emulate

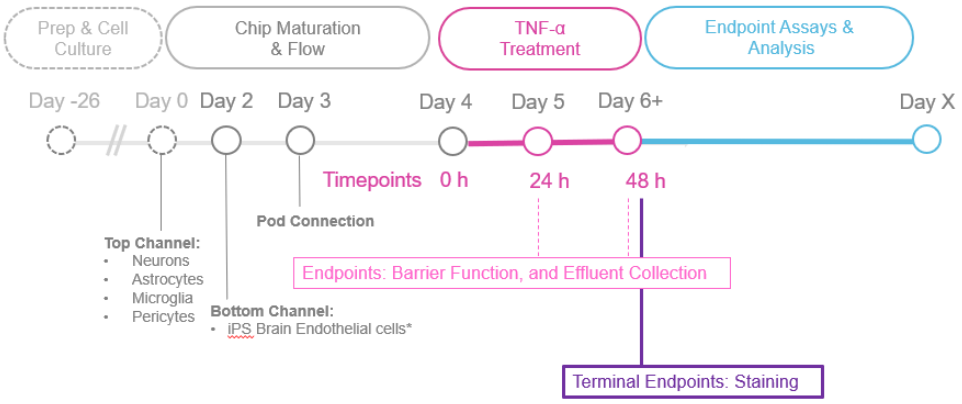
Brain-Chip
Neuroinflammation Protocol

EP213 Rev. A

Table of Contents

Experimental Overview

Experimental Overview – Neuroinflammation Application



**Brain Endothelial Cells will remain two days in flasks before the seeding on Chips (Day 2)*

NOTE: Follow all steps through Day 3 Chips to Pods and Pods to Zoë from the Brain-Chip Protocol (EP203), then continue below.

Day 4+: Chip Maintenance, Sampling, and TNF-α Treatment

Goals:

- Maintain chips in Zoë
- Inspect cell culture
- Collect samples for analysis
- Dose chips with TNF-α
- Assess barrier function

Key Steps:

- Observe cell morphology
- Maintenance
- Sample and replenish media
- Make appropriate concentration of TNF-α in medium

Required Materials:

- Chips in Pods
- Human Endothelium Serum-Free Media (SFM)

- Short-Term Maintenance Medium
- Recombinant Human TNF-α Protein
- DPBS (1x)
- Serological pipettes
- Pipettes, multichannel and filtered tips
- 37°C water or bead bath
- 70% ethanol
- 3KDa Dextran Cascade Blue
- 0.5KDa Lucifer Yellow
- 96 well tissue culture plates
- Optical Microscope

Observe Cell Morphology

1. Pause Zoë by pressing the silver “Activation” button.
2. Remove the trays and place them in the Biosafety Cabinet (BSC).
3. Visually inspect each chip for bubbles.
4. Using a microscope, inspect cells in the chips to assess morphology and viability. Capture representative images at 10x magnification, followed by 20x magnification at the following locations (see figure 1):
   - Inlet junction
   - Center of channel
   - Outlet junction

   Refer to figure 2 as a reference.

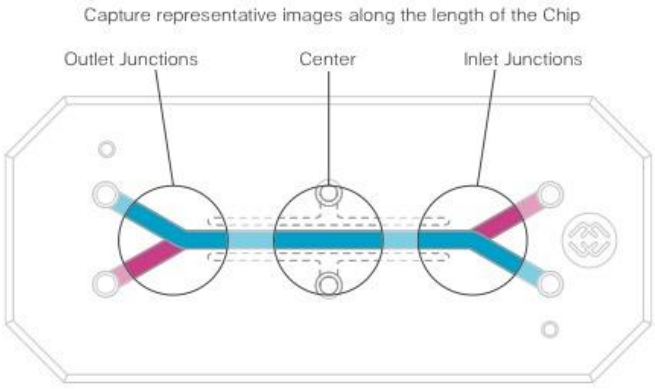


Figure 1: Chip image capture points

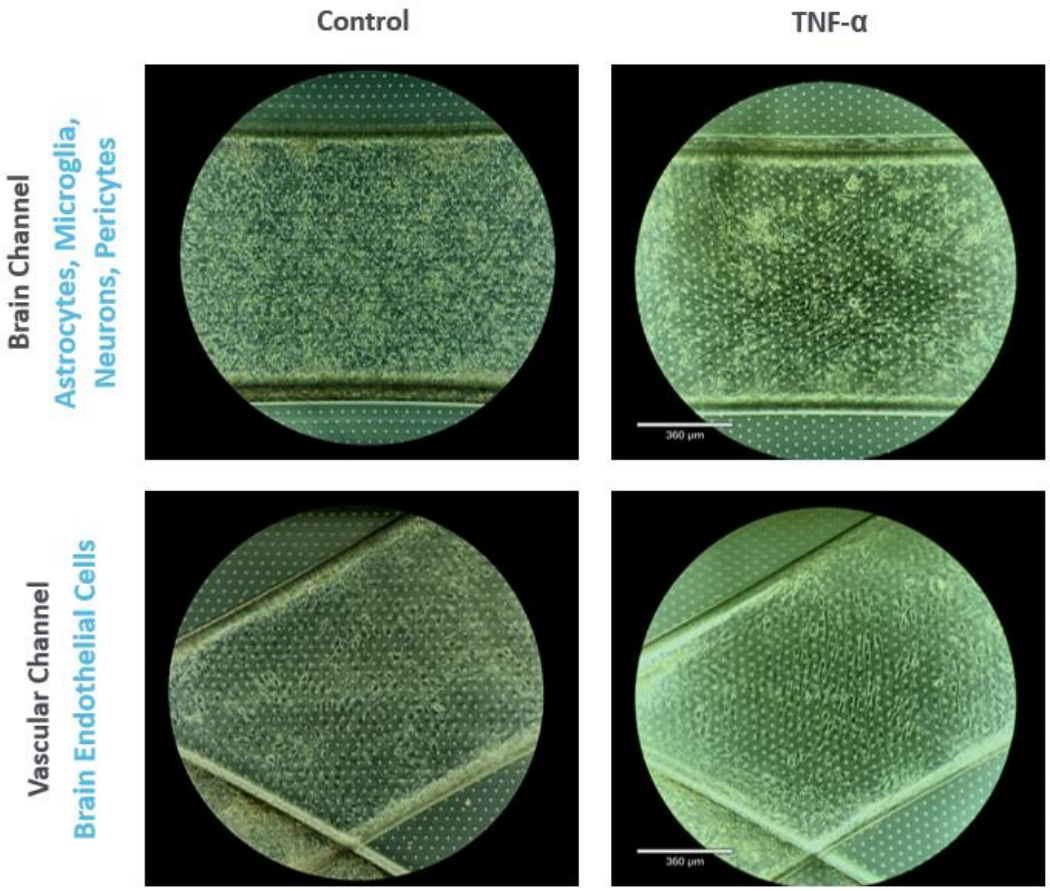


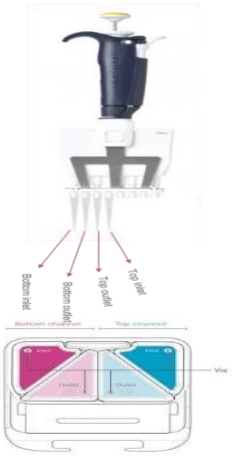
Figure 2: Brain-Chip after 48 hours of TNF-α treatment compared to healthy control. Treated chips have increased cell stress and depleted monolayers.

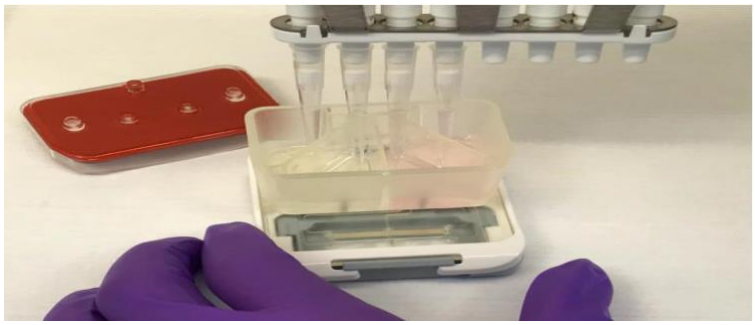
Effluent Sampling

1. Remove the Pod lids and collect effluent medium from Pod outlet reservoirs for analysis.
    a. Collect effluent from the indicated regions. Avoid disturbing the Pod reservoir vias. Refer to Figure 3 below.
2. Gently aspirate any medium not collected for analysis, ensuring that a thin liquid film still covers the reservoir vias so that no air is introduced into them.
3. Refill the Pod media reservoirs with the appropriate fresh cell culture medium and perform a via wash:
    a. Using medium within the Pod reservoir, pipette 1 mL of medium directly over the top of the via to dislodge any bubbles that may be present.
4. Replace the Pod lids and return trays to Zoë.

5. Press the silver “Activation” button to resume pre-set Organ-Chip culture conditions. Zoë will engage when the “Activation” button glows blue.

A





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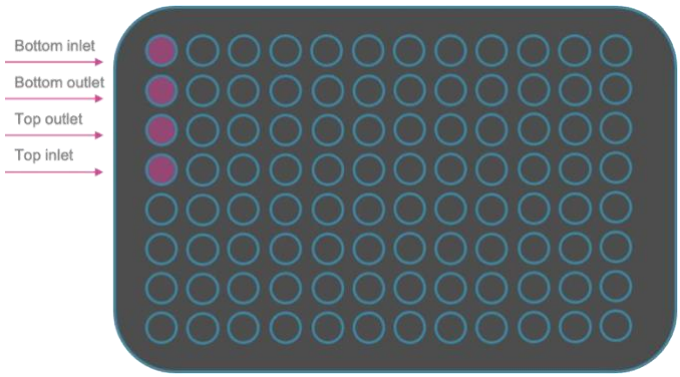

Figure 3: Effluent Sampling. A. Multichannel collecting effluent from the Pod. B. Medium dispensed in the 96-well black bottom plate.

Barrier Function Readout

1. Prepare plate maps to determine which wells of a 96-well tissue culture plate receive effluent or standard samples.
2. Adjust the volume to 50 µL and, using a standard multichannel pipette, collect effluent and media from all four reservoirs simultaneously by placing the pipette tips into the Pods, ensuring one tip is in each reservoir as depicted in the figure above.
3. Dispense the collected effluents into an appropriately labelled 96-well plate. Change tips between Pods to avoid cross-contamination.
4. Add 100 µL of PBS to the effluent collected on the plate for a final dilution of 1:3.
5. To prepare the standard curves, collect 300 µL from the bottom inlet reservoir and add to designated wells. This will be referred to as the “donor” standard curve. Also, collect 300 µL from the top inlet reservoir and add to the designated wells. This will be referred to as the “receiver” standard curve.
6. Dispense 150 µL of 1x DBPS to the empty standard wells.

7. Perform serial dilution, collecting 150 µL from the first well with medium and dispensing it into the well beneath. Repeat these for wells 1-7. On the 7th well, collect 150 µL and dispense it in a waste container, ensuring each well as a final volume of 150 µL. Well 8 should only contain 1x DPBS and will serve as a blank.
8. Perform serial dilution collecting 150 µL from the first well with medium and dispensing it into the well beneath. Repeat this step for wells 1-7. In the 7th well, collect 150 µL and dispense it into a waste container, ensuring each well has a final volume of 150 µL. Well 8 should only contain 1x DPBS, as it will serve as a blank.
9. Save the plate wrapped in foil at room temperature to read later or freeze it at -20°C to read at the end of the experiment. Using a Spectrophotometer Microplate Reader, adjust the Ex/Em based on the fluorescent tracer used (e.g., for Dextran Cascade Blue and Lucifer Yellow, set Ex/Em at 375/420, gain 60).
10. Perform assay daily to monitor barrier function and test for when/if it remains below $2x10^{-6}$ cm/s. For more information, refer to Emulate website's Barrier Function Readout Analysis to download permeability and standard curve.

Acceptance Criteria for Neuroinflammation

Barrier Function

On Day 6 or after 48 hours of treatment, the barrier function apparent permeability (Papp) levels for the TNF-α-treated group are expected to reach above ~$2x10^{-6}$ cm/s when using 3KDa Dextran Cascade Blue or 0.5KDaLucifer yellow. Further details on barrier function readouts and analysis can be found in the Suggested Readouts section near the end of the protocol.

Dosing with Recombinant Human TNF-α Protein

Recombinant human TNF-α protein (R&D Systems cat: 210-TA-020/CF) acts as the neuroinflammatory agent and is effective in this system. It should be thawed and made fresh on each dosing day. Dilute the 20 µg stock vial with 2 mL of DPBS (1x), making a 10 µg / mL stock concentration. Add the calculated amount of the TNF-α stock into the degassed *top* channel Short-Term media after steriflipping. Refer to "Gas Equilibration of Media" section in the Brain-Chip Protocol for more details on degassing.

After effluent sampling, replenish the Pods with media with or without TNF-α protein according to the experimental plan above. Repeat this process as needed.

Calculation Example

TNF-α protein [stock] = 10 µg / mL or 10,000 ng / mL (C_1)

TNF-α protein [final] = 100 ng / mL (C_2)

Total volume of Short-Term Media = 100 mL (V_2)

$C_1V_1 = C_2V_2$, solve for V_1

(10,000 ng / mL) x (X mL) = (100 ng / mL) x (100 mL)

X = 1 mL TNF-α protein stock solution

Dosing the Cells in the Brain-Chip

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

   Note: If there are multiple collection time points in the experiment, it helps to organize experimental conditions such that there is one time point per tray. This allows pausing flow only on the tray from which effluent is being collected while the other trays flow uninterrupted, resulting in a more accurate assessment of elapsed time.

2. Fully aspirate both inlet and outlet reservoirs of each Pod, avoiding direct contact with the Pod reservoir vias.
3. Add the calculated volume of warm, freshly prepared treatment media to the appropriate channel. Ensure both media are properly degassed.
4. Once all Pods have been refreshed, ensure that all trays are returned to the appropriate Zoë. Note the time as the experimental start time (t=0).
5. Each outlet reservoir can be sampled independently at each timepoint following Protocol EP124 Effluent Sampling.
6. Replenish Pods with freshly prepared treatment media daily, regardless of the collection timepoints, until the end of the treatment period or experiment. Ensure both media are properly degassed.

Test Article	Concentration	Starting Day	Treatment Channel	Treatment Duration	Flow rate
TNF-α	100 ng/mL	Day 4	Top	24-48 hours	60 µL/h

Cytokine Secretion

Using V-PLEX Human Proinflammatory Panel I (4-Plex) and the effluent collected previously, the levels of these prominent proinflammatory cytokines (IFNγ, IL-6, and IL-1β) are expected to be significantly increased for TNF-α treated groups when compared to untreated groups by Day 6. Further details on acceptance range of cytokines can be found below.

Cytokine	Acceptance range for proinflammatory cytokines when measuring effluent from brain-side
IL-6	> 750 pg/mL
IFNγ	> 3 pg/mL
IL-1β	> 0.75 pg/mL

Suggested Readouts:

- Barrier Function Readout Analysis
- Isolation and Purification of RNA Samples
- Reverse Transcription
- Quantitative Polymerase Chain Reaction (qPCR)
- Protein Sample Isolation
- Total Protein Quantification
- Quantification of Cytokines and Acute Inflammatory Phase Proteins Secretion
- Immunofluorescence (IF) Staining

Troubleshooting

Hardware & Consumables

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

Brain-Chip Workflow

Issue	Step	Recommendation	Section
Insufficient cells	Chip Seeding	Cells to Chips	During cell suspension preparation, calculate the minimum volume required for seeding (i.e., if the bottom channel can be seeded with a 10-20 µL, seed with 10 µL per chip). Resuspending cells in less volume increases seeding density.
Endothelial cells in the bottom channel not confluent	Chip Seeding	Cells to Chips	Seeding density is not optimal. Ensure that cell suspensions prior to seeding are prepared at the required seeding densities. To ensure proper density, visually, inspect the first seeded chip immediately after seeding it and prior to seeding the remainder.
Uneven/or spotty coating of 6-well plate	6-well plate preparation	iPSCs Culture	Ensure an even distribution of Matrigel coating in all wells by moving the plate vertically and horizontally. Store on top of a flat surface at 4°C.
Dried plate coating	6-well plate preparation	iPSCs Culture	Wrap the plate carefully in parafilm prior to overnight incubation at 4°C.

Potential Root Causes of Bubbles

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

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