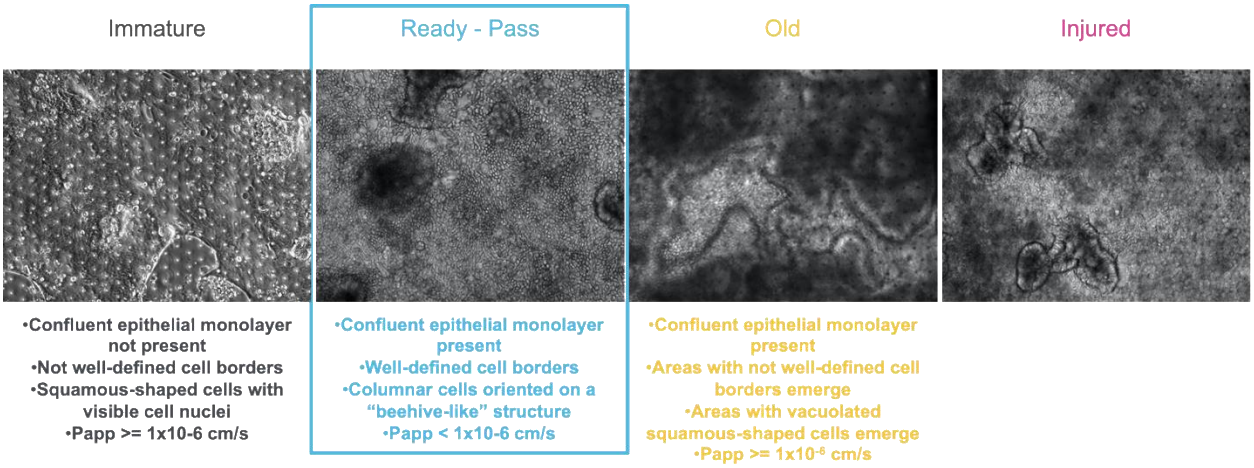
1. Pause Zoë by pressing the silver “Activation” button.
2. Using the rotary dial, highlight the “Stretch” field.
3. Press the dial to select “Stretch” and rotate the dial clockwise to increase stretch to “10.0%”.
4. Press the dial to select “Freq.” and rotate the dial clockwise to increase stretch to 10% and the frequency to 0.15 Hz.
5. Press the “Activation” button.





Acceptance Criteria

Morphology Acceptance Criteria



Barrier Function Acceptance Criteria

Between day 4 and 5, the barrier function Papp levels are expected to reach below $1x10^{-6}$ cm/s, when using 3KDa Dextran Cascade Blue. If so, the chips are ready for the experimental phase.

Troubleshooting

Issue	Section	Step	Recommendation
Bubbles are present in channel	Chip Preparation	Introduce ER-1 Solution to Channels; Activate Chips; Coat Chips with ECM	Dislodge by washing the channel with appropriate solution until all bubbles have been removed. If bubbles persist, it may be helpful to aspirate the channel dry and slowly re-introduce the solution.
Bubbles in the ports upon introduction of media into the chip	Chips to Pods and Pods to Zoë	Wash Chips	Since the chip material is hydrophobic, bubbles could get trapped at the ports. Dislodge bubbles using pipette tip or aspirate the channels and reintroduce equilibrated media.
Media take too long to pass through Steriflip	Chips to Pods and Pods to Zoë	Equilibration of Media	Vacuum pressure is not reaching -70kPa. Need to find an alternate vacuum source.
Pods do not prime	Chips to Pods and Pods to Zoë	Prime Pods	Ensure that medium is directly over all Pod vias. If problem persists, record the Pod lot number and replace with new a Pod.
Screen is frozen or unresponsive	Chips to Pods and Pods to Zoë; Maintaining and Sampling	Pod to Zoë; Maintenance and Regulate	Power off Zoë and then turn Zoë on again; contact our support team if problem persists.
Pods stuck in Zoë	Maintaining and Sampling	Maintenance and Regulate	Pod lid not secured on Pod. Try wiggling tray to the right and left as you slide it out while keeping it level. If problem persists, contact our support team.
Pods not flowing properly or evenly; Bubbles observed in chip	Maintaining and Sampling	Maintenance and Regulate	There is inherent variability with Zoë, however large fluctuations and major flow issues primarily result from bubbles. Remove chip from Pod; flush chip with media; re-prime Pod with degassed media; connect chip to Pod; and run the Regulate cycle.

Potential Root Causes of Bubbles

Check the following if a high failure rate due to bubbles is observed or if bubbles are persistent, despite following the mitigation steps above.

- Medium not sufficiently equilibrated before adding to Pods: be sure to follow media preparations steps in section Gas Equilibration of Media.
- Vacuum for Steriflip too weak: ensure that media is passing through the Steriflip in about 10 seconds.
- Incorrect Steriflip used: confirm correct Steriflip unit (Millipore SE1M003M00).
- Medium not warmed correctly before Steriflip step: Be sure to follow media preparations steps in section Gas Equilibration of Media.
- Insufficient priming: disconnect chip and reprime Pod.

Appendices

Reagent Aliquots

Aliquot reagents including media supplements and ECM prior to use and store at -20°C to avoid multiple freeze-thaw cycles.

Y-27632 (ROCK inhibitor)

Reagent	Conc. [Stock]	Amount	Volume	Solvent
Y-27632	10 mM	5 mg	1.56 mL	0.1% BSA in DPBS

- Resuspend 5 mg of Y-27632 compound in 1.56 mL of 0.1% BSA in DPBS according to manufacturer's instructions. yielding a stock concentration of 10 mM.
- The final concentration of Y27632 used in organoid medium will be 10 µM.
- Aliquot reconstituted Y-27632 to single-use volumes and store at -20°C.

CHIR99021 (GSK3 inhibitor)

Reagent	Conc. [Stock]	Amount	Volume	Solvent
CHIR99021	5 mM	10 mg	4.29 mL	DMSO

- Resuspend 10 mg of CHIR99021 compound in 4.29 mL of DMSO according to manufacturer's instructions, yielding a stock concentration of 5 mM.
- The final concentration of CHIR99021 used in organoid medium will be 5 µM.
- Aliquot to single-use volumes and store at -20°C.

Matrigel – Growth Factor Reduced (GFR)

Reagent	Volume [Stock]
Matrigel Growth Factor Reduced	10 mL

- The stock bottle of Matrigel must be thawed overnight on slushy ice in a 2°C to 6°C refrigerator (in the back) or cold room and handled on slushy ice at all times. (Maintain Matrigel on ice at all times, as this solution gels rapidly at temperatures above 8°C.)
- After thawing, aliquot Matrigel to suitable single-use volumes based on the specific stock concentration and amount needed in experiment.

- Keep all materials on ice at all times.
- Use pre-chilled pipettes, tips, and tubes at -20°C prior to aliquoting.
- Freeze aliquots immediately at -20°C.
- Thaw aliquots on ice just prior to use.
- Once aliquots are thawed, do not re-freeze. Thaw aliquots on ice prior to use
- Do not freeze/thaw

Organoid Maintenance Medium (100 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
IntestiCult™ OGMH Component A	50 mL	-	-	Stemcell Technologies	06010
IntestiCult™ OGMH Component B	50 mL	-	-	Stemcell Technologies	06010
Primocin	200 µL	50 mg / mL	100 µg / mL	InvivoGen	Ant-pm-1

- Aseptically combine all media components in Intesticult™ OGMH Component A bottle
- Store at 4°C
- Use within one week of preparation

Organoid Growth Medium (100 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
IntestiCult™ OGMH Component A	50 mL	-	-	Stemcell Technologies	06010
IntestiCult™ OGMH Component B	50 mL	-	-	Stemcell Technologies	06010
Primocin	200 µL	50 mg / mL	100 µg / mL	InvivoGen	Ant-pm-1
Y-27632	100 µL	10 mM	10 µM	Stemell Technologies	72302
CHIR99021	100 µL	5 mM	5 µM	Reprocell	04-0004-10

- Store at 4°C.
- Use within 7 days of preparation.

Dissociation Solution (10 mL)

Reagent	Volume	Source	Cat. No.
TrypLE	5 mL	GIBCO	12604013
DPBS	5 mL	Sigma	D8537
Y-27632	10 µL	Sigma	72302

- Prepare fresh for each use.

Complete HIMEC Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Endothelial Cell Basal Medium	500 mL	-	-	PromoCell	C-22121 (kit); C-22221
Endothelial Cell Growth Medium MV2 Supplement Pack	-	-	-	PromoCell	C-39221
Primocin	500 µL	50 mg / mL	50 µg / mL	Invivogen	Ant-pm-1

- Aseptically transfer all components of Endothelial Cell Growth Medium MV2 SupplementPack to the Endothelial Cell Basal Medium bottle
- Store at 4°C.

Colon Intestine-Chip Timeline