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Dear editor,

We deeply appreciate the constructive editorial and reviewers' comments for our manuscript titled "Protocol for the Generation of a human iPSC-based Blood-Brain Barrier-Chip (BBB-Chip)" by Jagadeesan *et al*. We adressed all comments in the revised manuscript. Changes within the manuscript are underlined in the submitted file.
In addition, we provide a point-by-point Response letter, in which responses are marked in *italics*.

We hope that you find the improved manuscript suitable for publication and we appreciate the opportunity to submit to your journal.

Respectfully,

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TITLE:

Generation of a Human iPSC-Based Blood-Brain Barrier Chip

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KEYWORDS:

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SUMMARY:

The blood-brain barrier (BBB) is a multicellular neurovascular unit tightly regulating brain homeostasis. By combining human iPSCs and organ-on-chip technologies, we have generated a personalized BBB chip, suitable for disease modeling and CNS drug penetrability predictions. A detailed protocol is described for the generation and operation of the BBB chip.

ABSTRACT:

The blood brain barrier (BBB) is formed by neurovascular units (NVUs) that shield the central nervous system (CNS) from a range of factors found in the blood that can disrupt delicate brain function. As such, the BBB is a major obstacle to the delivery of therapeutics to the CNS. Accumulating evidence suggests that the BBB plays a key role in the onset and progression of neurological diseases. Thus, there is a tremendous need for a BBB model that can predict penetration of CNS-targeted drugs as well as elucidate the BBB's role in health and disease. We have recently combined organ-on-chip and induced pluripotent stem cell (iPSC)

technologies to generate a BBB chip fully personalized to humans. This novel platform displays cellular, molecular, and physiological properties that are suitable for the prediction of drug and molecule transport across the human BBB. Furthermore, using patient-specific BBB chips, we have generated models of neurological disease and demonstrated the potential for personalized predictive medicine applications. Provided here is a detailed protocol demonstrating how to generate iPSC-derived BBB chips, beginning with differentiation of iPSC-derived brain microvascular endothelial cells (iBMECs) and resulting in mixed neural cultures containing neural progenitors, differentiated neurons, and astrocytes. Also described is a procedure for seeding cells into the organ chip and culturing of the BBB chips under controlled laminar flow. Lastly, detailed descriptions of BBB chip analyses are provided, including paracellular permeability assays for assessing drug and molecule permeability as well as immunocytochemical methods for determining the composition of cell types within the chip.

INTRODUCTION:

The BBB is a highly selective barrier that separates the CNS from the circulating blood. It protects critical brain functions from potentially disruptive substances, factors, and xenobiotics while also allowing the influx of nutrients and other metabolites required to maintain brain homeostasis[1]. The BBB is a multicellular NVU in which pericytes, astrocyte endfeet, and neuronal processes directly contact brain microvascular endothelial cells (BMECs). These interactions allow BMECs to form specialized barrier properties that are supported by tight and adherens junctions[2,3]. The formation of this barrier limits the paracellular passage of molecules, but it contains polarized transporters to actively transport molecules into the CNS or back into the blood[1]. Due to these unique barrier properties, the BBB constitutes a major obstacle to the delivery of biopharmaceuticals into the brain, and it is estimated that less than 5% of FDA-approved small molecules can reach the CNS[4].

Animal models have been widely used to study BBB penetrance and the molecular mechanisms involved in BBB development[5]. While animal models faithfully represent the complex multicellular in vivo environment, differences in expression and activity of BBB transporters as well as substrate specificity across species often preclude accurate extrapolation of animal data to humans[6]. Thus, human-based models are critical to study the human BBB and for use in the development of drugs designed to target the CNS. This need becomes even more apparent with the increasing dominance of biological, human-specific drugs in the pharmaceutical development field. Accumulating evidence suggests that a compromised BBB is associated with a number of severe CNS disorders, including brain tumors and neurological diseases[7-9]. Human models faithfully reflecting these diseases have the potential to both 1) identify novel pathways that could be targeted for drug development and 2) predict CNS penetrance, thus reducing time and resources in preclinical studies and possibly decreasing failure rate in clinical trials.

In vitro models have been widely implemented to study interactions between BMECs and other cells of the NVU and conduct screens for prospective BBB-permeable drugs[10]. To recreate key aspects of the human BBB, in vitro models must display physiologically relevant properties (i.e., low paracellular permeability and physiologically relevant transendothelial electrical resistance

[TEER] across the endothelial monolayer). In addition, the molecular profile of an in vitro system must include expression of representative functional transport systems. Typically, in vitro models are composed of endothelial cells that are co-cultured on a semipermeable membrane with combinations of other NVU cells to enhance BBB properties[11]. This approach allows simple and relatively rapid assessment of barrier functionality and molecule permeability. Such cell-based BBB models can be established with animal or human cell sources, including cells isolated from surgical excisions or immortalized BMEC lines.

Recently, protocols to differentiate human pluripotent cells into BMECs were introduced as an attractive source for in vitro human BBB models[12,13]. Induced pluripotent stem cell (iPSC)-derived BMECs (iBMECs) are highly scalable, demonstrate crucial morphological and functional characteristics of the human BBB, and carry the genetics of the patient. In culture, iBMECs form a monolayer that expresses tight junction markers and displays in vivo-like tight junction complexes. These cells also express BBB markers, including the BBB glucose transporter, glucose transporter 1 (GLUT1). Importantly, and unlike other alternative cell sources for human BMECs, iBMECs acquire barrier properties with values as high as those measured in vivo[14], polarize along the basolateral axis, and express functional efflux pumps. Furthermore, the use of iPSCs from various subjects both 1) welcomes the opportunity to test aspects of the BBB in a personalized medicine manner and 2) provides a flexible source for generating additional cell types of the NVU. Generating these cells from an isogenic cell source to create personalized BBB chips would also aid in understanding inter individual differences in drug responses, which is a major cause for resistance or compromised response to treatment observed in clinical studies.

Use of iBMECs as monolayers in a dish or on a semi permeable transwell insert represents a powerful approach for BBB modeling. These systems tend to be robust, reproducible, and cost-effective. In addition, functional analyses such as TEER and permeability are relatively simple to perform. However, two-dimensional (2D) systems fail to recapitulate the 3D nature of in vivo tissue, and they lack the physiological shear stress forces provided by circulating blood and blood cells. This limits the ability of the vascular endothelium in these models to develop and maintain intrinsic BBB properties and functions.

Microengineered systems lined by living cells have been implemented to model various organ functionalities in a concept called organ-on-chips. By recreating in vivo-like multicellular architecture, tissue-tissue interfaces, physicochemical microenvironments, and vascular perfusion, these microengineered platforms generate levels of tissue and organ functionality not possible with conventional 2D culture systems. They also enable high resolution, real-time imaging, and analysis of biochemical, genetic, and metabolic profiles similar to living cells in the in vivo tissue and organ context. However, a particular challenge of the organ-on-chip is that the design, fabrication, and application of these microengineered chips requires specialized engineering expertise that is usually lacking in biologically oriented academic labs.

We have recently combined iPSC and organ-on-chip technologies to generate a personalized BBB chip model[15,16]. In order to overcome the technological challenges described, the

commercially available Chip-S1 is used together with the culture module, an instrument designed to automate the maintenance of the chips in a simple and robust manner (Emulate Inc.). The BBB chip recreates interactions between neural and endothelial cells and achieves physiologically relevant TEER values, which is measured by custom made organ chips with integrated gold electrodes[17]. Additionally, the BBB chip displays low paracellular permeability, responds to inflammatory cues at the organ level, expresses active efflux pumps, and exhibits predictive transport of soluble biomarkers and biopharmaceuticals. Notably, BBB chips generated from several individuals captures the expected functional differences between healthy individuals and patients with neurological diseases[15].

The protocol detailed below describes a reliable, efficient, and reproducible method for the generation of human iPSC-based BBB chips under dynamic flow conditions. Guidance is provided on the type of assays and endpoint analyses that can be performed directly in the BBB chip or from sampling effluent. Thus, the protocol demonstrates the spectrum of techniques that can be applied for evaluating biological and functional properties and responses in a human-relevant model.

A brief description of the iPSC-based BBB chip is provided here. Human iPSCs are initially differentiated and propagated in tissue culture flasks as free-floating aggregates of neural progenitors, termed EZ-spheres. The top channel of the Chip-S1[16,18,19] is seeded with dissociated EZ-spheres that form the "brain side" of the chip, as cells differentiate over 7 days into a mixed culture of neural progenitor cells (iNPCs), iAstrocytes, and iNeurons. Human iPSCs are also differentiated in tissue culture plates into iBMECs. The bottom channel of the chip is seeded with iBMECs to form the "blood side" as they develop to form an endothelial tube (**Figure 1**). The porous extracellular matrix (ECM)-coated membrane that separates the top and bottom channels 1) permits the formation of cell-to-cell interactions between channels and 2) allows the user to run permeability assays and image cells in either channel using a conventional light microscope.

PROTOCOL:

1. Generation of iPSC-derived neural progenitor cells (iNPCs)

1.1. Produce EZ-spheres from iPSC colonies as described below and as previously published[20-22].

1.1.1. Culture iPSC colonies to confluency on basement membrane matrix-coated 6 well plates (0.5 mg/plate) in mTESR1 or other commercial media (see **Table of Materials**).

1.1.2. Remove iPSC medium and replace with 2 mL of EZ-sphere medium [ESM; DMEM:F12 7:3 supplemented with 100 ng/mL basic fibroblast growth factor (bFGF), 100 ng/mL epidermal growth factor, 5 µg/mL heparin, and 2% B27 supplement].

1.1.3. Scrape the bottom of each confluent well with the back of a sterile 1000 µL pipette tip or cell scraper.

1.1.4. Collect all cells and place in an ultra-low attachment T25 flask to allow the spontaneous formation of free-floating spheres. Incubate overnight at 37 °C.

1.1.5. Feed the spheres every 2–3 days when the medium turns yellow by replacing half of the medium with fresh ESM. This allows for spheres to remain in conditioned medium, which is highly important for their growth and maintenance.

1.1.5.1. Lean the flask on a tube rack and allow the spheres to settle by gravity for 1–2 min down to the corner of the flask.

1.1.5.2. Once settled, aspirate half of the supernatant with a 5 mL or 10 mL serological pipette and replace with fresh, pre-warmed ESM.

1.1.6. Passage EZ-spheres weekly by chopping spheres to 200 µm diameters as previously described[23,20,21]. EZ-spheres can be maintained for up to 25 passages and are ideal when used between passages 8–25.

1.2. Prepare single cell suspension of iNPCs:

1.2.1. To induce neural differentiation, dissociate EZ-sphere into single cells.

1.2.2. Collect spheres 3–4 days post-chopping from a T75 flask and transfer into a 15 mL conical, then let it stand for 2 min or until all spheres are settled at the bottom.

1.2.3. Slowly remove ESM with a 5 mL pipette without disrupting the settled spheres. Add 1 mL of dissociation solution (see **Table of Materials**) and incubate for 10 min at 37 °C.

1.2.4. Swirl the dissociation solution and spheres after 5 min of incubation to ensure that any settled spheres are treated.

1.2.5. Slowly remove the dissociation solution. Add 1 mL of neural differentiation medium [NDM; DMEM: F12 with 2% B27 minus vitamin A, 1% N2 supplement, and human brain-derived neurotrophic factor (hBDNF, 20 ng/mL)].

1.2.6. Triturate the spheres into single cells by pipetting using a 1 mL pipette followed by 200 µL pipette, until all spheres have dissociated. Avoid bubble formation during the trituration procedure.

1.2.7. Count dissociated cells using a hemocytometer and dilute cells to a final density of 1 x 10^6 cells/mL. It is possible to change the density depending on the application.

NOTE: Higher densities (up to 6 x 10^6 cells/mL) are recommended for short-term cultures of up to 3 days, and lower densities are recommended for long-term applications of up to 3 weeks.

NOTE: Cells are now ready to seed into the top channel of the chip to form the "brain side".

2. Differentiation of iPSCs into iBMECs

2.1. Passage iPSCs from a single confluent well of a 6 well plate at a 1:6 ratio into a 6 well basement membrane matrix-coated plate. Let cells adhere for 24 h. Change iPSC medium daily.

2.2. Count cells daily using a hemocytometer.

2.3. When cells reach a density of 1.5–3.0 x 10^5 cells/well, replace iPSC medium with 3 mL of unconditioned medium without bFGF [DMEM:F12 1:1, with 10% knockout serum replacement (KOSR), 1% non-essential amino acids (NEAA), 0.5% glutamine supplement (**Table of Materials**), and 100 µM β-mercaptoethanol]. Replace medium daily for 6 days[24].

2.4. At day 6, replace medium with endothelial cell (EC) medium [human endothelial serum free medium (hESFM) supplemented with 1% platelet-poor plasma-derived bovine serum, 20 ng/mL bFGF, and 10 µM all-trans retinoic acid (RA)]. Leave medium for 2 days.

2.5. Remove EC medium and add 1 mL of dissociation solution per well. Incubate at 37 °C for 35 min.

NOTE: While this is considered a long incubation time in the dissociation solution used here, iBMECs can endure this treatment with cell viability higher than 90%.

2.6. Detach the cells from the well by gently pipetting the cell suspension and collecting all cells into a 15 mL conical tube.

NOTE: Avoid harsh pipetting. If cells do not detach easily, incubate for additional 5 min.

2.7. Add 1 volume of EC medium into the 15 mL conical to inactivate Accutase, centrifuge at 200 x *g* for 5 min, remove medium, and replace with 1 mL of EC medium (without bFGF and RA).

2.8. Count cells using a hemocytometer and adjust cell density to 14–20 x 10^6 cells/mL.

NOTE: Cells are now ready to be seeded into the bottom channel of the chip to form the "blood side.

3. Microfabrication of the organ chip

3.1. Use an organ chip for the BBB chip model and its production as done previously[16,18,19]. The tall channel organ chip (see **Table of Materials**) is fabricated from a highly flexible polydimethylsiloxane (PDMS) elastomer that contains two superimposed and parallel micro-

scale channels separated by a flexible porous membrane. The top and bottom microchannel sizes are 1 mm x 1 mm and 1.0 mm x 0.2 mm, respectively. The two channels are separated by a 50 µm thick PDMS-made flexible porous membrane, containing 7 µm diameter pores with 40 µm spacing. The surface area of the porous membrane that separates the channels is 0.171 cm^2.

4. Chip preparation

4.1. Organ chips are supplied prepackaged within the chip carrier, eliminating the need to disturb or distort the chip alignment during handling. In addition, the chip carrier connects securely to a portable module ("Pod") that acts as the interface between the culture module and chip.

4.1.1. Spray the packaging of the chips with 70% ethanol and bring into the biosafety cabinet (BSC).

4.1.2. Open the packaging and lay out the organ chip in a sterile Petri dish. Handle the chip carrier only by the sides to avoid direct contact with the chip.

4.1.3. Ensure that the tab of the carrier is facing to the right (**Figure 2**), and when using multiple chips, align them all in the same orientation.

4.1.4. Label each chip on the carrier tab (complete chip preparation and workflow is shown in **Figure 3)**.

5. Surface activation and ECM coating

5.1. Preparation of surface activation solution

5.1.1. Emulate reagent 1 (ER-1), provided in a vial containing 5 mg, is light-sensitive. Prepare fresh ER-1 solution immediately before use. ER-1 integrity is crucial in successful preparation of the chips.

5.1.2. Turn off the light in the BSC when handling ER-1.

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

5.1.3. Allow the ER-1 and ER-2 reagents to equilibrate to room temperature (RT) before use.

5.1.4. Protect solution from light by wrapping an empty sterile 15 mL conical tube with foil.

5.1.5. In the BSC, briefly tap the ER-1 vial to settle the powder at the bottom.

5.1.6. Add 1 mL of ER-2 buffer to the vial, and immediately transfer its content to the bottom of the 15 mL wrapped conical tube. Do not pipette to mix. The color of the solution transferred to the conical tube will be red.

5.1.7. Repeat step 5.1.6 3x. On the last round, cap the ER-1 vial and invert to collect any remaining powder from the lid, then transfer the solution to the conical tube; this will bring the total volume to 4 mL of ER-1 solution.

5.1.8. Add 6 mL of ER-2 solution to the 4 mL of ER-1 solution in the 15 mL conical tube to a final concentration of 0.5 mg/mL. ER-1 should be fully dissolved within the ER-2 solution.

5.2. Surface activation

5.2.1. Utilizing a P200 pipette and a sterile 200 µL filtered pipette tip, take up 200 µL of ER-1 mixture.

5.2.2. Place the pipette in the bottom inlet and push 20 µL of ER-1 mixture through the bottom channel until the mixture starts to flow out of the bottom channel outlet.

5.2.3. Add approximately 50 µL of ER-1 mixture and place it in the top channel inlet. Push the mixture through the top channel until it starts flow out of the top channel outlet.

5.2.4. Remove all excess ER-1 mixture from the surface of the chip by gentle aspiration. Make certain the ER-1 mixture is only removed from the chip surface and not from the channels.

5.2.5. Verify that the channels are free of air bubbles before ultraviolet (UV) activation. If air bubbles are detected, remove bubbles by washing the channel with ER-1 mixture.

5.2.6. Place the open dish containing the chips into the UV light box (provided by Emulate Inc.).

5.2.7. Set the switch at the back of the UV light box to the "Consistent" setting. Turn on the power and initiate UV activation. Leave the chips under UV light for 20 min.

NOTE: Avoid exposure of personnel to UV light.

5.2.8. Remove the ER-1 mixture from both channels.

5.2.9. Wash each channel with 200 µL of ER-2 solution.

5.2.10. Remove ER-2 from both channels.

5.2.11. Wash each channel with 200 µL of sterile cold Dulbecco's phosphate-buffered saline (DPBS).

5.2.12. Leave cold DPBS inside the channels until proceeding to the next step.

5.3. Extracellular matrix (ECM) preparation and coating

5.3.1. Prepare the ECM solution by combining the individual ECM components with cold DPBS, water, or other solvent to the final working concentrations. The ECM solution should be prepared fresh each time it is used.

5.3.2. Coat both top and bottom channels of the chip with ECM, with composition as determined by the cell type to be seeded. ECM mixture must be maintained on ice until use.

5.3.3. Use laminin (50 µg/mL) to coat the “brain side”, and collagen IV and fibronectin mixed at a 4:1 ratio (320:80 µg/mL) to coat the “blood side” as described in section 5.6.

5.4. Preparation of ECM aliquots

5.4.1. Dilute 1 mg/mL laminin in cold DPBS to a final concentration of 50 µg/mL. Aliquot and store at -20 °C until use.

5.4.2. Dissolve collagen IV in 0.1% acetic acid to a concentration of 1 mg/mL. Incubate the solution at 2–8 °C overnight or at RT for 1-3 h or until fully dissolved.

5.4.3. Prepare a 1 mL mixture of collagen IV:fibronectin (320 µL of collagen IV, 80 µL of fibronectin, 600 µL of sterile double-distilled H_2O). The mixture can be stored at -20 °C.

5.5. Coating the chips with ECM

5.5.1. Fully aspirate the cold DPBS from both channels. Set a P200 pipette to take up 100 µL of collagen IV:fibronectin solution.

5.5.2. Introduce the solution through the bottom channel inlet until a small droplet forms on the outlet. Leave a small droplet on the inlet after removing the pipette tip.

5.5.3. Introduce the laminin solution through the top channel inlet until a small droplet forms on the outlet. Leave a small droplet on the inlet after removing the pipette tip.

5.5.4. Look closely at the channels to ensure that no bubbles are present. If bubbles are present, wash the channel with the appropriate ECM solution until all bubbles are removed.

5.5.5. Repeat steps 5.5.1–5.5.4 for each chip.

5.5.6. Add 1.5 mL of DPBS to the cap of a 15 mL conical tube. Place the DPBS cap in the 150 mm culture dish with the chips to provide extra humidity and seal the dish with parafilm. For best results, incubate the chips at 4 °C overnight.

NOTE: If desired, cells can be seeded the same day as chip activation and ECM coating, though overnight incubation is preferred. Chips can be ready for seeding 4 h after adding the ECM and incubating chips at 37 °C.

6. Seeding the "brain side" channel and differentiating EZ spheres into mixed neural cultures

6.1. Bring the dish containing the prepared chips to the BSC. Gently wash both channels with 200 µL of NDM.

6.2. Avoiding contact with the ports, carefully aspirate excess media droplets from the surface of the chip. Gently agitate cell suspension before seeding each chip to ensure a homogeneous cell suspension

6.3. Seeding the iNPCs into the top channel to generate the "brain side"

6.3.1. Seed the cells (1 x 10^6 cells/mL) into the top channel of the chip. Add a P200 tip containing 30–100 µL of cells suspension to the top channel inlet and gently release the tip from the pipette. Take an empty P200 pipette, depress the plunger, insert into the top channel outlet and carefully pull the single cells suspension through the chip.

6.3.2. Cover the dish and transfer to the microscope to check the seeding density and homogenous distribution of cells within the top channel. Gently remove the pipette tip from the chip inlet and outlet ports.

6.3.3. Seeding density should appear as 20% coverage. If seeding density is higher or lower than expected or uneven, return the chips to the BSC, wash the channel 2x with 200 µL of fresh medium, and repeat step 6.3.1.

6.3.4. After confirming the correct cell density, immediately place the chips in the incubator for 2 h at 37 °C after seeding each batch of chips. Wash away the cells that do not attach with fresh NDM.

6.3.5. Keep cells under static conditions at 37 °C with a daily NDM replacement for at least 48 h before initiating flow. iBMECs can be seeded after iNPCs have attached or on a subsequent day following iNPC seeding.

7. Seeding iBMECs into the bottom channel to generate the "blood side"

7.1. Bring the dish containing the prepared chips to the BSC. Gently wash the bottom channel with 200 µL of EC medium.

7.2. Avoiding contact with the ports, carefully aspirate droplets of excess EC medium from the surface of the chip, making sure to leave medium in both channels. Gently agitate cell suspension before seeding each chip to ensure a homogeneous cell suspension.

7.3. Using a P200 pipette, draw up 30–100 µL of the iBMEC cell suspension (14–20 x 10^6 cells/mL) and place the tip into the bottom channel inlet. Gently disconnect the tip from the pipette, leaving the cell-containing tip in the inlet port.

7.4. Depress the plunger on a P200 pipette with an empty tip, insert into the bottom channel outlet, and carefully pull the single cell suspension through the bottom channel by slowly releasing the pipette plunger.

7.5. Aspirate excess cell suspension from the surface of the chip. Avoid direct contact with the inlet and outlet ports to ensure that no cell suspension is aspirated out of the channels.

7.6. Cover the chip and transfer it to the microscope to observe seeding density. The bottom channel should be filled with no observable gaps between cells when observed at 4x or 10x under a microscope (**Figure 4**).

7.7. If seeding density is below 90% coverage or is unevenly distributed, adjust cell density accordingly and repeat steps 7.2–7.6 until the correct density is achieved within the channel. After confirming the correct cell density (**Figure 4**), seed cells in the remaining chips. To attach cells onto the porous membrane, which is located on the top of the bottom channel, invert each chip and rest in a chip cradle.

7.8. Place a small reservoir (15 mL conical tube cap containing sterile DPBS) inside the 150 mm dish to provide humidity for the cells. Incubate chips at 37 °C for approximately 3 h, or until cells in the bottom channel have attached. Once iBMECs have attached (~3 h post-seeding), flip the chips back to an upright position to allow cell attachment to the bottom portion of the bottom channel.

8. Initiation of flow

8.1. Flow is typically initiated 48 h post-seeding of iBMECs. This time is required for the iBMECs to attach firmly to the chip.

8.2. To maintain laminar flow through the chip, it is important to degas and equilibrate the temperature of the medium. Medium must be pre-warmed in a 37 °C water bath for 1 h.

8.3. Up to 50 mL of warmed medium can be degassed by incubation under a vacuum-driven filtration system for 15 min.

8.4. Priming of portable modules

8.4.1. Sanitize the exterior of the portable module packaging and trays with 70% ethanol, wipe, and transfer to the BSC. Open the package and place the modules into the tray. Orient them with the reservoirs toward the back of the tray.

8.4.2. Pipette 3 mL of pre-equilibrated, warm media to each inlet reservoir. Add EC culture medium to the inlet reservoir of the bottom channel and NDM to the top channel inlet reservoir of the top channel (**Figure 5**).

8.4.3. Pipette 300 µL of pre-equilibrated, warm media to each outlet reservoir, directly over each outlet port (**Figure 5**).

8.4.4. Place up to six portable modules on each tray. Bring trays to the incubator and slide completely into the culture module with the tray handle facing outward.

8.4.5. Select and run the "Prime" cycle on the culture module. Close the incubator door and allow the culture module to prime the portable modules (takes ~1 min). The priming cycle is completed when the status bar reads "Ready". Remove the tray from the culture module and bring it to the BSC.

8.4.6. Verify that the portable modules were successfully primed by inspecting the underside of each portable module in the BSC. Look for the presence of small droplets at all four ports.

8.4.6.1. If any portable module does not show droplets, rerun the prime cycle on those modules. If any media dripped onto the tray (this may occur more often by the outlet ports), clean tray with 70% ethanol.

8.5. Connection of chips to portable modules, regulation, and initiation of flow

8.5.1. Gently wash both channels of each chip with warm, equilibrated cell-specific culture medium to remove any possible bubbles in the channel and place small droplets of media (according to the media in the channel) on the top of each inlet and outlet port.

8.5.2. Insert chips with carriers into the portable modules and place up to six on each tray. Insert trays into the culture module. Program the appropriate organ chip culture conditions (flow rate and stretch) on the culture module.

8.5.3. Programmed conditions will start as soon as the "Regulate" cycle is complete.

NOTE: The flow rates for each channel can be controlled independently and can be set to rates that range from 0–1,000 µL/h. The BBB chip is typically cultured at 30 µL/h. When flowing media such as EC media or ESM at 30 µL/h and 1000 µL/h, the shear forces are 0.01 dyn/cm^2 and 0.33 dyn/cm^2, respectively.

8.5.4. Run the “Regulate” cycle, which takes approximately 2 h, after which the culture module will begin flow at the preset organ chip culture conditions.

9. Blood-to-brain paracellular permeability assessment

9.1. Prepare NDM supplemented with 10 µg/mL dextran-FITC (4 kDa). This solution will be used as the input for the “blood side” channel.

9.2. Fill the bottom channel reservoirs of the portable modules with NDM supplemented with dextran-FITC. Fill the top channel reservoirs with NDM without tracer.

9.3. Perfuse both, the top and bottom channels at a flow rate of 30 µL/h for at least 4 h until enough media accumulates to be collected for assessment of fluorescence in a plate reader (typically 100 µL).

9.4. Collect media samples from the input and output reservoirs of the top and bottom channels. Protect the samples from light.

9.5. Serially dilute the NDM supplemented with 10 µg/mL dextran-FITC 1:1 using NDM without tracer to generate a 10–12 point calibration curve.

9.6. Take 100 µL of each sample, including the calibration curve, into a black 96 well plate and read fluorescence using a plate reader (485 nm excitation, 530 nm emission).

9.7. Use the measured values to compute P_{app} values as follows:

$$Papp = \frac{top\ output\ (\mu g/mL) - top\ input\ (\mu g/mL)}{bottom\ Input\ (\mu g/mL)} \times \frac{flow\ rate\ (mL/sec)}{membrane\ area\ (\mathrm{cm}^2)}$$

9.8. Take daily measurements to assess the barrier properties and confirm that the BBB chip is still functional.

10. Immunocytochemistry

10.1. Bring chips to a chemical fume hood. Using a P200 pipette, fix cells by perfusing both channels with 200 µL of 4% paraformaldehyde (PFA) in DPBS and incubate for 10 min at RT.

10.2. Following the fixation, perfuse each channel with 200 µL of DPBS and incubate for 5 min. Repeat DPBS washing 2x.

10.3. Block and permeabilize cells on the chip by perfusing primary blocking solution (PBS, supplemented with 5% normal donkey serum and 0.1% Triton X-100). Incubate at RT for 1 h.

10.4. Dilute primary antibodies in primary blocking solution and incubate overnight at 4 °C. BMECs markers are GLUT-1 (1:100 dilution), ZO-1 (1:300), PECAM-1 (1:250; CD31), and VE-cadherin (1:200). Neural markers are βIII-tubulin (1:1000; Tuj1α), S100β (1:500), nestin (1:1000) and GFAP (1:1000).

10.5. Wash the chips 3x with cold DPBS.

10.6. Dilute secondary antibodies in secondary blocking solution (DPBS with 5% normal donkey serum without Triton-X).

10.7. Perfuse the secondary antibody solution through both channels. Typically, fluorescent secondary antibodies are diluted 1:1000. Incubate for 1 h at RT protected from light.

10.8. Wash the chips 3x with DPBS.

10.9. Stain cell nuclei by perfusing chip with 100 µL of DAPI solution. Incubate at RT for 5 min.

10.10. Wash the chips 3x with DPBS.

10.11. The chip is ready for imaging using either an upright or an inverted fluorescent microscope. The PDMS transparency permits imaging in the intact organ chip. Magnifications above 10x may require long working distance objectives due to the thickness of the organ chip.

REPRESENTATIVE RESULTS:

Figure 6A,B,C represents a BBB chip seeded with EZ-spheres on the “brain side” top channel and iBMECs on the “blood side'” bottom channel. iBMECs were seeded first and allowed to attach overnight, after which EZ-spheres were seeded. Chips were then cultured under static conditions with daily media replacement for seven days. The BBB chip was then fixed using 4% PFA at RT for 10 min and washed 3x with DPBS. Immunocytochemistry was performed on the BBB chip using 1) nestin as a marker for neural progenitor cells, 2) S100β or GFAP as markers for astrocytes, and 3) βIII-tubulin as a marker for neurons. GLUT-1 and Pecam-1 were used as a marker for BMECs. Imaging was performed at 20x using a confocal microscope and images were processed using Fiji for ImageJ software.

Figure 6D represents a paracellular permeability assay that was performed on organ chips populated with iBMECs and EZ-spheres, iBMECs alone (without EZ-spheres), or EZ-spheres alone (without iBMECs). Following a “Regulate” cycle, a 4 kDa Dextran-FITC tracer was added to the reservoir of the “blood side” to a final concentration of 10 µg/mL, and chips were perfused at 30 µL/h overnight. Next, media was collected from inlet and outlet reservoirs of both the top and bottom channels. 100 µL of each sample was collected and examined for fluorescence using a plate reader.

Importantly, a 1:1 calibration curve was used to transform fluorescence values into

concentration values of Dextran FITC. Values were then used to calculate permeability (P_{app}). These results demonstrate that iBMECs form functional barrier properties, which are further tightened when iBMECs are co-cultured with EZ-spheres. Chips cultured with EZ-spheres only, fail to form a barrier. A similar approach can be used to examine the transport of any molecule across the BBB chip, depending on an available measurement method (e.g., fluorescence, ELISA, or mass spectrophotometry).

FIGURE LEGENDS:

Figure 1: Schematic of the iPSC-based BBB chip. Prior to seeding on the chip, iPSCs are differentiated in culture plates into (i) EZ-spheres (neural progenitor cells, iNPCs), which are grown in suspension as spheres, and into (ii) brain microvascular endothelial cells (iBMECs). EZ-spheres are dissociated into single cells, seeded on the top channel of the organ chip, where they further differentiate into mixed neural cultures to form the "brain side". iBMECs are seeded in the bottom channel of the organ chip to form a blood vessel-like structure on the "blood side".

Figure 2: Schematic of the top view of the chip in the chip carrier, with labeled ports.

Figure 3: Flowchart of the iPSC-based BBB chip preparation and workflow. Pre-differentiation of both neural and endothelial cells from iPSCs is required before initiation of the workflow.

Figure 4: Seeding of iBMECs in the bottom channel. Brightfield images of iBMECs seeded in the bottom channel (**A**) immediately after seeding or (**B**) 24 h post-seeding, after cells have attached.

Figure 5: Schematic of the connected chip and portable module with labeled inlet and outlet media reservoirs.

Figure 6: Representative results. Immunocytochemistry on the iPSC-based BBB chip 7 days post-seeding. EZ-spheres differentiated into a mixed neural cell population in the top "brain side" channel, including (**A**) S100β+ (green) astrocytes, Nestin+ (red) neural progenitor cells as well as (**B**) GFAP+ (red) astrocytes and βIII-tubulin+ (red) neurons. Scale bars = 200 µm. (**C**) iBMECs seeded in the bottom "blood side" channel expressed GLUT-1 and (green) PECAM-1 (CD31, red). Scale bar = 200 µm. (**D**) Evaluation of BBB chip permeability was performed by adding dextran-FITC (4 kDa) into the reservoir of the bottom channel. Results demonstrate that organ chips seeded with iBMECs and EZ-spheres display a tight barrier compared to organ chips seeded with iBMECs alone (*$p < 0.05$). Organ chips seeded with EZ-spheres alone do not display any barrier properties (***$p < 0.001$; one-way ANOVA with Tukey's multiple comparisons test).

DISCUSSION:

The combination of organ-on-chip technology and iPSC-derived cells in the NVU holds promise for accurate modeling of the human BBB. Here, we provide a detailed protocol for simple and

robust application of the recently published iPSC-based BBB chip[16]. An overview and timing of the seeding paradigm is shown in **Figure 3**. To obtain and maintain barrier functions that are suitable for BBB modeling, generating a homogenous iBMEC monolayer and retaining its integrity are critical. The first step towards the generation of a functional monolayer includes chemical activation of the nonpolar PDMS surface that allows attachment of ECM proteins. The surface activation reagents degrade rapidly upon reconstitution, which may eventually result in suboptimal attachment of cells. It is therefore important to keep the reagents fresh and protected from light throughout the process.

During UV activation (section 5.2), reagents must be evenly distributed within each channel in order to achieve consistent ECM coating and homogenous cell attachment. ECM compositions and concentrations that are provided in this protocol were optimized to the specific types of cells (i.e., iBMECs and EZ-sphere-derived neural progenitor cells). Changing cell composition is possible but may require differential ECM conditions, which will require optimization. Following ECM coating, it is crucial to seed iBMECs properly. High cell seeding density (>14 x 10^6 cells/mL) is essential for obtaining a complete monolayer. If failing to achieve full cell confluency, it is advised to further increase the density of the cell suspension up to 20 x 10^6 cells/mL during seeding.

Laminar flow was previously suggested to enhance iBMEC maturation[15] and is indispensable for fulfilling the advantages provided by the microfluidic platform. However, microbubbles flowing through the channels of the chip can physically stress and detach resident cells, which can lead to destruction of the BBB chip integrity. To avoid microbubble formation during laminar flow, it is critical to equilibrate the medium before perfusion is initiated. Equilibration requires pre-warming and degassing of the medium prior to use.

The generation of personalized chips is made possible using both human iPSC-derived iBMECs and iNPCs. While the differentiation of iBMECs is short and rather simple[24,22], differentiation of iPSCs into neural cells[21,25] is more challenging. However, the neural cells that reside in the "brain side" of the chip can be replaced by any neural cell type, such as primary neural cells or iPSC-derived motor neurons[16]. Similarly to "bona fide" endothelial cells, iBMECs display plasticity in gene expression in response to different neural co-cultures. Thus, this flexibility may result in future development of various NVUs on the chip. Additional flexibility of the system can be obtained by adjusting the cell seeding timing and order. iBMECs can be seeded prior to or after the neural cells, making it possible to seed iBMECs immediately following seeding and attachment of neural cells, or after EZ-spheres have differentiated into more mature neural cultures in the chip environment. This is of particular importance, given the immature nature of iPSC-derived neural cells. Given that EZ-spheres are early neural progenitors, longer differentiation periods may also result in an increased percentage of astrocytes and neurons.

One component that is missing from the protocol is perivascular pericytes, which provide a crucial component in the physiological NVU. Recent advancements in iPSCs differentiation into pericyte-like cells[26,27] will permit the introduction of this additional cell type, thereby providing

an improved replica of the BBB while preserving its personalized nature.

IBMECs have previously been shown to demonstrate various properties that are critical for BBB modeling including expression of cellular markers, the establishment of TEER and functional activity of efflux pumps[28,24,5]. However, molecular analyses have revealed that these cells also express several epithelial markers[15]. Advancements in iBMEC generation showing improved function and endothelial marker expression have recently been described[29,30]. Following the paradigm presented here, these potentially improved cells can easily be incorporated into the BBB chip model for future applications. The flexible yet robust platform described here may facilitate both disease modeling and the development and assessment of new CNS drugs.

While this protocol relies on a specific commercially available product, additional commercial companies offer diverse microphysiological platforms, which may offer alternative advantages[31]. In addition, protocols for "in house" manufacturing of microfluidic Organ chips are also available[32] and may offer more modularity including the integration of TEER electrodes[17], which is missing from the platform used here.

Organ-on-chips were introduced as an alternative approach to improve the physiological context of current cell cultures[33]. However, the application of this technology requires specialized engineering skills, which are often lacking in biologically oriented labs. A commercially available chip platform, such as the one employed here, provides less modularity and increased robustness and reproducibility, which can be applied by a wider array of users. Further, the application of laminar flow on a microfluidic chip relies on the application of syringe or peristaltic pumps, which introduces another level of complexity. This obstacle is now easier to overcome with the application of the culture module, which facilitates the simultaneous perfusion of multiple chips.

ACKNOWLEDGMENTS:

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DISCLOSURES:

Cedars-Sinai owns a minority stock interest in Emulate, the company that produces the study's microfluidic Organ chips. An officer of Cedars-Sinai also serves on Emulate's Board of Directors. Emulate provided no financial support for this research. Cedars-Sinai and Emulate have patents filed related to this work.

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Figure 1

Click here to access/download;Figure;Figure 1.pdf

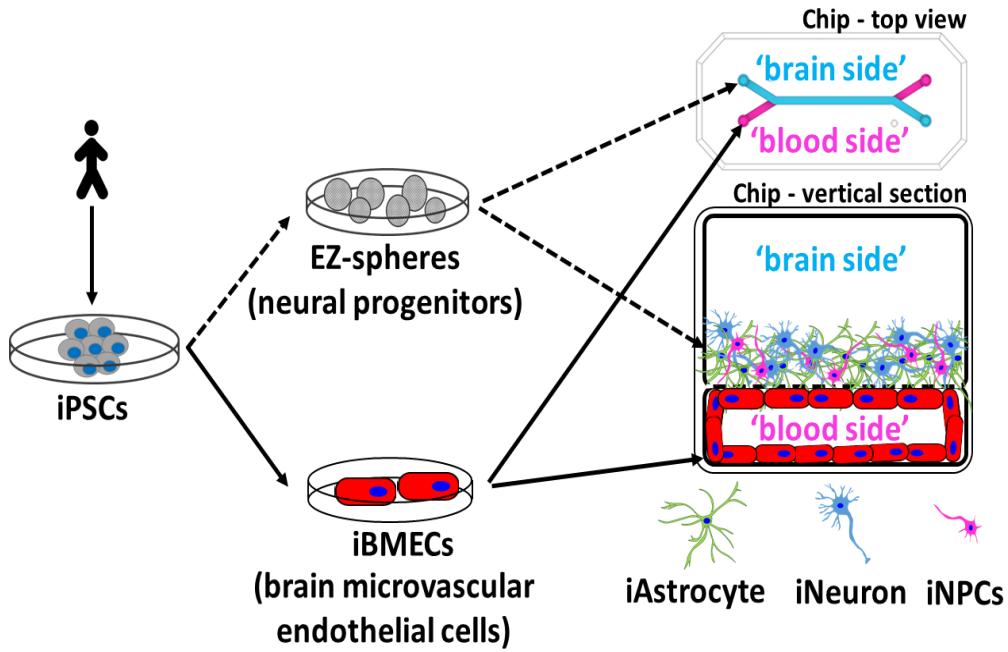

Figure 2

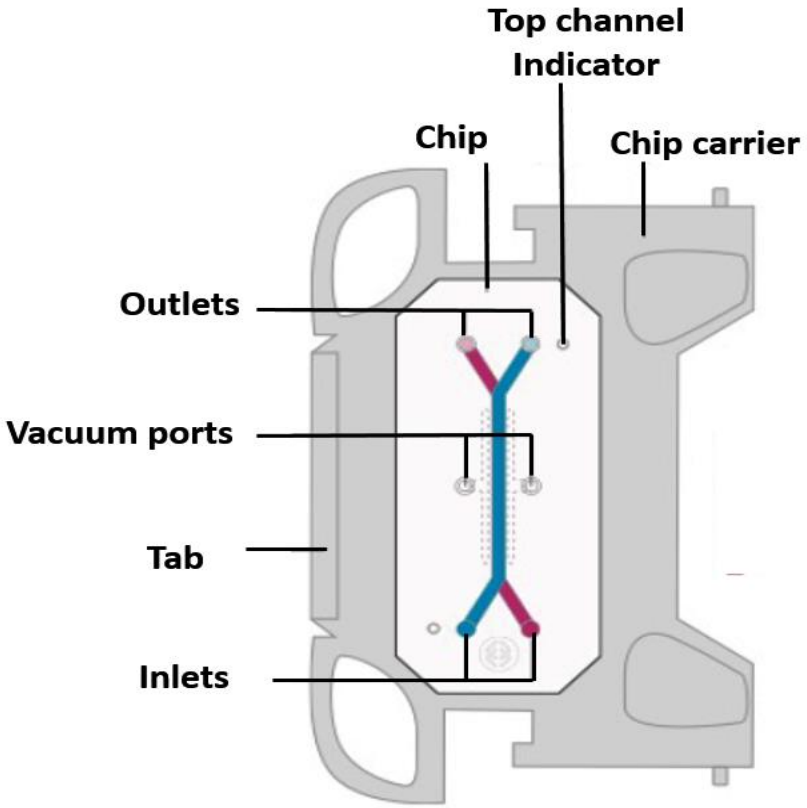

Figure 3

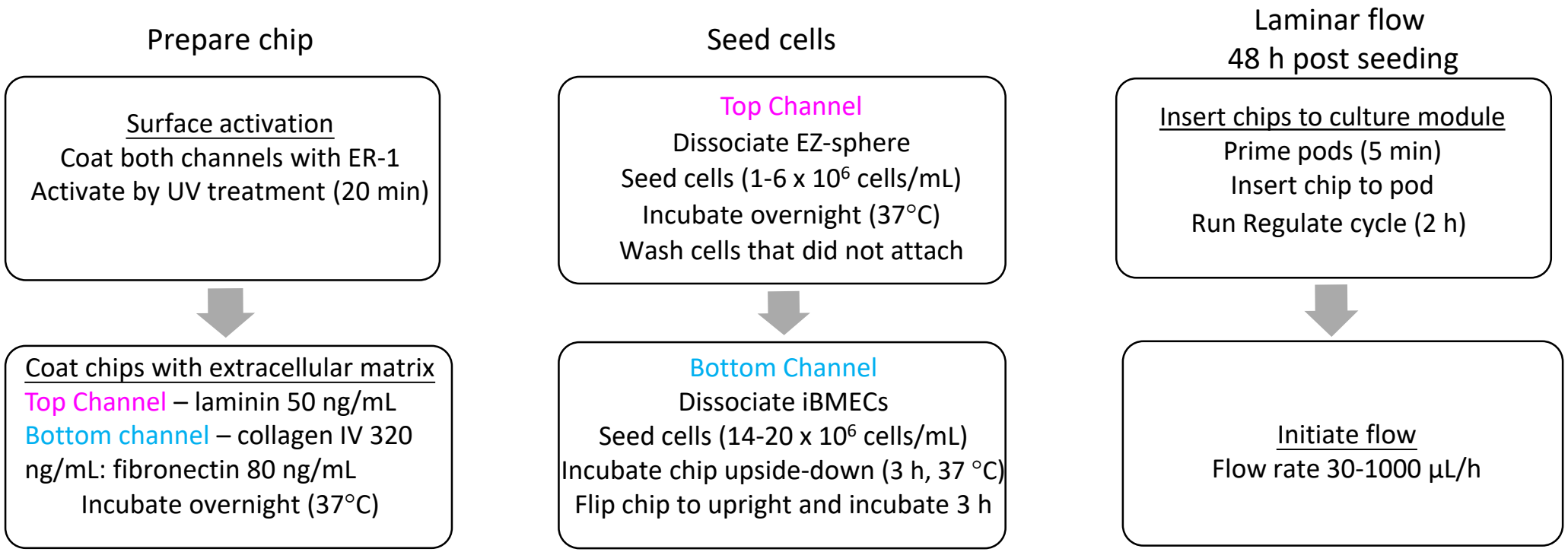

Figure 4

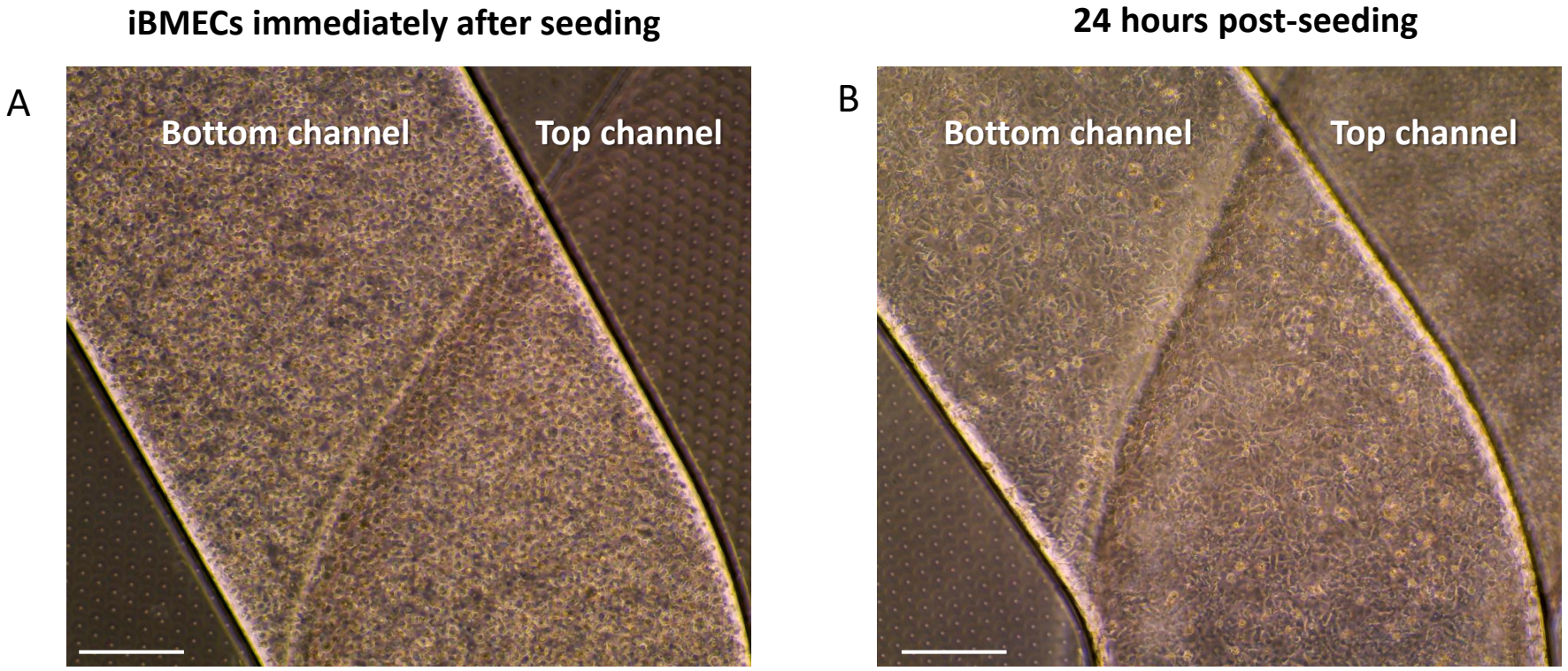

Figure 5

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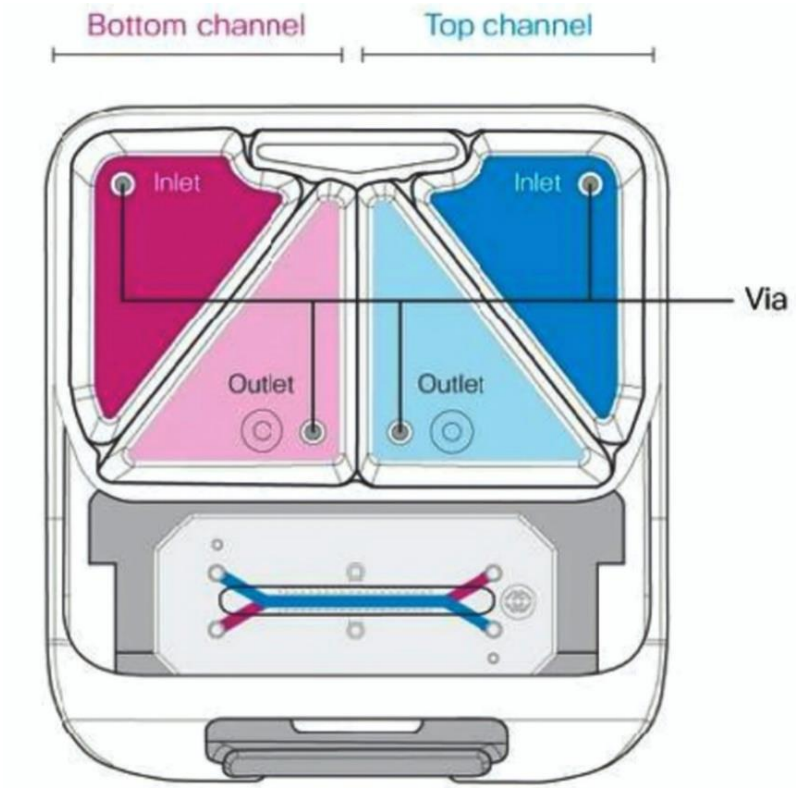
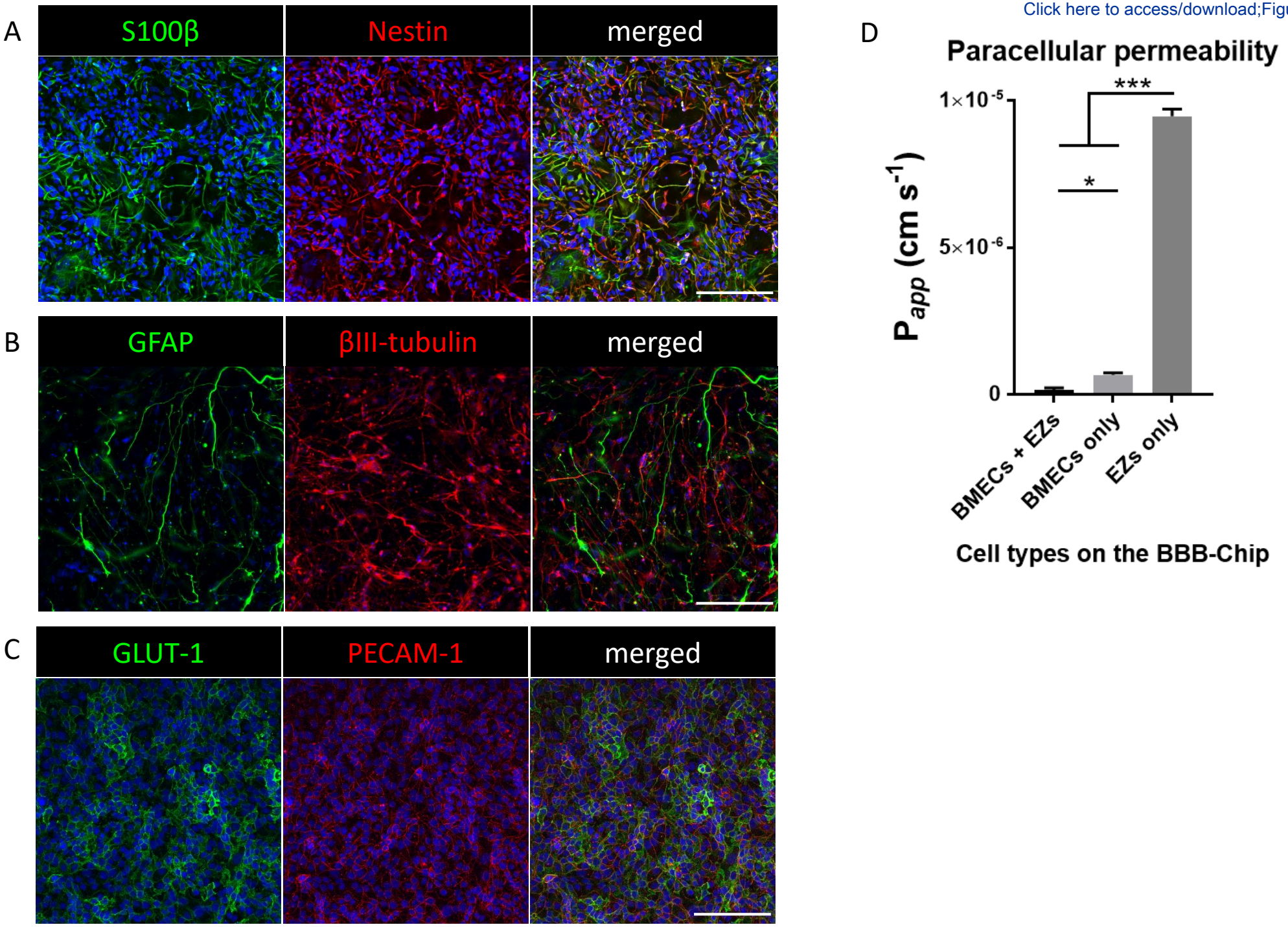


Figure 6



Name of Material/Equipment	Company	Catalog Number	Comments/Description
Accutase	EMD Millipore	SCR005	Dissociation solution
B27	Gibco	12587010	
Bfgf	Peprotech	100-18B	
Chip-S1	Emulate Inc	Chip-S1	Organ-Chip
Collagen IV	Sigma	C5533	
DAPI	Invitrogen	D3571	
Dextran-FITC	Sigma	46944	
DMEM: F12	Thermo Fisher scientic	31330038	
Donkey serum	Sigma	D9663	
Emulate Reagent 1 (ER-1)	Emulate Inc	ER-1	
Emulate Reagent 2 (ER-2)	Emulate Inc	ER-2	
Fibronectin	Sigma	F1141	
Glial Fibrillary Acidic Protein (GFAP)	Dako	Z0334	
GLUT-1	Invitrogen	MA5-11315	
Glutamax	Life Technologies	35050038	Glutamine supplement
hBDNF	Peprotech	450-02	
KOSR	Thermo Fisher scientic	10828028	
Laminin	Sigma	L2020	
Matrigel	Corning	354234	Basement membrane matrix
mTeSR1	StemCell Technologies, Inc.	85851	
NEAA	Biological industries	01-340-1B	
Nestin	Millipore	MAB353	
NutriStem	Biological industries	05-100-1A	Alternate media
PECAM-1	Thermo Fisher scientic	10333	

Platelet-poor plasma-derived bovine serum (PPP)	Biomedical Technologies	J64483AB
Retinoic acid (RA)	sigma	R2625
S100β	Abcam	ab6602
Steriflip-GP Sterile Centrifuge Tube Top Filter Unit	Millipore	SCGP00525
Triton X-100	Sigma	X100
ZO-1 Monoclonal Antibody	Invitrogen	33-9100
βIII-tubulin (Tuj1α)	Sigma	T8660
β-mercaptoethanol	Life Technologies	31350010

Response letter to editor and reviewers' comments

We thank the editor and reviewers for their constructive comments. We have addressed all concerns and our responses to the comments are provided below in *italics*. Changes to the manuscript are underlined in the manuscript file. We hope that the updated manuscript is now suitable for publication in Journal of Visualized Experiments.

Editorial comments:

Comment: 1. Please take this opportunity to thoroughly proof read the manuscript to ensure that there are no spelling or grammar issues.

Response: *The spelling and grammar were thoroughly checked and grammatical errors were corrected in the revised manuscript.*

Comment: 2. Please revise sections 4–8 of the protocol to avoid textual overlap with previous publications.

Response: *We have now modified the text to avoid textual overlapping with previously published protocols (Pages 7-13, sections 4-8).*

Comment 3. Please use Word's equation editor to write equations, instead of an image.

Response: *The equation for permeability calculation was now made in Word's equation (Page 13, line 542).*

Comment: 4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Matrigel, mTESR1, Nutristem, Accutase, Glutamax, Zoë Culture Module

Response: *All of the above terms are now changed to generic terms.*

Comment: Summary: 1. Please reduce the length of the protocol to 10-50 words.

Response: *We have now adjusted the summary to 47 words to read as follow (Page 1; Line 26):*

The blood-brain barrier (BBB) is a multicellular neurovascular unit tightly regulating brain homeostasis. By combining human iPSCs and Organ-on-Chip technologies, we have generated a personalized BBB-Chip, suitable for disease modeling and CNS-drug penetrability prediction. We provide a detailed protocol for the generation and operation of the BBB-Chip.

Protocol:
Comment:1. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Response*: The one case that we used 1000 RPM has now been changed to 200 RCF. (Page 6; Line 246).*

Comment: 2. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please reduce the highlighted length to no more than 2.75 pages of the protocol (including headers and spacing).

Response: *The highlighted length was now reduced to 2.75 pages of the protocol, which includes sections 6 and 8 (Page 10-13, Line 396-518).*

Comment: 3. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Response: *We have gone through the entire manuscript and separated or shortened sections that were too long according to the editor's request.*

Comment:4 Specific Protocol steps:
1. 5.5.5: Step 5.6 does not exist.

Response: *We thank the editor for noticing this mistake. Step '5.6' was now modified to '5.5.4.' Page 9; Line 386).*

Comment: Figures:

1. Please upload each Figure individually to your Editorial Manager account (6 files in total).

Response: *Each figure file is now uploaded separately.*

Comment: 2. Figure 3: Please use ‘h’ instead of ‘hrs’.

Response: *As requested 'hrs' was now changed to 'h' in figure 3.*

Table of Materials:

Comment: 1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Comment: 2. Please remove trademark (™) and registered (®) symbols from the Table of Materials.

Response: *We have verified that all items in the Table Materials have appropriate information. Trademarks and registered symbols are now removed.*

Reviewers' comments:
Reviewer #1:
Comment: Major Concerns:
1.1.5: Feed the spheres every 2-3 days by replacing half of the medium with fresh ESM. Please describe this in more details since the cells are cultured as free floating spheres. How do you remove half of the medium without removing the spheres?

Response: *We thank the reviewer for this comment. We have now added section 1.1.5 to describe the procedure (Page 5; Line 177):*

1.1.5. Feed the spheres every 2-3 days when the medium turns yellow by replacing half of the medium with fresh ESM. This allows for spheres to remain in conditioned medium, which is highly important for their growth and maintenance.

1.1.5.1. Lean the flask on a tube rack and allow the spheres to settle by gravity for 1-2 minutes down to the corner of the flask.

1.1.5.2. Once settled, aspirate half of the supernatant with a 5 ml or 10 ml serological pipette and replace with fresh, pre-warmed ESM.

Comment: 1.2.4: "gentle" pipetting is a very tricky term. Everybody's "gentle" pipetting is different and with iPSCs culture, small differences in this technique can yield vastly different results. Can the authors provide a video on this?

Response: *We thank the reviewer for the important note. We have now removed the term "gentle" from the sentence and added the underlined below at the end of the sentence (Page 5; Line 206), which now reads:*

1.2.6. Triturate the spheres into single cells by pipetting using a 1 ml pipette followed by a 200 µl pipette, until all spheres have dissociated. Avoid bubble formation during the trituration procedure.

Comment: 2.5: adding Accutase for 35 min is something of concern. Accutase can essentially kill the culture.

Response: *While most cells, such as neural cells cannot tolerate Accutase for 35 minutes, iBMECs can endure this treatment. This procedure was previously described in multiple publications (Lippmann et al., 2014, Lippmann et al., 2012, Canfield et al., 2016, Vatine et al., 2017, Lim et al., 2017, Vatine et al., 2019 and more). Surprisingly, cell viability following this treatment is above 90%. We have now added a note to this section to clarify (Page 6; Line 238):*

Note: While this is considered a long incubation time in Accutase, iBMECs can endure this treatment with cell viability higher than 90%.

Comment: 2.7: please report centrifuge in RCF.

Response*: We have now modified 1000 RPM to 200 RCF (Page 6; Line 245):*

2.7. Add 1 volume of EC medium into the 15 ml conical to inactivate Accutase, centrifuge at 200 RCF for 5 minutes, remove medium and replace with 1 ml of EC medium (without bFGF and RA).

Comment: Figures are not labeled. The quality of Figures 4 and 6 (staining) are too low.

Response: *We agree and thank the reviewer for this comment. We have now included labeled figures with improved resolution (Figure 4 & 6) in the revised submission.*

Comment: An endothelial cell marker like CD31 or VE-cadherin staining is suggested to show the proper differentiation.

Response: *We agree with the reviewer that iBMECs also express CD31 and VE-cadherin as markers of endothelial cells. Pecam-1 (CD31) staining was now included in the following sections of the revised manuscript*

Figure Legend 6C (Page 15; Line 639):iBMECs seeded in the bottom 'blood side' channel expressed GLUT-1 and (green) PECAM-1 (CD31, red)

Representative Results (Page 14; Line 598):

GLUT-1 and Pecam-1 were used as a marker for BMECs

Section 10.4 (Page 13; Line 561):

10.4. Dilute primary antibodies in primary blocking solution and incubate overnight at 4°C. BMECs markers: GLUT-1 1:100 (Thermo Fisher, Cat# MA5-11315); ZO-1 (1:300 Thermo Fisher, Cat# 33-9100; RRID: AB-87181); PECAM-1 (CD31, 1:250; Thermo Cat# 10333); VE-Cadherin (1:200; eBioscience, Cat# 14-1449). Neural markers: βIII-Tubulin 1:1000 (Tuj1α, Sigma-Aldrich, T8660);S100β, Abcam, Cat# ab66028; Nestin 1:1000 (Millipore, MAB353); GFAP 1:1000 (Dako, Z0334).

Reviewer #2:

Manuscript Summary

This paper provides a protocol for development of a microfluidic blood-brain barrier model from human iPSC-derived bMEC and brain cells. With poor feasibility of collection of primary brain cells in live human donors, this prospect of patient-specific blood-brain barrier screening models have significant impact for the future of personalized medicine. This manuscript is well-written, without the need for copy-editing. While I would recommend it for publication in its current form, there are some additional details I think would be helpful for the readers to ensure good reproducibility of the Authors' work.

Major Concerns:

-No major concerns

Response: *We thank the reviewer for their positive comments and have addressed the minor concerns outlined below.*

Minor Concerns:

Comment: -130: The introduction describes this model as having physiologically relevant TEER values, but there is no discussion of the method of TEER measurement in this protocol. If this is an automated function provided by the Zoë platform, please specify.

Response: *We thank the reviewer for noticing that this sentence might have been misleading. In this protocol, we focused on the commercially available chips from Emulate Inc.. The TEER*

analysis that was performed in Vatine et al., 2019 and Park et al., 2019 were both made with custom made Organ-Chips with embedded gold electrodes that permitted these measurements. This feature is not available on the Emulate chip. We have now made the underlined addition to clarify this issue (Page 3; Line 126):

The BBB-Chip that was developed recreates interactions between neural and endothelial cells and achieves physiologically relevant TEER values, which was measured by custom made Organ-Chips with integrated gold electrodes[17]. Additionally, the BBB-Chip displays low paracellular permeability, responds to inflammatory cues at the organ level, expresses active efflux pumps, and exhibits predictive transport of soluble biomarkers and biopharmaceuticals.

In addition, we have added the following sentence in the bottom of the Discussion section (Page 17; Line 707):

In addition, protocols for "in house" manufacturing of microfluidic Organ-Chips are also available[32], and may offer more modularity including the integration of TEER electrodes[17], which are missing from the platform used here.

Comment: -169: Is there a particular benefit to using a P1000uL pipette tip, or would a cell scraper be suitable for this step?

Response: *We thank the reviewer for this question. While we have only tried using the back of P1000 µl pipette tip, a cell scraper would also be suitable. We have now made the underlined addition to section 1.1.3. (Page 4; Line 166):*

1.1.3. Scrape the bottom of each confluent well with the back of a sterile P1000 µl pipette tip or a cell scraper.

Comment: -213: Do the cells need to be re-suspended for cell counting?

Response: *Once the cells are detached from the matrigel coated well and after centrifugation cells are re-suspended in EC medium for counting. We have clarified the procedure for resuspension and cell counting in sections 1.2.5 to 1.2.7.*

1.2.5. Slowly remove the Accutase. Add 1 ml Neural Differentiation Medium [NDM; DMEM: F12 with 2% B27 minus vitamin A, 1% N2 Supplement, and human brain-derived neurotrophic factor (hBDNF, 20ng/ml; Peprotech)].

1.2.6. Triturate the spheres into single cells by pipetting using a 1 ml pipette followed by a 200 µl pipette, until all spheres have dissociated. Avoid bubble formation during the trituration procedure.

1.2.7. Count dissociated cells using a hemocytometer and dilute cells to a final density of 1 x 106 cells/ml. It is possible to change the density depending on the application.

Note: Higher densities (up to 6 x 106 cells/ml) are recommended for short-term cultures of up to three days and lower densities are recommended for long-term applications of up to three weeks.

Comment: -247: It would also be helpful to the reader to include the length of the channels separated by the membrane, especially since the area is required for the equation on line 574.

Response: We *thank the reviewer for the useful comment. It is indeed necessary to specify the area that is separated by the membrane for* P_{app} *calculation. We have now added the area in the bottom of section 3.1 (Page 7; Line 263):*

3.1. The basic structure of the Organ-Chip utilized for the BBB-Chip model and its production is based on previous protocols.[16-18] *The tall channel Organ-Chip (Chip-S1, Emulate Inc.,) is fabricated from a highly flexible polydimethylsiloxane (PDMS) elastomer that contains two superimposed and parallel micro-scale channels separated by a flexible porous membrane. The top and bottom micro-channels sizes are 1×1 mm and 1 × 0.2 mm, respectively. The two channels are separated by a 50 μm thick PDMS-made flexible porous membrane, containing 7 μm diameter pores with 40 μm spacing. The surface area of the porous membrane that separates the channels is 0.171 cm*^{2}*.*

Comment: -420: Though this information is included for the iBMEC-loading step, what is meant by "optimal or uneven" seeding density? For example, approximate % cell coverage.

Response: *We thank the reviewer for the useful comment. We have now made two changes to clarify the required density (Page 10; Line 416 and Page 11; Line 450):*

6.3.3. Seeding density should appear as 20% coverage. If seeding density is higher or lower than expected or uneven, return the chips to the BSC, wash the channel 2 times with 200 μl of fresh medium, and repeat Step 6.3.1.

7.7. If seeding density is below 90% coverage or unevenly distributed, adjust cell density accordingly and repeat steps 7.2 through 7.6 until the correct density is achieved within the channel.

Comment: -468: What is the optimal amount of time to wait before inverting the chips for attachment to the underside of the membrane?

Response: The *incubation time before inverting the cells is approximately 3 hours, as specified in section 7.8 (Page 11; Line 457):*

7.8. Incubate chips at 37°C for approximately 3 hours, or until cells in the bottom channel have attached.

Comment:-546: It may be useful to some readers to include the surface shear stress at the recommended flow rate.

Response: *The authors thank the reviewer for the useful comment. We have now added the shear stress values in a note in section 8.5.3. (Page 12; Line 515):*

Note: When flowing media such as EC media or ESM at 30 and 1000 µl/hr the shear forces are 0.01 and 0.33 dyn/cm^2, respectively.

Comment: -613: Can imaging be done directly on the chip, or does it need to be destroyed so the membrane can be placed on a slide? If the thickness of the PDMS is the reason for long-distance working objective being required, please specify. Is an inverted microscope required?

Response: We *thank the reviewer for the constructive comment. We have now clarified why the long-distance objectives are required and that both, upright or inverted microscopes can be used in section 10.11 (Page 14; Line 584):*

10.11. The chip is ready for imaging using either an upright or an inverted fluorescent microscope. The PDMS transparency permits imaging in the intact Organ-Chip. Magnifications above 10X may require long working distance objectives due to the thickness of the Organ-Chip.

Comment: -665: If this data is available, it would be more informative to also include Dextran P(app) values for iBMEC-only chips (to demonstrate the value of including EZ's) and blank chips.

Response: *We thank the reviewer for this comment. We have now added a condition with iBMECs only (without EZ-spheres). Consequently, we have modified the manuscript as follow:*

Figure legend 6D (Page 15; Line 642):

*Results demonstrate that Organ-Chips seeded with iBMECs and EZ-spheres display a tight barrier compared to Organ-Chips seeded with iBMECs alone (*P<0.05). Organ-Chips seeded with EZ-spheres alone do not display any barrier properties (***P<0.001). One-way ANOVA with Tukey's multiple comparisons test.*

Representative Results section (Page 15; Line 608):

These results demonstrate that iBMECs form functional barrier properties, which are further tightened when iBMECs are co-cultured with EZ-spheres. Chips cultured with EZ-spheres only, fail to form a barrier.

Comment: -705: If the authors have any perspective to provide about differences in performance when using primary neurons or iPSC-derived motor neurons (either with results or from the cited literature), it may be helpful to include this in the discussion section.

Response: *Unfortunately, we were not able to find corresponding values for primary cells, and have not tried this in our lab.*

Reviewer #3:

Manuscript Summary:

In this manuscript, Vatine et al. describe in great detail a protocol combining organ-on-a-chip and iPSC technologies for the generation of a 3D blood-brain-barrier (BBB) chip model. The authors present specific protocols to i) differentiate both neural cells and BMEC from iPSC; ii) the ECM coated membrane for the formation of cell-to-cell interactions; iii) chip preparation including how to seed the cells; and iv) how to perform permeability and immunofluorescence assays on those chips. The protocol described here should be of interest to researchers in a variety of fields. Given that 2D-BBB models, such as transwell inserts, have a rigid surface that hampers direct cell-cell interactions between different cells to create the neurovascular unit, and the absence of mechanical forces such as blood flow, the 3D model presented here will allow further progress in drug screening and research on the role of the BBB in neurological disorders in a more physiological context. The usefulness of this protocol has been recently demonstrated in Vatine et al., Cell Stem Cell. 24:6 995-1005; 2019. The introduction section is very complete; no key references are missing. The discussion is also very complete, critically stating the strengths and weaknesses of the protocol, such as the fact that pericytes are missing. The degree of "brain" maturation could be further discussed, a the authors use rather immature neural cells from only 7 days of differentiation.

Response: *We thank the reviewer for their positive feedback on the manuscript and have addressed major and minor concerns outlined below.*

Major Concerns:

Comment: My only major concern is that some aspects of the protocol (especially those dealing with the actual chip device and the culture module) rely on specific commercial products (by Emulate Inc.). It would be desirable that the authors described other non-proprietary alternatives alongside the commercial ones.

Response: *We understand the reviewer concern regarding the specific commercial product that is described in this manuscript. We have now added a paragraph at the end of the Discussion section in which we mention another commercially available alternative and a detailed published protocol for "in house" manufacturing of chips (Page 17; Line 705):*

While this protocol relies on a specific commercially available product, additional commercial companies offer diverse microphysiological platforms, which may offer alternative advantages[31]. In addition, protocols for "in house" manufacturing of microfluidic Organ-Chips are also available[32] and may offer more modularity including the integration of TEER electrodes[17], which is missing from the platform used here

Minor Concerns:
- The authors seed "pre-rosette" spheres on the chip and let them differentiate for 7 days to obtain iNPC, astrocytes and neurons. What is the percentage of each type of cell in the mixed culture? Using such a short time of differentiation, the authors could be using immature cells, not reflecting the in vivo physiology. This should be analyzed and/or discussed in the manuscript.

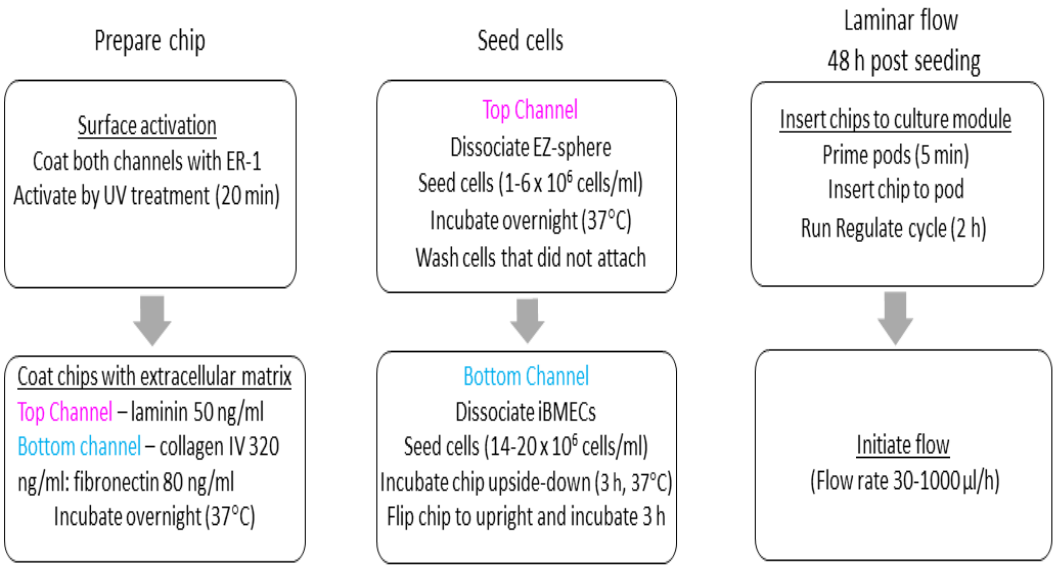
Response: *As this reviewer comments, the cells that are used in this protocol are early neural progenitors. Therefore, it is not surprising that they are immature cells. These cells reflect a more immature stage of neural cells. We have addressed this in the Discussion section and now added a sentence to emphasize this (Page 16; Line 684):*

iBMECs can be seeded prior to or after the neural cells, making it possible to seed iBMECs immediately following seeding and attachment of neural cells, or after EZ-spheres have differentiated into more mature neural cultures in the chip environment. This is of particular importance given the immature nature of iPSC-derived neural cells. Given that EZ-spheres are early neural progenitors, longer differentiation periods may also result in increased percentage of astrocytes and neurons.

-Comment: Figure 3 showing the scheme of the protocol should be more detailed.

Response: *Yes we agree with the reviewer comment. As per the suggestion, the modification has been made in the revised manuscript.*

Modification:



Comment; - Figure 6 shows only GFAP and DAPI from the brain part of the chip. Some neuronal marker (e.g. MAP2 or TUJ1) should also be shown.

Response: *We appreciate the reviewer's concern. Additional glial and neuronal markers were now added both, to Figure 6 and the text of the revised manuscript:*

Representative Results (Page 14; Line 596):

Immunocytochemistry was then performed on the BBB-Chip using Nestin as a marker for neural progenitor cells, S100ß or GFAP as markers for astrocytes and βIII-tubulin as a marker for neurons.

Section 10.4. (Page 14; Line 561):

Neural markers: βIII-Tubulin 1:1000 (Tuj1α, Sigma-Aldrich, T8660); Nestin 1:1000 (Millipore, MAB353);GFAP 1:1000 (Dako, Z0334).