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Investigating the Physiological Use of an Organ-on-a-Chip Device through the Design and Fabrication of a micro-Electrical Blood-Brain Barrier (µE-BBB) System

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ABSTRACT:

Aging is considered the main risk factor for various neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD). Aging is associated with an increase in neuroinflammation, which is a significant contributor to cognitive impairment observed in these age-related neurodegenerative diseases. However, studying brain behavior remains a challenge due to the complex nature of isolating brain tissue from an organism. Modeling the brain allows for further study of these neurodegenerative diseases. Organ-on-a-chip (OoC), a system containing engineered or natural tissues grown within microfluidic chips, has emerged as a promising tool for mimicking human physiology in a controlled microenvironment. A healthy blood-brain barrier (BBB) is essential for maintaining brain tissue, while disruption of the BBB results in toxins and immune cells entering the central nervous system. This impairment is associated with neurodegenerative diseases like AD and PD. Here, a brain-on-a-chip system has been created to mimic the human BBB. Results were validated through light microscopic imaging of the triple co-culture of cells associated with the BBB, namely, microglial, endothelial, and astrocyte cells, in the device. The development of this device can be an effective platform for studying neurodegeneration and testing new therapeutics.

INTRODUCTION:

Neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), are a major global health concern due to their increasing prevalence among aging populations. Despite efforts to understand the etiology of these diseases, the underlying mechanisms remain unclear. Organ-on-a-chip devices may be a useful platform for modeling the microenvironment of the brain, providing more accurate and relevant models of neurodegenerative disease that may further inform our understanding of neurodegenerative diseases and lead to the discovery of new treatment options.

The Blood-Brain Barrier (BBB) Microenvironment

The blood-brain barrier (BBB) is a network of blood vessels and tissue made up of tightly packed cells that keeps toxins from reaching the brain. The BBB allows some substances, such as water, oxygen, and carbon dioxide, to pass into the brain. Microglial, endothelial, and astrocyte cells are found in the BBB. Microglia, a type of glial cell, are located throughout the brain and spinal cord.[1] These cells account for 7% of the cells found within the brain.[2] They serve as the main form of immune defense in the central nervous system (CNS). Microglia (SIM-A9) play an important role in cellular repair after brain damage and are thus particularly sensitive to small pathological changes in the CNS.[3] Their sensitivity is demonstrated by their potassium channels that respond to changes in ion concentration. In addition to microglia, astrocytes and brain endothelial cells are two other important cell types that constitute the blood-brain barrier

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[1]Ginhoux F, Lim S, Hoeffel G, Low D, Huber T (2013). "Origin and differentiation of microglia". *Frontiers in Cellular Neuroscience*. **7**: 45. doi:10.3389/fncel.2013.00045

[2]Dos Santos SE, Medeiros M, Porfirio J, Tavares W, Pessôa L, Grinberg L, et al. (2020). "Similar Microglial Cell Densities across Brain Structures and Mammalian Species: Implications for Brain Tissue Function". *The Journal of Neuroscience*. **40** (24): 4622–4643. doi:10.1523/JNEUROSCI.2339-19.2020

[3]Dissing-Olesen L, Ladeby R, Nielsen HH, Toft-Hansen H, Dalmau I, Finsen B (2007). "Axonal lesion-induced microglial proliferation and microglial cluster formation in the mouse". *Neuroscience*. **149** (1): 112–122. doi:10.1016/j.neuroscience.2007.06.037

(BBB). Astrocytes (C8-D1A), located on the basolateral chamber of the BBB, have been shown to contribute to both neuroinflammation and cell signaling through modulation of the tight junctions between endothelial cells. On the other hand, brain endothelial (bEnd3) cells, located on the lumen channel of the BBB, play a critical role in maintaining the selective permeability of the BBB through the formation of tight junctions between adjacent endothelial cells (Figure 1).

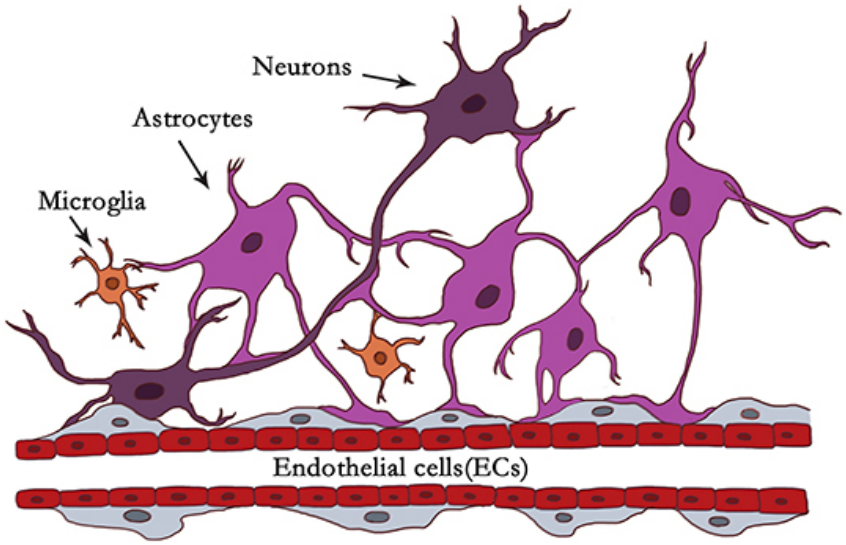

Figure 1. Interaction of microglia and astrocytes with brain endothelial cells adapted from Liu et al., 2020[4]

Under normal conditions, the CNS does not come into contact with pathogenic factors because of the endothelial cells that make up the BBB. The BBB maintains brain homeostasis and protects neurons by preventing pathogens from reaching nervous tissue.[5] Microglia and astrocytes are layered and overlapped throughout the CNS.[6] The BBB integrity is determined by

[4]Liu, L. R., Liu, J. C., Bao, J. S., Bai, Q. Q., & Wang, G. Q. (2020). Interaction of Microglia and Astrocytes in the Neurovascular Unit. Frontiers in immunology, 11, 1024. https://doi.org/10.3389/fimmu.2020.01024
[5]Takata, F. Nakagawa, S. Matsumoto, J. Dohgu, S. (2021) Blood-brain barrier dysfunction amplifies the development of neuroinflammation: Understanding of cellular events in brain microvascular endothelial cells for prevention and treatment of BBB Dysfunction. *Frontiers in Cellular Neuroscience*, *15*.
[6]Kreutzberg GW (1995). "Microglia, the first line of defense in brain pathologies". *Arzneimittel-Forschung*. **45** (3A): 357–360.

signaling pathways between astrocytes and microglia with endothelial cells.[7] Signaling molecules in brain endothelial cells and inflammatory mediators released by astrocytes and microglia work together to prevent BBB disruption. However, when the barrier is impaired, infectious agents can cross the BBB, thus activating neuroinflammation, which is seen in many neurodegenerative diseases.

While microglia have been well studied, their role in the pathology of many CNS diseases remains unclear. Several studies have revealed that microglial-mediated inflammation contributes to the progression of Alzheimer's disease (AD).[8] However, the specific mechanisms through which these cells contribute to AD remain unclear. An understanding of intracellular interactions that disrupt the BBB may elicit insight into treatments for neurodegenerative diseases characterized by neuroinflammation.

Organ-on-a-Chip Devices

In the past, cell culture models, the process by which cells are grown under controlled conditions, were developed to create an in vitro model to study neurodegenerative diseases. Cell culture models can be 2D, where cells are in contact with nutrients. They can also be 3D, where cells are grown in an extracellular matrix.[9] 3D cell culture models allow for the study of brain processes in the context of the human genetic background and species-specific developmental mechanisms.

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[7]Takata, F. Nakagawa, S. Matsumoto, J. Dohgu, S. (2021) Blood-brain barrier dysfunction amplifies the development of neuroinflammation: Understanding of cellular events in brain microvascular endothelial cells for prevention and treatment of BBB Dysfunction. Frontiers in Cellular Neuroscience, 15.

[8]Takata, F. Nakagawa, S. Matsumoto, J. Dohgu, S. (2021) Blood-brain barrier dysfunction amplifies the development of neuroinflammation: Understanding of cellular events in brain microvascular endothelial cells for prevention and treatment of BBB Dysfunction. Frontiers in Cellular Neuroscience, 15.

[9]Brown, J., Quadrato, G., & Arlotta, P. (2018). Studying the brain in a dish: 3D cell culture models of human brain development and disease. Current Topics in Developmental Biology, 99–122. https://doi.org/10.1016/bs.ctdb.2018.03.002

Different co-culture models have been developed to study the interaction between glial and BBB cells. A recent study developed a neuron, microglia, and astrocyte triple co-culture model on an ELISA plate to study the mechanisms present in AD neurodegeneration.[10] However, this model was not capable of real-time monitoring. Thus far, most studies have shown that real-time monitoring of the data can only be conducted with a single cell line rather than three.[11] Goshi et al. 2020 developed a 3D cell culture model with neurons, astrocytes, and microglia.[12] While these studies are valuable in eliciting background information during preliminary studies, these studies are limited in their ability to mimic the tissue environment as blood flow and cellular tension cannot be controlled.

Organ-on-a-chip (OoC) devices can recreate a physiologically relevant environment that can mimic human tissues and organs, accounting for the limitations present in cell culture models. Thus, they are particularly useful for drug discovery, toxicity testing, and disease modeling. Brain OoCs have been specifically developed to study neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD).[13]

OoC devices are extremely useful for mimicking the brain tissue microenvironment, demonstrating the potential of brain OoCs for modeling neurodegenerative diseases. Microglia are the immune cells of the central nervous system (CNS) that play a critical role in

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[10]Luchena, C., Zuazo-Ibarra J., Valero J., Matute C., Alberdi E., and Estibaliz Capetillo-Zarate. (2022) "A Neuron, Microglia, and Astrocyte Triple Co-Culture Model to Study Alzheimer's Disease." *Frontiers in Aging Neuroscience* 14 . https://doi.org/10.3389/fnagi.2022.844534.

[11]Luchena, C., Zuazo-Ibarra, J., Valero, J., Matute, C., Alberdi, E., & Capetillo-Zarate, E. (2022). A Neuron, Microglia, and Astrocyte Triple Co-culture Model to Study Alzheimer's Disease. Frontiers in aging neuroscience, 14, 844534. https://doi.org/10.3389/fnagi.2022.844534

[12]Goshi, N., Morgan, R.K., Lein, P.J. et al. A primary neural cell culture model to study neuron, astrocyte, and microglia interactions in neuroinflammation. J Neuroinflammation 17, 155 (2020). https://doi.org/10.1186/s12974-020-01819-z

[13]Ma, C., Peng, Y., Li, H., & Chen, W. (2021). Organ-on-a-Chip: A New Paradigm for Drug Development. Trends in pharmacological sciences, 42(2), 119–133. https://doi.org/10.1016/j.tips.2020.11.009

neuroinflammation and neurodegenerative diseases. Shi et al., 2013 recreated a brain microenvironment using microglial and neuronal cells. The study examined the use of a brain OoC microfluidic device to investigate the potential mechanisms behind synapses.[14] Shi et al., 2013 discovered that communication between neurons and glia is critical for the formation and stability of synapses, demonstrating the importance of microfluidic platforms as co-culture systems.

OoCs have also been used to model the progression of PD by co-culturing brain endothelial cells and astrocytes in a microfluidic device, which allowed for the assessment of drug toxicity and neuroprotection, both of which are highly associated with the presence of neurodegenerative diseases.[15] 3D printing has been used to fabricate microfluidic devices with embedded microelectrodes that can measure the electrical activity of neurons in real time.[16] Despite these recent advancements, there are still limitations in the device design that need to be addressed.

One of the main challenges with the current OoC devices is the complexity of the brain tissue, which makes it difficult to mimic. For example, the BBB is a highly specialized barrier that restricts the passage of molecules from the blood to the brain. Several studies have attempted to model the BBB in OoCs, but there are still limitations in the reproducibility and scalability of these models. These limitations highlight the need for further development and optimization of OoC designs for brain research.

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[14]Shi, M., Majumdar, D., Gao, Y., Brewer, B. M., Goodwin, C. R., McLean, J. A., Li, D., & Webb, D. J. (2013). Glia co-culture with neurons in microfluidic platforms promotes the formation and stabilization of synaptic contacts. Lab on a chip, 13(15), 3008–3021. https://doi.org/10.1039/c3lc50249j

[15]Choi, J. H., Santhosh, M., & Choi, J. W. (2019). In Vitro Blood-Brain Barrier-Integrated Neurological Disorder Models Using a Microfluidic Device. Micromachines, 11(1), 21. https://doi.org/10.3390/mi11010021

[16]Hiramoto, K., Ino, K., Nashimoto, Y., Ito, K., & Shiku, H. (2019). Electric and Electrochemical Microfluidic Devices for Cell Analysis. Frontiers in chemistry, 7, 396. https://doi.org/10.3389/fchem.2019.00396

In conclusion, OoC devices represent a powerful tool for studying biological systems in vitro. By mimicking the structure and function of human tissues or organs, OoC devices provide a more accurate representation of human physiology than traditional in vitro models. OoC devices have numerous applications in drug development, disease modeling, and toxicology testing. The use of OoC devices for studying the BBB and microglia interactions can help in understanding the mechanism of neuroinflammation, which is a critical step in the development of therapies for neurodegenerative diseases. Previously, Payam et al., 2021 created a first-generation micro-electrical blood-brain barrier (μE-BBB) device that addressed some of the limitations mentioned above; namely, the ability to seed cells into the device with real-time monitoring.[17]

μE-BBB Device

Payam et al., 2021 μE-BBB device contains a trans-endothelial electrical resistance (TEER) system which allows for real-time monitoring of physiological conditions through integrated gold electrodes. Payam et al., 2021 discovered that TEER measurements are inversely proportional to BBB permeability, thus providing a mechanism to investigate BBB resistance. The device also contains a 0.4 μm pore membrane which is sandwiched between a luminal channel and a basolateral chamber. Both sides of the membrane contain inlets and outlets that are made of polyurethane tubing. Under the tubing, there are gold contact pads where wires were soldered onto the pads to allow for connection to an epithelial volt ohmmeter system (EVOM) for TEER measurements. I collaborated with researchers at the Aran Lab to optimize this device

[17]Amiri, P., DeCastro, J. Littig, J. Lu, H. Liu, C. Conboy, I. and Aran, K. (2021). "Erythrocytes, a New Contributor to Age-Associated Loss of Blood–Brain Barrier Integrity." Advanced Science 8, no. 20: 2101912. https://doi.org/10.1002/advs.202101912.

to mimic the BBB environment with a microfluidic triple co-culture model that demonstrates real-time monitoring on a mass-scale level.

I hypothesize that the μE-BBB organ-on-a-chip device can be used to mimic the BBB microenvironment through the triple co-culture of brain cells. To assess the device, microglia, astrocytes, and endothelial cells were co-cultured in a BBB, organ-on-a-chip model. Through this investigation, I determined how an OoC device can be used for the triple co-culture of astrocytes, endothelial cells, and microglia while also optimizing the device fabrication protocol for this experiment (Figure 2). I assessed the validity of the μE-BBB platform by staining and imaging the cells from the membrane via a light microscope.

MATERIALS AND METHODS:

*μ*E-BBB Device Fabrication

The fabrication process of the *μ*E-BBB device involves several steps (Figure 2).

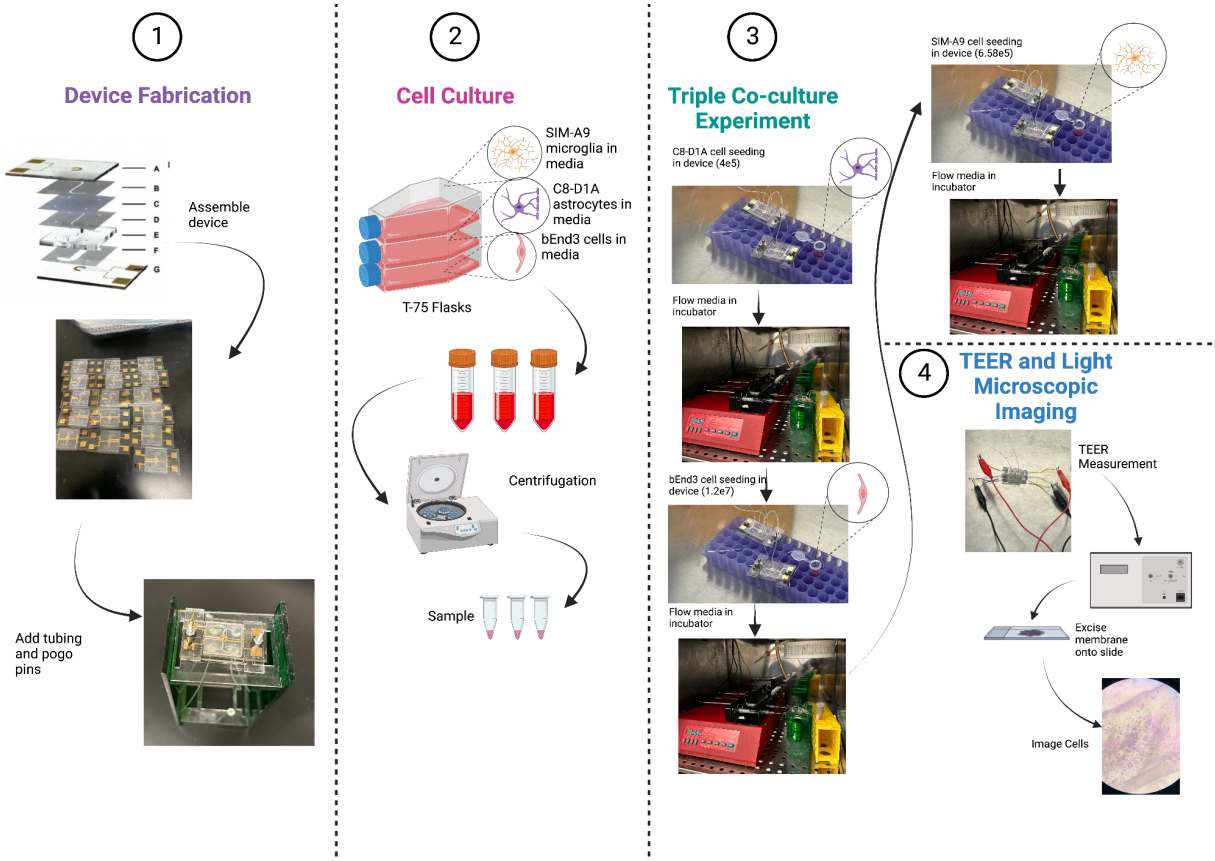


Figure 2. Fabrication and experimentation with the *μ*E-BBB device. Step 1: Device fabrication using polycarbonate, tubing, pogo pins and gold sputtering to assemble the device. Step 2: Cell culture and preparation of SIM-A9, C8-D1A, and bEnd3 cells. Step 3: Triple co-culture experiment of all cell lines in *μ*E-BBB device. Step 4: TEER measurement of experiment and H&E staining of membrane to visualize cells.

Initially, the polycarbonate pieces and the membrane were prepared. The top electrode, bottom electrode, and basolateral membrane pieces were cut using a Roland Modela Milling Machine. To achieve this, the drill was cleaned, and the residue was removed via vacuuming. The pieces were then filed and cleaned so that double-sided tape can be applied to stick them onto the grid. The Roland MDX panel was set to the appropriate z, x, and y axes, and the pieces

were drilled into rectangular shapes. Subsequently, a laser cutter was used to cut tape for the gold electrode top and bottom pieces. The laser cutter was set to the appropriate parameters, including 30.00 X 45.72 cm, uncertified material, 1 mm thickness, 500 speed, and 35 precision power. The tape was placed on the plastic pieces for gold plating. Once the tape was secured to the plastic, the pieces were placed in the gold sputter machine. Argon gas was released, and the gold sputter machine was turned on to deposit the gold. The piece was then cleaned with alcohol, DI water, and nitrogen.

The membrane (0.4 micrometers) was made by taping blue and then transparent paper on a metal block using forceps. Then, a micro-torch was used to heat a needle to cut four corner holes on the "i" sticker. The "i" sticker was placed onto white paper and arbor pressed four times on each side. The arbor press is a small, manually operated press used to squash the device pieces together. Then, using a razor blade, the tape was cut away. Next, the membrane was flipped over leaving the blue paper on the metal block. The small "S" sticker was taped onto the membrane in the same direction as the "i" sticker. The membrane was arbor-pressed four times and finally, the membrane was excised with a razor blade.

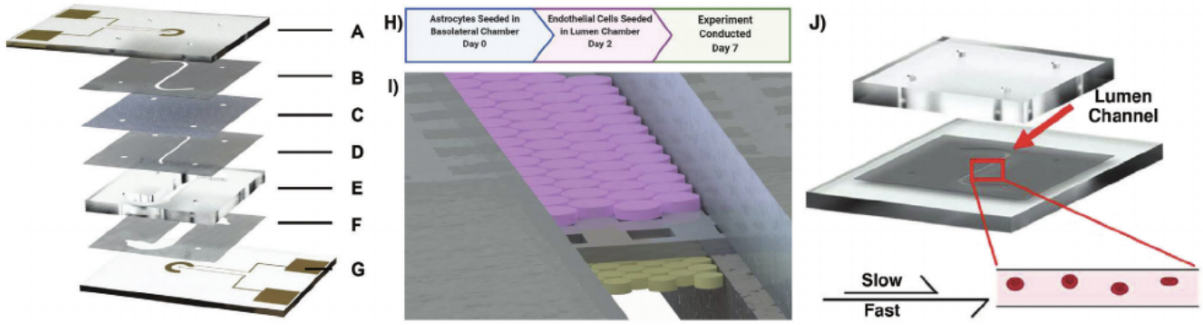


Figure 3. The μE-BBB is comprised of A) a lumen electrode, B) a lumen channel, C) a PC membrane, D) a membrane-basolateral adhesive layer, E) a basolateral layer, F) a basolateral electrode adhesive layer, and G) a basolateral electrode. H,I) The μE-BBB is seeded with astrocytes (yellow) on day, and with endothelial cells (pink) on day . By day , the μE-BBB is fully mature. J) To evaluate the behavior of erythrocytes in response to shear stress, a modified μE-BBB was utilized that only contains the shear inducing lumen channel. Schematic shows that old and young erythrocytes experience either a slow flow rate ($\mu Lmin^{-1}$) for low shear or high flow rate ($\mu Lmin^{-1}$) for physiological shear in BBB integrity studies, adapted from Payam et al., 2021.[18]

After all the individual (A-G) pieces were fabricated (Figure 3), the device was assembled from top to bottom. Before assembling the pieces, the gold electrodes were tested with an ohmmeter to confirm electrical flow. Then, a wire was poked through the holes to ensure the device can be assembled. Next, the pins were placed onto a square block. The top piece (layer A) was placed on the metal block on top of the four pins. Then, four holes were poked into the membrane and the "i" channel was confirmed to not be torn under the microscope. The bottom film layer was removed, and forceps were used to line up the membrane to the pins on top of the top piece. The membrane (layer B-D) was pushed down at an angle to push the air out towards the square electrodes. The "i" sticker was placed directly on the skinny gold electrode. Next, the top piece was taken off from the pins and the arbor press was used to get rid of the air

[18]Amiri, P., DeCastro, J. Littig, J. Lu, H. Liu, C. Conboy, I. and Aran, K. (2021). "Erythrocytes, a New Contributor to Age-Associated Loss of Blood–Brain Barrier Integrity." Advanced Science 8, no. 20: 2101912. https://doi.org/10.1002/advs.202101912.

bubbles in the membrane. After arbor pressing, the piece was placed back on the metal block with the pins. A clean-shaven basolateral membrane (layer E) was obtained and cleaned with alcohol, deionized water, and nitrogen gas. The top film of the membrane was removed using a razor blade and tweezers. Then, the basolateral membrane was placed in the opposite direction of the small S on the membrane. The basolateral membrane was secured to the membrane using forceps. Then, the big S membrane sticker (layer F) was placed on top of the basolateral membrane in the same direction by removing the film. Air bubbles were removed using forceps. Then, the second sticker layer of the big S membrane was removed using a razor blade, and the bottom piece (layer G) was placed on top. Finally, the entire device was arbor pressed four times to ensure adequate adhesion and no leakage.

Once the device was fabricated from top to bottom, tubes were placed into the device. Tubing was prepared by using a razor blade to cut the tube at one end, ensuring it was in a sharp diagonal. The tubes were secured into each hole of the device using epoxy (Figure 4). Then, the device was hung upside down using magnets so that the resin did not obstruct the tubes.

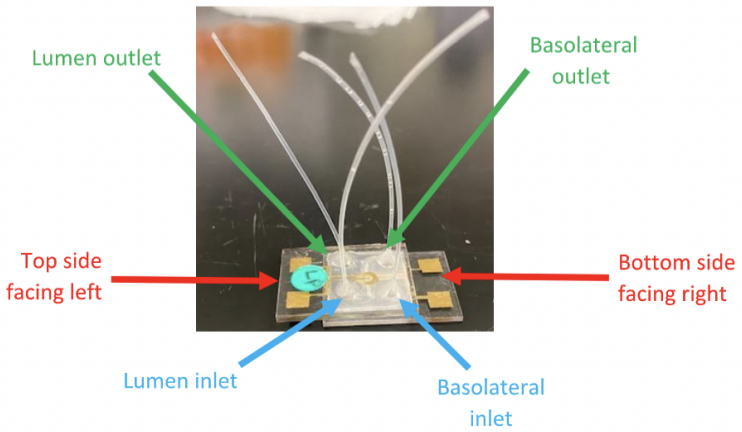


Figure 4. Orientation of the first generation μE-BBB device with tubes

After the tubes were secured to the device, wires were soldered and placed onto the gold electrodes as shown below (Figure 5).

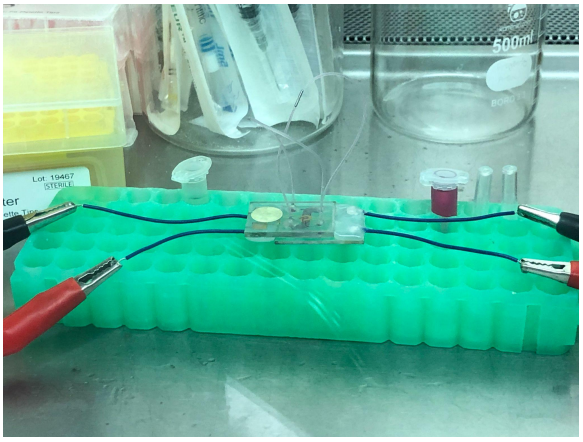


Figure 5. μE-BBB device with tubes and wires

After the devices were fabricated, they were validated to ensure water-tight conditions with no obstruction.

Cryo-Handling and Expansion Procedure: bEnd3 and C8-D1A

In collaboration with Noor Chaudhry, cell culture was conducted. Before the addition of the cells, 9mL of complete DMEM growth media was added to a T-75 flask and placed in the incubator at 37°C for 15 minutes to allow the medium to reach its normal pH (7.0-7.6). Then, the cell vial was thawed via gentle agitation in a 37°C warm bath. After 15 minutes, the T-75 flask was removed from the incubator and 9 mL of DMEM growth media were transferred to a centrifuge tube. Then, the cell vial contents were transferred to the same centrifuge tube and spun at 125 x g for 5-7 minutes. The cell pellet and orientation were noted when taking out the tube from the centrifuge. Next, the supernatant was suctioned out with 100 μL of media left. The cell pellet was then resuspended with 5 mL of complete DMEM. 5 mL of the cell suspension was pipetted into a T-25 flask and labeled. Cells were confirmed and imaged from a light microscope. Healthy cells were light and circular, while dead cells were gray and dense.

The culture was incubated at 37°C. The cell flask was removed from the incubator within 24 hours and visualized and imaged under a microscope. The dead cells were removed by first suctioning out the spent media. Then, the bottom adherent surface was washed with 2-3 mL of 0.22 μm filtered PBS by pipetting in PBS, flipping the T-25 flask, pooling PBS to the top corner surface, and pipetting again. The PBS was suctioned again by pooling it to the top corner surface. Then, the DMEM was replaced with 5 mL of fresh complete media, placed back into the incubator, and left for 48 hours. The media was then renewed every 2 to 3 days.

To passage bEnd3 and C8-D1A cells, the appropriate PPE was first worn. Then, trypsin-0.03% EDTA, complete growth media (DMEM+ 10% FBS - fetal bovine serum + 1X AA - antibiotic antimycotic), and 0.22 micrometer filtered sterile PBS were placed in a hot bath for 20 to 30 minutes. The microscope and incubator were sprayed down with alcohol and a Kim wipe. Then, the T-25 flask was obtained from the incubator and the cell morphology and confluency were checked.

Once the morphology and confluency were checked and noted, the cells were put back in the incubator while the sterile hood was prepared and cleaned. Then, the reagents and cells were obtained from the heat bath and incubator, respectively, and put into the hood. The media at the top of the T-75 flask was sucked. Then, the cells were washed with PBS to make sure no media was remaining. 1.5-2 mL of PBS was used to wash the cells. Then, the PBS was removed and suctioned again. Trypsin was added to digest the proteins that adhere the cell to the surface. Then the flask was placed back into the incubator for five minutes so that the trypsin can function under heat. A new flask was prepared and labeled with the cell line, passage, and concentration. The cells were obtained from the incubator and appeared as round circles. Then, trypsin was inactivated by placing double the amount of growth media. The FBS protein in the DMEM

saturates and inactivates trypsin. The volume and cell count were noted via a hemocytometer. Then, the new cell suspension volume of centrifuge liquid was pipetted into a new flask. The growth media was also added into a new flask. Cells were confirmed to be in the flask via a microscope. Then, the cells were placed back in the incubator and the medium was renewed every 2 to 3 days.

Cell seeding into *μ*E-BBB device

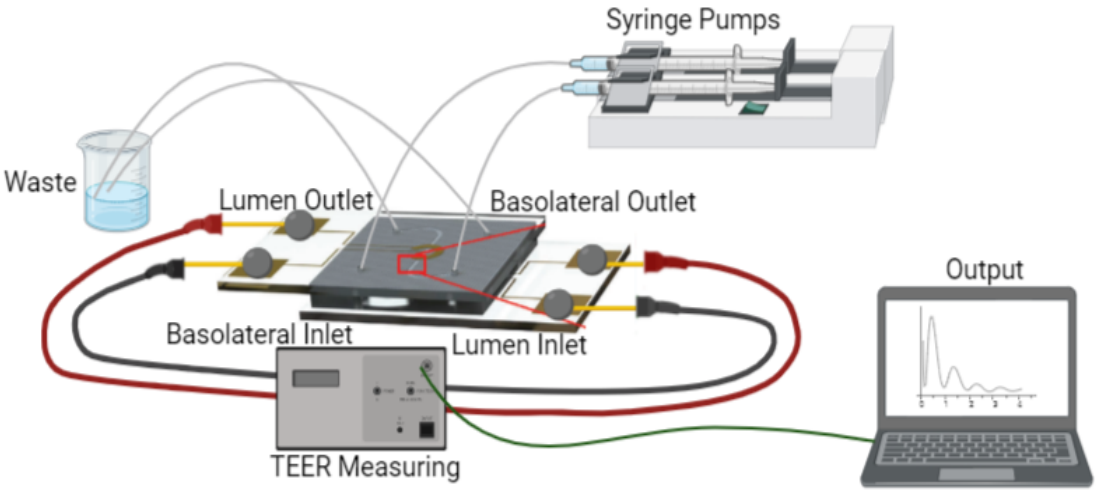


Figure 6. Experiment Set-Up with Soldered Wire Design, adapted from Jonalyn Herce and Mary Bessell

In collaboration with Noor Chaudhry, cell seeding into the device was conducted (Figure 6). To seed into the device, initially, metal plugs are used to plug the basolateral tubes and flush the lumen. Then, the air was vacuumed out of the device by filling an Eppendorf with PBS/EtOH and withdrawing the liquid from the outlet to the inlet. Then, the basolateral membrane was flushed out. The device was then sterilized by filling it with 70% EtOH. First, the lumen channel was filled and then the basolateral chamber of the membrane. Then, the device was flushed and filled with PBS with first the basolateral and then the lumen channel of the membrane. Next, the device was flushed and filled with fibronectin. The device was then incubated at 37 °C for 1

hour. The device was then flushed and filled with complete growth media, beginning with the basolateral and then the lumen channel of the membrane. The device was then incubated at 37 °C for 2 more hours. During the 2-hour incubation period, trans-epithelial electrical resistance (TEER) data were sequentially collected, and TEER values were calculated to measure resistance values in the blood-brain barrier. Then, C8-D1A cells were passaged by re-suspending cells to a concentration of $4 * 10^5$ cells/ml. Next, the basolateral chamber of the device was flushed and filled with C8-D1A cell suspension. The devices were connected to syringes filled with complete growth media at the inlets. They were placed upside down in the incubator with the outlets feeding into an open conical vial that has EtOH in it. Then, the device was left to adjust to normal pH for three hours. Three hours after the initial seed, the media was allowed to flow, and TEER measurements were collected (Figure 7).

Then, the process for seeding C8-D1A cells was repeated for bEnd3 cells. The cells were first resuspended in media to a concentration of $1.2 * 10^7$. Then, the device was flushed and filled from the lumen channel with bEnd3 cell suspension with the outlets feeding into a closed conical vial with ethanol. The device was then connected to a syringe filled with complete growth media from the basolateral inlet and it was placed into the incubator at 37 °C for 3 hours. Three hours after the initial seed, the media was allowed to flow, and TEER measurements were collected.

Finally, the process for seeding bEnd3 cells was repeated for SIM-A9 cells. The cells were first resuspended in media to a concentration of $1.2 * 10^7$. Then, the device was flushed and filled from the lumen channel with bEnd3 cell suspension with the outlets feeding into a closed conical vial with ethanol. The device was then connected to a syringe filled with complete growth media from the basolateral inlet and it was placed into the incubator at 37 °C for three

hours. Three hours after the initial seed, the media was allowed to flow, and TEER measurements were collected.

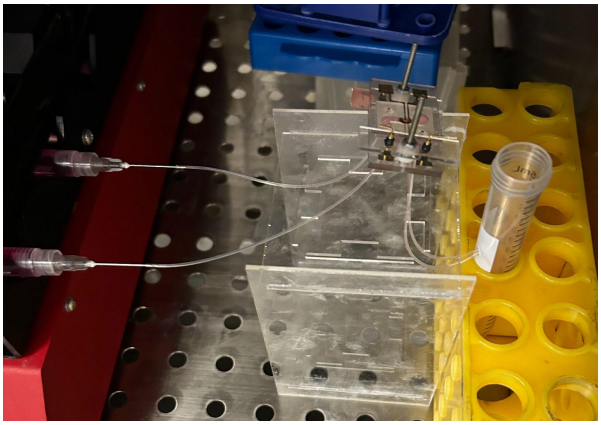


Figure 7. Triple co-culture experiment of the device in the incubator with media flowing

H&E Staining

To stain the cells seeded into the μE-BBB device, the membrane was first fixed to the glass slide with 4% paraformaldehyde for 15 minutes on ice. Then, the membrane insert was cut out from the device with IPA. Then, the membrane was cut out from the membrane insert. It was then rinsed with ice-cold PBS. An adequate amount of hematoxylin, Mayer's (Lille's Modification) was pipetted to completely cover the membrane. It was set to incubate at room temperature for five minutes and then washed with Scott's Tap Water. Then, an adequate amount of bluing reagent was added to completely cover the tissue. It was set to incubate for 10-15 seconds and then washed with Scott's Tap Water 2X. The slide was dipped in absolute alcohol and the excess was blotted off. Next, Eosin Y solution was added to completely cover the membrane. It was set to incubate at room temperature for 2-3 minutes and then the slide was rinsed with absolute alcohol. The slide was then dehydrated in three changes of absolute alcohol, cleared, and clear nail polish was applied.

RESULTS:

The soldered wire model was unstable and provided minimal support during experimentation as the hot glue would frequently fall off in the incubator (Figure 8).

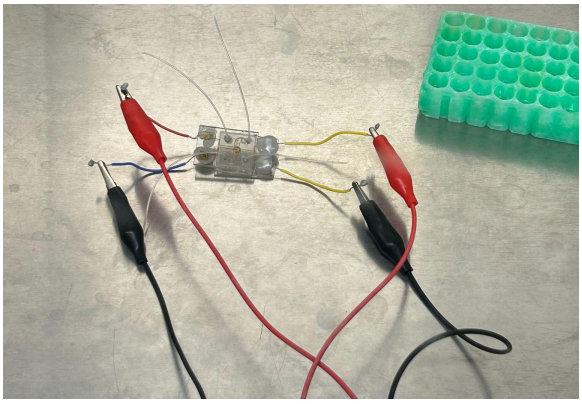


Figure 8. Completed μE-BBB device with tubing and soldered wires

The pogo-pin model minimized errors during experimentation by providing greater stability and accessibility (Figure 9).

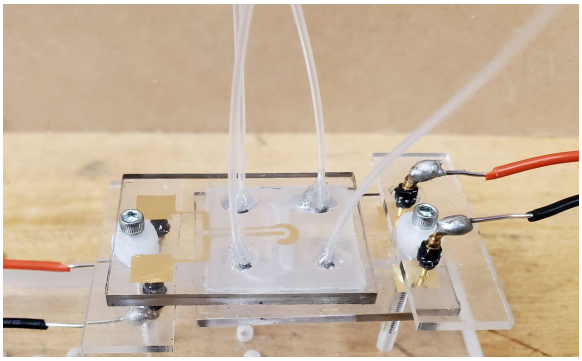
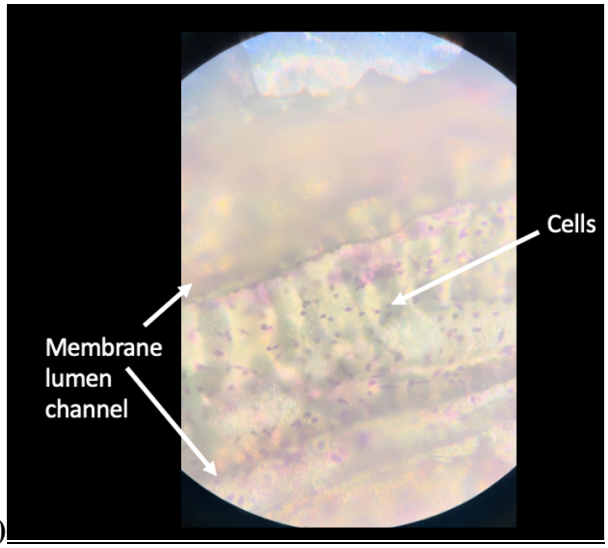
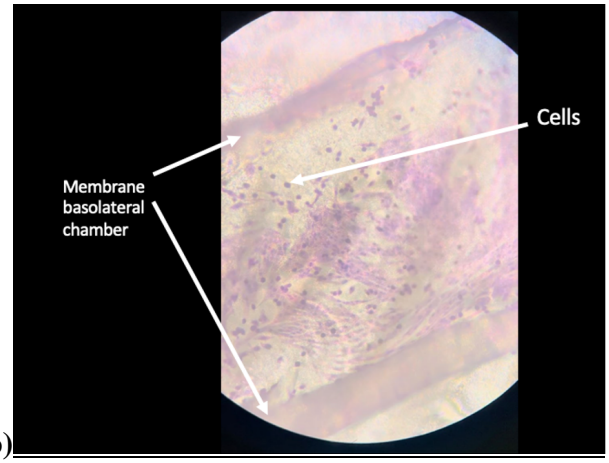


Figure 9. Completed μE-BBB device with tubing and pogo pin design

Cells were confirmed to be present in both the lumen channel and basolateral chamber of the device, proving the device's application in mimicking the blood-brain barrier microenvironment (Figure 10a and 10b).



a)



b)

Figure 10a. Cells observed in the lumen channel of device **b.** Cells observed in the basolateral chamber of device

DISCUSSION:

After a trial-and-error period while building the device, the device fabrication was optimized by perfecting various steps throughout the protocol of building a single device. First, the sizing of the laser-cut tape was adjusted to fit the top and bottom pieces ideally. Originally, computer-aided design (CAD) produced tapes with a thin gold electrode template. The tape would have to be cut around the electrode region before using the gold sputtering machine to deposit the gold. This procedure often deposited the gold inconsistently and led to inaccurate or no current at all. To make this process more efficient, the CAD file was redesigned to a larger electrode design so that the tape that was used for gold sputtering did not require further processing (Figure 11).

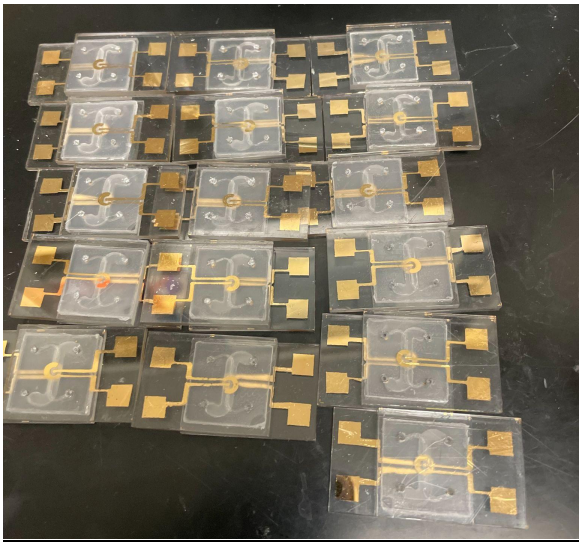


Figure 11. Completed μE-BBB devices without tubing and pogo pins

Another issue while fabricating the device pertained to device leakage. The device largely leaked because the membrane was either obstructed or not watertight. To fix this issue, an arbor press was used more frequently in the device fabrication process. Instead of arbor pressing the device once during device fabrication, it was pressed four times on each side for five

seconds after taping the membrane to the top thick gold electrode and four times after securing the thin gold electrode piece to the membrane. This greatly increased the success rate of water-tight membranes before the cells were seeded for experimentation.

While attaching the tubes to the device, there were sometimes blocked channels due to the epoxy occluding the channels. To minimize blocked channels, the epoxy was set for 1-2 minutes until tacky before it was applied to the tubes. Additionally, less epoxy per tube was used so that there was less of a chance that the glue would block the hole in the top gold electrode piece. The device was also hung immediately after the epoxy was applied because it typically takes a minimum of one hour to cure. This process ensured that the epoxy would not drip into the flow channel as it cures.

Another issue was soldering the wires to the gold electrode pads (Figure 8). The solder from the wire would not adhere to the gold pad electrodes on the device. Hot glue had to be used to additionally secure the device, but this method was also flimsy and would often lead to broken wires. To optimize this process, in collaboration with prospective M.S. student Allexa Ortiz, a pogo pin model was designed so wires did not have to be soldered to the gold pad electrodes. Instead, alligator clips from the EVOM were connected to the pogo pins to measure TEER (Figure 9). This method allowed for a cleaner, more durable, and more accessible device design during experiments. However, some issues that arose were greater leakage in the incubator with the pogo pin model. While the device initially passed through a membrane leakage test before experimental use, when it was in the incubator for 7 days, there was leakage through the membrane. This leakage could be due to a pressure difference in the tubes of the device. For future experiments, a solution could be to incorporate t-valves on the outlet or to plug the outlet tubes with a wire. Another solution could be to submerge the outlet with liquid like PBS so that

the membrane does not dry up. Future research can explore other alternatives to avoid leakage during experimentation in the incubator.

While figures 10a and b show the presence of cells, future experiments and studies should validate the presence of all three cell types. Whether the cell was microglia, astrocytes, or endothelial cells was unable to be determined due to limited characterization methods. However, visualization of cells was confirmed to be on both the lumen and basolateral side of the BBB. In the future, the use of antibodies and proteins can be implemented as markers to concretely identify the cell types present on either side of the membrane. Once cells were characterized, neuroinflammation could also be investigated by using neuro-inflammatory markers to induce an inflammatory response within SIM-A9 microglial cells. This study will allow for an exploration of how microglial-mediated inflammation affects BBB permeability.

In the future, the device fabrication process could be optimized by using PMMA instead of polycarbonate as the top, bottom, and BSL pieces. Polymethyl acrylate (PMMA) offers a better alternative to polycarbonate as it was faster to cut and isolate. The milling machine was used to cut the polycarbonate, but each piece would take around four hours for individual fabrication. In contrast, if PMMA was used, it could be laser cut which would take 10-15 minutes to fabricate at least 20 individual pieces. This method offers a faster way to cut individual pieces, allowing for mass-scale production of the μE-BBB device. Additionally, PMMA offers other benefits such as greater transparency to allow for better visualization of the cells before taking apart the device to extract the membrane.[19] It is also a tough, durable, and lightweight thermoplastic with scratch-resistant properties. Initial PMMA testing was conducted

[19]Bhushan, B., Burton, Z. (2005). Adhesion and friction properties of polymers in microfluidic devices. Nanotechnology, 16(4), 467–478. https://doi.org/10.1088/0957-4484/16/4/023

but future studies should concretely explore this material's potential in next-generation μE-BBB devices.

The device fabrication process could also be optimized with the addition of a chromium layer in between the gold electrodes and plastic pieces. The current device fabrication process directly sputtered gold onto the polycarbonate piece. An adhesion layer, like chromium, could offer many benefits including high binding strength with oxygen and thus the ability to depassivate material surfaces. However, there are some limitations including increased surface contamination and increased intrinsic stress which could lead to fracturing.[20] Regardless, it remains clear that further research can be done to best optimize this second-generation μE-BBB device.

[20]Li, Y., Yang, G., Deng, D., & Zhang, Y. (2021). Effect of chromium coating thickness on surface adhesion of polyethylene terephthalate optical film. Surfaces and Interfaces, 26, 101429. https://doi.org/10.1016/j.surfin.2021.101429

CONCLUSION:

In conclusion, the μE-BBB device fabrication process was optimized and confirmed to be an organ-on-a-chip system that has the potential to mimic the BBB microenvironment. In the future, concrete characterization of cells would be useful in identifying the success of the model and allowing for studies to explore other properties of the BBB like neuro-inflammation. Additionally, many steps of the device fabrication process can be further optimized to ensure the scalability and reproducibility of this OoC device. Regardless, this study demonstrates the physiological potential of the μE-BBB device in furthering knowledge on mechanisms and interactions in the brain and neurodegenerative diseases.

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