Guardians of the Gateway: Exploring Blood-Brain Barrier with Impedance Spectroscopy

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The Blood-Brain-Barrier

The blood-brain barrier (BBB) is a highly specialized and complex physiological structure that plays a crucial role in maintaining the homeostasis of the central nervous system (CNS) [1]. Grossly the BBB consists of three selective layers [2]. (1) The endothelial cells, which impede substances to infiltrate the brain by tight junctions. (2) The basement membrane containing pericytes provides structural support and contributes to selective permeability. (3) The astrocytes, specialized glial cells, enclose the basement membrane with their end feet and aid nutrient transfer. Lastly between the astrocytes microglia, brain-resident immune cells, can modulate BBB function during inflammatory responses, affecting the barrier's integrity [3]. A schematic representation of the BBB is depicted in **Figure 1**.

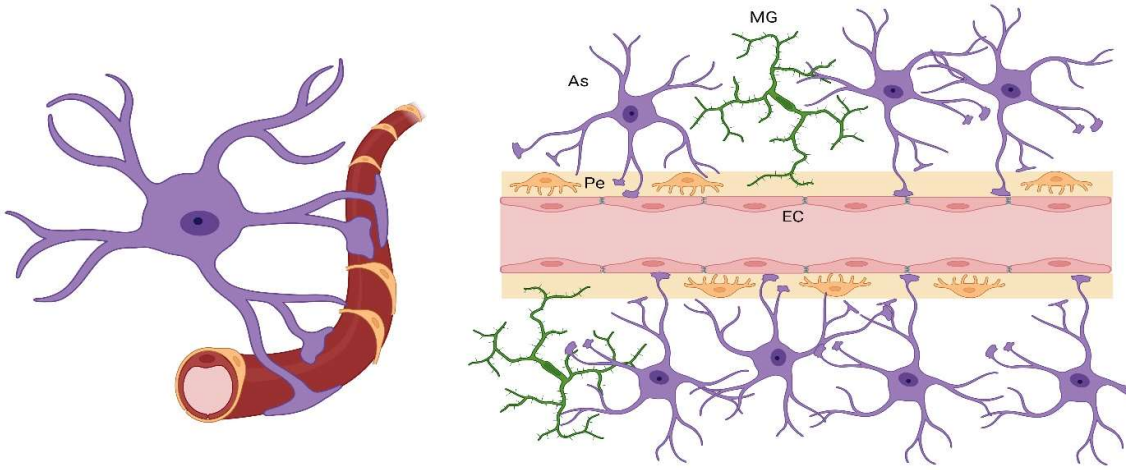

Figure 1. Blood-brain-barrier. *Endothelial cells (ECs) with tight junction form the first barrier. The ECs are covered in a basement membrane which contains the pericytes. This is ensheated by astrocytes. Beside astrocytes the lumen also contains microglia. EC, = endothelial cell, Pe = pericyte, As = astrocyte, MG = microglia*

Understanding the blood-brain barrier is paramount in neuroscience and pharmacology, as it significantly influences the delivery of drugs to the brain. Disorders affecting the integrity of the BBB, such as neuroinflammatory conditions, can have profound implications for neurological health [4]. Investigating the mechanisms that govern BBB function is an ongoing area of research, shedding light on potential therapeutic targets for various neurological diseases and disorders [5]. In order to do so, adequate *in vitro* models are necessary. In the development of an *in vitro* BBB model, analysis and quantification of key characteristics is essential. Naturally for the BBB this key characteristic is the highly selective barrier function, distinguishing it from the endothelium in the majority of organs where easy transport of nutrients, solutes, and macromolecules occurs [6]. A quantitative evaluation

of the barrier integrity is therefore crucial in all BBB research. Here, we showcase how trans endothelial electrical resistance (TEER) and impedance spectroscopy can quantify endothelial barrier integrity.

Human cerebral microvascular endothelial cell D3 (hCMEC/D3)

The hCMEC/D3 is an endothelial cell line, derived from the temporal lobe of the brain of a patient during epilepsy management surgery. Immortalization ensured reproducibility and scale-up, while maintaining the phenotypic profile. hCMEC/D3 are renowned for their resembles of the *in vivo* barrier in terms of adherence junction and tight junction formation ability, low permeability of both high and low molecular weight molecules, and expression of functional transporters. While the capacity to establish tight junctions exists, the formation observed is deficient when compared to the *in vivo* context, leading to a notable reduction in transendothelial electrical resistance (TEER) [7].

In the search to improve the TEER and tight junction formation [8], quantitative measurement of TEER is crucial. Here we demonstrate an automated, real-time impedance/TEER detector, compatible for the TEER measurement on transwell cultured hCMEC/D3 cells.

Culturing of hCMEC/D3

hCMEC/D3 (Merck Millipore) were cultured in endothelial growth medium 2 (EGM2; Cell Applications, Inc) on 100 µg/ml collagen I (rat tail, Corning, United States) coated culture flasks (Greiner, Austria) at 37°C and 5% CO_2. Cells were passaged when 95% confluency was reached. Here, cells with a passage number of 30–35 were used for all experiments.

Prior to cell seeding on transwells (24 wells, 3 µm pores, transparent, Cellqart, Germany), the apical side of the membrane was coated with collagen I for 1 h at 37°C and subsequently washed with phosphate buffered saline (PBS). hCMEC/D3 were singularized by incubation with trypsin for 3 min at 37°C, spun down, and seeded in 100 µl of $1.5*10^5$ cells/ml in EGM2 on the apical side of the transwell membrane. The basolateral compartment (24 wells, cell culture plate, Corning) was filled with 600 µl EGM2. During measurements medium volume is increased to 200 µL (apical compartment) and 800 µL (basolateral compartment) to ensure the electrodes were completely submerged in medium. The culture was kept in 37°C and 5% CO2 for 48 h total to obtain a hCMEC/D3 monolayer. Medium was refreshed daily.

After two days a tight monolayer of hCMEC/D3 was formed as depicted in **Figure 2**. Here, the DAPI (blue) clearly shows the nuclei of cell and F-actin (white) the cytoskeleton. Furthermore, vascular endothelium (VE)-cadherin (red) and zonula occludens (ZO)-1 (yellow) elucidate the formed junctions. VE-cadherin is an endothelial-specific adhesion molecule, located at the adherence junctions between endothelial cells, crucial for maintaining endothelial cell contacts and controlling vascular permeability [9]. ZO-1 is a tight junction protein that plays a central role in barrier formation and tunning cell-cell tension in endothelial cells [10]. Together these data indicate that culturing hCMEC/D3 cells on collagen I coated transwell membranes leads to a well-structured monolayer with intact adherence junctions and tight junctions.

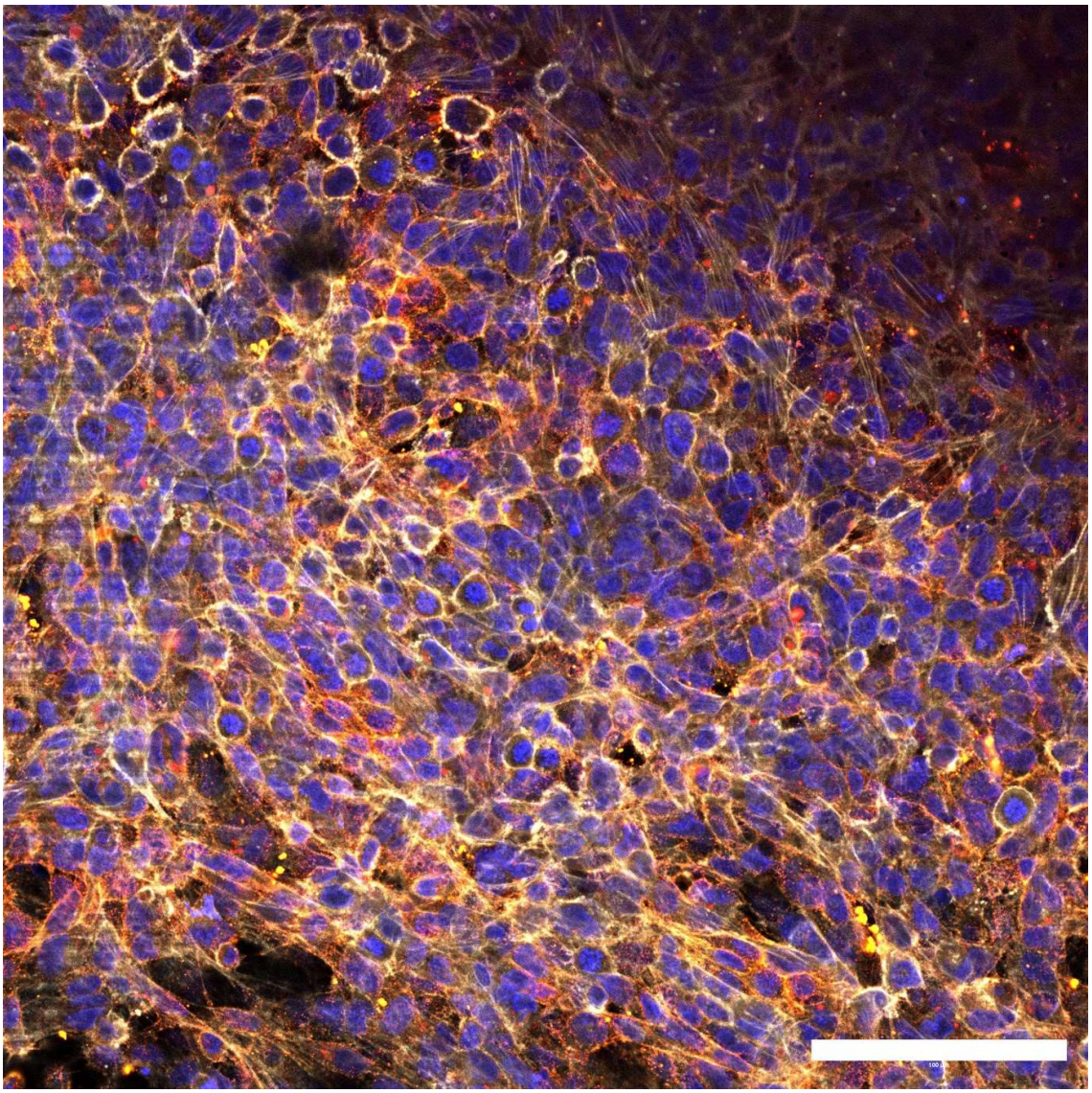

Figure 2. hCMEC/D3 monolayer at day 2. *Cells are stained for nucleus (blue), VE-cadherin (red), ZO-1 (yellow) and F-actin (white). Scale bar is 100 µm*

Impedance spectrometry

As an indicator for the quality and integrity of the monolayer, impedance spectroscopy measurements were performed. Therefore, we used a real-time, non-destructive impedance spectrometer, the Locsense Artemis.

A cell layer on a transwell can be considered as a complex electrical circuit, with multiple resistances and capacitors, which together form the impedance [11]. A simplified yet, adequate circuit is depicted in **Figure 3**, below. Here, R_{TEER} represents the transendothelial electrical resistance (TEER) of the tight junctions, consequently the BBB resistance. Additionally, R_{medium} signifies the resistance of the cell culture medium, C_{cell}, indicates the capacitance of the cell, which indicates the growth and differentiation of the cells and $C_{electrode}$ represents the capacitance of the electrode [12]. Since the total measured impedance is a complex sum of all resistors and capacitors, the R_{medium}, C_{cell}, and $C_{electrode}$ cannot be neglected when trying to measure the R_{TEER} [13].

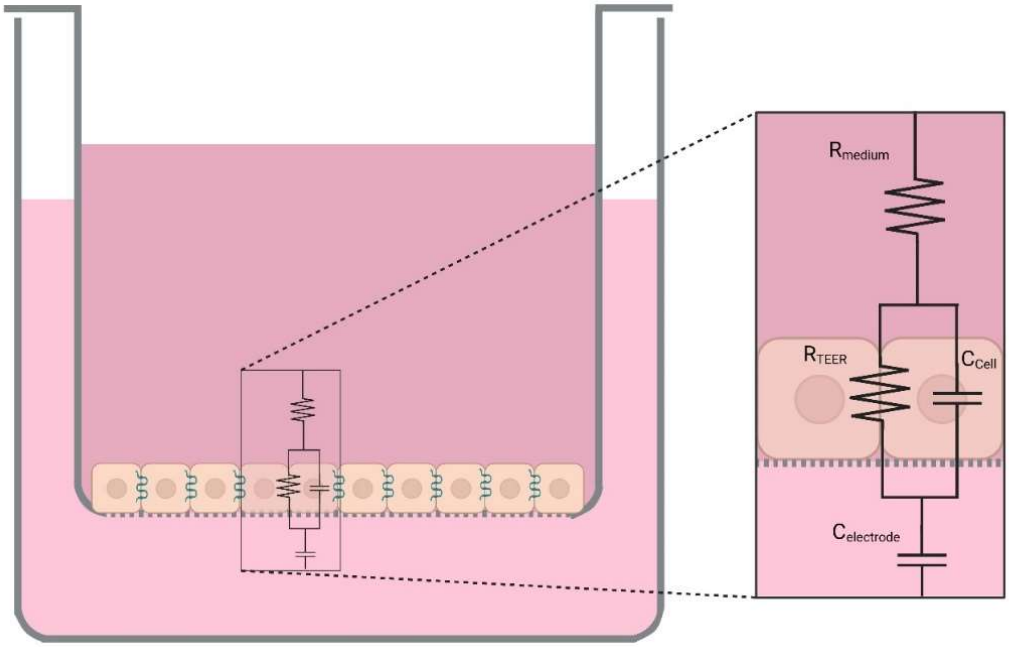

Figure 3. Simplified yet adequate representation of the electrical circuit of the impedance measurement*. R_{medium} is the resistance of the medium, R_{TEER} is the resistance of the tight junctions, C_{cell} is the capacitance of the cell, $C_{electrode}$ is the capacitance of the measurement electrodes.*

So, the measured impedance is a complex number, which can be described as the combination of the real part, the resistive contribution and the imaginary part the capacitive and inductive contribution [13]. Since the equivalent circuit does not contain any inductive elements, we consider only capacitive contributions to the latter. The relation can be visualized as an Argand diagram where the y axis is imaginary (capacitive) and the X axis is real (resistive). As the Argand diagram describes at 0° and 180° phase difference the contribution of the imaginary part is negligible, while at -90° and 90° phase difference the contribution of the real part is negligible. In order to find the TEER of the tight junctions, a crucial parameter when mimicking the BBB, a frequency should be used where the influence of the

capacitance is negligible. This is the case when the phase shift is close to either 0° or 180°. As seen in **Figure 4**, the phase shift is close to zero at a frequency of 23 kHz.

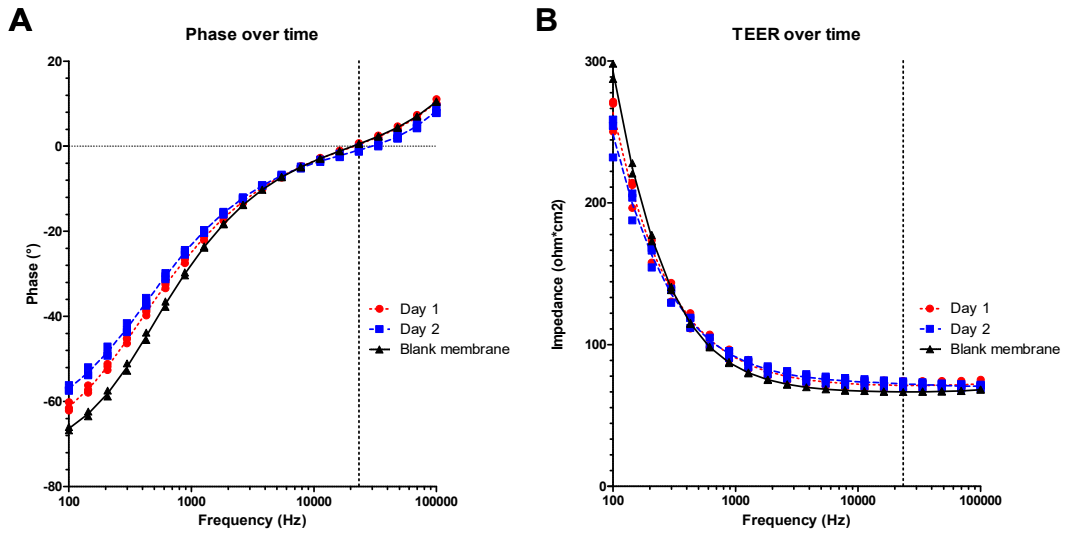

Figure 4. Impedance results of hCMEC/D3 on collagen I coated transwells. *A. Phase difference as function of frequency, a thick at 0°. B. Impedance as function of the frequency, with a thick at 0° where the influence of the imaginary part is negligible and the influence of the real part is dominant*

Therefore, we consider the impedance at this frequency to consist of the resistance of the medium and the resistance of the TEER, rather than the capacitances of the cells and electrodes. To make sure the resistance of the medium in the blank samples and at 24 h or 48 h is the same, the medium at the apical (200µL) and basal side (800µL) of the membrane is refreshed right before the measurement. Then by subtracting the impedance of the blank membrane from the total impedance at 48 h or 24 h, the impedance resembles the TEER.

TEER increased over time

Figure 5 clearly illustrate a consistent rise in TEER over time. At the 24 h mark, the impedance difference is approximately 5 Ωcm^2, and by 48 hours, it has steadily increased to over 11 Ωcm^2. This progressive elevation in TEER values serves as a positive indicator of the ongoing development and maturation of the endothelial cell monolayer. When hCMEC/D3 cells are seeded onto a culture insert or permeable membrane, their adherence and proliferation initiate the formation of a monolayer, mimicking the endothelial lining found in brain blood vessels. As time progresses, the hCMEC/D3 cells fortify these tight junctions, resulting in an enhancement of the physical barrier function. The rising TEER values serve as an indicative measure of the *in vitro* BBB model's maturation, with the increased number of tight junctions and adherence junctions contributing to heightened resistance across the cell monolayer, as demonstrated by the escalating TEER values.

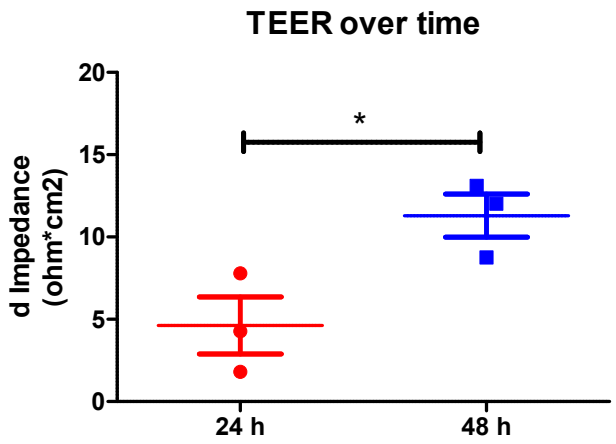

Figure 5. Transendothelial electrical resistance (TEER) over time. *The TEER significantly increases after 48 h. Results are presented as mean ± standard error of the mean (SEM) *p < 0.05, two-tailed t-test.*

TEER values *in vivo*

However, the measured TEER values do not correspond with the values found *in vivo* which are estimated well above 5000 Ωcm^2 [13], [14]. This discrepancy is widely recognized in literature and tremendous efforts have been put in increasing the TEER [7].

Among these efforts are the experimental set-ups with other types of brain cells, such as astrocytes and/or pericytes. Hatherrel found the TEER to be doubled when hCMEC/D3 were co-cultured with astrocytes. The astrocytes would lead to maturation of the tight junctions, and thereby increased TEER [15]. However this same goal has not been reached by other researchers, who found no change in the TEER when co-cultured with astrocytes [16], [17].

Another method that proved to be effective is the application of shear stress [18]. It is thought that shear stress can induce endothelial cell differentiation, which leads to the upregulation of tight and adherence junction proteins. This phenotype better mimics the *in vivo* BBB and can eventually lead to a 15-fold increase of TEER results [19], [20].

Lastly, (bio)chemical compounds can influence the barrier integrity of the BBB. Retinoic acid for example, can induce a higher expression of tight junction and adherence junctions and thereby increasing the TEER [21]. On the other hand, chemicals such as MMP3 [22], or histamine [23], can reduce the expression of tight junctions and thereby inhibit the barrier function and TEER values.

Conclusions

Taken together, the BBB is a functionally complex barrier, characterized by a high expression of tight junctions and adherence junctions. In order to understand disease mechanisms and find treatments for diseases a good understanding of this barrier is crucial. *In vitro* mimics of the BBB need to be physiologically representable with functional tight and adherence junctions. The functionality of these junctions can be measured using TEER. Here we show a how impedance spectroscopy can be used *in vitro* to measure TEER. We provided a concise method on how to culture hCMEC/D3 on transwells and measure the impedance using the Artemis. We elucidated how to interpretate complex impedance data and a possible strategy to extract the TEER, using the phase shift. Lastly we discussed the difference between *in vitro* and *in vivo* TEER results and possible strategies to increase the TEER and make a model more physiologically representative.

References

[1] Y. Persidsky, S. H. Ramirez, J. Haorah, and G. D. Kanmogne, "Blood-brain barrier: Structural components and function under physiologic and pathologic conditions," in *Journal of Neuroimmune Pharmacology*, Sep. 2006, pp. 223–236. doi: 10.1007/s11481-006-9025-3.

[2] H. Kadry, B. Noorani, and L. Cucullo, "A blood–brain barrier overview on structure, function, impairment, and biomarkers of integrity," *Fluids and Barriers of the CNS*, vol. 17, no. 1. BioMed Central Ltd, Dec. 01, 2020. doi: 10.1186/s12987-020-00230-3.

[3] R. Daneman and A. Prat, "The blood–brain barrier," *Cold Spring Harb Perspect Biol*, vol. 7, no. 1, Jan. 2015, doi: 10.1101/cshperspect.a020412.

[4] A. K. Dotiwala, C. McCausland, and N. S. Samra, *Anatomy, Head and Neck, Blood Brain Barrier*. StatPearls Publishing, Treasure Island (FL), 2022. [Online]. Available: http://europepmc.org/abstract/MED/30137840

[5] M. E. M. Stamp, M. Halwes, D. Nisbet, and D. J. Collins, "Breaking barriers: exploring mechanisms behind opening the blood–brain barrier," *Fluids and Barriers of the CNS*, vol. 20, no. 1. BioMed Central Ltd, Dec. 01, 2023. doi: 10.1186/s12987-023-00489-2.

[6] S. Yazdani, J. R. Jaldin-Fincati, R. V. S. Pereira, and A. Klip, "Endothelial cell barriers: Transport of molecules between blood and tissues," *Traffic*, vol. 20, no. 6, pp. 390–403, Jun. 2019, doi: 10.1111/TRA.12645.

[7] B. Weksler, I. A. Romero, and P.-O. Couraud, "The hCMEC/D3 cell line as a model of the human blood brain barrier FLUIDS AND BARRIERS OF THE CNS," *Fluids Barriers CNS*, vol. 10, p. 16, 2013, doi: 10.1186/2045-8118-10-16.

[8] K. Hatherell, P.-O. Couraud, I. A. Romero, B. Weksler, and G. J. Pilkington, "Development of a three-dimensional, all-human in vitro model of the blood-brain barrier using mono-, co-, and tri-cultivation Transwell models-brain barrier Human model Transwell model Human pericytes hCMEC/D3 Trans-endothelial electrical resistance," *J Neurosci Methods*, vol. 199, pp. 223–229, 2011, doi: 10.1016/j.jneumeth.2011.05.012.

[9] D. Vestweber, "VE-cadherin: The major endothelial adhesion molecule controlling cellular junctions and blood vessel formation," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 2. pp. 223–232, Feb. 2008. doi: 10.1161/ATVBAHA.107.158014.

[10] O. Tornavaca *et al.*, "ZO-1 controls endothelial adherens junctions, cell-cell tension, angiogenesis, and barrier formation," *Journal of Cell Biology*, vol. 208, no. 6, pp. 821–838, 2015, doi: 10.1083/jcb.201404140.

[11] K. H. Tu *et al.*, "Development of Real-Time Transendothelial Electrical Resistance Monitoring for an In Vitro Blood-Brain Barrier System," *Micromachines 2021, Vol. 12, Page 37*, vol. 12, no. 1, p. 37, Dec. 2020, doi: 10.3390/MI12010037.

[12] T. H. Phan *et al.*, "Advanced pathophysiology mimicking lung models for accelerated drug discovery," *Biomater Res*, vol. 27, no. 1, pp. 1–21, Dec. 2023, doi: 10.1186/S40824-023-00366-X/FIGURES/8.

[13] B. Srinivasan, A. R. Kolli, M. B. Esch, H. E. Abaci, M. L. Shuler, and J. J. Hickman, "TEER measurement techniques for in vitro barrier model systems," *J Lab Autom*, vol. 20, no. 2, pp. 107–126, Apr. 2015, doi: 10.1177/2211068214561025.

[14] M. A. Erickson, M. L. Wilson, and W. A. Banks, "In vitro modeling of blood-brain barrier and interface functions in neuroimmune communication," *Fluids and Barriers of the CNS*, vol. 17, no. 1. BioMed Central Ltd., Mar. 30, 2020. doi: 10.1186/s12987-020-00187-3.

[15] K. Hatherell, P.-O. Couraud, I. A. Romero, B. Weksler, and G. J. Pilkington, "Development of a three-dimensional, all-human in vitro model of the blood-brain barrier using mono-, co-, and tri-cultivation Transwell models-brain barrier Human model Transwell model Human pericytes hCMEC/D3 Trans-endothelial electrical resistance," *J Neurosci Methods*, vol. 199, pp. 223–229, 2011, doi: 10.1016/j.jneumeth.2011.05.012.

[16] D. E. Eigenmann, G. Xue, K. S. Kim, A. V Moses, M. Hamburger, and M. Oufir, "Comparative study of four immortalized human brain capillary endothelial cell lines, hCMEC/D3, hBMEC, TY10, and BB19, and optimization of culture conditions, for an in vitro blood–brain barrier model for drug permeability studies," *Fluids Barriers CNS*, vol. 10, no. 1, p. 33, 2013, doi: 10.1186/2045-8118-10-33.

[17] S. Hinkel, K. Mattern, A. Dietzel, S. Reichl, and C. C. Müller-Goymann, "Parametric investigation of static and dynamic cell culture conditions and their impact on hCMEC/D3 barrier properties," *Int J Pharm*, vol. 566, pp. 434–444, 2019, doi: https://doi.org/10.1016/j.ijpharm.2019.05.074.

[18] R. H. Booth and H. Kim, *A PARALLEL ARRAY MICROFLUIDIC BLOOD-BRAIN BARRIER MODEL FOR HIGH-THROUGHPUT QUANTITATION OF SHEAR STRESS EFFECTS*.

[19] L. Cucullo, M. Hossain, V. Puvenna, N. Marchi, and D. Janigro, "The role of shear stress in Blood-Brain Barrier endothelial physiology," 2011, doi: 10.1186/1471-2202-12-40.

[20] L. Cucullo *et al.*, "Immortalized human brain endothelial cells and flow-based vascular modeling: A marriage of convenience for rational neurovascular studies," *Journal of Cerebral Blood Flow and Metabolism*, vol. 28, no. 2, pp. 312–328, Feb. 2008, doi: 10.1038/sj.jcbfm.9600525.

[21] E. S. Lippmann, A. Al-Ahmad, S. M. Azarin, S. P. Palecek, and E. V Shusta, "A retinoic acid-enhanced, multicellular human blood-brain barrier model derived from stem cell sources", doi: 10.1038/srep04160.

[22] Q. Zhang *et al.*, “Research Article Increase in Blood-Brain Barrier (BBB) Permeability Is Regulated by MMP3 via the ERK Signaling Pathway,” 2021, doi: 10.1155/2021/6655122.

[23] R. Booth and H. Kim, “Lab on a Chip Characterization of a microfluidic in vitro model of the blood-brain barrier (mBBB),” vol. 12, no. 10, 2012, doi: 10.1039/c2lc40094d.