Basic Research Kit Protocol

EP223 Rev. A

Basic Research Kit Protocol

Introduction

This protocol describes the general steps for using the Emulate Basic Research Kit

In this protocol

This protocol contains the parts listed below.

Part I: Optimization of the Culture Environment

Part I Overview

Introduction

This section describes the key areas of focus for optimizing the culture environment.

Contents

Optimization Overview

Overview

Introduction

When developing new Organ-Chip models, several culture parameters should be considered, as they provide important information about cell morphology, viability, and function.

Defining Successful Criteria

Users should define the essential criteria for successfully culturing specific cell types, and they should choose readouts that accurately assess the phenotypes of interest. These readouts are organ-specific and will help identify the best conditions for culturing cells on chips.

Examples of Successful Criteria

Some examples of success criteria parameters are:

A. *In vivo*-relevant cell morphology
B. Expression pattern of specific genes by RNA or protein by western blot or immunofluorescence staining
C. Cell functionality (cell-type-dependent)
D. Permeability values in acceptable range for specific cells and tracer
E. Correct cell polarization according to *in vivo* findings

Example: Bile Canaliculi Network

Both albumin secretion by effluent analysis and live staining of the bile canaliculi network allow users to assess hepatocyte-specific functionality and confirm polarization.

Example: Colon Intestine-Chip

For example, in the Colon Intestine-Chip, the two most important factors needed to confirm *in vivo*-related cytoarchitecture are identification of:

- cell-type-specific markers with immunofluorescence analysis
- expected polarization of cells

Parameters Typically Optimized

Overview

Introduction

A brief description of the parameters that are typically optimized is provided below.

Parameters

Cells

Overview

Users can apply cell types that they have previously used in their research, such as:

- cell lines
- primary cells
- induced Pluripotent Stem Cells (iPSCs)
- organoids

Organoids

Organoids complement Organ-on-a-Chip technology by providing a quality source of human cells containing the relevant types from a specific tissue at the appropriate ratios; furthermore, they can be isolated from patients with or without disease. Organ-Chips can be populated with either biopsy-derived or stem cell (iPS cell)-derived organoids.

Variability

When comparing phenotypes across multiple donors, users should keep in mind the inter-subject variability that is expected from human or patient-derived samples. Features such as age, lifestyle, and health status can play a vital role in the cells' proliferation and differentiation capacities.

Key Parameters

When culturing multiple cell types on a chip, the key cell-related parameters that users must optimize are:

A. Seeding Density
B. Seeding Order

Continued on next page

Cells, continued

Parameter 1: Seeding Density

Users must empirically determine the optimal seeding density for each cell type, taking into consideration the cells' size and proliferation rate. The table below shows optimal seeding densities for several organ models.

Starting Guidance:

Model	Cell Type	Cell Density for Seeding Chip
Liver-Chip (Quad-Culture)	Primary Human Hepatocyte	3.5 x 10^6 cells / mL
	Primary Liver Sinusoidal Endothelial Cells	3 x 10^6 cells / mL
	Kupffer Cells	2 x 10^6 cells / mL
	Stellate Cells	0.1 x 10^6 cells / mL
Kidney-Chip	Kidney PT Epithelial Cells	1 x 10^6 cells / mL
	Kidney endothelial cells	2-4 x 10^6 cells / mL
Colon Intestine-Chip	Colon organoid fragments	2-3 wells from a 24-well plate, per chip
	Colon Human Intestinal Microvascular Endothelial Cells (cHIMECs)	8-10 x 10^6 cells / mL
Duodenum Intestine-Chip	Small Intestine Human Intestinal Microvascular Endothelial Cells (siHIMECs)	8-10 x 10^6 cells / mL
Alveolus Lung-Chip	Human Primary Alveolar Epithelial Cells	1 x 10^6 cells / mL
	Human Primary Alveolar Endothelial Cells	5 x 10^6 cells / mL
Airway Lung-Chip	Human Primary (small/large) Airway Epithelial Cells	3 x 10^6 cells / mL

Parameter 2: Seeding Order

Optimal seeding order depends upon the specific cell type's requirement, as some cells benefit from a period of on-chip monoculture. For example:

- In the Liver-Chip, the primary hepatocytes are first seeded in the top channel, and the liver sinusoidal endothelial cells (LSECs) are seeded in the bottom channel two days later to allow for hepatocyte stabilization.
- The Colon Intestine-Chip allows for same-day seeding of intestinal endothelial cells (HIMECs) on the bottom channel, followed by intestinal organoids.

Extracellular Matrix (ECM)

Overview

An Extracellular Matrix (ECM) is required to ensure optimal cell performance within the Human Emulation System. The ECM is a thin layer of extracellular matrix proteins that provides a scaffold, anchoring the cells to the culture surface while regulating a cell's dynamic behavior.

Starting ECM Optimization

Users can either start with an ECM they have used previously, or they can select the appropriate ECM based on published *in vitro* or *in vivo* data. Once an ECM is selected, it is recommended that users optimize the specific ECM components and concentration that best support the viability, morphology, and function of the cells.

Note: Using an unoptimized ECM will lead to cell loss when flow is initiated, poor cell morphology, uneven coverage of the chip channels, and poor functionality.

ECM Optimization Steps

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

Step	Action
1	Select one to three of the most relevant ECM types.
2	Choose two concentrations—one high and one low—to test.
3	Make different combinations (see table below for example).
4	Select optimal ECM that supports cell morphology and function.

ECM Type and Test Conditions

ECM Type	Collagen I		Fibronectin		Matrigel	
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		✓		✓		
Test condition 7		✓		✓		✓

Media

Overview

Since the Chip-S1 experiences laminar flow, its top and bottom channels are fluidically independent. Selecting an optimal cell culture medium for each channel is crucial for a successful chip culture.

Getting Started Optimizing Media

To start, users can choose medium conditions for each channel based on what will work for each cell type. It is important to confirm that all cell types are viable and exhibit the expected phenotypes for the chosen media combination. This will help to prevent specific media components from interfering with cell performance in the adjacent channel (e.g., retinoic acid effects on proliferation and differentiation of multiple cell types).

In these cases, users need to optimize the best medium condition to support cells in the top and bottom channels simultaneously. Typically, users can:

- Test 1:1 mixture of two different medium conditions.
- Adjust the concentration of medium supplements (e.g., growth factors for specific cell type) to find an optimal culture medium condition.

Media Optimization for ALI Models

For models that require Air-Liquid Interface (ALI) culture, such as the Lung-Chip, only one medium composition provides nutrients for cells in both channels (e.g., medium on the vascular channel for Lung-Chip). Consequently, it is essential for successful ALI co-cultures to determine the specific medium composition that enables viability and proper phenotype of all cells on the chip.

Note: Media Storage and Preparation

Users should follow instructions from individual media suppliers for proper storage and preparation. Typically, it is recommended to first prepare 500 mL of base media. Afterwards, users should add the necessary supplements and prepare 50 mL of complete media for short-term use.

Mechanical Forces

Overview

As media flow through the chip, cells are exposed to mechanical forces via shear stress. The exact level of shear stress is determined by the flow rate and the channel dimensions. The flow rates for each channel can be independently set between 0-1000 µL / hour. (For most organ models, a flow rate between 20-200 µL / h is ideal). An increase in flow rate will cause an increase in the shear forces exerted on the cell monolayer.

Stretch

The Human Emulation System enables cells to experience additional dynamic forces via cyclic mechanical deformation of the chip's flexible material. Stretch parameters (i.e., amplitude and frequency) are entirely dependent upon the organ type and application; therefore, they must be optimized for the tissue of interest. Not all cell types require stretching, but some organ systems—such as the Colon Intestine-Chip and Alveolus Lung-Chip—benefit from stretch to replicate the motions of peristalsis and breathing.

Note on Mechanical Forces

If mechanical forces that are not physiologically relevant are applied to Organ-Chip models, cell viability and function may be negatively impacted.

Optimizing Time

Users should optimize the timing for introducing dynamic culture conditions. In some cases, flow can be initiated soon after cell attachment; in others, it may only be initiated after two days in culture. Similarly, stretch can be initiated or changed at different points in time depending on the organ type and application.

Continued on next page

Mechanical Forces, Continued

Examples of Key Parameters

Key experimental parameters for two example Organ-Chip models are listed below:

Parameters	Liver Quad-Culture	Intestine (Colon)
ECM (top channel)	Collagen I (100 µg / mL) and Fibronectin (25 µg / mL)	Matrigel™ (100 µg / mL) and Collagen IV (200 µg / mL)
ECM (bottom channel)	Collagen I (100 µg / mL) and Fibronectin (25 µg / mL)	Collagen IV (200 µg/mL) and Fibronectin (30 µg / mL)
Cell type and density in top channel	Hepatocytes: 3×10^6 cells / mL	Colon organoids: 2-3 wells of organoid culture per chip (from 24-well plate culture)
Cell type and density in bottom channel	Liver sinusoidal endothelial cells (LSEC): 3×10^6 cells / mL Stellate cells: 0.1×10^6 cells / mL Kupffer cells: 0.5×10^6 cells / mL	Human Intestinal Microvascular Endothelial cells (HIMEC): $8\text{-}10 \times 10^6$ cells / mL
Flow rate	30 µL / h	30 µL / h
Stretching	Not applied, as it is not physiologically relevant	Applicable - 10% strain, 0.15 Hz
Cell culture medium for top channel	Hepatocyte culture medium	Intestinal Organoid Culture medium
Cell culture medium for bottom channel	1:1 mixture of hepatocyte culture medium and LSEC culture medium omitting dexamethasone	HIMEC 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 (HIMEC and Organoid seeding), Day 1 (Chips to Zoë)

Part II: Experimental Overview

Part II Overview

Introduction	This section gives an overview of the experimental workflow.
Day -X: Reagent Preparation	• Prepare and aliquot reagents (media supplements, ECM, etc.)
Day -X to 0: Prepare cells for chip culture as needed	A Cell lines and primary cells: Thaw and expand cells B iPSCs: Thaw, expand, and differentiate iPSCs C Tissue biopsy: Prepare spheroid culture from biopsy tissue and expand culture D Terminally differentiated cells: Thaw cells and seed directly on chips
Day -1: Chip Preparation	• Unpack and label chips • Prepare ER-1 solution • Introduce ER-1 solution to channels • Activate and wash chips • Prepare ECM solution • Coat chips with ECM
Day 0: Seed Cells on Chips	• Wash chips • Prepare cells for seeding on chip • Seed cells into bottom channel • Seed cells into top channel • Seed a well plate (quality control of cell morphology) • Gravity wash chips (2-18 hours post-seeding)
Day 1: Chips to Pods and Pods to Zoë	• Prepare complete culture media for connection and aliquot • Wash chips • Gas equilibration of media using Steriflip® workflow • Prime Pods • Connect chips to Pods • Connect Pods to Zoë • Run Regulate Cycle • Begin experimental flow

Continued on next page

Part II Overview, Continued

Day 2+: Organ-Chip Culture Maintaining and Sampling	• Inspect chips for bubbles and cell morphology • Via wash • Run 2nd Regulate Cycle • Add stretch (if relevant to model or application) • Media replenishment • Effluent sampling
Day X: Chip Termination	• Terminate Organ-Chip culture and process for various downstream analyses

Part III: Equipment and Materials Provided

Part III Overview

Requirement

Ensure all equipment, materials, and reagents provided with the system are accessible prior to beginning each experiment.

Provided Equipment and Materials

Equipment and materials provided with the Basic Research Kit (12-pack product code: OBKWER12, 24-pack product code: OBKWER24) provided below:

Equipment	Description	Quantity – 12-pack	Quantity – 24-pack
Chip-S1™ Stretchable Chip	Organ-chip consumable. Each kit comes with 3 extra chips for seeding optimization and redundancy	15	27
Pod™ Portable Modules	Provides reservoirs for media	12	24
ER-1™ surface activation reagent	5 mg powder	1	1
ER-2™ surface activation reagent	50 mL bottle	1	1
Steriflip®-HV Filters	0.45 µm PVDF filter sterile for Gas Equilibration of Media	4	8

Part IV: Equipment and Materials Required

Part IV Overview

Requirement

Ensure all required equipment, materials, and reagents are accessible prior to beginning each experiment.

Note on Catalog Numbers

Exact catalog numbers are not provided for some required materials, as several brands and models are acceptable.

Required Materials

A list of equipment and materials needed for this protocol in addition to the Emulate Basic Research Kit is provided below:

Equipment	Subscription	Supplier	Catalog Number
Square Cell Culture Dish (120 x 120 mm)	Sterile, 1 per 6 chips	VWR	82051-068
Handheld vacuum aspirator	-	Corning	4930
Aspirating pipettes	2 mL, polystyrene, individually wrapped	Corning / Falcon	357558
Aspirating tips	Sterile (autoclaved)	-	-
Serological pipettes	2 mL, 5 mL, 10 mL, and 25 mL low endotoxin, sterile	-	-
Pipette	P20, P200, 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 Flask	-	-	-
T75 Flask	-	-	-
Ice bucket	-	-	-
Parafilm®	-	-	-
70% ethanol and wipes	For surface sterilization	-	-
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
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	-	-

Notes for ER-1™ and ER-2™ reagents

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

- Both ER-1 and ER-2 reagents should be discarded if stored at room temperature for over 3 weeks, as this can compromise the performance of the reagents.
- If additional ER-1 and ER-2 are needed, these can be purchased separately from Emulate. Product codes: ER-1: RGT-ER1-5, ER-2: RGT-ER2-50.

Notes on Fungin™

- To prevent fungal contamination, add 0.1% of Fungin™ to any Organ-Chip culture when necessary.
- 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.

Part V. Chip Handling Techniques

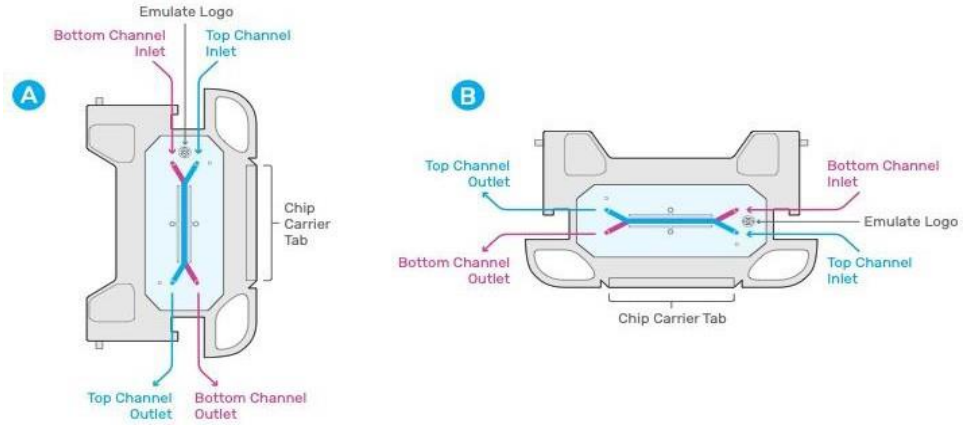
Part V Overview

Requirements

- Always work with chips in a sterile environment, such as the biosafety cabinet (BSC).
- 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 during an experiment.

Possible Chip Orientations

The two possible chip orientations are displayed below:



Orientation A

In this orientation, the bottom channel inlet will be on the top left of the chip, while the top channel inlet will be on the top right of the chip. Conversely, the bottom channel outlet will be on the bottom right of the chip, while the top channel outlet will be on the bottom left of the chip.

Orientation B

In this orientation, the bottom channel inlet will be on the top right of the chip, while the top channel inlet will be on the bottom right of the chip. Conversely, the bottom channel outlet will be on the bottom left of the chip, while the top channel outlet will be on the top left of the chip.

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Part V Overview, Continued

Pipetting

When pipetting to fill each channel, a volume of 50 µL 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.

Pipetting: Range of Acceptable Volumes

While 50 µL (top channel) and 20 µL (bottom channel) are the 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

Channel and Membrane Dimensions

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 the bottom of the chip to the top of membrane (See Figure 1)	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

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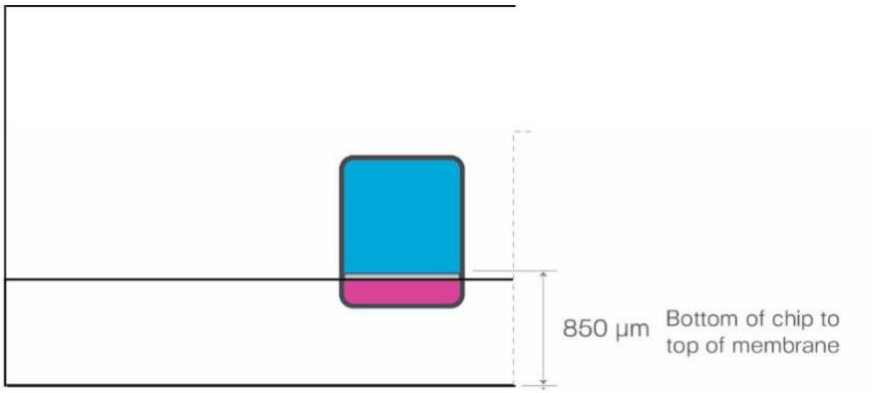
Part V Overview, Continued

Pipetting Solution into Channels

Use a P200 pipette with a sterile pipette tip to add solution directly to the channels of the chip as well as to coat, wash, and seed cells prior to connecting the chip to flow on 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.

Note: Always introduce liquid to the bottom channel before pipetting into the top channel (See Figure1).

Figure 1 Bottom Channel Dimensions



Part VI: Inspecting Chips for Bubbles Throughout the Experiment

Part VI Overview

Workflow steps that can create bubbles downstream and damage Zoë Culture Module

The table of contents below contains critical workflow steps that can impact bubble formation downstream and cause damage to Zoë Culture Module. These steps are identified on their respective pages with CAUTION statements.

Potential Bubble Issues and Solutions

Issue	Section	Step	Recommendation
Bubbles are present in chip ports or channel (Figure 2)	Any step related to chip handling, such as Chip Activation, washing, ECM coating, and cell seeding	Any step related to chip handling, such as Chip Activation, washing, ECM coating, and cell seeding	Wash the channel with the appropriate solution until all bubbles have been removed. If bubbles persist, it may be helpful to aspirate the channel dry and slowly re-introduce solution
Bubbles are in the ports upon introduction of media into the chip	Any step related to chip handling, such as Chip Activation, washing, ECM coating, and cell seeding	Any step related to chip handling, such as Chip Activation, washing, ECM coating, and cell seeding	Since the chip material is hydrophobic, bubbles could get trapped at the ports. Dislodge bubbles using the pipette tip or aspirate the channels and reintroduce appropriate solution.

Media takes too long to pass through Steriflip®	Chips to Pods and Pods to Zoë	Gas Equilibration of Media	Vacuum pressure is not reaching -70 kPa. Find and use an alternative vacuum source with the appropriate pressure. If this solution is unavailable, leave the media in the incubator with the caps loose for at least 2 hours before adding to Pods.
Pods do not prime	Chips to Pods and Pods to Zoë	Prime Pods	If Pods do not prime on the first attempt, ensure that medium covers all Pod Vias, and run the Prime Cycle again. If the problems persist, record the Pod lot number, and replace it with a new Pod.
Screen is frozen or unresponsive	Chips to Pods and Pods to Zoë; Maintaining and Sampling	Any step related to Organ-Chip culture on Zoë	Power off Zoë and turn it on again. If the problem persists, contact our support team.
Pods are stuck in Zoë	Maintaining and Sampling	Any step related to Organ-Chip culture on Zoë	The Pod lid is not secured. Try wiggling the tray to the right and left as you slide it out while keeping it level. If the problem persists, contact our support team.
Pods are not flowing properly or evenly/ Bubbles observed in chip	Maintaining and Sampling	Any step related to Organ-Chip culture on Zoë	There is inherent variability with Zoë; however, large fluctuations and major flow issues primarily result from bubbles. To remove

			bubbles and allow for flow, remove the chip from the Pod, flush the chip with media, re-prime the Pod with media, and reconnect the chip to the Pod.

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Part VI Overview, Continued

Potential Root Cause of Bubbles

If a high failure rate due to bubbles is observed, or if bubbles remain after following the mitigation steps above, check for the following:

If ...	Then ...
Medium is not sufficiently equilibrated before adding to Pods	Be sure to follow media preparations steps in the section “Gas Equilibration of Media.”
Vacuum for Steriflip® is too weak	Ensure that 50 mL of media can pass completely through the Steriflip in about 10 seconds.
Incorrect Steriflip® was used	Confirm the correct Steriflip® unit is being used (Millipore SE1M003M00).
Medium was not warmed correctly before the Steriflip® step	Be sure to follow media preparation steps in the section “Gas Equilibration of Media.”
Insufficient priming occurring	Disconnect chip and reprime Pod. Ensure media droplets are present in all ports.

Notes on Bubble Issues and Solutions:

- It is recommended that users take note of specific lot numbers for Pods and chips and report them to Emulate for further investigation.
- Ensure there is enough media in pod reservoirs for culture duration and flow rate. For example, for a flow rate of 60 µL / h, users need to have at least 1.5 mL of media for 24 h to prevent the media from depleting and air from being introduced into the chip cultures. Please refer to details in the section “Media Replenishment.”

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Part VI Overview, Continued

Figure 2 Top view of chip inside chip carrier depicting different types of bubbles that can occur during Organ-Chip culture.

Part VII: Basic Research Kit Protocol

Protocol Overview

Introduction

This section lists the basic steps for using Organ-Chips in experiments

Timeline

Day -X: Reagent Preparation

Day -X Procedure

Prepare and aliquot reagents (media supplements, ECM, etc.)

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

Day -X to 0: Prepare Cells for Chip Culture as Needed

Day -X to 0 Procedure

Introduction

Prior to chip seeding, the cells should be prepared depending on the organ model and the cell source type. When deciding the best way to prepare the cells, users should consider specific cell types' capacities to proliferate and differentiate. Typical procedures are listed below.

A. Thaw and expand cells

Cell lines and most primary cells can be cryopreserved and further expanded in flasks prior to chip seeding (e.g., LSECs, HIMECs, Caco2 cells, HepG2 cells, and HUVECs). In general, cells can be thawed 2-7 days before chip seeding to allow for expansion of up to 85-90% confluency.

Furthermore, thawing cells directly onto the T75 flask with 30 mL of media and letting them attach (for at least 6 hours and at most overnight) before media change, is generally better than performing centrifugation at the time of thawing. Nevertheless, cells should be thawed, handled, and expanded according to the appropriate Emulate culture protocol, cell manufacturer's instructions, or user's specific routine protocols. Users should ensure that cell quality and cell-specific phenotypes are preserved after repeated freezing and expansion.

B. Thaw, expand, and differentiate iPSCs

When iPSCs are used as a cell source for an Organ-Chip, differentiation into particular cell lineages requires specific procedures, reagents, and timelines. Consequently, the exact timing required to achieve specific stages can vary greatly between models.

Users should ensure that cell quality and cell-specific phenotypes are preserved after repeated freezing and expansion. Moreover, iPSC-derived cells can be seeded on the chip at the final differentiation stage (e.g., microvascular endothelial cells for Brain-Chip) or at an intermediate differentiation stage (e.g., intermediate mesoderm cells seeded on glomerulus kidney-chip) (Musah, et al 2018).

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Day -X to 0 Procedure, Continued

C. Prepare spheroid culture from biopsy tissue and expand culture

Since spheroids isolated from patient-derived biopsies are composed of multiple cell types, they are a great cell source for chips. Standard methods can be used to generate tissue fragments of adequate size and culture them in 3D Matrigel systems for *in vitro* expansion prior to seeding them on chips.

D. Thaw cells and directly seed on chips

Some cells are terminally differentiated and cannot be expanded *in vitro* (e.g., human primary hepatocytes, human Kupffer cells, and human Stellate cells). In those cases, cells can be cryopreserved from the initial biopsy and thawed only on the day of chip seeding. Upon thawing, cells are washed and counted in the presence of Trypan Blue to estimate cell viability. A cell solution, prepared with the seeding density of choice, is used to seed the top or bottom channel of ECM-coated chips. Chips are incubated at 37°C undisturbed for at least 2 hours and at most overnight in order to allow the cells to attach to the membrane (incubation time is cell-type dependent).

Day -1: Chip Preparation

Day -1 Procedure

Introduction

Chip preparation includes activating the chip surface with UV light and ER-1 solution before coating it with ECM—a thin layer of extracellular proteins that anchors the cells to the chip surface. The use of an ECM is required to ensure optimal cell performance with the Human Emulation System. Users can choose the optimal ECM for the cell types being used in the chips.

Goals

- Activate the inner surface of the chip channels for proper ECM coating
- Coat the inner channels with ECM proteins for cell attachment

Required Materials

- Chip-S1™ (12 chips per Zoë™ culture module)
- Chip Cradle (2 are shipped per Zoë™ culture module)
- ER-1™ powder
- ER-2™ buffer
- 15 mL conical tubes
- DPBS (- / -) on ice 70% ethanol
- ECM components
- 120 X 120 mm cell culture dish
- Ice and ice bucket
- Pipettes and filtered tips
- Aspirator and sterile tips
- Aluminum foil
- UV light box
- UV safety glasses

Key Steps

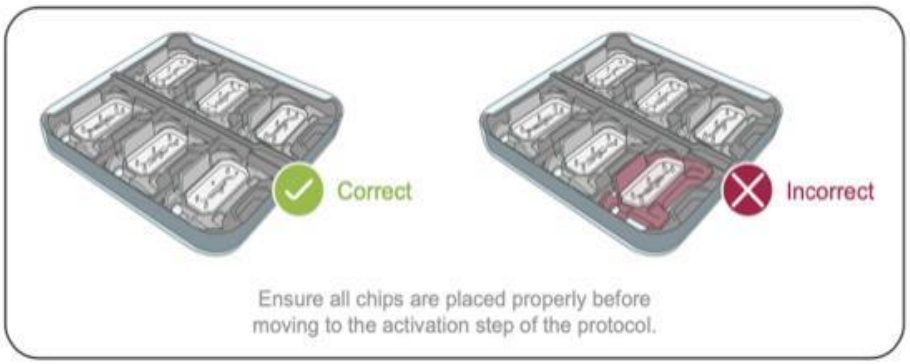
Unpack and label chips

Steps

Step	Action
1	Spray the chip packaging, square cell culture dish packaging, and autoclaved Chip Cradle with 70% ethanol, and bring them into the BSC.
2	Open the Chip Cradle sterile packaging. 2 Chip Cradles are shipped 2 per Zoë Culture Module. If necessary, additional cradles can be purchased from Emulate.
3	Place the Chip Cradle into the square dish, making sure it is oriented properly (corners facing up).
4	Open the chip packaging carefully and pick up the chip.
5	Label the chip carrier tab with ID numbers for each chip needed in the experiment. Place the first chip into the Chip Cradle by sliding the back of the carrier under the tabs on the cradle (See Figure 3).
6	Open the remaining chips necessary for the experiment and place them into the cradle. Note that 1 Chip Cradle can support up to 6 chips.

Figure 3

Place chips by sliding them under the tabs of the Chip Cradle.



Prepare ER-1™ solution

ER-1 Overview

ER-1 is light-sensitive. Prepare ER-1 solution immediately before use and discard any remaining ER-1 solution 1 hour after reconstitution. Using ER-1 solution that has been exposed to light or that has not been freshly prepared will lead to chip failure.

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

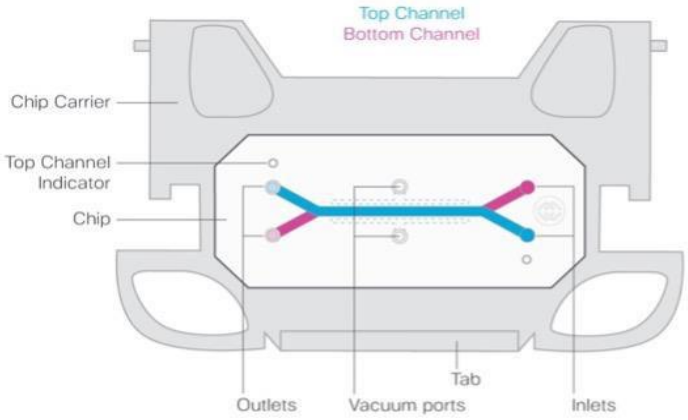
Steps

The steps for Preparing ER-1 Solution are as follows:

Step	Action
1	Turn off the BSC light, and allow the ER-1 and ER-2 reagents to equilibrate to room temperature (which will take approximately 10 to 15 minutes) before use.
2	Wrap an empty, sterile, 15-mL conical tube with foil to protect it from light.
3	In the BSC, remove the small vial of ER-1 powder from the packet. Briefly tap the vial to concentrate the powder at the bottom.
4	Add 1 mL of ER-2 buffer to the vial, and transfer the 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.
6	Repeat Step 5 twice more, adding another 1 mL of ER-2 buffer each time. **Note:** On the last addition of 1 mL ER-2 to the ER-1 bottle, cap and invert the bottle to collect any remaining ER-1 powder in the lid. Transfer the collected solution to the conical tube, bringing the total volume in the tube to 4 mL of ER-1 solution.
7	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 the channels

Figure 4 Top view of a chip with labelled ports in a chip carrier:



Steps for Introducing ER-1 to channels

Step	Action
1	Using a P200 pipette and a sterile 200 µL filtered pipette tip, collect 200 µL of ER-1 solution. **Note:** 200 µL of ER-1 solution will fill approximately 3 chips.
2	Working with one chip at a time, carefully introduce ER-1 solution through the bottom channel inlet 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 place it on the top channel inlet to introduce ER-1 solution.
4	Repeat steps 2 and 3 for each chip.
5	Gently aspirate all ER-1 solution from the surface of the chip. Be sure to remove ER-1 solution only 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 them by washing the channel with ER-1 solution until they have all been removed. If bubbles persist, it may be helpful to aspirate the channel until it is dry and then slowly re-introduce the ER-1 solution

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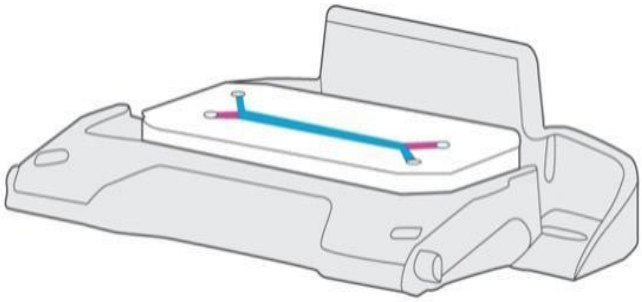
Introduce ER-1 solution to the channels, Continued

Figure 5 View of a chip with no excess ER-1 solution on its surface:



Activate and wash chips

Steps

The steps for activating and washing chips are as follows:

Step	Action
1	Bring the square dish containing the ER-1-filled chips to the UV light box.
2	Remove the cover from the dish. Place the uncovered dish in the UV light box.
3	Set the switch at the back of the UV light box to the “Constant” setting. Turn the device power on, and press the red “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 the ECM Solution. (For more information, see the next section)
6	After UV treatment, close the dish, and bring the chips back to the BSC. **Note:** The light in the BSC may be on from this point forward.
7	Fully aspirate the ER-1 solution from both channels.
8	Wash each channel with 200 µL of ER-2 solution.
9	Fully aspirate the ER-2 from the channels.
10	Wash each channel with 200 µL of sterile cold DPBS.
11	Leave cold DPBS inside the channels.

Prepare ECM solution

ECM Overview

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

Note on Coating

In some models, such as the human Liver-Chip and human Brain-Chip, the same solution is used to coat both channels. In other models, such as the human Colon and Duodenum Intestine-Chips, the channels must be coated with different solutions to ensure proper cell functionality.

Example: ECM for Liver-Chip

ECM composition and concentration should be optimized for any new Organ-Chip model prior to beginning studies. Below is an example of proper ECM solution preparation under optimal conditions for the human Liver-Chip.

ECM working concentration is:

Collagen I: 100 µg / mL

Fibronectin: 25 µg / mL

Step	Action
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 solutions on ice at all times.
3	Calculate the total volume of ECM solution needed to coat all chips. • Volume required per chip = approximately 100 µL • For every 12 chips to be coated, 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 it is ready to use.

Continued on next page

Prepare ECM solution, Continued

Example Calculation: Liver-Chip

ECM Calculation Example (C1V1 = C2V2)

Solution	Concentration
Collagen I stock concentration	8.41 mg / mL (C1)
Collagen I final concentration	0.1 mg / mL (C2)
Fibronectin stock concentration	1 mg / mL (C1)
Fibronectin final concentration	0.025 mg / mL (C2)
Stock volume	collagen I (X) or fibronectin (Y) (V1)
Final volume of ECM solution	1.5 mL (V2)

Collagen calculation:

(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 calculation:

(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

Steps

The steps for coating chips with ECM are as follows:

Step	Action
1	Fully aspirate the cold DPBS from both channels.
2	Set a P200 pipette to take up 200 µL of ECM solution. **Note:** If using the same ECM solution for both channels, it is recommended to fill the bottom channel and the top channel—in that order—before moving to the next chip. However, if using two different ECM solutions, it is recommended to fill the bottom channels of each chip within one cradle first, then change pipette tips to fill the top channels.
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 the remaining ECM solution to the next inlet (this may be either a top or bottom inlet depending on the number of ECM solutions used per chip). Introduce ECM solution through the next inlet, leaving small droplets of excess ECM solution on both ports in both channels (See Figure 6).
5	Inspect each channel to ensure there are no bubbles. If bubbles are present, wash the channel with ECM solution until they have all been removed.
6	Repeat steps 1 through 6 for each chip.
7	Fill the chip cradle reservoir with 750 mL of DPBS to provide extra humidity (See Figure 7). **Note:** The ECM droplets may combine if the same components are being used in both channels. Care should be taken when using different solutions in order to prevent them from mixing.

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Coat chips with ECM, Continued

Figure 6 Image of a chip carrier containing a chip with small droplets of ECM solution at its ports:

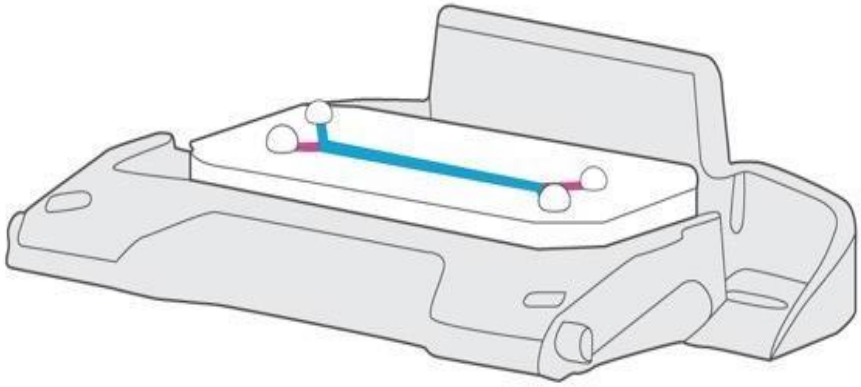
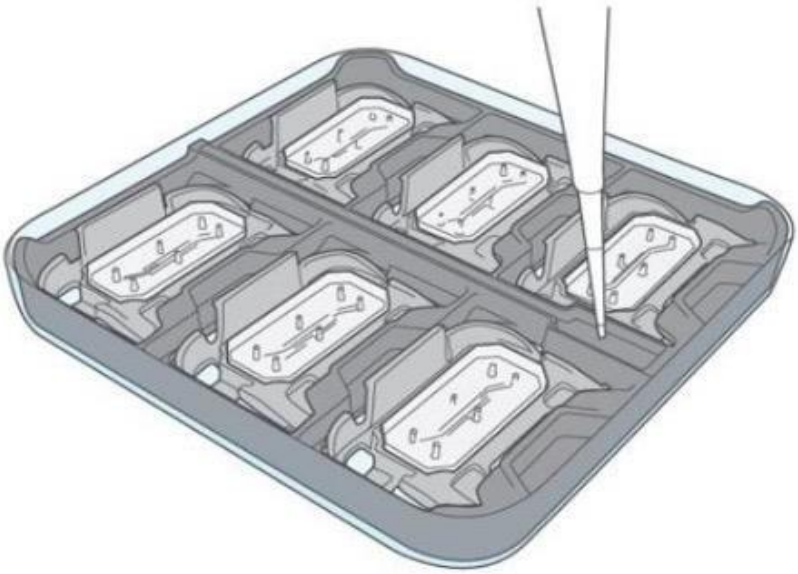


Figure 7 Fill the central reservoir of the Chip Cradle with 0.75 mL of DPBS:



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Coat chips with ECM, Continued

Next Step

Use the table below to determine next steps after coating the chips with ECM:

If the…	Then…	And on seeding day…
Cells will be seeded the following day (preferred method with best results)	Incubate the chips containing ECM solution overnight at 37°C	Wash chips with cell seeding media and place them in the incubator until cells are ready to be seeded
Cells will be seeded the same day as chip activation and ECM coating	Incubate the chips containing ECM solution for at least 2 h at 37°C prior to seeding	
Cells will be seeded after 2-5 days (Chips can be stored at 4°C for up to 1 week as long as they are kept moist)	A. Seal the dish with parafilm, and B. Incubate the chips at 4°C	A. Remove the parafilm wrap B. Incubate the chips containing ECM solution for at least 2 h at 37°C C. Wash chips with cell seeding media, and place them into the incubator until cells are ready to be seeded

Day 0: Seed Cells on Chips

Day 0 Procedure

Introduction

Chips can be seeded with a variety of cell types and cell sources. The seeding order and density should be empirically determined by the user.

Considerations

Considerations for seeding cells on chips:

If ...	Then ...
The bottom channel is being seeded	Use 15-20 µL
The top channel is being seeded	Use 35-40 µL
The cell suspension is ready for chip seeding	Seed only one chip and inspect under the microscope to confirm proper cell density. Adjust accordingly, if needed, before seeding the remaining chips
The bottom channel has just been seeded	Quickly invert the chip to allow the cells to adhere to the opposite side of the porous membrane. Users can invert all chips simultaneously by inverting the Chip Cradle (See Figure 8).
The application requires obtaining the vascular channel's full lumen (i.e., cells attached to upper and lower surface of the bottom channel)	Users can seed the bottom channel in two consecutive steps using two flasks of cells, allowing them to attach to both the upper and lower surfaces at each step.
More than one cell type is being seeded into either channel	The model must first be optimized. To find more information and example protocols, refer to the Emulate website for specific Organ-Chip Culture protocols at https://emulatebio.com/support/

Continued on next page

Day 0 Procedure, Continued

Tips

Tips for successful chip seeding:

- Inject cells into the chip quickly (1-second technique) so they distribute evenly throughout the channel. A slow injection will generate a gradient of cells that puts higher densities towards inlets and lower densities towards outlets.
- Ensure all media is aspirated from the chip surface before and after injecting the cells. Culture media surrounding ports can cause local gradients on inlet and outlet regions.
- Use the aspirator to hold the chip in place while removing the pipette. This will prevent chip displacement in the chip carrier as well as uneven cell distribution within channels.

Key Steps

Required Materials

- Complete cell-seeding media for all cell types (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
- Cell culture microscope

Wash chips

Steps

The steps for washing chips are as follows:

Step	Action
1	Transfer ECM-coated chips from the incubator into the BSC.
2	Gently wash both channels of the chip with 200 µL of complete cell culture medium for the first cell type to be seeded. Aspirate the excess medium outflow on the surface of the chips, leaving enough medium to cover the inlet and outlet ports.
3	Cover the square dish. Place the chips in the incubator until the cells are ready to be seeded.

Prepare cells for seeding on chip

Collecting Cells

Regardless of the cells' type or source, they will require collection from flask, tube, or plate, washing and density adjustment.

Reagents

Reagents such as Trypsin, TrypLE, Accutase, and Cell recovery solution can be used to obtain single cell suspensions of samples expanded *in vitro* prior to seeding chips.

Counting Cells

We recommend counting cells in the presence of Trypan Blue to estimate the viability. For the best chip performance, only seed cell populations exhibiting 85% viability or higher.

Adjusting Cell Density

Following cell counting and viability determination, adjust the cell solution to the desired density. Ensure that enough seeding solution is prepared to seed all chips within an experiment.

Note: Adjusting Cell Density

If there is not enough volume of cell suspension available, you may pipette additional media and count the cells again. This will allow all chips to be seeded at a cost of decreased cell density. This approach is not recommended when seeding non-proliferative cells, as they will not form a confluent monolayer.

Continued on next page

Prepare cells for seeding on chip, Continued

Example: Cell Density Adjustment

Example of cell density adjustment:

Variable	Definition
C1	viable cell yield
C2	optimal final density
V1	volume of cell suspension to dilute
V2	volume of cell suspension needed to seed all chips

Determine volume of cell suspension to dilute by solving for V1 using the equation C1V1 = C2V2

1) Determine the values for C1, C2, and V2
    a. C1: for this example, let's assume **10 x 10^6 cells / mL**)
    b. C2: (**2 x 10^6 cells / mL**)
    c. V2:
        i. Number chips seeded = 20
        ii. Volume of cell suspension to seed one chip (~35 µL)
        iii. 20 x 35 = **700 µL or 0.700 mL**
2) Use the equation to solve for V1:
    a. (10 x 10^6 cells / mL) x V1 = (2 x 10^6 cells / mL) x (0.7 mL)
    b. V1 = 0.140 mL or 140 µL
3) Determine media volume needed by subtracting V1 from V2
    a. 700 µL - 140 µL = 560 µL
    b. Dilute 140 µL of cell suspension with additional 560 µL of media to obtain 2 x 10^6 cells / mL final concentration

Seed cells into bottom channel

Overview

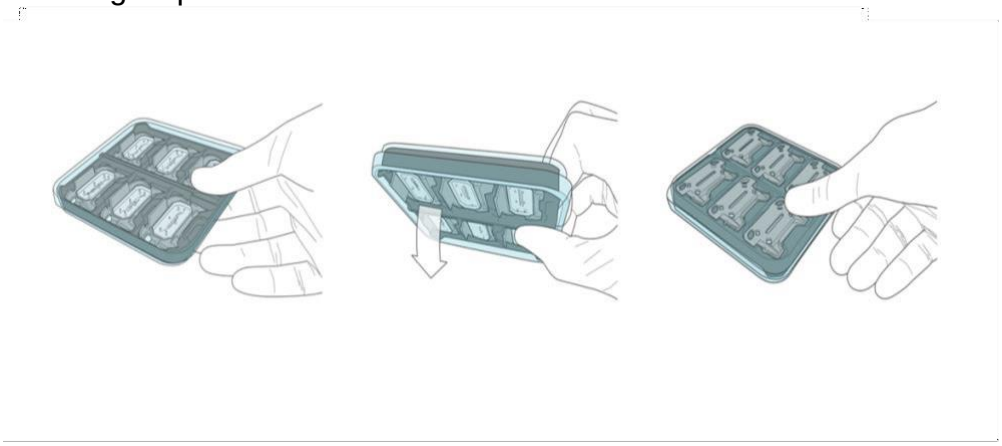
For most Organ-Chip models, the bottom channel is populated by tissue-specific endothelial cells to emulate the vascular lumen. However, other cell types can be used in addition to, or instead of, endothelial cells. In the Liver-Chip Quad-culture, for example, Kupffer cells and Stellate cells are seeded simultaneously with LSECs, and all cells are co-cultured in the bottom channel.

Steps

Step	Action
1	Bring the square dish containing the ECM-coated, washed chips to the BSC.
2	While avoiding contact with the ports, gently aspirate medium droplets from the surface of one chip.
3	Gently agitate the cell suspension before seeding each chip to ensure a homogeneous cell suspension.
4	Seed 15 to 20 µL of the cell suspension into the bottom channel while aspirating the outflow.
5	Cover the dish and transfer it to the microscope to confirm proper seeding density within the chip.
6	If the seeding density is not optimal or cell distribution is not even, return the chips to the BSC and wash the channel with 200 µL of fresh medium twice. 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 the remaining chips in one dish. **Note:** Minimize the amount of time the cells are outside the incubator by seeding no more than 6 chips at a time and by immediately placing the chips in the incubator at 37°C after seeding each batch of 6.
8	Once all 6 chips have been seeded in the cradle, cover the dish and then carefully invert it (Figure 8). **Note:** After seeding the bottom channel, chips should be inverted as soon as possible to ensure cells attach to the upper side of the bottom channel.
9	To prevent evaporation during incubation, fill the central reservoir with 0.75 mL of DPBS and then place the cover onto the square dish.
10	Seed the remaining chips.
11	Incubate chips in the 37°C incubator for approximately 2 hours or until cells have attached.

12	Upon cell attachment, aspirate DPBS from the central reservoir, and flip the dish back to an upright position. **Note 1:** It is essential to achieve the correct seeding density to prevent chip failure. **Note 2:** If full vascular lumen is desired, repeat the steps above with additional endothelial cells, and incubate chips in the upright position to allow the cells to attach in the bottom of the channel.

Figure 8 Inverting chips before endothelial cell attachment:



Seed cells into top channel

Overview

The top channel of any Organ-Chip model is typically populated by tissue-specific epithelial cells that provide the characteristic function of the organ of interest (e.g., Intestine-Chip, Kidney PT-Chip and Lung-Chip). However, other cell types can be seeded alongside, or instead of, epithelial cells.

Example: Brain-Chip

On the Brain-Chip, neurons, astrocytes, pericytes, and microglia are seeded simultaneously, and all cells are co-cultured in the top channel.

Steps

Step	Action
1	Bring the square dish containing the ECM-coated, washed chips to the BSC.
2	While avoiding contact with the ports, gently aspirate medium droplets from the surface of one chip.
3	Gently agitate the cell suspension before seeding each chip to ensure a homogeneous cell suspension.
4	Seed 35 to 50 µL of the cell suspension into the top channel while aspirating the outflow.
5	Cover and transfer the dish to the microscope to confirm proper seeding density within the chip.
6	If the seeding density is not optimal or cell distribution is not even, return the chips to the BSC and wash the channel with 200 µL of fresh medium twice. 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 the remaining chips in one dish. **Note:** Minimize the amount of time the cells are outside the incubator by seeding up to 6 chips at a time and then immediately placing them into the 37°C incubator.
8	To prevent evaporation during incubation, fill the central reservoir with 0.75 mL of DPBS and then place the cover onto the square dish.
9	Seed the remaining chips
10	Incubate chips at 37°C for approximately 2 hours or until cells have attached.

11	Upon attachment, aspirate DPBS from the central reservoir, and flip the dish back to an upright position. **Note:** Correct seeding density is essential to prevent chip failure.

Seed a well plate (quality control of cell morphology)

Overview

It is recommended to always seed any remaining cells into a 24-well plate as a control for cell quality. Ideally, plates should be coated with the same ECM solution applied to the chips, but collagen I pre-coated plates can also be used.

Steps

Use the steps below to empirically find adequate cell seeding density for a 24-well plate:

Step	Action
1	Further dilute the cell suspension with warm 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, respectively.
3	Mix each well to ensure an even suspension. Allow the cells to settle for 5 minutes on the microscope stage with the light off. Inspect densities under the microscope.
4	Determine which of the wells depicts the optimal seeding density and then seed the remaining cells using the chosen volume into as many wells as desired.
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. Once the cells are dispersed, do not disturb the plate until the next day to allow the cells to fully attach.

Gravity wash chips (2-18 hours post seeding)

Overview

Once the cells in the chip have attached, a gentle gravity wash should be performed. The specific timing of the wash is cell-type-dependent and should be determined by the user. This step is important to ensure that nutrients are replenished and that the channels do not dry out; therefore, each channel should be washed with the relevant media for the particular cell being seeded. Since two media are being used, they must be separated by keeping them in filtered tips.

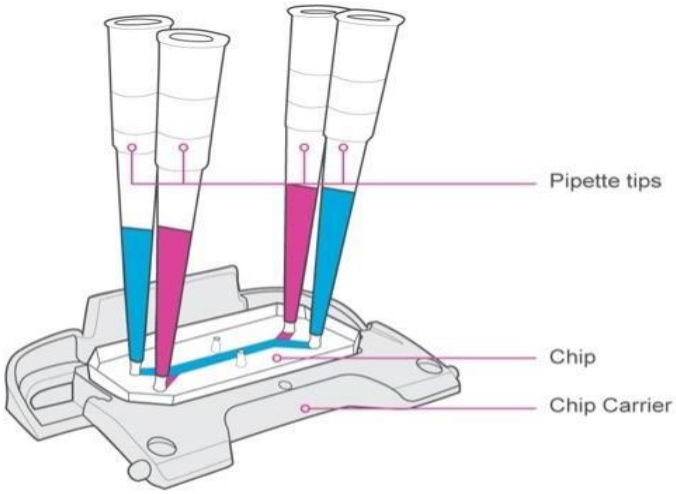
Steps

The steps for performing a gravity wash on the chips is as follows:

Step	Action
1	Add empty tips to both outlet ports.
2	With a P200 pipette, gently introduce media into the inlet ports one channel at a time—starting with bottom channel—until medium is observed flowing into the empty tip in the outlet port.
3	Disconnect pipette from tip, leaving the tip inserted in chip ports (See Figure 9).
4	Incubate the chips overnight at 37°C.
5	Maintain the cells in static culture within the chips until connecting them to Pods and Zoë. **Note:** If cells are fully attached, the chips can be connected 2 hours after seeding.

Figure 9

Chips with filtered tips inserted into ports with respective media:



Day 1: Chips to Pods, and Pods to Zoë

Day 1 Procedure

Goals

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

Key Steps

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

Prepare complete culture media for connection and aliquot

Overview

On the day of chip connection, prepare a sufficient volume of complete cell culture medium for each chip channel (e.g., epithelial cell and endothelial cell culture media). The amount of medium needed depends on how many chips will be connected to the flow. It is generally recommended to prepare at least 3.5 mL of each medium type per chip; however, users are encouraged to make slightly more to account for possible pipetting errors.

Steps for Aliquoting

Aliquot the media as follows:

Step	Action
1	Aliquot 500 µL or more of complete medium for each channel per chip into fresh tubes and warm at 37°C to wash chips.
2	Aliquot 3 mL or more of complete medium for each channel, per chip, into fresh 50 mL conical tubes for chip connection to flow. Warm these media at 37°C for at least 1 hour before gas equilibration.

Wash chips

Steps

The steps for washing chips are as follows:

Step	Action
1	Transfer the seeded chips in the square dish from the incubator to the BSC.
2	Remove the pipette tips from the chip inlet and outlet ports.
3	Gently wash both channels of each chip with 100-200 µL of the appropriate warm cell culture medium to remove any possible bubbles in the channels.
4	For every chip, place small droplets of medium onto the top of each inlet and outlet port.
5	Place chips back in incubator until the Pods are ready for connection.

Note on Media Mixture

It is fine if the media mixes at this step. The most important thing is to ensure that all ports are covered with media so that a liquid-liquid interface will be formed when connecting to Pod.

Gas Equilibration of Media

CAUTION

The media equilibration step is imperative to successfully culture Organ-Chips. Omitting this step will cause bubbles to form in the chip, in the Pod, or both, which will in turn negatively impact cell viability and result in irregular flow.

Overview

Once the culture media for both chip channels have been warmed for at least 1 h, the gas equilibration step can be performed.

Note on Work Environment

Work quickly and ensure the medium is not outside of a warmed environment—such as an incubator or bath—for longer than 10 minutes, as gas equilibrium can become compromised when the medium is allowed to cool.

Note on Cooling

If the vacuum pump is not located close to the water bath (e.g., inside the BSC), it is recommended to place some clean water warmed at 37°C inside the BSC in order to minimize cooling during the media equilibration step.

Steps

The steps for equilibrating media are as follows:

Step	Action
1	Bring tubes containing warm media to BSC.
2	Immediately connect each 50 mL tube to a Steriflip® unit (See Figure 10).
3	With the unit “right-side up” (medium in the bottom conical tube), apply vacuum for 10 seconds.
4	Invert the Steriflip-connected tubes, and check that the medium begins to pass from the top conical tube to the lower tube. **Note 1:** 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 section— “medium not equilibrated properly”. Leave the filtered medium under vacuum for 5 minutes. **Note 2:** If possible, keep media warm at 37°C (on beads or water bath) during the degassing step to ensure best efficiency.
5	Remove the vacuum tubing from the Steriflip® units.
6	Separate the conical tubes containing media from the Steriflip® unit. Immediately place the conical tubes

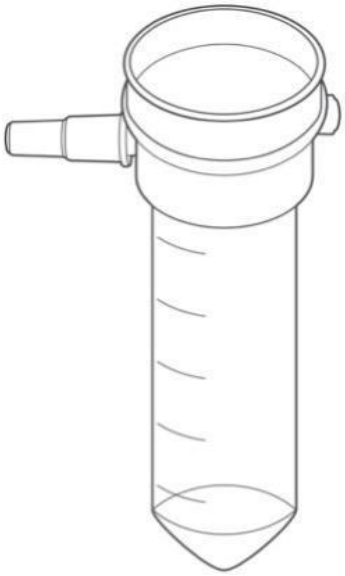
	containing media in the incubator with the caps loose to ensure that media remains equilibrated with incubator gas conditions.

Note on Media Temperature

Minimize the time media is outside of the incubator during Pod preparation to maintain temperature. This is a very important step to prevent chip failure.

Figure 10

Illustration of Steriflip® unit:



Prime Pods

CAUTION

Ensuring successful Pod priming is best practice for successful liquid-liquid connection between the chip and Pod. Failure to do so can cause bubbles to form in the chip, in the Pod, or both, which will in turn negatively impact cell viability and result in irregular flow.

Priming Steps

The steps for priming Pods are as follows:

Step	Action
1	Sanitize the exterior of the Pod packaging and Zoë trays with 70% ethanol, wipe it, and transfer it to the BSC.
2	Open the Pod package and then place the Pods into the trays. Orient the Pods with the reservoirs toward the back of the tray (See Figure 11).
3	Pipette 3 mL of pre-equilibrated, warm media to each inlet reservoir, ensuring to add the appropriate cell type-specific media to the correct pod inlet reservoir.
4	Pipette 300 µL of pre-equilibrated, warm media to each outlet reservoir, directly over each Via (See Figure 12).
5	Bring the trays containing the Pods to the incubator to keep media warm until all Pods have been filled.
6	Slide one tray completely into Zoë so that the handle is facing outward.
7	Run the Prime Cycle on Zoë. (See details on Page 55) **Note:** It is recommended to run the Prime Cycle on one tray at a time to ensure the prime droplets do not dry while in the incubator.
8	Close the incubator door and allow Zoë to prime the Pods (this process takes approximately 1 minute). **Note:** Once “Start” is selected, there will be an audible sound as Zoë engages the Pods.
9	Remove the tray from Zoë, and then bring it to the BSC.
10	Verify that all Pods within the tray were fully primed. This is very important for successful chip connection.

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Prime Pods, Continued

Figure 11 Pods in tray:

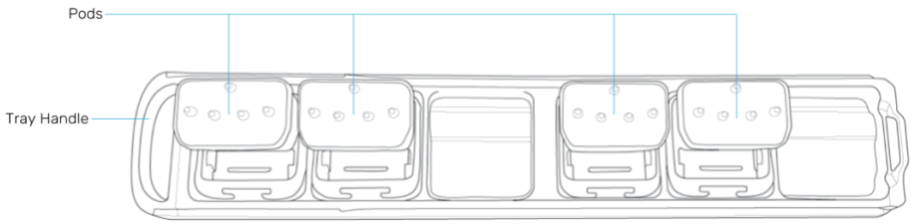
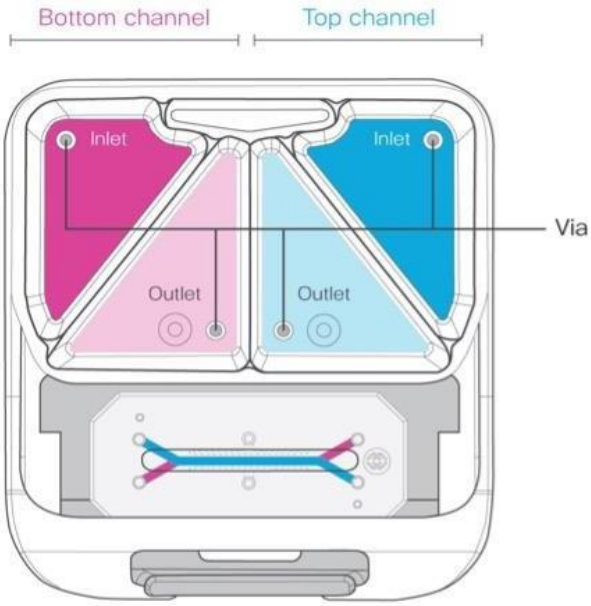


Figure 12



Running the Prime Cycle

The steps for running the Prime Cycle on Zoë are as follows:

Step	Action
1	Rotate the Dial to highlight “Prime” on the display.
2	Use the Dial Button to select “Prime.”
3	Rotate the Dial clockwise to highlight “Start.”
4	Press the Dial Button again to select “Start,” beginning the Prime Cycle. **Note:** Once “Start” is selected, there will be an audible sound as Zoë engages the Pods.

Continued on next page

Prime Pods, Continued

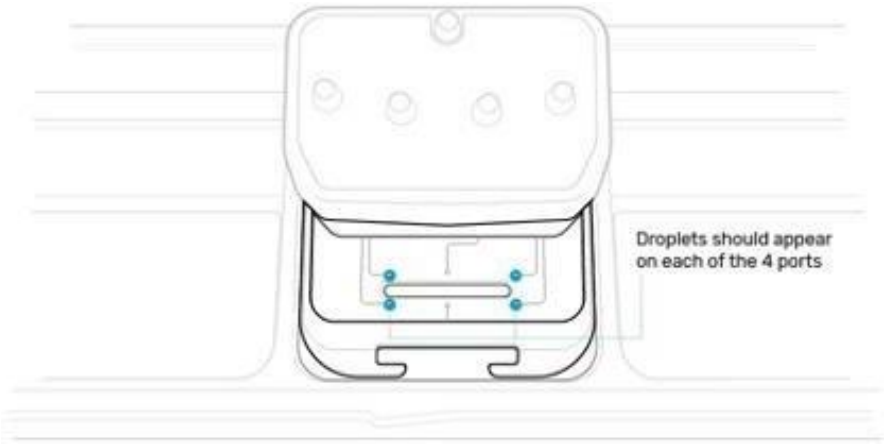
Pod Priming Verification

Take out the tray and inspect the top of the Pods (See Figure 13) to verify the presence of small media droplets through the Pod window at all four fluidic ports.

If ...	Then ...
Droplets are not visible through the top window	Rerun the Prime Cycle on those Pods. If the issue persists, contact Emulate Support.
Any outlet port does not show a droplet, but the inlet port does	Ensure step 4 of “Priming Steps” has been performed correctly.
Any media escaped onto the tray (this may occur more often by the outlet ports)	Clean the tray using a wipe sprayed with 70% ethanol.

Figure 13

Top view of chip in Pod with fluidic ports covered with droplets:



Connect chips to Pods

Steps

The steps for connecting chips to pods are as follows:

Step	Action
1	While holding one chip in the dominant hand (maintaining it in the chip carrier) and one Pod in the non-dominant hand, slide the chip carrier into the tracks on the Pod's underside until the chip carrier has seated fully.
2	Place a thumb on the chip carrier tab. Gently but firmly press the tab in and up to fit into the chip carrier's tab with the Pod.
3	Place the Pod with the connected chip onto the tray.
4	Aspirate any excess media on the chip surface from the Pod window. Additionally, clean all excess media from the trays and bottoms of pods using a wipe sprayed with 70% ethanol.
5	Repeat steps 1 through 4 for each Pod, chip carrier, and tray.
6	Confirm that there is sufficient media in each Pod inlet and outlet reservoir and that the Pod lids are flat and secure.
7	Bring the tray to the incubator until all samples are connected in order to keep the equilibrated media warm.

Connect Pods to Zoë

Overview

Different Organ-Chips will require exposure to different types of mechanical forces. Zoë enables physiologically relevant mechanical forces to be applied via flow and/or stretch. The type and optimal level of these forces will be determined depending on the cells of interest and the desired physiologically relevant microenvironment.

Before Running the Regulate Cycle

Once all chips are connected to Pods:

Step	Action
1	Place the trays into Zoë. **Note:** One Zoë can run 12 chips simultaneously.
2	Program the appropriate Organ-Chip culture conditions on Zoë. These conditions will start as soon as the Regulate Cycle is complete. See the example below.

Example Culture Conditions

To culture the human Liver-Chip, set the flow rate to 30 µL / h for both the top and bottom channels, and set the stretch parameters to 0.

Steps for Running the Regulate Cycle

The steps for running the Regulate Cycle are as follows:

Step	Action
1	Rotate the Dial to highlight the “Regulate” field.
2	Press the Dial Button to select “Regulate.”
3	Rotate the Dial clockwise to highlight “Start.”
4	Press the Dial Button 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.
5	At this point, see that the “Activation” button glows blue.
6	The Regulate Cycle lasts for 2 h. After the cycle has finished, Zoë will begin flow and/or stretch at the preset Organ-Chip culture conditions.

Canceling the Regulate Cycle

You can cancel the Regulate Cycle on Zoë, but only as necessary. To do so, highlight the “Regulate” field with the Dial, and press the Dial Button to select it. Rotate the Dial counterclockwise to highlight over “Cancel.” Press the Dial Button once more. Wait 1 minute for the cycle to end, after which the tray can be removed. If the Regulate Cycle is cancelled, always rerun it fully before proceeding.

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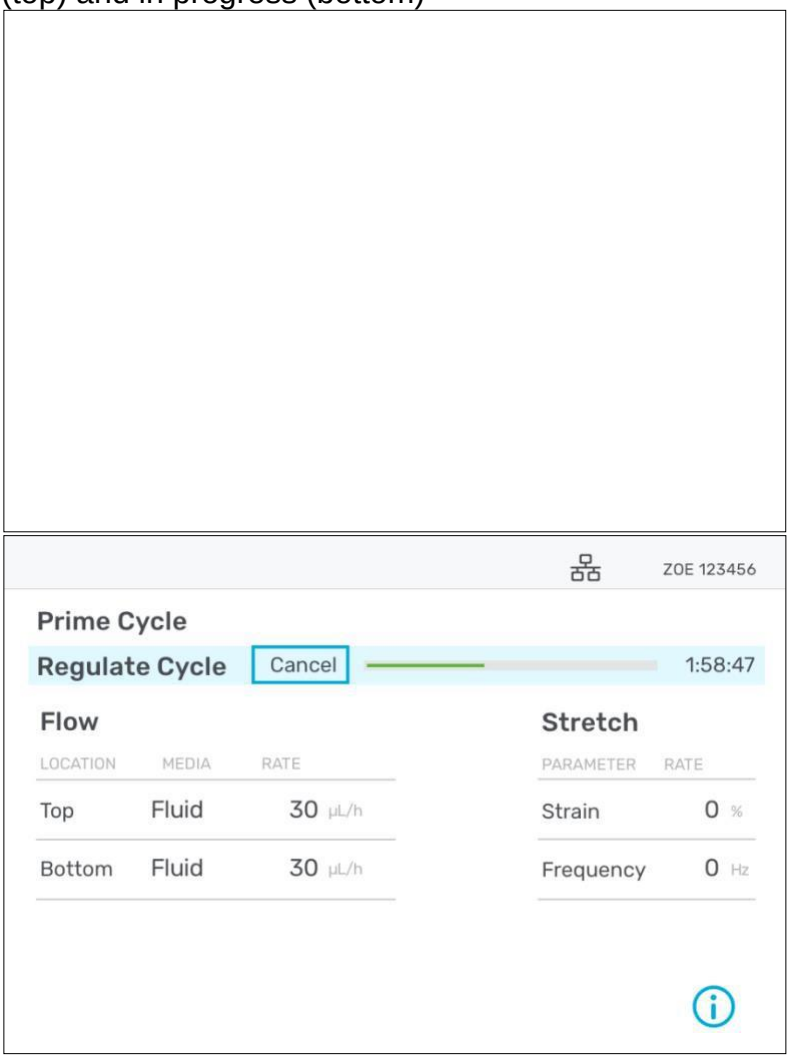
Connect Pods to Zoë, Continued

Figure 14 Representation of Zoë screen showing the Regulate Cycle starting (top) and in progress (bottom)



Day 2+: Organ-Chip Culture Maintenance and Sampling

Day 2+ Procedure

Goals

- Maintain chip culture in Zoë over time
- Collect effluent samples for various analyses

Key Steps

Required Materials

- Chips in Pods
- Cell Culture Medium
- Serological pipettes
- Pipettes with multichannel and filtered tips
- Aspirator and sterile tips
- Cell culture microscope
- 96-well plates or tubes to collect and store effluent

Inspect chips for bubbles and cell morphology

Overview

The day after connecting chips and Pods to Zoë, which begins the process of Organ-Chip culture, pause Zoë by pressing the silver “Activation” button located above the tray bays. This stops flow and releases the Pods.

Steps

The steps for inspecting the chips are as follows:

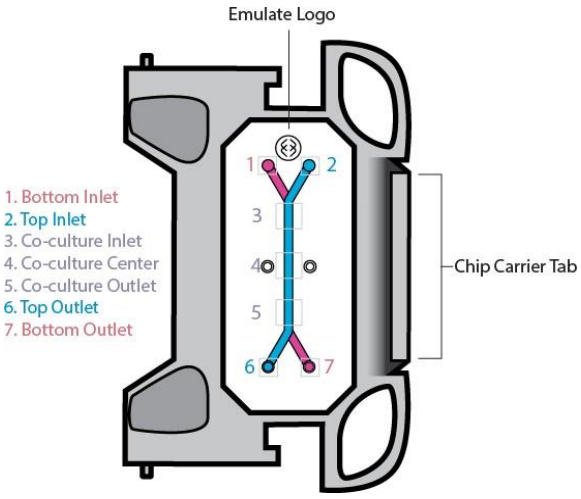
Step	Action
1	Remove the trays and then place them into the BSC.
2	Visually inspect each chip for bubbles and flow issues.
3	Using a microscope, inspect cells in the chips to assess morphology and viability. Capture representative images at 10X or 20X magnification along the length of the chip, as shown in Figure 15.

Note on Imaging

For some models, such as the Colon Intestine-Chip and the Liver-Chip, images taken at the co-culture area will be useful in showing the morphology of the cells in the top channel at the interface with the cells in the bottom channel (positions 3,4,5,). However, for other models like the Brain-Chip, the best cell resolution for the top channel will be achieved in the inlet and outlet areas to avoid cell interference from the bottom (positions 2, 6). Bottom channel images should always be acquired in monoculture areas (positions 1,7).

Figure 15

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



Via wash

Steps

On the day after connecting chips and Pods to Zoë, perform a Via wash on each Pod inlet and outlet reservoir to dislodge any bubbles that may be present on top of the Vias:

Step	Action
1	Remove the Pod lids.
2	Using a 200-µL pipette, pipette 200 µL of medium directly over the top of the Via.
3	Repeat this wash step for all four reservoirs in each Pod. **Note:** The Via wash should also be performed on inlet Vias after every media change.

Run 2nd Regulate Cycle

Overview

After the Via wash, it is recommended to run a 2nd Regulate Cycle to prevent flow issues. This step is not mandatory but will help media flow properly for long-term Organ-Chip culture.

Note on 2nd Regulate Cycle

In some instances, you may skip the 2nd Regulate Cycle and move forward with next steps (i.e., short cell viability over time).

Running 2nd Regulate Cycle

Follow the steps below to run the Regulate Cycle again:

Step	Action
1	Rotate the Dial to highlight the “Regulate” field.
2	Press the Dial Button to select “Regulate,” and rotate the Dial clockwise to highlight “Start”.
3	Press the Dial Button 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 is complete, Zoë will begin flow at the preset Organ-Chip culture conditions.

Add stretch (if relevant to model or application)

Overview

Cyclical chip stretching can be programmed on Zoë to emulate mechanical forces that naturally occur in the body, such as peristalsis within the intestine and breathing motions within the lungs. The frequency and amplitude of stretching can be adjusted depending on experimental needs (see example below).

Example

For the Colon Intestine-Chip, we recommend starting with 2% stretching at 0.15 Hz and then increasing to 10% for higher *in vivo* relevance.

Steps

Follow the steps below to add stretch to the culture:

Step	Action
1	Pause Zoë by pressing the silver “Activation” button.
2	Rotate the Dial to highlight the “Stretch” field.
3	Press the Dial Button to select “Stretch,” and rotate the Dial clockwise to increase stretch to “2%”.
4	Press the Dial Button to select “Freq.” and rotate the Dial clockwise to increase stretch to “0.15 Hz”.
5	Press the “Activation” button.

Media Replenishment

CAUTION

Failure to replenish media on time will cause air to be introduced into the Pod and chip, which ultimately results in cell death and may cause damage to Zoë Culture Module. Do not fill reservoir past 3.5 mL of the total volume during replenishment.

Timing

The timing for media replenishment will depend on the specific flow rate used for the experiment. For example, when using a flow rate of 30 µL / h, 720 µL of media will take 24 h to flow. We recommend adding a safety factor of 1.5x the expected media volume.

Note on Culture Media

Ensure that enough culture media is contained within the Pod inlet reservoir to ensure that cultures do not deplete.

Steps

The steps for replenishing media are as follows:

Step	Action
1	Pause Zoë by pressing the silver “Activation” button.
2	Remove the trays and then place them into the BSC.
3	Refill the Pod media reservoirs with appropriate fresh cell culture medium. Then, perform a Via wash by pipetting 200 µL of medium directly over the top of the Via to dislodge any bubbles that may be present.
4	Replace the Pod lids and then return trays to Zoë.
5	Ensure media is collected from the indicated regions, avoiding disturbing the Pod reservoir Vias.
6	Dispense the collected effluents into the appropriate well plates or tubes (Figure 17). Change tips between Pods to avoid cross contamination.
7	Cover the Pod and then move on to the next, collecting medium from all your chips. **Note:** For more information, check the "Effluent sampling protocol".

Effluent Sampling

Overview

Medium effluent samples can be easily collected from Pod reservoirs over time for many types of analyses such as ELISA, permeability, LCMS, and others. Samples can be collected on 96-well plates or tubes and stored at -80°C or according to specific kit manufacturer's instructions.

Steps

Follow the steps below to collect effluent from all reservoirs at once:

Step	Action
1	Pause Zoë by pressing the silver “Activation” button.
2	Remove the trays and place them in the BSC.
3	Remove the Pod lid to collect effluent, one pod at time.
4	Use a standard multichannel pipette; adjust the volume to 50-200 µL to collect effluent and media from all the four reservoirs simultaneously by placing the pipette tips into the Pods such that one tip is in each reservoir, as depicted in Figure 16.
5	Ensure media is collected from the indicated regions, avoiding disturbing the Pod reservoir Vias.
6	Dispense the collected effluents in appropriate well plates or tubes (Figure 17). Change tips between Pods to avoid cross contamination.
7	Cover the Pod and move to the next, collecting medium from all your chips.

Note: For more information, check the “Effluent sampling protocol”.

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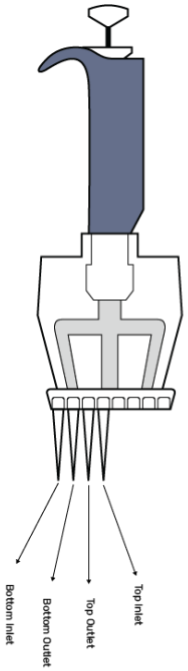
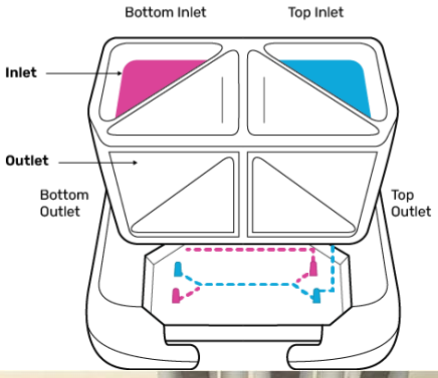
Effluent Sampling, continued

Figure 16 Multichannel collection of effluent from the Pod:





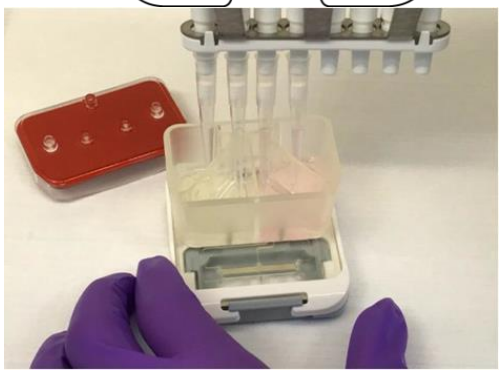
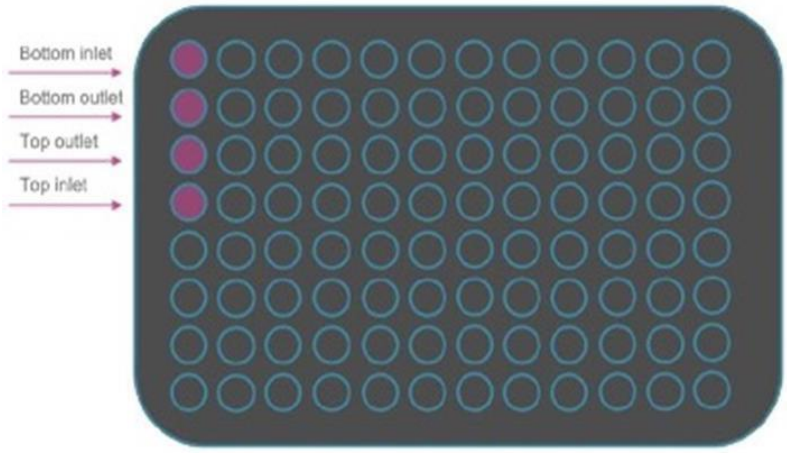


Figure 17 Effluent media dispensed in a 96-well plate:



Day X: Chip Termination

Day X Procedure

Overview

Once the Organ-Chip experiment is finished, chips can be processed for a variety of analyses, including:

- Preparation of lysates for RNA-based assays
- Live staining or fixation for immunofluorescence imaging
- Harvesting cells for obtaining a single-cell suspension

Information about Endpoint Assays

Please refer to Part VIII for additional information on representative validated endpoint assays.

Part VIII: Useful Protocols and References

Protocols

List of Protocols

Protocol Subject	ID Number
Liver-Chip Co-Culture Protocol	EP008
Liver-Chip Tri-Culture Protocol	EP080
Liver-Chip Quad-Culture Protocol	EP079
Colon Intestine-Chip Culture Protocol	EP203
Duodenum Intestine-Chip Culture Protocol	EP203(2)
Kidney-Chip Co-Culture Protocol	EP169

Guidelines

Guideline Subject	ID Number
Alveolus Lung-Chip Culture Guideline	EG180

Resources

Endpoint and Readout Protocols

Effluent Assay References	
Document	ID Number
Effluent Sampling	EP124
Barrier Function Analysis Protocol	EP187
Albumin Quantification Assay	EP139

Lysate Isolation	
Document	ID Number
Isolation and Purification of RNA Samples (Trizol method)	EP216
Cell Lysis for RNA Isolation (Non-trizol method)	EP161
Protein Sample Isolation	EP210

Imaging Chip References	
Document	ID Number
Bright Field Phase Contrast Imaging	EP123
Fluorescence Imaging	EP126
Live Staining of Cells	EP155
Fixation and Immunofluorescence-IF-Staining	EP137
Immunofluorescence Staining - Colon Intestine-Chip	EP217

References

Publications

Organ-Specific Publication:
Musah, S. et al. “Directed differentiation of human induced pluripotent stem cells into mature kidney podocytes and establishment of a Glomerulus Chip”. Nature Protocols. 2018. (13)
https://www.nature.com/articles/s41596-018-0007-8