emulate

Brain-Chip Culture Protocol

EP212 Rev. A

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Introduction to the Human Emulation System®

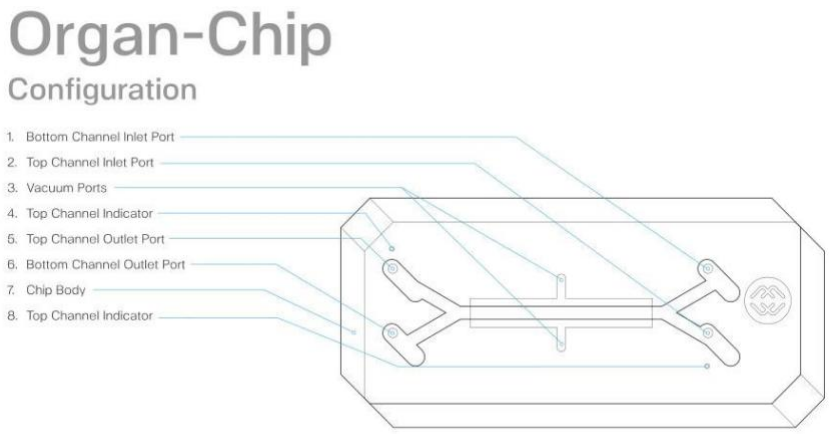
This section provides an overview of key components of the Human Emulation System®, including the Chip-S1® Stretchable Chip, Pod® Portable Module, Zoë® Culture Module, and Orb® Hub Module.

An Organ-Chip is a living, micro-engineered environment that recreates the natural physiology and mechanical forces cells experience within the human body. The Pod provides the media to the Organ-Chip needed to promote cell growth; the Pod also acts as the interface between the Organ-Chip and the Zoë Culture Module. Zoë delivers 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)

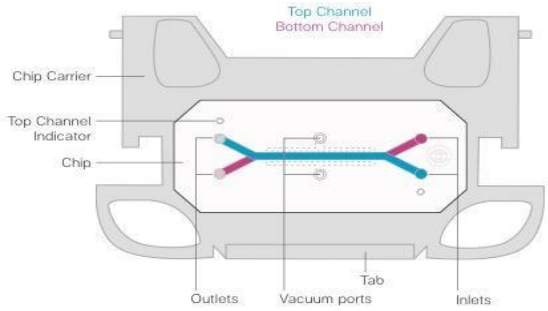
Our Chip-S1 can be configured to emulate different organ tissues, including lung, liver, intestine, kidney, and brain. The chip, when connected to the Pod and Zoë, recreates the body's dynamic cellular microenvironment, including tissue-to-tissue interfaces, blood flow, and mechanical forces. This protocol describes the methodology for the Brain-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-to-cell interactions like those observed *in vivo.* These two channels are fluidically independent. Cells in each channel are seeded with organ- and cell-specific extracellular matrix proteins (ECM) and can be maintained in static culture for up to four days—depending on cell type—before being connected to Zoë, which provides continuous flow of cell culture media.



Chips are supplied pre-packaged in the chip carrier and should be **maintained in the chip carrier at all times**. The chip carrier is designed to hold chips in place, eliminating the need to disturb or distort the

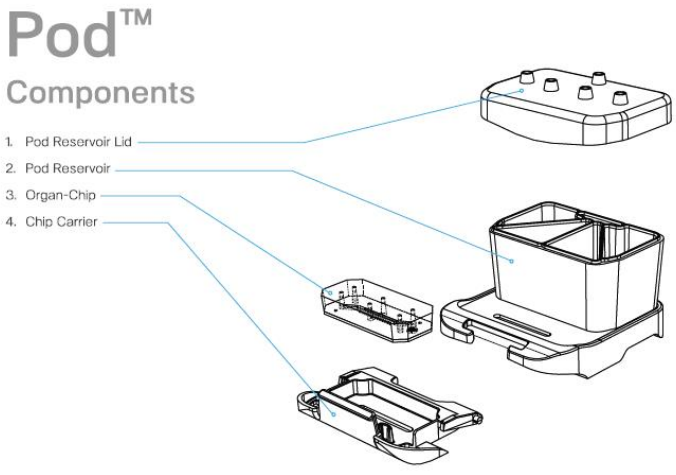
chip during preparation. In addition, the chip carrier connects securely to the Pod, which acts as the interface between Zoë and the chip. Use sterile tweezers when handling chips in chip carriers. Removal of the chip from the chip carrier prior to or during an experiment could result in chip failure. The only appropriate time to remove the chip from the carrier is when it is no longer being used for live culture of the cells in the experiment.

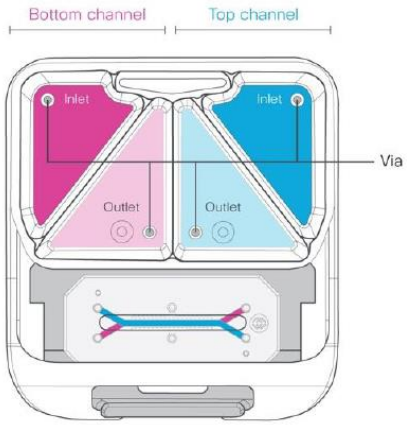


Pod

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, sample chip effluent, and precisely control dosing to test drugs or other inputs. Each Pod reservoir holds up to 4 mL.





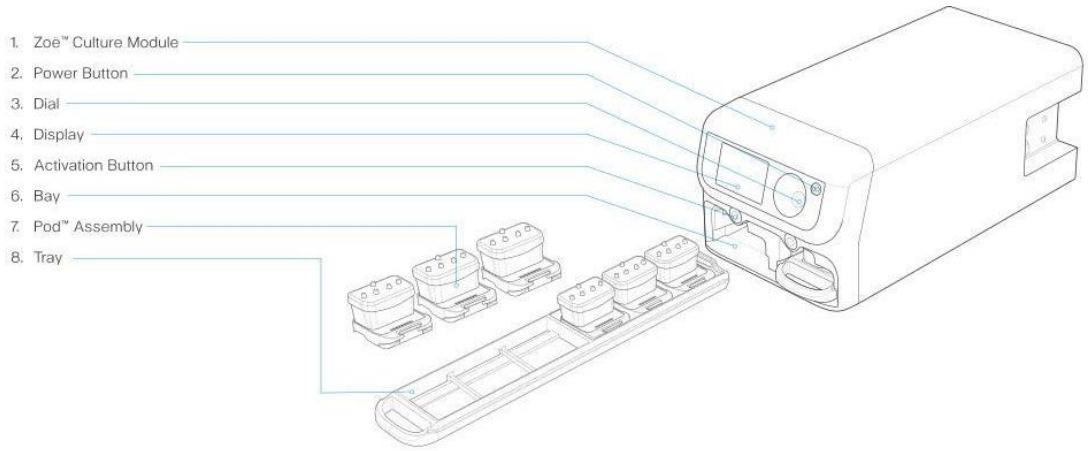
Zoë Culture Module

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 to recreate the microenvironment cells experience *in vivo*. The instrument automates the precise conditions needed for the simultaneous cell culture of up to 12 Organ-Chips.

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

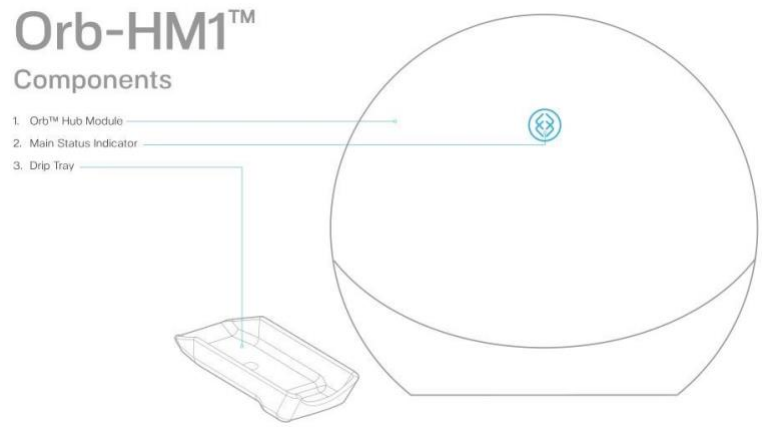
Zoë Culture Module

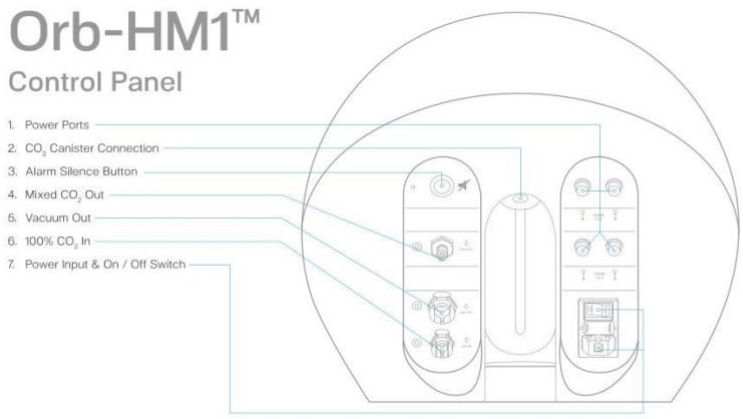
Components



Orb Hub Module

The Orb is a central hub that connects to up to four Zoë Culture Modules. It allows for simple installation and operation of 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 through its four individual power ports. It generates a 5% CO_2 supply gas to Zoë by combining air with an external 100% carbon dioxide supply (or from a portable CO_2 canister for increased flexibility) in a controlled mixture. The Orb also generates the vacuum required to apply Organ-Chip stretching (-70 kPa).

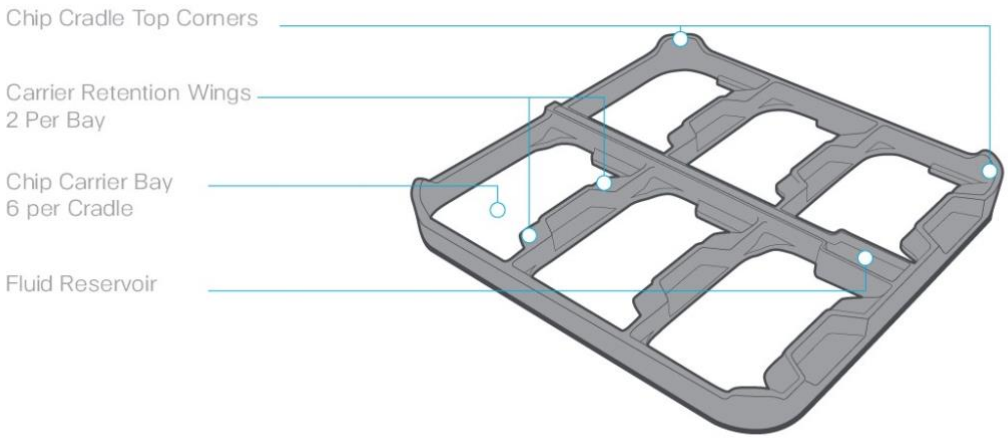

Orb-HM1™
Components
1. Orb™ Hub Module
2. Main Status Indicator
3. Drip Tray


Orb-HM1™
Control Panel
1. Power Ports
2. CO_2 Canister Connection
3. Alarm Silence Button
4. Mixed CO_2 Out
5. Vacuum Out
6. 100% CO_2 In
7. Power Input & On / Off Switch

Chip Cradle

The Chip Cradle is an autoclavable, plastic accessory that holds and organizes up to six chips, offering several benefits:

- Allows inversion of the chips during seeding.
- Keeps the chips humidified through a DPBS reservoir.
- Contains slots with numbered labels to help keep chips organized.



Cells

The Emulate Brain Bio-Kit contains human primary astrocytes, primary pericytes, microglia (SV40 immortalized human embryonic cells), and induced pluripotent stem cell (iPSC)-derived GABAergic and iPSC-derived glutamatergic neurons that are seeded into the top channel of the Chip-S1. iPSCs are included for differentiation into human brain microvascular endothelial cells (hBMECs) that are seeded in the bottom channel.

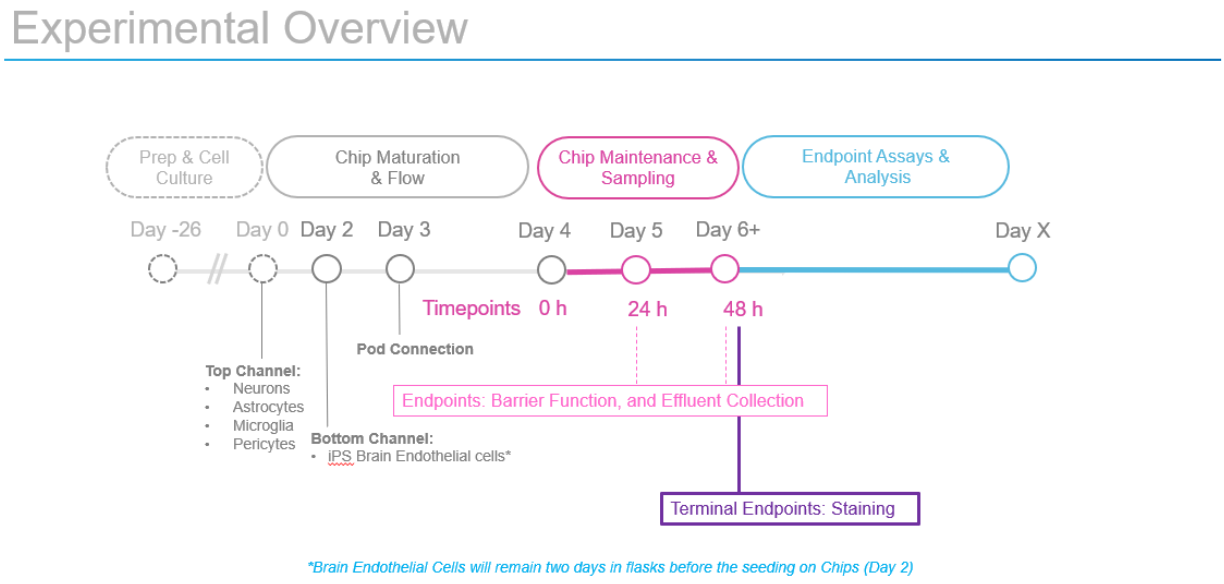
Media and Gas Equilibration

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

It is 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 -26: Reagent Preparation

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

Day -25: Thaw, Expand, and Passage iPSCs

Day -10: Differentiate iPSCs

Day -3: Thaw Pericytes and Microglia

- Prepare appropriate culture mediums and flasks

Day -1: Final Differentiation of iPSCs, Chip and Flask Preparation

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

Day 0: hBMECs to Flasks and Brain Cells to Chips

Replating hBMECs from plates to flasks

- Prepare seeding medium
- Prepare flasks
- Prepare Cell Counting Solution
- Harvest hBMECs from 6-well plates
- Seed hBMECs to flasks
- Allow cell attachment for 20 minutes
- Refresh with seeding media

Brain Cells to Chips

- Prepare Seeding Medium
- Prepare chips
- Prepare Cell Counting Solution
- Harvest pericytes and microglia
- Count cells and assess viability
- Seed the above cell types to the top channel

Day 1: Refresh Media of Flasks and Chips

- Prepare Short-Term Maintenance Medium
- Refresh both channels of chips and flasks

Day 2: hBMECs to Chips

- Prepare Endothelial Medium
- Harvest hBMECs from flasks
- Adjust cell density
- Seed hBMECs to bottom channel and invert chip
- Incubate for 2 hours
- Flip chips back and refresh media

Day 3: Chips to Pods and Pods to Zoë

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

Days 4+: Chip Maintenance and Sampling

- Media replenishment and sampling

Required Materials

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

Equipment and Materials

Equipment	Description	Supplier	Catalog Number
Brain Bio-Kit	Brain Bio-Kit contains all 5 qualified cell types, Organ-Chips, reagents (ER1, ER2, Seeding Media, and Short-term Media), Pods, & Steriflips for 6 or 12 Organ-Chip replicates. For research use only.	Emulate	BIO-BR1-COR6 (pk of 6) BIO-BR1-COR12 (pk of 12)
Zoë® Culture Module	Zoë is an instrument that can hold and support biology in up to 12 Chips-S1 and Pods. It is used for the application of physiologically relevant mechanical forces through the membrane.	Emulate	ZOE-CM1
Orb-HM1® Hub Module	The Orb Hardware Module 1 supplies Zoë with power and pressure. One Orb can support up to four Zoës.	Emulate	ORB-HM1
UV Light Box	1 per Zoë.	Emulate	-
Chip Cradle	The Chip Cradle kit contains 2 cradles and 1 pack of 10 square dishes. The cradle is a tool that holds 6 chips and simplifies the ECM activation and cell seeding workflow.	Emulate	CHIP-CRD

Equipment and Materials (continued)

Equipment	Description	Supplier	Catalog Number
Handheld vacuum aspirator	N/A	Corning	4930
Aspirating pipettes	2 mL, polystyrene, individually wrapped	Corning / Falcon	357558
Aspirating tips	Sterile (autoclaved)	N/A	N/A
Serological pipettes	2 mL, 5 mL, 10 mL, and 25 mL low endotoxin, sterile	N/A	N/A
Pipette	P20, P200, P1000, and standard multichannel	N/A	N/A
Pipette tips	P20, P200, and P1000. Sterile, low-adhesion	N/A	N/A
Conical tubes	15 mL and 50 mL polypropylene, sterile	Eppendorf	15 mL – 0030122216 or 50 mL - 0030122240
Eppendorf Tubes®	1.5 mL, sterile	Eppendorf	022431081
96 wells black walled plate	For permeability assessment	N/A	N/A
Microscope (with camera)	For bright-field imaging	N/A	N/A
Water bath (or beads)	Set to 37°C	N/A	N/A
Vacuum set-up	Minimum pressure: -70 kPa	N/A	N/A
T-75 flasks	N/A	BD Falcon	353136
Ice bucket	N/A	N/A	N/A
70% ethanol and wipes	For surface sterilization	N/A	N/A
Cell Strainer	40 µm	N/A	N/A
Vacuum Filter/Storage Bottle System	0.22 µm pore, sterile	Corning	430758
Square Cell Culture Dish (120 x 120 mm)	Sterile, 1 per 6 chips	VWR	82051-068

Reagents, Media & Supplements

Reagent	Description	Supplier	Catalog Number
ER-1	ER-1 is a required reagent to activate chips for cell seeding (5 mg powder)	Emulate	ER-105
ER-2	ER-2 is a required reagent to activate chips for cell seeding (25 mL bottle)	Emulate	ER-225
Dulbecco's PBS (DPBS - / -) (without Ca^{2+}, Mg^{2+})	1x	Corning	21-031-CV
Trypan blue	0.4% solution	Sigma	93595
TrypLE Express	Dissociation reagent	ThermoFisher Scientific	12604013
DMEM/F12, HEPES, no phenol red	Medium	ThermoFisher Scientific	11039021
mTeSR™ Plus Kit	Medium and Supplement	Stemcell Technologies	100-0276
ReLeSR™	Cell Detachment Solution	Stemcell Technologies	05872
Human Endothelial Serum-Free Media (SFM)	Basal Medium	ThermoFisher Scientific	11111044
Fetal bovine serum (FBS)	Sterile, heat-inactivated	Sigma	F4135
Complete Classic Medium with CultureBoost™	Medium and Supplement	Cell Systems	4Z0-500
Attachment Factor™	1x	Cell Systems	4Z0-210
Seeding Media with Supplement	SM1 Brain-Chip Seeding Media, 50 mL bottle	Emulate	RGT-SM1-50
Short-Term Maintenance Basal Media with Supplement	SM2 Brain-Chip Short-term Media, 250 mL bottle	Emulate	RGT-SM2-250

Matrigel™ – hESC-Qualified Matrix, LDEV-Free	Plate Coating	Corning	354277
Collagen IV	1 mg / mL	Sigma	C5533
Fibronectin	1 mg / mL	Corning	356008
Y-27632	ROCK inhibitor 10 mM	Stemcell Technologies	72304
Penicillin-Streptomycin	Antibiotic	Sigma	P4333-100ML
Eagle's Minimum Essential Medium (EMEM)	Medium	ATCC	30-2003
Ethyl alcohol, 200 proof, for molecular biology	Molecular grade	Sigma	E7023
Cell Culture Grade Water	Sterile, Water	Corning	MT25055CV
Human Serum from platelet-poor human plasma	Sterile, supplement for medium	Sigma	P2918
ACCUTASE™	Cell Detachment Solution	Stemcell Technologies	07920
MEM Non-Essential Amino Acids Solution	Supplement for medium, 100x	ThermoFisher Scientific	11140050
2-Mercaptoethanol	14.3 M	Sigma	M3148
B-27 Supplement, serum free	Supplement for medium, 50x	ThermoFisher Scientific	17504044
Recombinant Human FGF basic/FGF2/bFGF (146 aa) Protein	25 µg stock	R&D Systems	233-FB-025/CF
Retinoic Acid	50 mg powder	Sigma	R2625-50MG
Laminin Mouse Protein, Natural	0.5 - 2.0 mg/mL	ThermoFisher Scientific	23017015

CHIR99021	GSK3 enzyme inhibitor 6 µM	Reprocell	04-0004-10
3kDa Dextran Cascade Blue	10 mg powder	Invitrogen	D7132
0.5kDa Lucifer Yellow CH, Lithium Salt	25 mg power	Invitrogen	L453

Notes for ER-1 and ER-2

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

Aseptic Technique

- Prepare and sanitize the work surface of the biosafety cabinet (BSC) prior to beginning. Ensure that the workspace 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.
- Always avoid touching the chip directly.
- Handle the chip carrier with gloves and only by the sides or tab.
- Never remove the chip from the chip carrier prior to an experiment.

Cell Storage

Cryopreserved cells must be stored accordingly. Severe damage to cell membrane and cytoskeletal components can result from chronic temperature fluctuations. iPSC-derived Glutamatergic and GABAergic neurons must be stored in -80°C for up to 6 months. The remaining cell types, primary pericytes, astrocytes, microglia (SV40 immortalized human embryonic cells), and iPSCs must be stored in liquid nitrogen.

Chip Handling Techniques

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

When pipetting to fill each channel, 35 µL volume is generally used for the top channel, and 15 µL is used for the bottom channel. These volumes allow for simple pipetting and a slight overfill to avoid bubbles or

dry channels. All wash steps, unless otherwise stated, are performed using 200 µL of the specific wash solution.

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 µL
Imaging distance from bottom of chip to top of membrane	850 µm

Bottom Channel

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

Membrane

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

Co-Culture Region

Area	17.1 mm^2

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

Brain-Chip Protocol

Day -26: Reagent Preparation

Aliquot Reagents

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

1. Y-27632 (ROCK inhibitor)

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

- Resuspend 5 mg of Y-27632 compound in 1.56 mL of DPBS according to manufacturer's instructions, which will yield a stock concentration of 10 mM.
- The final concentration of Y-27632 used in medium will always be 10 µM.
- Aliquot reconstituted Y-27632 to single-use volumes of 20 µL and 100 µL and store at -20°C.

2. CHIR99021 (GSK-3 inhibitor)

Reagent	Conc. [Stock]	Amount	Volume	Solvent
CHIR99021	6 mM	10 mg	3.58 mL	DMSO

- Resuspend 10 mg of CHIR99021 compound in 3.58 mL of DMSO according to manufacturer's instructions, which will yield a stock concentration of 6 mM.
- The final concentration of CHIR99021 used in-medium will be 6 µM.
- Aliquot to single-use volumes and store at -20°C.

3. Recombinant Human Fibroblast Growth Factor (FGF) basic (146 aa) Protein, CF

Reagent	Conc. [Stock]	Amount	Volume	Solvent
Recombinant Human FGF	2.5 µg / mL	25 µg	10 mL	Sterile DPBS (1x)

basic (146 aa) Protein, CF				

- The stock vial of Recombinant human FGF basic protein is 25 µg and should be reconstituted in 10mL of DPBS (1x), creating a 2.5 µg/mL stock concentration.
- Aliquot the desired volume needed per differentiation day for a final concentration of 2.5 ng/mL.
- Store at -20°C immediately afterwards.
- Thaw aliquots just prior to use.

4. Matrigel – hESC-Qualifed Matrix, LDEV-free

Reagent	Volume
Matrigel - hESC-Qualifed Matrix, LDEV-free	5 mL

- The stock bottle of Matrigel may be thawed on ice overnight in a 2°C to 6°C refrigerator or in a cold room. Maintain Matrigel on ice at all times, as this solution gels rapidly at temperatures above 8°C.
- After thawing, aliquot Matrigel to suitable single-use volumes that are equivalent to the dilution factor specified by the manufacturer's instructions. This information can be found on the Corning's product data sheet after inputting the lot number. The protein concentration varies per lot, but each aliquot is typically around 270-350 µL. These aliquots need to be dissolved in 25 mL of complete DMEM/F12 medium. Smaller aliquots can be made to limit waste.
    - Keep all materials on ice at all times.
    - Use pre-chilled pipettes, tips, and tubes at -20°C prior to aliquoting.
    - Freeze aliquots immediately at -20°C.
- Thaw aliquots on ice just prior to use.
- Once aliquots are thawed, do not re-freeze.

5. Collagen IV

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

- Resuspend 5 mg of Collagen IV in 5 mL of sterile cell culture grade water and place at 4°C until dissolved or allow it to dissolve overnight.
- Prepare 1 mL aliquots and store aliquots at -20°C.

6. Fibronectin

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

- Resuspend 5 mg of Fibronectin in 5 mL of sterile cell culture grade water and leave the mixture at 4°C for 30 min to dissolve (avoid harsh agitation or vortexing). Swirl gently before aliquoting.
- Prepare 1 mL aliquots and store at -20°C.

7. 3kDa Dextran Cascade Blue

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

- In BSC, resuspend 10 mg of Dextran, Cascade Blue™, 3000 MW, Anionic, Lysine Fixable in 1 mL of sterile cell culture grade water to obtain 3kDa Dextran Cascade Blue Working Solution at 10 mg/mL concentration. The final concentration in medium is 100 µg/mL or 1:100 dilution. One vial of 10 mg of 3kDa Dextran Cascade Blue is sufficient for 100 mL of media.
- Freeze any remaining solution at -20°C.

8. 0.5kDa Lucifer Yellow CH, Lithium Salt

Reagent	Conc. [Stock]	Amount	Volume	Solvent
Lucifer Yellow CH, Lithium Salt	1 mg/mL	25 mg	25 mL	Sterile Water Cell Culture Grade

- In BSC, resuspend 25 mg of Lucifer Yellow CH, Lithium Salt in 25 mL of sterile cell culture grade water to obtain Lucifer Yellow Working Solution at 1 mg/mL concentration. The final concentration in medium is 20 µg /mL or 1:50 dilution.
- Freeze any remaining solution at -20°C.

Day -25: Thaw, Expand, and Passage iPSCs

Goals:

- Expand iPSC in plates prior to chip seeding as per established protocols.

Key Steps:

- Prepare mTeSR culture media.
- Thaw and plate iPSCs in 6-well plates.

Required Materials:

- Complete mTeSR Culture Medium (at 37°C)
- DMEM/F-12, HEPES, no phenol red medium
- Y-27632
- ReLeSR™
- Accutase
- DPBS (1x)
- Matrigel
- 15 mL conical tube
- 6-well plates
- T-75 flask
- Serological pipettes
- Pipettes and tips
- Aspirator
- Centrifuge
- 70% ethanol
- Microscope

Culture Media for iPSCs

mTeSR™ Plus Culture Media for iPSCs (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
mTeSR™ Plus Kit	495 mL	N/A	N/A	Stemcell Technologies	100-0276
Penicillin-Streptomycin	5 mL	10,000 U/mL; 10 mg/mL	1%	Sigma	P4333-100ML
Y-27632, “iRock”	Varies	10 mM	10 µM	Stemcell Technologies	72632

Culture Media for iPSCs is composed of mTeSR Plus growth medium (100-0274) and a 5x mTeSR Plus Supplement (100-0275). Stemcell Technologies supplies both as a kit (100-0276). Please reference the table above for more information.

1. Aseptically transfer 100 mL of the 5x mTeSR Plus Supplement to the mTeSR Plus medium bottle. This will make a total volume of 500 mL.
2. Penicillin-Streptomycin is a non-toxic antimicrobial agent for the cells. Remove 5 mL of the above medium, then add 5 mL of Penicillin-Streptomycin (P4333-100ML). This will make a 1% final concentration that is complete and ready for use.
3. Store complete media at 4°C for up to 30 days and bring to room temperature before use.
4. The addition of 10 µM Y-27632 to the complete mTeSR medium is only required on the days of thawing and passaging. To do this, perform a 1:1000 dilution using prior aliquots. On the following days, ensure Y-27632 is *not* added to the media.

Coat 6-well Plates with Matrigel

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
DMEM/F12, HEPES, no phenol red	495 mL	N/A	N/A	ThermoFisher Scientific	11039021
Penicillin-Streptomycin	5 mL	10,000 U/mL; 10 mg/mL	1%	Sigma	P4333-100ML
Matrigel – hESC-Qualified Matrix, LDEV-Free	Varies	Varies	Varies	Corning	354277

Aseptically, combine 5 mL from the Penicillin-Streptomycin stock bottle into a 495 mL bottle of DMEM/F12, making a complete DMEM/F12 medium (please reference the table above for more information). This

process will need to be repeated before the passage of iPSCs. Proportionally scale the volumes up or down as needed. Properly sealed and coated plates can last up to a week in 4°C.

1. Label one 6-well tissue culture plate with relevant information (e.g., cell type, date, initials, etc.)
2. Warm sufficient mTeSR Plus medium to room temperature (see section below).
3. Dissolve 1 aliquot Matrigel in appropriate volume of complete DMEM/F12 medium (cat: 11039021) on ice. The single-use aliquot needs to be dissolved in 25 mL of complete DMEM/F12 medium. The final concentration varies per lot; however, a sample calculation is as follows:
   Single-Use Aliquot: 181 µL
   Volume of DMEM/F12: 25 mL
   Final Concentration: 181 µL / 25 mL = 7.24 µL / mL
4. Add 1 mL of Matrigel solution into each well of the 6-well plate. Pipette on the side of the wells.
5. Ensure an even distribution of Matrigel coating in all wells by moving the plate vertically and horizontally. Wrap the plate carefully using Parafilm®.
6. Store overnight at 4°C, and on day of use, place in BSC for at least one hour before use.
7. Carefully aspirate Matrigel solution from each well. Do not disturb the coating in the well.
8. Gently wash each well with 1 mL of complete mTeSR Plus medium.
9. Aspirate mTeSR Plus medium and add 1.5 mL of complete mTeSR + 10 µM iRock media to each well that will be seeded.

Thaw iPSCs as Colonies

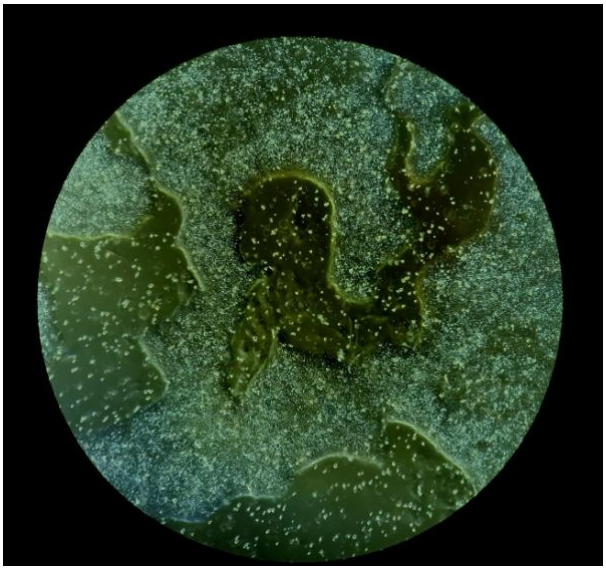
iPSCs require expansion and differentiation in culture prior to seeding in the chip.

1. Thaw the vial(s) of cells by immersing them in a 37°C water bath for approximately 60 to 90 seconds. Closely observe the thawing process while gently agitating the vial. Remove it from the water bath just before the last ice pellet disappears.
2. Spray vial(s) with 70% ethanol and wipe dry before placing them in the BSC.
3. Immediately and gently transfer the contents of the vial using a P1000 pipette into the prepared conical tube containing warm complete mTeSR Plus medium with 10 µM Y-27632.
4. Rinse the vial with 1 mL of complete mTeSR Plus medium with 10 µM Y-27632 and add to the same conical. Bring up the total volume of the conical tube to 7 mL with complete mTeSR medium with 10 µM Y-27632.
5. Centrifuge the conical tube at 200g for 5 minutes at 24°C or according to vendor's instructions.
6. Aspirate supernatant and gently break up the pellet to keep colonies intact.
7. Resuspend in the minimum amount of complete mTeSR medium with 10 µM Y-27632 needed for thawing (0.5 mL per well).
8. Add 0.5 mL of cell suspension dropwise into each well of the previously Matrigel-coated 6-well plate. You will need one well for each vial thawed, performing a 1:1 dilution.
9. Check under the microscope for suspended colonies in desired wells.
10. Incubate overnight at 37°C and 5% CO_2.

11. The following day, refresh colonies with 2 mL of complete mTeSR medium *without* Y-27632. Repeat this step every two days until cells are at least 70% confluent. A sample image of what this will look like is below.

    Note: Continue with passaging once cells reach at least 70% confluency (refer to image below). This will likely fall before or after the designated timeline. It is expected to take 4-5 days for the cells to reach the desired confluency; it is acceptable if your cells expand slower or quicker than this rate. Proceed with the first round of passaging.

    Try to coat new plates with Matrigel the day before cells reach 70% confluency. Prepare one 6-well plate with Matrigel for each vial thawed using the previous instructions. If two vials were used, prepare two 6-well plates to perform a 1:6 dilution. Proportionally scale up or down accordingly. Properly sealed and coated plates can last up to a week in 4°C.



D-20: Sample image of colonies of iPSCs >70% confluency after media wash

First Passage of iPSCs as Colonies

1. On the day before the cells reach 70% confluency, coat the necessary number of 6-well plates with Matrigel using the previous instructions. Ensure that all reagents and the new Matrigel-coated plates are at room temperature on the day of passaging.
2. Aspirate, wash, and replenish each well as instructed previously. They should end with a volume of 1.5 mL of complete mTeSR + 10 µM iRock media.
3. Carefully aspirate complete mTeSR Plus medium from the plate *with cells*.

4. Wash each well with 1 mL of DPBS (1x) and aspirate afterwards.
5. Carefully pipette 500 µL of ReLeSR™ to each well, ensuring that a thin layer of detachment reagent covers the entire surface of the well.
6. Incubate at 37°C and check for detachment after 3 minutes.
7. Once cells are detached, gently tap the plate so that the colonies detach without being broken down and collect them in the conical tube. Bring the total volume up to 10 mL using complete mTeSR medium with 10 µM Y-27632. Spin at 200g for 5 minutes at 24°C or according to manufacturer's instructions.
8. Aspirate the supernatant and do not disturb the pellet.
9. Carefully break the pellet using 1 mL of complete mTeSR Plus medium with 10 µM Y-27632; use the minimum amount of pipetting possible to keep colonies intact.
10. Resuspend cells in the minimum volume of complete mTeSR with 10 µM Y-27632 needed (0.5 mL per well).
11. Add 0.5 mL of cell suspension dropwise to each well of the already-coated 6-well plates to make a final volume of 2 mL.
12. Incubate overnight at 37°C and 5% CO_2.
13. The following day, refresh the colonies with 2 mL of complete mTeSR Plus medium *without* Y-27632. Repeat this step every two days until cells reach at least 70% confluency. Refer to image above.

Note: Continue with passaging once cells reach at least 70% confluency (refer to previous image). This will likely fall before or after the designated timeline. It is expected to take an additional 4-5 days for the cells to reach the desired confluency; it is acceptable if your cells expand slower or quicker than this rate. Proceed with the second round of passaging.

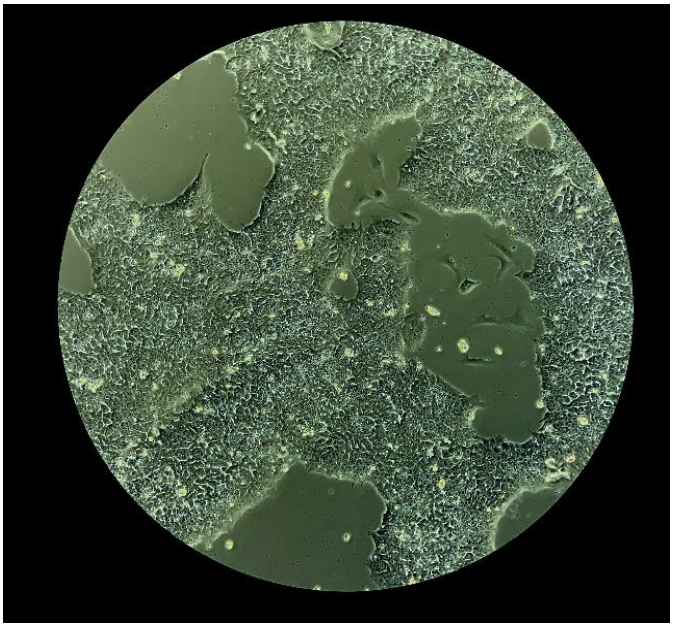
Try to coat new plates with Matrigel the day before cells reach 70% confluency. Prepare three 6-well Matrigel-coated plates for each plate currently with cells. If two full plates currently have cells, coat six 6-well plates with Matrigel as instructed previously. This creates a 1:3 dilution. Proportionally scale up or down accordingly. Properly sealed and coated plates can last up to a week in 4°C.

Second Passage of iPSCs as Single Cells

1. On the day before cells reach 70% confluency, prepare the necessary number of 6-well Matrigel-coated plates for each plate currently with cells. Ensure that all reagents and the new Matrigel-coated plates are at room temperature on the day of passaging.
2. Aspirate, wash, and replenish each well as instructed previously. They should end with a volume of 1.5 mL of complete mTeSR + 10 µM iRock media.
3. Carefully aspirate complete mTeSR Plus medium from the plates with *cells*.
4. Wash each well with 1 mL of DPBS (1x) and aspirate afterwards.
5. Add 1 mL of Accutase to the cells.
6. Incubate at 37°C and check for detachment every 5 minutes.

7. Place a 40 µm cell strainer on top of a 50 mL conical tube.
8. Add a base layer of complete mTeSR Plus medium with 10 µM Y-27632 to the conical tube.
9. Once the cells begin to detach, collect the cells into the conical tube. Rinse each well with 1mL of complete mTeSR Plus medium and add to the conical tube. Use force to detach any remaining cells. Ensure the cells are passed through the cell strainer.
10. Bring up conical tube to 50 mL using complete mTeSR Plus medium with 10 µM Y-27632. Spin at 200g for 5 minutes at 24°C.
11. Aspirate the supernatant and do not disturb the pellet.
12. Carefully break the pellet using 1 mL of complete mTeSR Plus medium with 10 µM Y-27632 and continue to pipette up and down to ensure that mostly single cells remain.
13. Resuspend cells in the minimum volume of complete mTeSR Plus medium with 10 µM Y-27632 needed (0.5 mL per well.)
14. Add 0.5 mL of cell suspension dropwise to each well of the already coated 6-well plates, making a final volume of 2 mL.
15. Incubate overnight at 37°C and 5% CO_2.
16. The following day, refresh cells with 2 mL of complete mTeSR Plus medium *without* Y-27632. Repeat this step every two days until cells reach at least 70% confluency. Refer to image below.

    Note: Continue to the next section once cells reach at least 70% confluency. This will likely fall before or after the designated timeline. It is expected that it will take an additional 3-4 days for the cells to reach this stage. Differentiation will occur in these same plates.



D-11: Sample image of single-celled iPSCs > 70% confluency after media wash

Day -10: Differentiate iPSCs

Goals:

- Differentiate iPSCs to hBMECs in plates prior to chip seeding as per established protocols.

Key Steps:

- Prepare specific differentiation medium per day.

Required Materials:

- DMEM/F-12, HEPES, no phenol red medium
- Human Endothelial SFM medium
- Y-27632
- Accutase
- DPBS (1x)
- Cell Culture Grade Water
- MEM Non-Essential Amino Acids Solution (100X)
- β-mercaptoethanol,14.3 M
- CHIR99021
- B-27 Supplement (50x), serum free
- Recombinant Human FGF Basic (146 aa) Protein, CF
- Retinoic Acid, 50 mg
- Ethyl Alcohol, pure
- 50 mL conical tube
- 6-well plates
- T-75 flask
- Serological pipettes
- Pipettes and tips
- Aspirator
- Centrifuge
- 70% ethanol
- Microscope

DeSR1 Culture Media

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
DMEM/F12, HEPES, no phenol red	495 mL	N/A	N/A	ThermoFisher Scientific	11039021
Penicillin-Streptomycin	5 mL	10,000 U/mL; 10 mg/mL	1%	Sigma	P4333-100ML
MEM Non-Essential Amino Acids Solution	800 µL	100x	1%	ThermoFisher Scientific	11140050
2-Mercaptoethanol	7 µL	14.3 M	0.1 mM	Sigma	M3148
CHIR99021	80 µL	10 mg	6 µM	Reprocell	04-0004-10

Once the cells reach at least 70% confluency, the first day of differentiation can begin in the same plates. The differentiation medium should be made fresh before use and at the specified final concentrations. Aseptically combine 5 mL from the Penicillin-Streptomycin stock bottle into a 495 mL bottle of DMEM/F12, making a complete working medium. Dilute 7 µL of 2-Mercaptoethanol stock with 1 mL of PBS in the fume hood, making a 100 mM mixture. Lastly, in the filtration portion of the 0.22 µm filter bottle, combine 79.11 mL of complete DMEM/F12 medium with 800 µL of MEM Non-Essential Amino Acids stock, 8 µL of 2-Mercaptoethanol mixture (0.1 mM) and 80 µL of the CHIR99021 aliquot (6 µM). Filter the bottle to create DeSR1 medium. Please reference the table above for more information.

D-10: First Day of Differentiation

1. Bring the necessary reagents to room temperature in the BSC.
2. Aseptically prepare DeSR1 culture medium as described above.
3. Aspirate the mTeSR Plus medium from the cells.
4. Carefully add 2 mL of DeSR1 to each well.
5. Incubate for 24 hours at 37°C and 5% CO_2.

DeSR2 Culture Media

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete DMEM/F12, HEPES, no phenol red	495 mL	N/A	N/A	ThermoFisher Scientific	11039021
Penicillin-Streptomycin	5 mL	10,000 U/mL; 10 mg/mL	1%	Sigma	P4333-100ML
MEM Non-Essential Amino Acids Solution	800 µL	100x	1%	ThermoFisher Scientific	11140050
2-Mercaptoethanol	7 µL	14.3 M	0.1 mM	Sigma	M3148
B-27 Supplement, serum free	1.6 mL	50x	1x	ThermoFisher Scientific	17504044

The differentiation medium should be made fresh before use and at the specified final concentrations. Aseptically combine 5 mL from the Penicillin-Streptomycin stock bottle into a 495 mL bottle of DMEM/F12, making a complete working medium. Dilute 7 µL of 2-Mercaptoethanol stock with 1 mL of PBS in the fume hood, making a 100 mM mixture. Lastly, in the filtration portion of the 0.22 µm filter bottle, combine 77.59 mL of Complete DMEM/F12 medium with 800 µL of MEM Non-Essential Amino Acids stock, 8 µL of 2-Mercaptoethanol mixture (0.1 mM), and 1.6 mL of B-27 Supplement. Filter the bottle to create the DeSR2 working medium. Please reference the table above for more information.

D-9 to -5: Next Five Days of Differentiation

1. Bring the necessary reagents to room temperature in the BSC.
2. Aseptically prepare DeSR2 culture medium as described above.
3. Aspirate the DeSR1 medium from the cells.
4. Carefully add 2 mL of DeSR2 to each well.
5. Incubate for 24 hours at 37°C and 5% CO_2.
6. Repeat these steps for four additional days.

hECSR1 Culture Media

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Human Endothelial SFM	495 mL	N/A	N/A	ThermoFisher Scientific	11111044
Penicillin-Streptomycin	5 mL	10,000 U/mL. 10 mg/mL	1%	Sigma	P4333-100ML
Recombinant Human FGF basic (146 aa) Protein, CF	640 µL	25 µg	20 ng	R&D Systems	233-FB-025/CF
Retinoic acid	80 µL	50 mg	10 µM	Sigma	R2625-50MG
B-27 Supplement, serum free	1.6 mL	50x	1x	ThermoFisher Scientific	17504044
Ethyl alcohol, Pure	16.6 mL	N/A	N/A	Sigma	E7023

The differentiation medium should be made fresh before use and at the specified final concentrations. Aseptically combine 5 mL from the Penicillin-Streptomycin stock bottle into a 495 mL bottle of Human Endothelial SFM to make a complete working medium. Dilute the 50 mg stock vial of Retinoic Acid with 16.6 mL of pure ethyl alcohol in the BSC. In a separate container, combine 77.68 mL of Complete Human Endothelial medium with 640 µL of the Recombinant Human FGF basic protein aliquots, 80 µL of retinoic acid mixture (10 µM), and 1.6 mL of B-27 Supplement. This creates the hECSR1 working medium. Please reference the table above for more information.

D-4 to D-2: Next Three Days of Differentiation, D-3: Thaw Microglia and Pericytes (see below)

1. Bring the necessary reagents to room temperature in the BSC.
2. Aseptically prepare hECSR1 culture medium as described above.
3. Aspirate the DeSR2 medium from the cells.
4. Carefully add 2 mL of hECSR1 to each well.
5. Incubate for 48 hours at 37°C and 5% CO_2.
6. After 48 hours, refresh cells with new hECSR1 medium.
7. Incubate for 24 hours at 37°C and 5% CO_2.

Day -3: Thaw Pericytes and Microglia

Goals:

- Expand human brain microglia (SV40 immortalized embryonic cells) and primary brain vascular pericytes in flasks prior to chip seeding.

Key Steps:

- Prepare microglia and pericyte culture media.
- Thaw microglia and pericytes in flasks.

Required Materials:

- Complete cell culture medium for each cell type at 37°C
- Attachment Factor
- Fetal bovine serum (FBS)
- 15 mL conical tubes
- T-75 flasks
- Serological pipettes
- Pipettes and tips
- Aspirator
- Centrifuge
- 70% ethanol

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Classic Medium with CultureBoost™	495 mL	N/A	N/A	Cell Systems	4Z0-500
Eagle's Minimum Essential Medium (EMEM)	495 mL	N/A	N/A	ATCC	30-2003
Penicillin-Streptomycin	5 mL	10,000 U/mL; 10 mg/mL	1%	Sigma	P4333-100ML
Fetal bovine serum (FBS)	Varies	N/A	10%	Sigma	F4135

To ensure sufficient cell numbers, Microglia (ATCC cat: CRL-3304) and Pericytes (Cell Systems cat: ACBRI 498) must be thawed starting on D-3: Differentiation of iPSCs. Eagle's Minimum Essential Medium is used for the microglia while the Complete Classical Medium is used for the pericytes. In the BSC, combine one vial of the Cell System's CultureBoost™ to one full bottle of Complete Classic Medium.

Separately, combine 5 mL from the Penicillin-Streptomycin stock bottle into a 495 mL of Complete Classic Medium with CultureBoost™ and Eagle's Minimum Essential Medium. Determine the volume of prior medium needed for each cell type and separately add this to two storage bottles. Lastly, combine FBS to both mediums, making a final concentration of 10%. For example, 45 mL of complete medium with 5 mL of FBS. These are the working mediums. Please reference the table above for more information.

1. Prepare a flask as per cell vendor's specifications and place in an incubator at 37°C. Pre-treat flasks designated for expansion of pericytes with 5 mL of Attachment Factor for 15 seconds, ensure an even coating across the flask and then aspirate afterwards. Flasks designated for microglia do not need any pre-treatment.
2. Thaw the vial(s) of cells by immersing in a 37°C water bath. Closely observe while gently agitating the vial. Remove it from the water bath just before the last of the ice pellets disappears.
3. Spray vial(s) with 70% ethanol and wipe dry prior to placing them in the BSC.
4. Immediately transfer the contents of the vial into 3 mL of warm complete media for the respective cell type in separate sterile 15 mL conical tubes.
5. Rinse the vial with an additional 1 mL of warm medium and add it to their respective conical tube.
6. Bring the volume to 15 mL with their respective working medium.
7. Centrifuge as per vendor's instructions.
8. Aspirate and discard supernatant, leaving approximately 100 µL of medium covering the cell pellet.
9. Loosen the cell pellet by gently flicking the tube.
10. Re-suspend cells in 10-15 mL of their respective warm complete working medium.
11. Add the cell suspension to their own separately prepared flask.
12. Incubate overnight at 37°C and 5% CO_2.
13. Every two days, refresh each flask with the appropriate working medium—pre-warmed to 37°C—until flasks are confluent, or until there are approximately enough cells to seed the chips needed for experiments.

    Note: On the following day (D-2), remember to refresh iPSCs with new hECSR1 medium for 24 hours. Refer to page 29 for additional information.

Day -1: Final Differentiation of iPSCs, Chip and Flask Preparation

Goals:

- Create hECSR2 culture media for final differentiation of iPSCs.
- Activate the inner surface of the chip channels for proper ECM attachment.
- Coat top and bottom channels with an ECM mixture of collagen IV, fibronectin, and laminin.
- Coat six T-75 flask with an ECM mixture of collagen IV, fibronectin, and laminin.

Key Steps:

- Prepare hECSR2 media
- Differentiate iPSCs
- Prepare chips
- Prepare ER-1 solution
- Introduce ER-1 solution to channels
- Activate chips
- Prepare ECM solutions
- Coat chips with ECM
- Coat flasks with ECM

Required Materials:

- Human Endothelial SFM
- Penicillin-Streptomycin
- B-27 Supplement, serum free
- Chip-S1 (12 chips per Zoë)
- T-75 flasks
- ER-1 reagent
- ER-2 buffer
- 50 mL conical tubes
- DPBS (- / -) on ice
- Collagen IV
- Fibronectin
- Laminin
- 70% ethanol
- 120 x 120 mm cell culture dish
- Ice and ice bucket
- Pipettes and filtered tips
- Aspirator and sterile tips
- UV light box
- UV safety glasses

hECSR2 Culture Media

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Human Endothelial SFM	495 mL	N/A	N/A	ThermoFisher Scientific	11111044
Penicillin-Streptomycin	5 mL	10,000 U/mL; 10 mg/mL	1%	Sigma	P4333-100ML
B-27 Supplement, serum free	1.6 mL	50x	1x	ThermoFisher Scientific	17504044

The differentiation medium should be made fresh before each use and at the specified final concentrations. Aseptically combine 5 mL from the Penicillin-Streptomycin stock bottle into a 495 mL bottle of Human Endothelial SFM, making a complete working medium. In a separate container, combine 78.4 mL of Complete Human Endothelial medium with 1.6 mL B-27 Supplement. This creates the hECSR2 working medium. Please reference the table above for more information.

D-1: Final Day of Differentiation, Chip and Flask Preparation (see next section)

1. Bring the necessary reagents to room temperature in the BSC.
2. Aseptically make hECSR2 culture medium as described above.
3. Aspirate the hECSR1 medium from the cells.
4. Carefully add 2 mL of hECSR2 to each well.
5. Incubate for 24 hours at 37°C and 5% CO_2.

Chip and Flask Preparation

Prepare Chips

1. Spray chip packaging of 120 x 120 mm square cell culture dish and Chip Cradle with 70% ethanol and bring into the BSC.
2. Open the Chip Cradle sterile packaging and place the cradle into the culture dish, making sure the Chip Cradle is oriented properly with the corners facing up.
3. Carefully open the chip packaging and place the first chip into the cradle by sliding the back of the carrier under the tabs on the cradle.
4. Repeat as necessary for each of the chips included in the experiment (See Figure 1).
5. Label each chip with ID numbers on the chip carrier tab.

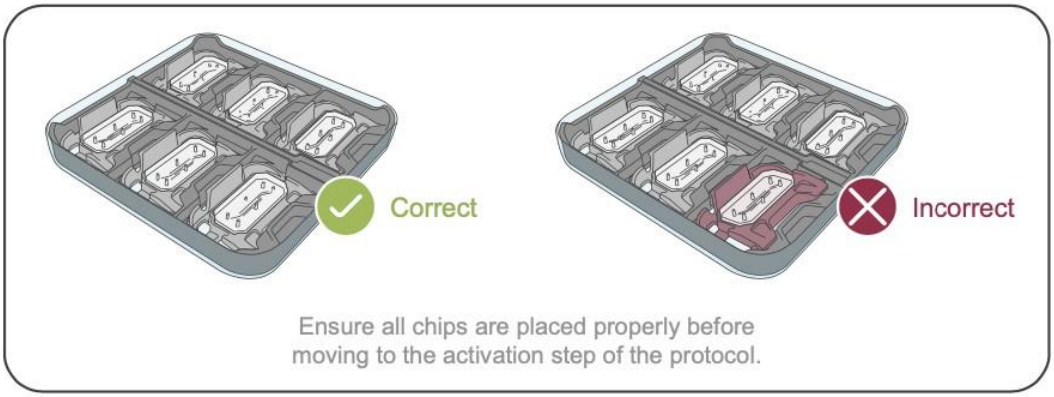


Figure 1. Place chips by sliding under the tab of the Chip Cradle.

Prepare ER-1 Solution

Prepare ER-1 solution immediately before use and discard any remaining ER-1 solution one hour after reconstitution. ER-1 is light-sensitive. Failure to protect ER-1 solution from light, or the 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 glove 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. 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 two more times 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 it to collect any remaining ER-1 powder in the lid. Transfer the collected solution to the conical tube, bringing the total volume in the tube to 4 mL ER-1 solution.
8. Add 6 mL of ER-2 solution to the 4 mL of ER-1 solution in the 15 mL conical tube for a final working concentration of 0.5 mg / mL. Pipette gently to mix without creating bubbles. ER-1 should be fully dissolved within the ER-2 solution prior to use.

Introduce ER-1 Solution to Channels

1. Using a P200 pipette and a sterile 200 µL filtered pipette tip, pipette 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 (See Figure 2 below).

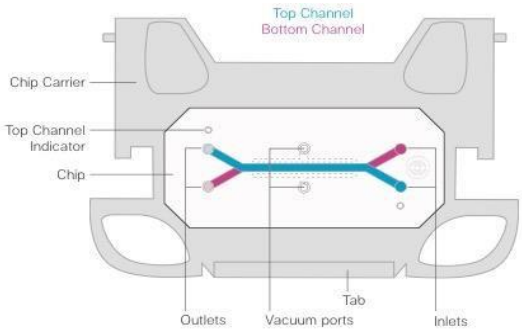


Figure 2: Top view of chip, with labelled ports in Chip Carrier

5. Remove all excess ER-1 solution from the surface of the chip by gently aspirating. Be sure only to remove ER-1 solution from the chip surface—do not aspirate ER-1 from the channels (See Figure 3 below).
6. Repeat Steps 1 through 5 for each chip.
7. Inspect the channels for bubbles prior to UV activation. If bubbles are present, dislodge by washing the channel with ER-1 solution until all bubbles have been removed. If bubbles persist, it may be helpful to completely aspirate the channel and slowly re-introduce ER-1 solution.

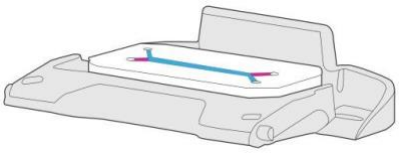


Figure 3: View of chip with no excess ER-1 solution on surface

Activate Chips

1. Bring the 120 x 120 mm square culture dish containing the ER-1-coated chips to the UV light box.
2. Before placing the square culture dish into the UV light box, be sure to remove the cover from the dish. **Note:** If the lid is not removed prior to placing the dish in the UV light box, the chips will not activate properly. This could result in poor cell attachment.
3. Select the “Constant” setting on the back of the UV light box. Press the “Power” button followed by the “On” button to begin UV activation.
4. Allow the chips to activate under UV light for 15 minutes.
5. While the chips are being treated, prepare the ECM Solution. (For more information, refer to the next section: Prepare ECM Solution.)

6. After UV treatment, bring chips back to the BSC.
7. Fully aspirate the ER-1 solution from both channels.
8. Wash each channel with 200 µL of ER-2 solution.
9. Fully aspirate the ER-2 from the channels.
10. Wash 2x each channel with 200 µL of sterile DPBS.
11. Leave DPBS inside the channels.

Prepare ECM Solutions

The ECM solution should be freshly prepared by combining the individual ECM components with cold DPBS to the final working concentrations.

For human Brain-Chips, as well as T-75 flasks, the final concentration of proteins in the ECM working solution is:

Collagen IV: 400 µg / mL

Fibronectin: 100 µg / mL

Laminin: 20 µg / mL

1. Bring a full ice bucket full into the BSC.
2. On ice, thaw one aliquot each of fibronectin (1 mg / mL), Collagen IV (1 mg / mL), and Laminin (the concentration is dependent on the specific lot used). Always maintain all individual ECM components and mixture on ice.
3. Calculate total volume of ECM solution needed to coat all flasks and chips.
    a. Volume required per flask = approximately 3 mL.
    b. For each plate with cells, prepare one flask. For example, if six plates contain cells, then prepare six flasks; therefore, 18 mL of ECM minimum is needed ((6 flasks x 3 mL) + extra 2mL = 20 mL of ECM solution). See calculation example below.
    c. Volume required per channel = approximately 50 µL
    d. For every 12 chips to coat, prepare 1.5 mL of each ECM solution (12 chips x 100 µL per chip + extra 300 µL = 1.5 mL of ECM solution). Add these two volumes together to determine total volume of ECM needed for both flasks and chips. See calculation example below.
4. Combine components to prepare ECM working solutions.
5. Keep the ECM solution on ice at all times.

Calculation Example

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

Collagen IV [final] = 0.4 mg / mL (C_2)

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

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

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

Laminin [final] = 0.02 mg / mL (C_2)

Total volume of ECM solution = 1.5 mL (V_2)

Collagen IV

$C_1V_1 = C_2V_2$, solve for V_1

(1 mg / mL) x (V_1 mL) = (0.4mg / mL) x (1.5 mL)

V_1 = 0.6 mL = 600 µL of Collagen IV stock solution

Fibronectin

$C_1V_1 = C_2V_2$, solve for V_1

(1 mg / mL) x (V_1 mL) = (0.1 mg / mL) x (1.5 mL)

V_1 = 0.15 mL = 150 µL of Fibronectin stock solution

Laminin

$C_1V_1 = C_2V_2$, solve for V_1

(1 mg / mL) x (V_1 mL) = (0.02 mg / mL) x (1.5 mL)

V_1 = 0.03 mL = 30 µL of Laminin stock solution

DPBS

DPBS = (total volume of ECM needed) – (volume of Collagen IV) – (volume of Fibronectin) - (volume of Laminin)

= 1500 µL – 600 µL – 150 µL – 30 µL

= 720 µL of DPBS

Coat Chips with ECM

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

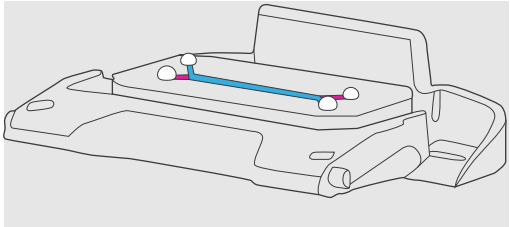


Figure 4. Chip inserted into chip carrier with small droplets of ECM solution at ports

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

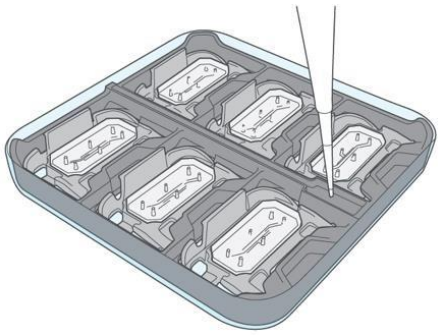


Figure 5. Fill central reservoir of Chip Cradle with 0.75 mL of DPBS

Coat Flasks with ECM

1. Spray packaging of T-75 flasks with ethanol before bringing into the BSC.
2. Wash each flask with 5 mL of sterile DPBS.
3. Fully aspirate water.
4. Pipette 3 mL of ECM mixture into each flask and ensure even distribution across the flasks.
5. Store flat overnight at 4°C.

Day 0: hBMECs to Flasks and Brain Cells to Chips

Goals:

- Plate hBMECs to flasks.
- Seed brain cells to top channel of chip.

Key Steps:

hBMECs to Flasks

- Prepare hBMEC Culture Medium
- Prepare flasks
- Harvest hBMECs
- Seed hBMECs to flasks

Brain cells to Top Channel of Chips

- Thaw cells
- Prepare Seeding Medium
- Detach Microglia and Pericytes
- Prepare chips
- Prepare cell counting solution
- Adjust cell density
- Seed brain cells to the top channel

Required Materials:

- 120 x 120 mm Square culture dishes (1 dish per 6 chips)
- Autoclaved chip cradle (1 cradle per 6 chips)
- Endothelial SFM culture medium
- Seeding medium (at 37°C)
- Human Serum from platelet-poor human plasma, sterile-filtered, “PPP”
- 15 mL and 50 mL conical tube
- Dissociation Solution
- TrypLE Express
- Y-27632
- Mini Cell Scraper
- 1X DPBS (at room temperature)
- Aspirator and sterile tips
- Trypan blue counting solution
- Hemocytometer
- Installed and qualified Zoë
- Serological pipettes
- Pipettors and filtered tips
- 70% ethanol

- 37°C water or bead bath
- Centrifuge

hBMECs to Flasks

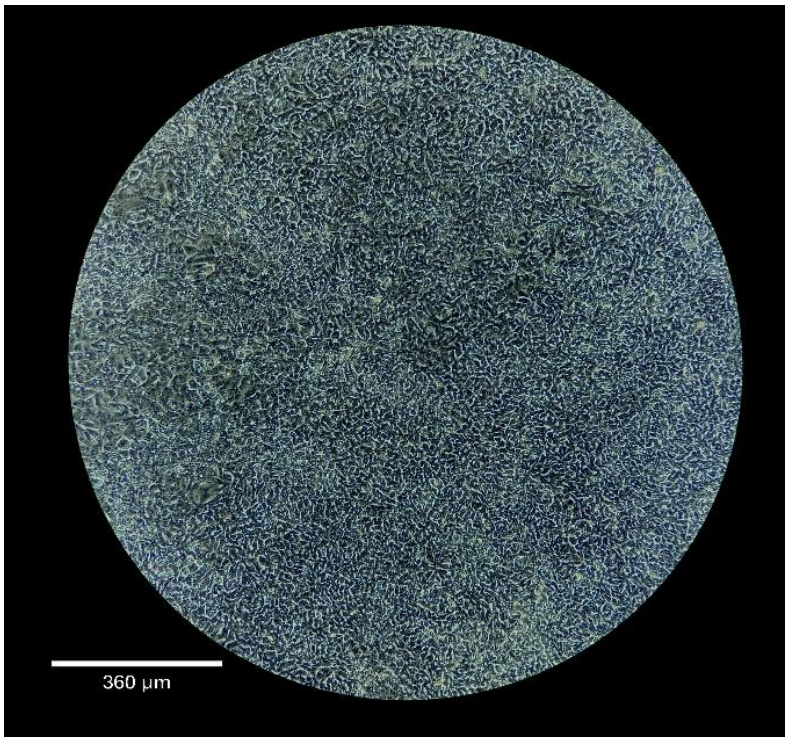
Replating hBMECs from Plate to Flask

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Human Endothelial SFM	495 mL	N/A	N/A	ThermoFisher Scientific	11111044
Penicillin-Streptomycin	5 mL	10,000 U/mL; 10 mg/mL	1%	Sigma	P4333-100ML
ACCUTASE™ Cell detachment	36 mL	N/A	N/A	STEMCELL Technologies	07920
Human Serum from platelet poor human plasma, sterile-filtered, “PPP”	10 mL	N/A	5%	Sigma	P2918
Y-27632	200 µL	10 mM	10 µM	STEMCELL Technologies	72632

Aseptically, combine 5 mL from the Penicillin-Streptomycin stock bottle into a 495 mL bottle of Human Endothelial SFM; this creates a complete Endothelial medium. In a separate container, combine 189.8 mL of complete Endothelial medium with 10 mL of Human Serum from platelet-poor human plasma (PPP) and 200 µL from the Y-27632 aliquot. This creates the working media used for today. Please reference the table above for more information.

Detaching hBMECs and Replating to Flasks

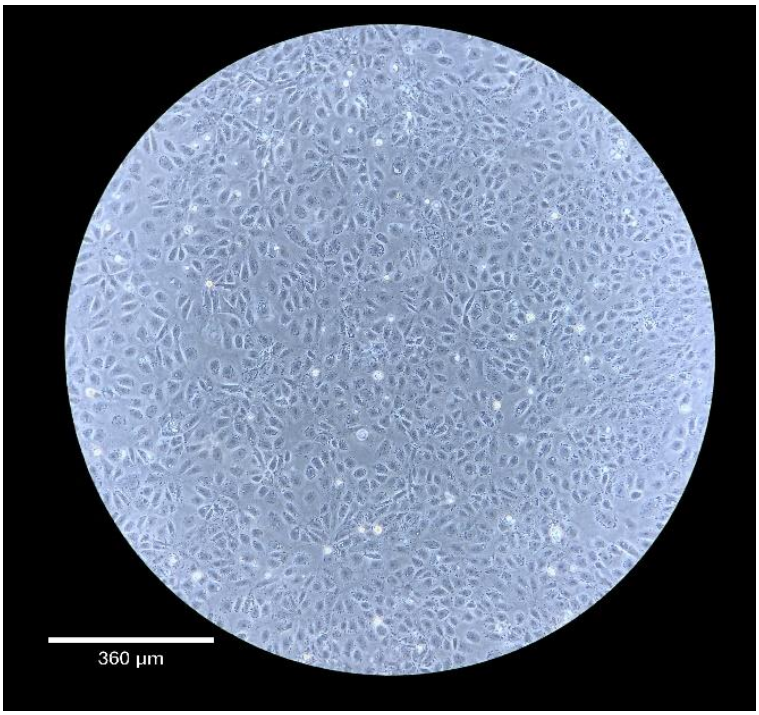
See image below for morphology of what the cells should look like before proceeding.



D-1: Final day of Differentiation in Plates after media wash

1. Bring the necessary reagents and flasks to room temperature in the BSC.
2. Create working media by adding 5% PPP and 10 µM of Y-27632 to Human Endothelial SFM medium.
3. Aspirate hECSR2 medium from 6-well plates.
4. Wash each well with 1 mL of DPBS and aspirate afterwards.
5. Add 1 mL of Accutase to each well, place plates in incubator and check for cell detachment every 5 minutes.
6. Once detached, forcefully collect the cells into a 50 mL conical tube. To capture any remaining cells, add 1 mL of complete Endothelial medium with 5% PPP and Y-27632 to the first well of each plate and collect any additional cells. Add this cell suspension to the next well, collect the cells, and repeat until all wells are collected.
7. Add the cell suspension to the conical tube and bring the total volume up to 50 mL with complete Endothelial medium with 5% PPP and Y-27632. Spin cells at 200g for 5 minutes at 24°C or according to vendor's instructions.
8. Prepare flasks by gently aspirating ECM mixture, washing with 7 mL of Endothelial SFM stock medium, aspirating afterwards, and adding 5 mL of complete Endothelial medium with 5% PPP and Y-27632.
9. Once the spin is complete, carefully aspirate the supernatant, break up the pellet, and finally resuspend with 30 mL of complete Endothelial medium with 5% PPP and Y-27632 to the conical tube.
10. Add 5mL of cell suspension to each flask and incubate for 20 minutes at 37°C and 5% CO_2.

11. After 20 minutes, monitor attachment under the microscope and aspirate the medium from the flask.
12. Refresh the flask with 15 mL of complete Endothelial medium with 5% PPP and Y-27632 and incubate flasks overnight at 37°C and 5% CO_2.
13. The following day, aspirate the medium and refresh with 15 mL of Endothelial medium and 5% PPP *without* Y-27632. Refresh the medium every two days until ready for use. See image below for morphology in the flask.



D1: hBMECs in flask after 24-hour incubation and media wash

Prepare Seeding Media

Bring supplement and seeding media bottle (NeuCyte) to room temperature in the BSC. Combine the appropriate volume of supplement to the media bottle according to manufacturer's instructions. This is the complete medium that is used for seeding both channels today. Since the media is light-sensitive, wrap it with aluminum foil.

Prepare Chips

1. Transfer ECM-coated chips from 4°C refrigerator into the BSC.

2. Flush ECM from both channels by gently washing each channel with 200 µL of Seeding medium.
3. Aspirate the medium outflow at the surface of the chips, leaving the medium in the channels.
4. Repeat the wash with an additional 200 µL of seeding medium per channel, leaving the excess medium outflow covering the inlet and outlet ports.
5. Cover the square dish and place the chips in the incubator until the cells are ready for seeding.

Prepare Cell Counting Solution

Trypan Blue Cell Counting Solution

Reagent	Volume	Source	Cat. No.
Cells Suspension	5 µL	N/A	N/A
Trypan blue	5 µL	Sigma	93595
Seeding Media	40 µL	NeuCyte	102.CUS

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

Microglia, Pericytes, Astrocytes, GABAergic, and Glutamatergic Neurons are seeded as a mixture of cells in the top channel at the following final seeding densities:

Microglia, 0.2 x 10^6 cells/mL

Pericytes, 0.2 x 10^6 cells/mL

Astrocytes, 2 x 10^6 cells/mL

GABAergic, 2 x 10^6 cells/mL

Glutamatergic, 4 x 10^6 cells/mL

The final seeding density needs to be optimized within this range depending on the proliferation rate and profile of the cells, the morphology and function of the tissue, and the viability of the cells. After determining cell count and viability, make calculations for all five cell types to correctly add the appropriate number of cells. Keep the cells at room temperature in the BSC until all cell types are ready. Cell preparation and counting is covered in the next sections.

Thaw Astrocytes, GABAergic, and Glutamatergic Neurons

Cryovials of Astrocytes, Glutamatergic, and GABAergic neurons should have sufficient cell numbers, but cells need to be collected in a labeled 15 mL conical tube using seeding medium. If not, the use of additional vials or expansion according to manufacturer's instructions may be necessary.

1. Carefully submerge cryovials under a warm water bath for 90 seconds or until only one small ice pellet remains. Do not let the water reach the neck of the cap.
2. Spray each cryovial with 70% ethanol and wipe dry before placing in BSC.

3. Gently collect cells and place them into three separately labelled 15 mL conical tubes.
4. Using 1 mL of warm seeding media, gently rinse each cryovial to collect any remaining cells and transfer them to respective conical tubes from before.
5. Bring the total volume up to 15 mL with warm seeding medium. Leave them in BSC at room temperature until next section is complete.

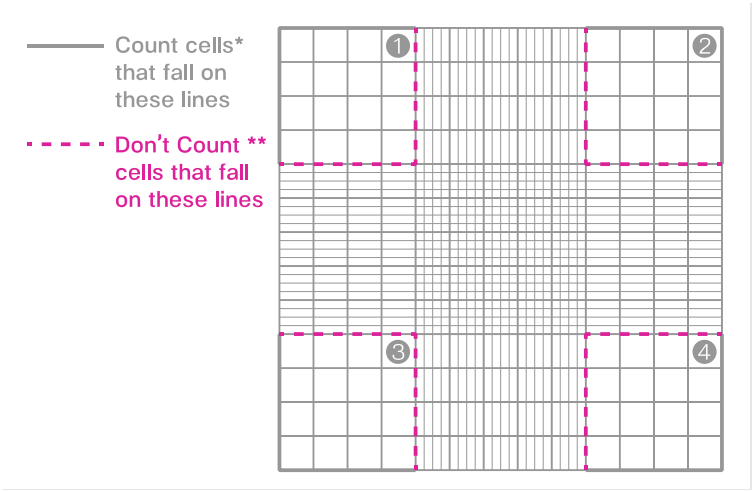
Harvest Microglia and Pericytes

Microglia and pericytes must be harvested and counted before top channel seeding.

1. Bring the culture flasks containing microglia and pericytes from the incubator into the BSC.
2. Aspirate culture media and add 10 mL of 1X DPBS to wash the culture surface in each flask.
3. Aspirate the DPBS wash.
4. Add 3 mL of TrypLE Express to each flask. Incubate for 5 to 10 minutes at 37°C and 5% CO_2.
5. Tap the side of the flasks gently and inspect the culture under the microscope to assess whether cells have completely detached from the culture surface. If cells have not detached, return the flask to the incubator for 5 additional minutes, and repeat again if necessary.
6. Add 9 mL of warm seeding medium to each flask and gently mix to collect all cells from the culture surface.
7. Transfer the contents of each flask (12 mL) into two individual sterile 15 mL conical tubes. At this stage, there should be one conical tube of microglia in suspension and one conical tube of pericytes in suspension.
8. Add 3 mL of warm seeding medium to each tube to bring the total volume of the tubes to 15 mL.

Count the Five Cell Types and Create One Cell Suspension

1. Centrifuge the conical tubes of microglia, astrocytes, glutamatergic, and GABAergic neurons at 300 x g for 4 minutes at room temperature, or as per cell vendor's recommendations. Separately, centrifuge the conical tube containing the pericytes at 900 x g for 10 minutes at 4°C or as per vendor's recommendations.
2. Carefully aspirate the supernatant, leaving approximately 100 µL of medium above each of the cell pellets. **Note:** The cell pellet will be very small. Aspirate carefully.
3. Loosen the cell pellets by flicking the tube gently. Stop when each pellet has loosened.
4. Using a P1000 pipette, gently resuspend the cells in each tube by adding 500 µL of warm seeding medium.
5. Pipette gently to create a homogeneous mixture and transfer 5 µL from each of the cell suspensions to a labeled Eppendorf tube containing 5 µL trypan blue cell counting solution and 40 µL complete seeding medium (This will make a 1:10 dilution). Repeat steps for all cell types.
6. Mix the counting solution thoroughly and count all five cell types separately using a manual hemocytometer.



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

7. Following cell counting and viability determination, perform calculations for all five cell types using the information below. Keep cells at room temperature in the BSC until all cells are ready. **Note:** If there is not enough volume of cell suspension available, pipette additional media as needed and count the cells again.

Microglia

For this example, let's assume 95% viability of microglia.
Proportionally adjust desired seeding density by its viability count:
(Optimal Seeding Density / Cell Viability) = Adjusted Final Seeding Density
$0.2x10^6$ cells/mL / 95% = 0.21 x 10^6 cells/mL
$C_1V_1 = C_2V_2$, solve for V_1
C_1: Viable cell yield, for this example, let's assume $1x10^6$
V_1: ?
C_2: 0.21 x 10^6 cells/mL, adjusted final density for each chip
Volume of cell suspension to seed one chip: ~35 µL
Number of chips seeded: 20
V_2: 35 x 20 = 700 µL = 0.700 mL
$C_1V_1 = C_2V_2$, solve for V_1
($1x10^6$ cells/mL) x (V_1 mL) = (0.21 x 10^6 cells/mL) x (0.7 mL)
V_1 = .147 mL = 147 µL of microglia cell suspension

Repeat this calculation for all cell types

8. Combine the calculated volume of all five cell types into one sterile 15 mL conical tube. Ensure the total volume prepared is sufficient to seed all chips. If the total volume of the cell suspension mixture is less than the desired volume, calculate the difference and bring up the volume with seeding media.

Seed Astrocytes, Microglia, Pericytes, as well as Glutamatergic and GABAergic Neurons to the Top Channel

1. Retrieve the dish of chips from the incubator and bring it to a BSC. Remove the dish lid and keep sterile. Ensure the chips were previously washed twice with 200 µL of seeding media.
2. Quickly and steadily pipette 35 to 50 µL of the co-culture cell suspension into the top channel inlet port. Pipette rapidly without creating bubbles and aspirate the excess outflow.
3. Visually inspect the chips to ensure even distribution of cells across the length of the top channel. Once the top channels of all 6 chips in the dish have been seeded, pipette 1 mL of 1x DPBS into the chip carrier to provide humidity for the cells. Cover with the dish lid and place in the incubator at 37°C and 5% CO_2 until the cells in the top channel have attached to the membrane (this will take approximately 2-3 hours).
4. Once attached, perform a gravity wash by adding 200 µL of seeding media onto the inlets of the chip, ensuring media flows through the channel outlets (See Figure 6). If media does not flow through, carefully insert the pipette tip into both channels to push out any bubbles.

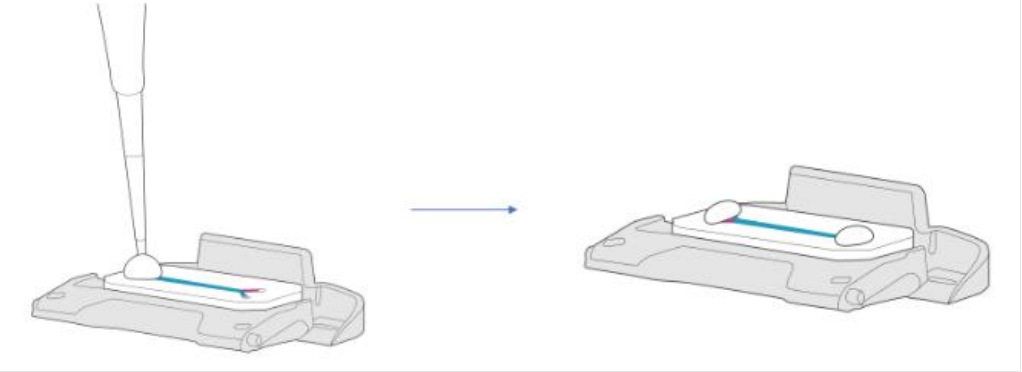


Figure 6: Chip with medium drops covering the inlet and outlet ports

Day 1: Refresh Media of Flasks and Chips

Goals:

- Refresh medium of flasks and chips.
- Ensure attachment of cells to top channel.

Key Steps:

- Prepare Short-Term Maintenance Medium and equilibrate to 37°C before use.
- Check each chip under microscope for proper attachment, cell density, and survival.

Required Materials:

- 120 x 120 mm Square culture dishes (1 dish per 6 chips)
- Autoclaved chip cradle (1 cradle per 6 chips)
- Human Endothelial SFM
- Penicillin-Streptomycin
- Human Serum from platelet-poor human plasma, sterile-filtered, “PPP”
- Short-Term Maintenance medium (at 37°C)
- 15 mL and 50 mL conical tube
- Aspirator and sterile tips
- Installed and qualified Zoë
- Serological pipettes
- Pipettors and filtered tips
- 70% ethanol
- 37°C water or bead bath

Refresh Media of Flasks

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Human Endothelial SFM	495 mL	N/A	N/A	ThermoFisher Scientific	11111044
Penicillin-Streptomycin	5 mL	10,000 U/mL; 10 mg/mL	1%	Sigma	P4333-100ML
Human Serum from platelet poor human plasma, sterile-filtered, “PPP”	5 mL	N/A	5%	Sigma	P2918

Aseptically combine 5 mL from the Penicillin-Streptomycin stock bottle into a 495 mL bottle of Human Endothelial SFM. The medium is now complete and ready for use. In a separate container, combine 95 mL of Complete Human Endothelial medium with 5 mL of Human Serum from platelet-poor human plasma

(PPP). This creates the working medium used to refresh flasks today. Please reference the table above for more information.

1. Bring all flasks containing hBMECs from the incubator to BSC.
2. Carefully aspirate media by tilting the flasks sideways.
3. Add 15 mL of complete Endothelial medium with 5% PPP to each flask.
4. Place the flasks into the incubator overnight at 37°C and 5% CO_2.

Prepare Short-Term Maintenance Media

Bring the supplement and the Short-Term Maintenance media bottle (NeuCyte) into the BSC and allow it to come to room temperature before use. Be mindful to protect it from light with aluminum foil. Determine the volume needed for today—likely around 20 mL—and pipette it into a separate, sterile conical tube. Combine the appropriate volume of supplement to the media in the conical, according to manufacturer's instructions. This is the complete medium that is used for refreshing both channels today, but it is used to refresh only the top channel tomorrow.

Refresh Media of Both Channels of Chip

1. Bring chips into the BSC and carefully remove the pipette tips from each channel if necessary.
2. Gently wash out any cellular debris and seeding medium from each channel with 200 µL of supplemented Short-Term maintenance medium. Repeat again per chip.
3. Insert the pipette tip into the outlet of bottom channel and pipette 100 µL of supplemented Short-Term maintenance medium into the inlet of the bottom channel, leaving the pipette tip inserted afterwards. Repeat for the top channel.
4. Aspirate excess media from chip.
5. Place chips into the incubator overnight at 37°C and 5% CO_2.

Day 2: hBMECs to Chips

Goals:

- Seed hBMECs on upper and lower surfaces of the bottom channel of the chip.

Key Steps:

- Prepare Endothelial Medium (Equilibrate to 37°C before use)
- Harvest hBMECs from flask
- Adjust cell density
- Seed hBMECs to upper surface of the bottom channel
- Gently wash chips 2-3 hours post-seeding
- Check each chip under microscope for proper attachment as well as cell density and survival
- Refresh both channels with the appropriate medium

Required Materials:

- 120 x 120 mm Square culture dishes (1 dish per 6 chips)
- Autoclaved chip cradle (1 cradle per 6 chips)
- Endothelial SFM medium (at 37°C)
- Short-Term Maintenance medium (at 37°C)
- Y-27632
- Human Serum from platelet-poor human plasma, sterile-filtered, “PPP”
- TrypLE Express
- 15 mL and 50 mL conical tube
- Aspirator and sterile tips
- Installed and qualified Zoë
- Serological pipettes
- Pipettors and filtered tips
- 70% ethanol

Prepare hBMEC Culture Medium for Seeding

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Human Endothelial SFM	495 mL	N/A	N/A	ThermoFisher Scientific	11111044
Penicillin-Streptomycin	5 mL	10,000 U/mL; 10 mg/mL	1%	Sigma	P4333-100ML
Human Serum from platelet poor human plasma, sterile-filtered, “PPP”	Varies	N/A	5%	Sigma	P2918

Y-27632	Varies	10 mM	10 µM	STEMCELL Technologies	72632

First, prepare the Human Endothelial SFM Medium by combining 5 mL of the stock bottle of Penicillin-Streptomycin, making a 1% final concentration. Using this medium, determine the volume needed for seeding and add the appropriate amount to create a final concentration of 5% PPP and 10 µM of Y-27632. This is the media used for seeding the hBMECs. Please refer to the table above for more information.

Trypan Blue Cell Counting Solution

Reagent	Volume	Source	Cat. No.
hBMEC Cell Suspension	5 µL	N/A	N/A
Trypan blue	5 µL	Sigma	T8154
Human Endothelial SFM Medium	40 µL	ThermoFisher Scientific	11111044

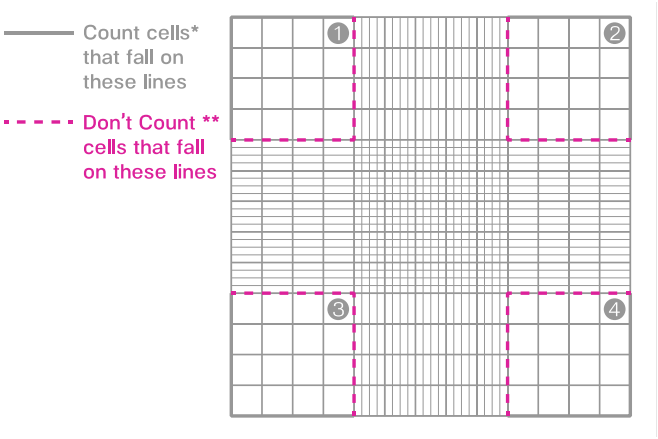
- Maintain cell counting reagents at room temperature prior to use.
- Prepare fresh cell counting solution for each use.

Harvest hBMECs from Flask and Adjust Cell Density

hBMECs in culture must be harvested and counted for bottom channel seeding. hBMECs are adjusted to a density between 12 x 10^6 – 16 x 10^6 cells / mL prior to seeding in the bottom channel. The final seeding density needs to be optimized within this range depending on the proliferation rate and viability of the cells as well as the morphology and function of the endothelial monolayer formed by these cells.

1. Bring the culture flask containing hBMECs from the incubator into the BSC.
2. Aspirate the medium from the flask and add 10 mL of 1X DPBS to wash the culture surface. Aspirate the DPBS wash.
3. Add 3 mL of TrypLE Express to the flask. Incubate for 5 to 10 minutes at 37°C and 5% CO_2.
4. Tap the side of the flask gently and inspect the culture under the microscope to assess complete detachment of cells from the culture surface.
5. Add 3 mL of warm hBMEC culture medium with 5% PPP and 10 µM of Y-27632 to the flask and pipette gently to mix while collecting all cells from the culture surface.
6. Transfer the contents of the flask (6 mL) into a sterile 50 mL conical tube. Repeat these steps for each flask.
7. Add 2 mL of the above hBMEC culture medium to bring the total volume of the tube to 50 mL.
8. Centrifuge the hBMECs at 200 x g for 5 minutes at room temperature, or as per cell vendor's recommendations.

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 hBMEC culture medium.
12. Pipette gently to create a homogeneous mixture and transfer 5 µL of the cell suspension to an Eppendorf tube containing 5 µL trypan blue cell counting solution and 40 µL of medium (this will make a 1:10 dilution).
13. Mix the counting solution thoroughly and count cells using a manual hemocytometer.



    a. Count both viable and non-viable cells in each quadrant of the hemocytometer.
        Live Cell Count; Dead Cell Count; Total Cell Count
    b. Calculate percent viability of the cell solution.
        (Live Cells) ÷ (Total Cells) x 100 = % Viability
    c. Calculate viable cell concentration. The dilution factor is 10 when prepared in the trypan blue solution above.
        (Live Cell Count x 10 x 10^4) ÷ 4 = Viable Cell Concentration (cells / mL)
    d. Calculate viable cell yield.
        (Viable Cell Concentration) X (Cell Suspension Volume) = Viable Cell Yield (cells)
14. Dilute the hBMECs to a final concentration between 12 x 10^6 cells / mL – 16 x 10^6 cells / mL in above hBMEC culture medium. Ensure sufficient volume is available for seeding all the chips. See previous microglia calculation for an example.

Prepare Chips

1. Transfer ECM-coated chips containing top channel cells from incubator into the BSC.
2. Gently wash the bottom channel of the chip with 200 µL of complete Endothelial culture medium containing 5% PPP and 10 µM Y-27632. Aspirate the medium outflow at the surface of the chips,

leaving the medium in the channels and a small volume of excess medium covering the inlet and outlet ports.

3. Cover the 120 x 120 mm dish and place the chips in the incubator until the cells are ready for seeding.

Seed hBMECs to Upper Surface of the Bottom Channel

Work with one chip at a time. After seeding the first chip, assess cell density within the channel through a microscope, then invert the chip on the Chip Cradle as quickly as possible. If inappropriate cell density is observed, adjust it accordingly for all subsequent chips. If the density of the first chip needs to be changed, wash the bottom channel twice with complete hBMEC culture medium with 5% PPP and 10 µM Y-27632, then re-seed the chip using the adjusted cell suspension. Assess the chip once again through a microscope to confirm the suitability of the seeding density, then return to the BSC and invert the chip on the Chip Cradle. As it is important to invert the chips as soon as possible after seeding them, we recommend checking the seeding density of only 1 chip out of every set of 6. If a high magnitude of chips is being seeded simultaneously, seeding density can be assessed for 1 chip out of every set of 12.

1. Bring the 120 x 120 mm dish containing the prepared chips to the BSC.
2. Avoiding contact with the ports, carefully aspirate droplets of excess hBMEC culture medium from the surface of one chip, ensuring the presence of medium in both the channels.
3. Very gently agitate the cell suspension before seeding each chip to ensure homogeneity.
4. Quickly and steadily pipette 15 to 20 µL of the cell suspension into the bottom channel inlet port while aspirating the outflow at a slight distance from the outlet port. Avoid direct contact with the outlet port to ensure no suspension gets aspirated out of the channel.
5. Cover the dish and transfer to the microscope to check the seeding density within the chip.
   Note: At this stage, optimal seeding density should form an even layer of cells dispersed homogenously along the length of the bottom channel of the chip.
6. If seeding density is not optimal, return the chips to the BSC and wash the channel with 200 µL of fresh medium twice. Adjust cell density accordingly and repeat steps 3 through 5 until the desired density is achieved within the channel.
7. After confirming the correct cell density, invert each chip and rest the edge of the chip carrier on the chip cradle immediately after seeding to maximize attachment of cells on the upper surface of the bottom channel.
   Note: Each chip cradle can support up to 6 chips inside a 120 x 120 mm cell culture dish.
8. Seed cells in the remaining chip.
9. Fill the Chip Cradle reservoir with 1 mL of 1x DPBS to provide extra humidity. Cover dish with lid.
10. Invert chips immediately after (See Figure 7 below).

11. Place the dish with seeded chips at 37°C for 2-3 hours or until cells have attached.

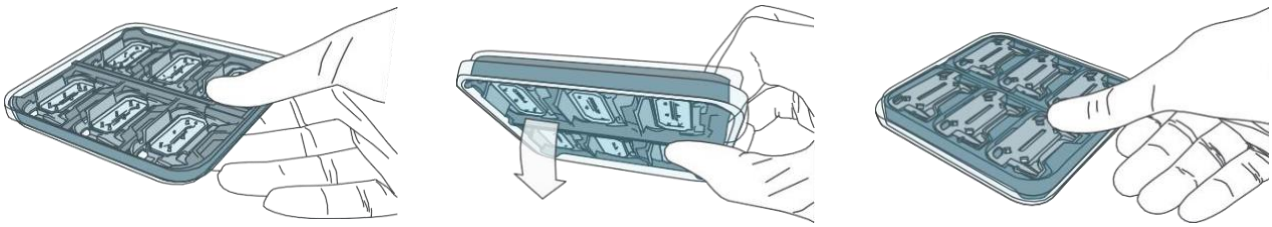


Figure 7: Inverting chips during endothelial attachment

Note: Minimize the amount of time the cells are outside the incubator by seeding no more than 12 chips at a time and immediately placing the chips in the incubator at 37°C and 5% CO_2 after seeding each batch.

Gravity Wash and Overnight Static Culture

1. Approximately 2-3 hours after seeding the bottom channel, visually inspect the chips to ensure the cells in culture have attached in the bottom channel.
2. Gently wash the bottom channel of the chip with 200 µL of hBMEC culture medium with 5% PPP and 10 µM Y-27632 and the top channel with 200 µL with complete Short-Term medium (from the day before). Since there are 2 different media being used, they must be separated by keeping them in filtered tips instead of droplets (See Figure 8 below).
3. Return chips, with pipette tips inserted in each inlet and outlet port, to the incubator overnight.
4. Maintain cells in static culture in the chips until connecting to the Pods and Zoë the next day.

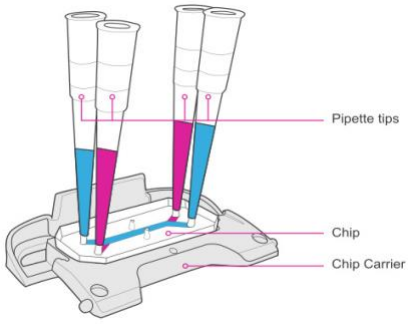


Figure 8: Chip with filtered tips inserted into ports with respective media

Day 3: Chips to Pods and Pods to Zoë

Goals:

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

Key Steps:

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

Required Materials:

- Steriflip-HV Sterile Centrifuge Tube Top Filter Unit, 0.45 µm
- Short-Term Maintenance Culture Medium
- Human Endothelial SFM Medium
- Human Serum from platelet-poor human plasma, sterile-filtered, “PPP”
- Installed and qualified Zoë
- Prepared chips
- Pods (sterile)—1 per chip
- Tray—1 per 6 chips
- Serological pipettes
- Pipettors and filtered tips
- 37°C water or bead bath
- 70% ethanol
- 3kDa Dextran Cascade Blue
- 0.5kDa Lucifer Yellow

Prepare hBMEC Culture Medium for Bottom Channel Reservoirs of Pods

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Human Endothelial SFM	495 mL	N/A	N/A	ThermoFisher Scientific	11111044

Penicillin-Streptomycin	5 mL	10,000 U/mL; 10 mg/mL	1%	Sigma	P4333-100ML
Human Serum from platelet poor human plasma, sterile-filtered, "PPP"	Varies	N/A	2%	Sigma	P2918
3kDa Dextran Cascade Blue	Varies	10 mg	100 µg/mL	ThermoFisher Scientific	D7132
0.5kDa Lucifer Yellow	Varies	25 mg	20 µg/mL	ThermoFisher Scientific	L453

Prepare the Human Endothelial SFM Medium by first combining 5 mL of the stock bottle of Penicillin-Streptomycin, making a 1% final concentration. Determine the total volume needed for connection and maintenance. Degas this media *before* adding any additional components (see section below).

Ensure the media is warmed to 37°C by placing the sufficient volume of media in an incubator for at least one hour, then degas via steriflipping the media following the "Gas Equilibration of Media" section further below. After gas equilibration, add the appropriate amount of PPP to create a final concentration of 2%. Please reference the table above for more information.

Note: For barrier function analysis, 3kDa Dextran Cascade Blue at final concentration of 100 µg/mL or 0.5kDa Lucifer Yellow at a final concentration of 20 µg/mL can be added to the medium. If barrier assessment is not necessary, these tracer molecules do not need to be added. These tracers can be kept in the culture medium through the course of experiment. To assess barrier function throughout the entire experiment, continue to add these molecules to future hBMEC culture medium at the same concentration.

Prepare Short-Term Maintenance Media for Top Channel Reservoirs of Pods

Bring the remaining supplement and Short-Term Maintenance media bottle (NeuCyte) up to 37°C before use and then aseptically, transfer to BSC. Determine the total volume needed for connection and maintenance. Degas via steriflipping the Short-Term base media *before* adding any components (refer to "Gas Equilibration of Media" section below).

After degassing, determine the appropriate volume of the supplement to add to the media, according to the manufacturer's instructions. This is the complete medium that is used for refreshing the top channel today and for maintaining the top chambers of Pods afterward. Ensure the media is warmed to 37°C before use by placing media in an incubator for at least one hour and degassed following the protocol below. Protect both media from light by wrapping them in aluminum foil throughout the experiment.

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, in turn causing irregular flow and negatively impacting cell viability. Ensure the medium is outside of a warmed environment—such as an incubator or bath—for no longer than 10 minutes, as gas equilibrium can become compromised when the medium is allowed to cool. Repeat these steps for both the top and bottom media.

1. Place at least 3.3 mL of complete Human Endothelial medium for each chip in a 50 mL. conical tube.
2. Place at least 3.3 mL of complete Short-Term 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 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 is 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 one.
   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.
   4. Leave the filtered medium under vacuum for 15 minutes.
5. Remove the vacuum tubing from the Steriflip units.
6. Bring media into the BSC and separate the conical tubes containing media from the Steriflip.
7. Add the appropriate components to both mediums as mentioned in the tables above.
8. Immediately place the conical tubes containing media in the incubator with the caps loose. Once warmed, both media are ready for use. If more of either medium is needed, repeat all steps again.

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 both 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 (See Figure 9).

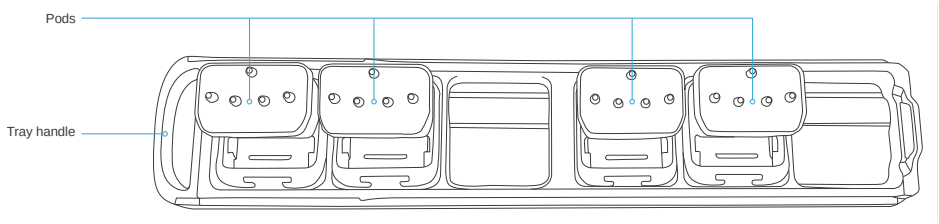


Figure 9: Pods in Tray

3. Pipette 3 mL of pre-equilibrated, warm media to each inlet reservoir. Add the complete hBMEC culture medium to the inlet reservoir of the bottom channel and the Short-Term maintenance medium to the inlet reservoir of the top channel.
4. Pipette the corresponding 300 µL of pre-equilibrated, warm media to each outlet reservoir, directly over each outlet via (See Figure 10).

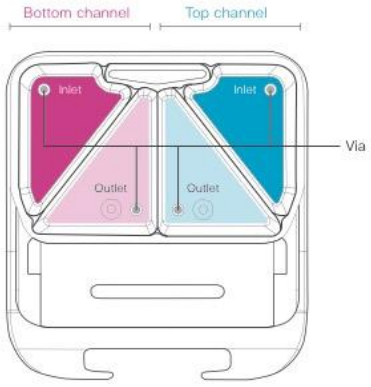


Figure 10: Pod

5. Bring trays containing Pods to the incubator and slide them completely into Zoë with the tray handles facing outward.
6. Run the Prime Cycle on Zoë.
   a. Use the rotary dial to highlight “Prime” on the display.

b. Press the rotary dial to select “Prime.”
c. Rotate the dial clockwise to highlight “Start.”
d. Press the dial again to select “Start” and begin.
Note: Once “Start” is selected, listen for a sound as Zoë engages the Pods.

7. Close the incubator door and allow Zoë to prime the Pods; this process takes approximately one minute. When the status bar reads “Ready,” the Prime Cycle is complete.
8. Remove the tray from Zoë and bring to the BSC.
9. Verify that the Pods were successfully primed. This is very important for success.
   a. Inspect the underside of each Pod to look for the presence of small droplets at all four fluidic ports. Droplets will vary in size—often, droplets on the outlet ports will be larger.
   b. If any Pod does not show droplets, re-run the Prime Cycle on those Pods.
   c. If any media dripped onto the tray (this occurs more often by the outlet ports), clean the tray with a wipe sprayed with 70% ethanol.
10. Once it is confirmed that all Pod ports are wet with droplets, leave the tray with Pods in the incubator while washing the chips until you are ready to connect the chips to the Pods (See Figure 11).

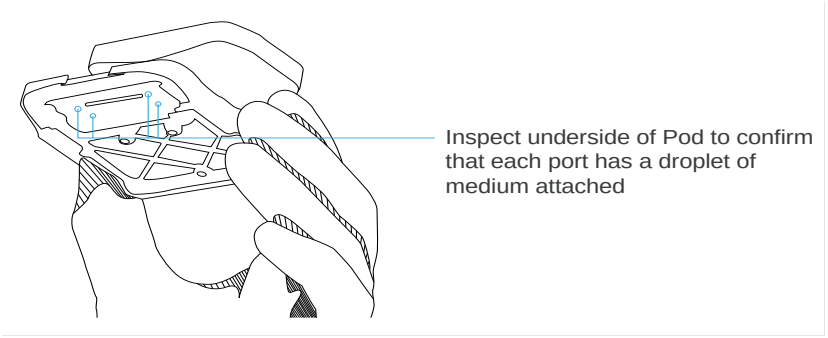


Figure 11: Correct way to hold Pod while inspecting

Wash Chips

1. Transfer the seeded chips in a 120 x 120 mm dish from the incubator to the BSC.
2. Gently wash the bottom channel of each chip with warm, equilibrated hBMEC culture medium and the top channel of each chip with warm, equilibrated top channel culture medium. Be careful to leave small droplets of media on the chip surface above the ports to remove any possible bubbles in the channel.

Chips to Pods

1. Bring one tray at a time to the BSC. 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 is fully seated.

2. Place thumb on the chip carrier tab and gently but firmly push the tab inward to engage 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 chips 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 reservoir, that a droplet is over the via in each Pod outlet reservoir, and that the Pod lids are flat and secure.
7. Return the tray to Zoë, bring out the next tray, and repeat steps 1-6 for each Pod.

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 (flow rate and stretch) on Zoë. These conditions will start as soon as the Regulate Cycle is complete.
   Note: It is recommended to flow media in both channels at 60 µL / hr and to set stretch to 0% at 0.0 Hz (no stretch).
3. Run Regulate Cycle (See Figure 12)
   a. Using the rotary dial, highlight the “Regulate” field.
   b. Press the dial to select “Regulate” and rotate the dial clockwise to “Start.”
   c. Press the dial again to select “Start” and begin the Regulate Cycle.
      Note: Once “Start” is selected, listen for a sound as Zoë engages the Pods.
   d. At this point, the “Activation” button will glow blue.

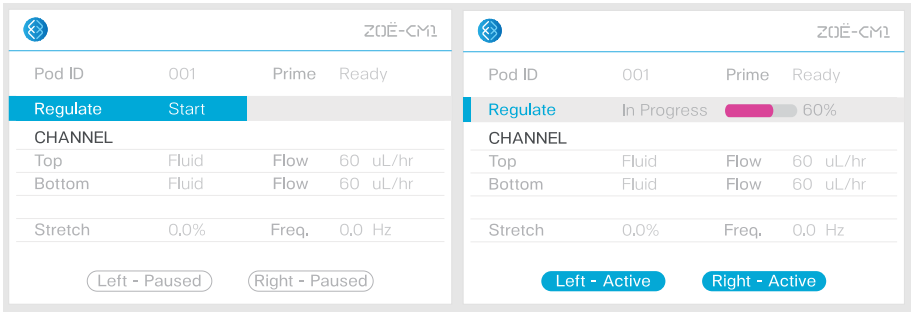


Figure 12: Zoë UI while running Regulate Cycle

Note: The Regulate Cycle lasts 2 hours. After it has finished, Zoë will begin flow at the preset Organ-Chip culture conditions. If there is an urgent need to cancel the Regulate Cycle, wait until at least 5% of the cycle has been completed as per the progress bar, then select the “Regulate” field by rotating and pressing the dial. Rotate the dial counterclockwise to the “Cancel” field and press it to select. Press the dial once more and wait one minute for the cycle to end, after which the tray can be removed. If the Regulate Cycle is cancelled, always rerun a complete cycle before proceeding.

Day 4+: Chip Maintenance and Sampling

Goals:

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

Key Steps:

- Observe cell morphology
- Maintenance
- Sampling and media replenishment
- Barrier Function assessment

Required Materials:

- Chips in Pods
- Cell culture media
- Serological pipettes
- Pipettes, Multichannel and filtered tips
- 37°C water or bead bath
- 70% ethanol
- 3kDa Dextran Cascade Blue
- 0.5kDa Lucifer Yellow
- 96 wells tissue culture plates
- Microscope

Observe Cell Morphology

1. Pause Zoë by pressing the silver “Activation” button.
2. Remove the trays and place them into the BSC.
3. Visually inspect each chip for bubbles.
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 (See Figure 13):
    - Inlet junction
    - Center of channel
    - Outlet junction

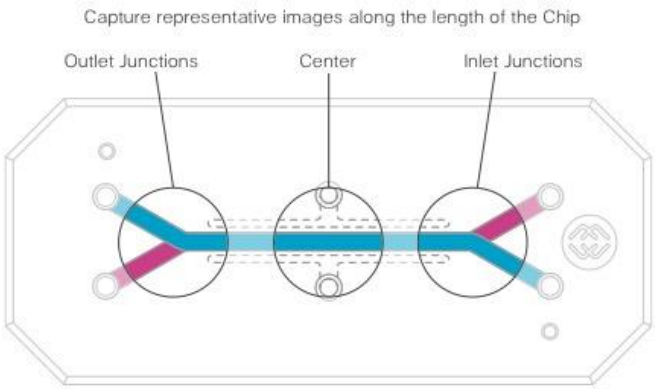


Figure 13: Chip image capture points

Morphology Acceptance Criteria

Representative images of both the top and bottom channels are pictured below (Figure 14):

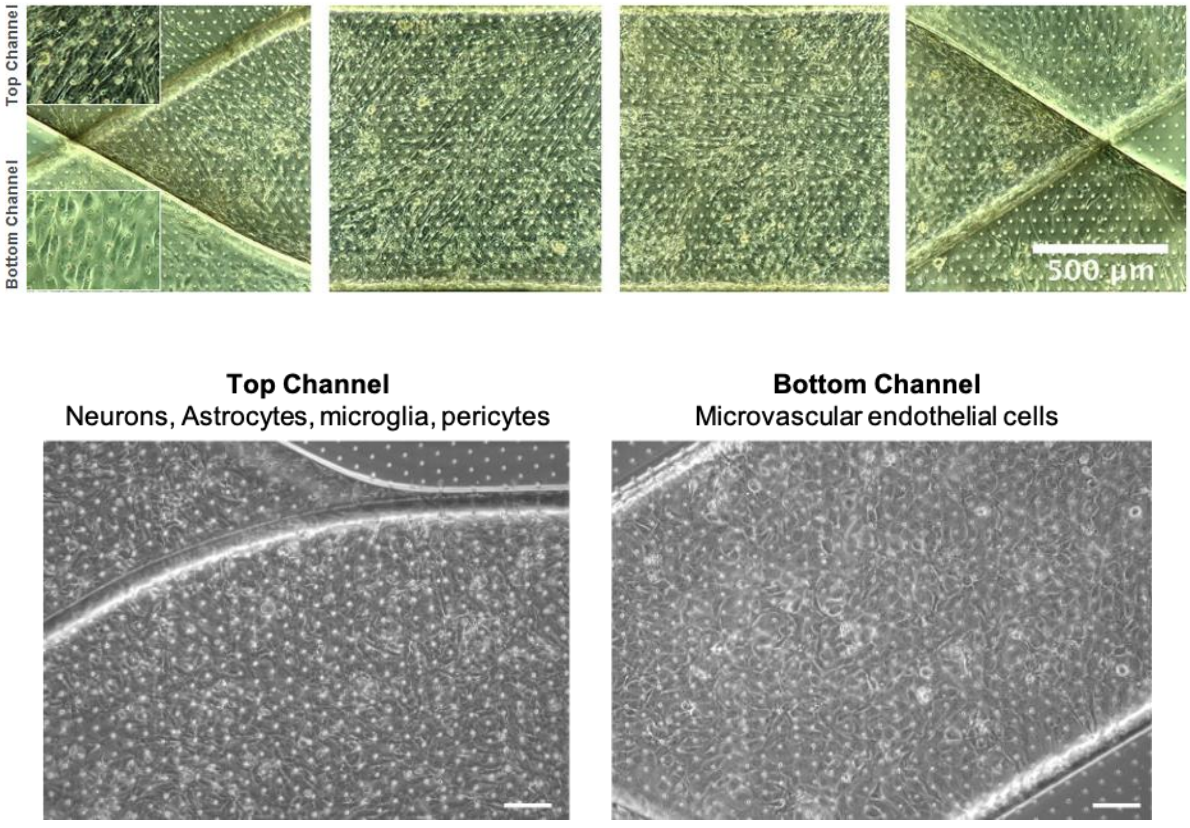
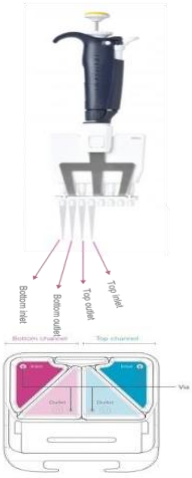


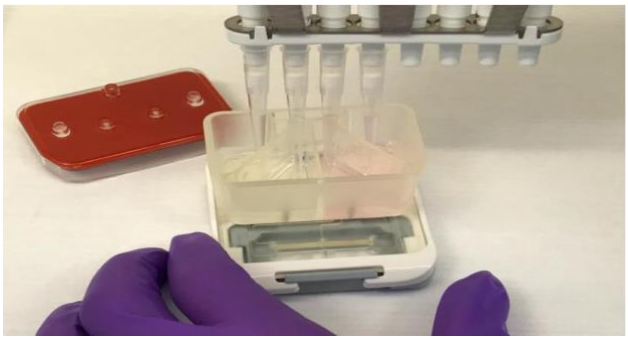
Figure 14: Morphology of both channels

Effluent Sampling

1. Remove the Pod lids and collect effluent media from Pod outlet reservoirs for analysis.
    a. Collect effluent from the indicated regions; avoid disturbing the Pod reservoir vias (See Figure 15).
2. 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 them.
3. Refill the Pod media reservoirs with appropriate fresh cell culture medium and perform a via wash (see next section).
    a. 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.
4. Replace the Pod lids and return trays to Zoë.
5. Press the silver “Activation” button to resume pre-set Organ-Chip culture conditions.
    a. Zoë will engage when the “Activation” button glows blue.

A





B

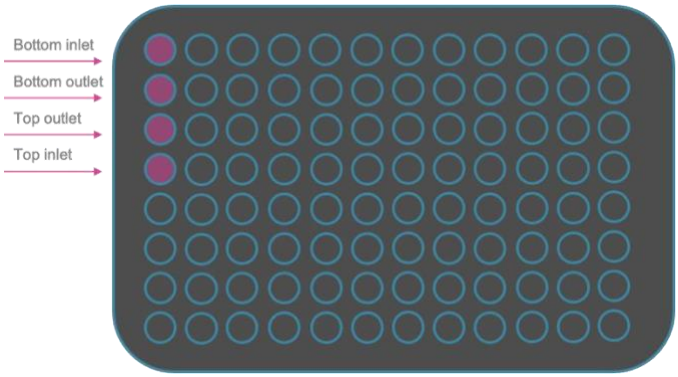


Figure 15: Effluent Sampling. A. Multichannel collecting effluent from the Pod. B. Medium dispensed in the 96 well black bottom plate.

Refresh media of the Brain-Chip

1. Carefully remove trays with Pods from the Zoë™ and transfer them to the BSC. We recommend removing one tray at a time to minimize stress experienced by cells due to fluctuations in temperature. If you are working with a light-sensitive compound, ensure that the biosafety cabinet light is turned off.

   Note: If you have multiple collection time points in your experiment, it helps to organize your conditions so that you have one time point per tray. This will allow you to pause flow only on the tray from which you are collecting effluent while letting the other trays flow uninterrupted, resulting in a more accurate assessment of elapsed time.

2. If necessary, fully aspirate both inlet and outlet reservoirs of each Pod after effluent collection. Avoid direct contact with the Pod reservoir vias.
3. Add the calculated volume of warm, freshly equilibrated media to the appropriate channel using the same steps as before. Refer to the Gas Equilibration of Media section for additional details.
4. Once all Pods have been refreshed, return all trays to the appropriate Zoë and note the time that the Zoë was restarted as the new experimental start time.
5. Each outlet reservoir can be sampled independently at each timepoint following Protocol EP124 Effluent Sampling.
6. Replenish Pods with freshly prepared treatment medium as needed, regardless of the collection timepoints, until the end of your treatment period or experiment.

Barrier Function Readout

1. Prepare plate maps to determine which wells of a 96-well tissue culture plate will receive effluent or standard samples.

2. Adjust the volume to 50 µL and use a standard multichannel pipette to collect effluent and media from all the four reservoirs simultaneously by placing the pipette tips into the Pods, ensuring one tip is in each reservoir (see Figure 15 above).
3. Dispense the collected effluents in an appropriately labelled 96-well plate. Change tips between Pods to avoid cross-contamination.
4. Add 100 µL of PBS to the effluent collected on the plate for a final dilution of 1:3.
5. To prepare the standard curves, collect 300 µL from the bottom inlet reservoir and add to designated wells. This will be referred to as the "donor" standard curve. Also, collect 300 µL from the top inlet reservoir and add to designated wells. This will be referred to as a "receiver" standard curve.
6. Dispense 150 µL of 1x DBPS to the empty standard wells.
7. Perform serial dilution by collecting 150 µL from the first well with medium and dispensing it into the well beneath. Repeat this step for wells 1-7. In the 7^{th} well, collect 150 µL and dispense it into a waste container, ensuring each well has a final volume of 150 µL. Well 8 should only contain 1x DPBS, as it will serve as a blank.
8. Save the plate wrapped in foil at room temperature to read after all chips have been sampled and refreshed or freeze at -20°C to read at the end of the experiment. Using a Spectrophotometer Microplate Reader, adjust the Ex/Em based on the fluorescent tracer used. (e.g. For Dextran Cascade Blue and Lucifer Yellow, set Ex/Em at 375/420, gain 60).
9. Perform assay daily to monitor barrier function and to test if the Papp remains below $2x10^{-6}$ cm/s. For more information, refer to the Emulate website's Barrier Function Protocol and Permeability and Standard Curve Calculator page to download permeability and standard curves.

Barrier Function Acceptance Criteria

On day 4, the barrier function Papp levels are expected to reach below $2x10^{-6}$ cm/s, when using 3kDa Dextran Cascade Blue. If this is the case, the chips are ready for the experimental phase. If the Papp value exceeds this, please reach out to Emulate technical support for troubleshooting.

Suggested Readouts:

- Barrier Function Readout Analysis
- RNA Isolation and Purification Protocol
- cDNA Preparation Protocol
- Quantitative Polymerase Chain Reaction (qPCR) Protocol
- Protein Samples Isolation Protocol
- Total Protein Quantification Protocol
- Quantification of Cytokines Secretion Protocol

Troubleshooting

Issue	Section	Step	Recommendation
Bubbles are present in the channel	Chip Preparation	Introduce ER-1 Solution to Channels; Activate Chips; Coat Chips with ECM	Dislodge by washing 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 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 can get trapped at the ports. Dislodge bubbles using pipette tip, or aspirate the channels and reintroduce equilibrated media.
Media takes too long to pass through Steriflip	Chips to Pods and Pods to Zoë	Equilibration of Media	Vacuum pressure is not reaching -70kPa. Find an alternate vacuum source.
Pods do not prime	Chips to Pods and Pods to Zoë	Prime Pods	Ensure that medium covers all Pod vias. If the problem persists, 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	Pod to Zoë; Maintenance and Regulate	Power off Zoë and then turn it on again. Contact our support team if the problem persists.
Pods stuck in Zoë	Maintaining and Sampling	Maintenance and Regulate	The Pod lid is not secured on the Pod. 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.

Issue	Section	Step	Recommendation
Pods not flowing properly or evenly; Bubbles observed in chip	Maintaining and Sampling	Maintenance and Regulate	There is inherent variability with Zoë; however, large fluctuations and major flow issues primarily result from bubbles. Remove the chip from the Pod, flush the chip with media, re-prime the Pod with degassed media, connect the chip to the Pod, and run the Regulate Cycle.
Insufficient cells	Chip Seeding	Cells to Chips	During cell suspension preparation, calculate the minimum volume required for seeding (i.e., if the bottom channel can be seeded with 10-20 µL, seed with 10 µL per chip). Resuspending cells in less volume increases seeding density.
Endothelial cells in the bottom channel not confluent	Chip Seeding	Cells to Chips	Seeding density is not optimal. Ensure that cell suspensions prior to seeding are prepared at the required seeding densities. To ensure proper density, visually inspect the first seeded chip immediately after seeding it and prior to seeding the remainder.
Uneven or spotty coating of 6-well plate	6-well plate preparation	iPSCs Culture	Ensure an even distribution of Matrigel coating in all wells by moving plate vertically and horizontally. Store on top of a flat surface at 4°C.
Dried plate coating	6-well plate preparation	iPSCs Culture	Wrap plate carefully in parafilm prior to overnight incubation at 4°C.

Potential Root Causes of Bubbles

If a high failure rate due to bubbles is observed or if bubbles are persistent despite following the mitigation steps above, check for these possible causes.

- Medium not sufficiently equilibrated before adding to Pods.
- Insufficient priming: disconnect chip and reprime Pod.

TITLE	DOCUMENT	REVISION
Brain-Chip Culture Protocol	EP212	A
	DATE 01-Jan-2022	PAGE 69 OF 69