emulate

Liver-Chip Tri-Culture Protocol

April 4, 2019

EP080 v1.0

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Introduction to the Human Emulation System

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

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

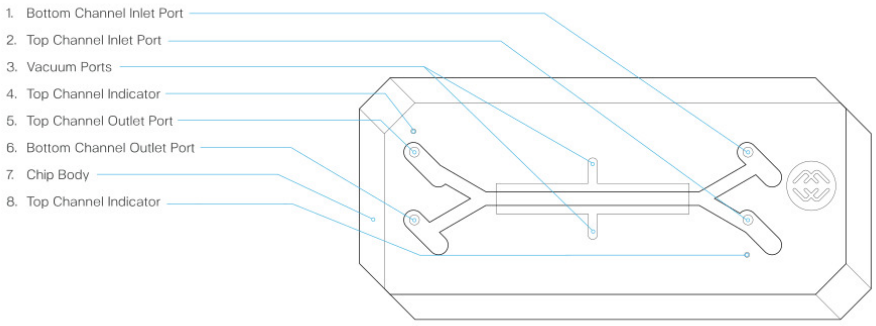
Organ-Chip (Chip-S1)

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

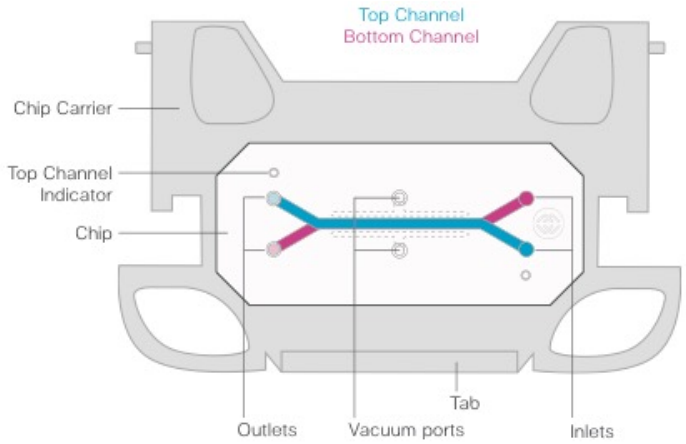
The microenvironment created within each Chip-S1 includes epithelial cells in the top channel and endothelial cells in the bottom channel. Top and bottom channels are separated by a porous membrane that allows for cell-cell interaction like those that are seen *in vivo*. These two channels are fluidically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

Organ-Chip

Configuration



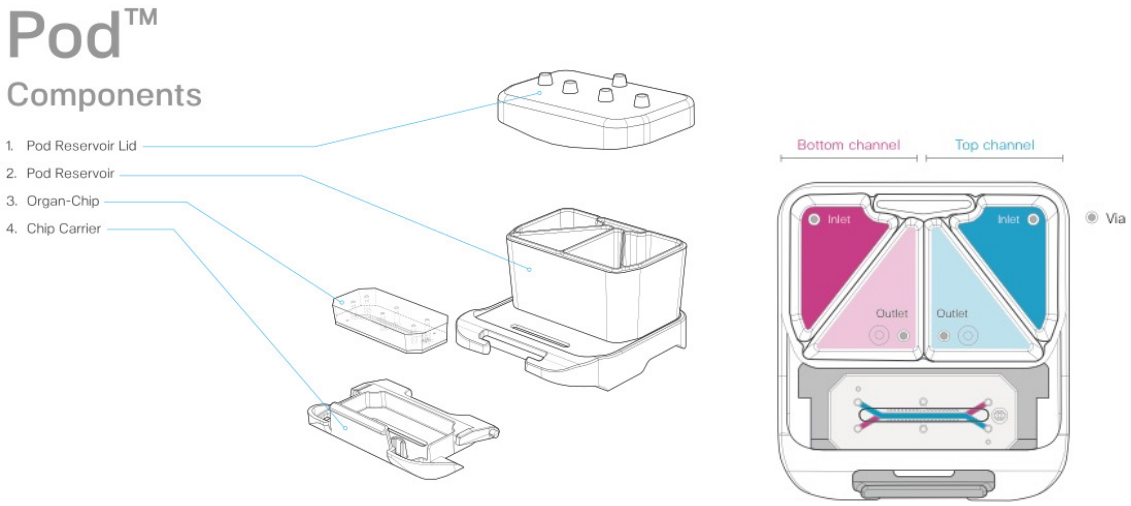
Chips are supplied pre-packaged in the chip carrier and should be maintained in the chip carrier at all times. The chip carrier is designed to hold chips in place, eliminating the need to disturb or distort the chip during preparation. In addition, the chip carrier connects securely to the Pod, which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



Pod

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

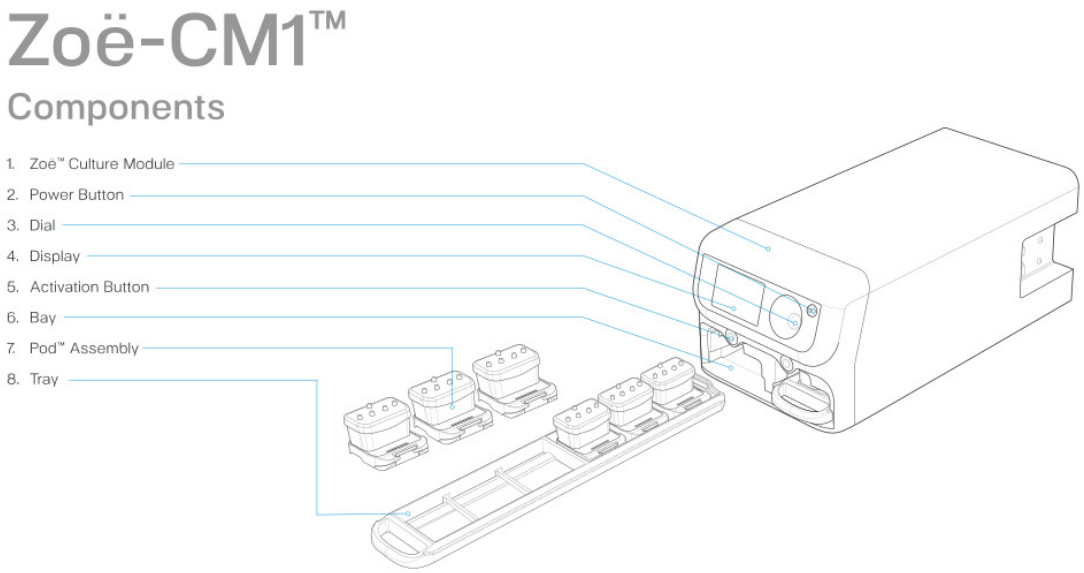
The Pod's reservoirs allow users to introduce nutrient media or blood, precisely control dosing to test drugs or other inputs, and sample chip effluent. Each Pod reservoir holds up to 4 mL.



Zoë Culture Module

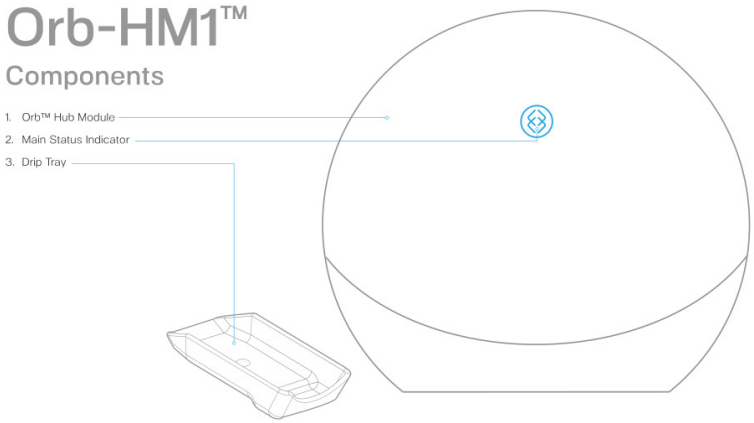
The Zoë Culture Module is designed to sustain the life of cells within Emulate's Organ-Chips. It provides the dynamic flow of media and the mechanical forces that help recreate the microenvironment cells experience *in vivo*. The instrument automates the precise conditions needed for simultaneous cell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

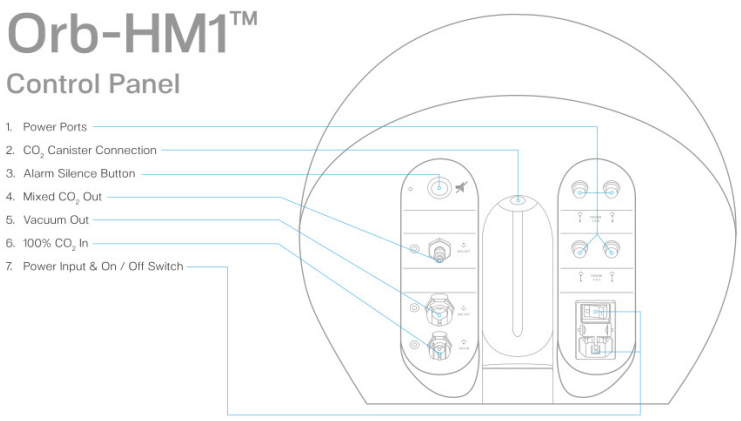
Zoë enables independent control of the flow rate of media through the top and bottom channels of the Organ-Chips, as well as stretch parameters — including frequency and amplitude — of the Organ-Chip. Additionally, Zoë has automated algorithms to prime the fluidic channels of Pods with media, and programming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



Orb Hub Module

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





Cells

The Liver Bio-Kit includes pre-qualified primary human liver cells. Cells are shipped in a cryogenic storage vacuum flask and must be stored in liquid nitrogen until use.

The Liver Tri-Culture Bio-Kit includes the following cells:

Parenchymal epithelial cells (top channel):

- Hepatocytes

Non-parenchymal cells (NPC) (bottom channel):

- Liver sinusoidal endothelial cells (LSECs)
- Kupffer cells

Media and Gas Equilibration

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

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

Experimental Overview

Workflow Overview

Day X: Reagent Preparation
- Aliquot reagents (media supplements, ECM, Matrigel, etc.)

Day -2: Thaw LSECs
- Prepare LSEC culture medium and flask
- Thaw and plate LSECs

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: Hepatocytes to Chips
- Prepare hepatocyte seeding medium
- Prepare chips
- Thaw hepatocytes
- Adjust cell density
- Seed hepatocytes to top channel
- Seed a well plate
- Gravity wash chips (3 hours post-seeding)

Day 1: Hepatocyte Overlay
- Prepare overlay medium
- Overlay hepatocytes

Day 2: Non-Parenchymal Cells (NPC) to Chips
- Prepare NPC seeding medium
- Wash chips
- Harvest LSECs
- Thaw Kupffer cells
- Combine NPC mixture
- Seed non-parenchymal cells (LSECs and Kupffer cells) to bottom channel
- Gravity wash with tips (4 hours post-seeding)

Day 3: Chips to Pods and Pods to Zoë
- Gas equilibration of media
- Prime Pods

- Wash chips
- Chips to Pods
- Pods to Zoë

Day 4+: 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
Human Liver Bio-Kit	Tri-Culture 12- or 24-pack	Emulate	-
Zoë CM-1™ Culture Module	1 per 12 chips	Emulate	-
Orb-HM1™ Hub Module	1 per 4 Zoës	Emulate	-
Chip-S1™ Stretchable Chip	12 per Zoë	Emulate	-
Pod™ Portable Modules	1 per Chip-S1	Emulate	-
UV Light Box	1 per Zoë	Emulate	-
Chip Cradle	Autoclaved, 1 per 6 chips	Emulate	-
Steriflip®-HV Filters	0.45 µm PVDF filter sterile	EMD Millipore	SE1M003M00
150 mm cell culture dish	Sterile, 1 per 6 chips	Corning / Falcon	353025
Collagen type-1 coated plates	24 well, flat-bottom TC-treated	Corning	356408
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	-	-	-
Parafilm®	-	-	-

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	5 mg powder	Emulate	-
ER-2	25 mL bottle	Emulate	-
Dulbecco's PBS (DPBS - /-) (without Ca^{2+}, Mg^{2+})	1X	Corning	21-031-CV
10X DPBS (- / -) (without Ca++, Mg++)	10X	Corning	20-031-CV
Trypan blue	0.4% solution	Sigma	93595
Percoll® solution	100% stock solution	Sigma	P4937
Trypsin-EDTA solution	0.05% trypsin	Sigma	T3924
WEM medium (+)	Williams' medium E with phenol red (+)	Sigma	W4128
WEM medium (-)	Williams' medium E no phenol red (-)	Sigma	W1878
CSC medium (Kit)	LSEC medium & supplements	Cell Systems	4Z3-500
Culture boost™	50X supplement	Cell Systems	4CB-500
Attachment Factor™	1X	Cell Systems	4Z0-210
Cell freezing medium	1X	Cell Systems	4Z0-705
Matrigel®	LDEV-free	Corning	354234
Fibronectin	Bovine protein, plasma	ThermoFisher	33010-018
Collagen type I	Rat tail; HC	Corning	354249

Penicillin-streptomycin	10,000 U/mL; 10 mg/mL	Sigma	P4333
L-GlutaMax™	200 mM	ThermoFisher	35050-061
L-Ascorbic Acid	100 mg powder	Sigma	5960
Dexamethasone	100 mg Powder	Sigma	D4902
Fetal bovine serum (FBS)	Sterile heat-inactivated	Sigma	F4135 or F8317
ITS+	Premix supplement	Corning	354352
Fungin™	10 mg / mL	InvivoGen	ANT-FN-1

Notes for ER-1 and ER-2

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

Notes for Fungin

- Addition of 0.1% of Fungin to any Liver-Chip culture medium is essential to prevent fungal contaminations.
- 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. Never store in dry ice or -80°C freezer.

Chip Handling Techniques

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

When pipetting to fill each channel, 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.

Liver-Chip Culture Protocol

Day X: Reagent Preparation

Aliquot Reagents

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

Fibronectin (ECM)

Reagent	Conc. [Stock]	Amount	Volume	Solvent
Fibronectin	1 mg / mL	1 mg	1 mL	Cell culture grade water

- Resuspend 1 mg fibronectin in 1 mL of cell culture grade water according to manufacturer's instructions.
- Aliquot to single-use volumes and store at -20°C.

Matrigel (Overlay)

Reagent	Amount	Volume
Matrigel	5 mg per aliquot	Varies per lot

Matrigel bottle must be thawed overnight on slushy ice in a 2°C to 6°C refrigerator (in the back), or cold room and handled on slushy ice at all times. (Do not only use ice — add some water to make it slushy, as this solution gels rapidly at temperatures above 10°C.) Use pre-chilled pipettes, tips, and tubes at -20°C prior to aliquoting.

- After thawing, aliquot Matrigel to 5 mg aliquots based on the specific stock concentration. For example, if the stock concentration is 10 mg / ml, 500 µL of solution will yield 5 mg of Matrigel.
- Store aliquots at -20°C.

Culture Supplements

Reagent	Conc. [Working]	Volume	Solvent
L-Ascorbic acid	50 mg / mL	Calculate based on amount measured	Cell culture grade water
Dexamethasone	10 mM	Calculate based on amount measured	Cell culture grade DMSO
Dexamethasone	1 mM	Calculate based on amount measured	Cell culture grade DMSO

- Resuspend each supplement to the working concentration in the table above.
- Aliquot each supplement to single-use volumes.
- Store aliquots at -20°C.

Day -2: Thawing Liver Sinusoidal Endothelial Cells (LSECs)

Goals:

- Expand LSECs in flask prior to chip seeding

Key Steps:

- Prepare LSEC culture media and flask
- Thaw and plate LSECs

Required Materials:

- Complete LSEC culture medium (at 37°C)
- 15 mL conical tube
- Attachment Factor™
- T-75 flask
- Serological pipettes
- Pipettes and tips
- Aspirator
- Centrifuge
- 70% ethanol

Prepare LSEC Culture Media and Flask

LSEC Culture Media

Base LSEC Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
CSC basal medium	485 mL	-	-	Cell Systems	4Z3-500
Culture-boost	10 mL	-	2%	Cell Systems	4CB-500
Pen / strep	5 mL		1%	Sigma	P4333

- Store at 4°C.
- Use within 30 days of preparation.

Complete LSEC Culture Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base LSEC Culture Medium	45 mL	-	-	Recipe Above	-
FBS	5 mL	-	10%	Sigma	F4135

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

Prepare Flask

1. Warm sufficient amount of Complete LSEC Culture Medium and Attachment Factor™ to 37°C.
2. Label the culture flask with relevant information (e.g., cell type, passage number, date, initials).
3. Pipette Attachment Factor onto growth surface of flask ensuring full coverage. (5 mL of Attachment Factor is used per T75 flask.)
4. Place prepared flask into 37°C incubator to coat surface and maintain temperature until plating cells.

Thaw and Plate LSECs

LSECs are the only cells in the tri-culture Liver-Chip that require prior plating and expansion before seeding in the chip.

Thawing and Maintaining Cells

1. 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.
2. Spray vial(s) with 70% ethanol and wipe dry prior to placing them in the BSC.
3. Immediately transfer the contents of the vial into 3 mL of warm Complete LSEC Culture Medium in a sterile 15 mL conical tube.
4. Rinse the vial with 1 mL of Complete LSEC Culture Medium and collect in the 15 mL tube.
5. Bring the volume to 15 mL with Complete LSEC Culture Medium.
6. Centrifuge 200 x g for 5 minutes at room temperature.
7. Aspirate and discard supernatant, leaving approximately 100 µL of medium covering the pellet.
8. Loosen the cell pellet by gently flicking the tube.
9. Re-suspend cells in 15 mL of Complete LSEC Culture Medium.
10. Aspirate and discard excess Attachment Factor from the T75 flask that was prepared earlier. Note: Rinsing and / or drying the flask prior to adding cells is not necessary.
11. Add the LSEC suspension to the freshly coated T75 flask.
12. Incubate overnight at 37°C and 5% CO_2.
13. Exchange with fresh Complete LSEC 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 a mixture of collagen I and fibronectin 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)
- ER-1 powder
- ER-2 solution
- 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

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.

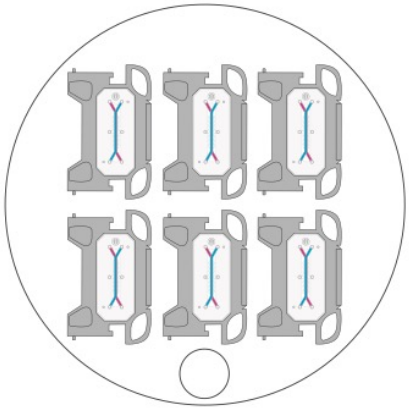


Figure 1: Proper orientation of Organ-Chips in 150 mm dish

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 ER-1 and ER-2 to equilibrate to room temperature before use (Approximately 10 to 15 minutes).
2. Wrap an empty sterile 15 mL conical tube with foil to protect it from light.
3. In the BSC, remove the small vial of ER-1 powder from the packet, and briefly tap the vial to concentrate the powder at the bottom.
4. Add 1 mL of ER-2 to the vial, and transfer contents directly to the bottom of the 15 mL conical tube. Do not pipette to mix. Note: The color of the solution transferred to the conical tube will be deep red.
5. Add an additional 1 mL of ER-2 to the ER-1 vial to collect any remaining material, and transfer the solution directly to the 15 mL conical tube. Note: The color of the transferred ER-1 solution will become lighter with each additional wash of the ER-1 bottle.
6. Repeat Step 5 twice more, with an additional 1 mL of ER-2 each time.

7. On the last addition of 1 mL ER-2 to the ER-1 bottle, cap the bottle and invert to collect any remaining ER-1 powder from the lid. Transfer the collected solution to the conical tube, bringing the total volume in the tube to 4 mL ER-1 solution.
8. Add 6 mL of ER-2 solution to the 4 mL of ER-1 solution in the 15 mL conical tube (for a final working concentration of 0.5 mg / mL). Pipette gently to mix without creating bubbles. ER-1 should be fully dissolved within the ER-2 solution prior to use.

Introduce ER-1 Solution to Channels

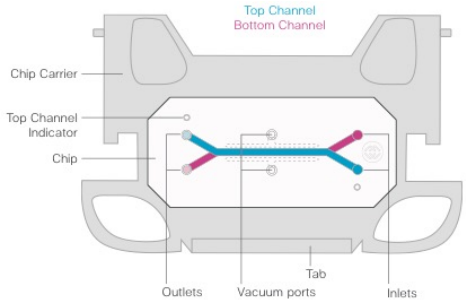


Figure 2: Top view of chip, with labelled ports, in chip carrier

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.

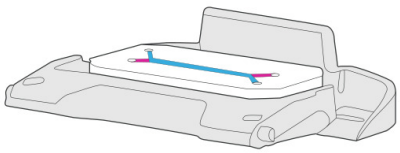


Figure 3: View of chip with no excess ER-1 solution on surface

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 DPBS to the final working concentrations. The ECM solution will be used to coat both the top and bottom channels.

For human Liver-Chips, the 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.
    1. Volume required per chip = approximately 100 µL
    2. 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 example 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 Fig. 4 below.)
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.

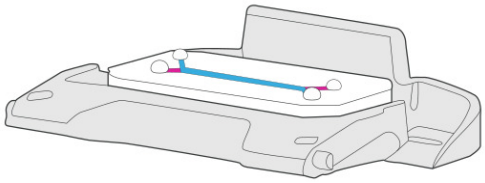


Figure 4: Chip in chip carrier with small droplets of ECM solution at ports

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

Day 0: Hepatocytes to Chip

Goals:

- Seed hepatocytes in chip

Key Steps:

- Prepare hepatocyte seeding medium
- Prepare chips
- Thaw hepatocytes
- Adjust cell density
- Seed hepatocytes to top channel
- Seed a well plate
- Gravity wash chips (3 hours post-seeding)

Required Materials:

- Complete hepatocyte seeding medium (at 37°C)
- Percoll solution (at room temperature)
- 10X DPBS (at room temperature)
- 1X DPBS (at room temperature)
- Serological pipettes
- Pipettes and filtered tips
- Aspirator and sterile tips
- 50 mL conical tubes
- Diluted trypan blue counting solution
- Hemocytometer
- 24-well collagen I-coated plate
- 70% ethanol
- Microscope

Hepatocyte Seeding Media

Base Hepatocyte Seeding Medium (500 mL bottle)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
WEM + (with phenol red)	490 mL	-	-	Sigma	W4128
Pen / Strep	5 mL	-	1%	Sigma	P4333
L-GlutaMAX	5 mL	-	1%	Gibco	35050-61

- Store at 4°C.
- Use within 30 days of preparation.

Complete Hepatocyte Seeding Medium (200 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base Hepatocyte Seeding Medium	187.78 mL	-	-	Recipe Above	-
ITS+ premix	2 mL	-	1%	Corning	354352
Ascorbic acid	200 µL	50 mg /mL	0.05 mg / mL	Sigma	5960
Dexamethasone	20 µL	10 mM	1 µM	Sigma	D4902
FBS	10 mL	-	5%	Sigma	F4135

- Store at 4°C.
- Use within 3 days of preparation.

90% Percoll Solution

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Percoll solution	18 mL	100%	90%	Sigma	P4937
10X DPBS (- / -)	2 mL	100%	10%	Corning	20-031-CV

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

Trypan Blue Cell Counting Solution

Reagent	Volume	Source	Cat. No.
Complete Hepatocyte Seeding Medium	400 µL	Recipe Above	-
Trypan blue	50 µL	Sigma	93595

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

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 Hepatocyte Seeding 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 Hepatocyte Seeding 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.

Thaw Hepatocytes

Prior to thawing cryopreserved hepatocytes, ensure all equipment and materials are ready to use, organized, and that all required reagents are prepared and are at the appropriate temperature.

If needed, up to 3 vials of cryopreserved hepatocytes can be thawed at the same time. The contents of each of the 3 cryovials should be combined in the same 50 mL conical tube (step 6), and further processed as one sample.

When thawing cells, it is critical to work as quickly but gently as possible to maximize viable cell recovery and minimize damage to the hepatocytes.

Do not allow cells to thaw at room temperature or on ice. Once hepatocytes are thawed, it is critical that they are diluted in cell culture medium as soon as possible to prevent toxicity from DMSO within the cryoprotectant.

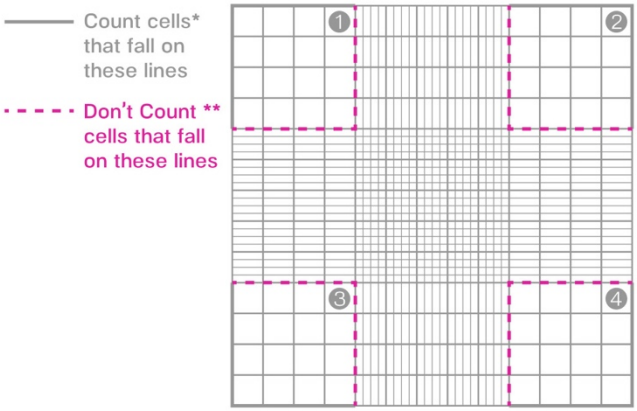
1. Place 3 mL of warm Complete Hepatocyte Seeding Medium into a sterile 50 mL conical tube.
2. Remove required number of vials of cryopreserved hepatocytes from liquid nitrogen.
3. Spray the cryovial with 70% ethanol and wipe dry. Briefly twist the cap a quarter turn to relieve any internal pressure, then re-tighten. Note: Adjusting the cap in this manner will prevent potential popping of the cryovial as a result of the rapid expansion of any liquid nitrogen that may have been trapped inside the vial.

4. Immediately place the frozen vial in a 37 °C water bath, without submerging the cap. Rapidly thaw hepatocytes by gently swirling the vials in the water bath until only a small ice pellet remains. This process should take only 60 to 90 seconds — thawing at 37°C for longer time periods will result in decreased viability and cell yield.
5. Once only a small ice pellet remains, immediately remove the vial from water bath, wipe dry, spray with 70% alcohol, and wipe dry again before placing in the BSC.
6. Quickly transfer the contents of the vial into the 3 mL of Complete Hepatocyte Seeding Medium in the 50 mL tube prepared in Step 1.
7. Rinse the cryovial with 1 mL of Complete Hepatocyte Seeding Medium, and transfer to the 50 mL conical tube.
8. With gentle agitation and swirling, slowly and gradually add sufficient Complete Hepatocyte Seeding Medium to bring the total volume to 35 mL.
9. Add 15 mL of 90% Percoll solution to bring up the total volume to 50 mL.
10. Cap the tube tightly and gently invert 3 times to mix the cell solution.
11. Centrifuge cells at 96 x g for 6 minutes at room temperature.
12. Return the tube to the BSC and carefully aspirate the supernatant, leaving 3 to 5 mL. Ensure the pellet remains undisturbed.
13. Tilt and rotate the tube to gently re-suspend the cell pellet in the remaining medium.
14. Gently add sufficient Complete Hepatocyte Seeding Medium to bring up the total volume to 50 mL.
15. Centrifuge the cells at 72 x g for 4 minutes at room temperature.
16. Return the tube to the BSC and carefully aspirate the supernatant, leaving 1 to 2 mL. Ensure the pellet remains undisturbed.

Adjust Cell Density

Human hepatocytes seeded in the Liver-Chip must be seeded at a density of 3.5 x 10^6 cells / mL. An accurate seeding density is critical to ensure viable and functional cells within the Liver-Chip for long-term culture.

1. Tilt and rotate the tube to gently resuspend the cell pellet.
2. Measure the total suspension volume using a 5 mL pipette.
3. After sufficient rotation to homogenize the cell suspension, transfer 50 µL of the cell suspension to the trypan blue cell counting solution, generating a 1:10 dilution.
4. Mix the trypan blue solution thoroughly and count cells using a 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)

Dilute the hepatocytes with warm Complete Hepatocyte Seeding Medium to the required final cell density of 3.5 x 10^6 cells / mL. Note: If the viable cell concentration is less than 3.5 x 10^6 cells / mL, allow the hepatocyte cell suspension to sit undisturbed at room temperature for at least 5 minutes for the cells to settle to the bottom of the tube. Once settled, very gently remove enough volume from the top of the supernatant to decrease the total cell suspension volume. Recount the cell suspension and recalculate

the appropriate seeding density accordingly (steps 5 and 6 above). This is to avoid additional centrifugation steps, as hepatocytes are sensitive to mechanical stress.

Seed Hepatocytes to Top Channel

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

1. Bring the 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. Very gently agitate cell suspension before seeding each chip to ensure a homogeneous cell suspension.
4. Quickly and steadily pipette 35 to 50 µL of the cell suspension (at 3.5 x 10^6 cells / mL) into the top channel inlet port, while aspirating the outflow fluid from the chip surface. Avoid direct contact with the outlet port.
5. Cover the dish and transfer to the microscope to check the seeding density within the chip. Note: Cells at the optimal seeding density at this stage will form an even cell layer with a distance between individual cells that is about half of a cell radius. (See Figure 5 below.)
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 3 hours, or until cells have attached. Note: Correct seeding density is essential for success of the chips

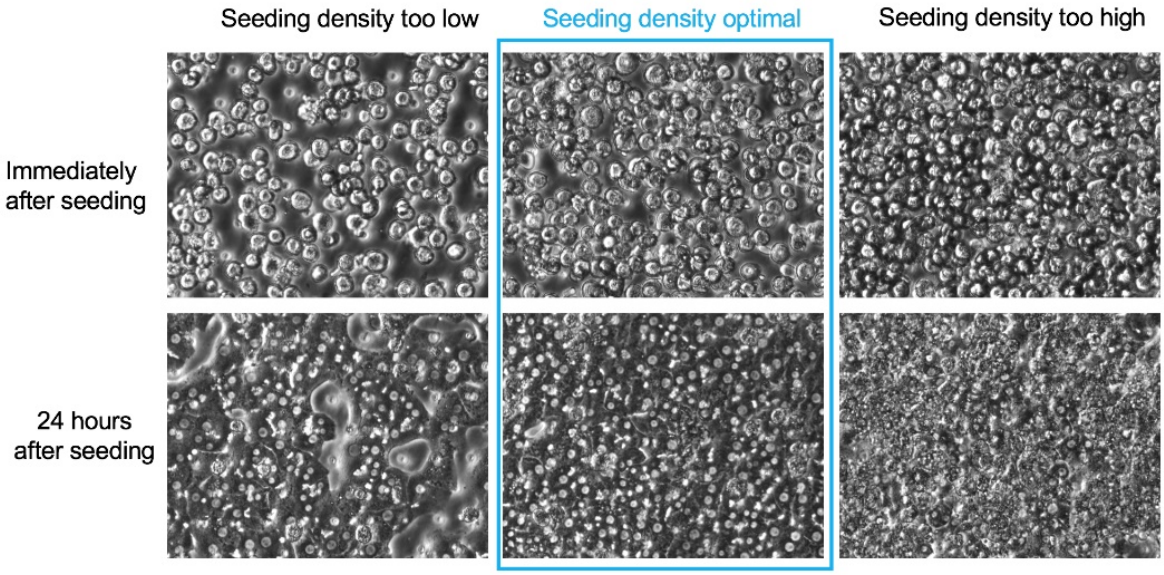


Figure 5: Hepatocyte seeding density reference chart

Seed a Well Plate

It is recommended to always seed any remaining hepatocytes into a plate as control for cell quality. Hepatocytes are seeded on a 24-well plate pre-coated with collagen I.

1. Further dilute hepatocyte suspension with warm Complete Hepatocyte Seeding Medium to a final cell density of 0.8 x 10^6 cells / mL.
2. Add 400, 500, and 600 µL of the cell suspension to 3 separate wells of the 24-well plate.
3. Mix each well to ensure an even suspension, and allow the cells to settle for 5 minutes on the microscope stage with the light off. Inspect densities under the microscope.
4. Determine which well depicts the optimal seeding density — either the well containing 400, 500, or 600 µL of the cell suspension — and plate the remaining cells using that volume into individual wells of the 24-well plate.
5. In the incubator, disperse the cells evenly across the bottom of the culture wells by moving the plate in a figure eight motion across the shelf at least 3 times, while keeping the plate flat on the surface of the incubator. Finally, move the plate in a crisscross pattern to evenly disperse the cells at least 3 times. Once the cells are dispersed, do not disturb the plate until the next day to allow for cells to fully attach.

Gravity Wash

A gentle gravity wash is performed 3 hours post-seeding, after the hepatocytes 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 Figure 6).
4. Incubate chips overnight at 37°C.

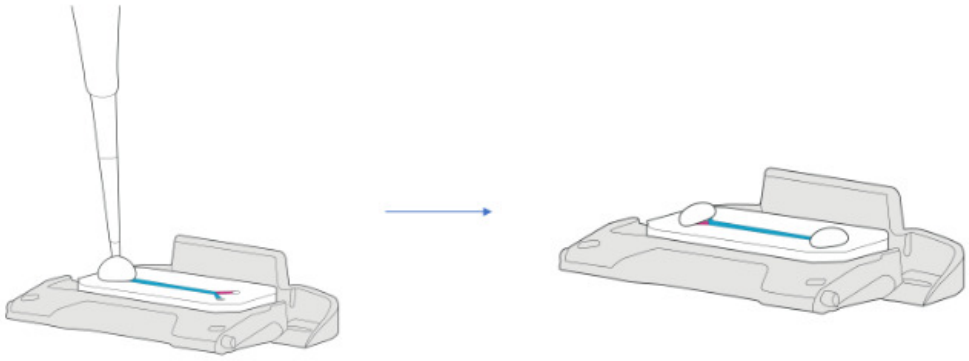


Figure 6 Chip with medium drops covering the inlet and outlet ports

Day 1: Hepatocyte Overlay

Key Steps:

- Prepare hepatocyte maintenance media
- Prepare overlay medium
- Overlay hepatocytes

Required Materials:

- Complete Hepatocyte Maintenance Medium (at 4°C)
- Complete Hepatocyte Maintenance Medium (at 37°C)
- Hepatocyte Overlay Medium (at 4°C)
- Matrigel aliquot (at 4°C in slushy ice)
- 50 mL conical tube
- Serological pipettes
- Pipettes and filtered tips
- Aspirator and sterile tips
- Ice bucket and ice
- 70% ethanol
- Microscope

Prepare Hepatocyte Maintenance Media

Hepatocyte Maintenance Media

Base Hepatocyte Maintenance Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
WEM (without phenol red)	490 mL	-	-	Sigma	W4128
Pen / Strep	5 mL	100X	1%	Sigma	P4333
L-GlutaMAX	5 mL	100X	1%	Gibco	35050-61

- Store at 4°C.
- Use within 30 days of preparation.

Complete Hepatocyte Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base Hepatocyte Maintenance Medium	49.445 mL	-	-	Recipe above	-
ITS+ premix	500 µL	-	1%	Corning	354352
Ascorbic acid	50 µL	50 mg/mL	50 µg/mL	Sigma	5960
Dexamethasone	5 µL	1 mM	100 nM	Sigma	D4902

- Store at 4°C in well-labeled tubes.
- Use within 3 days of preparation.

Prepare Overlay Medium

Matrigel aliquot must be thawed slowly on slushy ice for 30+ minutes and kept in slushy ice at all times, as this solution gels rapidly at temperatures above 10°C. (Do not use ice alone — you must add some water to make it slushy.) In order to maintain even coating, use pre-chilled pipettes, tips, and tubes stored at -20°C prior to use and cold medium during medium preparation and overlay.

Hepatocyte Overlay Medium (20 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium	19.5 mL	-	-	Recipe Above	-
Matrigel	0.5 mL	10 mg/mL	0.25 mg/mL	Corning	354234

- Keep both Matrigel aliquot and Hepatocyte Overlay Medium on slushy ice.

1. Prepare Hepatocyte Overlay Medium by diluting ice cold thawed Matrigel into ice cold Complete Hepatocyte Maintenance Medium using cold tips, at a final concentration of 250 µg / mL, as directed above.

2. Mix well and keep the Hepatocyte Overlay Medium on ice at all times.

   Note: Solidified Matrigel introduced to the chip channels can cause uneven distribution and may impact cell viability and chip performance. If any traces of solidified Matrigel are observed in the Hepatocyte Overlay Medium, discard and prepare new medium following the steps above, ensuring all reagents and equipment are kept at 4°C during this step.

Overlay Hepatocytes

In Chips

1. In the BSC, quickly pipette 200 µL of warm Complete Hepatocyte Maintenance Medium through the top channel of each chip to remove any cell debris from the hepatocyte monolayer.
2. Aspirate the media outflow at the outlets, leaving media within the channel.
3. Gently pipette 200 µL of the cold Hepatocyte Overlay Medium using cold tips to the top channel of each chip, leaving droplets of Hepatocyte Overlay Medium covering both the inlets and outlets (see Figure 7).
4. Incubate the chips overnight at 37°C.

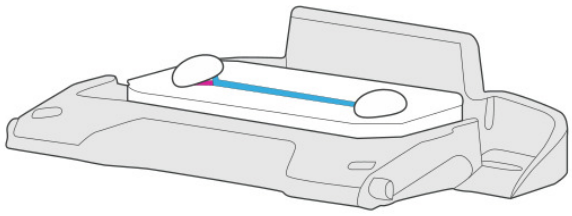


Figure 7 Chip with Hepatocyte Overlay Medium covering ports

In Well Plates

1. In the BSC, swirl the 24-well plate of hepatocytes with a vigorous motion to release any cell debris and unattached cells from the monolayer.
2. Aspirate the medium from each well.
3. Add 500 µL of cold Hepatocyte Overlay Medium to each well and return to the incubator overnight.

Day 2: Non-Parenchymal Cells (NPC) to Chip

Goals:

- Seed mixture of non-parenchymal cells (NPC: LSECs and Kupffer) in chip

Key Steps:

- Prepare NPC seeding medium
- Wash chips
- Harvest LSECs
- Thaw Kupffer cells
- Combine NPC mixture
- Seed NPC mixture to bottom channel
- Gravity wash with tips

Required Materials:

- NPC Seeding Medium (at 37°C)
- NPC Seeding Medium (at 4°C)
- Complete Hepatocyte Maintenance Medium (at 37°C)
- Trypan blue counting solution
- Hemocytometer
- 15 mL conical tubes
- Serological pipettes
- Pipettes and filtered tips
- Aspirator and sterile tips
- Ice bucket and ice
- 70% ethanol
- Microscope

Prepare NPC Seeding Medium

The LSECs and Kupffer cells are seeded in the bottom channel in NPC Seeding Medium.

NPC Seeding Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium omitting dexamethasone	22.5 mL	-	-	-	-
Base LSEC Culture Medium	22.5 mL	-	-	-	-
FBS	5 mL	-	10%	Sigma	F4135

- Store at 4°C.
- Use within 3 days of preparation.

Trypan Blue Cell Counting Solution

Reagent	Volume	Source	Cat. No.
NPC Seeding Medium	40 µL	Recipe Above	-
Trypan blue	5 µL	Sigma	93595

- Maintain at room temperature prior to use.
- Prepare 3 tubes of cell counting solution
- Prepare fresh for each use.

Wash Chips

1. Gently pipette 200 µL of warm Complete Hepatocyte Maintenance Medium to the top channel of each chip to wash. Aspirate the outflow, leaving media in the channel.
2. Pipette 200 µL of warm NPC Seeding Medium to the bottom channel of each chip. Aspirate the outflow, leaving media in the channel.
3. Return chips to the incubator until NPCs are ready for seeding.

Harvest LSECs

LSECs in culture must be harvested and counted for bottom channel seeding. LSECs are adjusted to a density of 6 x 10^6 cells / mL (2 times the final seeding concentration) prior to combining with Kupffer cells to generate the bottom channel co-cell mixture. If the LSECs are not as proliferative as expected, the concentration can be increased from up to 8 x 10^6 cells / mL (2 times the final seeding concentration), in order to achieve a confluent monolayer within the channel.

1. Bring the culture flask containing LSECs from the incubator into the BSC.
2. Aspirate culture media and add 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 NPC Seeding 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 NPC Seeding Medium to bring the total volume of the tube to 15 mL.
8. Centrifuge LSECs at 200 x g for 5 minutes at room temperature.
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 cold NPC Seeding 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).
13. Mix the counting solution thoroughly and count cells using a manual hemocytometer. (See Cell Counting and Viability Assessment notes below.)
14. Dilute the LSECs to 6 x 10^6 cells / mL (2 times the final seeding concentration) in cold NPC Seeding Medium.
15. Keep the LSEC cell suspension on ice until the rest of the cells (Kupffer cells) are ready.

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)

Thaw Kupffer Cells

Kupffer cells must be thawed and counted for bottom channel seeding. Kupffer cells are adjusted to a density of 1 x 10^6 cells / mL prior to combining with LSECs to generate the bottom channel co-cell mixture.

Kupffer cells are very sticky at physiological temperature of 37°C. If the medium is warmed to 37°C, the Kupffer cells will attach to any substrate including the walls of the conical tube and pipette tip. Therefore, use cold NPC Seeding Medium and pre-chilled tips throughout the thawing process.

1. Place 3 mL of cold NPC Seeding Medium into a sterile 15 mL conical tube on ice.
2. Remove required number of vials of cryopreserved Kupffer cells from liquid nitrogen.
3. Spray the cryovial with 70% ethanol and wipe dry. Briefly twist the cap a quarter turn to relieve any internal pressure, then re-tighten. Note: Adjusting the cap in this manner will prevent potential popping of the cryovial as a result of the rapid expansion of any liquid nitrogen that may have been trapped inside the vial.
4. Immediately place the frozen vial in a 37°C water bath, without submerging the cap. Rapidly thaw Kupffer cells by gently swirling the vials in the water bath until only a small ice pellet remains. This process should take only 60 to 90 seconds — thawing at 37°C for longer time periods will result in decreased viability and cell yields.
5. Once only a small ice pellet remains, immediately remove the vial from water bath, wipe dry, spray with 70% alcohol, and wipe dry again before placing in the BSC.
6. Quickly transfer the contents of the vial into the 3 mL of cold NPC Seeding Medium in the 15 mL tube on ice prepared in Step 1.
7. Rinse the cryovial with 1mL of cold NPC Seeding Medium, and transfer to the 15 mL conical tube.
8. Bring the volume to 15 mL with cold NPC Seeding Medium.
9. Centrifuge Kupffer cells at 500 x g for 5 min at 4 °C.
10. Carefully aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet. Note: The cell pellet will be very small. Aspirate carefully.
11. Loosen the cell pellet by flicking the tube gently.
12. Using a P1000 pipette, gently resuspend the cells by adding 200 µL of cold NPC Seeding Medium.
13. Pipette gently to create a homogeneous mixture, and transfer 5 µL of the cell suspension to the trypan blue cell counting solution (1:10 dilution).
14. Mix the counting solution thoroughly and count cells using a manual hemocytometer. (See Cell Counting and Viability Assessment notes below.)
15. Dilute the Kupffer cells to 2 x 10^6 cells / mL (2 times the final seeding concentration) in cold NPC Seeding Medium and keep on ice until 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)

Combine NPC Mixture

With all two cell types prepared and at the proper density, prepare the cell suspension mixture that will be seeded into the bottom channel of the chip.

The final density of each cell type in the bottom channel will be:

LSECs: 3 x 10^6 cells / mL

Kupffer cells: 0.5 x 10^6 cells / mL

1. Mix the three NPC cell suspensions in a 1:1 ratio (v/v/v) in a sterile 15 mL conical tube on ice. Ensure that enough seeding solution is prepared to seed all chips.

Seed NPC Mixture 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. Seed 15 to 20 µL of the combined NPC cell suspension into the bottom channel, while aspirating the outflow.
2. After seeding, 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 8).
3. 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.
4. Place the chips still in the dish in the 37°C incubator for approximately 4 hours, or until cells in the bottom channel have attached.

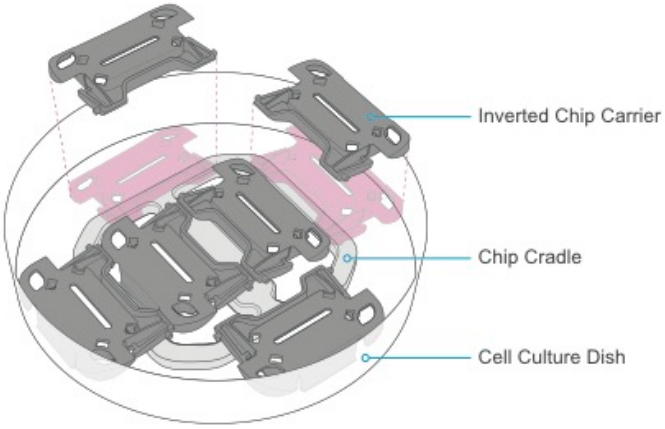


Figure 8: Inverting chips during endothelial attachment

Prepare NPC Maintenance Medium

NPC Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium omitting dexamethasone	24.5 mL	-	-	-	-
Base LSEC Culture Medium	24.5 mL	-	-	-	-
FBS	1 mL	-	2%	Sigma	F4135

- Store at 4°C.
- Use within 3 days of preparation.

Gravity Wash with Tips

1. Once the NPCs have attached (approximately 4 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.
2. A gravity wash with 200 µL of Hepatocyte Maintenance Medium for the top channel and NPC Maintenance 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 9).
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.

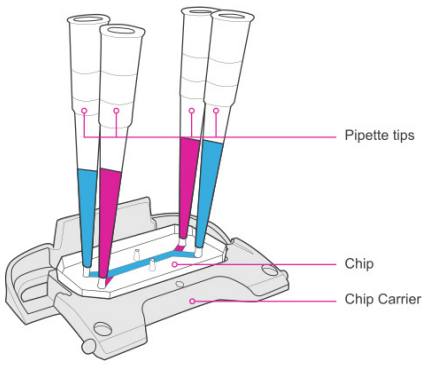


Figure 9: Chip with filtered tips inserted into ports with respective media

Day 3: 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:

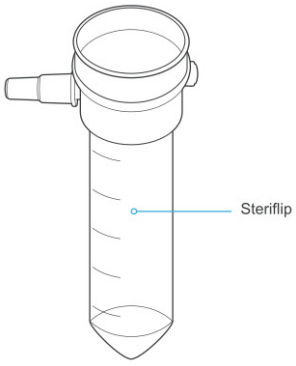
- Installed and qualified Zoë
- Prepared chips
- Pods (sterile) — 1 per chip
- Tray — 1 per 6 chips
- Steriflip filtration unit: PVDF filter 0.45 µm (sterile)
- Vacuum source (minimum -70 kPa)
- Serological pipettes
- Pipettors and filtered tips
- 37°C water or bead bath
- 70% ethanol

Gas Equilibration of Media

The media equilibration step is critical to the success of Organ-Chip culture. Omission of this step will cause the eventual formation of bubbles in the chip, the Pod, or both, which will in turn cause irregular flow and negatively impact cell viability. Minimize the time medium is outside — 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.

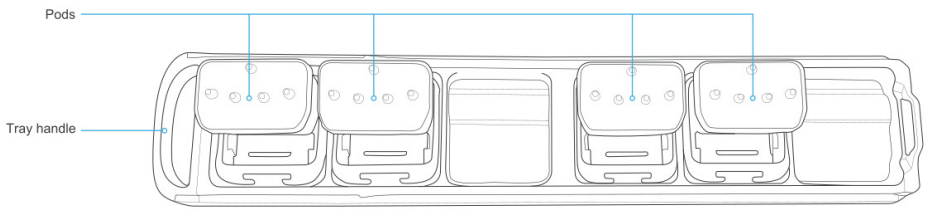
1. Place at least 3.3 mL of Complete Hepatocyte Maintenance Medium for each chip in a 50 mL conical tube.
2. Place at least 3.3 mL of NPC Maintenance Medium for each chip in a separate 50 mL conical tube.
3. Warm both 50 mL conical tubes of media at 37°C in a water or bead bath for at least 1 hour.
4. Immediately connect the 50 mL tube containing each warmed medium to a Steriflip unit.
    1. Attach each conical tube containing warmed medium to a Steriflip unit.
    2. With the unit "right-side-up" (medium in the bottom conical tube), apply vacuum for 10 seconds.
    3. 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.
    4. Leave the filtered medium under vacuum for 5 minutes.
5. Remove the vacuum tubing from the Steriflip units.
6. Separate the conical tubes containing media from the Steriflip unit and immediately place the conical tubes containing media in the incubator with the caps loose.

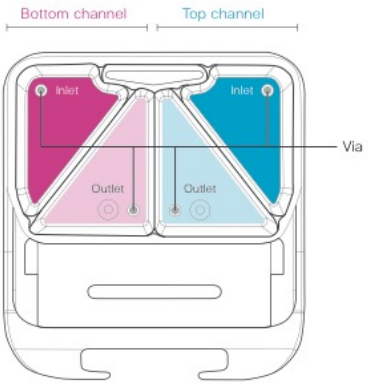
    As noted above, minimize the time media is outside of the incubator during Pod preparation to maintain temperature. This is a critical step to ensure success of the chips.

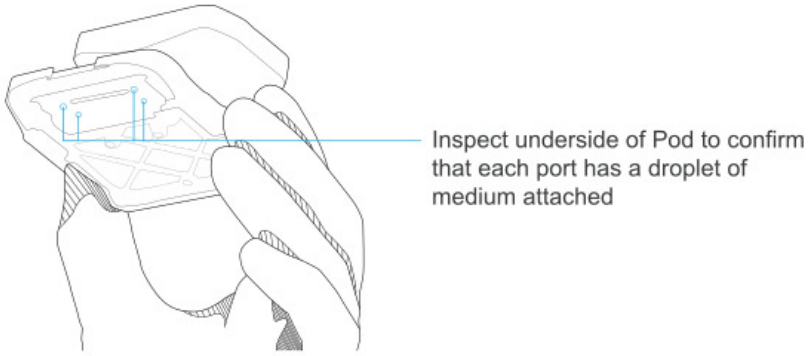


Prime Pods

1. Sanitize the exterior of the Pod packaging and trays with 70% ethanol, wipe, and transfer to the BSC.
2. Open the Pod package and place the Pods into the trays. Orient the Pods with the reservoirs toward the back of the tray.
3. Pipette 3 mL of pre-equilibrated, warm media to each inlet reservoir. In the top channel inlet reservoir add Complete Hepatocyte Maintenance Medium; in the bottom channel inlet reservoir add NPC Maintenance Medium.
4. Pipette 300 µL of pre-equilibrated, warm media to each outlet reservoir, directly over each outlet via.
5. Bring trays containing Pods to the incubator and slide completely into Zoë with the tray handle facing outward.
6. Run the Prime cycle on Zoë.
    1. Use the rotary dial to highlight “Prime” on the display.
    2. Press the rotary dial to select “Prime.”
    3. Rotate the dial clockwise to highlight “Start.”
    4. 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 critical for success.
    1. 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.
    2. If any Pod does not show droplets, re-run the “Prime” cycle on those Pods.
    3. If any media dripped onto the tray (this may occur more often by the outlet ports), clean tray with a wipe sprayed with 70% ethanol.
10. Once it is confirmed that all Pod ports are wet with droplets, put the tray of Pods to the side in the BSC.







Wash Chips

1. Transfer the seeded chips in a 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 Hepatocyte Maintenance Medium to remove any possible bubbles in the channel.
4. Place small droplets of equilibrated Complete Hepatocyte Maintenance 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 NPC Maintenance Medium to remove any possible bubbles in channel.
6. Place small droplets of equilibrated NPC Maintenance Medium on the bottom channel inlet and outlet ports of each chip.

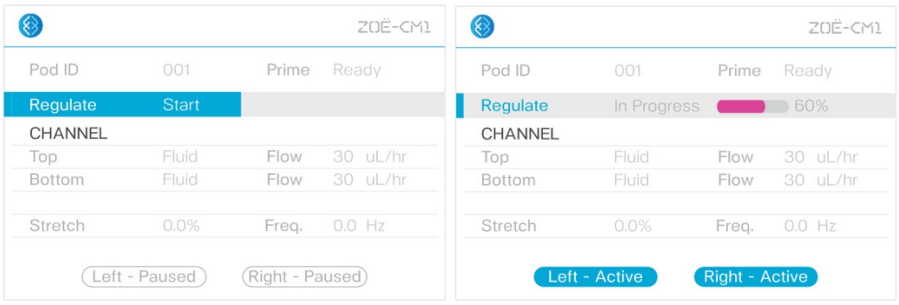
Chips to Pods

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

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 human tri-culture Liver-Chips, set the flow rate to 30 µL / hour for both top and bottom channels.
3. Run Regulate cycle.
   1. Using the rotary dial, highlight the “Regulate” field.
   2. Press the dial to select “Regulate,” and rotate the dial clockwise to “Start.”
   3. 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.
   4. 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.



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:

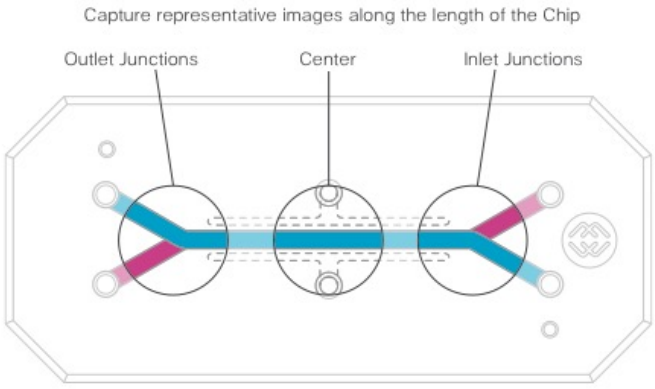
- Chips in Pods
- Cell culture media

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:
    1. 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.
    2. 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.
    1. Using the rotary dial, highlight the “Regulate” field.
    2. Press the dial to select “Regulate,” and rotate the dial clockwise to “Start”.
    3. 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.
    4. 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 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 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.



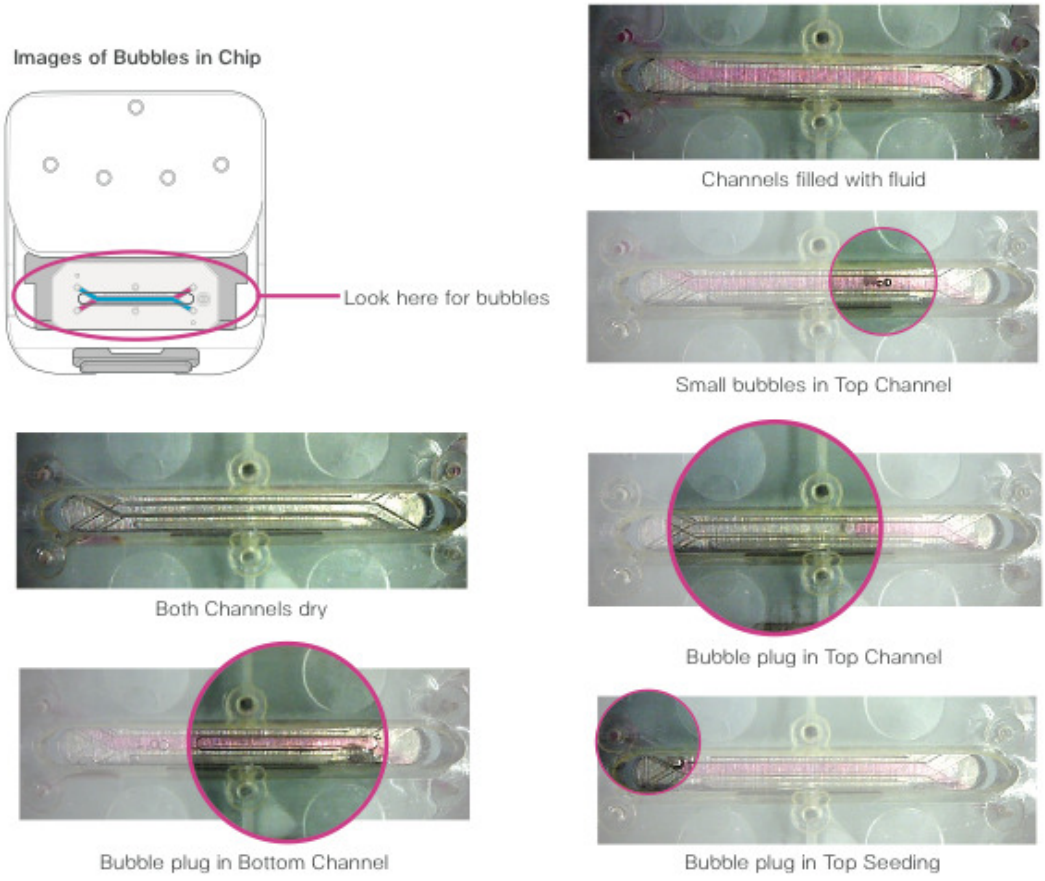
Troubleshooting

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

Potential Root Causes of Bubbles

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

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



Appendices

Reagent Aliquots

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

Fibronectin (ECM)

Reagent	Conc. [Stock]	Amount	Volume	Solvent
Fibronectin	1 mg / mL	1 mg	1 mL	Cell culture grade water

- Resuspend 1 mg fibronectin in 1 mL of cell culture grade water according to manufacturer's instructions.
- Aliquot to single-use volumes and store at -20°C.

Matrigel (Overlay)

Reagent	Amount	Volume
Matrigel	5 mg per aliquot	Varies per Lot

Matrigel must be thawed and handled at 4°C or in slushy ice at all times, as this solution gels rapidly at temperatures above 4°C. Use pre-chill pipettes and Eppendorf Tubes on ice or at -20°C prior to aliquoting.

- After thawing on slushy ice, aliquot Matrigel to 5 mg aliquots based on the specific stock concentration. For example, if the stock concentration is 10 mg / ml, 500 µL of solution will yield 500 mg of Matrigel.
- Store aliquots at -20°C.

Culture Supplements

Reagent	Conc. [working]	Volume	Solvent
L-Ascorbic Acid	50 mg / mL	Calculate based on amount measured	Cell culture grade water
Dexamethasone	10 mM	Calculate based on amount measured	DMSO
Dexamethasone	1 mM	Calculate based on amount measured	DMSO

- Resuspend each supplement to the working concentration in table above.
- Aliquot each supplement to single-use volumes.
- Store aliquots at -20°C.

Media Recipes

LSEC Culture Media

Base LSEC Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
CSC Basal Medium	485 mL	-	-	Cell Systems	4Z3-500
Culture-Boost	10 mL	-	2%	Cell Systems	4CB-500
Pen / Strep	5 mL	-	1%	Sigma	P4333

- Aseptically combine all media components in CSC Basal Medium bottle.
- Store at 4°C.
- Use within 30 days of preparation.

Complete LSEC Culture Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base LSEC Culture Medium	45 mL	-	-	Recipe Above	-
FBS	5 mL	-	10%	Sigma	F4135

- Store at 4°C in well-labeled tubes.
- Use within 7 days of preparation.

Hepatocyte Seeding Media

Base Hepatocyte Seeding Medium (500 mL bottle)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
WEM + (with phenol red)	490 mL	-	-	Sigma	W4128
Pen / Strep	5 mL	-	1%	Sigma	P4333
L-GlutaMAX	5 mL	-	1%	Gibco	35050-61

- Store at 4°C.
- Use within 30 days of preparation.

Complete Hepatocyte Seeding Medium (200 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base Hepatocyte Seeding Medium	187.78 mL	-	-	Recipe Above	-
ITS+ Premix	2 mL	-	1%	Corning	354352
Ascorbic Acid	200 µL	50 mg /mL	0.05 mg / mL	Sigma	5960
Dexamethasone	20 µL	10 mM	1 µM	Sigma	D4902
FBS	10 mL	-	5%	Sigma	F4135

- Store at 4°C.
- Use within 3 days of preparation.

90% Percoll Solution

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Percoll Solution	18 mL	100%	90%	Sigma	P4937
10X DPBS (-/-)	2 mL	100%	10%	Corning	20-031-CV

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

Trypan Blue Cell Counting Solution

Reagent	Volume	Source	Cat. No.
Complete Hepatocyte Seeding Medium	400 µL	Recipe Above	-
Trypan blue	50 µL	Sigma	93595

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

Hepatocyte Maintenance Media

Base Hepatocyte Maintenance Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
WEM - (without phenol red)	490 mL	-	-	Sigma	W4128
Pen / Strep	5 mL	100X	1%	Sigma	P4333
L-GlutaMAX	5 mL	100X	1%	Gibco	35050-61

- Store at 4°C.
- Use within 30 days of preparation.

Complete Hepatocyte Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base Hepatocyte Maintenance Medium	49.445 mL	-	-	Recipe Above	-
ITS+ Premix	500 µL	-	1%	Corning	354352
Ascorbic Acid	50 µL	50 mg / mL	50 µg / mL	Sigma	5960
Dexamethasone	5 µL	1 mM	100 nM	Sigma	D4902

- Store at 4°C in well-labeled tubes.
- Use within 3 days of preparation.

Hepatocyte Overlay Medium (20 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium	19.5 mL	-	-	Recipe Above	-
Matrigel	0.5 mL	10 mg / mL	0.25 mg / mL	Corning	354234

- Keep both Matrigel aliquot and Overlay Medium on slushy ice.

NPC Media

NPC Seeding Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium omitting dexamethasone	22.5 mL	-	-	-	-
Base LSEC Culture Medium	22.5 mL	-	-	-	-
FBS	5 mL	-	10%	Sigma	F4135

- Store at 4°C.
- Use within 3 days of preparation.

Trypan Blue Cell Counting Solution

Reagent	Volume	Source	Cat. No.
NPC Seeding Medium	40 µL	Recipe Above	-
Trypan blue	5 µL	Sigma	93595

- Maintain at room temperature prior to use.
- Prepare 3 tubes of cell counting solution
- Prepare fresh for each use.

NPC Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium omitting dexamethasone	24.5 mL	-	-	-	-
Base LSEC Culture Medium	24.5 mL	-	-	-	-
FBS	1 mL	-	2%	Sigma	F4135

- Store at 4°C.
- Use within 3 days of preparation.