Basic Organ-Chip Culture Protocol

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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.

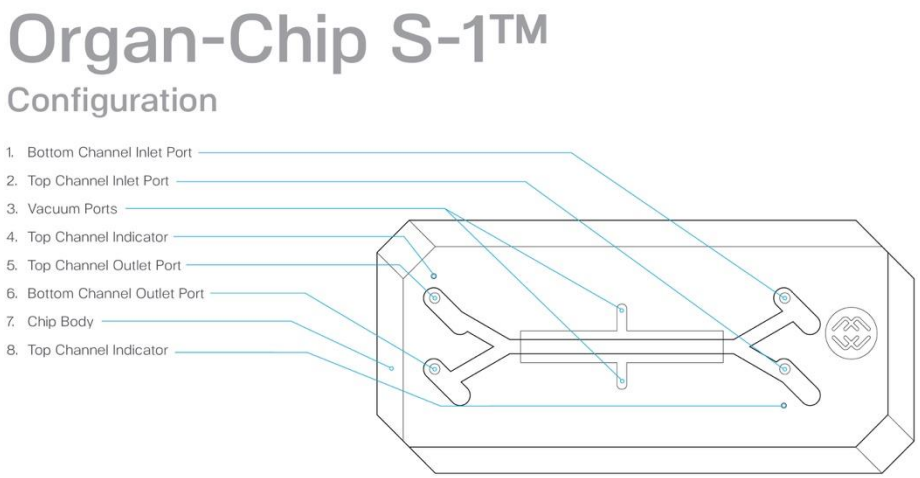
An Organ-Chip is a living, micro-engineered environment that recreates the natural physiology and mechanical forces that cells experience within the human body. The Pod module provides the cell culture media to the Organ-Chip; the Pod also acts as the interface between the Organ-Chip and the Zoë Culture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

The Emulate team can provide additional guidelines for optimization of different Organs-Chips using different types of cell sources such as cell lines, primary cells, and organoids.

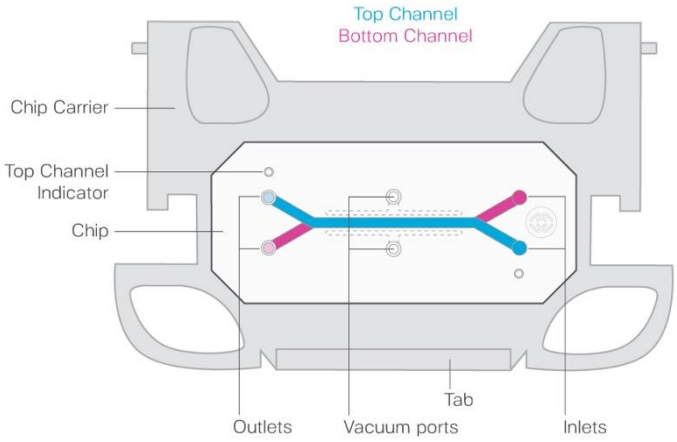
Organ-Chip (Chip-S1™ stretchable chip)

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.

The microenvironment created within each Chip-S1 includes typically 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.



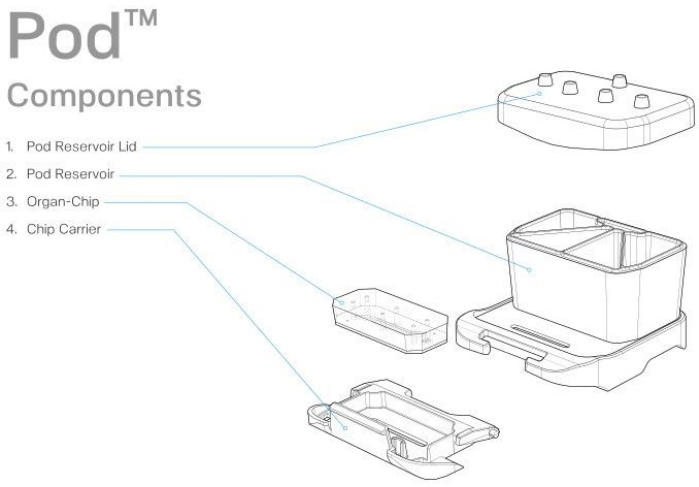
Chips are supplied pre-packaged in the chip carrier and should be maintained in the chip carrier at all times. The chip carrier is designed to hold chips in place, eliminating the need to disturb or distort the chip during preparation. In addition, the chip carrier connects securely to the Pod, which 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.)

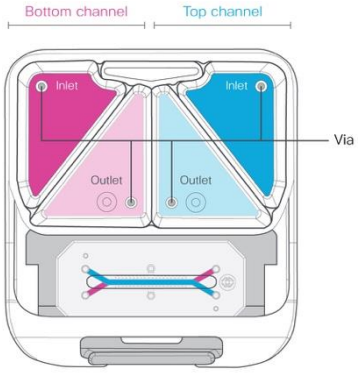


Pod™ Portable Module

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 lab equipment.

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

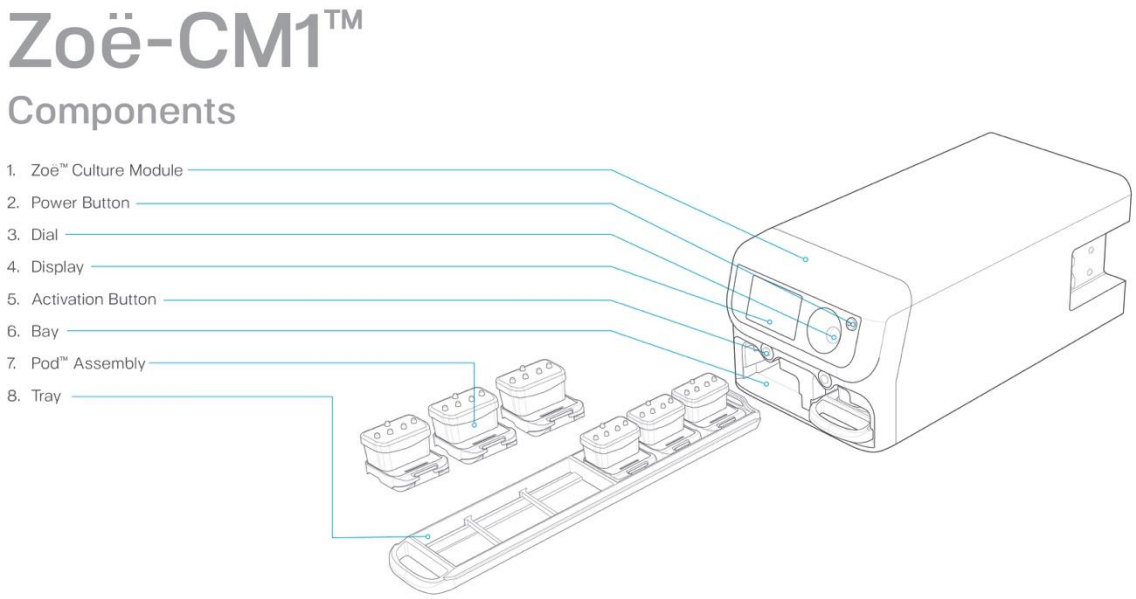




Zoë™ Culture Module

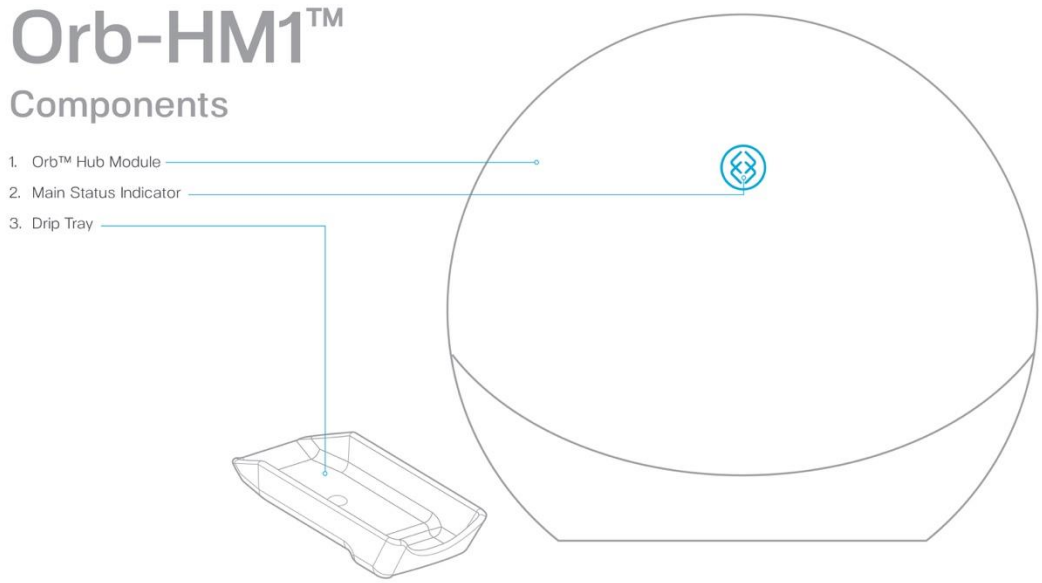
The Zoë Culture Module is designed to sustain the viability of cells within Emulate's Organ-Chips. It provides the dynamic flow of media and the mechanical forces that help recreate the microenvironment cells experience *in vivo*. The instrument automates the precise conditions needed for simultaneous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

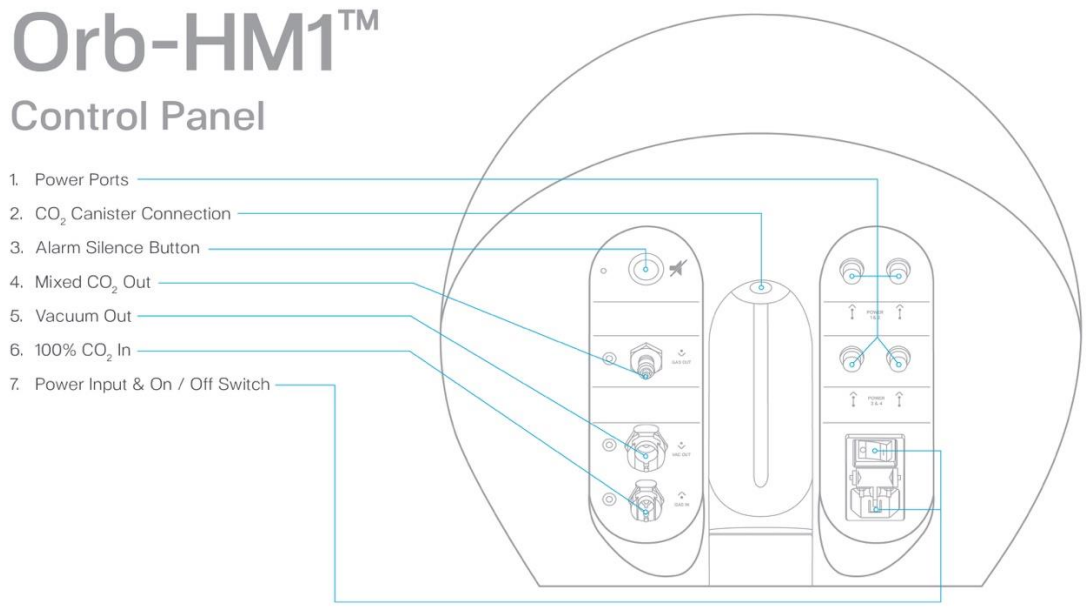
Zoë enables independent control of the flow rate of media through the top and bottom channels of the Organ-Chips, as well as stretch parameters — including frequency and amplitude — of the Organ-Chip. Additionally, Zoë has automated algorithms to prime the fluidic channels of Pods with media, and programming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



Orb™ Hub Module

The Orb Hub Module 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. The Orb also generates the vacuum required to apply Organ-Chip stretching (-70 kPa), and contains four individual power ports.





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.

Optimization of Culture Environment in Chip

Cells

Users can use any cell type that they have previously used for their research, such as cell lines, primary cells, iPSCs, and organoids. The key cell-related parameters that users need to optimize for chip culture are seeding density and seeding order if using multiple cell types. We have found that organoids provide an excellent cell source for Organ-Chips. Organoids complement the Emulate technology in that they provide a good source of human cells that contain all the relevant cell types from a specific tissue at the relevant ratios from both normal and disease patients. We can take biopsy-derived or stem cell (iPS-cell)-derived organoids and populate our Organ-Chips.

ECM

The use of an ECM is required to ensure optimal cell performance with the Human Emulation System. The ECM is a thin layer of extracellular matrix proteins that provide a scaffold and function to anchor the cells to the culture surface. Users can start with an ECM that they have previously used with a particular cell type, or select the appropriate ECM based on *in vivo* data. We then recommend that users optimize the ECM condition that best supports the viability, morphology, and function of the cells. If the ECM is not optimized, this will lead to poor cell morphology, uneven coverage of the chip channels, and often result in loss of cells from the chip upon initiation of flow.

Typically, users can optimize the ECM condition through the following steps:

1. Select one to three most relevant ECM types.
2. Choose two different concentrations to be tested (high and low).
3. Make different combinations. See table below.
4. Select optimal ECM that supports cell morphology and function.

ECM type	ECM type 1		ECM type 2		ECM type 3	
Concentration	High	Low	High	Low	High	Low
Test condition 1	√					
Test condition 2	√		√			
Test condition 3	√		√		√	
Test condition 4	√			√		√
Test condition 5		√		√		√
Test condition 6		√		√		

Medium

Selecting an optimal culture medium for the cells in the top and bottom channels is crucial step for successful chip culture. Since the chips experience laminar flow, the top and bottom channels are fluidically independent. Users can start with medium conditions that they know to work well for each cell type of interest in both top and bottom channels, respectfully. If different cell types from each channel survive and function well with two different media condition per channel, continue using these two different media. If not, users need to optimize the best medium condition to support different cells in both top and bottom channels as needed. Typically, users can test 1:1 mixture of two different media conditions, or adjust the concentration of medium supplements (e.g., growth factors) to find an optimal culture medium condition.

Mechanical forces

Flow through the chip exposed the cells to mechanical forces via shear stress. The level of shear stress is determined by the flow rate and dimensions of the channel dimension. The flow rates for each channel can be controlled independently and can be set to rates that range from 0 µL to 1000 µL per hour. (Typically between 20 to 200 µL per hour is the ideal flow rate for most of organ models). Flow rates

should be optimized for each cell type to enable the shear stress forces that resemble those experienced by the cells *in vivo*. Timing on connecting to flow should be optimized. In some cases, flow can be initiated soon after cell attachment; in others it may be after two days in culture. Stretching percentage and frequency is entirely dependent on the cell population and chip application. Not all cell types require stretching, but some organ systems — such as Lung-Chips and Intestine-Chip — benefit from stretch to replicate the motions of breathing or peristalsis. The stretching permeameter must be optimized for the tissue of interest. Note that if mechanical forces are applied to chips that are not physiologically relevant, these forces may negatively affect cell viability and function.

Examples of key experimental parameters for two different Organ-Chips:

Organ type	Liver	Intestine (Caco-2)
ECM	Collagen I (100 µg/ml) and Fibronectin (25 µg/ml)	Matrigel™ (100 µg/ml) and collagen I (30 µg/ml)
Cell type and density in top channel	Hepatocytes (3 M / ml	Caco-2 (2 M / ml)
Cell type and density in bottom channel	Liver sinusoidal endothelial cells (3 M / ml), stellate cells (0.1 M / ml), Kupffer cells (0.5 M / ml)	HUVEC (6 ~ 8 M / ml)
Flow rate	30 µL / hour	30 µL / hour
Stretching	Not applied as not physiologically relevant	Applicable - 10% strain, 0.15 Hz
Cell culture medium for top channel	Hepatocyte culture medium	Caco-2 culture medium
Cell culture medium for bottom channel	1:1 mixture of hepatocyte culture medium and LSEC culture medium omitting dexamethasone	HUVEC culture medium
Timeline	Day -1 (coating), Day 0 (hepatocyte seeding), Day 1 (hepatocyte overlay), Day 2 (bottom channel cell seeding), Day 3 (Chips to Zoë)	Day -1 (coating), Day 0 (HUVEC seeding), Day 1 (Caco-2 seeding), Day 2 (Chips to Zoë)

Experimental Overview

Workflow Overview

Day X: Reagent Preparation

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

Day -2: Thaw Epithelial and Endothelial cells

- ☐ Thaw and plate epithelial cells
- ☐ Thaw and plate endothelial cells

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: Endothelial Cells to Chips

- ☐ Prepare chips
- ☐ Harvest endothelial cells
- ☐ Seed endothelial cells to bottom channel
- ☐ Gravity wash chips (2 hours post-seeding)

Day 1: Epithelial Cells to Chips

- ☐ Wash chips
- ☐ Harvest epithelial cells
- ☐ Seed epithelial cells to top channel
- ☐ Gravity wash chips (2 hours post-seeding)

Day 2: Chips to Pods and Pods to Zoë

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

Day 3+: Maintaining and Sampling

- ☐ Maintenance and the Regulate cycle
- ☐ Sampling and media replenishment

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
Basic Bio-Kit	12- or 24-pack	Emulate	-
Zoë-CM1™ 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
150 mm cell culture dish	Sterile, 1 per 6 chips	Corning / Falcon	353025
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, and P1000	-	-
Pipette tips	P20, P200, and P1000 sterile, low-adhesion	-	-
Conical tubes	15 mL and 50 mL polypropylene, sterile	-	-
Eppendorf tubes	1.5 mL, sterile	-	-
Aluminum foil	-	-	-
Microscope (with camera)	For bright-field imaging	-	-
Hemocytometer	-	-	-
Manual counter	-	-	-
Water bath (or beads)	Set to 37°C	-	-
Vacuum set-up	Minimum pressure: -70 kPa	-	-

T25 flasks	-	-	-
T75 flasks	-	-	-
Ice bucket	-	-	-
70% ethanol and wipes	For surface sterilization	-	-

Reagents, Media & Supplements

Reagent	Description	Supplier	Catalog Number
ER-1™ surface activation reagent	5 mg powder	Emulate	-
ER-2™ surface activation reagent	25 mL bottle	Emulate	-
Trypan blue	0.4% solution	Sigma	93595
Trypsin-EDTA solution	0.05% trypsin	Sigma	T3924
Cell culture medium for cell type 1 (epithelial)	-	-	-
Cell culture medium for cell type 2 (endothelial)	-	-	-
ECM (dependent on cell type)	-	-	-
Penicillin-streptomycin	10,000 U/mL; 10 mg/mL	Sigma	P4333
Fetal bovine serum (FBS)	Sterile heat-inactivated	Sigma	F4135 or F8317
Fungin™	10 mg / mL	InvivoGen	ANT-FN-1

Notes for ER-1™ and ER-2™ reagents

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

Notes for Medium selection

- Users can start with medium for specific cell types that they have been using previously. We recommend users to follow instructions from media suppliers for storage and preparation.
- We typically prepare base media in 500 mL of media bottle first, then prepare 50 mL of complete media aliquot after adding all supplements.

Notes for Fungin™

- Addition of 0.1% of Fungin to any Organ-Chip culture medium is essential to prevent fungal contamination.
- This antimycotic compound kills yeasts, molds, and fungi by disrupting ionic exchange through the cell membrane. This reagent has no effect on eukaryotic cell viability or function due to its mechanism of action.

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.

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, 50 µL volume is generally used for the top channel, and 20 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.

While 50 µL (top channel) and 20 µL (bottom channel) are standard volumes used throughout the protocol, there can be some flexibility in the actual volumes used.

Top Channel: 35–50 µL

Bottom Channel: 15–20 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 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. 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.

Basic Organ-Chip Culture Protocol

Day X: Reagent Preparation

Aliquot Reagents

Aliquot reagents including media supplements and ECM prior to use and store at -20°C to avoid multiple freeze-thaw cycles. Reagents will be dependent upon the cell type being used for the Organ-Chips, for reference on types of reagents that you may need refer to Emulate protocol EP079 Liver-Chip Quad-Culture.

Day -2: Thawing Epithelial and Endothelial Cells

Goals:

- Expand epithelial or endothelial cells in flask prior to chip seeding as per established protocols.

Key Steps:

- Thaw and plate epithelial cells
- Thaw and plate endothelial cells

Required Materials:

- Complete epithelial cell culture medium (at 37°C)
- Complete endothelial cell culture medium (at 37°C)
- 15 mL conical tube
- T-25 or T-75 flask
- Serological pipettes
- Pipettes and tips
- Aspirator
- Centrifuge
- 70% ethanol

Tips:

- If cells need to be seeded directly from cryopreserved vial to chip, user can skip this step.

Thaw and Plate Epithelial cells

1. Prepare epithelial cell seeding medium.
2. Thaw the vial(s) of cells by immersing in a 37°C water bath. Closely observe while gently agitating and remove from the water bath just before the last of the ice disappears.
3. Spray vial(s) with 70% ethanol and wipe dry prior to placing them in the BSC.
4. Immediately transfer the contents of the vial into 3 mL of warm complete epithelial cell culture medium in a sterile 15 mL conical tube.
5. Rinse the vial with 1 mL of complete epithelial cell culture medium and collect in the 15 mL tube.
6. Bring the volume to 15 mL with complete epithelial cell culture medium.
7. Spin down cells.
8. Aspirate and discard supernatant, leaving approximately 100 µL of medium covering the pellet.
9. Loosen the cell pellet by gently flicking the tube.
10. Re-suspend cells in 15 mL of complete epithelial cell culture medium.
11. Add the epithelial cell suspension to T-75 (or T-25) flask.
12. Incubate overnight at 37°C and 5% CO_2.
13. Exchange with fresh complete epithelial cell culture medium every other day until use for seeding in the chip.

Thaw and Plate Endothelial cells

1. Prepare endothelial cell seeding medium.
2. Thaw the vial(s) of cells by immersing in a 37°C water bath. Closely observe while gently agitating and remove from the water bath just before the last of the ice disappears.
3. Spray vial(s) with 70% ethanol and wipe dry prior to placing them in the BSC.
4. Immediately transfer the contents of the vial into 3 mL of warm complete endothelial cell culture medium in a sterile 15 mL conical tube.
5. Rinse the vial with 1 mL of complete endothelial cell culture medium and collect in the 15 mL tube.
6. Bring the volume to 15 mL with complete endothelial cell culture medium.
7. Spin down cells.
8. Aspirate and discard supernatant, leaving approximately 100 µL of medium covering the pellet.
9. Loosen the cell pellet by gently flicking the tube.
10. Re-suspend cells in 15 mL of complete endothelial cell culture medium.
11. Add the endothelial cell suspension to T-75 (or T-25) flask.
12. Incubate overnight at 37°C and 5% CO_2.
13. Exchange with fresh complete endothelial cell culture medium 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 inner channels with ECM proteins for cell attachment

Key Steps:

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

Required Materials:

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

Tips:

- The use of an ECM is required to ensure optimal cell performance with the Human Emulation System. The ECM is a thin layer of extracellular proteins that function to anchor the cells to the culture surface. Users can choose the optimal ECM for the cell types being used in the chips.

Prepare Chips

1. Spray the packaging of the chips with 70% ethanol and bring into the BSC.
2. Open the packaging carefully, and lay out 6 chips in each sterile 150 mm dish. Note: For ease of workflow, align all chips facing the same direction in the 150 mm dish. To properly orient the chips, ensure the tab of the carrier is facing to the right, and all chips are in the same orientation. (See Figure 1)
3. Label each chip with ID numbers on the chip carrier tab.

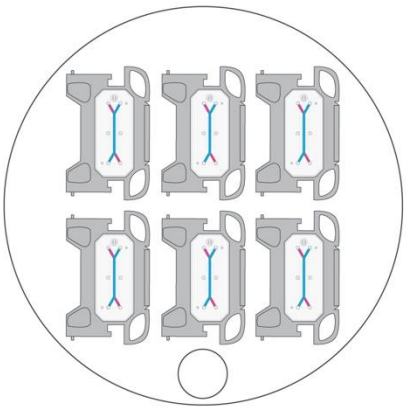


Figure 1 Proper orientation of Organ-Chips in 150 mm dish

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 the ER-1 and ER-2 reagents 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 buffer 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 buffer 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 buffer 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

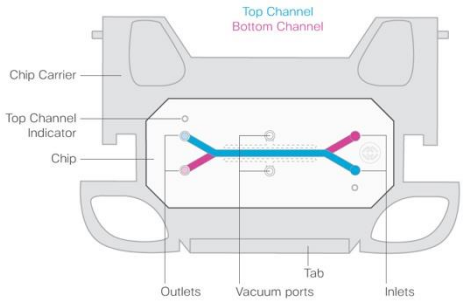


Figure 2 Top view of chip, with labelled ports, in chip carrier

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.
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.

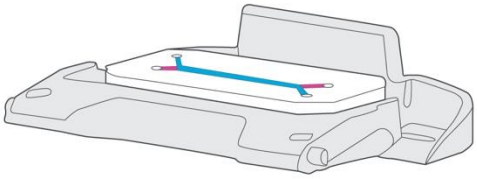


Figure 3: View of chip with no excess ER-1 solution on surface

Activate and Wash Chips

1. Bring the 150 mm dish containing the ER-1-coated chips to the UV light box.
2. Remove the cover from the 150 mL dish and place the open dish in the UV light box.
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 20 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 each channel with 200 µL of sterile cold DPBS.
11. Leave cold DPBS inside the channels.

Prepare ECM Solution

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

ECM should be optimized for any new Organ-Chip prior to starting your studies. An example of how to prepare ECM solution is below using the optimized conditions for the human Liver-Chip:

ECM working concentration is:

Collagen I: 100 µg / mL

Fibronectin: 25 µg / mL

1. Bring an ice bucket and ice to the BSC.
2. Thaw one aliquot of fibronectin (1 mg / mL) 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.
   - Volume required per chip = approximately 100 µL
   - For every 12 chips to coat, prepare 1.5 mL of ECM solution (12 chips x 100 µL / chip + extra 300 µL = 1.5 mL of ECM solution). See calculation examples below.
4. Combine components to prepare ECM working solution.
5. Keep the ECM solution on ice until ready to use.

Calculation Example

ECM Calculation Example ($C_1V_1 = C_2V_2$)

Collagen I stock concentration: 8.41 mg/ mL (C_1)

Collagen I final concentration: 0.1 mg / mL (C_2)

Fibronectin stock concentration: 1 mg / mL (C_1)

Fibronectin final concentration: 0.025 mg / mL (C_2)

Stock volume: collagen I or fibronectin (V_1)

Final volume of ECM solution: 1.5 mL (V_2)

Collagen

(8.41 mg / mL) x (X mL) = (0.1 mg / mL) x (1.5 mL)

X = 17.83 µL of collagen I stock solution

Fibronectin

(1 mg / mL) x (Y mL) = (0.025 mg / mL) x (1.5 mL)

Y = 37.5 µL of fibronectin

DPBS

Volume DPBS =

(total volume of ECM needed) – (volume of collagen I) – (volume of fibronectin)

= 1500 µL – 17.83 µL – 37.5 µL

= 1444.67 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 ECM solution. (100 µL volume total will be used per chip.)
3. Carefully introduce ECM solution through the bottom channel inlet until a small ECM droplet forms on the outlet.
4. Without releasing the pipetting plunger, take the pipette out from the bottom channel inlet, and move the pipette containing remaining ECM solution to the top channel inlet.
5. Introduce ECM solution through the top channel inlet, leaving small droplets of excess ECM solution on both ports in both channels. (See Figure 4.)
6. 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.
7. Repeat steps 1 through 6 for each chip.
8. Add 1.5 mL of DPBS to the cap of a 15 mL conical tube. Place the PBS cap in the 150 mm culture dish with the chips to provide extra humidity and seal the dish.

For best results, incubate the chips at 4°C overnight, then at 37°C for 1 hour the following day. Note: If desired, cells can be seeded the same day as chip activation and ECM coating, though incubation overnight is preferred. Chips can be ready for cell seeding 4 hours after adding the ECM and incubating chips at 37°C.

If chips will be stored longer than overnight, store the chips at 4°C for up to 2 days.

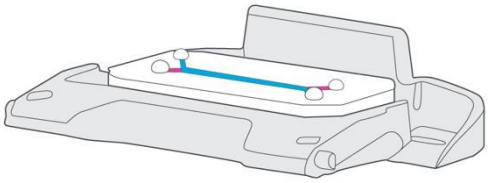


Figure 4: Chip in chip carrier with small droplets of ECM solution at ports

Day 0: Endothelial Cells to Chip

Goals:

- Seed endothelial cells in chip

Key Steps:

- Prepare chips
- Harvest endothelial cells
- Adjust cell density
- Seed endothelial cells to bottom channel
- Seed a well plate
- Gravity wash chips (2 hours post-seeding)

Required Materials:

- Complete endothelial cell seeding medium (at 37°C)
- 1X DPBS (at room temperature)
- Serological pipettes
- Pipettes and filtered tips
- Aspirator and sterile tips
- 15 mL conical tubes
- Diluted trypan blue counting solution
- Hemocytometer
- 70% ethanol
- Microscope

Tips:

- It is possible to seed epithelial cells first in the top channel. The order of cell seeding must be optimized and will be dependent on tissue type being studied. In order to find an optimal seeding order for specific cell types, it is recommend to test different seeding orders between epithelial and endothelial cells first.
- More than one cell type can be seeded in bottom channel and this will require a model optimization. To find more information, refer to Emulate protocol EP079 Liver-Chip Quad-Culture.
- After constructing endothelial cell layer, users can flow blood or immune cells in the endothelial cell channel. To find more information, visit our publications on our website.

Prepare Chips

1. Transfer ECM-coated chips from incubator into the BSC.
2. Fully aspirate ECM from both channels.

3. Gently wash each channel of the chip with 200 µL of complete endothelial cell culture medium. 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 endothelial cell culture medium per channel, leaving the excess medium outflow covering the inlet and outlet ports.
5. Cover the 150 mm dish and place the chips in the incubator until the cells are ready for seeding.

Harvest Endothelial Cells

Endothelial cells that have been expanded in culture must be harvested and counted for bottom channel seeding. Typically endothelial cells are adjusted to a density range of 3 ~ 9 x 10^6 cells / mL prior to seeding in the bottom channel. We recommend users to test different densities of endothelial cells to find an optimal seeding density for their specific endothelial cells.

1. Bring the culture flask (e.g., T-75) containing endothelial cells from the incubator into the BSC.
2. Aspirate culture media and add 5 to 15 mL of 1X DPBS to wash the culture surface. Aspirate the DPBS wash.
3. Add 3 mL of trypsin-EDTA to the flask. 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 9 mL of warm complete endothelial cell culture medium to the flask and pipette gently to mix, while collecting all cells from the culture surface.
6. Transfer the contents of the flask (12 mL) into a sterile 15 mL conical tube.
7. Add 3 mL of complete endothelial cell culture medium to bring the total volume of the tube to 15 mL.
8. Spin down endothelial cells.
9. Carefully aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet. Note: The cell pellet will be very small. Aspirate carefully.
10. Loosen the cell pellet by flicking the tube gently.
11. Using a P1000 pipette, gently resuspend the cells by adding 200 µL of complete endothelial cell culture medium.
12. Pipette gently to create a homogeneous mixture and transfer 5 µL of the cell suspension to the trypan blue cell counting solution. This will make a 1:10 dilution. (See trypan blue counting solution table below.)
13. Fix the counting solution thoroughly and count cells using a manual hemocytometer. (See Figure 5 below.)

Trypan Blue Cell Counting Solution

Reagent	Volume	Source	Cat. No.
Endothelial Cell Culture Medium	40 µL	Recipe Above	-
Trypan blue	5 µL	Sigma	93595

- Maintain at room temperature prior to use.
- Prepare fresh for each use.

Cell Counting and Viability Assessment

1. Count both viable and non-viable cells in each quadrant of the hemocytometer.
   Live Cell Count; Dead Cell Count; Total Cell Count
2. Calculate percent viability of the cell solution.
   (Live Cells) ÷ (Total Cells) x 100 = % Viability
3. Calculate viable cell concentration. The dilution factor is 10 when prepared in the trypan blue solution above.
   (Live Cell Count x 10 x 10^4) ÷ 4 = Viable Cell Concentration (cells / mL)
4. Calculate viable cell yield.
   (Viable Cell Concentration) ÷ (Cell Suspension Volume) = Viable Cell Yield (cells)

Dilute the endothelial cells to desired seeding density in complete endothelial cell culture medium.

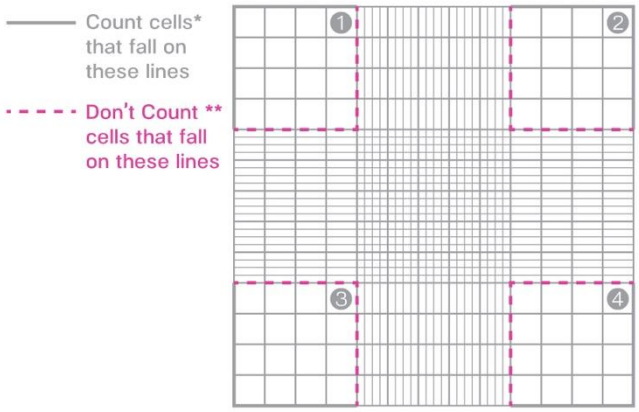


Figure 5: Manual hemocytometer

Seed Endothelial Cells 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 150 mm 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. Seed 15 to 20 µL of the endothelial cell suspension into the bottom channel using one chip first, while aspirating the outflow.
5. Cover the dish and transfer to the microscope to check the seeding density within the chip.

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.
7. 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 150 mm cell culture dish. (See Figure 6.)
8. Place the small reservoir (15 mL conical tube cap containing sterile DPBS) inside the 150 mm dish 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.
9. Place the chips still in the dish in the 37°C incubator for approximately 2 hours, or until cells in the bottom channel have attached. Note: Correct seeding density is essential for success of the chips.
10. Once endothelial cells have attached (approximately 2 hours post-seeding), flip the chips back to an upright position. Note: Remove the chip cradle, wipe with 70% ethanol to clean, and autoclave for use in next experiment.

Note: It is recommended to always seed any remaining cells into a plate as control for cell quality.

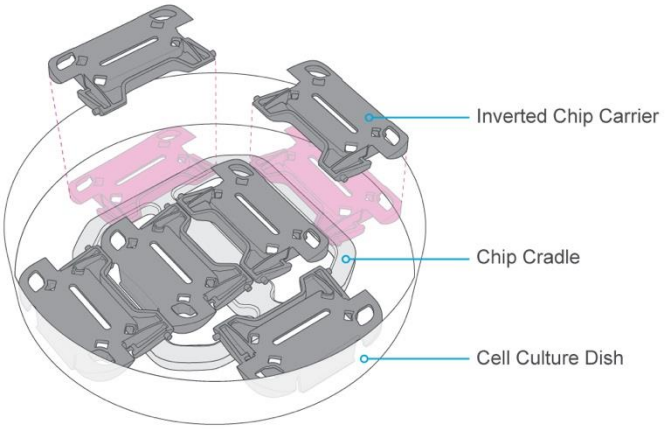


Figure 6: Inverting chips during endothelial attachment

Gravity Wash Chips

A gentle gravity wash is performed 2 hours post-seeding, after the endothelial cells in the chip have attached, to ensure that nutrients are replenished and the channels do not dry out. During a gravity wash the medium should be observed to flow through the channel and outflow from the outlets.

1. With a P200 pipette, gently drop 200 µL on top of both inlet ports of top and bottom channels. This should cause medium to gently flow through the channel, spilling out of the outlets.
2. If the media does not flow through the channel, very gently pipette a small amount of medium into the inlets, until a small droplet appears on the outlet, or until a bubble is ejected through the outlet.
3. Place additional droplets of media to fully cover all inlet and outlet ports to prevent evaporation from the ports. (See **Error! Reference source not found.**7.).
4. Incubate chips overnight at 37°C. Note: if desired, epithelial cells can be seeded at least 4 hours post-attachment of endothelial cells

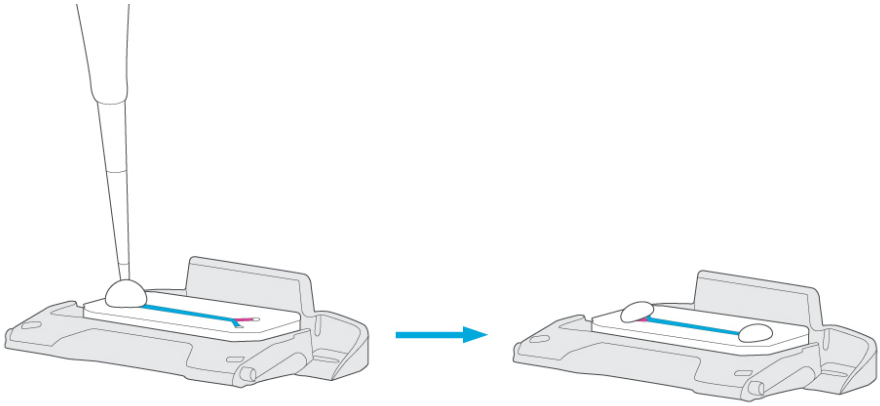


Figure 7 Chip with medium drops covering the inlet and outlet ports

Day 1: Epithelial Cells to Chip

Goals:

- Seed epithelial cells in chip

Key Steps:

- Wash chips
- Harvest epithelial cells if using cell lines
- Seed epithelial cells to top channel
- Gravity wash with tips (2 hours post-seeding)

Required Materials:

- Complete epithelial cell seeding medium (at 37°C)
- 1X DPBS (at room temperature)
- Serological pipettes
- Pipettes and filtered tips
- Aspirator and sterile tips
- 15 mL conical tubes
- Diluted trypan blue counting solution
- Hemocytometer
- 70% ethanol
- Microscope

Tips:

- More than one cell type can be seeded in top channel and this will require a model optimization. To find more information, refer to Emulate protocol EP079 Liver-Chip Quad-Culture.
- Organoids can be cultured in chip. More information can be found in research we published on the topic.

Wash Chips

1. Gently pipette 200 µL of warm complete endothelial cell medium to the bottom channel of each chip to wash. Aspirate the outflow, leaving media in the channel.
2. Return chips to the incubator until epithelial cells are ready for seeding.

Harvest Epithelial Cells

Epithelial cells that have been expanded in culture must be harvested and counted for bottom channel seeding. Typically epithelial cells are adjusted to a density range of 1 to 3.5 x 10^6 cells / mL prior to seeding in the top channel. We recommend users to test different densities of epithelial cells to find an optimal seeding density for their specific epithelial cells.

1. Bring the culture flask (e.g., T-75) containing epithelial cells from the incubator into the BSC.
2. Aspirate culture media and add 5 to 15 mL of 1X DPBS to wash the culture surface. Aspirate the DPBS wash.
3. Add 3 mL of trypsin-EDTA to the flask. 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 9 mL of warm complete epithelial cell culture medium to the flask and pipette gently to mix, while collecting all cells from the culture surface.
6. Transfer the contents of the flask (12 mL) into a sterile 15 mL conical tube.
7. Add 3 mL of complete epithelial cell culture medium to bring the total volume of the tube to 15 mL.
8. Spin down epithelial cells.
9. Carefully aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet. Note: The cell pellet will be very small. Aspirate carefully.
10. Loosen the cell pellet by flicking the tube gently.
11. Using a P1000 pipette, gently resuspend the cells by adding 200 µL of complete epithelial cell culture medium.
12. Pipette gently to create a homogeneous mixture, and transfer 5 µL of the cell suspension to the trypan blue cell counting solution. This will make a 1:10 dilution. (See trypan blue counting solution table below.)
13. Mix the counting solution thoroughly and count cells using a manual hemocytometer. (see Figure 8 below)
14. Dilute the epithelial cells to desired seeding density in complete epithelial cell culture medium.

Trypan Blue Cell Counting Solution

Reagent	Volume	Source	Cat. No.
Epithelial Cell Culture Medium	40 µL	Recipe Above	-
Trypan blue	5 µL	Sigma	93595

- Maintain at room temperature prior to use.
- Prepare fresh for each use.

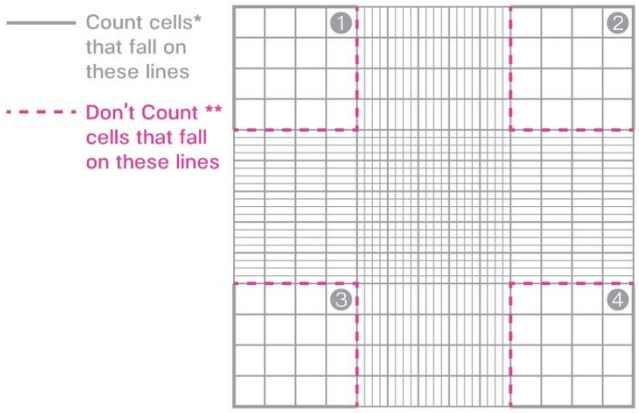


Figure 8: Manual hemocytometer

Cell Counting and Viability Assessment

1. Count both viable and non-viable cells in each quadrant of the hemocytometer.
   Live Cell Count; Dead Cell Count; Total Cell Count
2. Calculate percent viability of the cell solution.
   (Live Cells) ÷ (Total Cells) x 100 = % Viability
3. Calculate viable cell concentration. The dilution factor is 10 when prepared in the trypan blue solution above.
   (Live Cell Count x 10 x 10^4) ÷ 4 = Viable Cell Concentration (cells / mL)
4. Calculate viable cell yield.
   (Viable Cell Concentration) ÷ (Cell Suspension Volume) = Viable Cell Yield (cells)

Seed Epithelial Cells 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 next chips if necessary.

1. Bring the 150 mm 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. Seed 35 to 50 µL of the epithelial cell suspension into the top channel, while aspirating the outflow.
5. Cover the dish and transfer to the microscope to check the seeding density within the chip.

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.
7. After confirming the correct cell density, 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.
8. Place the chips (with the DPBS reservoir) at 37°C for 2 hours, or until cells have attached. Note: Correct seeding density is essential for success of the chips

   Note: It is recommended to always seed any remaining cells into a plate as control for cell quality.

Gravity Wash

When both endothelial and epithelial cells are cultured in the same media, use the gravity wash method described in the endothelial cell culture section (See Figure 6).

When both cells are cultured in different media, use the following instruction using tips.

1. Once epithelial cells have attached (approximately 2 hours post-seeding), bring the dish containing the chips to the BSC.
2. A gravity wash with 200 µL of complete epithelial cell culture medium for the top channel and complete endothelial cell culture medium for the bottom channel per chip will provide nutrients to cells. Since there are 2 different media being used, these two media must be separated by keeping them in filtered tips instead of drops (see Figure 7).
3. Return chips with pipette tips inserted in each inlet and outlet port to the incubator overnight.
4. Maintain cells in static culture in chips until connecting to Pods and Zoë the next day. Note: If desired, chips can be connected at least 2 hours post-attachment.

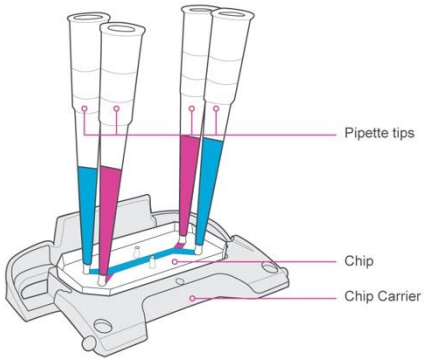


Figure 9: Chip with filtered tips inserted into ports with respective media

Day 2: Chips to Pods and Pods to Zoë

Goals:

- De-gas and equilibrate media
- Connect chips to Pods
- Connect Pods to Zoë

Key Steps:

- Gas equilibration of media
- Prime Pods
- Wash chips
- Connect chips to Pods
- Connect Pods to Zoë
- Run Regulate cycle
- Begin experimental flow

Required Materials:

- 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

Gas Equilibration of Media

The media equilibration step is very important 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 — no more than 10 minutes — of a warmed environment (such as an incubator or bath), as gas equilibrium can become compromised when medium is allowed to cool.

1. Place at least 3.3 mL of complete epithelial cell culture medium for each chip in a 50 mL conical tube.
2. Place at least 3.3 mL of complete endothelial cell culture medium for each chip in a separate 50 mL conical tube.
3. Warm both 50 mL conical tubes of 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. (See Figure 10 below)
    - Attach each conical tube containing warmed medium to a Steriflip® unit.
    - With the unit "right-side-up" (medium in the bottom conical tube), apply vacuum for 10 seconds.
    - 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.
    - 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 very important step to ensure success of the chips.

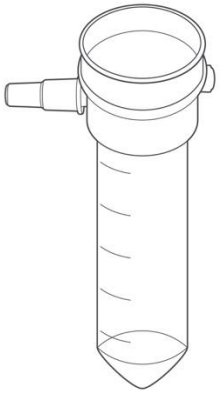


Figure 10 Steriflip® unit

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. (Reference Figure 11 below)
3. Pipette 3 mL of pre-equilibrated, warm media to each inlet reservoir. In the top channel inlet reservoir add complete epithelial cell culture medium; in the bottom channel inlet reservoir add complete endothelial cell culture Medium.
4. Pipette 300 µL of pre-equilibrated, warm media to each outlet reservoir, directly over each outlet via. (Reference Figure 12 below)
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.
    - Use the rotary dial to highlight “Prime” on the display.
    - Press the rotary dial to select “Prime.”
    - Rotate the dial clockwise to highlight “Start.”
    - 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. This is very important for success.
    - 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.
    - If any Pod does not show droplets, re-run the “Prime” cycle on those Pods.
    - 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.

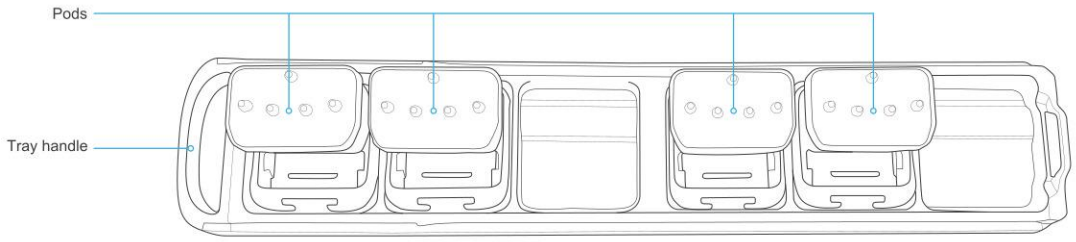


Figure 11: Pods in tray

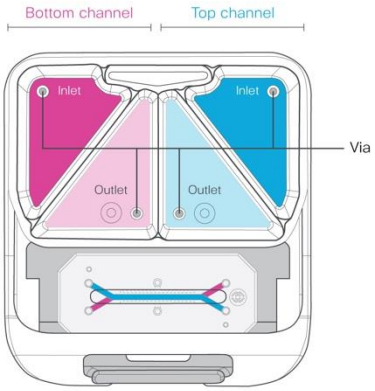
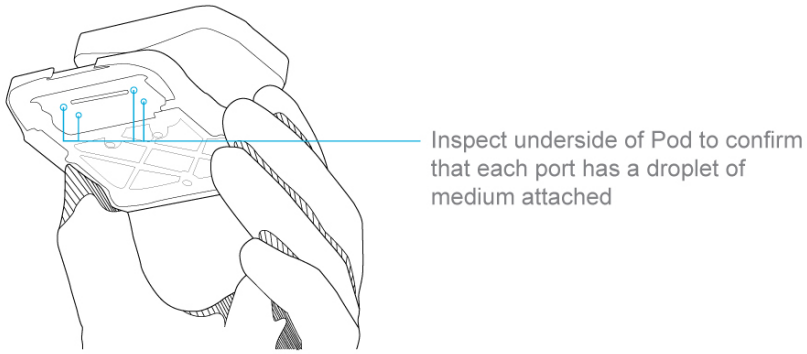


Figure 12: Pipette 300 µL of pre-equilibrated, warm media to each outlet reservoir, directly over each outlet via



Wash Chips

1. Transfer the seeded chips in a 150 mm dish from the incubator to the BSC.
2. Remove the pipette tips from the chip inlet and outlet ports.
3. Gently wash the top channel of each chip with warm, equilibrated complete epithelial cell culture medium to remove any possible bubbles in the channel.
4. Place small droplets of equilibrated complete epithelial cell culture medium on the top of each inlet and outlet port of each chip.
5. Gently wash the bottom channel of each chip with warm, equilibrated complete endothelial cell culture medium to remove any possible bubbles in channel.
6. Place small droplets of equilibrated complete endothelial cell culture medium on the bottom channel inlet and outlet ports of each chip.

Chips to Pods

1. Holding one chip (while it remains in the chip carrier) in the dominant hand and one Pod 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

Different Organ-Chips will require exposure to different types of mechanical forces. Zoë enables the use to apply physiologically relevant mechanical forces via stretch and/or flow. The type and optimal level of mechanical force will be determined by the cells of interest and the physiologically-relevant microenvironment you aim to recreate.

Flow rates will need to be optimized depending on cell types used in the chips and the physiologically relevant mechanical forces required by the cells.

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 example, set the flow rate to 30 µL / hour and without stretch for both top and bottom channels for human Liver-Chip culture.
3. Run Regulate cycle.
    - Using the rotary dial, highlight the “Regulate” field.
    - Press the dial to select “Regulate,” and rotate the dial clockwise to “Start.”
    - 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.
    - At this point the “Activation” button will glow blue.
    - 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.

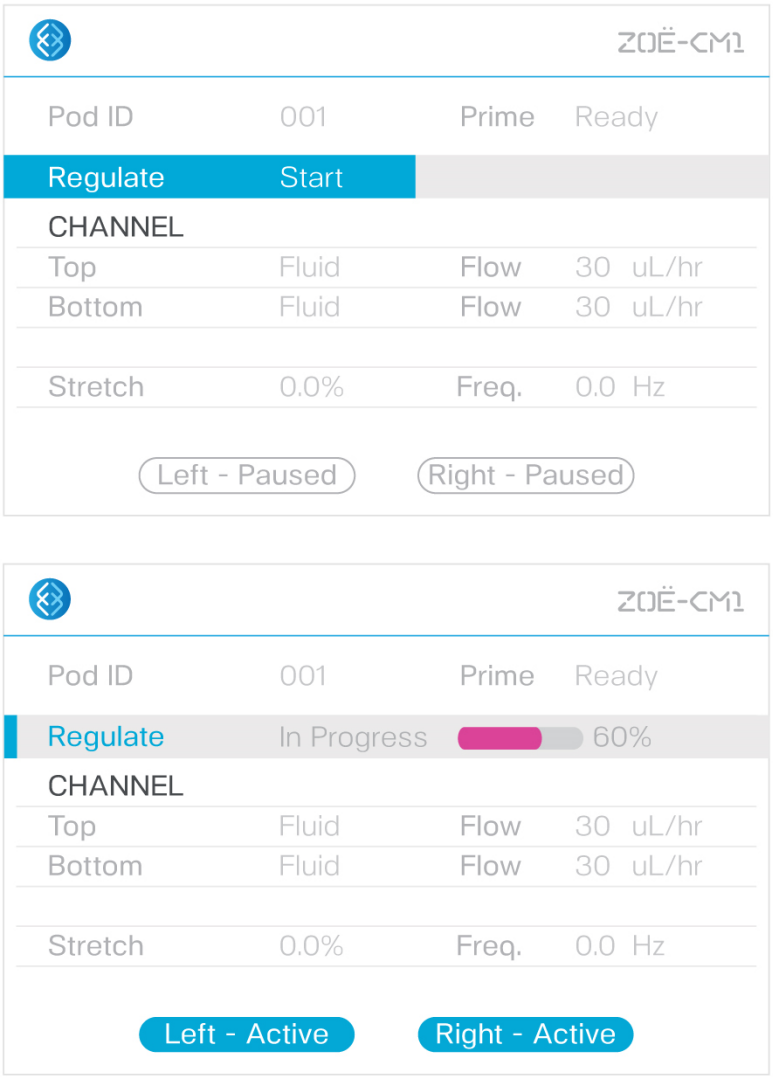


Figure 13–14: Regulate cycle

Day 4: Chip Maintenance and Sampling

Goals:

- Maintain chips in Zoë
- Cell culture inspection
- Collect samples for analysis

Key Steps:

- Maintenance and the Regulate cycle
- Sampling and media replenishment

Required Materials:

- Chips in Pods
- Cell culture media

Maintenance and the Regulate Cycle

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:
   - Using media within the Pod reservoir, pipette 200 µL of media directly over the top of the via to dislodge any bubbles that may be present.
   - Repeat this wash step for each of the four Pod reservoirs.
4. Replace Pod lids and return the trays to Zoë.
5. Run the Regulate cycle again.
   - Using the rotary dial, highlight the “Regulate” field.
   - Press the dial to select “Regulate,” and rotate the dial clockwise to “Start”.
   - 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. At this point the “Activation” button will glow blue.
   - The Regulate cycle lasts 2 hours. After the cycle completes, Zoë will begin flow at the preset Organ-Chip culture conditions.

Sampling and Media Replenishment

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 (Reference Figure 15 below)
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.
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:
   - Using medium within the Pod reservoir, pipette 1 mL 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.

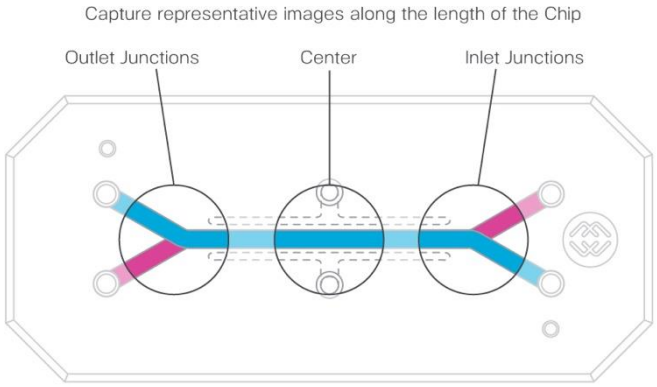


Figure 15: Capture representative images at 10X or 20X magnification at the following locations:

- Inlet junctions
- Center of channel
- Outlet junctions

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.