EP-239 Liver-Chip Array Quad-Culture Workflow Protocol

Rev E

Experimental Overview

Pre-Experiment: Reagent Preparation

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

Day -2: Thaw LSECs

- Prepare LSEC culture flasks
- Thaw and plate LSECs

Day -1: Chip-Array Preparation

- Prepare Chip-Arrays
- Prepare ECM solution
- Coat chips with ECM

Day 0: Hepatocytes to Chip-Arrays, Hepatocyte Overlay

- Prepare hepatocyte seeding medium
- Prepare Chip-Arrays
- Thaw hepatocytes
- Adjust cell density
- Seed hepatocytes to top channel
- Seed a well plate
- Prepare overlay medium
- Overlay hepatocytes

Day 1: Non-Parenchymal Cells (NPCs) to Chip-Arrays, Chip-Array Prime, Regulate, and Flow

- Prepare NPC seeding medium
- Wash Chip-Arrays
- Harvest LSECs
- Thaw Stellate cells
- Thaw Kupffer cells
- Combine NPC mixture
- Seed non-parenchymal cells (LSECs, Stellate cells, Kupffer cells) to bottom channel

- Gas equilibration of media
- Prime Chip-Arrays
- Regulate Chip-Arrays, Flow

Day 2+: Maintaining and Sampling

- Sampling and media replenishment
- Routine automated imaging of biology

Timepoint Day 0: Quality Check and Dosing

- Cell culture quality check and inclusion/exclusion from study
- Assign conditions to each Chip-Array Emulation
- Prepare dosing media and dose into Chip-Array inlet reservoirs

Equipment and Materials Required

Introduction

Ensure all equipment, materials, and reagents are accessible prior to each experiment. Below are the catalog numbers for the specific materials needed.

Note: Exact catalog numbers are not provided for some required materials, as several brands and models are accepted. For those materials with specific catalog numbers we recommend purchasing this exact item in order to guarantee success.

Required Emulate Products

A list of Emulate products needed for this protocol is provided below:

Item	Quantity/Description	Vendor	Cat #
AVA™ Emulation System	1 Instrument	Emulate	AVA-ES1
Orb-HM1® Hub Module	1 Module	Emulate	ORB-HM1
Liver-Chip Array Quad-Culture BioKit	• Chip-Array Organ-Chip consumables (8) • Pipette Guides (3 per Chip-Array; 24 total) • Flow Guides (3 per Chip-Array; 24 total) • Pipette Guide Covers (1 per Chip-Array; 8 total) • Hepatocytes • Kupffer Cells • Stellate Cells • Liver Sinusoidal Endothelial Cells (LSECs)	Emulate	BIO-LH-QUAD96CA

Required Lab Equipment and Consumables

A list of lab equipment and consumables needed for this protocol is provided below:

Item	Quantity/Description	Vendor	Cat #
Steriflip-HV Filters (Sterile, 0.45 µm PVDF-filter)	25/pack	EMD Millipore	SE1M003M00
Collagen type-I coated plates; 24-well, flat-bottom, TC-treated	1 control plate per hepatocyte donor	Corning	354408
Handheld vacuum aspirator	1 or 2 aspirators for BSC	Corning	4930
Aspirating pipettes (2-mL, polystyrene, individually wrapped)	-	VWR	53106-450
Aspirating tips	-	-	-
Serological pipettes (2-mL, low-endotoxin, sterile)	-	VWR	55300-363
Serological pipettes (5-mL, low-endotoxin, sterile)	-	VWR	55300-421
Serological pipettes (10-mL, low-endotoxin, sterile)	-	VWR	55300-523
Serological pipettes (25-mL, low-endotoxin, sterile)	-	VWR	55300-567
Serological pipettes (50-mL, low-endotoxin, sterile)	-	VWR	53106-441

P20 Single Channel Pipette	-	-	-
P200 Single Channel Pipette	-	-	-
P1000 Single Channel Pipette	-	-	-
P20 Multi Channel Pipette	-	-	-
P200 Multi Channel Pipette	-	-	-
P1000 Multi Channel Pipette	-	-	-
P20 sterile, low-adhesion, filtered pipette tips*	-	VWR	76322-134
P200 sterile, low-adhesion, filtered pipette tips	-	VWR	76322-150
P1000 sterile, low-adhesion, filtered pipette tips*	-	VWR	76322-154
15 mL Falcon Conical Tubes	-	VWR	21008-918
50 mL Falcon Conical Tubes	-	VWR	21008-940
50 mL Disposable Pipetting Reservoirs	5/pack	VWR	89094-682
Nunc™ 96-Well Polystyrene Round Bottom Microwell Plates	10/pack	ThermoFisher	268200
Nunc™ 96-Well Polypropylene DeepWell™ Sample Plates, Square, Conical Bottom	50/pack	ThermoFisher	95040462
Eppendorf tubes	-	-	-
Aluminum foil	-	-	-

Parafilm M Sealing Film	-	VWR	52858-076
Microscope (with camera), for phase contrast imaging	-	-	-
Hemocytometer	-	-	-
Water bath (or bead bath)	-	-	-
Vacuum set-up	-	-	-
T75 TC-Treated cell culture flask with vented cap	Minimum 6 flasks required	Corning	353136
Centrifuge	-	-	-
Ice bucket	-	-	-
70% ethanol	-	-	-

***Disclaimer: The listed P20 and P200 filtered pipette tips are recommended due to their validated robustness in interfacing with the Chip-Array.**

Required Cells

A list of cells needed for this protocol is provided below (as included as part of the Liver-Chip Array Quad-Culture BioKit):

Item	Quantity/Description	Vendor	Cat #
Primary Human Hepatocytes	2 vials for seeding into the top channel of Chip-Array	Emulate	BIO-LH-QUAD96CA
Liver Sinusoidal Endothelial Cells	6 vials for seeding into the bottom channel of Chip-Array	Emulate	BIO-LH-QUAD96CA
Kupffer Cells	5 vials for seeding into the bottom channel of Chip-Array	Emulate	BIO-LH-QUAD96CA
Stellate Cells	1 vial for seeding into the bottom channel of Chip-Array	Emulate	BIO-LH-QUAD96CA

Required Reagents, Media, and Supplements

A list of reagents, media, and supplements needed for this protocol is provided below:

Item	Description	Vendor	Cat #
1X Dulbecco's PBS (DPBS -/-) (without Ca2+, Mg2+)	500 mL bottle	Corning	21-031-CV
10X Dulbecco's PBS (DPBS -/-) (without Ca2+, Mg2+)	500 mL bottle	Corning	20-031-CV
Cell Culture Grade Water	500 mL bottle; for fibronectin solution preparation	Corning	25-055-CV
Cell culture grade Dimethyl Sulfoxide (DMSO)	100 mL bottle; for dexamethasone	Sigma	D2650
0.4% Trypan Blue Solution	50 mL bottle	Sigma	93595
Percoll Solution (100% stock solution)	100 mL bottle	Sigma	P4937

Trypsin-EDTA solution (0.05% trypsin)	100 mL bottle; aliquoted	Sigma	T3924
William's Medium E with phenol red (+)	500 mL bottle	Sigma	W4128
William's Medium E without phenol red (-)	500 mL bottle	Sigma	W1878
CSC medium (Kit)	Comes with 500 mL Medium without Serum, Attachment Factor, Culture Boost	Cell Systems	4Z3-500
Culture Boost	50X supplement, 5 mL bottle, comes with CSC Kit	Cell Systems	4CB-500
Attachment Factor	1X, 10 mL bottle, comes with CSC Kit	Cell Systems	4Z0-210
Matrigel Basement Membrane Matrix, LDEV-Free	10 mL bottle; aliquoted	Corning	354234
Fibronectin, bovine protein, plasma	1 mg; aliquoted	ThermoFisher	33010-018
Collagen type I, rat tail, high concentration	100 mg bottle	Corning	354249
Penicillin-streptomycin	10,000 U/mL, 10 mg/mL; aliquoted	Sigma	P4333
L-GlutaMax	200 mM; 100 mL bottle	ThermoFisher	35050-061
L-Ascorbic Acid	100-mg powder	Sigma	A5960-100MG
Dexamethasone	100-mg powder	Sigma	D4902-25MG
Fetal bovine serum (FBS), sterile, heat-inactivated	100 or 500 mL bottle; aliquoted	Sigma	F4135
Corning® ITS+ Premix Universal Culture Supplement	20 mL bottle	Corning	354352

Workstation Preparation and Chip-Array Handling Techniques

Overview

Aseptic Techniques

- Always work with chips in a sterile environment, such as the biosafety cabinet (BSC).
- Before beginning the experiment, prepare, sanitize, and organize the work surface of the BSC. Arrange tips, media, and other necessary materials in the sterile field so they are easily within reach but not blocking the path of airflow.
- Use 70% ethanol to thoroughly sanitize all reagents and materials before placing them into the BSC.
- Always avoid touching the Chip-Array Emulations directly. Handle the Chip-Arrays only by the sides, by the lid, or by the tab, with gloves.

Cell Storage

Always store cryopreserved cells in the vapor phase of liquid nitrogen. Never store them in dry ice or in a -80°C freezer. Chronic temperature fluctuations can cause severe damage to cell membranes and cytoskeletal components.

Chip-Array Handling Techniques

Chip-Array Design

Figure 1 shows the Chip-Array. It can be divided into two separate sections: the “Reservoir” section and the “Emulations” section.

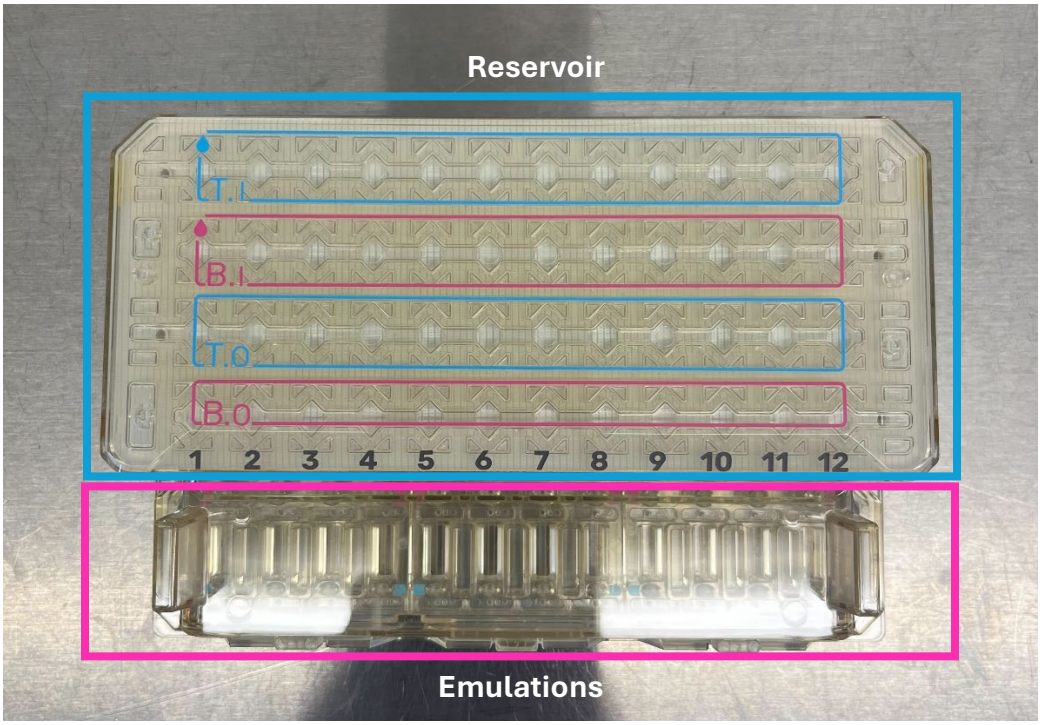

Figure 1. Chip-Array consumable design.

One Chip-Array contains twelve “Emulations” (see Figure 2 below). An Emulation is an independent Organ-Chip sample; each Emulation has its own top and bottom microfluidic channel, which is separated by a porous membrane. Each Emulation is compartmentalized from one another, meaning that each Emulation can be treated individually from each other. Each Emulation is labeled 1-12 on the lid of the reservoir.

Additionally, each Emulation has its own set of 4 reservoirs: a top channel inlet reservoir (TI), a top channel outlet reservoir (TO), a bottom channel inlet reservoir (BI), and a bottom channel outlet reservoir (BO). This is indicated by the label on the reservoir lid.

The inlet reservoirs are where media is added during media replenishment and dosing, which will be supplied to the biology seeded in the Emulations via unidirectional flow facilitated by the AVA instrument. Because each Emulation’s reservoirs are compartmentalized from each other, different compounds and concentrations can be dosed into each Emulation independently from one another, allowing users to have multiple dosing conditions on a single Chip-Array.

Each Emulation can be broken down into separate inlet and outlet ports for the top and bottom channels. The inlet sides of each channel are towards the reservoir, while the outlet sides are oriented away from the reservoir. Inlet and outlet orientation are based

on the direction of flow once the Chip-Array is under flow in the AVA instrument. Media will flow unidirectionally from inlet to outlet, from the inlet reservoir through the Emulation then into the outlet reservoir.

Additionally, a viewing window is cut into the gasket on each Emulation, allowing users to image directly into the channels. Each Emulation is labeled with its own chip number (1 through 12) and is visible under the microscope.

Chip-Arrays come with tape on the reservoir lid. This helps to prevent the lid from accidentally falling off when users are handling the Chip-Array and any inversion steps when seeding the bottom channel. This tape can be removed once users are ready to start adding media to the reservoir during the Prime step.

See Figure 2 for a graphic that displays the orientation of each Emulation.

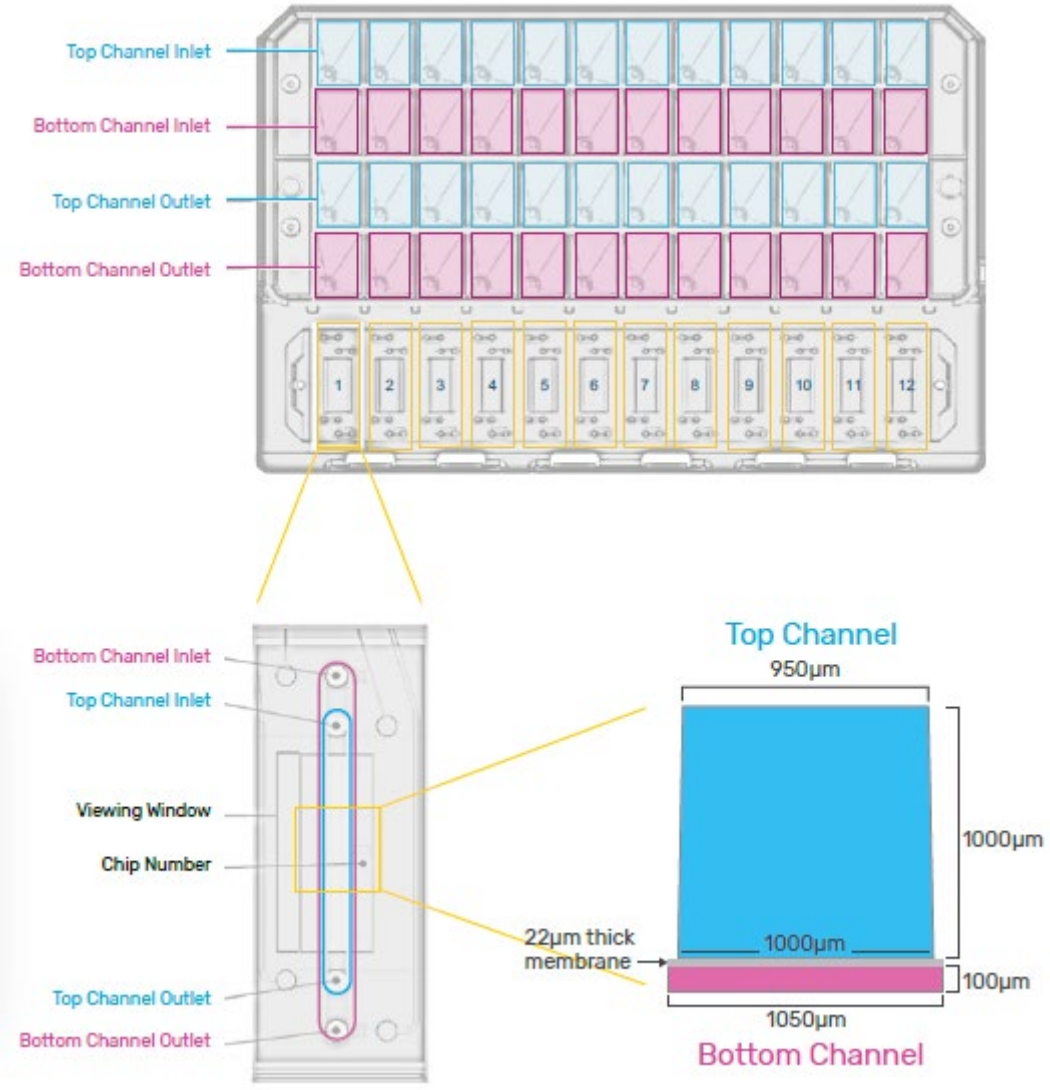

Figure 2. Schematic of an Emulation within the Chip-Array consumable.

Components of the Chip-Array

One Chip-Array will come in its package with three pipette guides, three flow guides, and one pipette guide cover. See Figure 3 for what these Chip-Array components look like.

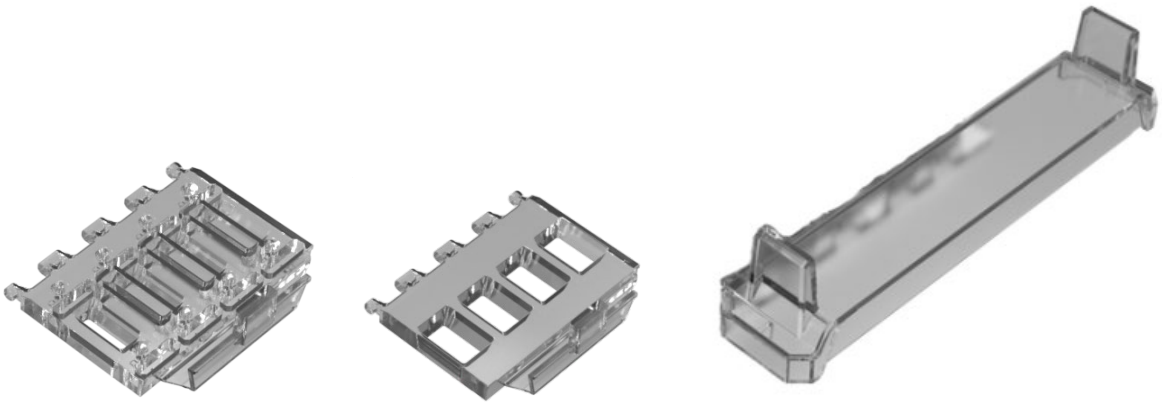

Figure 3. Chip-Array accessories: pipette guide (left), flow guide (middle), and pipette guide cover (right).

Pipette Guides

Pipette guides facilitate manual pipetting with micropipettes into the top and bottom channels of each Emulation on the Chip-Arrays. These channels are microfluidically separated and therefore have separate pipetting/seeding ports that allow users to pipette into each channel individually. Single channel and multichannel pipettes are compatible with the Chip-Array pipette guides. Each pipette guide interfaces with 4 Chip-Array Emulations, meaning that one Chip-Array accommodates three pipette guides.

For each Emulation, the pipette guide contains two seeding ports, which allow the user to manually pipette solution and cell suspension into the top and bottom channels using a single channel or multichannel pipette. **These seeding ports are compatible with P20 and P200 pipette tips. Refer to the Required Lab Equipment and Consumables Section for recommended pipette tips.** The top channel seeding port is at the bottom of the pipette guide, while the bottom channel seeding port is at the top of the pipette guide. On each pipette guide, the magenta color indicates the location of

the seeding ports used for pipetting into the bottom channel, while the blue color indicates the location of the seeding ports used for pipetting into the top channel.

Additionally, the pipette guide contains outflow troughs for each channel, which collects the outflow from each channel as users pipette into them. The bottom channel trough is on the opposite side of the bottom channel seeding port, while the top channel trough is on the opposite side of the top channel seeding port. These troughs can be filled with excess media or ECM when pipetting in order to prevent evaporation of the liquid in the Emulations during long periods of incubation such as overnight incubation after hepatocyte attachment in the top channel.

Pipette guides also contain humidity PBS reservoirs between each Emulation (3 humidity reservoirs per pipette guide). These reservoirs should be filled with PBS to ensure minimal evaporation of liquid in the Emulation channels.

See Figure 4 for the orientation of the different ports and wells of the pipette guide.

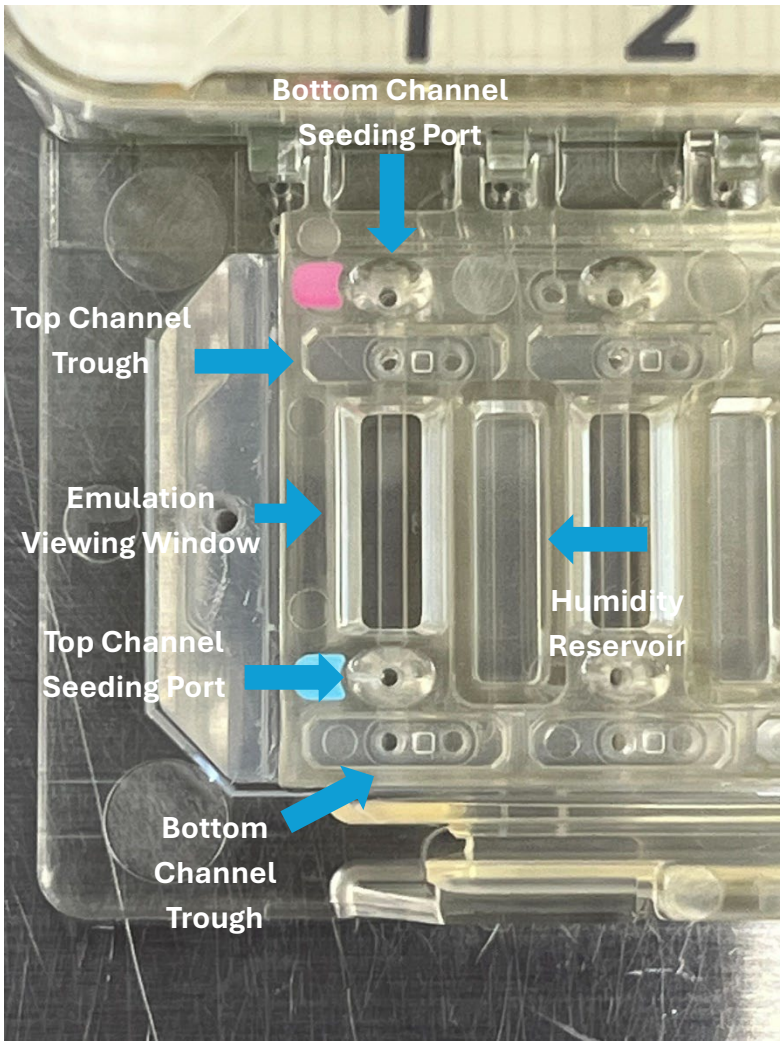

Figure 4. Orientation and components of the pipette guide.

Pipette Guide Covers

Pipette guide covers act as a shield to help maintain sterility while pipette guides are attached to the Emulations. Pipette guide covers should always be used when pipette guides are attached to the Chip-Array and should only be removed when users are handling the Chip-Array in the BSC. The covers are not required once flow guides are attached to the Chip-Array, as the flow guides provide a complete seal that prevents exposure of biology in the culture channels to the outside environment.

It should be noted that pipette guide covers have a specific orientation. The larger, flat side of the pipette guide cover should face the user, while the shorter, angled side should face the Chip-Array reservoir. This specific orientation allows the pipette guide cover to properly slot over the Emulations and remain flush. See Figure 5 for the proper orientation of the pipette guide cover.

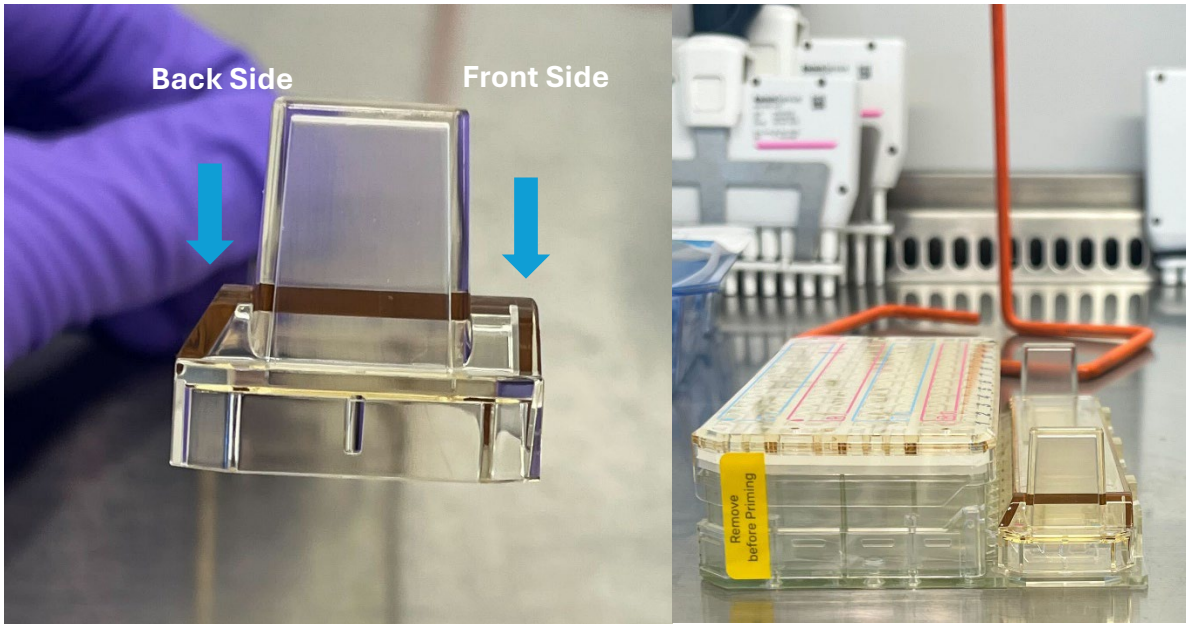

Figure 5. Proper orientation of the pipette guide cover.

Flow Guides

Flow guides are used to fluidically connect the Emulations to the reservoirs filled with media, allowing the AVA to flow media from the reservoir inlets to the Emulations.

Pipetting Solution into Channels

All wash steps are typically performed with a P200 multichannel pipette, using 50-100 µL of the specific solution, which is enough to fill the channels and leave excess in the troughs. While the Chip-Array is compatible with multichannel pipettes using 12 tips at a time, it is recommended for users to use 6 tips at a time during these wash steps. When using multiple tips at a time, users must ensure that all tips are properly interfacing with the seeding port, and enough force is applied to the tips. If there is not a proper seal, liquid will not flow through the channel.

Follow the steps below for pipetting solution into each Chip-Array using a multichannel P200 pipette.

1. Take a multichannel P200 pipette with up to 6 sterile pipette tips and collect the solution to be added to the Chip-Array Emulations. Ensure that all tips on the pipette are properly secure and attached. Failure to do so can cause tips to fall off during handling or for tips to collect the incorrect volume of solution.
2. Align all pipette tips with the specific channel seeding port and insert all tips perpendicular to the port, ensuring that the tips are secure in the port.
    a. Users must ensure that all tips are properly secured in the seeding port and enough force is applied by the user during insertion of tips. Lack of a proper seal will cause improper liquid dispensing.
    b. When pipetting with multiple tips, users can use their offhand to steady the pipette with their fingers on the pipette shoulders to distribute force before pipetting. Always make sure tips are perpendicular when inserted into the pipette guide; failure to do so can cause tips to become stuck.
3. Steadily dispense the liquid through the channel. See Figure 6 for proper placement of tips into seeding port and dispensing of liquid through the channels.
4. Excess liquid will flow into the trough of the channel that is being washed. Users can either keep the excess liquid in the through or aspirate the liquid.
5. If aspirating liquid from the troughs, users must avoid touching the port in the trough, as this will aspirate the liquid that is in the Emulation channels. It is recommended for users to place their aspirating tips along the left or rightmost walls of the trough that is being aspirated. See Figure 7 for proper placement of tips when aspirating from troughs.

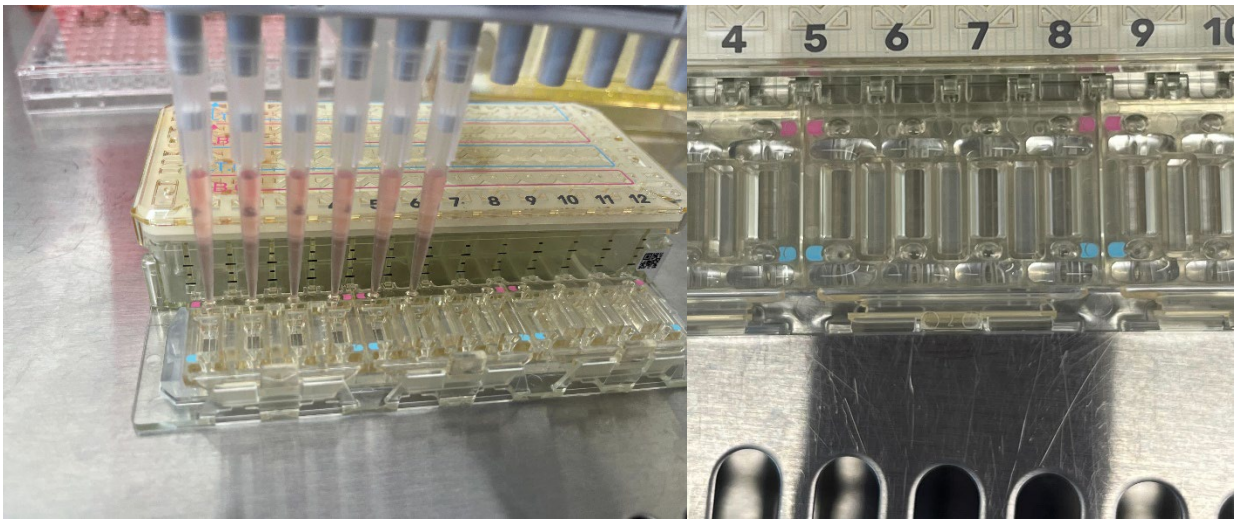

Figure 6. Left image shows proper placement of tips into seeding ports and dispensing of liquid. Right image shows collection of excess outflow into the trough.

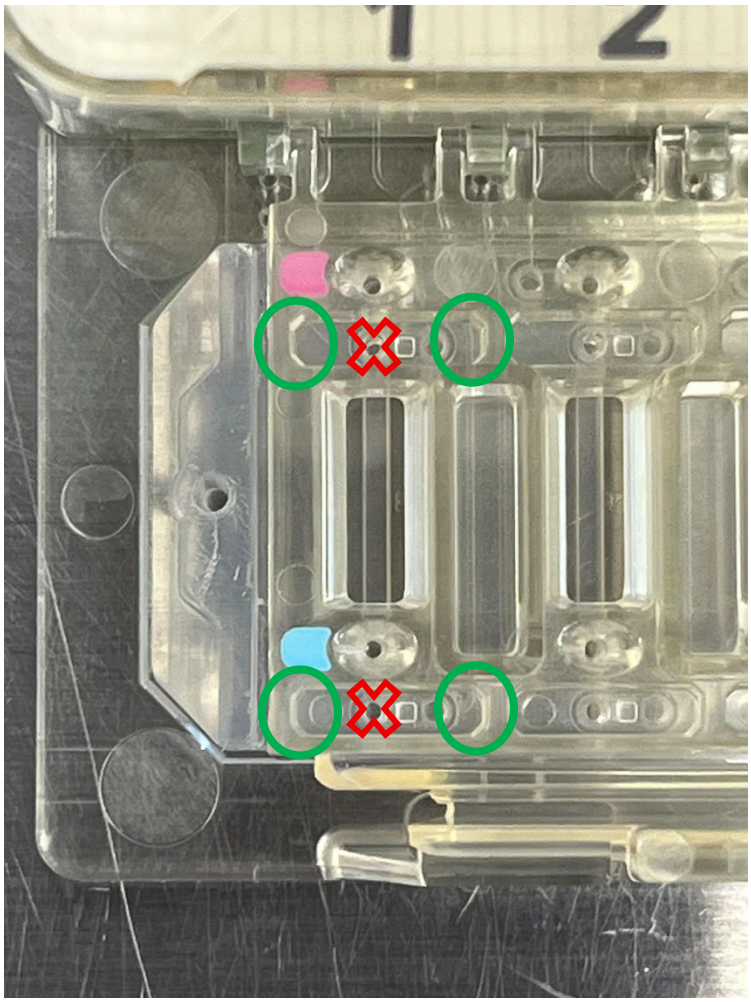

Figure 7. Proper placement of tip when aspirating from trough.

CAUTION: If excess liquid during pipetting falls into the viewing window of the Emulation, take precaution NOT to make direct contact with the viewing window with the aspirating pipette tip. The top gas exchanger film can be easily punctured by a pipette tip. Tips should be placed in the corner of the viewing window and gently lowered until all excess liquid is aspirated from the viewing window.

All seeding steps are performed with a P20 pipette using the same steps above. **It is recommended that users seed a maximum of 6 Emulations at a time. Seeding with more than 6 at a time increases the likelihood of variability between**

Emulations. The table below highlights the seeding volumes recommended per channel.

Channel	Volume
Top Channel	20 µL
Bottom Channel	10 µL

CAUTION: Users must be careful when using P20 pipette tips during cell seeding steps. P20 pipette tips are more likely to get stuck in the seeding ports during seeding compared to P200 tips during washing. If a tip gets stuck, users can pull the tip out of the seeding port. However, it is likely that this will compromise cell distribution within the channel of that specific Emulation, likely leading to a re-seed of that Emulation. Users should follow these guidelines to prevent tips getting stuck:

- Always make sure the pipette tips and multichannel pipette are perpendicular to the seeding port and vertical. Removing tips from the seeding ports at an angle can increase likelihood of the tips getting stuck.
- When seeding several Emulations at a time with P20 pipette tips, users must be careful with the amount of force applied to the multichannel P20 tips. Inserting the tips is enough to create a seal with the seeding port to ensure proper flow. Additional excessive force when pipetting multiple Emulations at a time (i.e. using your offhand to apply force to the multichannel pipette) can cause tips to become stuck in the pipette guide seeding port.
- Refer to the Required Lab Equipment and Consumables Section for recommended pipette tips. These pipette tips are recommended as the tip shape is most compatible with the pipette guide seeding ports and has been thoroughly tested.

CAUTION: When seeding, take care to aspirate all excess liquid from the trough of the specific channel that is being seeded. This should be done before and after pipetting cells through the specific channel seeding port. Any excess liquid in the trough may contribute to improper cell distribution and gradients during seeding.

NOTE: When seeding, it is recommended to leave the P20 pipette tip in the seeding port until the outflow volume in the trough is aspirated, then the user may remove the pipette tip.

Attachment and Detachment of Pipette Guides and Flow Guides onto Chip-Array

There are three sections of the Chip-Array that allow connection with a pipette guide or flow guide: left, right, and center. The left pipette guide or flow guide will interface with Emulations 1-4, the center pipette guide or flow guide with Emulations 5-8, and the right pipette guide or flow guide with Emulations 9-12. This means that each Chip-Array accommodates three pipette guides or flow guides at a time. See Figure 8 for a breakdown of these sections.

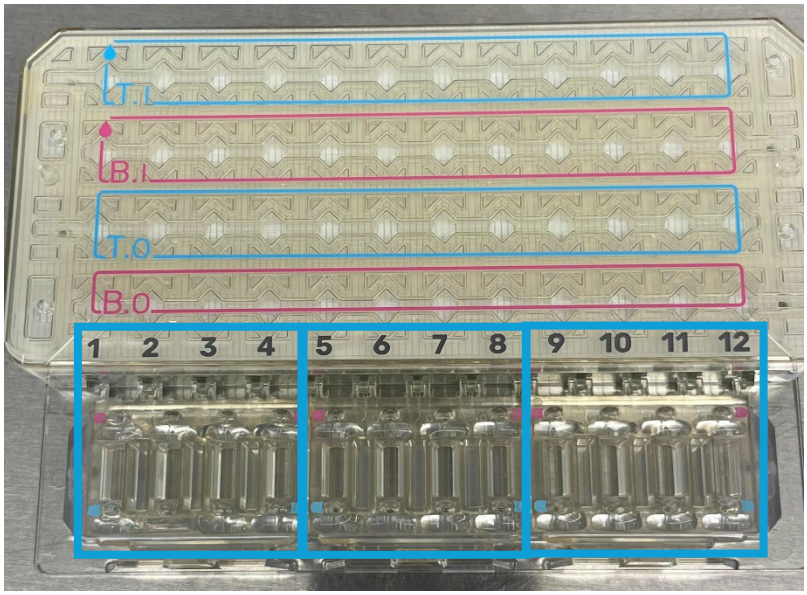

Figure 8. The three different sections of the Chip-Array for pipette guides or flow guides.

All Chip-Arrays will come packaged with three pipette guides attached to the consumable, meaning users will not have to attach the pipette guides and can immediately begin pipetting solution into the Emulations. Pipette guides should be detached and re-attached if any leakages are observed during ECM coating/washing steps. The following steps, while particular for flow guide attachment, are also applicable to pipette guide attachment.

Each guide is connected to the Chip-Array via hinges on the Chip-Array. The guides have connection points that interface with these hinges. See Figure 9, which highlights the connection points on the guides and the hinges on the Chip-Array.

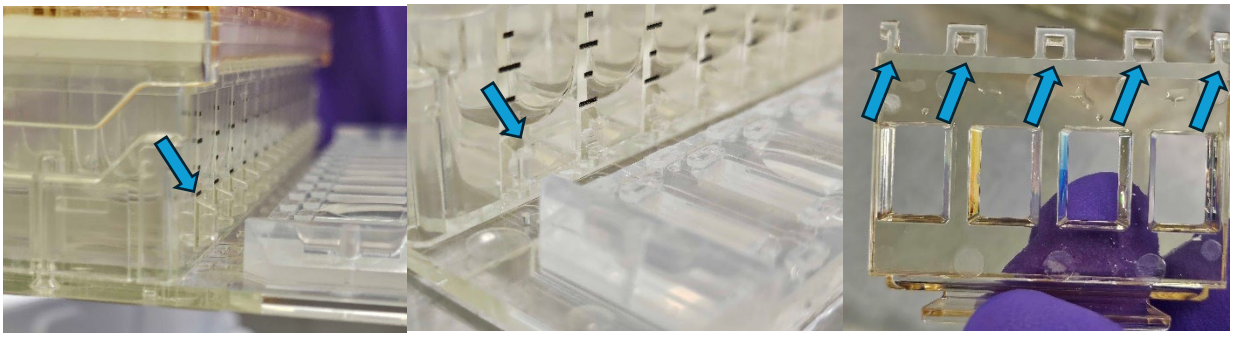

Figure 9. Pipette or flow guide connection points and hinges on Chip-Array.

It is recommended to attach the left guide first, followed by the middle guide, then lastly the right guide. Follow the steps below:

1. Align each guide connection point with the hinges on the Chip-Array. Push these connection points into the hinges; the user should be able to feel or hear a very light snapping noise, which indicates proper connection.
2. Once connected, gently snap the flow guides in place one at a time, using two fingers to hold onto the flow guide tab while pushing downwards, so that it interfaces with its respective Emulations. Users should hear an audible click when snapping the guide into place over its Emulations. See Figures 10 and 11 for visuals on connected guides and then snapping them into place
3. For the left guide, make sure to align the guide as flush as possible to the leftmost hinge connector. When connecting the right guide, make sure to align the guide as flush to the rightmost hinge connector as possible. When connecting the middle guide, use the middle hinge on the guide and the middle hinge connector on the Chip-Array as reference points.

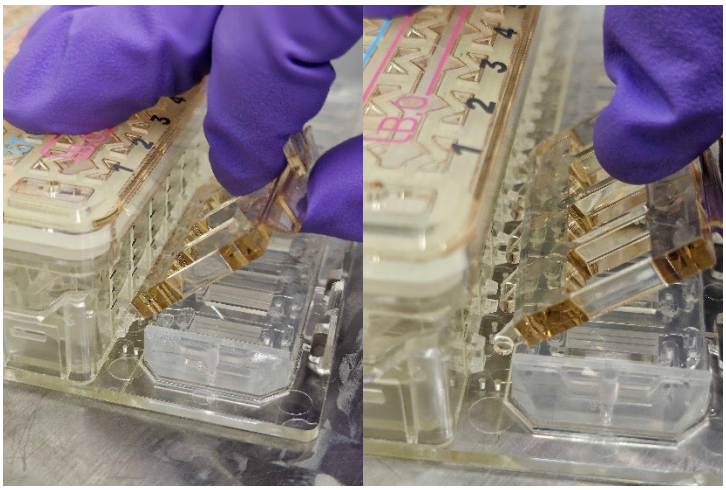

Figure 10. Connecting pipette or flow guide onto Chip-Array.

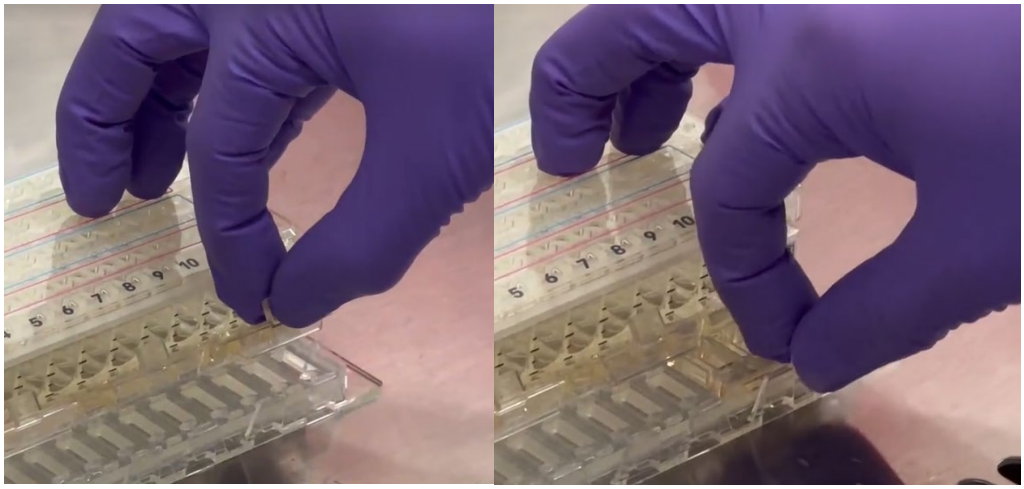

Figure 11. Snapping pipette or flow guide into place over Emulations.

Pipette and flow guides can also be removed from the Chip-Arrays after attachment. All Chip-Arrays come packaged with pipette guides attached to the consumables. Pipette guides should only be removed either if a leak is observed during pipetting, as this may indicate misalignment of the pipette guide with the Emulation, or during the Prime step which requires removal of pipette guides for the attachment of flow guides.

Remove guides by pushing on the tab that secures the guides inwards, then lifting the guide upwards. Once the clip is at a 45-degree angle, lightly tug on the clip to remove it. See Figure 12 for removal of the pipette guide.

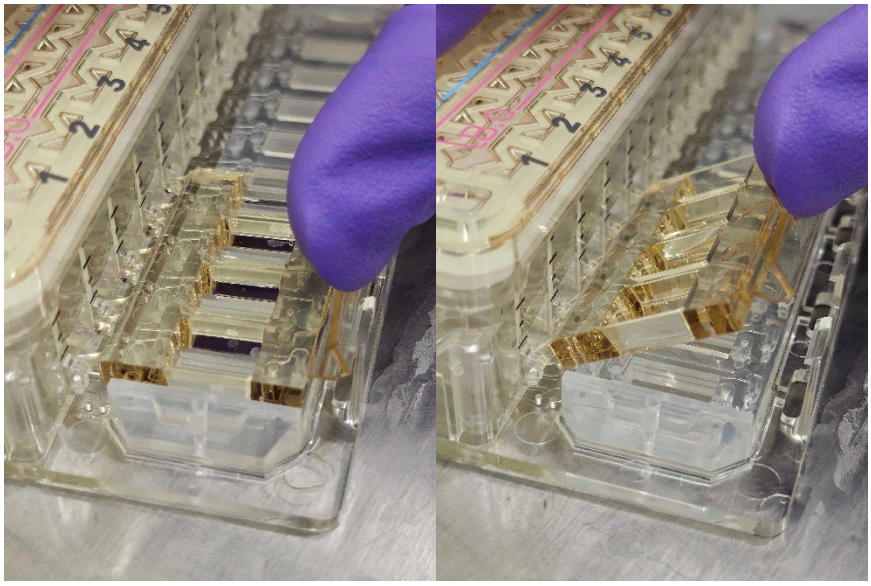

Figure 12. Removal of the pipette guide.

Pre-Experiment: Reagent Preparation

Aliquot Reagents

Introduction

Aliquot reagents prior to use so that the stock solutions do not undergo multiple freeze-thaw cycles.

Fibronectin (ECM)

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

- Resuspend 1 mg fibronectin in 1 mL of cell culture grade water according to manufacturer's instructions
- Create single-use volume aliquots and store them at -20°C. For one Chip-Array, this equates to 50 µL of fibronectin per final ECM volume needed per Chip-Array

Matrigel (Overlay)

Reagent	Amount	Volume
Matrigel	5 mg per aliquot	Varies per lot

The Matrigel bottle must be thawed overnight on ice either in the back of the 2-6°C refrigerator or in a cold room. Add water to ensure the ice is slushy, as the solution gels rapidly at temperatures above 10°C. For aliquoting, use pipettes tips and tubes prechilled to -20°C, maintain aliquots on slushy ice, and transfer the aliquots immediately to -20°C.

- Calculate and prepare the aliquot volume needed to achieve a concentration of 0.25 mg/mL in the overlay media
- Store single-use aliquots of 500 µL at -20°C

Culture Supplements

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

- Resuspend each supplement to the working concentration in the table above
- Aliquot each supplement to single-use volumes
    - L-Ascorbic acid: 200 µL
    - 10 mM Dexamethasone: 50 µL
    - 1 mM Dexamethasone: 50 µL
- Store aliquots at -20°C

Day -2: Liver Sinusoidal Endothelial Cell (LSEC) Thawing and Plating

Overview

Goals

- Thaw and expand LSEC culture media in flask

Required Materials

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

Prepare LSEC Culture Media

Base LSEC Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
CSC Basal Medium	490 mL	-	-	Cell Systems	4Z3-500
Culture Boost	5 mL	-	2%	Cell Systems	4CB-500
Pen/Strep	5 mL	-	1%	Sigma	P4333

- Store the Base LSEC Culture Medium at 4°C.
- Use the Base LSEC Culture Medium within 30 days of preparation.

Complete LSEC Culture Medium (50 mL)

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

- Store the Complete LSEC Culture Medium at 4°C.
- Use the Complete LSEC Culture Medium within 7 days of preparation.

Preparation of Flasks

Steps

1. Prepare and warm a sufficient amount of Complete LSEC Culture Medium (CLCM) and Attachment Factor to 37ºC. 15 mL of medium per 3 vials is needed for thawing, and an additional 15 mL is needed for each T75 flask.
    a. The number of flasks to coat and vials of LSECs to thaw is dependent on the number of Chip-Array Emulations that will be seeded. Six vials (and therefore six T75 flasks) of LSECs are recommended to seed 96 Emulations.
2. Label the culture flask with relevant information (e.g., cell type, passage number, date initials, experiment ID).
3. Pipette Attachment Factor onto the growth surface of each flask until it is fully covered. 4 mL of Attachment Factor is used for each T75 flask.
4. Incubate the prepared flask in a 37°C incubator to coat the flask surface. Maintain this temperature until the cells are plated (about 5 minutes).

Thawing LSECS

Note on LSECs

LSECs are the only cells in this protocol that must be plated before being seeded in the Chip-Array. LSECs' initial passage number may vary per Liver Chip-Array Quad-Culture BioKit. Reach out to your designated Scientific Liaison or to Emulate Support for further guidance based on the lot and passage of LSECs you received.

If needed, LSECs can be further expanded by passaging prior to chip seeding but should not exceed passage 5 from the initial BioKit. Reach out to Emulate support (support@emulatebio.com) for any questions related to LSEC seeding density optimization.

Steps

1. Thaw six vials of LSECs by immersing the vials in a 37°C water bath without submerging the cap. Closely observe and gently agitate the vials. Remove them from the water bath just before the last of the ice disappears.
2. Wipe the vials dry, then sterilize the outside of the vial by wiping with a 70% ethanol-soaked wipe before placing into the BSC.
3. Immediately transfer the contents of the vials into a sterile 15-mL conical tube containing 3 mL of warm Complete LSEC Culture Medium.

a. It is recommended to thaw batches of 3 LSEC vials into a single conical tube. For example, for six vials, thaw 3 LSEC vials into one conical tube, and the remaining 3 LSEC vials into another conical tube.

4. Rinse the vials with 1 mL Complete LSEC Culture Medium and collect the run-off in the 15-mL conical tube.
5. Bring the volume of each conical tube to 15 mL with Complete LSEC Culture Medium.
6. Centrifuge the conical tubes at 200 x g for 5 minutes at room temperature.
7. Aspirate and discard the supernatant, leaving approximately 100 µL of medium covering the pellet.
8. Resuspend the cell pellet in each conical tube in 3 mL of Complete LSEC Culture Medium.

9. Aspirate the Attachment Factor from each T75 flask that was prepared and add 14 mL of Complete LSEC Culture Medium to each flask.

    NOTE: it is unnecessary to rinse or dry the flask prior to adding cells.
10. Distribute LSEC suspensions equally (1mL per flask) to the freshly coated T75 flasks.
11. Inspect T75 flasks on microscope to ensure cells have been added to each flask.
12. Incubate T75 flasks overnight at 37ºC and 5% CO_2.

Day -2: AVA Setup

Overview

Goals

- Prepare the AVA instrument and software

AVA Decontamination

It is recommended by Day -2 to have the AVA decontaminated via Mycofog.

This is approximately a 4-4.5 hour activity that requires heating the AVA to 37°C, then decontaminating via Mycofog.

AVA Environmental Setup

After decontamination, it is recommended to have the AVA set to these environmental parameters for a Liver-Chip Quad-Culture experiment.

- Temperature: 37°C
- CO2: 5%
- Ensure the water pan is filled to the 100% fill volume

Ensure that the environmental conditions of the AVA reach these specific setpoints and remain stable by Day 0.

AVA Experimental Design Setup

Steps

1. Open the Experiment Tab and create a new experiment from the Liver-Chip Quad-Culture Template
    a. This template will come pre-populated with all necessary medias, cells, and ECM reagents, as well as activities, schedule imaging, and timepoint days tied to readouts displayed in a calendar view
2. In the Conditions window, input Experiment Name, Start Date of study and ensure that all cell, media, reagent, and ECM lot information is updated to reflect the materials used for the study
3. Input any necessary conditions, along with relevant information and number of replicates associated with each condition
4. In the Protocol window, double check that the experimental design incorporates scheduled imaging routines on days 5, 6, 7 and 12 (timepoint days 0, 1, 3 and 7)
    a. These are the recommended days for imaging representative Fields of View (FOVs) of Liver-Chip Emulations to assess morphology and health
    b. Users can modify or add in additional scheduled imaging routines to their discretion
5. Input the number of Chip-Arrays required, assigning names and serial numbers to each Chip-Array
6. Ensure experiment days in calendar view correctly align with expected timeline of activities

Day -1: Chip-Array Preparation

Overview

Goals

- Change media in T75 flasks
- Coat the inner channels of Chip-Arrays with a mixture of collagen I and fibronectin ECM proteins for cell attachment

Required Materials

- Complete LSEC Culture Medium (at 37°C)
- Chip-Arrays
- 15 mL conical tubes
- DPBS (-/-) at 4°C
- Collagen I
- Fibronectin
- 50 mL disposable pipetting reservoirs
- 70% ethanol
- Ice and ice bucket
- Single channel and multichannel pipettes and filtered tips
- Aspirator and sterile aspirating tips
- Parafilm

LSEC Inspection

Steps

1. Inspect T75 flasks on lab microscope and take representative images of attached LSECs.
2. If LSEC density or morphology is not ideal, thaw additional LSEC vial(s) using the procedure described on Day -2.

LSEC Media Change

Steps

1. Prep 15 mL of Complete LSEC Culture Medium per T75 flask and warm media to 37ºC on bead bath.
2. Aspirate and discard old media from flasks and replenish with new media.
3. Refresh the Complete LSEC Culture Medium every other day until the cells are seeded in the chip.

Chip-Array Preparation

Steps

1. Spray the Chip-Array packaging with 70% ethanol and bring into the BSC.
2. Open the packaging and take out all contents. This should include one Chip-Array, three pipette guides, three flow guides, and one pipette guide cover.
    a. Pipette guides facilitate manual pipetting with micropipettes into the top and bottom channels of each Emulation on the Chip-Arrays
        i. Single channel and multichannel pipettes are compatible with the Chip-Array pipette guides.
    b. Flow guides are used to fluidically connect the Emulations to the reservoirs filled with media, allowing the AVA to flow media from the reservoir inlets to the Emulations.
    c. Pipette guide covers act as a cover to help maintain sterility while pipette guides are attached to the Emulations. These are not necessary once flow guides are attached, as the flow guides provide a complete seal that prevents exposure of biology in the culture channels to the outside environment.
3. Chip-Arrays will already come with three pipette guides attached, so there is no need to attach the pipette guides.
4. Label each Chip-Array with its corresponding ID or label.

ECM Preparation

Before you begin

Prepare fresh extracellular matrix (ECM) before each use by combining the individual ECM components with cold DPBS to reach the final working concentrations. The ECM solution will be used to coat both top and bottom channels. Prepare and maintain on ice.

Needed volumes

For human Liver-Chips, the ECM working concentrations are:

Reagent	Concentration
Collagen I	100 µg/mL
Fibronectin	25 µg/mL

Steps

1. Bring a full ice bucket to the BSC.
2. Calculate the volume of ECM solution needed to coat all Emulations.
    a. Volume required per Emulation: ~140 µL
    b. For every Chip-Array, prepare 2 mL of ECM solution:
        i. 12 Emulations x 140 µL/Emulation = 1.68 mL of ECM solution
        ii. 1.68 mL + extra 320 µL = 2.0 mL of ECM solution
3. Thaw the necessary number of aliquots of fibronectin (1 mg/mL) on ice. Always maintain each ECM component and mixture on ice.
4. Combine the components to prepare the ECM working solution. See Example ECM Calculation section on next page for example of calculating ECM volumes.
5. Keep the ECM solution on ice until it is used.

Example ECM Calculation

ECM Calculation example for 8 Chip-Arrays

Solution	Concentration
Collagen I stock concentration	11.61 mg/mL (C1)
Collagen I final concentration	0.1 mg/mL (C2)
Fibronectin stock concentration	1 mg/mL (C1)
Fibronectin final concentration	0.025 mg/mL (C2)
Stock Volume	Collagen I (X) or fibronectin (Y) (V1)
Final volume of ECM solution	16 mL (V2)

Collagen Calculation:

(11.61 mg/mL) x (X mL) = (0.1 mg/mL) (16 mL)

X = 137.81 µL of collagen I stock solution

Fibronectin Calculation:

(1 mg/mL) x (X mL) = (0.025 mg/mL) (16 mL)

Y = 400 µL of fibronectin

DPBS Calculation:

Volume DPBS =

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

= 16000 µL – 137.81 µL – 400 µL

= 15462.19 µL of DPBS

Coat Chip-Arrays with ECM

Steps

1. Pour ECM solution into a disposable pipetting reservoir on ice to allow pipetting with multichannel pipette.
2. Using a multichannel P200 pipette, add 60 µL of ECM solution to the bottom channel, pipetting through the bottom channel seeding port.
    a. This volume is enough to fill the bottom channel as well as the bottom channel trough with ECM. The troughs should be full but not overflowing.
    b. It is recommended to add ECM solution with 6 tips at a time using a multichannel pipette.
    c. Double check that ECM flowed through all 12 Emulations for each Chip-Array. If there is no flow, this is likely due to an improper interface of pipette tip with the bottom channel seeding port, or there is misalignment of the pipette guide. Attempt to add ECM solution to the bottom channel using a single channel P200 pipette. If this still does not properly flow, then detach and reattach the pipette guide and attempt re-coating that specific Emulation. See Chip-Array Handling section for steps on pipette guide detachment and attachment.
3. Using a multichannel P200 pipette, add 60 µL of ECM solution to the top channel, pipetting through the top channel seeding port.
    a. This volume is enough to fill the top channel as well as the top channel trough with ECM. The troughs should be full but not overflowing.
    b. It is recommended to add ECM solution with 6 tips at a time using a multichannel pipette.
    c. Double check that ECM flowed through all 12 Emulations for each Chip-Array. If there is no flow, this is likely due to an improper interface of pipette tip with the bottom channel seeding port, or there is misalignment of the pipette guide. Attempt to add ECM solution to the top channel using a single channel P200 pipette. If this still does not properly flow, then detach and reattach the pipette guide and attempt re-coating that specific Emulation. See Chip-Array Handling section for steps on pipette guide detachment and attachment.
4. Repeat steps 2-3 for all Chip-Arrays.

5. Visually inspect the Emulations and wash any bubbles from the channel with the ECM solution.
6. To prevent evaporation during incubation, add PBS to the PBS humidity troughs between each Emulation (each PBS trough is filled with ~150 µL).
7. Place pipette guide cover over the Emulations, ensuring that the cover is placed properly and flush. Keep this lid on whenever moving the consumable from the AVA, tissue culture incubator, and the biosafety cabinet. Failure to do so will compromise sterility.
8. Wrap a piece of Parafilm around edges of each Chip-Array and pipette guide cover to help maintain sterility when Chip-Arrays are incubating overnight.
9. Incubate all coated Chip-Arrays overnight at 4ºC.
    a. *Note:* Chip-Arrays can be stored at 4°C for up to 2 days if kept moist.

Day 0: Hepatocytes to Chip-Arrays & Matrigel Overlay

Overview

Goals

- Thaw and seed hepatocytes in chip.

Required Materials

- Complete Hepatocyte Seeding Medium (at 37°C)
- Percoll Solution (at room temperature)
- 10X DPBS (at room temperature)
- 1X DPBS (at room temperature)
- Serological pipettes
- Single channel and multichannel pipettes and filtered tips
- Aspirator and sterile aspirating tips
- 50 mL conical tubes
- 50 mL disposable pipetting reservoirs
- Diluted trypan blue counting solution
- Hemocytometer
- 24-well collagen I-coated plate
- Nunc™ 96-Well Polystyrene Round Bottom Microwell Plates
- 70% ethanol
- Microscope
- Complete Hepatocyte Maintenance Media (at 4°C)
- Complete Hepatocyte Maintenance Media (at 37°C)
- Hepatocyte Overlay Medium (at 4°C)
- Matrigel aliquot (at 4°C in slushy ice)
- Ice bucket and ice

Prepare Hepatocyte Seeding Medium

Base Hepatocyte Seeding Medium (500 mL)

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

- Store the Base Hepatocyte Seeding Medium at 4°C.
- Use the Base Hepatocyte Seeding Medium within 30 days of preparation.

Complete Hepatocyte Seeding Medium (150 mL)

This amount is sufficient for seeding 8 Chip-Arrays

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base Hepatocyte Seeding Medium	140.835 mL	-	-	Recipe Above	-
ITS+ premix	1.5 mL	-	1%	Corning	354352
Ascorbic Acid	150 µL	50 mg/mL	0.05 mg/mL	Sigma	A5960
Dexamethasone	15 µL	10 mM	1 µM	Sigma	D4092
FBS	7.5 mL	-	5%	Sigma	F4135

- Store the Complete Hepatocyte Seeding Medium at 4°C.
- Use the Complete Hepatocyte Seeding Medium within 3 days of preparation.

90% Percoll Solution (20 mL)

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

Trypan Blue Cell Counting Solution

Reagent	Volume	Source	Cat. No.
Complete Hepatocyte Seeding Medium	40 µL	Recipe Above	-
Trypan Blue	5 µL	Sigma	93595

- Maintain the Trypan Blue Cell Counting Solution at room temperature prior to use.
- Prepare the Trypan Blue Cell Counting Solution fresh for each use.

Prepare Chip-Arrays

Steps

1. Transfer all ECM-coated Chip-Arrays from 4ºC to the AVA to incubate at 37ºC for one hour.
2. Prepare and warm Hepatocyte Seeding Media.
3. Prepare 90% Percoll solution and keep at room temperature.
4. Fully aspirate the ECM solution from the top and bottom channel troughs, then from each channel by gently inserting aspirating pipette in top and bottom channel seeding ports. All Emulations should be dry after this step with no remaining ECM in the channels.
5. Wash the bottom channel, then the top channel twice with 50 µL of warm Complete Hepatocyte Seeding Media, slowly pipetting and aspirating the media coming out of the trough for each channel.
    a. It is recommended to wash channels with 6 tips at a time using a multichannel pipette.
    b. After the first wash, make sure to fully aspirate the troughs so that they are dry, while being careful not to aspirate media from the channels.
    c. Hold the aspirator along the far edge of the trough and avoid the port when aspirating the top channel outflow.
    d. After the second wash, leave the outflow volume of media in the top and bottom channel troughs to keep the Emulations hydrated until seeding.
6. Thoroughly dry the area around the top channel seeding port with an aspirating pipette to prepare for hepatocyte seeding, being careful not to aspirate the channel.
7. Cover the Emulations with the pipette guide cover and return Chip-Arrays to the AVA until the cells are ready for seeding.

Thaw Hepatocytes

Before you begin

Before thawing the cryopreserved hepatocytes, make sure all equipment is organized and ready for use. Also, be sure that all required reagents are prepared and have reached the appropriate temperature.

Tips for thawing cells

- Up to three vials of cryopreserved hepatocytes can be thawed at the same time. Once they are thawed, the contents for each should be combined into one 50 mL conical tube and processed as one sample. For seeding 8 Chip-Arrays, it is recommended to thaw two vials of hepatocytes.
- As the cells are thawing, it is critical to work as quickly but as gently as possible. This will help maximize cell recovery and minimize damage to the hepatocytes.
- Do not allow the cells to thaw at room temperature or on ice.
- Once the hepatocytes are thawed, dilute them in the Complete Hepatocyte Seeding Medium as soon as possible to prevent DMSO toxicity within the cryoprotectant.

Steps

1. It is recommended to thaw 2 vials of hepatocytes when seeding 8 Chip-Arrays. This number can be adjusted accordingly based on number of Chip-Arrays to be seeded.
2. Place 3 mL of warm, Complete Hepatocyte Seeding Medium into a sterile 50 mL conical tube.
3. Remove the required number of cryovials.
4. Spray each cryovial with 70% ethanol and wipe it dry. Twist the cap a quarter of a full turn to relieve any internal pressure, then re-tighten it.
5. Immediately place the frozen vial(s) in a 37°C water bath without submerging the cap. Rapidly thaw hepatocytes by gently swirling the vials in the water bath until only a small ice pellet remains. This process should take only 60–90 seconds. Thawing any longer will decrease viability and cell yield.

6. When one small ice pellet remains, immediately remove the vial(s) from the water bath, wipe it dry, spray it with 70% alcohol, and wipe it dry again before placing it into the BSC.
7. Quickly transfer the contents of the vial(s) into the 3 mL of Complete Hepatocyte Seeding Medium in the sterile 50 mL conical tube prepared in Step 2.
8. Rinse the cryovial(s) with 1 mL of Complete Hepatocyte Seeding Medium and transfer it to the 50 mL conical tube.
9. With gentle agitation and swirling, slowly add enough of the Complete Hepatocyte Seeding Medium to bring the total volume to 35 mL.
10. Add 15 mL of 90% Percoll Solution, bringing the total volume to 50 mL.
11. Cap the tube tightly and slowly invert it three times to mix the cell solution.
12. Centrifuge the cells at 96 x g for 6 min at room temperature.
13. Return the tube to the BSC. Carefully aspirate the supernatant, leaving 3–5 mL. Ensure the pellet remains undisturbed.
14. Tilt and rotate the tube to gently re-suspend the cell pellet in the remaining medium.
15. Gently add enough Complete Hepatocyte Seeding Medium to bring the total volume to 50 mL.
16. Centrifuge the cells at 72 x g for 4 min at room temperature.
17. Return the tube to the BSC. Carefully aspirate the supernatant, leaving 1–2 mL. Ensure the pellet remains undisturbed

Adjusting Cell Density

Overview

Human hepatocytes must be seeded in the Liver-Chip at a density of 2.8 x 10^6 cells / mL. It is essential to ensure the seeding density is accurate for viable, functional cells and long-term culture.

Note: This seeding density was optimized with Hepatocyte donor 2214423. The seeding density may vary and must be optimized based on the donor that is part of your BioKit. Reach out to Emulate support (support@emulatebio.com) for any questions related to hepatocyte seeding density optimization.

Steps

1. Tilt and rotate the tube to gently resuspend the cell pellet.
2. Measure the total suspension volume using a 2 mL or 5 mL pipette.
3. Confirm the cell pellet has disappeared, sufficiently rotate to homogenize the cell suspension and transfer 5 µL of the cell suspension to the Trypan Blue Cell Counting Solution, generating a 1:10 dilution.
4. Mix the trypan blue solution thoroughly and count the cells using a hemocytometer.

Cell Counting and Viability Assessment

1. Count both viable and non-viable cells in each quadrant of the hemocytometer.
   Live Cell Count, Dead Cell Count, Total Cell Count.

2. Calculate the percentage viability of the cell solution.
   Live Cells / Total Cells x 100 = % Viability

3. Calculate the viable cell concentration. The dilution factor is 10 when prepared in the trypan blue solution above.
   Live Cell Count x (1 x 10 x 10^4) / 4 = Viable Cell Concentration (cells / mL)

4. Calculate the viable cell yield.
   Viable Cell Concentration x Cell Suspension Volume = Viable Cell Yield (cells)
   Viable Cell Yield / Desired Density = Reconstitution Volume

Diluting Hepatocytes

After calculating the Viable Cell Yield, dilute the hepatocytes with warm Complete Hepatocyte Seeding Medium to the required final cell density: 2.8 x 10^6 cells / mL (if using hepatocyte donor 2214423).

Additional Steps

If the Viable Cell Concentration is less than 2.8 x 10^6 cells / mL (when using hepatocyte donor 2214423):

1. Leave the hepatocyte cell suspension undisturbed at room temperature for at least 5 minutes. This will allow the cells to settle at the bottom of the tube.
2. Gently remove enough from the top of the supernatant to decrease total cell suspension volume.
3. Re-count the cell suspension and recalculate the appropriate seeding density accordingly. This will help to avoid subjecting hepatocytes to mechanical stress with centrifugation.

Seed Hepatocytes to the Top Channel

Overview

Work with one Chip-Array at a time per user. After seeding the first Chip-Array Emulation, use a microscope to assess the cell density within the channel. Adjust the density of the cell suspension as necessary for the rest of the chips.

Steps

1. Transfer the prepared Chip-Arrays from the AVA to the BSC.
    a. When seeding, work with one Chip-Array at a time per user. Leave other Chip-Arrays in a 37°C incubator or the AVA.
2. Carefully aspirate media from the top channel troughs for all Emulations, ensuring not to touch the trough port linked to the top channel.
    a. *NOTE:* Having a completely dry top channel trough before seeding is essential in ensuring uniformity and even distribution of hepatocytes in the top channel. Having any liquid in the top channel trough before seeding may encourage movement of cells due to hydrostatic pressure.
3. Designate the first Emulation on a Chip-Array to test the seeding density.
4. Very gently agitate the cell suspension before seeding to ensure a homogeneous cell suspension.
5. Quickly and steadily pipette 20 µL of hepatocyte cell solution with a single channel P20 pipette in the top channel through the top channel seeding port, aspirating the outflow from the top channel trough immediately without touching the trough port.
    a. When seeding, it is recommended to leave the P20 pipette tip in the seeding port until the outflow volume in the trough is aspirated, then the user may remove the pipette tip.
6. Cover the Chip-Array with the pipette guide cover and visually inspect the seeded Emulation under a lab microscope to check if the density and distribution look appropriate (see Figure 13 below). Hepatocytes should not be overcrowded and should have an even layer with ~one cell radius between individual hepatocytes. Troubleshoot by readjusting density if not.

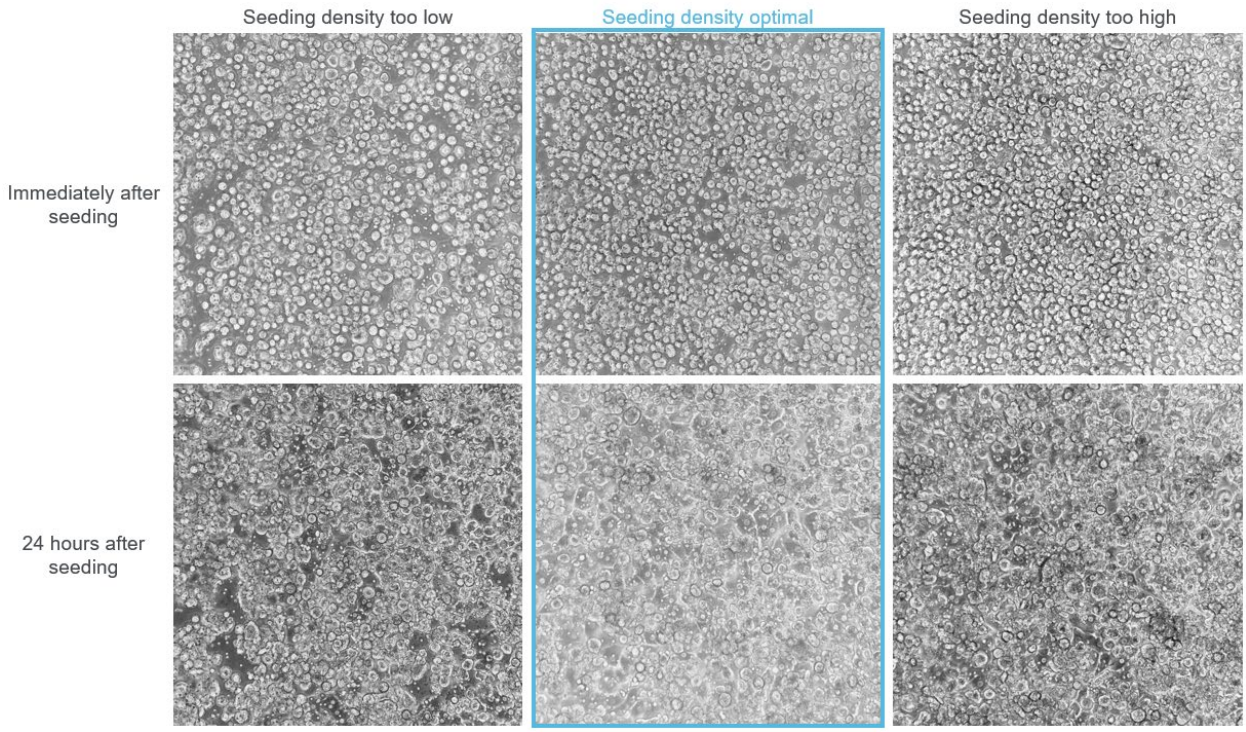

Figure 13. Hepatocyte seeding density reference chart

a. If cell distribution is not uniform due to gaps or gradients present in the top channel, vacuum aspirate the top channel of the specific Emulation, then flush the top channel twice with 50 µL Hepatocyte Seeding Media while aspirating the outflow.
b. Re-seed the Emulation again with 20 µL hepatocyte suspension with a single channel P20 pipette and double check cell distribution.
c. If the seeding density is too dense or not optimal, wash the test Emulation according to Step 6a, adjust the cell density accordingly, and re-seed the test Emulation until the density in the channel is correct.
d. If excessive fluid flow is observed during cell settling (referred to as “cell racing”), an extra step is required to ensure that the hepatocytes settle on the top channel membrane evenly. Add 500 µL of Hepatocyte Seeding Media to the top outlet reservoir of the Emulation experiencing cell racing. Then proceed with the washing and re-seeding steps as described in Steps 6a and 6b, respectively.

7. After confirming the correct cell density, agitate the cell suspension to ensure a homogenous cell suspension, then transfer 400 µL of cell solution to 6 wells of a 96 well Ultra-Low Attachment (ULA) plate.
8. Resuspend the hepatocyte suspension in the wells using a multichannel P200 pipette.
    a. Due to the weight and size of hepatocytes, these cells will readily settle to the bottom of the wells. It is recommended to resuspend the hepatocyte suspension in these wells before seeding each Chip-Array.
9. On the same Chip-Array, seed Emulations 2-6, then Emulations 7-12 with a multichannel P20 pipette, pipetting quickly and steadily 20 µL of hepatocyte cell solution through the top channel seeding port. Allow cells to settle for a minute before checking for appropriate distribution using a lab microscope.
    a. *NOTE:* When seeding several Emulations at a time, users must be careful with the amount of force applied to the multichannel P20 tips. Inserting the tips is enough to create a seal with the chip gasket to ensure proper flow. Additional excessive force when pipetting multiple Emulations at a time (i.e. using your offhand to apply force to the multichannel pipette) can cause tips to become stuck in the pipette guide.
    b. Always make sure tips are perpendicular when inserted into the pipette guide; failure to do so can cause tips to become stuck.
    c. It is recommended to seed no more than 6 Chip-Array Emulations at a time. Seeding more than 6 Emulations simultaneously can potentially lead to variability and pipetting issues.
10. After confirming the correct cell density and seeding the first Chip-Array, seed all remaining Chip-Arrays using a multichannel P20 pipette, working in groups of 6 Emulations at a time.
    a. *Note*: Minimize the amount of time the cells are outside the incubator by seeding one Chip-Array at a time per user and then immediately placing each seeded Array into the AVA at 37°C or a 37°C incubator.
11. Capture representative images on a lab microscope (3-4 Emulations per Chip-Array).
    a. If any Emulations need to be reseeded, follow the protocol outlined in Step 6
12. If needed, top off the bottom channel trough with warm Hepatocyte Seeding Media to ensure the bottom channel does not dry out during incubation.

13. Add any additional PBS to the PBS troughs if needed.
14. Incubate Chip-Arrays in the AVA at 37ºC for 4 hours. See Figure 14. for examples of attachment.

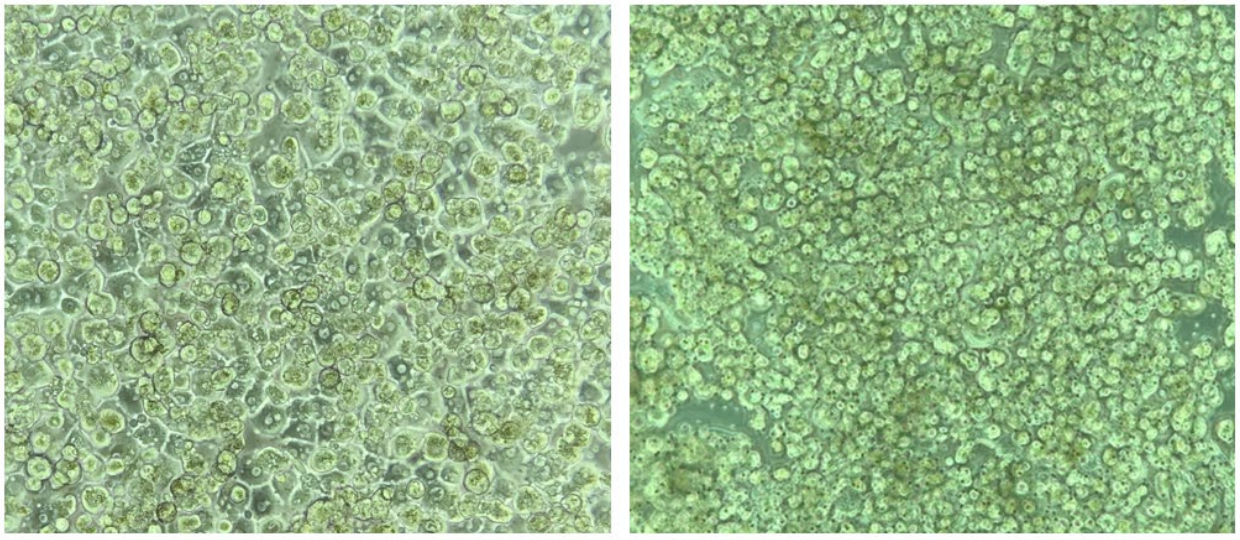

Figure 14. Left: example of appropriate attachment. Right: example of poor attachment.

Seed Well Plate

Overview

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

Steps

1. Dilute the hepatocyte suspension with warm Complete Hepatocyte Seeding Medium to a final density of 0.8 x 10^6 cells/mL.
2. Seed 400, 500, and 600 µL suspension to three separate wells of the 24-well plate.

3. Mix each well to ensure an even suspension and allow the cells to settle for 5 minutes on the microscope stage. After this, inspect the densities under a microscope.
4. Determine which of the three wells depicts the optimal seeding density. Then, using that well's volume, plate the remaining cells into individual wells until no cells remain.
5. Place the well plate onto an incubator shelf. To ensure even cell distribution across the bottoms of the culture wells, first move the plate in a figure-eight motion across the shelf at least three times. Then, move it in a crisscross pattern at least three times. Afterward, leave the plate undisturbed for 4 hours to allow the cells to fully attach.

Prepare Hepatocyte Maintenance Medium

Base Hepatocyte Maintenance Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No
William's Medium E - (without phenol red)	490 mL	-	-	Sigma	W1848
Pen/Strep	5 mL	100X	1%	Sigma	P4433
L-GlutaMAX	5 mL	100X	1%	Gibco	35050-061

- Store the Base Hepatocyte Maintenance Medium at 4°C.
- Use the Base Hepatocyte Maintenance Medium within 30 days of preparation.

Complete Hepatocyte Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No
Base Hepatocyte Maintenance Medium	49.445 mL	-	-	Recipe Above	-
ITS+ Premix	500 µL	-	1%	Sigma	354352
Ascorbic Acid	50 µL	50 mg/mL	0.05 mg/mL	Sigma	A5960
Dexamethasone	5 µL	1 mM	100 nM	Sigma	D4902

- Store the Complete Hepatocyte Maintenance Medium at 4°C.
- Use the Complete Hepatocyte Maintenance Medium within 3 days of preparation.

Prepare Overlay Medium

Before You Begin

Slowly thaw the Matrigel aliquot on ice (which should be made slushy with water) for 30 minutes or until thawed. Keep the Matrigel aliquot in slushy ice at all times, as this solution gels rapidly at temperatures above 10°C. To maintain an even coating, use pre-chilled pipettes, tips, and tubes stored at -20°C prior to use as well as cold medium during preparation and overlay.

Prepare Hepatocyte Overlay Medium by adding Matrigel to the Complete Hepatocyte Maintenance Medium to achieve a final Matrigel concentration of 0.025 mg/mL.

Example calculation is below:

Hepatocyte Overlay Medium (20 mL)

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

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

Steps

1. Prepare Hepatocyte Maintenance Media.
    a. Aliquot Hepatocyte Maintenance Media and keep at 4°C. This media will be used for the Matrigel Overlay solution and must remain cool.
    b. Warm rest of Hepatocyte Maintenance Media to 37°C on bead bath.
2. When the 4 hour hepatocyte incubation period is complete, slowly thaw a Matrigel aliquot on ice (which should be made slushy with water) for 30 minutes or until thawed.

3. Prepare fresh Hepatocyte Overlay Medium by diluting ice-cold, thawed Matrigel into ice-cold Complete Hepatocyte Maintenance Medium using pre-chilled tips to achieve a final Matrigel concentration of 0.25 mg/mL, as directed above.
4. Gently mix the overlay medium well and keep the Hepatocyte Overlay Medium on ice at all times

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

Wash and Overlay Hepatocytes

Steps

1. When the 4 hour hepatocyte incubation period is complete, image several representative Emulations to ensure proper attachment is observed.
2. In the BSC, wash the top channel **very gently** twice with 50 µL of warm Complete Hepatocyte Maintenance Media, pipetting through the top channel seeding port and aspirating the media coming out of the top channel trough. This wash is done to remove cell debris from the hepatocyte monolayer.
    a. Make sure to fully aspirate the troughs so that they are dry, while being careful not to aspirate media from the channels.
    b. Hold the aspirator along the far edge of the trough and avoid the port when aspirating the top channel outflow.
    c. Wash should be gentle to minimize detaching cells (~several seconds for washing).
3. Using cold tips, very **gently and slowly** pipette 75 µL of ice cold Matrigel Overlay solution through the top channel seeding port. Do not aspirate the outflow in the top channel troughs, as this will ensure the channel remains hydrated overnight.
4. If needed, add additional Hepatocyte Maintenance Media to the bottom channel trough to ensure the bottom channel does not dry overnight.
5. Add any additional PBS to the PBS troughs if needed.
6. Incubate Chip-Arrays in the AVA at 37ºC overnight.

Overlay Hepatocytes in Well Plate

Steps

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

Day 1: NPC Seeding and Connection

Overview

Goals

- Seed mixture of non-parenchymal cells (LSECs, Kupffer cells, and Stellate cells) in the Chip-Array
- De-gas and equilibrate media
- Prime Chip-Arrays
- Regulate Chip-Arrays and begin overnight flow

Materials

- NPC Seeding Medium (at 37°C)
- NPC Seeding Medium (at 4°C)
- Complete Hepatocyte Maintenance Medium (at 37°C)
- Trypan blue counting solution
- Hemocytometer
- 50 mL conical tube
- Serological pipettes
- Single channel and multichannel pipettes and filtered tips
- Aspirator and sterile tips
- 50 mL disposable pipetting reservoir
- Nunc™ 96-Well Polystyrene Round Bottom Microwell Plates
- Ice bucket and ice
- 70% ethanol
- Microscope
- Installed and qualified AVA instrument
- Steriflip® filtration unit: PVDF filter 0.45 µm (sterile)

Prepare NPC Seeding Medium

Before You Begin

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

NPC Seeding Medium (150 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No
Complete Hepatocyte Maintenance Medium (omitting dexamethasone)	67.5 mL	-	-	Prepared above, but omit dexamethasone	-
Base LSEC Culture Medium	67.5 mL	-	-	Prepared above	-
FBS	15 mL	-	10%	Sigma	F4135

- Maintain 50 mL at 4°C and 100 mL at 37°C.
- Store any remaining NPC Seeding medium at 4°C.
- Use the NPC Seeding Medium within 3 days of preparation.

Trypan Blue Cell Counting Solution (45 µL)

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

- Maintain the Trypan Blue Cell Counting Solution at room temperature prior to use.
- Prepare three tubes of the Trypan Blue Cell Counting Solution.
- Always prepare the solution fresh before each use.

Wash Chips

Steps

1. Prepare Hepatocyte Maintenance Media and NPC Seeding Medium and warm to 37ºC in bead bath. Aliquot NPC Seeding Medium to keep cool at 4ºC.
2. Image representative Emulations to capture hepatocyte morphology 24h after seeding.
3. **Gently** wash bottom channel by pipetting twice with 50 µL of warm Complete NPC Seeding Medium through the bottom channel seeding port, aspirating the media coming out of the trough and being careful not to aspirate media from the channel.
4. **Gently** wash top channel by pipetting twice with 50 µL of warm Complete Hepatocyte Maintenance Media through the top channel seeding port, aspirating the media coming out of the trough and being careful not to aspirate media from the channel.
5. Aspirate PBS from PBS troughs and refill each PBS trough to exactly 100 µL. This is enough volume to partially fill the troughs while also not spilling when inverting the Chip-Array.
6. Use previous techniques for multichannel P200 pipette and ensure port area and bottom channel trough is dry for seeding. Return the Chip-Arrays to the incubator until the NPCs are ready for seeding.

Harvest LSECs

Before You Begin

To seed the bottom channel, LSECs in culture must be harvested and counted. LSECs are adjusted to a density of 13.5 x 10^6 cells/mL (3 times the final seeding concentration) before being combined with Stellate and Kupffer cells.

Steps

1. Bring all culture flasks containing LSECs from the incubator to the BSC.
2. Aspirate culture media and add 15 mL of 1X DPBS to wash the culture surface. Aspirate the DPBS wash.
3. Add 3 mL of trypsin-EDTA to each flask. Incubate for 2–3 min at 37°C.
4. Tap the side of the flask gently. Inspect the culture under the microscope to verify complete cell detachment from the culture surface.
5. Add 5 mL of warm NPC Seeding Medium to each flask, and pipette gently to mix while collecting all cells from the culture surface.
6. Transfer the contents of each flask (8 mL) into a single, sterile 50 mL conical tube.
7. Add 2 mL of NPC Seeding Medium the tube, bringing the total volume to 50 mL.
8. Centrifuge LSECs at 200 x g for 5 min at room temperature.
9. Carefully aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet.
    a. *Note:* The cell pellet will be very small, so be sure to aspirate gently
10. Loosen the cell pellet by flicking the tube gently.
11. Using a P1000 pipette, gently resuspend the cells by adding 100 µL of cold NPC Seeding Medium.
12. Pipette gently to create a homogeneous mixture and transfer 5 µL of the cell suspension to the Trypan Blue Counting Solution. This will create a 1:10 dilution.
13. Mix the counting solution thoroughly. Count the cells using a hemocytometer.
14. Dilute the LSECs to 13.5 x 10^6 cells / mL (3 times the final seeding concentration) in cold NPC Seeding Medium.
15. Keep the LSEC cell suspension on ice until the Stellate and Kupffer cells are ready.

Thaw Stellate Cells

Before You Begin

Stellate cells must be thawed and counted before bottom channel cell seeding. Stellate cells are adjusted to a density of 0.45 x 10^6 cells / mL (3 times the final seeding concentration) prior to combining with LSECs and Kupffer cells.

Steps

1. Place 3 mL of warm NPC Seeding Medium into a sterile 15 mL conical tube.
2. Remove the required number of cryopreserved stellate cell vials from the liquid nitrogen.
    a. *NOTE:* It is recommended to thaw 1 vial of stellate cells when seeding up to 96 Chip-Array Emulations.
3. Spray the cryovial(s) with 70% ethanol and wipe it dry. Untwist the cap a quarter of a full turn to relieve any internal pressure, then retighten it.
    a. *NOTE*: Adjusting the cap this way will prevent the cryovial from popping due to rapid expansion of any liquid nitrogen that may have been trapped inside the vial.
4. Immediately place the frozen vial in a 37ºC water bath without submerging the cap. Rapidly thaw the stellate cells by gently swirling the vial in the water bath until only one small ice pellet remains.
    a. *NOTE*: This should take 60–90 seconds. Thawing for longer will result in decreased cell viability and yields.
5. Immediately remove the vial from the water bath, wipe it dry, spray it with 70% alcohol, and dry it once more before placing it into the BSC.
6. Quickly transfer the vial's contents into the 15 mL conical tube prepared in Step 1.
7. Rinse the cryovial with 1 mL of warm NPC Seeding Medium and transfer it to the 15 mL conical tube.
8. Bring the volume within the conical tube to 15 mL using cold NPC Seeding Medium.
9. Centrifuge stellate cells at 250 x g for 5 min at room temperature. Once done, cool centrifuge to 4°C to prepare for Kupffer cell seeding.

10. Carefully aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet.
    a. *NOTE*: The cell pellet will be very small. Aspirate carefully.
11. Gently flick the tube to loosen the cell pellet.
12. Using a P1000 pipette, carefully resuspend the cells by adding 400 µL of cold NPC Seeding Medium.
13. Pipette gently to recreate a homogenous mixture, and transfer 5 µL of the cell suspension to the trypan blue cell counting solution (1:10 dilution).
14. Mix the counting solution thoroughly. Count the cells using a manual hemocytometer.
15. Dilute the stellate cells to 0.45 x 10^6 cells / mL (3 times the final seeding concentration) in cold NPC Seeding Medium and keep them on ice until the rest of the cells (Kupffer) are ready.

Thaw Kupffer Cells

Before You Begin

Kupffer cells must be thawed and counted for bottom channel seeding. Kupffer cells are adjusted to a density of 9 x 10^6 cells / mL (3 times the final seeding concentration) prior to combining with LSECs and stellate cells.

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

Steps

1. Place 3 mL of cold NPC Seeding Medium into a sterile 15 mL conical tube.
2. Remove the required number of cryopreserved Kupffer cell vials from the liquid nitrogen.
    a. *NOTE:* It is recommended to thaw 5 vials of Kupffer cells when seeding up to 96 Chip-Array Emulations.
3. Spray the cryovial(s) with 70% ethanol and wipe it dry. Untwist the cap a quarter of a full turn to relieve any internal pressure, then retighten it.
    a. *NOTE*: Adjusting the cap this way will prevent the cryovial from popping due to rapid expansion of any liquid nitrogen that may have been trapped inside the vial.
4. Immediately place the frozen vial in a 37ºC water bath without submerging the cap. Rapidly thaw the stellate cells by gently swirling the vial in the water bath until only one small ice pellet remains.
    a. *NOTE*: This should take 60–90 seconds. Thawing for longer will result in decreased cell viability and yields.
5. Immediately remove the vial from the water bath, wipe it dry, spray it with 70% alcohol, and dry it once more before placing it into the BSC.
6. Quickly transfer the vial's contents into the 15 mL conical tube prepared in Step 1.
7. Rinse the cryovial with 1 mL of warm NPC Seeding Medium and transfer it to the 15 mL conical tube.

8. Bring the volume within the conical tube to 15 mL using cold NPC Seeding Medium.
9. Centrifuge the Kupffer cells at 500 x g for 5 min at 4°C.
10. Carefully aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet.
    a. *NOTE*: The cell pellet will be very small. Aspirate carefully.
11. Gently flick the tube to loosen the cell pellet.
12. Using a P1000 pipette, carefully resuspend the cells by adding 100 µL of cold NPC Seeding Medium.
13. Pipette gently to recreate a homogenous mixture, and transfer 5 µL of the cell suspension to the trypan blue cell counting solution (1:10 dilution).
14. Mix the counting solution thoroughly. Count the cells using a hemocytometer.
15. Dilute the Kupffer cells to 9.0 x 10^6 cells / mL (3 times the final seeding concentration) in cold NPC Seeding Medium and keep them on ice until use.

Combine NPC Mixture

Before You Begin

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

Combining to Final Densities

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

Cell Type	Densities
LSECs	4.5 x 10^6 cells / mL
Stellate cells	0.15 x 10^6 cells / mL
Kupffer cells	3 x 10^6 cells / mL

- Mix all 3 cell suspensions on ice in a 1:1:1 ratio (v/v/v) inside a sterile, 15 mL conical tube on ice to achieve final seeding densities of each cell type in the bottom channel. Ensure there is enough seeding solution for all chips – a minimum of 1200 µL NPC suspension (400 µL from each cell suspension) is recommended to seed 96 Chip-Array Emulations.

Seed NPC Mixture to Bottom Channel

Before You Begin

Work with one Chip-Array at a time. Leave other Chip-Arrays in a 37°C incubator or in the AVA. After seeding the first Chip-Array Emulation, assess the cell density within the channel using a microscope. Adjust the density of the cell suspension for the next Chip-Arrays if necessary. After all Emulations in a Chip-Array have been seeded, immediately invert the Chip-Array.

Steps

1. Transfer the prepared Chip-Arrays from the AVA to the BSC.
2. Carefully aspirate media from the bottom channel troughs for all Emulations, ensuring not to touch the trough port linked to the bottom channel.
    a. *NOTE:* Having a completely dry bottom channel trough before seeding is essential in ensuring uniformity and even distribution of NPCs in the bottom channel. Having any liquid before seeding may encourage movement of cells due to hydrostatic pressure.
3. Designate the first Emulation on a Chip-Array to test the seeding density.
4. Very gently agitate the cell suspension before seeding to ensure a homogeneous cell suspension.
5. Quickly and steadily pipette 10 µL of NPC solution with a single channel P20 pipette in the bottom channel through the bottom channel seeding port, aspirating the outflow from the bottom channel trough immediately without touching the trough port.
    a. When seeding, it is recommended to leave the P20 pipette tip in the seeding port until the outflow volume in the trough is aspirated, then the user may remove the pipette tip.
6. Cover the Chip-Array and quickly visually inspect under a lab microscope if the density and distribution look appropriate (see Figure 15). Troubleshoot by readjusting density if not.
    a. If distribution isn't uniform due to gaps or gradients present in the bottom channel, flush the bottom channel with 50 µL NPC Seeding Media twice,

while aspirating the outflow. Do not vacuum aspirate the bottom channel, as this may remove media from the top channel.

b. Re-seed the Emulation again with 10 µL NPC suspension with a single channel pipette.
c. If the seeding density is not optimal, wash the test Emulation according to Step 6a. above, adjust the cell density accordingly, and re-seed the test Emulation until the density in the channel is correct.

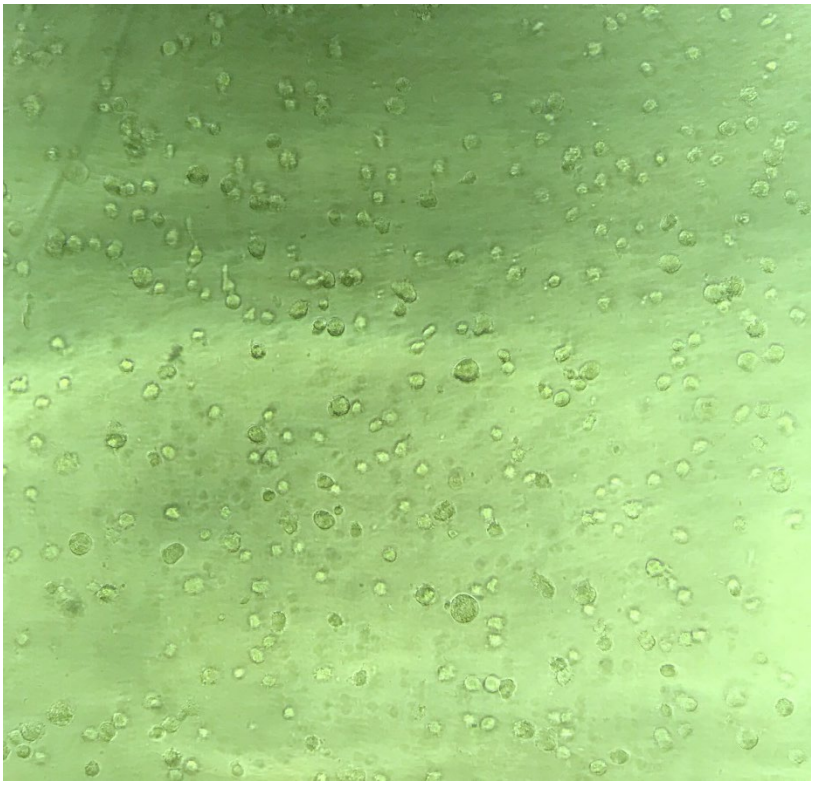

Figure 15. Optimal NPC seeding density in bottom channel.

7. After confirming the correct cell density, agitate the cell suspension to ensure a homogenous cell suspension, then transfer 150 µL to 6 wells in ULA plates.
    a. Place ULA plate on ice to keep the NPCs cool
8. Resuspend the NPC suspension in the wells using a multichannel P200 pipette.
    a. Take care while resuspending as such a small volume is prone to bubbles that reduce the total volume available.

   b. If static for long enough, the NPCs will settle to the bottom of the wells. It is recommended to resuspend the NPC suspension in these wells with a multi-channel pipette before seeding each Chip-Array.
9. On the same Chip-Array, seed Emulations 2-6, then Emulations 7-12 with a multichannel P20 pipette, pipetting quickly and steadily 10 µL of NPC cell solution through the bottom channel seeding port. Invert Array as swiftly as possible.
   a. *NOTE:* When seeding several Emulations at a time, users must be careful with the amount of force applied to the multichannel P20 tips. Inserting the tips is enough to create a seal with the chip gasket to ensure proper flow. Additional excessive force when pipetting 6 Emulations at a time (i.e. using your offhand to apply force to the multichannel pipette) can cause tips to become stuck in the pipette guide.
   b. Always make sure tips are perpendicular when inserted into the pipette guide; failure to do so can cause tips to become stuck.
   c. Be very careful when inverting Chip-Arrays, making sure that hands are gripping both the pipette guide cover and the reservoir lid. Failure to do so can cause either of the lids to fall off, potentially compromising sterility.
10. Seed all remaining Chip-Arrays using a multichannel P20 pipette working in groups of 6 Emulations at a time, imaging representatives immediately and inverting the Chip-Array as swiftly as possible.
   a. If any Emulations need to be reseeded, flush the bottom channel with 50 µL NPC Seeding Media twice, while aspirating the outflow. Re-seed the Emulation again with 10 µL NPC suspension with a single channel pipette.
   b. *Note*: Minimize the amount of time the cells are outside the incubator by seeding one Chip-Array at a time per user and then immediately placing each seeded, inverted Array into an incubator at 37°C.
11. Incubate inverted Chip-Arrays in an incubator at 37ºC for 4 hours.

Prepare NPC Maintenance Medium

NPC Maintenance Medium (100 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium omitting Dexamethasone	49 mL	-	-	-	-
Base LSEC Culture Medium	49 mL	-	-	-	-
FBS	2 mL	-	2%	Sigma	F4135

- Store at 4°C.
- Use the NPC Maintenance Medium within three days of preparation.

Note: If the seeding density is too low, you can use 10% FBS for 24-48h post-seeding then switch to 2% FBS.

Gas Equilibration of Media

CAUTION: The media equilibration step is imperative to successfully culture Organ-Chips. Omitting this step will cause bubbles to form in the Chip-Array, which will in turn negatively impact cell viability and can result in irregular flow.

Before You Begin

- Once the culture media for both chip channels have been warmed for at least 1h, the gas equilibration step can be performed.
- 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 medium is allowed to cool.
- If the vacuum pump is not located close to the water bath (e.g., inside the BSC), it is recommended to place some clean water warmed at 37°C inside the BSC to minimize cooling during the media equilibration step.

Steps

1. Prepare and warm Complete Hepatocyte Maintenance Media and Complete NPC Maintenance Media. Once the culture media for both chip channels have been warmed for at least 1 hour, the gas equilibration step can be performed.
2. Place at least 2 mL of Complete Hepatocyte Maintenance Medium for each Emulation in a 50 mL conical tube.
3. Place at least 2 mL of Complete NPC Maintenance Medium for each Emulation in a 50 mL conical tube.
4. Warm all conical tubes of media at 37ºC in water bath or bead bath for at least 1 hour.
5. Immediately connect the 50 mL tubes containing each warmed medium to a Steriflip unit using the following steps:
    a. Attach each conical tube containing warmed media to a Steriflip unit.
    b. With the unit “right-side up” (medium in the bottom conical tube), apply vacuum and wait for 10 seconds.
    c. Invert the Steriflip-connected tubes, and check that the medium begins to pass from the top conical tube to the bottom tube.

i. *Note*: The vacuum must operate at a minimum of -70 kPa. At this pressure, it should take about 2 seconds for 10 mL of media to flow through the filter. If it takes longer, stop and refer to “Media taking too long to pass through Steriflip” in the troubleshooting section.

d. Leave the filtered medium under vacuum for at least five minutes.

6. Remove the vacuum tubing from the Steriflip unit.
7. Separate the conical tubes containing media from the Steriflip unit and immediately place them into the incubator with the caps loose to maintain the dissolved gas equilibrium state and allow bubbles to escape.
    a. *Note*: Minimize the time media is outside of the incubator when the Chip-Arrays are being prepared to maintain the correct temperature. This is critical to ensure Chip-Array success.

Prime Chip-Arrays

CAUTION: Ensuring successful Chip-Array priming is best practice for successful liquid-liquid connection between the Emulation and the Chip-Array. Failure to do so can cause bubbles to form in the Chip-Array, which will in turn negatively impact cell viability and result in irregular flow.

Steps

For the following steps, handle Chip-Arrays one at a time, setting each Chip-Array aside in the incubator after media is added to reservoirs before Prime:

1. After incubating for 4 hours, return Chip-Arrays to the “right side up” by flipping consumable over.
    a. Be very careful when flipping Chip-Arrays, making sure that hands are gripping both the pipette guide cover and the reservoir lid. Failure to do so will cause either of the lids to fall off, potentially compromising sterility.
2. Image several representative Emulations to ensure proper NPC attachment is observed. See Figure 16. for examples of NPC attachment in the bottom channel.

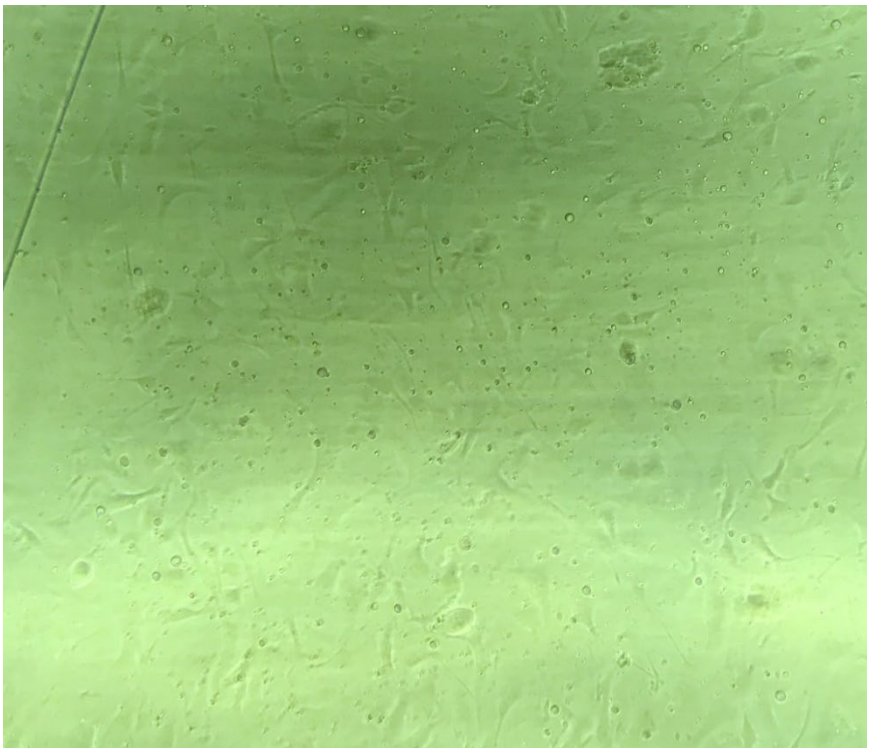

Figure 16. Optimal NPC attachment in the bottom channel.

3. Gently wash both top channel and bottom channel with 50 µL of their respective warmed and equilibrated media, leaving a small amount of media in the pipette tips after dispensing. Allow media to fill the pipette guide troughs – do not aspirate while dispensing media.
    a. Do not fully dispense media from the pipette tips. Leaving a small amount of media in the pipette tip rather than dispensing the full volume helps ensure large bubbles are not trapped in the seeding ports.
4. Aspirate media from the pipette guide troughs and humidity troughs (PBS filled).
    a. Place aspirating pipette tip in the corner of the troughs and avoid contact or proximity to the port leading to the emulations.
    b. Allow media to be fully aspirated from the troughs before moving to the next trough for aspiration. This may take over one second to achieve.
5. Place the pipette guide cover back over emulations.
6. Remove the tape tabs from the sides of the consumable and peel the plastic protector from the reservoir lid.

7. Fill the top channel inlet reservoirs by adding 1000 µL of warmed and equilibrated Hepatocyte Maintenance Media directly over the top channel inlet vias using a multichannel P1000 pipette.
    a. Reservoir vias are located in the bottom left corner of each reservoir compartment. The recommended technique is to hug the bottom left corner of the inlet reservoirs with the pipette tips, then allow liquid to dispense down the wall onto the via – this minimizes the risk of trapping air pockets in the vias.
    b. See Figure 17 for locations of reservoir vias.
8. Fill the top channel outlet reservoirs by adding 500 µL of warm and equilibrated Hepatocyte Maintenance Media directly over the top channel outlet vias using a multichannel P1000 pipette.
9. Fill the bottom channel inlet reservoirs by adding 1000 µL of warmed and equilibrated NPC Maintenance Media directly over the bottom channel inlet vias using a multichannel P1000 pipette.
    a. Reservoir vias are located in the bottom left corner of each reservoir compartment. The recommended technique is to hug the bottom left corner of the inlet reservoirs with the pipette tips, then allow liquid to dispense down wall onto the via – this minimizes the risk of trapping air pockets in the vias.
10. Fill the bottom channel outlet reservoirs by adding 500 µL of warmed and equilibrated NPC Maintenance Media directly over the bottom channel outlet vias using a multichannel P1000 pipette.
11. The final volume of media in the reservoirs at this step:
    a. All inlet reservoirs should be filled with 1000 µL of media.
    b. All outlet reservoirs should be filled with 500 µL of media.

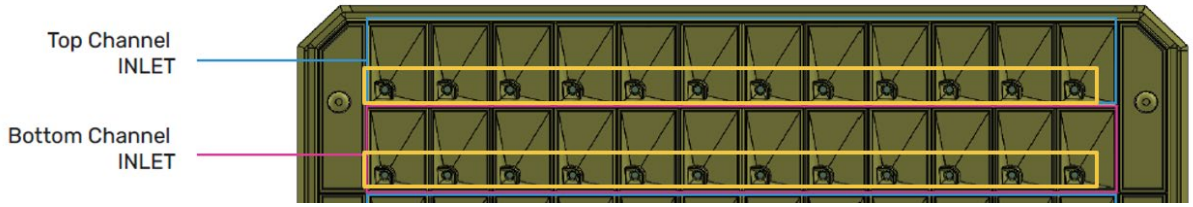

Figure 17. Location of reservoir vias.

12. Place the reservoir lid on top of the reservoirs. Minimize any motion that may splash media onto the reservoir lid.
13. Remove pipette guides by pushing on the clip then lifting upwards. Once the clip is at a 45-degree angle, lightly tug on the clip to remove it. Be careful not to drag your hand over media in the gasket ports.
    a. Refer to “Attachment and Detachment of Pipette Guides and Flow Guides onto Chip-Array” section for more guidance on pipette guide removal.
14. Aspirate any excess media from the viewing windows and on the gasket. Do not aspirate within the gasket ports. See Figure 18 for proper aspiration of excess media around the gasket ports.

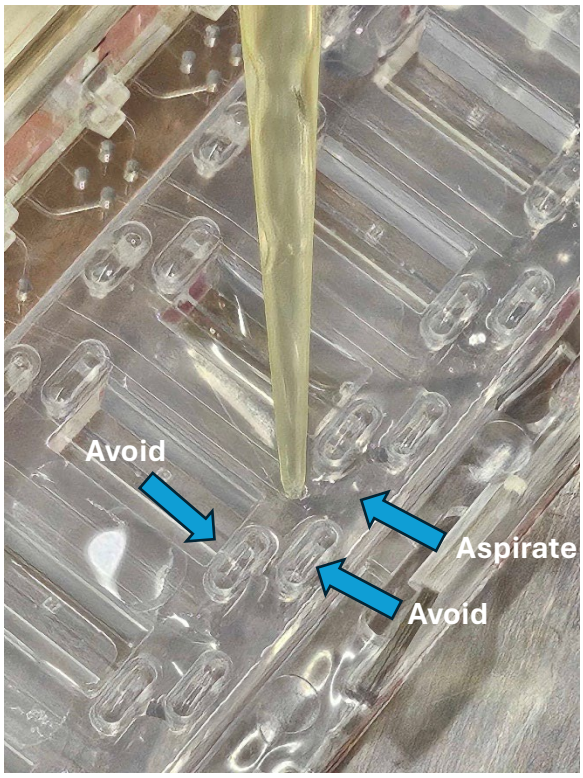

Figure 18. Proper aspiration of excess media around Emulation gasket ports.

15. Place the pipette guide cover back onto the Chip-Array.
    a. Do this one by one for all Chip-Arrays, setting each Chip-Array aside in the incubator when media has been added to the reservoirs.

b. Once all Chip-Arrays are ready for prime, bring them out of the incubator and to the AVA. Be careful not to tip or tilt the Chip-Arrays as this will cause media to splash onto the reservoir lids.

16. Eject the trays using the AVA tray button or using the AVA software.
17. Place the Chip-Arrays on the trays, making sure that the Emulations are facing inwards towards the AVA instrument. Double check that each Chip-Array is slotted flush against the pins on each tray.
    a. **Additionally, ensure that all reservoir lids are flat and secure on the Chip-Arrays before inserting the trays**. This can be checked by pushing forward on the corners of the lid facing the user as the Chip-Arrays sit on the AVA trays. Afterwards, press the lid flat. These steps will ensure that the lids are flush with the Chip-Arrays. Failure to perform this check can lead to flow issues. **Perform this check every time the Chip-Arrays are inserted into the AVA.**
18. Insert the trays using the AVA tray button or using the AVA software.
19. Click the “Flow Operations” dropdown menu and select Prime. In the box below the Prime button, the percent progress will appear.
    a. Prime Cycle parameters (Inlets 30 kPa, Outlets 0 kPa for 45 seconds).
    b. The Prime Cycle is performed to fill the microfluidic channels on the inlet and outlet sides of the Chip-Array Emulations with media from the Chip-Array inlet reservoirs.
20. After Prime Cycle has completed, eject the trays using the AVA tray button or using the AVA software.
21. Return to BSC and double check that priming was successful by observing media filling the gasket port cavities. See Figure 19 for an example of successful priming.

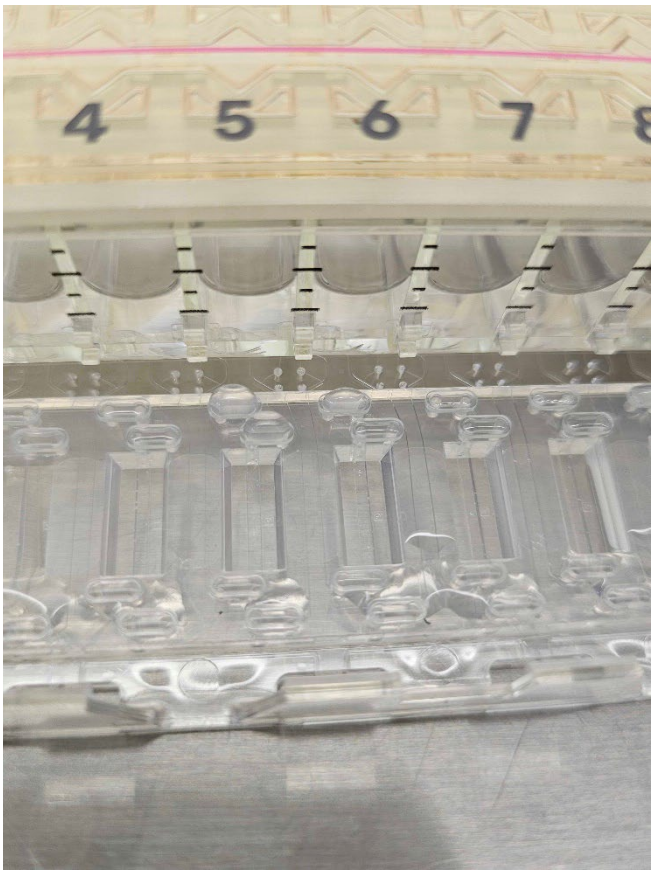

Figure 19. Successful priming indicated by media visible in the gasket port cavities.

22. “Flood” the inlet gasket ports by adding large droplets of media over the inlet gasket ports. This should create large pools of media over the inlet gasket ports. Any bubbles in the gasket ports post-Prime should now rise to the surface of these large pools of media.
23. Aspirate any excess bubbles on top of the large droplets of media around the gasket port cavities, ensuring that domed droplets still remain above the inlet gasket ports. Aspirate any excess media in the viewing windows of the Chip-Array. Do not aspirate within the gasket ports.
    a. The outlet port cavities may have a large droplet escaping the cavity. Typically, the media will be contained within the cavity and will either be level with the top of the gasket or protrude slightly above the top of the gasket.

b. Be careful to avoid aspirating media out of the top and bottom channels. Do not aspirate media from within the gasket ports.

24. Attach the flow guides to the Chip-Arrays, **but do not snap them closed. Leave the flow guides open at ~45° angle.**
    a. When attaching flow guides, a light “click” sound should be heard by the user when the guide is connected to the reservoir hinge.
    b. Once flow guides are attached, leave them open at an ~45° angle and do not push them closed downwards.
    c. Refer to “Attachment and Detachment of Pipette Guides and Flow Guides onto Chip-Array” section for more guidance on flow guide attachment and snapping into place.
25. Tilt Chip-Arrays forward at a 45° angle for 10 seconds, or until multiple large droplets form at the outlet gasket ports. Place the Chip-Array flat on the bench for 2 seconds, and then gently snap the flow guides in place one at a time, using two fingers to hold onto the flow guide tab while pressing downward. This process ensures consistent priming of the emulations.
    a. A loud click should be heard as the flow guides are snapped closed.
    b. Once the flow guides are connected to the Emulations, this creates a seal that prevents exposure of the Emulations to the outside environment, helping to ensure sterility. After flow guides are attached, there is no need to use the pipette guide cover anymore. These can be set aside.
    c. Double check that all flow guides are correctly snapped into place by pressing onto the flow guides.
    d. Double check the reservoir lid. If excess media is observed on the lid gasket, vacuum aspirate this excess media.
26. Move all Chip-Arrays to the AVA.

Regulate Chip-Arrays

Steps

1. Eject the trays using the AVA tray button or using the AVA software.
2. Place the Chip-Arrays on the trays, making sure that the Emulations are facing inwards towards the AVA instrument. Double check that each Chip-Array is slotted flush against the pins on each tray.
3. Insert the trays using the AVA tray button or using the AVA software.
4. Select the “Flow Operations” dropdown menu, select Regulate, select which manifolds should be lowered based on Chip-Array position in the AVA, and set flow rate parameters that the system will default to after Regulate is complete.
    a. Flow rates should be set to 15 µL/h in Top and Bottom Channels. These are the recommended flow rates for the Liver Chip-Array Quad-Culture model.
5. Manifolds will lower and Regulate will begin. In the box below the Regulate button, the percent progress will appear.
    a. Regulate Cycle parameters:
        i. Total Duration: ~4 hours

Day 2+: Chip-Array Maintenance and Sampling

Overview

Goals

- Perform 2nd Regulate on Chip-Arrays
- Maintain Chip-Arrays in AVA

Required Materials

- Chip-Arrays
- Cell culture media

Regulate Chip-Arrays

Steps

1. Prepare and warm Complete Hepatocyte Maintenance Media and Complete NPC Maintenance Media. Once the culture media for both chip channels have been warmed for at least 1 hour, the gas equilibration step can be performed according to the “Gas Equilibration of Media” section above.
2. Pause flow on the AVA and transfer two Chip-Arrays at a time from the AVA to the BSC.
3. Aspirate all inlet and outlet reservoirs.
4. Replenish inlet reservoirs with 1250 µL of respective, equilibrated medias and outlet reservoirs with 500 µL of respective, equilibrated medias.
5. Eject the trays using the AVA tray button or using the AVA software.
6. Place the Chip-Arrays on the trays, making sure that the Emulations are facing inwards towards the AVA instrument. Double check that each Chip-Array is slotted flush against the pins on each tray.
7. Insert the trays using the AVA tray button or using the AVA software.
8. Select the “Flow Operations” dropdown menu, select “Regulate”, select which manifolds should be lowered based on Chip-Array position in the AVA, and set flow rate parameters for the system to default to after Regulate is complete.

   a. Flow rates should be set to 15 µL/h in Top and Bottom Channels. These are the recommended flow rates for the Liver Chip-Array Quad-Culture model.
9. Manifolds will lower and Regulate will begin. In the box below the Regulate button, the percent progress will appear.
   a. Regulate Cycle parameters:
      i. Total duration: 4 hours
10. It is recommended after the 2nd Regulate is performed to remove all Chip-Arrays, aspirate the outlets, and top off the media in the inlets up to 1250 µL to facilitate flow over the next three days.

Day 5+: Chip-Array Maintenance and Sampling

Overview

Goals

- Maintain Chip-Arrays in AVA
- Cell culture inspection
- Collect samples for analysis

Required Materials

- Chip-Arrays
- Cell culture media

CAUTION: Failure to replenish media on time will cause air to be introduced into the Chip-Array, which ultimately results in cell death. Filling inlet reservoirs up to 1250 µL will ensure up to 72 hours of flow. Do not fill the inlet reservoirs past 1300 µL of the total volume during replenishment.

Changing Media

Steps

1. Media should be refilled every 2-3 days to ensure the inlet reservoirs do not deplete of media. At 15 µL/h, the maximum time between media replenishments should be 72 hours.
2. Once compound dosing begins (see Dosing section), make sure the proper dosing media is added to the correct Chip-Array Emulations.
3. Warm Complete Hepatocyte Maintenance Media and NPC Maintenance Media for 1 hour and dose with compound if necessary.
4. Pause flow on the AVA and transfer two Chip-Arrays at a time from the AVA to the BSC.
5. Remove Chip-Array lid and collect effluent from the Chip-Array inlet and outlet reservoirs, taking care not to disturb the reservoir vias.
6. Any media not collected for analysis should be aspirated by aspirating from the top right corner of the reservoirs farthest from the reservoir via while the

consumable lies flat. This ensures that a thin liquid film still covers the reservoir Vias so that no air is introduced into the fluidic channels of the Chip-Array.

a. When aspirating reservoirs, it is recommended to use a multichannel aspirator to streamline the aspirating workflow. This will help to prevent cross-contamination of dosing medias across different conditions. Users should also prevent cross-contamination of top and bottom channel medias when aspirating.

7. Refill the Chip-Array inlet reservoirs with 1250 µL of respective top and bottom channel warmed media.
8. Place the Chip-Array reservoir lids on and transfer the Chip-Arrays back to the AVA (two at a time).
9. Once all Chip-Arrays have been replenished with fresh media/dosing media, press the “Flush” Flow Operation function, either on the embedded AVA UI or the external companion laptop.
    a. Set the Flush parameters to:
        i. Top Channel Flow Rate: 1000 µL/h
        ii. Bottom Channel Flow Rate: 1000 µL/h
        iii. Duration: 6 minutes
        iv. Select Stop Flow once Flush is complete
    b. ***NOTE***: This flush step is critical to ensure that that cells are instantly exposed to the freshly prepared dosing media on each dosing day.
10. After the Flush step is complete, remove the Chip-Arrays from the AVA and aspirate the excess media pushed to the outlet reservoirs.
    a. Follow Step 6 above for proper outlet aspiration technique.
    b. ***NOTE***: This outlet aspiration step is critical after the Flush step is performed to ensure that excess media does not compromise any analysis performed on collected effluent in subsequent timepoint days.
11. Return all Chip-Arrays back to the AVA to resume 15 µL/h flow.

Automated Imaging

Steps

1. Automated imaging protocols should be assigned for every day required; it is recommended to image Liver-Chips on Timepoint Days 0, 1, 3 and 7 (Experiment Days 5, 6, 8 and 12).
    a. **Timepoint Day 0 is essential for quality checking every Emulation. The AVA software allows users to exclude Emulations if an Emulation is deemed unsuitable for assigning to a condition. Reference Timepoint Day 0 for more detail regarding this quality check**
2. Users should not eject the trays or remove the Chip-Arrays when an automated imaging protocol is running. As such, it is recommended that users schedule Chip-Arrays to be imaged in a manner so that on the specified Timepoint Days, the duration of the imaging does not interfere with lab activities.
3. It is recommended that at least 4 fields of view (FOVs) be captured for every Emulation: 2 FOVs in the bottom channel monoculture regions for assessment of NPC health, and 2 FOVs in the top channel for assessment of hepatocyte health.
4. After each automated imaging session, these image sets can be viewed in the Image Reviewer tab.

Timepoint Day 0 – Quality Checking and Dosing

Overview

Goals

- Cell culture quality check and inclusion/exclusion from study
- Assign conditions to each Chip-Array Emulation
- Prepare dosing media and dose into Chip-Array inlet reservoirs

Quality Check

Steps

1. Five days after hepatocyte seeding is considered Timepoint Day 0, meaning that this is the first timepoint where hepatocytes and NPCs are stable and healthy enough on the Chip-Arrays to begin dosing of compounds.
2. It is recommended that the Timepoint Day 0 automated imaging session be scheduled before the start of the workday, so that users can come into the lab and begin reviewing images to QC each Emulation using the AVA Image Reviewer software. Users should assess Emulation QC prior to condition assignment and dosing. QC assessment can done using either live streaming by conducting a Manual Imaging session or using the images acquired from the automated imaging session.
    a. Users should assess hepatocyte and NPC morphology based on the QC scale shown below in Figure 21, excluding any Emulations from their study that fails the quality check.

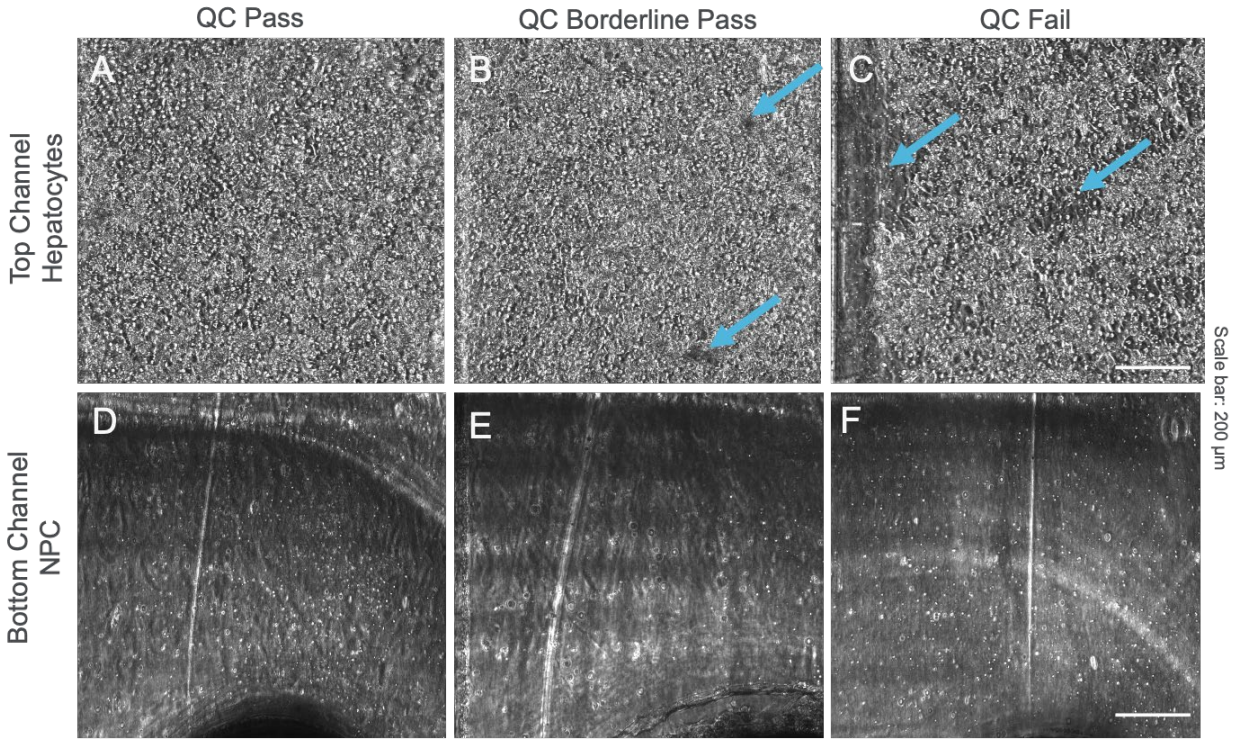

Figure 21. Examples of QC Pass (A&D), Borderline QC Pass (B&E) and QC Fail (C&F) when viewing imaged Emulations using Image Reviewer. (A&B) Emulations with clearly visible Hepatocytes with complete monolayer, or with minimal small gaps (arrows in B), and with minimal cell debris throughout the channel should be considered passing QC. (C) Emulations with presence of large gaps in the Hepatocyte monolayer (arrows) adjacent to channel walls, or in the middle of the channel, would fail QC and should be excluded. (D&E) Emulations with presence of clearly defined NPC, with minimal elongated morphology, across the bottom channel, should be considered passing. (F) Emulations completely denude of NPCs or with less than 50% confluency, elongated or stressed NPCs, should be considered QC fail.

b. Users can exclude Emulations that do not pass QC by using the Notes tab within the Image Reviewer software.
c. After QC of every Emulation is complete, users should go back into the Experiment tab and begin assigning conditions to the Chip-Array Emulations that have passed QC.
    i. It is recommended that conditions be randomly assigned across all Chip-Arrays and Emulations.

Dosing

Steps

1. Once all conditions have been assigned, begin aspirating media from the Chip-Arrays (handle Chip-Arrays two at a time to minimize time outside of AVA or BSC) and filling the reservoir inlets with their corresponding dosing media based on the condition assigned to the specific Emulation.
2. When dosing on specified timepoint days, refer to the “Changing Media” section of **Day 5+: Chip-Array Maintenance and Sampling** for proper aspiration and dosing technique and performing the required Flush step on each dosing day.

Sampling

Steps

1. Starting on Timepoint Day 0 (5 days after hepatocyte seeding), Liver-Chips are considered stable and healthy enough to begin analysis.
2. Effluent can be collected from the Chip-Array outlet reservoirs and transferred to a collection plate for analysis.
    a. It is recommended to handle consumables two at a time when sampling, returning consumables to the AVA with flow set to “0” immediately after taking samples.
3. Remove Chip-Array lid and collect effluent from the Chip-Array inlet and outlet reservoirs, taking care not to disturb the reservoir vias.
    a. *Note:* It is recommended to freeze the effluent at -80°C if effluent will not be analyzed immediately. It is also recommended to reduce the number of freeze-thaw cycles to prevent degradation of proteins or cytokines in the effluent, such as albumin.

Troubleshooting

Bubbles and Flow Issues

If a bubble is present in the channel during ECM coating, washing, or cell seeding, it is recommended to wash the channel with the appropriate solution until all bubbles have been removed. If bubbles persist, it may be helpful to aspirate the channel dry and slowly re-introduce solution.

After flow has been initiated on the AVA, it is possible that users may run into issues related to bubble formation and blockage in the fluidic elements of the consumables. If these are severe enough, they may cause lower than expected flow or no flow at all. This can usually be detected by eye when assessing volume in the top or bottom channel outlets. If this occurs, this can potentially be resolved via numerous methods based on the issue:

1. If a bubble is observed visually in the top and/or bottom channel, this bubble potentially can be removed by:
    a. Flushing the Chip-Array at 1,000 µL/h for 6 minutes. This should push the bubble out of the channel.
    b. If Step 1a does not work and bubble is still present, then detach the flow guide for the specific Emulation, reattach the pipette guide, and manually flush the bubble out with a pipette.
        i. Afterwards, it is highly recommended to detach the pipette guide, re-prime the Chip-Array, reattach the flow guide, and re-run Regulate on the Chip-Array.
        ii. Due to this manual intervention, b. should be considered a last resort if a. does not work.
2. If a bubble is not observed visually in the top and/or bottom channel, this indicates the bubble is likely in the fluidic elements of the consumable. This bubble can potentially be removed by:
    a. Flushing the Chip-Array at 1,000 µL/h for 6 minutes. This should push the bubble out of the channel.

   b. If the flow issue persists afterwards, re-run Regulate on the Chip-Array. There is no need to manually detach the flow guide for manual flushing with a pipette.
3. If media appears to be collecting in the outlet reservoirs during sampling, this could indicate the Chip-Array reservoir lid has media splashed on it and media is wicking out of the reservoirs. To mitigate, remove the reservoir lid and wipe clean with a cleanroom wipe sprayed with ethanol. Do not fill reservoirs beyond 1mL during media replenishment. Handle the Chips-Arrays in such a way so as to minimize inadvertent contact of media with the reservoir lid (e.g. tilting the Chip-Array during transport). Minimize time consumables are outside the AVA.

Media Taking Too Long to Pass Through Steriflip

If media takes too long to pass through Steriflip unit when equilibrating the dissolved gas in the media before Prime, it is likely that vacuum pressure is not reaching -70kPa. It is recommended to find an alternate vacuum source with the appropriate pressure.

Priming Issues

If Chip-Arrays do not properly prime, ensure that media are covering the reservoir Vias and run the Prime Cycle again on the AVA.

Environmental Controls

The AVA is set to maintain temperature and %CO_2 concentration with tolerances around these set points. If the AVA instrument is outside of these tolerances, users will be notified via an alert on the external workstation UI.

If these issues persist, contact Emulate Support to troubleshoot and diagnose root cause.

Automated Imaging

The AVA software allows users to schedule “Automated Imaging” sessions, where the AVA microscope will image specific FOVs on specific Emulations designated by the user at a certain time/date.

If the AVA fails to capture these images, attempt to open a “Live Imaging” session and manually capture images to determine if this is a microscope failure.

If “Live Imaging” works, then this means the microscope is functional and the failed “Automated Imaging” session is likely to be related to the scheduling software. Refer to the AVA software manual or contact Emulate Support for further troubleshooting.