emulate

Duodenum Intestine-Chip Culture Protocol

EP203 Rev. A

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Introduction to the Human Emulation System

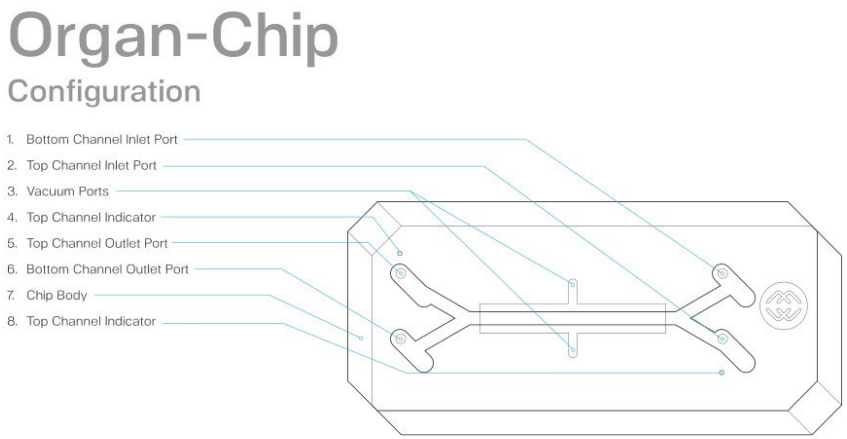
This section provides an overview of key components of the Human Emulation System®, including the Chip-S1® Stretchable Chip, Pod® Portable Module, Zoë® Culture Module, and Orb™ Hub Module.

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

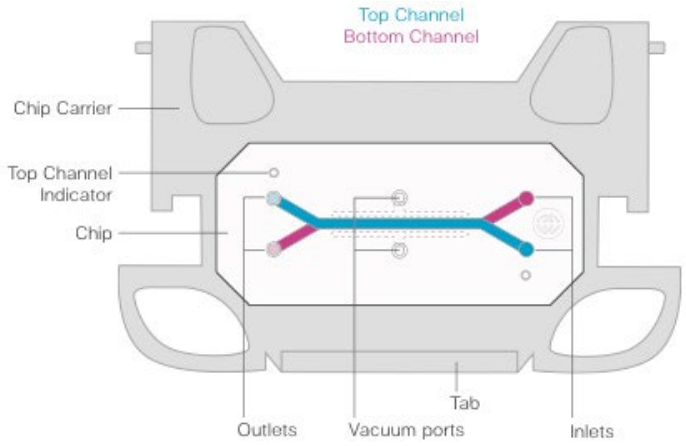
Organ-Chip (Chip-S1)

Our Chip-S1 can be configured to emulate multiple different organ tissues, including, but not limited to, brain, kidney, intestine, and liver. When connected to the Pod and Zoë, the chip recreates the body's dynamic cellular microenvironment, including tissue-to-tissue interfaces, blood flow, and mechanical forces. This protocol describes the methodology for the Duodenum Intestine-Chip.

The microenvironment created within each Chip-S1 includes epithelial cells in the epithelial channel and endothelial cells in the endothelial channel. The channels are fluidically independent, separated by a thin, porous membrane that allows for cell-cell interactions similar to those observed *in vivo*. 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. After this, it is connected to Zoë to receive continuous flow of cell culture media.



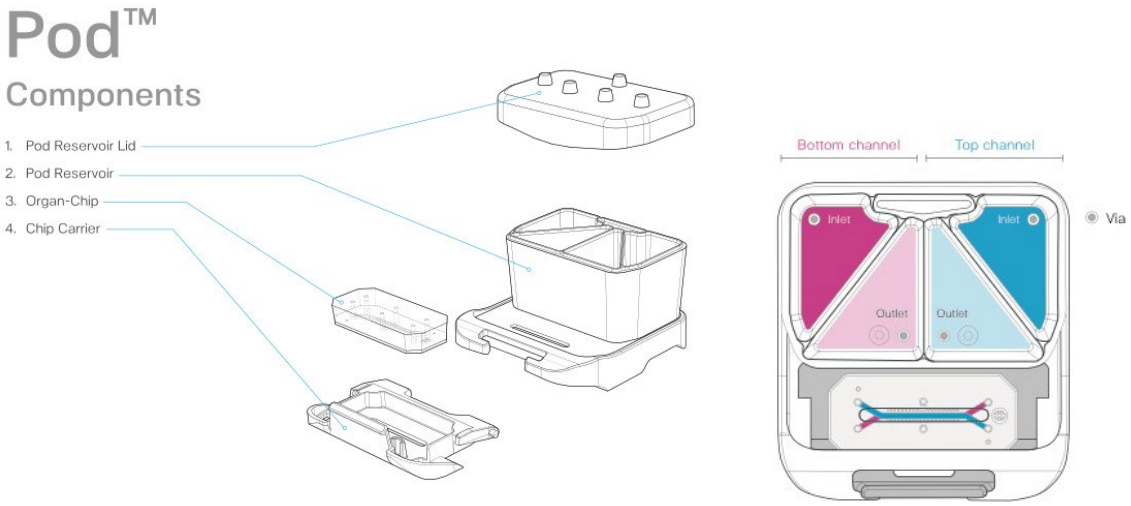
Chips are supplied in the chip carrier and should be maintained in the chip carrier at all times during the experiment. The chip carrier is designed to hold chips in place, eliminating the need to disturb or distort them during preparation. Removal of the chip from the chip carrier prior to or during 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.) 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.



Pod

Pod houses the chip, supplies media, and enables analysis with microscopes and other equipment. Each Pod has a compact height of 41.5 mm, enabling Organ-Chips to be easily transported and placed on standard microscopes for imaging as well as between the 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.

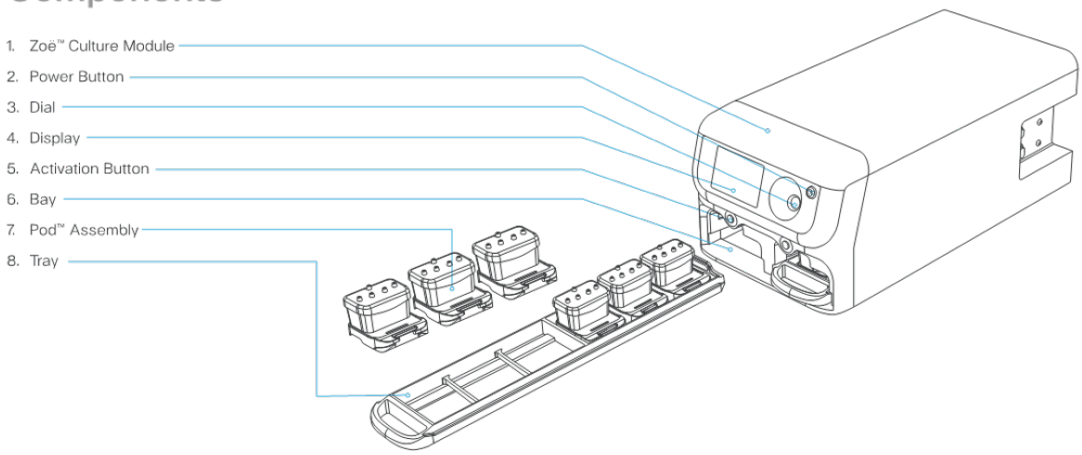


Zoë Culture Module

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

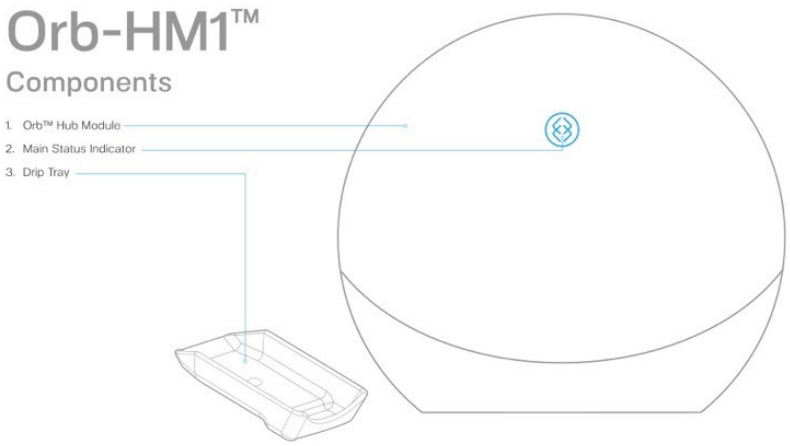
Zoë enables independent control of the media flow rate through the top and endothelial channels of the Organ-Chips. It also allows for independent adjustment of the chip's stretch parameters—that is, its frequency and amplitude. Additionally, Zoë has automated algorithms to prime the Pods' fluidic channels with media and programming to maintain the culture microenvironment for optimal cell performance.

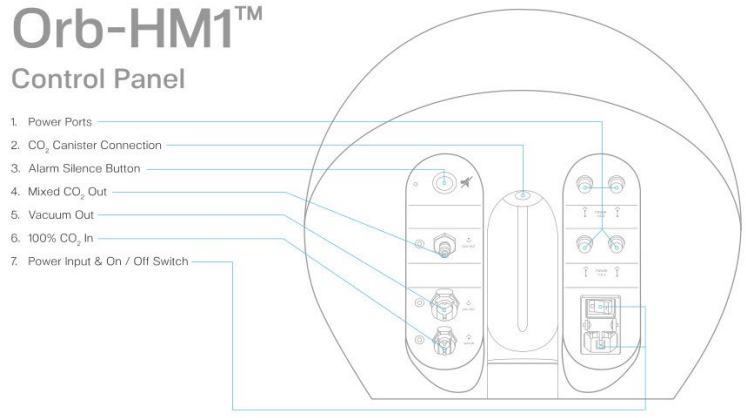
Components



Orb Hub Module

The Orb is the central hub that connects to a maximum of four Zoë Culture Modules. It allows for simple installation and operation of the Human Emulation System within a 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.

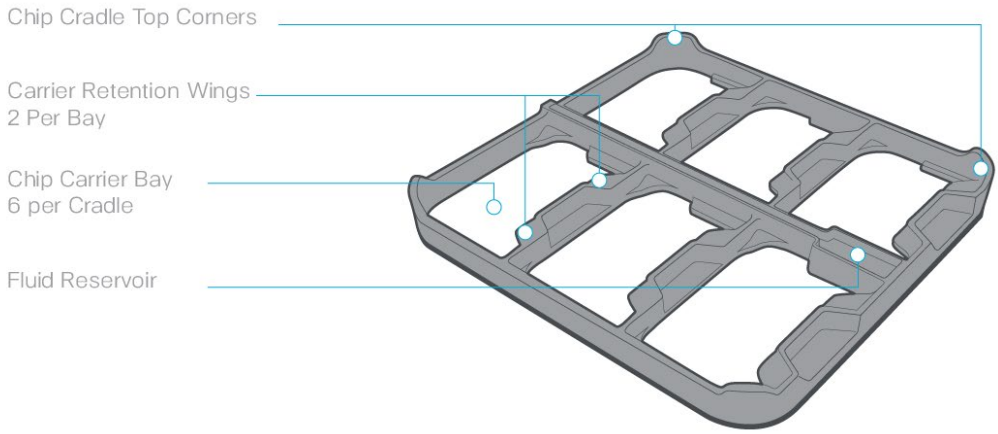




Chip Cradle

The Chip Cradle holds and organizes up to six chips. This accessory is made from autoclavable plastic and allows for inversion during seeding. It also contains slots with numbered labels to keep chips organized and a DPBS reservoir to keep them humidified.

Configuration



Cells

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

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 from the medium before it is introduced to the chip and Zoë. To do this, medium is warmed to 37°C and placed under vacuum using the 0.45 µm PVDF filter of a Steriflip® conical filter unit. Neglecting to carry out this step could lead to chip failure in the experiment.

It is also important to monitor 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 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 endothelial channel
- Wash chips

Organoids to Chips

- Prepare Organoid Growth Medium
- Prepare Dissociation Solution
- Recover organoids from Matrigel
- Adjust cell density
- Seed Duodenal organoids to the epithelial 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
- Replenish Medium, omit CHIR99021 and Y27632 from the supplements
- 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 each experiment. Below are exact catalog numbers for the specific materials needed in successful experiments.

Equipment & Materials

Equipment	Description	Supplier	Catalog Number
Intestine Bio-Kit: Duodenum	Co-Culture (12- or 24-pack)	Emulate	BIO-DH1-CO12
Zoë-CM2™ Culture Module	1 per 12 chips	Emulate	ZOE-CM2
Orb-HM1™ Hub Module	1 per 4 Zoës	Emulate	ORB-HM1
UV Light Box	1 per Zoë	Emulate	-
Chip Cradle	Autoclaved, 1 per 6 chips	Emulate	CHIP-CRD
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	-	-
24-well Clear TC-treated Multiple Well Plates	For organoid culture	- Costar	- 3526

Reagents, Media & Supplements

Reagent	Description	Supplier	Catalog Number
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 Alternative: 3KDa Dextran Texas Red	10 mg powder (optional for permeability assay)	Invitrogen	D7132 D3329

Notes for ER-1 and ER-2 (Supplied in the Bio-Kit)

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

Cell Storage

Cryopreserved cells must be stored in liquid nitrogen (gas phase). Chronic temperature fluctuations can cause severe damage to cell membranes and cytoskeletal components. Never store them in dry ice or in a freezer colder than -80°C.

Chip Handling Techniques

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

When pipetting to fill each channel, a volume of 35 µL is generally used for the epithelial channel, and 15 µL is used for the endothelial channel. This allows 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.

Epithelial Channel: 30-35 µL

Endothelial channel: 10-15 µL

The specific channel and membrane dimensions are outlined below:

Epithelial **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

Endothelial 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

Use a P20 or a P200 pipette with a sterile tip when adding solution directly to the channels of the chip as well as when coating, washing, and seeding cells prior to connecting the chip to Zoë. Before seeding the cell suspension into the chips, empty the channel into which the cells will be seeded. To introduce solution to the channels, place the pipette tip perpendicular to the chip channel inlet, ensuring that the tip is securely in the port, and steadily dispense liquid through the channel. Always introduce liquid to the endothelial channel before pipetting into the epithelial channel.

Duodenum Intestine-Chip Culture Protocol

Day -4: Reagent Preparation

Aliquot Reagents

Aliquot reagents, including media supplements and ECM, before use to avoid multiple freeze-thaw cycles of the stock solutions. Store the aliquots 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 to yield 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 to yield 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 the back of a 2-6°C refrigerator or cold room. When outside of the freezer, it must be handled on slushy 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 the experiment.
  - Keep 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 them

Note: Prepare 1.4 mL aliquots for organoids expansion and 100 µL aliquots 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. Incubate it at 4°C until the Collagen has dissolved.
- After 24 h, aliquot the substance into 300 µL volumes. Store the 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. Leave the mix to dissolve at room temperature for 30 min. Swirl gently before aliquoting. Avoid harsh agitation or vortexing.
- Store aliquots at -20°C.

6. 3KDa Dextran Cascade Blue (optional, recommended only for barrier assessment)

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

- In the 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 10 mg / mL concentration. The final concentration in medium is 10 ug / mL or 1:100 dilution. One vial of 10 mg of 3KDa Dextran Cascade Blue is sufficient for 100 mL
- Any remaining working solution can be frozen at -20°C.

Day -3: Thaw HIMEC

Goals:

- Expand HIMECs in flasks prior to seeding into chips, as per the established protocol below.

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 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

The HIMEC Culture Medium is prepared using the Endothelial Cell Growth Medium Kit (C-22121), which contains 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.
- Store complete media at 4°C for up to 30 days.
  Note: Primocin is a non-toxic antimicrobial agent for primary cells.

Prepare Flask

1. Warm 50 mL of HIMEC Culture Medium to 37°C.
2. Label the culture flask with the relevant information (e.g., cell type, passage number, date, initials).
3. Pipette the Attachment Factor onto the growth surface of the flask until it is fully covered (5 mL of Attachment Factor used per T-75 flask).
4. Ensure that the coating solution evenly covers the culture surface of the flask. Incubate the flask at room temperature for 5 minutes.
5. Aspirate excess coating solution from the surface after incubation.
6. Add 15 mL of HIMEC Culture Medium to the flask, and leave it in the incubator at 37°C until it is ready for plating.

Thaw and Plate HIMEC

After being thawed, HIMECs require one passage of expansion in culture before being seeded onto the chip.

1. Thaw the vial(s) of cells by immersing it in a water bath at 37°C for approximately 60 to 90 seconds. Closely observe the thawing process while gently agitating the vials. Just before the last ice pellet disappears, remove the vial from the water. These steps will help ensure cell viability.
2. Spray vial(s) with 70% ethanol, wipe them dry, and place them into the BSC.
3. Using a P1000 pipette, immediately transfer the contents of the vial into the prepared flask containing warm HIMEC Culture Medium.

4. Rinse the vial with 1 mL of HIMEC Culture Medium and add the contents of the vial to the flask.
5. Incubate the flask at 37°C for 6 hours.
6. Aspirate the medium, and carefully add 15 mL of fresh HIMEC Culture Medium.
7. Return the flask to the incubator set to 37°C and 5% CO_2. Leave it overnight under these conditions.
8. Exchange the medium in the flask with fresh HIMEC Culture Medium every other day until it is needed for chip seeding.
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. Replenish the media the next day.
10. Exchange the medium in the flask with fresh HIMEC Culture Medium the following day, and then every other day, until it is needed for chip seeding.

Day -1: Chip Preparation

Goals:

- Activate the inner surface of the chip channels so the ECM properly attaches
- Coat epithelial channels with an ECM mixture of collagen IV and Matrigel
- Coat endothelial 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 the chip packaging, the 120-mm square cell culture dish, and the Chip Cradle with 70% ethanol, and bring them 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. Carefully open the chip packaging, place the first chip into the cradle by sliding the back of the carrier under the tabs on the cradle (See Figure 1).

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

4. Repeat for each chip included in the experiment.
5. Label the chip carrier tap with ID numbers for each chip.

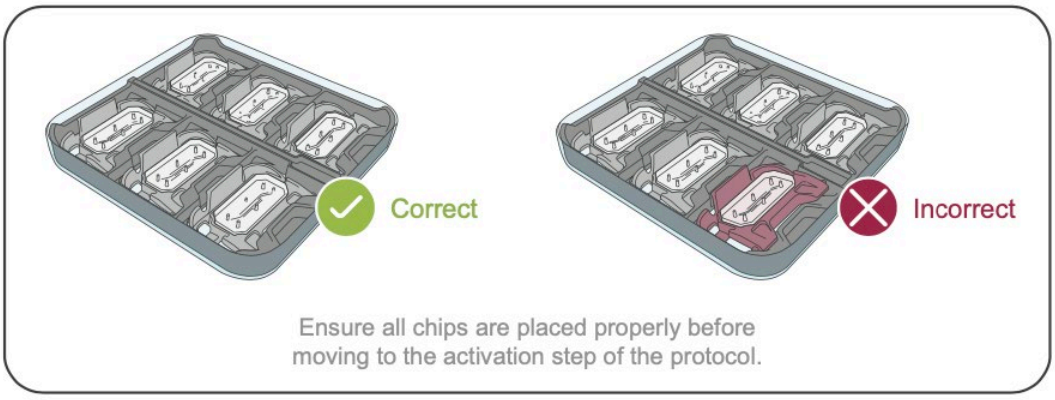


Figure 2. Proper chip placement within the chip carrier

Prepare ER-1 Solution

ER-1 is light sensitive. Failure to protect ER-1 from light, or using ER-1 solution that has not been freshly prepared, will lead to chip failure. Prepare the ER-1 solution immediately before use, and discard any remaining ER-1 solution 1 hour after reconstitution.

Note: ER-1 is an eye irritant and must be handled in the BSC with proper gloves and eye protection.

1. Turn off the BSC light, and allow both ER-1 and ER-2 to equilibrate to room temperature before use. This will take 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. Briefly tap the vial to concentrate the powder at the bottom.
4. Add 1 mL of ER-2 to the vial. Transfer the vial's contents directly to the bottom of the 15 mL conical tube. Do not pipette to mix.
   Note: 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, adding 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 in 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 the contents of conical tube without creating bubbles. ER-1 should be fully dissolved in the ER-2 solution prior to use.

Introduce ER-1 Solution to Channels

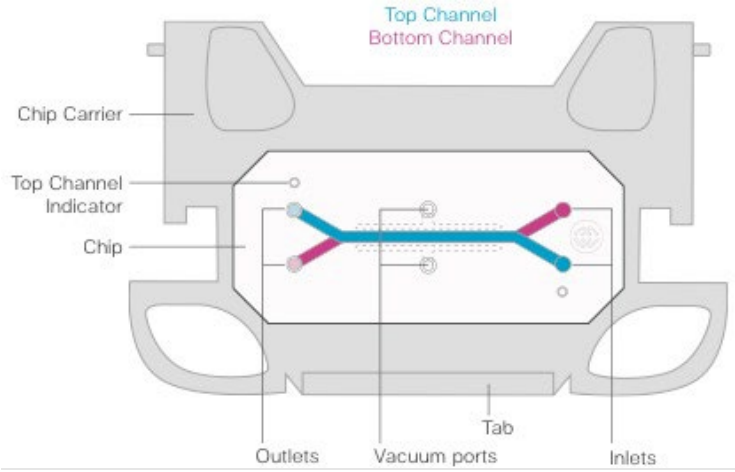


Figure 3. Top view of chip, with labelled ports, in the chip carrier

1. Using a P200 pipette and a sterile 200-µL filtered pipette tip, collect 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 endothelial channel inlet, pipetting until the solution begins to exit the endothelial channel outlet.
3. Without releasing the pipetting plunger, take the pipette out from the endothelial channel inlet. Then, move the pipette containing the remaining ER-1 solution to the epithelial channel inlet.
4. Introduce approximately 50 µL of ER-1 solution to the epithelial channel inlet, pipetting until the solution begins to exit the epithelial channel outlet.
5. Gently aspirate excess ER-1 solution from the surface of the chip. Remove ER-1 solution from the chip surface only—do not aspirate ER-1 from the channels (See Figure 4).
6. Repeat Steps 1 through 5 for each chip.
7. Inspect the channels for bubbles prior to UV activation. If bubbles are present, wash the channel with ER-1 solution until they have all been removed. If bubbles persist, it may be helpful to aspirate the channel dry and slowly re-introduce ER-1 solution.

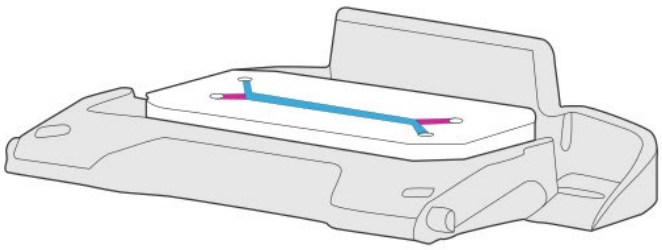


Figure 4. View of chip with no excess ER-1 solution on surface

Activate Chips

1. Bring the 120-mm square dish containing the ER-1-coated chips to the UV light box.
2. Remove the cover from the 120-mm square dish before placing it into the UV light box.
   Note: If the lid is not removed prior to placing the dish in the UV light box, the chips will not activate properly. This could result in improper cell attachment.
3. Set the switch at the back of the UV light box to “Constant.” To begin UV activation, turn the “Power” switch to “On”, and then press the “On” button.
4. Allow the chips to activate under UV light for 15 minutes.
5. While the chips are being treated, prepare the ECM Solution. (For more information, see the section “Prepare ECM Solution.”)
6. After UV treatment, bring the chips back to the BSC.
   Note: The BSC light may be left on 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 each channel twice with 200 µL of sterile DPBS.
11. Leave DPBS inside the channels.

Prepare ECM Solutions

The ECM solution is freshly prepared for each use 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 endothelial channels.

For the human Duodenum Intestine-Chips, the final protein concentration in the ECM working solution is:

Epithelial channel: Collagen IV: 200 µg / mL

Matrigel: 100 µg / mL

Endothelial channel: Collagen IV: 200 µg / mL

Fibronectin: 30 µg / mL

1. Bring ice and a bucket into the BSC.
2. Thaw one aliquot of fibronectin (1 mg / mL), collagen IV (1 mg / mL), and Matrigel (concentration is dependent on specific lot used, see Corning website) on ice.
   Note: Maintain ECM components and the mixture on ice at all times.
3. Calculate the total ECM solution volume needed to coat all of the chips.
   1. Volume required per channel = approximately 50 µL.
   2. For every 12 chips that will be coated, prepare 1.5 mL of each ECM solution (12 chips x 100 µL / chip + extra 300 µL = 1.5 mL of ECM solution). See the calculation example below for more detail.

4. Combine the components to prepare ECM working solutions.
5. Keep the ECM solutions on ice until they are needed.

Calculation Example – Epithelial 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

$C_1V_1 = C_2V_2$ 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

$C_1V_1 = C_2V_2$ 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

= 1185 µL of DPBS

Calculation Example – Endothelial 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

$C_1V_1 = C_2V_2$ 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

$C_1V_1 = C_2V_2$ 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

= 1155 µL of DPBS

Coat Chips with ECM

1. Fully aspirate the cold DPBS from both channels.
2. With a P200 pipette, collect 100 µL of the ECM solution for the endothelial channel.
   Note: 50 µL of ECM solution will be used per channel on each chip.
3. Carefully introduce the solution through the endothelial channel inlet until a small ECM droplet forms on the endothelial channel outlet.
4. Introduce the epithelial ECM solution through the epithelial channel inlet, leaving small droplets of excess ECM solution on both ports for both channels (See Figure 5).

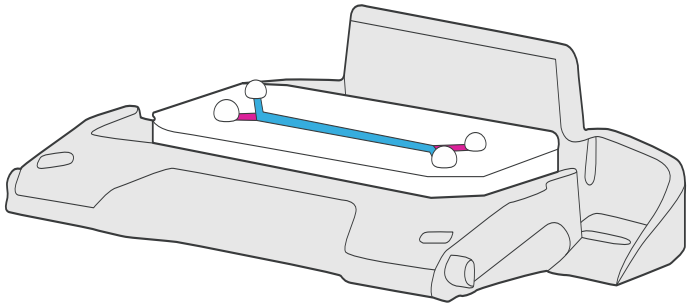


Figure 5. Chip in the chip carrier with small droplets of ECM solution at the ports

5. Inspect both channels to ensure that no bubbles remain. If a channel does contain bubbles, wash it 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 the central reservoir with 0.75 mL of DPBS (See Figure 6), place the lid onto the 120-mm square dish, and incubate it overnight at 37°C and 5% CO2.
8. Aspirate the BBS to avoid spillage.

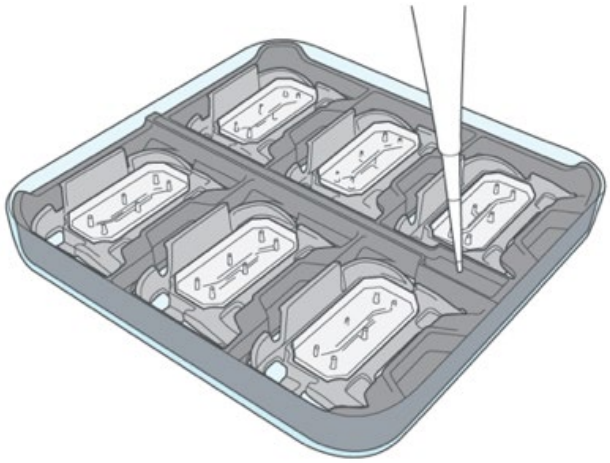


Figure 6. Fill the central reservoir of the Chip Cradle with 0.75 mL of DPBS

Day 0: Cells to Chips

Goals:

- Seed HIMECs in the endothelial channel
- Seed organoids in epithelial channel

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 endothelial channel
- Wash chips

Organoids to Chips

- Prepare Organoid Growth Medium
- Prepare Dissociation Solution
- Recover organoids from Matrigel
- Adjust cell density
- Seed Duodenal organoids in the epithelial 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 Culture Module
- 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 the ECM-coated chips from the incubator to the BSC.
2. Flush the ECM from both channels by gently washing the endothelial channel of the chip with 200 µL of complete HIMEC medium and the epithelial channel of the chip with 200 µL of Organoid Growth Medium.
3. Aspirate the medium outflow on the surface of the chips, leaving media in both 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 containing the chips and place it into the incubator. Leave it until the cells are ready for seeding.

Prepare Cell Counting Solution

Trypan Blue Cell Counting Solution

Reagent	Volume	Source	Cat. No.
Cell 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

Once HIMECs have expanded in culture, they can be harvested, counted, and seeded into the endothelial channel. Prior to seeding, HIMECs are typically adjusted to a density of range of 8 x 10^6 cells / mL to achieve a complete monolayer.

1. Bring the culture flasks containing HIMECs from the incubator into the BSC.
2. Aspirate the culture media and add 15 mL of DPBS to wash the culture surface. Aspirate the DPBS from the surface.
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 whether the cells have completely detached from the culture surface.
5. Add 3 mL of warm HIMEC Culture Medium to the flasks. Pipette the solution in the flasks gently to mix while collecting all the cells from the culture surface.
6. Transfer the contents of the flasks into a sterile 15-mL conical tube.
7. Transfer 20 µL of the cell suspension into a 1.5-mL tube containing 20 µL of trypan blue cell counting solution.
8. Count the cells using a manual hemocytometer (See Figure 7).
9. Transfer the cell suspension from the 15 mL tube to the centrifuge. Set and run it at 150 x g for 5 minutes.
10. Aspirate the supernatant carefully to avoid disturbing the small cell pellet while leaving approximately 100 µL of medium above it.
11. Gently flick the tube to loosen the cell pellets.
12. Using a P1000 pipette, gently resuspend the cells by adding 200 µL of complete HIMEC culture medium.

Cell Counting and Viability Assessment

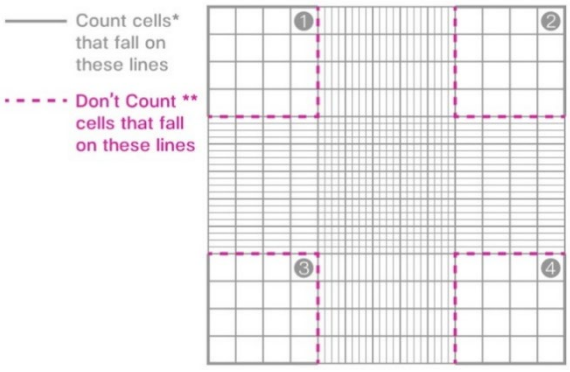


Figure 7. Hemocytometer

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

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

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

Seed HIMECs to Endothelial Channel

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

1. Bring the square dish containing the prepared chips to the BSC.
2. While carefully avoiding contact with the ports, aspirate excess medium droplets from the surface of one chip.
3. Gently agitate the cell suspension to ensure it is homogenous before seeding each chip.
4. Pipette the medium from the endothelial channel until it is completely empty.
5. Seed 10 to 15 µL of the HIMEC suspension into the endothelial channel of one chip.
6. Cover the dish, transfer it to the microscope, and check the seeding density within the chip.
7. If seeding density is not optimized (i.e. close to 8 M / mL) return the chip to the BSC and wash the channel with 200 µL of fresh medium twice (See Figure 8). 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 successful chip cultures.
8. After confirming the correct cell density, seed the 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 in a square cell culture dish (See Figure 9).
9. Place DPBS at the cradle to provide humidity for the cells, and replace the dish lid.
   Note: Minimize the amount of time the cells are outside the incubator by seeding no more than 12 chips at a time and by immediately placing the chips in the incubator at 37°C after seeding each batch of 12.
10. Place the dish with the chips into the 37°C incubator for approximately 30 minutes to 1 hour, or until cells in the endothelial channel have attached.
11. Once HIMECs have attached (approximately 1 hour after seeding), orient the chips in an upright position.

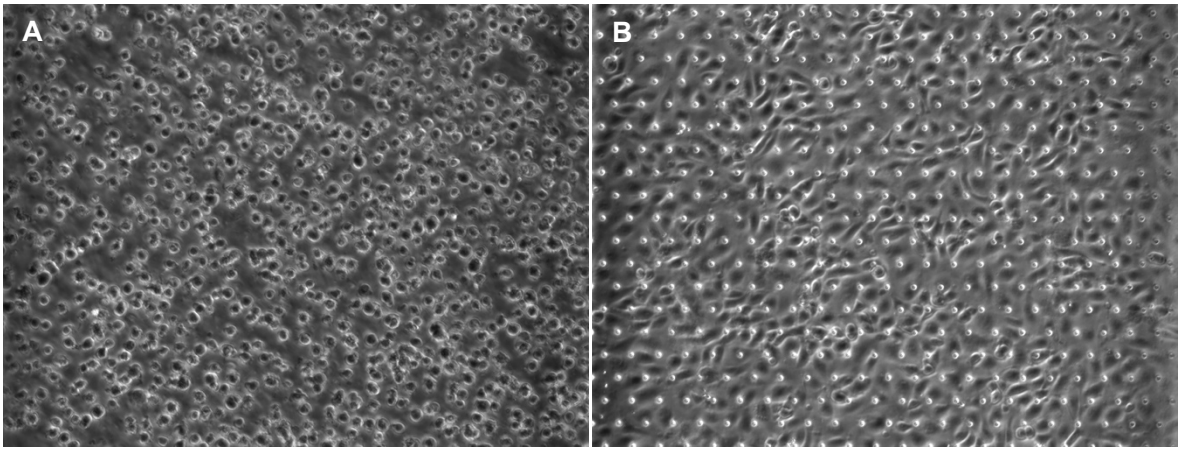


Figure 8.
A. HIMEC optimum cell seeding density
B. HIMEC attached on the chip 2 hours post-seeding

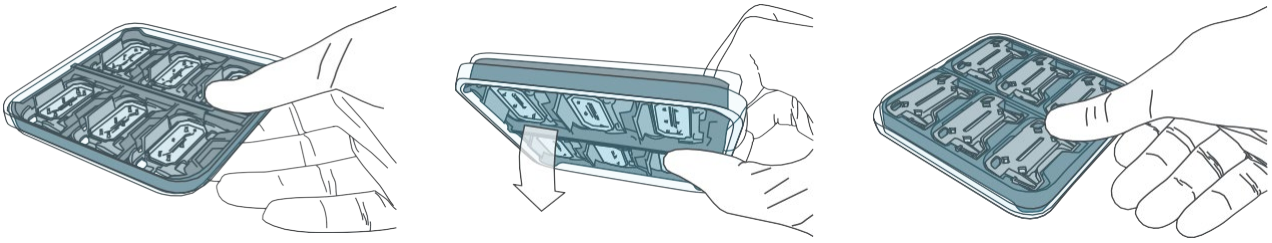


Figure 9. Inverting chips during endothelial attachment

Wash Chips

To ensure that nutrients are replenished and that the channels will not dry out, the chip must be washed after the HIMECs have attached (approximately 30 minutes to 1 hour after seeding). During the wash, ensure that media can flow through the channels and exit from the outlets.

1. With a P200 pipette, gently wash the endothelial channel of each chip with warm, equilibrated HIMEC Culture Medium to remove any possible bubbles.
2. Repeat Step 1 using complete Organoid Growth Medium for the epithelial channel.

Organoids to Chips

Prepare 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 only added to the medium until the day after chips are seeded (when they are connected to flow). The following day, when replenishing the medium, they should be removed.

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 fresh Dissociation Solution 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 for the dissociating 50 µL of organoids/Matrigel in 24-well plates.

Recover Organoids from Matrigel

Prior to recovering the organoids from the Matrigel, organize all equipment and materials so they are ready for use. Additionally, check that all required reagents are prepared and maintained at the appropriate temperature. Finally, transfer the 24-well plate containing organoids into the BSC.

1. Carefully aspirate the 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 the contents of each well, and transfer them to a cold, 15 mL conical tube.
   Note: It is highly recommended to use Low Protein Binding tubes (Lo Bind Eppendorf) to prevent organoids from sticking to the tube and to minimize organoid loss.
5. Incubate the conical tube on ice for 45 minutes, inverting it every 2-to-5 minutes throughout.
6. While the 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 that the Matrigel has completely disappeared and that there is clear cell pellet formation.
   Note: If a thin layer of Matrigel is present, use a P1000 pipette to carefully remove the supernatant without disrupting the pellet. Then, add 5 mL of new Cell Recovery Solution. Incubate for 5 minutes on ice, then repeat the centrifugation in step 8. If a defined cell pellet is not observed, repeat step 8.
9. Once a defined cell pellet is observed, aspirate the supernatant, gently flick the tube to disrupt the pellet, and then add 2 mL of prepared Dissociation Solution 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, they should **not** be incubated for longer than 2 minutes, as this could cause the organoids to dissociate into single cells, in turn leading to decreased seeding efficiency.
11. Dilute with at least 2X 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 the seeding density by suspending the pellet in the Organoid Growth Medium.

Adjust Cell Density

The seeding density depends on the size and density of organoids cultured on the 24-well plate. It is recommended to use 2 to 3 wells of organoids per chip (See Figure 10).

To calculate the volume for the suspension of the 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 until the entire mixture has reached the desired final cell density (See Figure 10).

Seed Duodenal Organoids to the Epithelial Channel

Work with one chip at a time. After seeding the first chip, use the microscope to assess the cell density within the epithelial channel. If necessary, adjust the density of the cell suspension for the subsequent chips.

1. Bring the square dish containing the prepared and washed ECM-coated chips to the BSC.
2. Using the pipette, empty the medium from the epithelial channel.
3. Carefully 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 epithelial channel inlet. Avoid directly touching the aspirator tip to the outlet port.
5. Cover the dish, transfer it to the microscope, and check the seeding density within the chip.
   Note: At the optimal seeding density, the organoid fragments will form an even cell layer on the epithelial channel of the chip that covers the whole chip membrane (See Figure 10).
6. If the seeding density is not optimal, return the chips to the BSC, and wash the epithelial channel with 200 µL of fresh medium twice. Do not aspirate the medium from the channel. Adjust the cell density accordingly, and repeat steps 3 through 5 until the correct density is achieved.
14. After confirming the cell density, transfer ~ 300 µL aliquots of the cell suspension to 1.5 mL Low Protein Binding tubes to avoid the formation of a density gradient.
15. Seed cells in the remaining chips.
    Note: Minimize the amount of time the cells are outside the incubator by seeding batches with no more than 12 chips and by immediately placing the chips into the incubator at 37°C after seeding each batch.
16. Fill the central reservoir of the Chip Cradle with 0.75 mL of DPBS.
17. Leave the dish of chips in the incubator set to 37°C overnight, undisturbed.

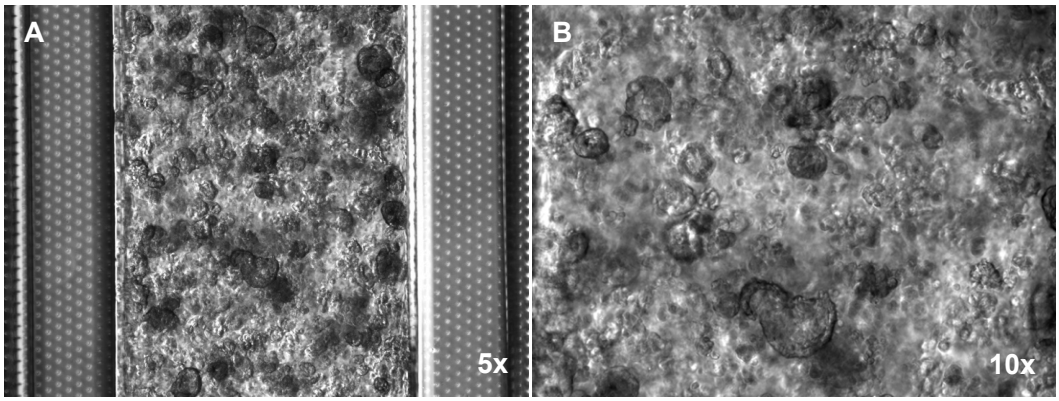


Figure 10. Optimal single and fragments organoids density
A. Low magnification
B. Higher Magnification
Note that the porous membrane is completely covered by the cells

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 for successful Organ-Chip culture. Omitting this step will lead to bubble formation in the chips, Pods, or both, which will cause irregular flow and suboptimal cell viability. Ensure media is outside of a warmed environment, such as an incubator or bath, for no more than 10 minutes to prevent the gas equilibrium from becoming compromised.

1. For every batch of 12 chips, prepare 50 mL of HIMEC Culture Medium in separate 50-mL conical tubes.
2. For every batch of 12 chips, prepare 50 mL of Organoid Growth Medium in separate 50-mL conical tubes with the addition of 10 µM Y-27632, 5µM CHIR99021, and a tracer of your choice to check the permeability. The most common tracer used is the 3KDa Dextran Cascade Blue at a final concentration in medium of 100 ug / mL. For temporal assessment of the barrier function, the tracer can be kept in the culture medium for the course of experiment. (For further instructions, see the section “Permeability Assay.”)
   Note: Use approximately 3 mL of medium for each chip reservoir. For every batch of 12 chips, prepare around 40 mL of medium for the epithelial channels and another 40 mL for the endothelial channels.
3. Warm the conical tubes with media to 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 reoriented such that the medium is 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 a minimum of -70 kPa. At this pressure, it should take about 2 seconds for every 10 mL of medium to flow through the filter. If it takes longer, stop the vacuum, and see the step labeled “Media take too long to pass through Steriflip” in the troubleshooting section.
   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.

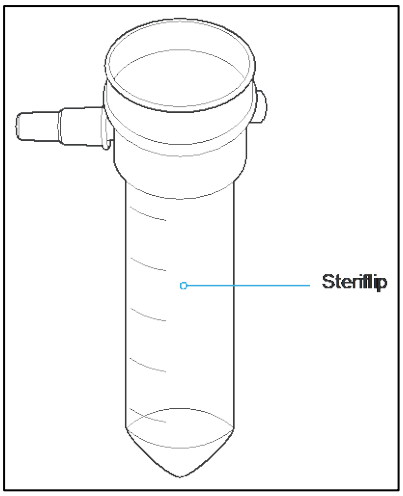


Figure 11. Steriflip Diagram

Prime Pods

1. Sanitize the exterior of the Pod packaging and trays with 70% ethanol, wipe it, and transfer it to the BSC.
2. Open the Pod package, and place the Pods into the trays. Orient the Pods so the reservoirs face the back of the tray.
3. For each Pod, place 3 mL of pre-equilibrated, warm Organoid Growth Medium in the top inlet reservoir and the same amount of HIMEC Culture Medium in the bottom inlet reservoir.
4. For each pod, pipette 300 µL of pre-equilibrated, warm Organoid Growth Medium to the top outlet reservoir and the same amount of HIMEC Culture Medium to the bottom outlet reservoir, ensuring the media is added directly over each outlet via.
5. Bring the trays containing Pods to the incubator, and slide them completely into Zoë so the tray handle faces outward.
6. Run the Prime Cycle on Zoë.
   a. Use the Dial to highlight “Prime” on the display.
   b. Press the Dial Button to select “Prime.”
   c. Rotate the Dial clockwise to highlight “Start.”
   d. Press the Dial Button again to select “Start” and begin the Prime Cycle.
      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 takes approximately 1 minute). When the status bar reads “Ready,” the Prime Cycle is complete.
8. Transfer the tray from Zoë to the BSC.
9. Verify that the Pods were successfully primed.
   a. Inspect the underside of each Pod, looking for the presence of small droplets at all four fluidic ports.
      Note: Droplets will vary in size from a small meniscus to larger droplets. Often, droplets on the outlet ports will be larger.
   b. Re-run the Prime Cycle on Pods that do not display droplets.
   c. If any media dripped onto the tray, clean it with a wipe sprayed with 70% ethanol.
      Note: Media often drips onto the tray from the outlet ports.
10. Once it is confirmed that all Pod ports are wet with droplets, place the tray of Pods to the side in the BSC.

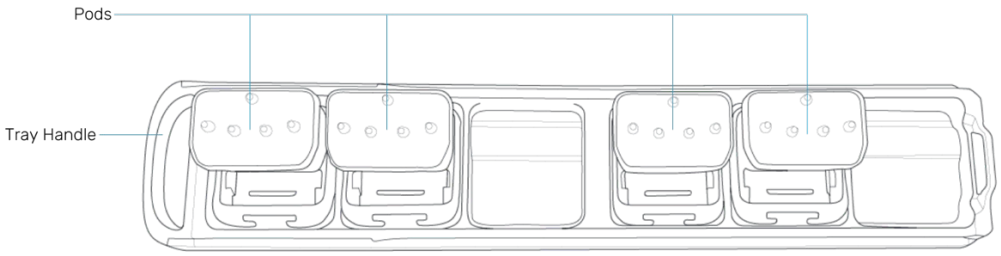


Figure 12. Pods in tray

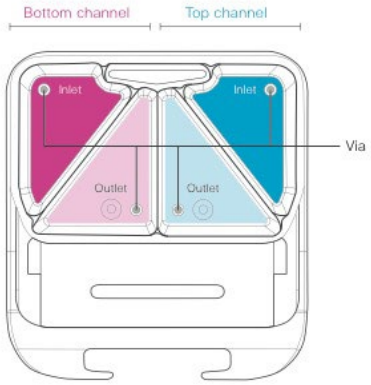


Figure 13. Top of Pod

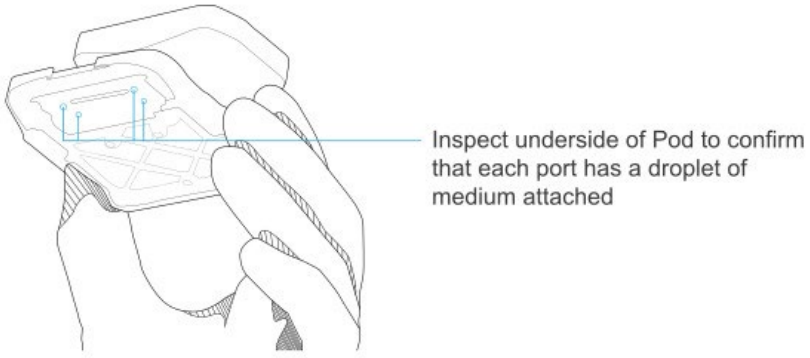


Figure 14. Bottom of Pod

Wash Chips

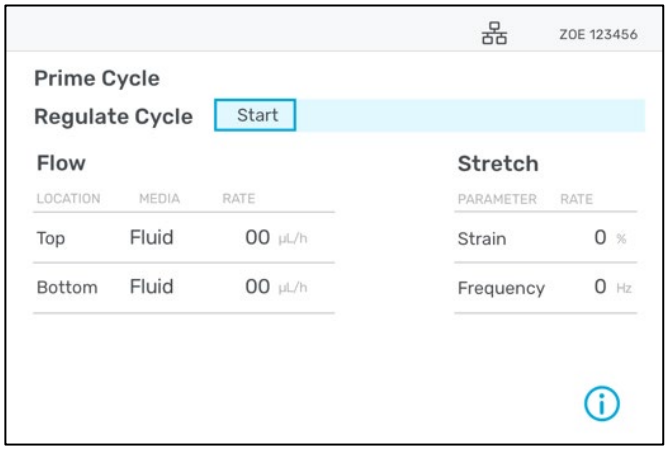
1. Transfer the seeded chips in a 120-mm square dish from the incubator to the BSC.
2. Wash the epithelial channel of each chip twice with 200 µL of warm, equilibrated Organoid Growth Medium, aspirating the outflow from the chip surface.
3. Wash the endothelial channel of each chip with warm, equilibrated HIMEC Culture Medium, aspirating the outflow from the chip surface.
4. Place small droplets of equilibrated Organoid Growth Medium on each of the epithelial channel's inlet and outlet ports. Similarly, place small droplets of HIMEC Culture Medium on the inlet and outlet ports of the endothelial channel.
5. Repeat steps 2 through 4 for each chip.
6. Remove the Chip Cradle from the dish, wipe with 70% ethanol to clean, and autoclave for use in next experiment.

Chips to Pods

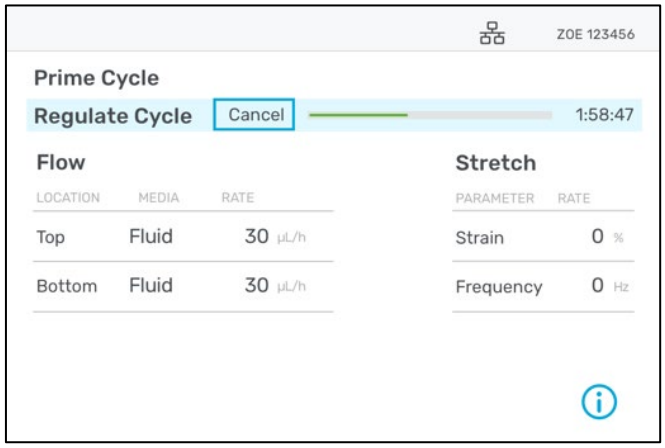
1. Use the dominant hand to hold one chip, still in the chip carrier, and use the non-dominant hand to firmly hold one Pod. Slide the chip carrier into the tracks on the underside of the Pod until the chip carrier has fully seated.
2. Place a thumb on the chip carrier tab. Gently, but firmly, press the tab in and up to secure it with the Pod.
3. Aspirate any excess medium on the chip surface from the Pod window.
4. Place the Pod with the connected 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 reservoir and that the Pod lids are flat and secure.

Pods to Zoë and Regulate

1. Immediately place the trays holding Pods and chips into Zoë to prevent the media from cooling and losing its gas equilibration.
2. On Zoë, program the appropriate Organ-Chip culture conditions. These will activate once the Regulate Cycle is complete.
   a. For Duodenum Intestine-Chips, set each channel's flow rate to 30 µL / h.
3. Run Regulate Cycle.
   a. Using the Dial, highlight the "Regulate" field. Then, press the Dial Button to select "Regulate."
   b. Rotate the Dial clockwise to highlight the "Start" field.



   c. Press the Dial Button 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 “Activation” button will glow blue to signify that the Regulate Cycle has started. The Regulate Cycle lasts for 2 hours, after which Zoë will initiate flow at the preset Organ-Chip culture conditions. To cancel the Regulate Cycle (which should only be done if necessary), use the Dial to highlight the “Regulate” field, and press the Dial Button to select it again. Next, rotate the Dial counterclockwise to highlight “Cancel.” Finally, press the Dial once more. Wait one minute for the cycle to end, after which the tray can be removed. If the Regulate Cycle is cancelled, always rerun a complete 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 omitting CHIR99021 and Y27632
- 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— the beginning of the Organ-Chip culture process—pause Zoë by pressing “Activation” button located above the tray bays. This will stop flow and release the Pods.
2. Slide the tray out of the bay, and transfer it to the BSC.
3. Remove the Pod lids. Using a 200-µL pipette, perform the following via wash step on each of the four Pod reservoirs:
   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.

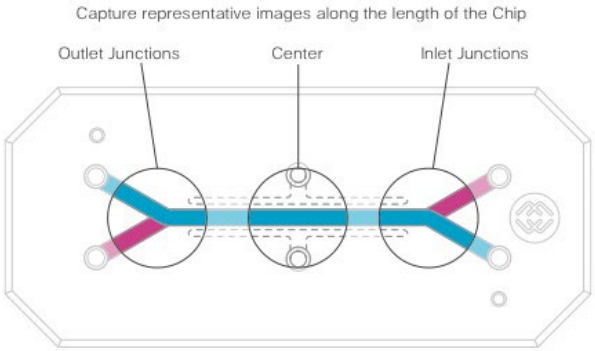
Replenish Medium omitting CHIR99021 and Y27632

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.
3. Replace Pod lids
4. 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

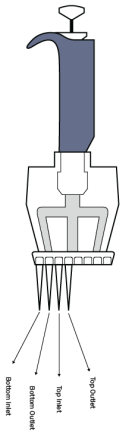
   Note: On the second day of culturing, if a confluent monolayer is not observed, the apparent permeability obtained will be higher than $1x10^{-6}$ cm/s. The optimum permeability will be obtained upon development of a confluent monolayer (day 4 or 5).

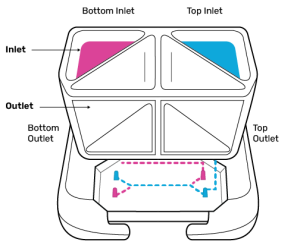


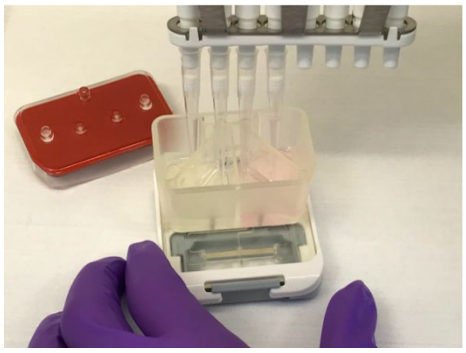
Effluent Sampling

1. Using a standard multichannel pipette adjusted to a volume of 50 µL, collect effluent and media from all the four reservoirs simultaneously by placing one tips into each of the Pod's reservoirs (See in Figure 15).
2. Dispense the collected effluents into an appropriately labelled 96-well black walled plate. Change tips between Pods to avoid cross contamination.
3. Cover the Pods. Move to the next Pod while collecting medium from all your chips.

A







B

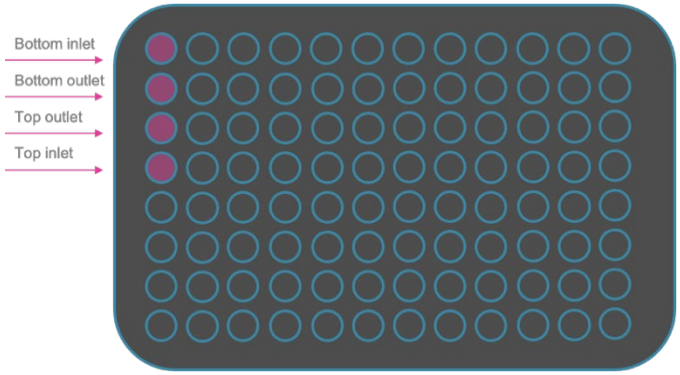
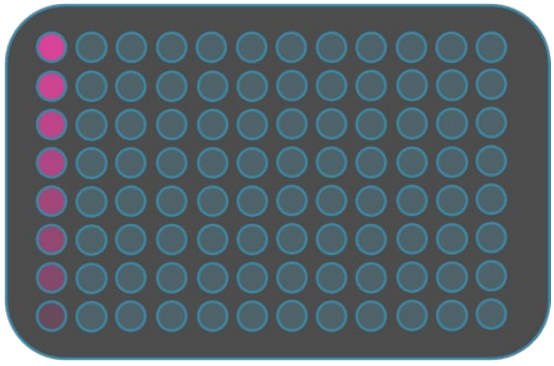


Figure 15. Effluent Sampling
A. Multichannel collecting effluent from the Pod

B. Medium dispensed in the 96 wells black walled plate.

4. Add 100 µL of PBS to the effluent collected on the plate for a final dilution of 1:3.
5. To prepare the standard curve, label two 1.5-mL tubes—one as “Diluent” and one as “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).
6. From the tube labeled diluent, dispense 150 µL of the diluted bottom reservoir medium to wells 1-7, and dispense the diluted medium from “Neat” tube into well 8 on the plate.
7. Perform serial dilution, collecting 150 µL from well 8 to 2 and leaving only the diluent medium on the last well (1). Well 1 will be used as your blank.
8. Maintain the foil-wrapped plate at room temperature to read later, or freeze the plate 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).
9. Perform an assay daily to monitor barrier function until 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. To remove cell debris, use the Dial to highlight the “Flow rate” field and set it to 1000 mL / h.
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 / h.
11. Press the silver “Activation” button to resume pre-set Organ-Chip culture conditions. Zoë will engage when the “Activation” button glows blue.
    Note: The flush cycle is performed on days 4, 6, and 8 for cellular debris removal. It is also used for priming during experimental treatment (e.g., introducing compounds, cytokine challenges.)

Day 3: Initiating Stretch

Goals:

- Set stretch to 2%, 0.2 Hz

Key Steps:

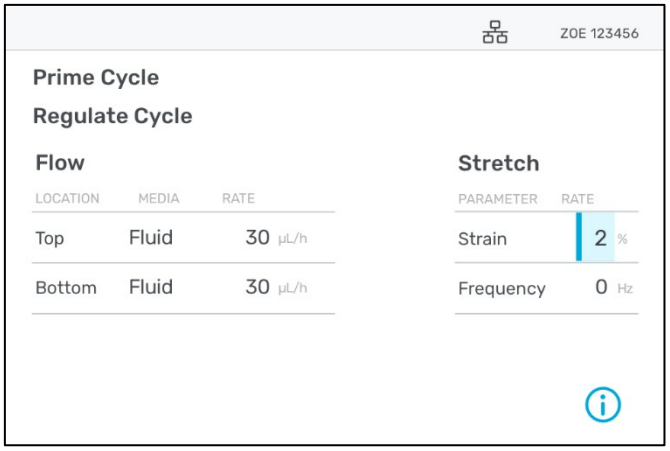
- Stretch introduction
- Effluent sampling

Required Materials:

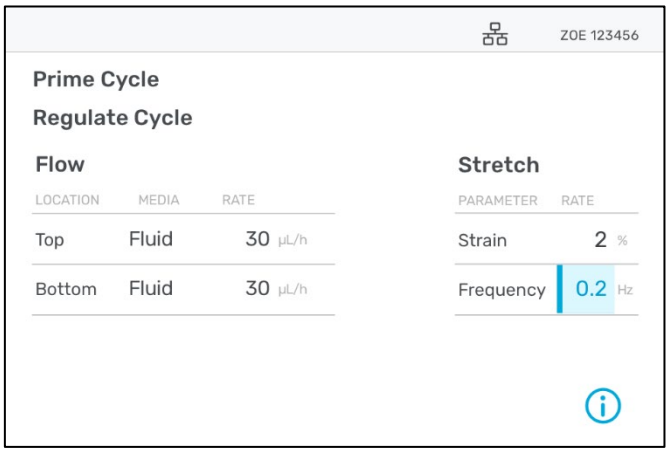
- Serological pipettes
- Pipettes, Multichannel and filtered tips
- 96 wells black walled plates

Stretch Introduction

1. Pause Zoë by pressing the silver “Activation” button.
2. Using the Dial, highlight the “Strain” field under “Stretch”.
3. Press the Dial Button to select “Strain,” and rotate the Dial clockwise to increase stretch to “2%”.



4. Press the Dial Button to select “Freq.” and rotate the Dial clockwise to increase stretch to “0.2 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 the 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%, 0.2 Hz

Key Steps:

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

Required Materials:

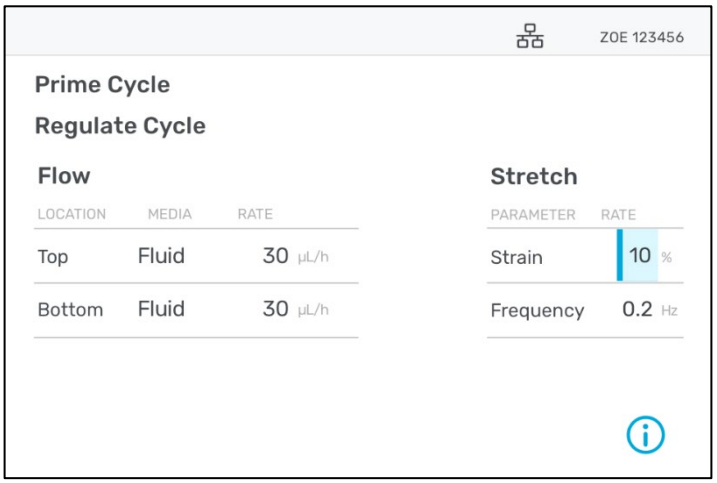
- HIMEC Culture Medium
- Organoid Growth Medium
- Serological pipettes
- Pipettes, multichannel and filtered tips
- 37°C water or bead bath
- 70% ethanol
- 3KDa Dextran Cascade Blue
- 96 wells black walled plate
- Microscope

Maintenance and Sampling of Chips

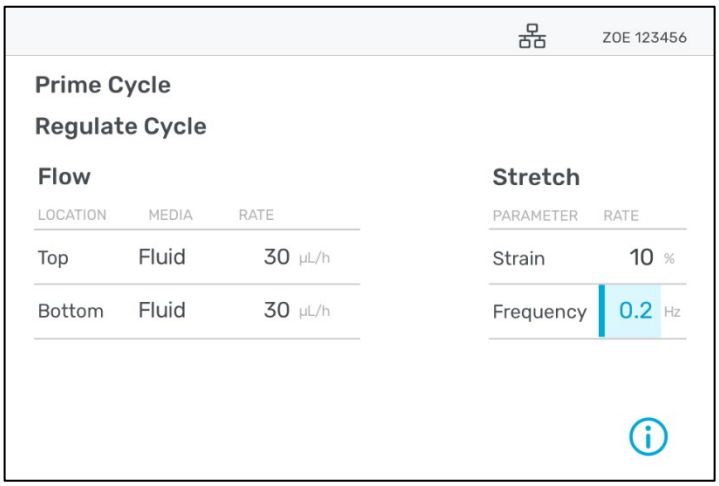
1. Pause Zoë by pressing the silver “Activation” button.
2. Remove the trays, and place them into the BSC.
3. Inspect each chip for bubbles by eye.
4. Using a microscope, inspect cells in the chips to assess morphology and viability. Capture representative images at 10X or 20X magnification at the following locations:
   - Inlet Junction
   - Center of channel
   - Outlet junction
5. Remove the Pod lids, and collect effluent medium from Pod outlet reservoirs for analysis.
   - Collect effluent from the indicated regions, avoiding disruption of the Pod reservoir vias.
   - Check on the links for effluent sampling and barrier function protocols for more information, or here to download the permeability or standard curve calculator.
6. Gently aspirate any medium that was not collected for analysis, ensuring that a thin liquid film still covers the reservoir vias to prevent air from entering.
7. Refill the Pod media reservoirs with the appropriate fresh cell culture medium. To remove any bubbles that may be present, perform a via wash by pipetting 200 µL of medium directly over the top of the Via.
8. Replace the Pod lids, and return the trays to Zoë.
9. Press the silver “Activation” button to resume the 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 Dial, highlight the “Strain” field under “Stretch”.
3. Press the Dial Button to select “Stretch,” and rotate the Dial clockwise to increase “Strain” to “10.0%”.



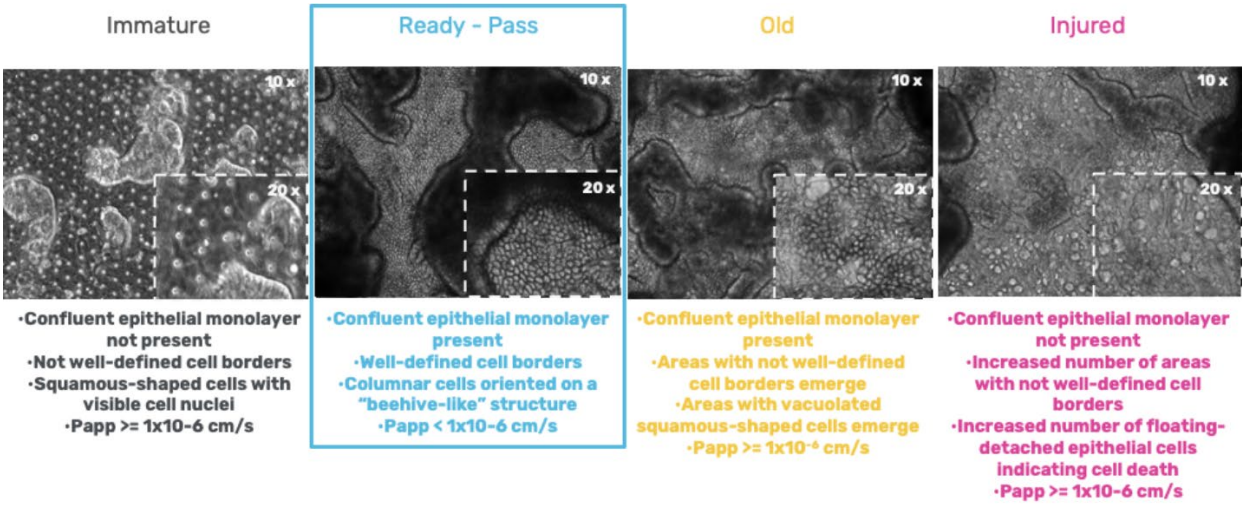
4. Use the Dial to select “Freq.” and ensure the frequency is set to 0.2 Hz.



5. Press the “Activation” button.

Acceptance Criteria

Morphology Acceptance Criteria



Barrier Function Acceptance Criteria

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

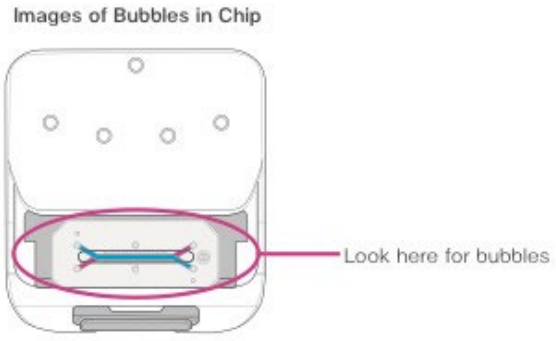
Troubleshooting

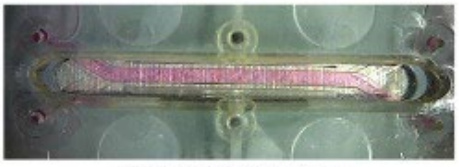
Issue	Section	Step	Recommendation
Bubbles are present in channel	Chip Preparation	Introduce ER-1 Solution to Channels; Activate Chips; Coat Chips with ECM	Dislodge bubbles by washing the channel with the appropriate solution until they have all been removed. If bubbles persist, it may be helpful to aspirate the channel dry and then 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 the pipette tip, or aspirate the channels and then 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. Find an alternate vacuum source.
Pods do not prime	Chips to Pods and Pods to Zoë	Prime Pods	Ensure that the medium is covering all Pod vias. If problem persists, record the Pod's lot number, and replace it with new one.
Screen is frozen or unresponsive	Chips to Pods and Pods to Zoë; Maintaining and Sampling	Pod to Zoë; Maintenance and Regulate	Power Zoë off and back on; if problem persists, contact our support team.
Pods stuck in Zoë	Maintaining and Sampling	Maintenance and Regulate	Pod lid is not attached securely. Try wiggling the tray to the left and right 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 the chip from the Pod; flush the chip with media; re-prime the Pod with degassed media; connect the chip to the Pod; and run the Regulate Cycle.

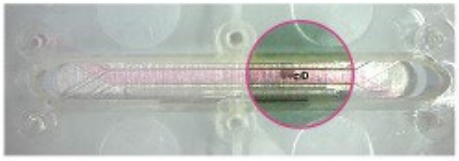
Potential Root Causes of Bubbles

If you've followed the mitigation steps above and bubbles remain and/or are observed to cause a high failure rate, check for the following:

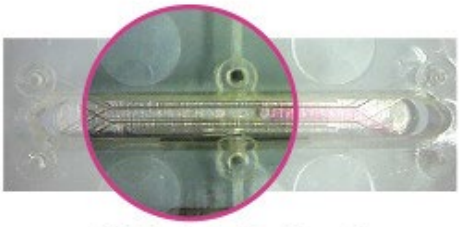
- Medium not sufficiently equilibrated before adding to Pods: be sure to follow the media preparation steps in section "Gas Equilibration of Media."
- Vacuum for Steriflip too weak: ensure that the media is passing through the Steriflip in about 10 seconds.
- Incorrect Steriflip used: confirm correct 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 the chip and reprime the Pod.

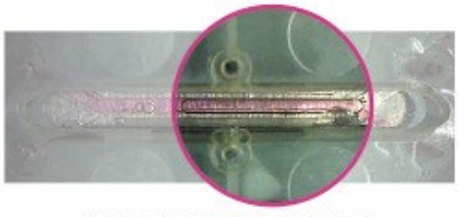



Channels filled with fluid


Small bubbles in Top Channel


Both Channels dry


Bubble plug in Top Channel


Bubble plug in Bottom Channel


Bubble plug in Top Seeding

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 to yield a stock concentration of 10 mM.
- The final concentration of Y27632 used in the 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 to yield 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 the back of a 2-to-6°C refrigerator or in a cold room. 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 the amount needed in the experiment.
- Keep 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 immediately before use.
- Once aliquots are thawed, do not re-freeze them. Thaw aliquots on ice prior to use.

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 if

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

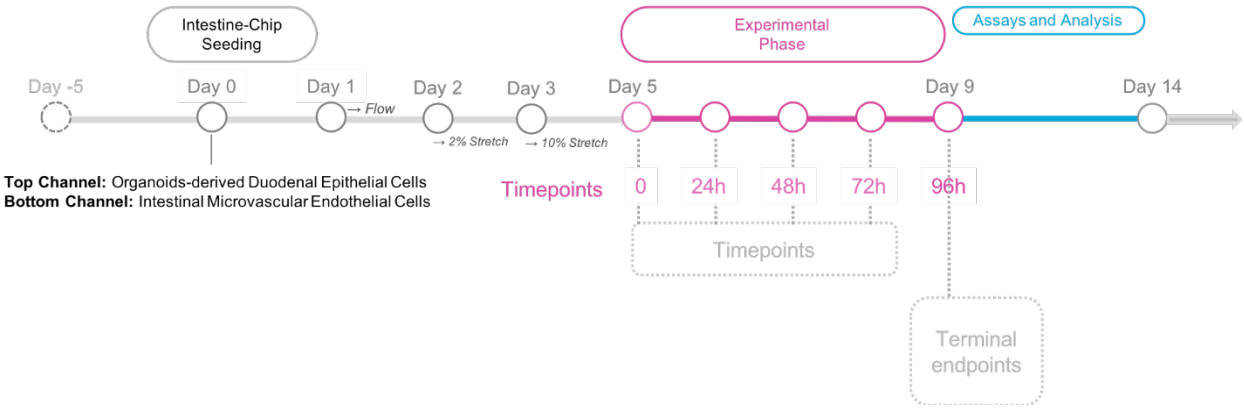
- Prepare fresh for each use.

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 Supplement Pack to the Endothelial Cell Basal Medium bottle.
- Store the bottle at 4°C.

Duodenum Intestine-Chip Timeline



Revision History

Version	CR#	Date	By	Description
A	CR-206	04Oct2022	J. Wells	Review/CR