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Protocol for Organ-Chips:

Basolateral Recruitment of Chimeric Antigen Receptor (CAR) T Cells in an Organ-Chip Model of Non-Small Cell Lung Cancer (NSCLC)

April 22, 2024

EP-231 Rev. B

Basolateral Recruitment of Chimeric Antigen Receptor (CAR) T Cells in an Organ-Chip Model of Non-Small Cell Lung Cancer (NSCLC)

Overview

Introduction

This protocol describes the steps for inducing the migration of chimeric antigen receptor T (CAR T) cells from the vascular endothelial (bottom) channel to the epithelial (top) channel in a cancer cell line Organ-Chip model of human non-small cell lung cancer (NSCLC). This protocol describes the steps for inflammatory treatment of the bottom channel, addition of chemoattractants to the top channel, preparation of buoyancy media, and administration of CAR T through the bottom channel. This protocol also describes steps for imaging recruited CAR T in the top channel and measuring target killing readouts. It also describes the steps for extracting recruited CAR T from the top channel for subsequent flow cytometric immunophenotyping to allow measurement of responses such as antigen-specific CAR T exhaustion. Finally, this protocol demonstrates the use of co-therapies (IL-2) to enhance CAR T recruitment and killing efficiency.

Contents

Topic	See Page
Experimental Timeline	4
Experimental Readouts	6
Master Materials List	7
Protocol for Culturing Organ-Chip Model of NSCLC	15
Adapting the Workflow to New CAR T, Targets, and Co-Therapies	66
Troubleshooting	78
Appendix	80

Goals

- Inflammation-specific migration of CAR T from the vascular (bottom) channel to the epithelial (top) channel
- Antigen-specific killing of target tumor cells by recruited CAR T and disruption of the epithelial barrier
- Assessment of antigen-dependent CAR T exhaustion marker expression
- Enhancement of CAR T recruitment and/or killing efficacy by co-therapies

Continued on next page

Overview, Continued

Associated Protocols

Effluent Sampling (EP124)
Fixation and Immunofluorescence (IF) Staining (EP137)

Experimental Timeline

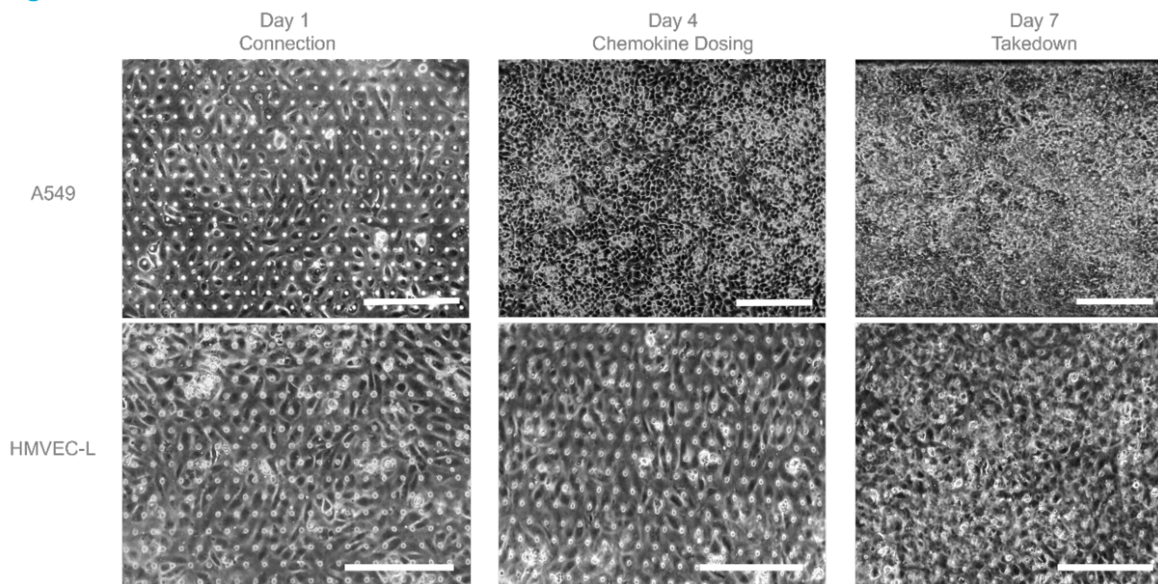
Overview

Introduction

The total timeline for this protocol is eight days, starting from seeding (Day 0) the NSCLC cell line on-chip. Inflammatory activation of the chip with tumor necrosis factor alpha (TNFα) is initiated once there is an acceptable epithelial and endothelial monolayer, as observed in figure 1 (this typically occurs by Day 3). TNFα is then administered alongside a cocktail of chemoattractants (defined in "Experimental Readouts"). The former is kept in the chip for a total of 24 h, while the latter is continually dosed until the end of the protocol. CAR T are thawed in-flask 4 h prior to administration and are then collected from the flask, mixed with buoyancy media, and introduced into the bottom channel. The CAR T are introduced to the bottom channel as a bolus at a high flow rate of 1,000 µL/h for 4 h, including a recommended hourly mixing of the CAR T suspension to avoid settling. The chips are then flushed with media at the same flow rate for 30 min. Finally, the flow rate is adjusted to 30 µL/h until the end of the protocol.

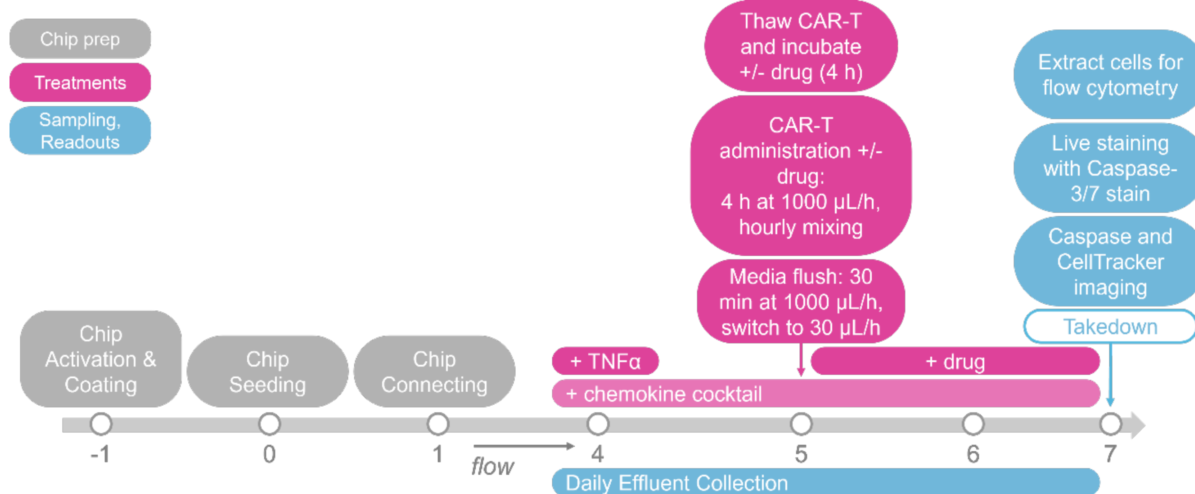
Exemplary phase contrast micrographs of endothelial and epithelial tissue are depicted in Figure 1, and a schematic timeline of the protocol is depicted in Figure 2, below.

Figure 1



Phase contrast images of untreated NSCLC cell line Organ-Chip model through culture. Human lung microvascular endothelial cells (HMVEC-L) in the bottom channel should maintain a monolayer of a "cobblestone" appearance until Day 7 of culture. A549 cell line in the top channel should maintain distinct epithelial junctions but will slowly grow 3D structures from Day 4 until Day 8. Treatment with TNFα or chemokines does not have a significant effect on morphology via phase contrast. Scale bar = 250 µm.

Figure 2



Schematic representation of this protocol's key steps following a typical timeline. The NSCLC cell line Organ-Chip is prepared, seeded, and grown to confluency by Day 5 (**Note:** Timeline can be adjusted depending on when the endothelium and epithelium form confluent monolayers on-chip). 24 h prior to administering CAR T into chip (Day 4), cytokines (TNFα in bottom channel, chemokine cocktail in top channel) are added to chip. On Day 5, CAR T are thawed in the flask and rested for 4 h. Next, administration is performed for 4 h at 1,000 µL/h with hourly mixing of the inlet CAR T suspension. A media wash is then performed for 30 min, and flow is returned to 30 µL/h for both channels. Flow is continued until takedown at 48 h, where chips are stained with a Caspase-3/7 stain and fixed for imaging analysis.

Experimental Readouts

Overview

Introduction

- CAR T localization by microscopy (and associated analysis)
- Caspase killing localization by microscopy (and associated analysis)
- Cytokine release through effluent analysis (performed according to manufacturer instructions)
- Optional: Extraction and immunophenotyping by flow cytometry of immune cells for activation and/or exhaustion markers

Master Materials List

Overview

Introduction

The below materials table is a complete account of all the materials required to successfully replicate this protocol. The displayed quantities correspond with a study incorporating 24 Chip-S1® Stretchable Chips. In cases where model information is omitted, the material supplier is subject to user preference.

Master List

	Material Name	Material Information	Quantity
Cells	A549 lung carcinoma epithelial cells	ATCC, Cat CCL-185	1
	CD19 CAR T cells	ProMab BioTechnologies, Cat PM-CAR1002	2
	HER2 CAR T cells	ProMab BioTechnologies, Cat PM-CAR1070	2
	Human lung microvascular endothelial cells (HMVEC-L)	Lonza, Cat CC-2527	2
Consumables	15 mL conical tubes (sterile)	--	2 (Pack of 50)
	50 mL conical tubes (sterile)	--	2 (Pack of 25)
	500 mL bottle with filtration	PVDF filter 0.45 µm, sterile	2
	70% ethanol and wipes	For surface sterilization	N/A
	96-well V-bottom plates	--	8
	Aspirator and sterile tips	--	-
	Bucket of ice	--	-
	Chip Cradle	Emulate, Autoclaved, 1 per 6 chips	4
	Chip-S1 Stretchable Chips	Emulate, 12 per Zoë	24
	Hemocytometer	--	1
	Microcentrifuge tubes	1.5 mL	15
	P20, P200, P1000 pipettes and filter tips	--	-
	Pod® Portable Modules	Emulate, 1 per Chip-S1	24
	Serological pipettes	--	-
	Square Cell Culture Dish (120 x 120 mm)	VWR, Cat 82051-068, Sterile, 1 per 6 chips	4
	Steriflip® Units	0.45 µm PVDF sterile filters, Millipore Sigma, Cat SE1M003M00	15
	Tissue culture flasks	T-25, T-75, T-150 tissue culture-treated, sterile	T-25: 10, T-75: 1, T-150: 4
	Trypan Blue Solution	0.4% solution, Sigma Cat 93595	1
Cytokines	Recombinant Human Fractalkine	CX3CL1, PeproTech, Cat 300-31	1
	Recombinant Human IL-2	PeproTech, Cat 200-02	1
	Recombinant Human IP-10	CXCL10, PeproTech, Cat 300-12	1

	Recombinant Human MCP-1	CCL2, PeproTech, Cat 300-04	1
	Recombinant Human MIG	CXCL9, PeproTech, Cat 300-26	1
	Recombinant Human I-TAC	CXCL11, Peprotech, Cat 300-46	1
	TNFα	R&D Systems, Cat 210-TA-020	1
Equipment	Autoclave	Capable of heating liquids to 121°C	1
	Magnetic hotplate stirrer	Capable of heating liquids to 90°C	1
	Microscope (with camera)	For bright-field imaging	1
	Orb-HM1® Hub Module	Emulate, 1 per 4 Zoës	1
	P20, P200, P1000 pipettes and filter tips	--	-
	P200 multichannel pipette	--	1
	UV Light Box	Emulate, 1 per Zoë	4
	Vacuum unit and tube connections	Minimum of -70 kPa operating pressure	1
	Water bath (or beads)	Set to 37°C	1
	Zoë-CM1® or CM2® Culture Module	Emulate, 1 per 12 chips	2
Media & Supplements	100X GlutaMAX Supplement	Thermo Fisher Scientific Cat 35050061	1
	7.5% NaHCO3 Sodium Bicarbonate solution	Thermo Fisher Scientific, Cat 25080094	1
	EGM MV-2 complete media	PromoCell, Cat 22121	1
	Fetal Bovine Serum (FBS)	Millipore Sigma, Cat F4135-500ML	1
	Full Medium 199	Thermo Fisher Scientific, Cat 11150067	1
	Ham's F-12K (Kaighn's) Medium	Thermo Fisher Scientific, Cat 21127022	1
	Pen-Strep	Millipore Sigma, Cat P4333	1
	Primocin	VWR, MSPP-ANTPM1	1
	RPMI-1640 medium (1x)	Thermo Fisher Scientific, Cat 11875093	2
	RPMI-1640 media (10x)	Sigma, Cat R1145-500ML	1
	1 M NaOH	Thermo Fisher Scientific, Cat 124260250	1
	Folic acid, 5 g	Sigma, Cat F8758-5G	1
	Gelzan™ CM	Low acyl gellan gum; Millipore Sigma, Cat G1910, Cas# 71010-52-1, EC#:2740117-5, MLD#: MFCD00131909	1
	Percoll®	Millipore Sigma, CatP4937	1
Reagents	Bovine serum albumin (BSA)	Millipore Sigma, Cat A7030	1
	CellEvent™ Caspase-3/7 Detection Reagent	Thermo Fisher Scientific, Cat C10423	1
	Cell culture grade water	--	500 mL
	Cell Tracker stain	Deep Red, Thermo Fisher Scientific, Cat C34565	1

	Collagen I	Millipore Sigma, Cat CLS354249	1
	Collagen IV	Millipore Sigma, Cat 5533	1
	DMSO	--	1 mL
	Dulbecco's PBS (DPBS - / -) (without Ca2+, Mg2+)	Corning, Cat D8537	1
	ECMatrix™-511 E8 Laminin Substrate	Sigma, CC160-350UG	1
	ER-1™ Chip Activation Reagent	Emulate, 5 mg powder	1
	ER-2™ Chip Activation Reagent	Emulate, 25 mL bottle	1
	Human Fibronectin	Corning, Cat354008	1
	Paraformaldehyde (PFA, 4%)	--	20 mL
	PBS + 0.05% Sodium Azide (optional)	Teknova, Cat P0202	1
	TrypLE™ Express Enzyme (1X)	Thermo Fisher Scientific, Cat 12604013	1
	Trypsin-EDTA (0.25%)	--	25 mL

Preparation of Materials

Overview

Preparation of Collagen IV

Step	Action
1	Resuspend 5 mg of Collagen IV in 5 mL of sterile cell culture grade water and incubate for 24 h at 4°C until dissolved.
2	The next day, create 300 µL aliquots and store them at -20°C.

Preparation of Fibronectin

Step	Action
1	Resuspend 5 mg of Fibronectin in 5 mL of sterile cell culture grade water and leave the mix at room temperature for 30 min to dissolve (avoid harsh agitation or vortexing). Swirl gently before aliquoting.
2	Store aliquots at -20°C.

Preparation of EGM-MV2 Complete Media

Prepare EGM-MV2 complete media following manufacturer instructions. Briefly:

Step	Action
1	Thaw components of Endothelial Cell Basal Medium MV2 Supplement Pack and bring to room temperature.
2	Aseptically transfer all components of Endothelial Cell Basal Medium MV2 Supplement Pack to the Endothelial Cell Basal Medium bottle.
3	Supplement media with 0.1% Primocin (i.e., 500 µL per 500 mL media).
4	Sterile filter complete media through 0.45 µm PVDF filter.
5	Store complete media at 4°C for up to 30 days

EGM-MV2 Complete Media		
Reagent	**Volume (mL)**	**Final Percentage (%)**
Endothelial Cell Basal Medium MV2	500	-
Supplement Pack	Total volume of all components	-
Primocin	0.5	0.1 %

Continued on next page

Overview, Continued

Preparation of Complete F-12K Media

Step	Action
1	Obtain bottle of Ham's F-12K (Kaighn's) Medium.
2	Add 1% Pen-Strep.
3	Add 10% FBS.
4	Store complete media at 4°C for up to 30 days.

Complete F-12K Media		
Reagent	Volume (mL)	Final Percentage (%)
Ham's F-12K (Kaighn's) Medium	445	-
FBS	50	10%
Pen-Strep	5	1%

Preparation of Complete RPMI Media (1X)

Step	Action
1	Obtain bottle of RPMI-1640 medium (Thermo Fisher Scientific, Cat 11875093, 1X, 500 mL).
2	Add 1% Pen-Strep.
3	Add 10% FBS.
4	Store complete media at 4°C for up to 30 days.

Complete RPMI (1X) Media		
Reagent	Volume (mL)	Final Percentage (%)
RPMI-1640 media, 1X	445	-
FBS	50	10%
Pen-Strep	5	1%

Preparation of Complete Medium 199 (M-199)

Step	Action
1	Obtain bottle of full medium 199.
2	Add 1% Pen-Strep.
3	Add 5% FBS.
4	Store complete media at 4°C for up to 30 days.

Complete M-199 Media		
Reagent	Volume (mL)	Final Percentage (%)
Medium 199	470	-
FBS	25	5 %
Pen-Strep	5	1 %

Continued on next page

Overview, Continued

Preparation of 2X RPMI Media

Step	Action
1	Prepare Folic Acid following manufacturer protocol to 50 mg/mL: • Add 100 mL of 1M NaOH to 5g folic acid to make 50 mg/mL stock solution
2	In a sterile, 500 mL bottle, add 30 mL of 7.5% NaHCO3 solution
3	Add 20 µL of 50 mg/mL folic acid
4	Add 10 mL of 100X GlutaMAX
5	Add 112.5 mL of 10X RPMI-1640
6	Add 100 mL of FBS
7	Add 10 mL of Pen-Strep
8	Add 238 mL of culture grade water
9	Sterile filter the final solution using a PVDF filter 0.45 µm
10	Test the pH of the solution by taking a small aliquot and testing its pH with a probe or test strip to ensure pH 7.0–7.4. If the solution does not measure within this range, adjust the pH slowly with 1M NaOH or 1M HCl solutions. Use test aliquots to maintain sterility.
11	Store at complete RPMI (2X) media at 4°C, and aliquot when needed. • The solution can be stored at 4°C for up to 3 months

2X RPMI Media			
Reagent	**Volume (mL)**	**Final Percentage (%)**	**Final Concentrations**
7.5% NaHCO3	30 mL	5.99%	0.45% NaHCO3
Folic Acid	0.02 mL (20 µL)	0.004%	2 µg/mL Folic Acid
100X GlutaMAX	10 mL	2%	2X GlutaMAX
RPMI-1640 (10X)	112.5 mL	22.48%	2.25X RPMI-1640
FBS	100 mL	19.97%	2X final
Pen-Strep	10 mL	2.00%	2X final
Water	238 mL	47.55%	-
Total	**500.52 mL**	**-**	**-**

Continued on next page

Overview, Continued

Preparation of Gelzan 1% w/v Stock Solution:

Note: Gelzan™ product (powder) is not manufactured sterile; therefore, autoclaving the prepared solution is highly recommended.

Step	Action
1	Make 1% w/v Gelzan (Millipore Sigma, Cat. G1910) in distilled water (e.g., 1 g/100 mL).
2	Dissolve by stirring at 90°C with a magnetic stir bar.
3	Sterilize solution at 121°C for 20 min in autoclave.
4	Cool to room temperature and immediately seal the solution to maintain sterility. Avoid leaving overnight, as excessive evaporation may occur which can influence the final viscosity.
5	Store at room temperature or use immediately in buoyancy media. • Gelzan stock solution is stable for at least 3 months when stored at room temperature in aseptic conditions. However, for best results, prepare fresh buoyancy reagent for each experiment.

Preparation of IL-2 Cytokine

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Centrifuge each chemokine vial briefly to pellet lyophilized contents.</td></tr>
<tr><td>2</td><td>Follow manufacturer recommendation for reconstitution of stock vial.
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Reconstitute to 1 mg/mL concentration in water. Pipette to mix. Store at 2–8°C for up to 1 week.</td></tr>
<tr><td>2</td><td>For long-term storage, further dilute in a buffer containing stabilizing protein (i.e., 0.1% BSA). Aliquot stock solutions (5 µL/aliquot) and store at -20°C to avoid multiple freeze-thaw cycles. Store at -20°C for up to 3 months.</td></tr>
</table></td></tr>
</table>

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Overview, Continued

Preparation of TNFα Cytokine

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Prepare 0.1% (w/v) BSA solution:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Weigh 50 mg BSA into a 50 mL conical tube.</td></tr>
<tr><td>2</td><td>Add cell culture grade water up to 50 mL.</td></tr>
<tr><td>3</td><td>Put solution on shaker on low speed to mix until dissolved.</td></tr>
<tr><td>4</td><td>Sterile filter solution through a 0.2 µm filter.</td></tr>
</table></td></tr>
<tr><td>2</td><td>Centrifuge TNFα vial for a few seconds to pellet lyophilized contents.</td></tr>
<tr><td>3</td><td>Reconstitute TNFα using BSA solution to 1 mg/mL. Pipette to mix.
• Reconstituted stock can be stored at -20°C for up to 3 months. Aliquot solution to avoid multiple freeze-thaw cycles.</td></tr>
</table>

Preparation of Chemokine Stock Solution

Preparation of Chemokine Stock Solutions for CXCL9, CXCL10, CXCL11, CCL2 and Fractalkine.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Centrifuge each chemokine vial briefly to pellet lyophilized contents.</td></tr>
<tr><td>2</td><td>Follow manufacturer recommendation for reconstitution of stock vials of chemokines:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Reconstitute each chemokine individually to 1 mg/mL concentration in cell culture grade water. Pipette to mix. Store at 2–8°C for up to 1 week.</td></tr>
<tr><td>2</td><td>For long-term storage, further dilute in a buffer containing stabilizing protein (i.e., 0.1% BSA). Aliquot stock solutions (5 µL/aliquot) and store them at -20°C to avoid multiple freeze-thaw cycles. They can be stored at this temperature for up to 3 months.</td></tr>
</table></td></tr>
</table>

Protocol for Culturing Organ-Chip Model of NSCLC

Overview

Introduction

To establish the Organ-Chip model of NSCLC, A549 cells and HMVEC-L are cultured prior to chip preparation. Chips are prepared through an activation and coating procedure, which is tailored for lung-specific cells (Day -1). The following day (Day 0), Organ-Chips are seeded first with HMVEC-L in the bottom channel, which are allowed to adhere. Afterwards, A549s are seeded in the top channel. On Day 1, the seeded Organ-Chips are connected to Pod Portable Modules, which are then connected to Zoë® Culture Module to initiate media flow. Chip maintenance is performed as the cells form mature monolayers (Day 2–3). On Day 4, the CAR T basolateral recruitment protocol begins with the introduction of inflammatory cytokines. This, along with the rest of the protocol, is described below.

Contents

Topic	See Page
Day -4: Thawing and Maintenance of HMVEC-L and A549	16
Day -1: Activation and Coating	18
Day 0: Seeding Chips	22
Day 1: Connection	29
Day 2: Pod Maintenance and Second Regulate	34
Chip Sampling Readouts	35
Day 4: TNFα and Chemoattractant Treatment	37
Day 5: CAR T Administration onto NSCLC Organ-Chips	41
Day 6: 24 h Post Administration	58
Day 7: Takedown (Caspase Staining and Fixation)	62

Day -4: Thawing and Maintenance of HMVEC-L and A549

Thawing and Maintenance of HMVEC-L

Required Materials

Material Name	Model Information
50 mL conical tubes (sterile)	--
70% ethanol and wipes	For surface sterilization
Aspirator and sterile tips	--
EGM-MV2 complete media	See “Preparation of Materials” section
Hemocytometer	--
Microcentrifuge tubes	1.5 mL
P20, P200, P1000 pipettes and filter tips	--
Tissue culture flasks	T-75 tissue culture-treated, sterile
Trypan Blue Solution	0.4% solution, Sigma Cat 93595
Vacuum unit and tube connections	Minimum of -70 kPa operating pressure

Steps

Approximately 120,000 HMVEC-L will be needed for each seeded chip. It is recommended to thaw at least 2 x 10^6 HMVEC-L in a T-75 for a 24-chip study.

Step	Action
1	Warm a 50 mL aliquot of EGM-MV2 complete media for at least 30 min.
2	Thaw vial(s) of HMVEC-L in a 37°C water bath for approximately 60–90 seconds. Closely observe the thawing process while gently agitating. Remove the vial from the water bath just before the last ice pellet disappears.
3	Using a P1000 pipette, immediately transfer the cell suspension to a conical tube containing approximately 20 mL of warmed EGM-MV2 complete media. • Use 1 mL of the suspension to rinse out the vial thoroughly.
4	Centrifuge at 300 x *g* for 5 min.
5	Loosen the pellet and resuspend it in at least 1 mL EGM-MV2 complete media.
6	Take a cell count using a hemocytometer.
7	Seed 2 x 10^6 HMVEC-L per T-75 flask. Add 15 mL of EGM-MV2 complete media. The HMVEC-L should be given fresh media the day after seeding and every two days thereafter until seeding onto chip.

Thawing and Maintenance of A549s

Required Materials

Material Name	Model Information
50 mL conical tubes (sterile)	--
70% ethanol and wipes	For surface sterilization
Aspirator and sterile tips	--
Complete F-12K media	See “Preparation of Materials” section
Fetal Bovine Serum (FBS)	Millipore Sigma, Cat F4135-500ML
Hemocytometer	--
Microcentrifuge tubes	1.5 mL
P20, P200, P1000 pipettes and filter tips	--
Tissue culture flasks	T-75 tissue culture-treated, sterile
Trypan Blue Solution	0.4% solution, Sigma Cat 93595
Vacuum unit and tube connections	Minimum of -70 kPa operating pressure

Steps

Approximately 10,000 A549 will be needed for each seeded chip. It is recommended to thaw at least 2 x 10^6 A549 in a T-75 for a 24-chip study. Maintain A549 cells below passage 15 (as low as possible) to avoid phenotypic mutations over time.

Step	Action
1	Warm a 50 mL aliquot of complete F-12K media in a bead bath for at least 30 min
2	Thaw vial(s) of A549 in a 37°C water bath for approximately 60–90 seconds. Closely observe the thawing process while gently agitating and remove the vial from the water bath just before the last ice pellet disappears.
3	Using a P1000 pipette, immediately transfer the cell suspension to a conical tube containing 20 mL of warmed complete F-12K media. • Use 1 mL of the suspension to rinse out the vial thoroughly.
4	Centrifuge at 300 x *g* for 5 min.
5	Loosen the pellet and resuspend it in at least 1 mL of warmed complete F-12K media.
6	Take a cell count using a hemocytometer.
7	Seed 2 x 10^6 A549 per T-75 flask. Add 10 mL of complete F-12K medium. The A549 cells should be given fresh media the day after seeding and every other day thereafter until seeding onto chip.

Day -1: Activation and Coating

Overview

Required Materials

Material Name	Model Information
15 mL conical tubes	--
70% ethanol and wipes	For surface sterilization
Aspirator and sterile tips	--
Bucket of ice	--
Cell culture grade water	--
Chip Cradle	Emulate, Autoclaved, 1 per 6 chips
Chip-S1 Stretchable Chip	Emulate, 12 per Zoë
Collagen I	Millipore Sigma, Cat CLS354249
Collagen IV	Millipore Sigma, Cat 5533
Dulbecco's PBS (DPBS - / -) (without Ca2+, Mg2+)	Corning, Cat D8537
ECMatrix-511 E8 Laminin Substrate	Sigma, CC160-350UG
ER-1 Chip Activation Reagent	Emulate, 5-mg powder
ER-2 Chip Activation Reagent	Emulate, 25-mL bottle
Human Fibronectin	Corning, Cat354008
P20, P200, P1000 pipettes, and filter tips	--
Serological pipettes	--
Square Cell Culture Dish (120 x 120 mm)	VWR, Cat 82051-068, Sterile, 1 per 6 chips
UV Light Box	Emulate, 1 per Zoë
Vacuum unit and tube connections	Minimum of -70 kPa operating pressure

Continued on next page

Overview, Continued

Steps

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Place the bottoms of the new 120 mm square dishes into the UV light boxes (open side down). The square dishes containing chips will sit on these dishes so the chips are closer to the UV light during activation. NOTE: This activation protocol is specific for the HMVEC-L cells used in validation of the workflow. For guidance on chip activation for your cells of interest, please contact Emulate Customer Support: https://emulatebio.com/contact-support/</td></tr>
<tr><td>2</td><td>Pre-warm UV light boxes before activation:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Set the switch at the back of the UV light box to the “Constant” setting. Turn on the “Power,” and press the “On” button to begin UV activation.</td></tr>
<tr><td>2</td><td>Allow the UV light boxes to warm for at least 5 min before activation.</td></tr>
</table></td></tr>
<tr><td>3</td><td>Chill a 50 mL aliquot of DPBS to 4°C (on ice).</td></tr>
<tr><td>4</td><td>Prepare chips:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Spray the chip packaging, additional 120 mm square cell culture dishes, and Chip Cradle with 70% ethanol and bring them into the biosafety cabinet (BSC).</td></tr>
<tr><td>2</td><td>Open the Chip Cradle sterile packaging and place the Chip Cradle into the 120 mm square dish, making sure the cradle is oriented properly with the corners facing up.</td></tr>
<tr><td>3</td><td>Open the chip packaging carefully and place the first chip into the cradle by sliding the back of the carrier under the tabs on the cradle.</td></tr>
<tr><td>4</td><td>Repeat as necessary for all the chips included in the experiment.</td></tr>
<tr><td>5</td><td>Label each chip with ID numbers on the chip carrier tab.</td></tr>
</table></td></tr>
<tr><td>5</td><td>Prepare ECM coating solution during UV activation step:
<table>
<tr><td>Top Channel</td><td>Collagen IV [200 µg/mL], Fibronectin [30 µg/mL], Laminin [5 µg/mL] in DPBS</td></tr>
<tr><td>Bottom Channel</td><td>Collagen I [50 µg/mL], Fibronectin [60 µg/mL] in DPBS</td></tr>
</table></td></tr>
<tr><td>6</td><td>Prepare ER-1/ER-2 Solution:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Turn off the light in BSC and allow the ER-1 and ER-2 to equilibrate to room temperature before use (approximately 10–15 min).</td></tr>
<tr><td>2</td><td>Wrap an empty sterile 15 mL conical tube with foil to protect it from light.</td></tr>
<tr><td>3</td><td>In the BSC, remove the small vial of ER-1 powder from the packet, and briefly tap the vial to concentrate the powder at the bottom.</td></tr>
<tr><td>4</td><td>Add 1 mL of ER-2 to the vial, and transfer contents directly to the bottom of the 15 mL conical tube. Do not pipette to mix.</td></tr>
</table></td></tr>
</table>

<table>
<tr><td></td><td>
<table>
<tr><td></td><td>Note: The color of the solution transferred to the conical tube will be a deep red.</td></tr>
<tr><td>5</td><td>Add an additional 1 mL of ER-2 to the ER-1 vial to collect any remaining material and transfer the solution directly to the 15 mL conical tube.
Note: The color of the transferred solution will become lighter with each additional wash of the ER-1 bottle.</td></tr>
<tr><td>6</td><td>Repeat Step 5 twice more, with an additional 1 mL of ER-2 each time.</td></tr>
<tr><td>7</td><td>On the last addition of 1 mL ER-2 to the ER-1 bottle, cap the ER-1 bottle and invert it to collect any remaining ER-1 powder from the lid. Transfer the collected solution to the conical tube, bringing the total volume in the tube to 4 mL ER-1 solution.</td></tr>
<tr><td>8</td><td>Add 6 mL of ER-2 solution to the 4 mL of ER-1 solution in the 15 mL conical tube (for a final working concentration of 0.5 mg/mL). Pipette gently to mix without creating bubbles. ER-1 should be fully dissolved within the ER-2 solution prior to use.</td></tr>
</table>
</td></tr>
<tr><td>7</td><td>Introduce ER-1 solution to channels:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Using a P200 pipette and a sterile, 200-µL filtered pipette tip, take up 200 µL of ER-1 solution.
Note: 200 µL of ER-1 solution will fill approximately 3 chips.</td></tr>
<tr><td>2</td><td>Carefully introduce approximately 20 µL of ER-1 solution through the bottom channel inlet, pipetting until the solution begins to exit the bottom channel outlet.</td></tr>
<tr><td>3</td><td>Without releasing the pipetting plunger, take the pipette out from the bottom channel inlet, and move the pipette containing the remaining ER-1 solution to the top channel inlet.</td></tr>
<tr><td>4</td><td>Introduce approximately 50 µL of ER-1 solution to the top channel inlet, pipetting until the solution begins to exit the top channel outlet.</td></tr>
<tr><td>5</td><td>Remove all excess ER-1 solution from the surface of the chip by gentle aspiration. Be sure to only remove ER-1 solution from the chip surface—do not aspirate ER-1 from the channels.</td></tr>
<tr><td>6</td><td>Repeat Steps 1–5 for each chip.</td></tr>
<tr><td>7</td><td>Inspect the channels for bubbles prior to UV activation. If bubbles are present, dislodge them by washing the channel with ER-1 solution until all bubbles have been removed. If bubbles persist, it may be helpful to aspirate the channel dry and slowly re-introduce ER-1 solution.</td></tr>
</table>
</td></tr>
<tr><td>8</td><td>Bring the 120 mm square dishes containing the ER-1-coated chips to the UV light boxes. Flip the dishes upside-down and place them on top of the existing dishes in Step 1. Activate under UV for 5 min.</td></tr>
<tr><td>9</td><td>Following this, flip the dishes back right-side up and place them under UV for another 10 min.
Note: Ensure the lids of the square dishes are taken off and the light boxes stay on for the entire duration of the activation.</td></tr>
<tr><td>10</td><td>After UV treatment, bring the chips back to the BSC.
Note: The light may be on in the BSC from this point forward.</td></tr>
<tr><td>11</td><td>Fully aspirate the ER-1 solution from both channels.</td></tr>
<tr><td>12</td><td>Wash chips with 200 µL ER-2 for each channel.</td></tr>
</table>

13	Wash the chips with 200 µL of cold DPBS for each channel.
14	Aspirate the channels entirely and fill them with their respective ECM coating solution using a P200 pipette.
15	Inspect the channels to ensure that no bubbles are present. If bubbles are present, wash the channel with ECM solution until all bubbles have been removed.
16	Add DPBS to fill the central reservoir of Chip Cradle to reduce evaporation in channels.
17	Incubate chips at 4°C overnight (Optional: Wrap plates in Parafilm® to avoid any evaporation).

Day 0: Seeding Chips

Overview

Contents

Topic	See Page
Preparation for Seeding	23
Seeding HMVEC-Ls into Bottom Channel	24
Seeding A549 into Top Channel	27

Required Materials

Material Name	Model Information
15 mL conical tubes (sterile)	--
50 mL conical tubes (sterile)	--
500 mL bottle with filtration	PVDF filter 0.45 µm, sterile
70% ethanol and wipes	For surface sterilization
A549 lung carcinoma epithelial cells	ATCC, Cat CCL-185
Aspirator and sterile tips	--
Chip-S1 Stretchable Chips	Emulate, 12 per Zoë
Complete M-199 media	See “Preparation of Materials” section
Dulbecco’s PBS (DPBS - / -) (without Ca2+, Mg2+)	Corning, Cat D8537
EGM-MV2 complete media	See “Preparation of Materials” section
Fetal Bovine Serum (FBS)	Millipore Sigma, Cat F4135-500ML
Hemocytometer	--
Human Lung Microvascular Endothelial Cells (HMVEC-L)	Lonza, Cat CC-2527
Microscope (with camera)	For bright-field imaging
P20, P200, P1000 pipettes and filter tips	--
Pen-Strep	Millipore Sigma, Cat P4333
Primocin	VWR, MSPP-ANTPM1
Serological pipettes	--
Square Cell Culture Dish (120 x 120 mm)	VWR, Cat 82051-068, Sterile, 1 per 6 chips
Trypan Blue Solution	0.4% solution, Sigma Cat 93595
TrypLE™ Express Enzyme (1X)	Thermo Fisher Scientific, Cat 12604013
Trypsin-EDTA (0.25%)	--
Vacuum unit and tube connections	Minimum of -70 kPa operating pressure
Water bath (or beads)	Set to 37°C

Preparation for Seeding

Steps

Step	Action
1	Prepare 50 mL EGM-MV2 complete media (see “Preparation of Materials” section) for the bottom channel.
2	Prepare 50 mL complete M-199 media (see “Preparation of Materials” section) for the top channel.
3	Transfer Organ-Chips to culture incubator 1 h prior to washing to equilibrate them to 37°C.
4	Wash both channels with 200 μL of EGM-MV2 complete media.
5	Return Organ-Chips to 37°C incubator until cell seeding.

Seeding HMVEC-Ls into Bottom Channel

Steps

HMVEC-Ls that have been expanded in culture must be harvested and counted for bottom channel seeding. Typically, HMVEC-Ls are adjusted to a density range of 8 x 10^6 cells/mL to achieve a complete monolayer prior to seeding in the bottom channel.

Step	Action
1	Check for confluence of HMVEC-L under brightfield and ensure >70% confluency.
2	Aspirate the culture media and add 10 mL of DPBS to wash the culture surface. Aspirate the DPBS wash.
3	Add 5 mL of TrypLE to the flasks. Incubate for 5 min at 37°C.
4	Tap the side of the flask gently and inspect the culture under the microscope to confirm detachment of cells from the culture surface.
5	Add 5 mL EGM-MV2 complete media to neutralize.
6	Transfer the contents of the flask into a conical tube. **Note:** Depending on volume of contents, use a 15 or 50 mL tube. **Note:** If working with more than one flask of cells which are being pooled together, centrifuge at 300 x *g* for 5 min and resuspend the pellet in a smaller total volume of EGM-MV2. This will ensure that a more accurate cell count is obtained.
7	Transfer 20 µL of the cell suspension to a 1.5 mL microcentrifuge tube containing 20 µL of Trypan Blue Solution. Count cells using a hemocytometer.
8	Centrifuge at 300 x *g* for 5 min.
9	Loosen the pellet and resuspend to 8 x 10^6 cells/mL in EGM-MV2 complete media.
10	Bring the square dish containing the prepared chips to the BSC.
11	Gently agitate cell suspension before seeding each chip to ensure a homogeneous cell suspension. • Routinely mix the suspension between every ~6 chips to prevent clumping of cells.
12	Using a pipette, remove the medium from the bottom channel, leaving it empty.
13	First, seed 15 µL of the HMVEC-L suspension into the bottom channel of one chip.
14	Cover the dish and transfer to the microscope to check the seeding density within the chip (see Figure 3 for reference)

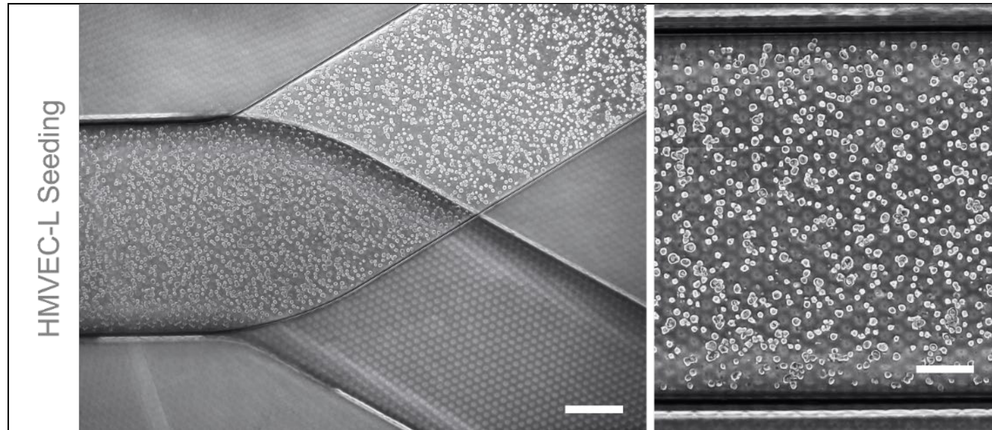


Figure 3. Seeding density reference for HMVEC-L (8 x 10^6 cells/mL)

15	If seeding density is not optimal, return the chip to the BSC and wash the channel with 200 µL of fresh medium twice. Do not aspirate the medium from the channel. Adjust the cell density accordingly and repeat steps 12–14 until the correct density is achieved within the channel. **Note:** Correct seeding density is essential for success of the chip cultures.
16	After confirming the correct cell density, seed cells in the remaining chips, and invert the 120 mm square dish containing the Chip Cradle and seeded chips (See Figure 4). **Note:** Each Chip Cradle can support up to 6 chips inside a 120 mm square dish.

Figure 4. Inverting chips during endothelial attachment.

17	Add 0.75 mL of DPBS into the central reservoir of the Chip Cradle to reduce evaporation in channels.
18	Incubate chips for 2–3 h at 37°C. See Figure 5 for representative morphology 3 h post-seeding

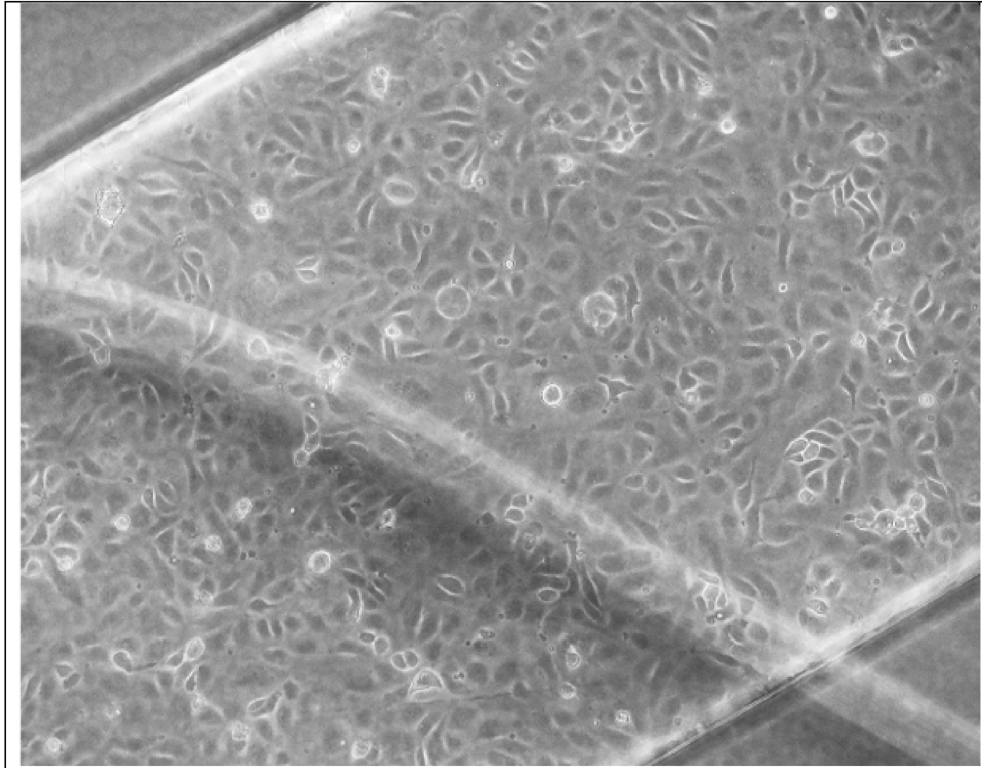


Figure 5. Representative HMVEC-L attachment 3 h post seeding.

Seeding A549 into Top Channel

Introduction

A549s that have been expanded in culture must be harvested and counted for top channel seeding. It is recommended to use A549 cells with as low a passage as possible (< passage 15) to avoid any phenotypic mutations over time. Typically, A549s are adjusted to a density of 10,000 cells per chip (~333,333 cells/mL) to achieve a complete monolayer.

Note that these instructions are tailored to growth characteristics of the A549 lung carcinoma cell line. If an alternative cell type is being used in the top channel as the tumor target (see "Adapting the Workflow to New CAR T, Targets, and Co-Therapies" section), empirical adjustments to the seeding density and maturation time on chip will need to be made. Optimal protocol would result in an ~80% confluent monolayer at seeding (Day 0) and avoid excessive overgrowth in the top channel by termination (Day 7). Additionally, morphological properties will also be expected to vary significantly among different cancer lines. For example, glioblastoma cells (astrocyte-derived cancer cells) will not be expected to form the organized cell-cell junctions seen with the A549 cells and will rather exhibit a spindly or dendritic morphology. Users should consult the literature to align morphological expectations with established norms for each cell line to be used.

Steps

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Prepare chips for seeding top channel:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Bring 120 mm square dishes to the biosafety cabinet and flip them right-side up.</td></tr>
<tr><td>2</td><td>Wash the top channels with 200 µL of warm complete M-199 media.</td></tr>
</table></td></tr>
<tr><td>2</td><td>Check for confluence of A549 in flask under brightfield and ensure >70% confluency.</td></tr>
<tr><td>3</td><td>Aspirate culture media and add 5 mL of DPBS to wash the culture surface. Aspirate the DPBS wash.</td></tr>
<tr><td>4</td><td>Add 5 mL of Trypsin + EDTA to the flasks. Incubate for 5 min at 37°C.</td></tr>
<tr><td>5</td><td>Tap the side of the flask gently and inspect the culture under the microscope to assess complete detachment of cells from the culture surface.</td></tr>
<tr><td>6</td><td>Add 5 mL of complete M-199 media to neutralize.</td></tr>
<tr><td>7</td><td>Transfer the contents of the flasks into a conical tube.
Note: Depending on volume of contents, use a 15 or 50 mL tube.</td></tr>
<tr><td>8</td><td>Transfer 20 µL of the cell suspension to a 1.5 mL tube containing 20 µL of Trypan Blue Solution. Count cells using a hemocytometer.</td></tr>
<tr><td>9</td><td>Centrifuge at 300 x g for 5 min.</td></tr>
<tr><td>10</td><td>Resuspend in complete M-199 media to seed 10,000 per chip (333,333 cells/mL), accounting for 30 µL of suspension per chip.</td></tr>
<tr><td>11</td><td>Remove the top channel media using a P200 or P1000 pipette.</td></tr>
<tr><td>12</td><td>Mix the A549 cell suspension before seeding:</td></tr>
</table>

	• Routinely mix the suspension between every ~6 chips to prevent clumping of cells.
13	First, seed 30 µL of the A549 cell suspension into the top channel of one chip.
14	Cover the dish and transfer to the microscope to check the seeding density within the chip (see Figure 6 for reference).

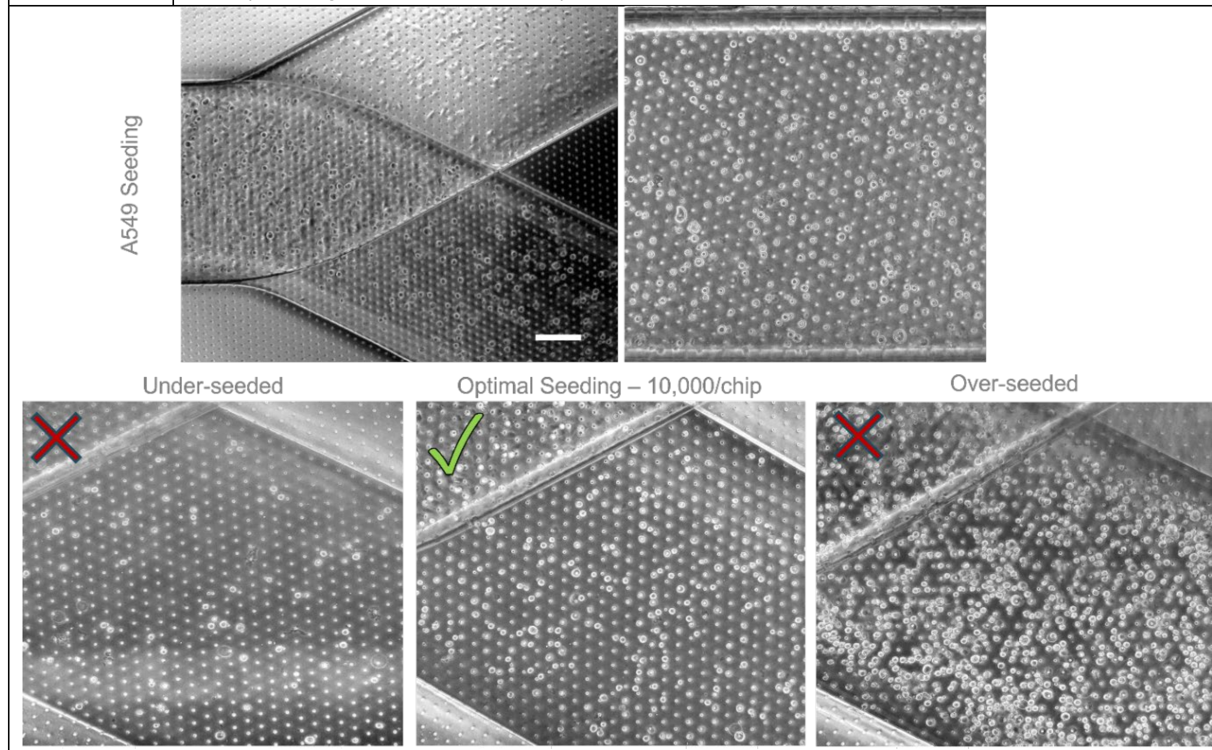


Figure 6. Seeding density reference for A549 (3.33 x 10^5 cells/mL).

15	If the seeding density is not optimal, return the chip to the BSC and wash the channel with 200 µL of fresh medium twice. Do not aspirate the medium from the channel. Adjust cell density accordingly and repeat steps 10–12 until the correct density is achieved within the channel. **Note:** Correct seeding density is essential for success of the chip cultures.
16	Repeat for all chips.
17	Add 0.75 mL of DPBS to the central reservoir of the Chip Cradle to reduce evaporation in channels.
18	Incubate overnight at 37°C.

Day 1: Connection

Overview

Contents

Topic	See Page
Gas Equilibration of Media	30
Wash Chips	31
Prime Pods	32
Pod to Zoë and Regulate	33

Required Materials

Material Name	Model Information
50 mL conical tubes (sterile)	--
70% ethanol and wipes	For surface sterilization
Aspirator and sterile tips	--
Complete M-199 media	See “Preparation of Materials” section
EGM-MV2 complete media	See “Preparation of Materials” section
Fetal Bovine Serum (FBS)	Millipore Sigma, Cat F4135-500ML
Microscope (with camera)	For bright-field imaging
Orb-HM1 Hub Module	Emulate, 1 per 4 Zoës
P20, P200, P1000 pipettes and filter tips	--
Pen-Strep	Millipore Sigma, Cat P4333
Pod Portable Modules	Emulate, 1 per Chip-S1
Primocin	VWR, MSPP-ANTPM1
Serological pipettes	--
Steriflip® Units	0.45 µm PVDF sterile filters, Millipore Sigma, Cat SE1M003M00
Vacuum unit and tube connections	Minimum of -70 kPa operating pressure
Water bath (or beads)	Set to 37°C
Zoë-CM1 or CM2 Culture Module	Emulate, 1 per 12 chips

Gas Equilibration of Media

Steps

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Prepare complete media for gas equilibration:

Note: Approximately 3–4 mL of media will be used per reservoir of each chip. For 24 chips, prepare around 100 mL of media for the top channel and 100 mL for the bottom channel.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Prepare complete M-199 media.</td></tr>
<tr><td>2</td><td>Prepare EGM-MV2 complete media.</td></tr>
</table></td></tr>
<tr><td>2</td><td>Equilibrate complete M-199 media and complete EGM-MV2 media:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Warm the aliquoted media at 37°C in a water bath or bead bath for at least 1 h for media to equilibrate and reach 37°C. Optional: Prior to equilibration, media can also be aliquoted and incubated overnight at 37°C.

Note: Warming media to 37°C for ≥ 1 h is crucial for proper equilibration and removal of dissolved gas. Failure to properly equilibrate media can create air nucleation sites in chip channels and can disrupt proper flow.</td></tr>
<tr><td>2</td><td>Immediately connect the 50 mL tubes containing each warmed medium to a Steriflip® unit.</td></tr>
<tr><td>3</td><td>With the unit "right-side-up" (medium in the bottom conical tube), apply vacuum for 10 seconds.</td></tr>
<tr><td>4</td><td>Invert the Steriflip®-connected tubes, and check that the medium begins to pass from the top conical tube to the lower tube.
Note: The vacuum source must operate at a minimum of -70 kPa. At this pressure, it should take about 2 seconds for every 10 mL of medium to flow through the filter. If it takes longer, stop, and refer to the troubleshooting section—Equilibration of Media.</td></tr>
<tr><td>5</td><td>Leave the filtered medium under vacuum for 5 min.</td></tr>
<tr><td>6</td><td>Remove the vacuum tubing from the Steriflip® units while keeping the vacuum on (to prevent any backflow or air), then turn off the vacuum source.</td></tr>
<tr><td>7</td><td>In a BSC, separate the conical tubes containing media from the Steriflip® unit and immediately place the conical tubes containing media in the incubator with the caps loose.

Note: Equilibrate no more than two conical tubes of media at a time and use within 10 min for Pod priming. It is imperative to use the media immediately to avoid media cooling and aeration.</td></tr>
</table></td></tr>
</table>

Wash Chips

Steps

Step	Action
1	Using complete, equilibrated media, wash each channel (EGM-MV2 for bottom channel, complete M-199 media for top channel) with 200 μL media with P200 pipette. Pipette slowly to avoid harming the cells and aspirate outflow.
2	Place small droplets of equilibrated complete M-199 media on all inlet and outlet ports of the top channel and EGM-MV2 complete media on the inlet and outlet ports of the bottom channel. Keep in 37°C incubator until Pod connection.

Prime Pods

Steps

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Using the equilibrated media, add approximately 3.5 mL of complete M-199 media and EGM-MV2 to the respective Pod inlets. In addition, dispense 300 µL of complete M-199 media and EGM-MV2 into the respective Pod outlets directly over the Vias.</td></tr>
<tr><td>2</td><td>Run the Prime Cycle on all the Pods:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Use the Dial to highlight “Prime” on the display.</td></tr>
<tr><td>2</td><td>Press the Dial Button to select “Prime.”</td></tr>
<tr><td>3</td><td>Rotate the Dial clockwise to highlight “Start.”</td></tr>
<tr><td>4</td><td>Press the Dial Button again to select “Start.”
Note: Once “Start” is selected, there will be an audible sound as Zoë engages the Pods.</td></tr>
<tr><td>5</td><td>After priming, check that liquid flowed through the Pod by observing media droplets. When the Prime Cycle is complete, there will be small droplets of medium formed over the Pod ports.</td></tr>
</table></td></tr>
<tr><td>3</td><td>Connect chips to Pods:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Holding one chip (while it remains in the chip carrier) in the dominant hand and one Pod firmly in the non-dominant hand, slide the chip carrier into the tracks on the underside of the Pod until the chip carrier has seated fully.</td></tr>
<tr><td>2</td><td>Place a thumb on the chip carrier tab and gently, but firmly, depress the tab in and up to engage the tab of the chip carrier with the Pod.</td></tr>
<tr><td>3</td><td>Aspirate any excess medium on the chip surface from the Pod window.</td></tr>
<tr><td>4</td><td>Place the Pod with the connected chip onto the tray.</td></tr>
<tr><td>5</td><td>Repeat steps 1–4 for each Pod and chip carrier.</td></tr>
<tr><td>6</td><td>Confirm that there is sufficient media in each Pod inlet reservoir. Ensure that 300 µL of media has been added to each Pod outlet and is covering the Pod outlet Vias. Check that the Pod lids are flat and secure.</td></tr>
</table></td></tr>
</table>

Pod to Zoë and Regulate

Steps

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Place trays holding Pods and chips immediately into Zoë to prevent media from cooling and losing its gas equilibration.</td></tr>
<tr><td>2</td><td>Program the appropriate Organ-Chip culture conditions on Zoë. These conditions will start as soon as the Regulate Cycle is complete.
For NSCLC Organ-Chips, set the flow rate to 30 µL / h for both top and bottom channels. Set to 0% stretch, 0.0 Hz frequency.</td></tr>
<tr><td>3</td><td>Run Regulate Cycle:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Using the Dial, highlight the “Regulate” field.</td></tr>
<tr><td>2</td><td>Press the Dial Button to select “Regulate,” and rotate the Dial clockwise to “Start.”</td></tr>
<tr><td>3</td><td>Press the Dial Button again to select “Start” and begin the Regulate Cycle.
Note: Once “Start” is selected, there will be an audible sound as Zoë engages the Pods.</td></tr>
<tr><td>4</td><td>At this point the “Activation” button will glow blue.</td></tr>
<tr><td>5</td><td>The Regulate Cycle lasts 2 h. After the cycle has finished, Zoë will begin flow at the preset Organ-Chip culture conditions. To cancel the Regulate Cycle (only if needed) on Zoë, hover over the “Regulate” field with the Dial and press the Dial Button to select. Rotate the Dial counterclockwise to select “Cancel.” Press the Dial Button once more and wait 1 min for the cycle to end, after which the tray can be removed. If cancelled, always rerun a complete Regulate Cycle before proceeding.</td></tr>
</table>
</td></tr>
</table>

Day 2: Pod Maintenance and Second Regulate

Overview

Required Materials

Material Name	Model Information
37°C water or bead bath	--
70% ethanol and wipes	For surface sterilization
Microscope (with camera)	For bright-field imaging
Orb-HM1 Hub Module	Emulate, 1 per 4 Zoës
P20, P200, P1000 pipettes and filter tips	--
P200 multichannel pipette	--
Pod Portable Modules	Emulate, 1 per Chip-S1
Serological pipettes	--
Water bath (or beads)	Set to 37°C
Zoë-CM1 or CM2 Culture Module	Emulate, 1 per 12 chips

Steps

Note: If any flow issues are observed on the Pods, see the “Troubleshooting” section.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Perform Via washes on Pod Vias, then collect any Pod effluent (if necessary):
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>The day after connecting chips and Pods to Zoë, which begins the process of Organ-Chip culture, pause Zoë by pressing the silver “Activation” button located above the tray bays. This stops flow and releases the Pods.</td></tr>
<tr><td>2</td><td>Slide the tray out of the bay and transfer to the BSC.</td></tr>
<tr><td>3</td><td>Remove the Pod lids. Using a 200 μL pipette, perform a Via wash on each Pod inlet and outlet reservoir. Using media within the Pod reservoir, pipette 200 μL of medium directly over the top of the Via to dislodge any bubbles that may be present.</td></tr>
<tr><td>4</td><td>Repeat this wash step for each of the four Pod reservoirs.</td></tr>
</table></td></tr>
<tr><td>2</td><td>Resume flow by starting a second Regulate Cycle at 30 μL/h for top and bottom channels at 0% stretch and 0.0 Hz frequency.</td></tr>
</table>

Chip Sampling Readouts

Effluent Collection for MSD Analysis (Day 4 through Takedown)

Required Materials

Material Name	Model Information
96-well V-bottom plates	--
P20, P200, P1000 pipettes and filter tips	--
P200 multichannel pipette	--
Optional: U-PLEX Custom Immuno-Oncology Assay	Meso Scale Discovery, cat. K151AEM-2

Effluent Collection

Step	Action
1	Using a P200 multichannel pipette, collect 200 µL from the Sampling/Aspirating zone of the Pod inlets and outlets. Follow Effluent Sampling (EP124) for detailed instructions.
2	Collect into 96-well V-bottomed plates and repeat for all samples. Optional: Centrifuge effluent collection plate at 300 x *g* for 5 min to pellet any residual cell debris. Transfer effluent (excluding debris pelleted at bottom) to a new 96-well V-bottomed plate and seal with adhesive film.
3	Cover the wells with plate sealer film or seal the plate with parafilm. Store the plate at -80°C for long-term storage before measuring.
4	Measure effluent for secreted markers. It is recommended to use U-PLEX Assay (Meso Scale Discovery) with the following panel on top channel effluent: • Granzyme B • IFN-γ • IL-2 • PD-1 (epitope 1) • PD-L1 (epitope 1) • Perforin

Continued on next page

Effluent Collection for MSD Analysis (Day 4 through Takedown), Continued

Continuation of Flow after Effluent Collection

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Aspirate Pod inlet reservoirs for top and bottom channels.</td></tr>
<tr><td>2</td><td>Aspirate Pod outlet reservoirs for top and bottom channels, avoiding the Vias and being cautious to leave ~200 µL of liquid remaining over the Vias.
Note: Aspiration of Pod reservoirs is not necessary if effluent is not being collected.</td></tr>
<tr><td>3</td><td>If necessary, replenish media to Pod inlets to ensure enough volume per chip for 30 µL/h flow:
<table><tr><th>Step</th><th>Action</th></tr><tr><td>1</td><td>Add complete M-199 media to the top channel Pod inlets.</td></tr><tr><td>2</td><td>Add equivalent volume of EGM-MV2 complete media to the bottom Pod inlets.</td></tr></table></td></tr>
<tr><td>4</td><td>Return the Pods to Zoë and continue flow for the top and bottom channels.</td></tr>
</table>

Day 4: TNFα and Chemoattractant Treatment

Overview

Goals

To induce endothelial cell activation with 24 h TNFα treatment in the bottom channel with simultaneous treatment of the top channel with a chemoattractant cocktail to create the appropriate inflammatory conditions for CAR T recruitment.

Notes

- TNFα treatment should not be started until complete monolayer of A549 epithelium is achieved.
- TNFα treatment must happen for a full 24 h to ensure sufficient inflammation of the endothelium. Thus, for logistical reasons, it is recommended to start TNFα treatment in the morning, which allows for a reasonably early start of CAR T administration the next day.

Contents

Topic	See Page
Cytokine Treatment with TNFα (24 h):	39
Chemokine Treatment with Cocktail (24 h):	40

Required Materials

Material Name	Model Information
15 mL conical tubes (sterile)	--
37°C water or bead bath	--
50 mL conical tubes (sterile)	--
500 mL bottle with filtration	PVDF filter 0.45 µm, sterile
70% ethanol and wipes	For surface sterilization
96-well V-bottom plates	--
Aspirator and sterile tips	--
Bovine serum albumin (BSA)	Millipore Sigma, Cat A7030
Cell culture grade water	--
Chip-S1 Stretchable Chip	Emulate, 12 per Zoë
Complete M-199 media	See “Preparation of Materials” section
Dulbecco’s PBS (DPBS - / -) (without Ca2+, Mg2+)	Corning, Cat D8537
EGM-MV2 complete media	See “Preparation of Materials” section
Fetal Bovine Serum (FBS)	Millipore Sigma, Cat F4135-500ML
Hemocytometer	--
Microscope (with camera)	For bright-field imaging
Orb-HM1 Hub Module	Emulate, 1 per 4 Zoës
P20, P200, P1000 pipettes and filter tips	--

P200 multichannel pipette	--
Pen-Strep	Millipore Sigma, Cat P4333
Pod Portable Modules	Emulate, 1 per Chip-S1
Primocin	VWR, MSPP-ANTPM1
Recombinant Human Fractalkine	CX3CL1, PeproTech, Cat 300-31
Recombinant Human IL-2	PeproTech, Cat 200-02
Recombinant Human IP-10	CXCL10, PeproTech, Cat 300-12
Recombinant Human MCP-1	CCL2, PeproTech, Cat 300-04
Recombinant Human MIG	CXCL9, PeproTech, Cat 300-26
RPMI-1640 media (1X)	Thermo Fisher Scientific, Cat 11875093
Serological pipettes	--
Tissue culture flasks	T-25 or T-75, tissue culture-treated, sterile
TNFα	R&D Systems, Cat 210-TA-020
Trypan Blue Solution	0.4% solution, Sigma Cat 93595
Water bath (or beads)	Set to 37°C
Zoë-CM1 or CM2 Culture Module	Emulate, 1 per 12 chips

Cytokine Treatment with TNFα (24 h):

Steps for Preparation of TNFα Dosing Media

Step	Action
1	Warm sufficient volume of EGM-MV2 complete media to 37°C. Account for at least 30 µL/h flow for 24 h, plus 200 µL per chip for effluent readouts (recommended at least 1.2 mL total per chip).
2	Add 1 mg/mL TNFα stock solution to the EGM-MV2 to a final concentration of 50 ng/mL. **Note:** If the necessary volume of TNFα is exceedingly small, dilute the stock 1:10 in 0.1% BSA solution to allow for larger pipetting volumes.

Chemokine Treatment with Cocktail (24 h):

Chemokine Cocktail Concentrations

Chemokine	Final Concentration in Media
CXCL9	100 ng/mL
CXCL10	100 ng/mL
CXCL11	10 ng/mL
CCL2	100 ng/mL
Fractaline	10 ng/mL

Preparation of Dosing Media with Chemokine Cocktail

Step	Action
1	Warm sufficient volume of complete M-199 media to 37°C (account for at least 30 µL/h for 24 h flow plus 200 µL per chip for effluent collection, i.e., at least 1.2 mL per chip).
2	Add chemokine stock solutions to complete M-199 media to achieve concentrations for each chemokine as specified in the above table, *Chemokine Cocktail Concentrations*. • If the necessary volume is exceedingly small, dilute the stock 1:10 in PBS to allow for larger pipetting volumes.

Administration of Dosing Media to Chip

Step	Action
1	Aspirate all four Pod reservoirs without drying the Vias.
2	Add the bottom dosing media (1.2 mL of EGM-MV2 complete media + TNFα) to the bottom inlet reservoir of appropriate chips.
3	Add the top dosing media (1.2 mL of complete M-199 media + chemokine cocktail) to the top inlet reservoir of appropriate chips.
4	Return chips to Zoë and resume flow for 24 h.

Day 5: CAR T Administration onto NSCLC Organ-Chips

Overview

Goals

Thaw CAR Ts and rest on flask for 4 h. Administer a bolus of CAR T cells to the bottom channel using buoyancy media, recirculating for 4 h. Continue chemokine administration to the top channel to induce CAR T recruitment into that channel. Validate on-target specificity of CAR T killing using CellEvent Green Caspase assay.

Contents

Topic	See Page
CAR T Thawing and Resting	42
CAR T Staining, Preparation, and Administration	46

Key Steps

- Thaw CAR T into flasks and rest for 4 h (with or without co-therapeutic).
- Prepare concentrated buoyancy solution of Percoll with 1.6% v/v Gelzan.
- Warm and equilibrate buoyancy media reagents (Percoll + Gelzan, and 2X RPMI).
- Collect CAR T from flasks and obtain cell counts.
- Incubate CAR T with Cell Tracker stain.
- Create concentrated cell suspension in 2X RPMI media.
- Add buoyancy solution to cell suspension.
- Add IL-2 to appropriate buoyancy media conditions.
- Replenish chemokine cocktail to media.
- Administer CAR T bolus while mixing buoyancy media.
- Administer media flush after administration to remove buoyancy media from chip.
- Perform Regulate Cycle and return to resting flow rate.

CAR T Thawing and Resting

Introduction

Allow the vials of commercially available frozen CAR T cells (ProMab) to rest for 4 h in culture media at 37°C after thawing. If fresh, expanded CAR T are to be used, they can be placed in culture media and used immediately.

Users can consider testing the functionality of the CAR T clones used by following the guidelines in "Adapting the Workflow to New CAR T, Targets, and Co-Therapies" section.

Contents

Topic	See Page
Cell Number and Volume Calculations	43
Thaw CAR T	44
CAR T Pre-Incubation with Therapeutics	45

Required Materials

Material Name	Model Information
15 mL conical tubes (sterile)	--
37°C water or bead bath	--
50 mL conical tubes (sterile)	--
70% ethanol and wipes	For surface sterilization
Aspirator and sterile tips	--
CD19 CAR T cells	ProMab BioTechnologies, Cat PM-CAR1002
Complete RPMI media (1X)	See "Preparation of Materials" section
Hemocytometer	--
HER2 CAR T cells	ProMab BioTechnologies, Cat PM-CAR1070
Microscope (with camera)	For bright-field imaging
P20, P200, P1000 pipettes and filter tips	--
Pen-Strep	Millipore Sigma, Cat P4333
Pod Portable Modules	Emulate, 1 per Chip-S1
Primocin	VWR, MSPP-ANTPM1
Recombinant Human IL-2	PeproTech, Cat 200-02
Complete RPMI (1X) media	See "Preparation of Materials" section
Serological pipettes	--
Tissue culture flasks	T-25 or T-75, tissue culture-treated, sterile
Trypan Blue Solution	0.4% solution, Sigma Cat 93595
Water bath (or beads)	Set to 37°C

Cell Number and Volume Calculations

Steps

Below are considerations for the total number of CAR T required:

Step	Action
1	It is recommended that 1.2 mL of CAR T cell suspension (at a density 1 x 10^6 cells/mL) is prepared and recirculated for each chip, which reduces the total CAR T required for an experiment.
2	Multiply the total number of chips (recommended at least four chips per condition) by the volume of CAR T suspension to be added per chip to determine the total volume needed.
3	Multiply the total volume needed by the recommended 1 x 10^6 cells/mL to determine the minimum number of CAR T required.
4	Increase the calculated number of required CAR T by ~20% to ensure all chips get adequate volume.

$$\textit{Total CAR T required} = \left(\textit{number of chips x} \frac{1\ x\ 10^6\ \textit{cells}}{1.2\ \textit{mL dosing volume}}\right) + 20\%\ \textit{extra}$$

Thaw CAR T

Steps

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Warm complete RPMI medium (1X) to 37°C in a bead bath.</td></tr>
<tr><td>2</td><td>Pipette 10 mL of warm complete RPMI medium (1X) into a 50 mL conical tube.</td></tr>
<tr><td>3</td><td>Partially immerse the vial of frozen CAR T cells in the 37°C water bath and gently agitate the vial until the residual frozen sphere is ~3 mm in diameter. The label on the vial can be removed to help visualize the size of the frozen sphere.</td></tr>
<tr><td>4</td><td>Transfer 0.5–1 mL of warm medium from the 50 mL tube into the vial of cells, then immediately pipette the contents of the vial back into the 50 mL tube containing the warm medium.</td></tr>
<tr><td>5</td><td>Centrifuge the 50 mL tube at 300 x g for 5 min at room temperature.</td></tr>
<tr><td>6</td><td>Discard supernatant and re-suspend cell pellet with pre-warmed medium (approx. 2 mL per 1 x 10^7 cells).</td></tr>
<tr><td>7</td><td>Count cells and determine viability using a hemocytometer.</td></tr>
<tr><td>8</td><td>Spin the cells: 300 x g for 5 min at room temperature. Remove supernatant by tube inversion or careful aspiration.</td></tr>
<tr><td>9</td><td>Gently disturb the cell pellet and resuspend in 1 mL complete RPMI (1X) media, using a P1000 pipette, then add media to adjust the volume to achieve a cell concentration of 2 x 10^6 cells/mL (for therapeutic treatment studies, replace this step with CAR T Pre-Incubation with Therapeutics, steps 1–7, the subsequent section).</td></tr>
<tr><td>10</td><td>Keep CAR T in a conical tube at 37°C with 5% CO_2 to split into treatment groups (See “Adapting the Workflow to New CAR T, Targets, and Co-Therapies” section for recommended treatment groups). Otherwise, transfer to the flask and incubate for 4 h.
• Recommended volume of 2 x 10^6 cells/mL suspension per flask:
<table>
<tr><th>Flask Size</th><th>Working Volume</th><th>Total Cells</th></tr>
<tr><td>T-25</td><td>5 mL</td><td>1–10 x 10^6 cells</td></tr>
<tr><td>T-75</td><td>15 mL</td><td>16–30 x 10^6 cells</td></tr>
<tr><td>T-175</td><td>50 mL</td><td>70–106 x 10^6 cells</td></tr>
</table>
• Once the appropriate number of cells is added to the flask, add complete RPMI media (1X) to bring up the volume to the appropriate working volume above, if necessary.</td></tr>
</table>

CAR T Pre-Incubation with Therapeutics

Steps

Step	Action
1	Separate the desired volume of freshly thawed CAR T suspension (prepared in preceding section) for co-therapeutic treatment volumetrically in conical tubes (ex. For 1 group getting co-therapeutic treatment out of 3 total groups, split 1/3 of the total suspension volume for the co-therapeutic treatment flask).
2	Centrifuge at 300 x *g* for 5 min.
3	Reconstitute the CAR T in complete RPMI media (1X) at 2 x 10^6 cells/mL density.
4	Distribute proportionally into tissue culture flasks. • Once the appropriate number of cells is added to the flask, add complete RPMI media (1X) to bring up the volume to the appropriate working volume specified above.
5	Into each flask, once brought up to the working volume specified above, add the appropriate amount of respective therapeutic to achieve the following final concentrations: **IL-2 = 12.7 ng/mL**
6	Gently mix the contents of the flask(s) with a serological pipette while avoiding bubbles.
7	Move to 37°C incubator for 4 h.

CAR T Staining, Preparation, and Administration

Sections

Topic	See Page
Chip Maintenance	48
Preparing Buoyancy Media Components	49
CAR T Staining with Cell Tracker	51
Preparation of CAR T in Buoyancy Media	53
CAR T Administration of the NSCLC Organ-Chips	54
Recirculation of CAR T During Administration	56
Media Flow and Regulate Post-Administration	57

Required Materials

Material Name	Model Information
15 mL conical tubes (sterile)	--
37°C water or bead bath	--
50 mL conical tubes (sterile)	--
70% ethanol and wipes	For surface sterilization
96-well V-bottom plates	--
Aspirator and sterile tips	--
CD19 CAR T cells	ProMab BioTechnologies, Cat PM-CAR1002
Cell culture grade water	--
Cell Tracker stain	Deep Red, Thermo Fisher Scientific, Cat C34565
Chip-S1 Stretchable Chip	Emulate, 12 per Zoë
Dulbecco's PBS (DPBS - / -) (without Ca2+, Mg2+)	Corning, Cat D8537
EGM-MV2 complete media	See "Preparation of Materials" section
Complete M-199 media	See "Preparation of Materials" section
Gelzan™ CM	Low acyl gellan gum; Millipore Sigma, Cat G1910, Cas# 71010-52-1, EC#:2740117-5, MLD#: MFCD00131909
Hemocytometer	--
HER2 CAR T-cells	ProMab BioTechnologies, Cat PM-CAR1070
Microscope (with camera)	For bright-field imaging
Orb-HM1 Hub Module	Emulate, 1 per 4 Zoës
P20, P200, P1000 pipettes and filter tips	--
Pen-Strep	Millipore Sigma, Cat P4333
Percoll®	Millipore Sigma, Cat P4937
Pod Portable Modules	Emulate, 1 per Chip-S1
Recombinant Human Fractalkine	See "Preparation of Materials" section
Recombinant Human IL-2	See "Preparation of Materials" section
Recombinant Human IP-10	See "Preparation of Materials" section
Recombinant Human MCP-1	See "Preparation of Materials" section

Recombinant Human MIG	See “Preparation of Materials” section
2X RPMI media	See “Preparation of Materials” section
Serological pipettes	--
Steriflip® Units	0.45 µm PVDF sterile filters, Millipore Sigma, Cat SE1M003M00
Tissue culture flasks	T-25 or T-75, tissue culture-treated, sterile
TNFα	R&D Systems, Cat 210-TA-020
Trypan Blue Solution	0.4% solution, Sigma Cat 93595
Vacuum unit and tube connections	Minimum of -70 kPa operating pressure
Water bath (or beads)	Set to 37°C
Zoë-CM1 or CM2 Culture Module	Emulate, 1 per 12 chips

Chip Maintenance

Steps

Step	Action
1	Pause flow on Zoë and remove 1–2 trays of Pods to biosafety cabinet.
2	Collect effluent from Pod reservoirs (see “Chip Sampling Readouts”).
3	Note any issues with flow, which can arise from insufficient media equilibrating or connection of chips to Pod. These issues include air bubbles visible in the chip channels and/or reduced outlet flow in either channel. **Note:** Follow Troubleshooting guide section “Media Flow Pre-Administration” if there are any flow issues observed. Mitigation of flow issues is critical for successful administration of CAR T on-chip.
4	Repeat for all trays of Pods. Return trays to incubator to keep warm once complete.

Preparing Buoyancy Media Components

Note

The buoyancy media described herein is required to overcome the effects of gravity and ensure that the CAR T cells remain evenly distributed within the bottom inlet reservoir and bottom channel during perfusion. These features are necessary for CAR T cells to interact with the endothelial cells on the top surface of the bottom channel that faces the epithelial compartment and is meant to mimic the distribution of CAR Ts in the blood. Buoyancy media is more viscous compared to standard media, so the overall flow through the Pods will be slightly lower in the administration channel.

Steps

Step	Action
1	To maintain temperature at 37°C while equilibrating buoyancy media components, ensure that the conical tubes can remain in a bead or water bath while equilibrating via vacuum. Otherwise, prepare a beaker of warm distilled water to hold conical tubes during equilibrating steps.
2	Prepare and equilibrate Percoll and Gelzan buoyancy media components (use a 50 mL conical tube):

Step	Action
1	Aliquot 2 mL of Percoll per chip into 50 mL conical tubes.
2	Calculate volume of Gelzan stock solution to prepare a 1.6% v/v solution in Percoll (e.g., 1.6 mL Gelzan + 98.4 mL Percoll).
3	Pipette Gelzan into Percoll suspension while swirling tube to ensure complete mixing. Additionally, mix with serological pipette to ensure homogeneity.
4	Warm Percoll and Gelzan in 37°C bead bath for at least 1 h.
5	Connect the warmed 50 mL tube(s) containing Percoll + 1.6% Gelzan to a Steriflip® unit.
6	With the unit kept "right-side-up" (liquid in the bottom conical tube), apply vacuum for at least 10 min. Do not flip the Steriflip unit or filter the solution as this will cause the Steriflip filter to clog. Tap the sides of the conical tube once complete to dislodge any bubbles that accumulate. **Note:** Applying vacuum in this step is only to equilibrate the solution.
7	Remove the vacuum tubing from the Steriflip units. Then, turn off the vacuum source. Gently tap the sides of the tube to release any remaining bubbles.
8	Separate the conical tubes containing Percoll and Gelzan from the Steriflip unit in a biosafety cabinet. Lightly tap the sides of the tube(s) to force any remaining bubbles out of the solution. Immediately place the conical tubes in the incubator with the caps loose. **Note:** Minimize the time the solution is outside of the incubator during Pod preparation to maintain temperature. This is a critical step to ensure the success of the chips.

<table>
<tr><td>3</td><td>Prepare and equilibrate 2X RPMI (use a 50 mL conical tube):
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Aliquot 2 mL of 2X RPMI per chip into 50 mL conical tubes.</td></tr>
<tr><td>2</td><td>Warm 2X RPMI in 37°C bead or water bath for at least 1 h.</td></tr>
<tr><td>3</td><td>With the unit "right-side-up" (medium in the bottom conical tube), apply vacuum for 10 seconds.</td></tr>
<tr><td>4</td><td>Invert the Steriflip-connected tubes, and check that the medium begins to pass from the top conical tube to the lower tube.
Note: The vacuum source must operate at a minimum of -70 kPa. At this pressure, it should take about 2 seconds for every 10 mL of medium to flow through the filter. If it takes longer, stop, and refer to the troubleshooting section—Equilibration of Media.</td></tr>
<tr><td>5</td><td>Leave the filtered medium under vacuum for 5 min.</td></tr>
<tr><td>6</td><td>Remove the vacuum tubing from the Steriflip units while keeping the vacuum on (to prevent any backflow or air), then turn off the vacuum source.</td></tr>
<tr><td>7</td><td>In a BSC, separate the conical tubes containing media from the Steriflip unit and immediately place the conical tubes containing media in the incubator with the caps loose.</td></tr>
</table>
</td></tr>
</table>

CAR T Staining with Cell Tracker

Steps

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Transfer the rested CAR T from flasks to separate 50 mL conical tubes:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Collect CAR T suspension using a 10 mL serological pipette. Pipette multiple times while washing the bottom flask surface.
Note: The CAR T are grown in a suspension culture, so there should be minimal cells adhered to the bottom of the flask.</td></tr>
<tr><td>2</td><td>Collect total volume of CAR T suspension from each flask into separate 50 mL conical tubes (labeled per condition).</td></tr>
<tr><td>3</td><td>Add a volume of PBS (without calcium and magnesium) to the empty flasks according to the working volumes outlined in table of “Thaw CAR T” section.</td></tr>
<tr><td>4</td><td>Incubate the flask for 5 min in incubator.</td></tr>
<tr><td>5</td><td>Collect the total volume in each flask, washing while collecting, into their respective 50 mL conical tubes.
Note: Wash thoroughly to ensure the collection of as many cells as possible from the flask.</td></tr>
<tr><td>6</td><td>Check the flask under the microscope for complete collection of cells. It is expected that some will adhere during this time.</td></tr>
</table></td></tr>
<tr><td>2</td><td>Count cells:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Invert the tubes gently to homogenize the cell suspension.</td></tr>
<tr><td>2</td><td>Count a small volume of the cell suspension with a hemocytometer.</td></tr>
</table></td></tr>
<tr><td>3</td><td>Centrifuge at 300 x g for 8 min.</td></tr>
<tr><td>4</td><td>Prepare Cell Tracker staining solution in PBS:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>For Cell Tracker Deep Red (20 mL final staining solution):
1. Prepare a working solution by adding 20 µL of DMSO to one 15 µg vial.
2. Dilute working solution in serum-free media to 1 µM (1:1000) final concentration (e.g., add 20 µL dye to 20 mL of PBS).</td></tr>
<tr><td>2</td><td>For all other Cell Tracker products (Thermo Fisher Scientific):
• Prepare a 10 mM working solution by adding 10.75 µL of DMSO to one 50 µg vial.
• Dilute the staining solution in serum-free media to 5 µM final concentration</td></tr>
</table></td></tr>
<tr><td>5</td><td>Remove the supernatant and gently loosen the CAR T pellets by tapping the tube.</td></tr>
<tr><td>6</td><td>Resuspend the separate CAR T tubes in 2 mL of staining solution per 10M cells using a P1000 pipette.</td></tr>
</table>

7	Incubate for 30 min at 37°C.
8	While the CAR T are incubating, prepare calculations for the volume needed to resuspend cells at 2 x 10^6 cells/mL in 2X RPMI during the "Preparation of CAR T in Buoyancy Media" steps.
9	After incubation, add 20 mL of DPBS to each tube to dilute stain.
10	Centrifuge at 300 x *g* for 8 min.
11	Move directly to the Preparation of CAR T in Buoyancy Media steps, or reconstitute CAR T at 2 x 10^6 cells/mL in complete RPMI media (1X) and store for up to 1 h at 37°C.

Preparation of CAR T in Buoyancy Media

Steps

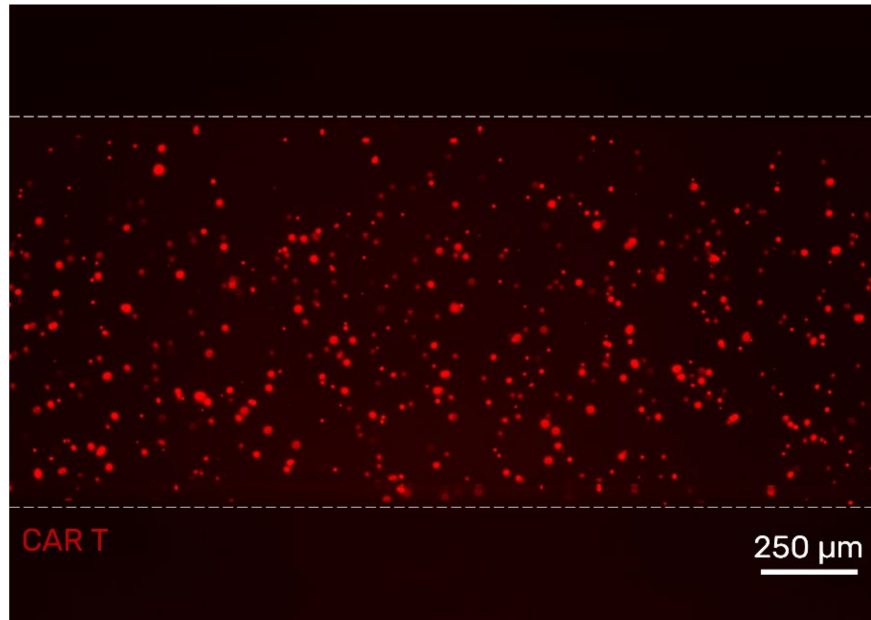
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Collect CAR Ts from resting into 50 mL conical tubes:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>a</td><td>If CAR Ts are already in 50 mL conical tubes, proceed to step 1c.</td></tr>
<tr><td>b</td><td>If CAR Ts are in flask:
• Collect the suspension from the flask using a serological pipette, rinsing the flask while collecting.
• Once the flask is empty, perform an additional rinse with PBS (half the original suspension volume) to collect any remaining CAR T adhered.</td></tr>
<tr><td>c</td><td>Centrifuge CAR Ts at 300 x g for 8 min, remove supernatant.</td></tr>
</table></td></tr>
<tr><td>2</td><td>Working with one tube at a time, loosen the pellet by gently tapping the tube, then resuspend at 2 x 10^6 cells/mL density based on prior cell counts in 2X RPMI media.
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Resuspend the pellet first with 1,000 µL of the volume using a P1000 pipette to homogenously incorporate the pellet.</td></tr>
<tr><td>2</td><td>Add the remaining volume of 2X RPMI to the pellet to achieve 2 x 10^6 cells/mL density.</td></tr>
</table>
Note: Immediately proceed to next steps to minimize CAR T exposure to 2X concentrated RPMI media.</td></tr>
<tr><td>3</td><td>Add buoyancy media to the CAR T suspension at a 1:1 (v/v) ratio:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Final solution: 1 x 10^6 cells/mL CAR T in 49.2% (v/v) Percoll and 0.8% (v/v) Gelzan with 50% (v/v) 2X RPMI. This makes 1X buoyancy media.</td></tr>
<tr><td>2</td><td>Mix gently and thoroughly with serological pipette to homogenize cells.</td></tr>
</table></td></tr>
<tr><td>4</td><td>Repeat for any remaining conical tubes of CAR T.</td></tr>
<tr><td>5</td><td>For chips receiving IL-2 therapeutic:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Thaw fresh aliquot of 1 mg/mL IL-2 (prepared and stored at -20°C previously).</td></tr>
<tr><td>2</td><td>Add IL-2 at a final concentration of 12.7 ng/mL, directly into the prepared buoyancy suspensions of appropriate groups. Add IL-2 at a final concentration of 12.7 ng/mL, directly into the prepared buoyancy suspensions of appropriate groups.</td></tr>
<tr><td>3</td><td>Mix suspension slowly with serological pipette.</td></tr>
</table></td></tr>
<tr><td>6</td><td>For any control groups not including CAR T, prepare aliquots of EGM-MV2 complete media (4 mL per chip) and complete M-199 media (4 mL per chip).
• Keep at 37°C until administration.</td></tr>
</table>

CAR T Administration of the NSCLC Organ-Chips

Steps

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Warm the required volume of complete M-199 media (4 mL per chip) at 37°C:
• Total volume required = 6 mL per chip (4 mL during administration + 2 mL post-administration flow)
• Add chemokines if being used:
<table>
<tr><th>Chemokine</th><th>Quantity</th></tr>
<tr><td>CXCL9</td><td>100 ng/mL</td></tr>
<tr><td>CXCL10</td><td>100 ng/mL</td></tr>
<tr><td>CXCL11</td><td>10 ng/mL</td></tr>
<tr><td>CCL2</td><td>100 ng/mL</td></tr>
<tr><td>Fractalkine</td><td>10 ng/mL</td></tr>
</table></td></tr>
<tr><td>2</td><td>Add 4 mL of plain, complete M-199 media or complete M-199 media with chemokines to the inlet of the top channel of each Pod.</td></tr>
<tr><td>3</td><td>Mix CAR T dosing solutions (cell suspensions in buoyancy media) with serological pipettes.</td></tr>
<tr><td>4</td><td>Add 1.2 mL of CAR T dosing solution to the inlet of the bottom channel of each Pod.</td></tr>
<tr><td>5</td><td>For controls not receiving CAR T, add 4 mL of EGM-MV2 complete media into the bottom inlet Pod reservoir.</td></tr>
<tr><td>6</td><td>Set Zoë flow rates to 1,000 µL/h for top and bottom channels.</td></tr>
<tr><td>7</td><td>Prior to starting flow, bring trays of Pods to the biosafety cabinet and, using a P1000 pipette, mix the buoyancy media in the bottom inlet twice (avoiding adding bubbles to the solution).</td></tr>
<tr><td>8</td><td>Return Pods to Zoës and run flow for 50 min (no more than 1 h or the channels will run dry). CAR T should flow through the bottom channel evenly distributed in buoyancy media, as shown in Figure 7. Continue directly to “Recirculation of CAR T During Administration,” below.</td></tr>
</table>

Figure 7



Representative immunofluorescent image of CAR T (red) density in buoyancy media at 1 x 10^6 cells/mL in the bottom channel. Dotted lines represent margins of chip channel.

Recirculation of CAR T During Administration

Introduction

The Gelzan component of buoyancy media allows for distributed cell suspension while not under flow; however, cells suspended in buoyancy media have been observed to float increasingly over the course of approximately 2 h. To combat any uneven cell distribution, we recommend pausing flow briefly to mix the suspension by recirculating the CAR T suspension. As buoyancy media is more viscous than standard cell culture media, the flow rate will be slightly lower, so expect less volume in the bottom channel outlet than the top channel outlet after each period of flow at 1,000 µL/h.

Technique Notes

- Flow is paused every 50 min as a safety factor to allow for a buffer volume in the inlet reservoirs. This buffer volume mitigates the risk of flowing the channels dry and causing damage to Zoë.
- Avoid extending the time Pods are off Zoë flow for this step. The lack of physiologically relevant shear over time may lead to over-attachment of CAR T, regardless of experimental condition.
- Ensure that mixing and recirculation is done consistently between trays and operators (i.e. similar pipetting speed and technique).
- The ideal speed of recirculation is < 5 seconds per Pod.

Steps

Step	Action
1	After 50 min of CAR T administration at 1,000 µL/h, pause flow on Zoës. Stopping flow on Zoës prevents Pod inlets from running dry if there is a pipetting error while dosing the inlets.
2	Bring one tray of Pods at a time to the biosafety cabinet (avoid time of Pods outside incubator).
3	Working one Pod at a time, use a P1000 pipette to mix the buoyancy media in the bottom outlet reservoir twice, avoiding adding air bubbles into the solution. **Note:** Avoid tilting the Pods excessively to prevent drying of the Pod Vias
4	Transfer outlet volume of buoyancy media to bottom inlet reservoir, while avoiding introducing air into the solution. Then, placing the P1000 tip into the buoyancy media, **mix two more times**.
5	Aspirate the top outlet reservoir, leaving behind ~200 µL to prevent drying of Pod Vias.
6	Continue with remaining Pods.
7	Once complete, return trays of Pods back to Zoë and resume flow at 1,000 µL/h.
8	Repeat this process every 50 min for a total of 4 h of administration.

Media Flow and Regulate Post-Administration

Description

This step washes out CAR T buoyancy media suspension from the bottom channel to remove any un-attached CAR T.

CAUTION

Ensure that flow rates are changed from 1,000 μL/h to 30 μL/h to avoid damage to Pod or instrument.

Steps

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>After CAR T administration, perform a media flush to remove non-adhered CAR Ts:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Aspirate both the top and bottom Pod inlets and outlets.</td></tr>
<tr><td>2</td><td>Aliquot 3 mL of EGM-MV2 complete media and complete M-199 media (+ chemokines when used) per chip.</td></tr>
<tr><td>3</td><td>Connect 50 mL conical tubes of media to Steriflip units and warm to 37°C for ≥ 1 h. Equilibrate following the procedure outlined in “Gas Equilibration of Media” steps in “Day 1: Connection.”</td></tr>
<tr><td>4</td><td>Add EGM-MV2 complete media to Pod bottom inlet.
• For groups receiving IL-2, add 12.7 ng/mL of IL-2 to EGM-MV2 complete media to flow through until Day 6 (24 h post-administration).</td></tr>
<tr><td>5</td><td>Add top channel media (complete M-199 media + chemokines where used) to Pod top inlet.</td></tr>
<tr><td>6</td><td>Flow both channels at 1,000 μL/h for 30 min.</td></tr>
<tr><td>7</td><td>Following the 30 min flush, aspirate the bottom and top outlet reservoirs of the Pods. Ensure that at least 200 μL of media remains in the outlets to cover the Pod outlet Vias.</td></tr>
</table></td></tr>
<tr><td>2</td><td>After flush, set flow rates for top and bottom channels to 30 μL/h (0% stretch).</td></tr>
<tr><td>3</td><td>Start a Regulate Cycle to run overnight.</td></tr>
</table>

Day 6: 24 h Post Administration

Overview

Steps

Step	Action
1	Pause flow on Zoës: <table><tr><th>Step</th><th>Action</th></tr><tr><td>1</td><td>Pause Zoë by pressing the silver “Activation” button located above the tray bays.</td></tr><tr><td>2</td><td>Slide the tray out of the bay and transfer to the BSC.</td></tr></table>
2	Perform Via washes on Pod inlet and outlet Vias for both the top and bottom channels (see “Day 2: Pod Maintenance and Second Regulate”).
3	Collect effluent from all Pods according to the steps provided in the “Chip Sampling Readouts” section of this protocol.
4	Aspirate Pod outlets for both the top and bottom channels, leaving behind at least 200 µL to cover the Pod outlet Vias.
5	For IL-2 groups, aliquot 3 mL per chip of EGM-MV2 media into 50 mL conical tubes.
6	Connect the 50 mL conical tubes of media to Steriflip units and warm to 37°C for ≥ 1 h. Equilibrate following procedure outlined in the “Gas Equilibration of Media” steps in “Day 1: Connection.”
7	For IL-2 groups, add 3 mL plain EGM-MV2 complete media to the bottom inlet and complete M-199 media with chemokines to the top inlet to flow through until the 48 h takedown.
8	Start a Regulate Cycle with flow set at 30 µL/h for both the top and bottom channels.
Note	The current protocol recommends maintaining media flow for 48 h to observe killing response. However, the user can reduce or extend this duration for desired endpoints.

Optional: Extraction of Cells for Flow Cytometry (24- or 48-hour Endpoint)

Introduction

After administration of CAR T, cells can be extracted from the top and bottom channels of the Organ-Chips to assess expression of CAR T surface or intracellular markers (i.e., exhaustion and activation) via flow cytometry. Note that this readout is a terminal endpoint, and dedicated Organ-Chips will need to be designated for this readout. We recommend running this readout at 24- or 48-h post-CAR T administration.

Surface Markers

We would recommend a combination of the following surface markers for evaluating CAR T killing activation and response:

Surface Marker	Fluorophore	Dilution
Live Dead	BV421	1:100
CD223 (LAG-3)	AF488	1:50
PD-1	PE	1:200
CD8	PE/Dazzle 594	1:200
CD152 (CTLA-4)	PerCP/Cy5.5	1:50
CD4	AF700	1:200
CD366 (Tim-3)	BV785	1:50

Procedure

The procedure follows steps from Fixation and Immunofluorescence (IF) Staining (EP137) for washing and staining chips outside of Pod.

Required Materials

Material Name	Model Information
15 mL conical tubes (sterile)	--
96-well V-bottom plates	--
Aspirator and sterile tips	--
Dulbecco's PBS (DPBS - / -) (without Ca2+, Mg2+)	Corning, Cat D8537
EGM-MV2 complete media	See "Preparation of Materials" section
Complete M-199 media	See "Preparation of Materials" section
Microscope (with camera)	For bright-field imaging
P20, P200, P1000 pipettes and filter tips	--
P200 multichannel pipette	--
Serological pipettes	--
Square Cell Culture Dish (120 x 120 mm)	VWR, Cat 82051-068, Sterile, 1 per 6 chips
TrypLE™ Express Enzyme (1X)	Thermo Fisher Scientific, Cat 12604013
Trypsin-EDTA (0.25%)	(optional)

Steps

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Prepare digestion solution:
<table><tr><th>Step</th><th>Action</th></tr><tr><td>1</td><td>Create an aliquot of TrypLE™ Express for 500 µL per chip.</td></tr><tr><td>2</td><td>Warm digestion solution to 37°C.</td></tr></table></td></tr>
<tr><td>2</td><td>Aliquot 500 µL per chip of respective top and bottom channel media, keep at 4°C.</td></tr>
<tr><td>3</td><td>Prepare microcentrifuge collection tubes for each chip:
<table><tr><th>Step</th><th>Action</th></tr><tr><td>1</td><td>For bottom channel, label a microcentrifuge tube for each chip and add 200 µL of bottom channel media.</td></tr><tr><td>2</td><td>Repeat for top channel, for each chip, adding 200 µL of top channel media.</td></tr></table></td></tr>
<tr><td>4</td><td>Spray the container of ice with 70% ethanol and place it in biosafety cabinet.</td></tr>
<tr><td>5</td><td>Wash chips with PBS on Zoë:
<table><tr><th>Step</th><th>Action</th></tr><tr><td>1</td><td>Remove Pod trays from Zoë and move them to the biosafety cabinet.</td></tr><tr><td>2</td><td>Aspirate Pod inlet reservoirs.</td></tr><tr><td>3</td><td>Add 300 µL of DPBS to bottom and top inlet reservoirs.</td></tr><tr><td>4</td><td>Return Pods to Zoë and set the flow rate to 1,000 µL/hr for both channels.</td></tr><tr><td>5</td><td>Run flow for 10 min to flush PBS through channels.</td></tr><tr><td>6</td><td>Stop flow and remove Pod trays from Zoë. Move them to the biosafety cabinet.</td></tr></table>Note: DPBS wash can also be performed manually (off-Zoë); however, the high pipetting shear may detach some cells.</td></tr>
<tr><td>6</td><td>Ensure all chip carriers are labeled and identify the different conditions clearly. Detach chips from Pods and organize them in square-well plates or petri dishes for handling.</td></tr>
<tr><td>7</td><td>Place 200 µL tips gently in the outlets of both channels. Be careful not to push the tips too hard against the bottom of the chip channel, as this could seal off the outlet and prevent reagents from going through the channel and outlet.</td></tr>
<tr><td>8</td><td>Add 200 µL each warm TrypLE to top and bottom channels with P200 pipette, leaving all the pipette tips in place.</td></tr>
<tr><td>9</td><td>Incubate at 37°C incubator for 5 min.</td></tr>
<tr><td>10</td><td>Starting with the bottom channel, connect a P200 pipette to the collection tips in the chip ports and push up and down repeatedly to dislodge cells, avoiding wetting the tip filter.</td></tr>
<tr><td>11</td><td>Check under the microscope to assess if most cells have been dislodged from the channel.
<table><tr><th>Step</th><th>Action</th></tr><tr><td>1</td><td>If there is insufficient dislodging of cells:
Collect volume inside the channel into a microcentrifuge tube</td></tr></table></td></tr>
</table>

<table>
<tr><td rowspan="3"></td><td></td><td>• Repeat steps 7–11.
Note: Ensure bottom and top channel suspensions are kept separate.
• Repeat for all bottom channels.</td></tr>
<tr><td>2</td><td>Repeat Steps 7–10 for all top channels.</td></tr>
<tr><td>3</td><td>If almost all of the cells are dislodged, continue to next step.</td></tr>
<tr><td>12</td><td colspan="2">Image the chips on the microscope to evaluate extraction efficiency. Repeat steps 7–9 if necessary.

Note: If cells are not detaching from chip after two rounds (or more than 20 min) of TrypLE digestion, refer to the section A in the Appendix.</td></tr>
<tr><td>13</td><td colspan="2">Centrifuge collection tubes to pellet cells (centrifugal speed dependent on cell type, typically 300 x g for 5 min is sufficient).</td></tr>
<tr><td>14</td><td colspan="2">Place collection tubes on ice for staining for flow cytometry.</td></tr>
</table>

Day 7: Takedown (Caspase Staining and Fixation)

Takedown of Chips for Immunofluorescence Imaging Readouts

Introduction

Termination of live samples in this protocol includes:

- Routine effluent collection
- Live staining of chips with CellEvent Green (off-Zoë)
- Fixation of chips with 4% PFA (off-Zoë)

Takedown, including live staining of caspase and PFA fixation, is recommended to be done Off-Zoë (i.e., manually with pipette) to ensure that each chip is stained thoroughly to fully capture the sensitivity of the caspase CellEvent Green dye.

Contents

Topic	See Page
Live Staining with Caspase Reagent (OFF-Zoë)	64
PFA Chip Fixation (OFF-Zoë)	65

Required Materials

Material Name	Model Information
15 mL conical tubes (sterile)	--
37°C water or bead bath	--
50 mL conical tubes (sterile)	--
70% ethanol and wipes	For surface sterilization
96-well V-bottom plates	--
Aspirator and sterile tips	--
Dulbecco's PBS (DPBS - / -) (without Ca2+, Mg2+)	Corning, Cat D8537
EGM-MV2 complete media	See "Preparation of Materials" section
Complete M-199 Media	See "Preparation of Materials" section
Microscope (with camera)	For bright-field imaging
Orb-HM1 Hub Module	Emulate, 1 per 4 Zoës
P20, P200, P1000 pipettes and filter tips	--
Paraformaldehyde (PFA, 4%)	--
PBS + 0.05% Sodium Azide (optional)	Teknova, Cat P0202
Pen-Strep	Millipore Sigma, Cat P4333
Primocin	VWR, MSPP-ANTPM1
Serological pipettes	--

Square Cell Culture Dish (120 x 120 mm)	VWR, Cat 82051-068, Sterile, 1 per 6 chips
Water bath (or beads)	Set to 37°C

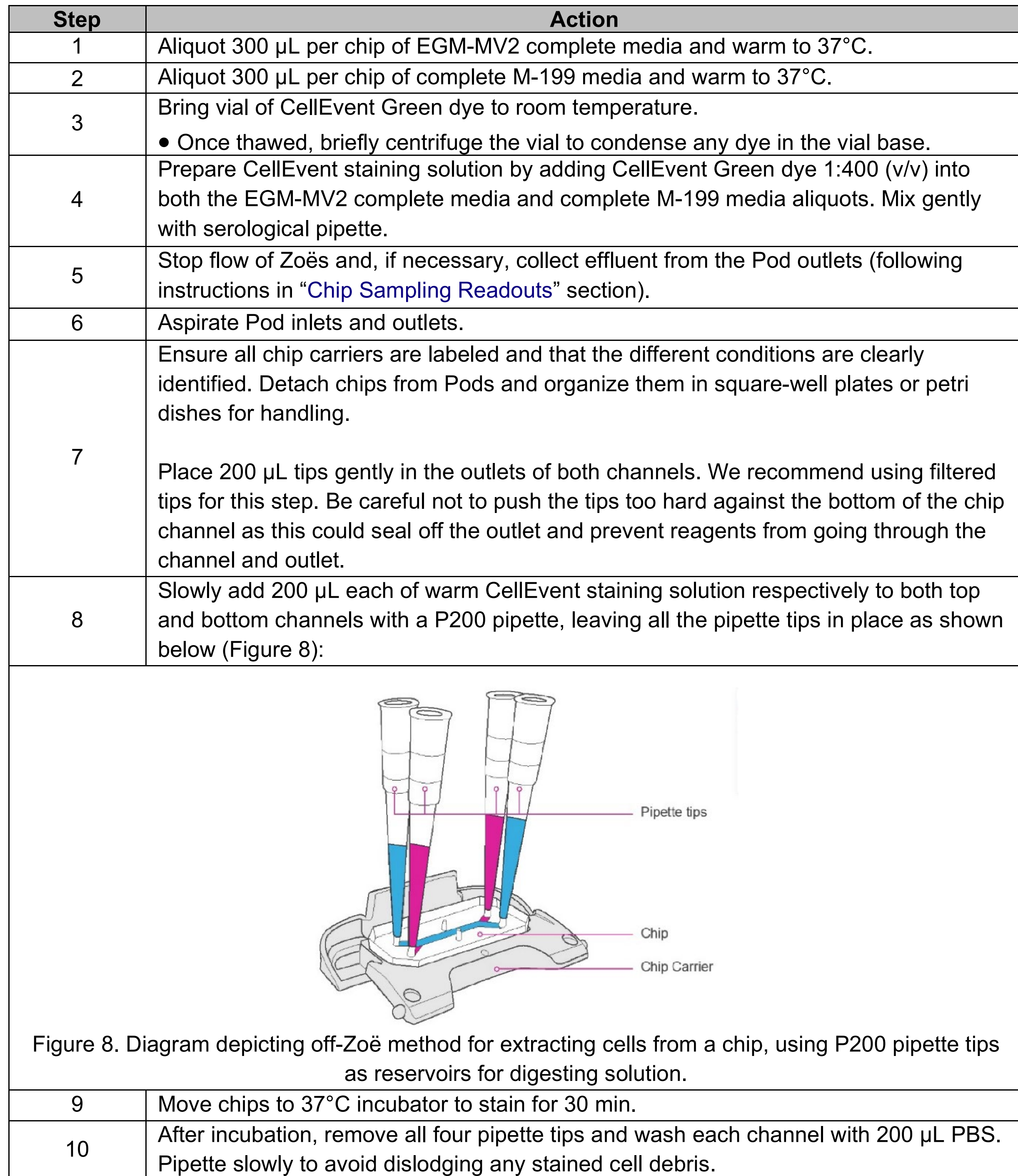
Note

Takedown OFF-Zoë is recommended for caspase live staining on chip. When pipetting, it is recommended to utilize gravity as much as possible to pull the solution through the channels instead of pushing solution through with the pipette to limit shear stress on live cells.

Live Staining with Caspase Reagent (OFF-Zoë)

Steps

Note: Complete washes with pipette slowly to avoid dislodging cells from channels.

Step	Action
1	Aliquot 300 µL per chip of EGM-MV2 complete media and warm to 37°C.
2	Aliquot 300 µL per chip of complete M-199 media and warm to 37°C.
3	Bring vial of CellEvent Green dye to room temperature. ● Once thawed, briefly centrifuge the vial to condense any dye in the vial base.
4	Prepare CellEvent staining solution by adding CellEvent Green dye 1:400 (v/v) into both the EGM-MV2 complete media and complete M-199 media aliquots. Mix gently with serological pipette.
5	Stop flow of Zoës and, if necessary, collect effluent from the Pod outlets (following instructions in "Chip Sampling Readouts" section).
6	Aspirate Pod inlets and outlets.
7	Ensure all chip carriers are labeled and that the different conditions are clearly identified. Detach chips from Pods and organize them in square-well plates or petri dishes for handling. Place 200 µL tips gently in the outlets of both channels. We recommend using filtered tips for this step. Be careful not to push the tips too hard against the bottom of the chip channel as this could seal off the outlet and prevent reagents from going through the channel and outlet.
8	Slowly add 200 µL each of warm CellEvent staining solution respectively to both top and bottom channels with a P200 pipette, leaving all the pipette tips in place as shown below (Figure 8):
 Figure 8. Diagram depicting off-Zoë method for extracting cells from a chip, using P200 pipette tips as reservoirs for digesting solution.	
9	Move chips to 37°C incubator to stain for 30 min.
10	After incubation, remove all four pipette tips and wash each channel with 200 µL PBS. Pipette slowly to avoid dislodging any stained cell debris.

PFA Chip Fixation (OFF-Zoë)

Steps

Step	Action
1	Place 200 µL tips gently in the outlets of both channels. We recommend using filtered tips for this step. Be careful not to push the tips too hard against the bottom of the chip channel, as this could seal off the outlet and prevent reagents from going through the channel and outlet.
2	Slowly add 200 µL of 4% paraformaldehyde into both channels of the chips. Let them sit for 15 min at 37°C.
3	Remove all four pipette tips and wash each channel twice with 200 µL PBS using a P200 pipette.
4	Place large droplets of PBS in all four ports and add PBS to the central reservoir of the Chip Cradle (if using) or square well plate to prevent drying.
5	Seal samples well and store chips in PBS at 4°C. Protect from light. • For long-term storage, store chips in PBS + 0.05% sodium azide solution at 4°C.

Adapting the Workflow to New CAR T, Targets, and Co-Therapies

Introduction

Adaptation Overview

Optimizing epithelial target cell, matched endothelial cells & associated priming stimulus

This workflow can be adapted to various target cancer cells to rank order CAR T clones and co-therapeutics of interest through migratory capability and killing efficacy of CAR T. Below are guidelines for adapting the workflow for new CAR-T/Target pairs based on the empirical approach to setting up the HER-2 CAR-T/A549 workflow. We specifically emphasize the process of selecting and optimizing extracellular matrix, priming factors, and endothelial cells that will create a model that is congruent with the cancer type of interest.

This section includes:

- Part 1. Plate Study Validation (if using alternative CAR T)
- Part 2: On-Chip Protocol Development (1 CAR T type)
- Part 3. On-Chip Validation Model (2 CAR T types)
- Part 4. On-Chip Co-Therapeutic Screening Mode

Part 1. Plate Study Validation (if using alternative CAR T)

Introduction

The experimental design outlined in the table below provides a complete set of controls to properly validate the application before executing an on-chip study. It is recommended to start with a plate study with the target (tumor) cells of interest to allow for rapid iteration of conditions.

Goals

The goals of these plate-based validation studies are to:

1. Ensure targeted CAR T cells can demonstrate measurable killing response when directly added to antigen-expressing target cells.
2. Ensure non-targeted CAR T cells do not demonstrate measurable killing response.
3. Assess any baseline effects of cytokine priming on potency of CAR T.
4. Assess functionality of co-therapies of interest when CAR T cells are directly interacting with the antigen.
5. Confirm Caspase dyes show adequate signal.

Additional Considerations

To treat target cells with media containing cytokines, it is recommended to follow the same treatment timeline as the on-chip studies (i.e., cytokine treatment for 24 h pre-administration).

Continued on next page

Part 1. Plate Study Validation (if using alternative CAR T), Continued

Plate Study 1: Epithelial Tumor Cell Seeding Density

Epithelial cells:

- Choose epithelial cells that are sourced from the organ of interest and known in the literature to express the target antigen.

Top channel (epithelial) chemokines:

- The ability of the anti-tumor immune response relies on the balance of chemokines directing their adhesion and migration. Chemokines added to the epithelial channel during administration facilitate migration of CAR T to the antigen-containing epithelium. Use literature as guidance to assess the optimal chemokine cocktail for T cells that are relevant to the solid tumor of interest.

Plate Study 1 — Epithelial Tumor Cell Seeding Density					
Group	**Description**	**Cell Type**	**Cell Density**	**Chemokines**	**# of wells**
1	Density 1 Resting	Tumor Epithelial	Density 1	**-**	≥ 3
2	Density 1 Primed	Tumor Epithelial	Density 1	**+**	≥ 3
3	Density 2 Resting	Tumor Epithelial	Density 2	**-**	≥ 3
4	Density 2 Primed	Tumor Epithelial	Density 2	**+**	≥ 3
5	Density 3 Resting	Tumor Epithelial	Density 3	**-**	≥ 3
6	Density 3 Primed	Tumor Epithelial	Density 3	**+**	≥ 3
				Total	≥ 18

Procedure:

- Day 0: Seed epithelial tumor cells into cell culture plate and maintain in culture.
- Day 4: Perform epithelial chemokine treatment.
- Day 7: (takedown) perform readouts.

Readouts:

- Caspase staining and imaging.
- Optional: Nucleic staining.
- Optional: Epithelial functional markers (specific to cell type).

Success Criteria:

- One or more density of tumor epithelial cells forms a healthy monolayer by Day 3 in culture.
- One or more density of tumor epithelial cells shows low caspase background.
- Chemokine treatment does not cause excessive inflammation as measured by increased caspase signal and/or reduced nucleic staining.

Continued on next page

Part 1. Plate Study Validation (if using alternative CAR T), Continued

Plate Study 2: Endothelial Cell Seeding Density and Activation

Endothelial cells:

- To model primary tumor, select tissue-matched endothelial cells that are sourced from the organ of interest (e.g., for a colorectal cancer cell target, use primary colon microvascular endothelial cells).

Endothelial cell cytokine treatment:

- By using literature as a guide, choose the optimal cytokine priming for endothelium. The priming of endothelial cells in the bottom channel upregulates adhesion molecules to allow CAR T attachment. Most likely, TNFα is an appropriate cytokine as it is relevant in most immune cell recruitment pathways; however, there are some exceptions.

Plate Study 2 – Endothelial Cell Seeding Density and Activation					
Group	**Description**	**Cell Type**	**Cell Density**	**Endothelial Cytokine Treatment**	**# of wells**
1	Density 1 Resting	Endothelial	Density 1	**-**	≥ 3
2	Density 1 Primed	Endothelial	Density 1	**+**	≥ 3
3	Density 2 Resting	Endothelial	Density 2	**-**	≥ 3
4	Density 2 Primed	Endothelial	Density 2	**+**	≥ 3
5	Density 3 Resting	Endothelial	Density 3	**-**	≥ 3
6	Density 3 Primed	Endothelial	Density 3	**+**	≥ 3
				Total	**≥ 18**

Procedure:

- Day 0: Seed endothelial cells into cell culture plate and maintain in culture.
- Day 4: Perform endothelial cytokine treatment.
- Day 7: (takedown) perform readouts.

Readouts:

- Caspase staining and imaging.
- Immunofluorescent staining for adhesion molecules (e.g., ICAM-1, LFA-1, VCAM-1, or PECAM-1).
- Optional: Endothelial functional markers (e.g., CD31, VE-Cadherin).
- Optional: Nucleic staining.

Success Criteria:

- One or more density of endothelial cells shows low caspase background.
- Cytokine treatment increases endothelial adhesion markers but does not cause excessive inflammation as measured by increased caspase signal and/or reduced nucleic staining.

Continued on next page

Part 1. Plate Study Validation (if using alternative CAR T), Continued

Plate Study 3: Target Expression on Epithelial Tumor Cells

Plate Study 3 – Target Expression on Epithelial Tumor Cells					
Group	**Description**	**Cell Type**	**Cell Density**	**Chemokines**	**# of wells**
1	Density 1 Resting	Tumor Epithelial	Density 1	**-**	≥ 3
2	Density 1 Primed	Tumor Epithelial	Density 1	**+**	≥ 3
3	Density 2 Resting	Tumor Epithelial	Density 2	**-**	≥ 3
4	Density 2 Primed	Tumor Epithelial	Density 2	**+**	≥ 3
5	Density 3 Resting	Tumor Epithelial	Density 3	**-**	≥ 3
6	Density 3 Primed	Tumor Epithelial	Density 3	**+**	≥ 3
				Total	**≥ 18**

Procedure:

- Day 0: Seed epithelial tumor cells into cell culture plate and maintain in culture.
- Day 4: Perform epithelial chemokine treatment.
- Day 7: (takedown) perform readouts.

Readouts:

- Flow cytometry or immunofluorescent staining for antigen of interest.
- Note: Antigen expression can also be assessed through RNA-sequencing and Proteomics.

Success Criteria:

- Antigen is present on epithelial tumor cells at one or more densities tested.
- Chemokine treatment does not significantly alter expression of antigen.

Continued on next page

Part 1. Plate Study Validation (if using alternative CAR T), Continued

Plate Study 4: CAR T Killing Efficacy on Epithelial Tumor Cells

CAR T Density:

- Optimal effector-to-target ratio ("CAR T Density") will vary depending on the efficiency of CAR T killing. It is recommended to assess a range of effector-to-target ratios to model a curve of CAR T functionality. Additionally, a few extra seeded wells of target cells can be used to digest and count to have an accurate target cell count per well.

Plate Study 3 – Target Expression on Epithelial Tumor Cells							
Group	**Description**	**Cell Type**	**CAR T**	**CAR T Density**	**Chemokines**	**Co-Therapeutic Treatment**	**# of wells**
1	Resting control	Tumor Epithelial	-	-	-	-	≥ 3
2	Resting + CAR T #1	Tumor Epithelial	CAR T #1	Ratio #1	-	-	≥ 3
3	Primed + CAR T #1	Tumor Epithelial	CAR T #1	Ratio #1	+	-	≥ 3
4	Primed + CAR T #1	Tumor Epithelial	CAR T #1	Ratio #1	+	Co-Therapeutic	≥ 3
5	Resting + CAR T #1	Tumor Epithelial	CAR T #1	Ratio #2	-	-	≥ 3
6	Primed + CAR T #1	Tumor Epithelial	CAR T #1	Ratio #2	+	-	≥ 3
7	Primed + CAR T #1	Tumor Epithelial	CAR T #1	Ratio #2	+	Co-Therapeutic	≥ 3
8	Resting + CAR T #1	Tumor Epithelial	CAR T #1	Ratio #3	-	-	≥ 3
9	Primed + CAR T #1	Tumor Epithelial	CAR T #1	Ratio #3	+	-	≥ 3
10	Primed + CAR T #1	Tumor Epithelial	CAR T #1	Ratio #3	+	Co-Therapeutic	≥ 3
						Total	≥ 30

Procedure:

- Day 0: Seed epithelial tumor cells into cell culture plate and maintain in culture.
- Day 4: Perform epithelial chemokine treatment.
- Day 5: Thaw CAR T (optional: stain with CellTracker dye) and administer onto wells suspended in epithelial (top) channel media.
- Day 7: (takedown) perform readouts.

Readouts:

- Caspase staining and imaging.
- If applicable: CellTracker immunofluorescent imaging.
- Optional: Nucleic staining.

Success Criteria:

- One or more ratio of CAR T demonstrates measurable killing response when directly added to tumor epithelial cells.
- Depending on mechanism of action of the co-therapeutic, CAR T killing is enhanced or unchanged, as measured by caspase or nucleic signal.

Part 2: On-Chip Protocol Development (1 CAR T type)

Introduction

To establish an on-chip protocol to assess CAR T recruitment and killing, below is a study design to test tumor-specific chemokine cocktails for CAR T recruitment (cocktail #1 and #2) and test optimal seeding density for epithelial cells (i.e., avoid overgrowth).

- Control group 1 establishes the baseline of caspase and cytokine release without CAR T treatment.
- Group 2 determines the primary signal of CAR T without priming stimulus (cytokines and chemokines), which is used as a comparator for the recruitment response with addition of endothelial cytokine treatment and top channel chemokine cocktails #1 and #2 (groups 3 and 4).
- Groups 5–8 allow for a similar comparison on epithelial Density #2.

To improve statistical significance in results and reduce variability, it is recommended to use n=4 chips per condition.

Chip Study 1: Establishing CAR T Controls (1 CAR T)

Chip Study 1 – Establishing CAR T Controls (1 CAR T)							
Group	**Description**	**Epithelial Cell Density**	**Endothelial Treatment (bottom channel)**	**Chemo-kines (top channel)**	**Chemokine Cocktail**	**CAR T**	**# of Chips**
1	Resting control	Density #1	-	-	-	-	4
2	Resting + CAR T #1		-	-	-	+	4
3	Primed + CAR T #1		+	+	Cocktail #1	+	4
4	Resting + CAR T #1		+	+	Cocktail #2	+	4
5	Resting control	Density #2	-	-	-	-	4
6	Primed + CAR T #1		-	-	-	+	4
7	Resting + CAR T #1		+	+	Cocktail #1	+	4
8	Primed + CAR T #1		+	+	Cocktail #2	+	4
						Total	**32**

Procedure:

- Day 0: Seed tumor epithelial cells and endothelial cells.
- Day 4: Perform epithelial chemokine treatment and initiate endothelial cytokine priming.
- Day 5: Thaw CAR T, then stain CAR T with CellTracker dye and administer onto chip.
- Day 7: (takedown) perform readouts.

Readouts

- Caspase staining and imaging.
- CellTracker immunofluorescent imaging.

Success Criteria:

- Demonstrate robust recruitment of CAR T from the endothelial (bottom) channel into the tumor epithelial (top) channel with cytokine and chemokine treatment compared to resting CAR T control groups.
- Targeted CAR T demonstrate measurable caspase killing response of the target tumor epithelial cells.
- Tumor epithelial cells for resting control for a seeding density tested (group 1 or group 5) shows monolayer formation by Day 4 and minimal overgrowth (caspase background) by Day 7.

Part 3. On-Chip Validation Model (2 CAR T types)

Introduction

The experimental design outlined in the table below provides a complete set of controls to properly validate the application in house in a 24-chip, 2-Zoë experiment.

- Groups 1 and 2 do not include CAR T administration and provide validation that the priming regime does not negatively impact the physiology of the model. It also captures any downstream cytokine production (by the epithelial and endothelial cells) that might be induced by priming. This is necessary to determine the degree to which cytokines detected in CAR T-containing groups can or cannot be directly attributed to the CAR T (i.e., versus priming).
- Groups 3 and 4 determine the primary signal, >4-fold selective recruitment of CAR T #1 on resting (Group 3) versus primed (Group 4) chips.
- Groups 5 and 6 use CAR T #2 on primed versus resting chips to compare responses.

To improve statistical significance in results and reduce variability, it is recommended to use n=4 chips per condition.

Establishing CAR T Controls Comparing 2 CAR T

Chip Study 2: Establishing CAR T Controls Comparing 2 CAR T					
Group	**Description**	**Endothelial Treatment (bottom channel)**	**Chemokines (top channel)**	**CAR T**	**# of Chips**
1	Resting control	-	-	-	**4**
2	Primed control	+	+	-	**4**
3	Resting + CAR T #1	-	-	+	**4**
4	Primed + CAR T #1	+	+	+	**4**
5	Resting + CAR T #2	-	-	+	**4**
6	Primed + CAR T #2	+	+	+	**4**
				Total	**24**

Procedure:

- Day 0: Seed tumor epithelial cells and endothelial cells.
- Day 4: Perform epithelial chemokine treatment and initiate endothelial priming.
- Day 5: Thaw CAR T, stain with CellTracker dye, and administer onto chip.
- Day 7: (takedown) perform readouts.

Readouts:

- Caspase staining and imaging.
- CellTracker immunofluorescent imaging.

Success Criteria:

- Demonstrate robust, priming-specific recruitment of CAR T from the endothelial (bottom) channel into the tumor epithelial (top) channel.
- Targeted CAR T cells demonstrate measurable Caspase killing response of the target tumor epithelial cells.

Part 4. On-Chip Co-Therapeutic Screening Mode

Introduction

Once the application has been validated in house, a modified experimental design can be implemented for screening of co-therapeutic "A" and "B", as indicated below.

In this example, a 36-chip, 3-Zoë experiment includes:

- Groups 1–3: Assess CAR T recruitment on resting and primed chip in the absence of therapeutic. Group 3 sets the primary response signal of recruitment on the primed chip.
- Groups 4–5: Recapitulate group 3 with the addition of two different co-therapies to assess killing responses.
- Groups 6–9: Test the same controls with CAR T #2. Groups 8–9: Assess therapeutic effects of co-therapies "A" and "B".

To improve statistical significance in results and reduce variability, it is recommended to use n=4 chips per condition.

On-Chip Co-Therapeutic Screening

Chip Study 3 – On-Chip Co-Therapeutic Screening						
Group	**Description**	**Endothelial Treatment (bottom channel)**	**Chemokines (top channel)**	**CAR T**	**Co-Therapy**	**# of Chips**
1	Resting control	-	-	-	-	**4**
2	Resting + CAR T #1	-	-	+	-	**4**
3	Primed + CAR T #1	+	+	+	-	**4**
4	Primed + CAR T #1	+	+	+	A	**4**
5	Primed + CAR T #1	+	+	+	B	**4**
6	Resting + CAR T #2	-	-	+	-	**4**
7	Primed + CAR T #2	+	+	+	-	**4**
8	Resting + CAR T #2	-	+	+	A	**4**
9	Primed + CAR T #2	+	+	+	B	**4**
					Total	**36**

Procedure:

- Day 0: Seed tumor epithelial cells and endothelial cells.
- Day 4: Perform epithelial chemokine treatment and initiate endothelial cytokine priming.
- Day 5: Thaw CAR T and, if applicable, incubate with co-therapeutic(s) of interest. Stain with CellTracker dye, and administer onto chip.
- Day 7: (takedown) perform readouts.

Readouts:

- Caspase staining and imaging.
- CellTracker immunofluorescent imaging.

Success Criteria:

- Demonstrate robust, priming-specific recruitment of CAR T from the endothelial (bottom) channel into the tumor epithelial (top) channel.
- Targeted CAR T cells demonstrate measurable caspase killing response of the target tumor epithelial cells.
- Depending on mechanism of action of the co-therapeutic(s), CAR T killing is enhanced or unchanged, as measured by caspase and CAR T recruitment to the tumor epithelial (top) channel.

Troubleshooting

Overview

Recommendations

Procedure	Possible Issue	Troubleshooting Recommendations
Equilibration of Media	Media takes too long to pass through the Steriflip	Vacuum pressure is not reaching -70kPa. Need to find an alternate vacuum source.
NSCLC Organ-Chip Culture	A549 epithelium has not reached acceptable monolayer growth (i.e., patches and incomplete coverage) by Day 3 of protocol (TNFα and Cytokine Treatment)	Delay TNFα and Cytokine Treatment for another 24 h, continuing culture under flow to allow for increased growth. Perform brightfield imaging the next day and assess. Repeat for another 24 h if necessary. If coverage does not improve, this is indicative of poor epithelial seeding, and the experiment will have to be repeated.
NSCLC Organ-Chip Culture	HMVEC-L endothelium has not reached acceptable monolayer growth (i.e., patches and incomplete coverage, or excessive cell debris) by Day 3 of protocol (TNFα and Cytokine Treatment)	Delay TNFα and Cytokine Treatment for another 24 h, continuing culture under flow to allow for increased growth. Perform brightfield imaging the next day and assess. Repeat for another 24 h if necessary. If coverage does not improve, this is indicative of insufficient density of HMVEC-L at seeding, and the experiment will have to be repeated. Confirm viability of cells are >80% at seeding and increase seeding density over 8 x 10^6 cells/mL if needed.
Media Flow Pre-Administration	Bubbles present in either channel, or low effluent in Pod outlet reservoirs after overnight flow.	An air bubble or debris may be present in the chip, obstructing flow. This could be due to improper media equilibrating during connection of chip to Pod. After effluent collection, aspirate the Pod outlet reservoirs. Perform a media flush of affected Pods at 1,000 µL/h on Zoë for 5 min. If no media flows to the outlet, perform a manual disconnection: 1. Disconnect chips from Pods 2. Using a P200 pipette, pipette respective media for bottom and top channel through the chip, observing if any bubbles are dislodged. Leave droplets of media on the chip ports 3. Take disconnected Pods and run a Prime Cycle on Zoë 4. Follow steps in “Day 1: Connection” to reconnect chips to Pods
Media Flow Post-Administration	Low effluent in Pod outlet reservoirs after overnight flow	An air bubble or debris may be present in the chip, obstructing flow. Check for bubbles in Pod Via or in coculture channel and perform Via

		washes on the Pod. After effluent collection, ensure that >3 mL of equilibrated media is present in the Pod inlets. Perform a media flush at 1,000 µL/h on Zoë for 5 min. If no media remains in the outlet after flush, run a Regulate Cycle on Zoë to remove any lodged air in Pod.
CAR T Culture	Low yield of CAR T collected from culture flask	Collect as much suspension as possible using serological pipette. Add PBS at half the working volume of the flask. Incubate at 37°C for 10 min. Gently tap the side of the flask and collect, washing the flask during collection. Check the flask under brightfield to ensure that nearly all the CAR T have been collected (up to 10% adhesion expected).
CAR T Administration	CAR T count post-24-h incubation is lower than needed for 4 h of flow at 1M cells/mL	The Pod bottom inlet reservoir can be dosed with as little as 800 µL of cell suspension per chip to flow for 1 h, due to the higher viscosity of the administration suspension with buoyancy media.
Post-Administration Media Wash	Flow issues/No flow	In the case of flow issues or no flow after CAR T administration day, Pods may need to be re-Primed. After effluent collection, proceed to disconnect the chip with flow issues from its Pod. Add 300µL of complete M-199 media directly onto the top outlet Via of the Pod. Add 300 µL of EGM-MV2 media directly onto the bottom outlet Via of the Pod. Make sure all inlets in the Pod have their respective media. Run the Prime Cycle once in Zoë. Add a droplet of appropriate media to the top of each chip Via. Reconnect chips to Pods. Run the Regulate Cycle on all Zoës.
Extraction of cells for flow cytometry	Cells are not detaching from chip after two rounds (or more than 20 min) of TrypLE digestion	With TrypLE still inside the bottom and top channels, use a P200 pipette to actively pipette up and down inside each channel (avoid wetting the filters) to provide shear stress for detachment. If TrypLE digestion is still unsuccessful, collect any liquid from the chip into tubes containing media prepared in the "Optional: Extraction of Cells for Flow Cytometry (24- or 48-hour Endpoint)" section, then repeat the procedure with a stronger enzymatic digestion solution (i.e., Trypsin-EDTA (0.25%)

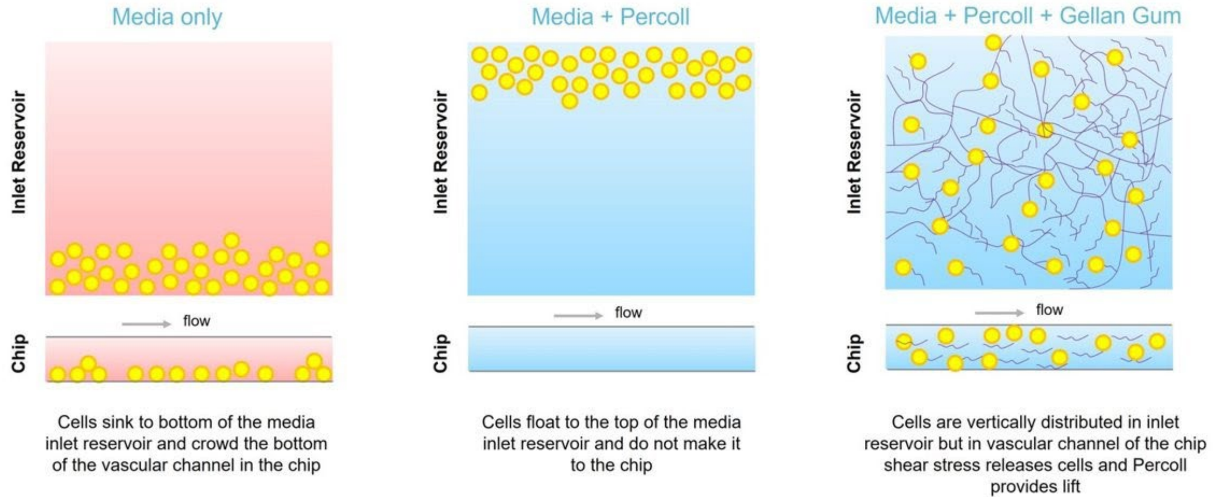
Appendix

Appendix A: Buoyancy Media

Buoyancy Media

The buoyancy media consists of cell culture media together with Percoll and Gellan Gum (Gelzan). The Percoll affords increased density to the media that prevents gravity-dependent sinking of CAR T to the bottom of the reservoir and channel. Gellan Gum provides a degree of cross-linking matrix that stabilizes the solution and promotes an even distribution of CAR T in the Pod reservoir (Figure 9). The Gellan Gum also is shear-thinning, which will release the CAR T from the matrix and allow them to float while under flow.

Figure 9



Scheme illustrating the functional rationale for the Buoyancy Media formulation (Media + Percoll + Gellan Gum).