UNIVERSITAT ROVIRA i VIRGILI

IBEC
Institute for Bioengineering of Catalonia

BLOOD-BRAIN BARRIER MONOLAYER MODEL FOR TRANSENDOTHELIAL TRANSPORT STUDIES

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BACHELORS'S THESIS FOR THE DEGREE OF BIOTECHNOLOGY

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1. IBEC Project

The research interests of the Neurobiotechnology group focus on three main aspects of developmental neurobiology and neurodegeneration. One of them is the search for new strategies to avoid the transport of α-synuclein and tau in neurons, which has been proposed as a cause of synucleinopathies such as Parkinson's disease.

The prion protein PrP^C and its functions are being studied in the IBEC Neurobiotechnology group. In 2018, they described that PrP^C is a new α-synuclein receptor involved in its spreading and propagation. Therefore, current objectives are aimed at blocking this interaction to reduce the neuropathological transport of α-synuclein. Similar experiments are also being carried out in the case of tau, one of the hallmarks of Alzheimer's disease, as tau also binds to PrP^C during its interneuronal spread. Additionally, it has been shown that PrP^c is expressed in endothelial cells in the blood-brain barrier and may be involved in clearance of accumulations of tau and amyloid beta peptides. The following work is a comparative study of different blood-brain barrier endothelial monolayer models in a transwell system, which will be needed to study PrP^c-mediated transendothelial transport mechanisms.

2. Abstract

Due to the interest for PrP^C protein and its transendothelial transport, it is necessary to develop *in vitro* blood-barrier models. In this study, we compare different endothelial cells, specifically bEnd.3 (mouse brain microvascular endothelial cells), and two human brain microvascular endothelial cells (HBMEC, from Cell Systems and Sciencell) in a monoculture model of the blood-brain barrier. We compare their barrier properties based on their transendothelial permeability and electrical resistance, as well as their morphology by immunocytochemical studies. This endothelial cell monolayer model is established in a transwell, which is a suitable system for transport studies. In the discussion of results, we conclude that HBMEC Sciencell cells are the most suitable cells for future experiments, due to their good results and morphology.

Keywords: blood-brain barrier; transendothelial transport; transwell; brain endothelial cells

INDEX

FIGURE INDEX

TABLE INDEX

BBB: Blood-brain barrier; **BM**: Basement membrane; **CNS**: central nervous system; **ECs**: endothelial cells; **ECGS**: endothelial cell growth supplement; **ECM**: extracellular matrix; **FBS:** fetal bovine serum; **HBMEC:** human brain microvascular endothelial cells; **JAM:** junctional adhesion molecules; **LY**: Lucifer Yellow; **P/S**: penicillin and streptomycin; **TEER**: transendothelial electrical resistance; **TJ**: tight junctions.

3. Background

3.1. Blood-brain barrier

Blood vessels are vital to delivering oxygen and nutrients to all the tissues and organs throughout the body. The human brain accounts for about 2% to 3% of the entire body mass, and yet, it consumes up to 50% of the total intake of oxygen and glucose. Such a high energy demand is only conceivable because of a controlled gating of mass exchange with the body across a network of barriers that are phenotypically regulated by the brain cells (1). The blood vessels that vascularize the central nervous system (CNS) possess distinctive properties which allow these vessels to tightly regulate the movement of ions, molecules, and cells between the blood and the brain. This control of CNS homeostasis allows for correct neuronal function and protects the neural tissue from toxins and pathogens, and alterations of these barrier properties are an important component of pathology and progression of different neurological diseases (2). This physiological barrier is the blood-brain barrier (BBB), it is mainly composed of polarized brain endothelial cells, pericytes, and astrocytes.

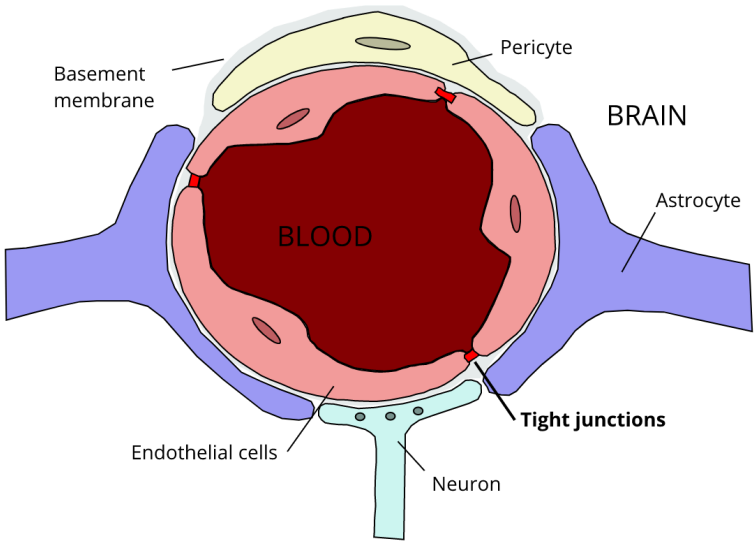

Fig. 1 | BBB scheme. Neurovascular unit: astrocytes, pericyte, basement membrane, neuron, endothelial cells, and their tight junctions. Made with Illustrator.

3.1.1. Endothelial cells

As mentioned above, the BBB protects the neural tissue from toxins and pathogens. For this, the first line of defense is the endothelial cells (ECs), phenotypically conditioned by the CNS (3). Unlike other tissue's vessels, they have unique properties such as: lack of fenestra, tight junctions (TJ) between adjacent endothelial cells, presence of solute carriers that regulate transport of ions and small molecules, expression of exit

transporters, low levels of pinocytosis, and receptor-mediated processes for specific uptake of macromolecules (4). Brain endothelial cells contain higher amounts of mitochondria compared to other ECs, which is thought to be critical to generate ATP to drive the ion gradients critical for transport functions (2).

3.1.2. Basement membrane

The next line of defense is the basement membrane (BM) formed by extracellular matrix (ECM) molecules. The BM consists of four major ECM proteins: collagen IV, laminin, nidogen and perlecan. The BM has many important functions including structural support, cell anchoring and signaling transduction (5). During the embryonic stage, ECs migrate and proliferate in a fibronectin-rich ECM, and shortly afterward a laminin-containing BM is formed. In brain capillary ECs, fibronectin signaling during angiogenesis switches to laminin signaling in adult stages (6).

3.1.3. Macrophages

Perivascular macrophages reside between the vascular cells and the glial endfeet, providing immune surveillance on the abluminal surface of the vessels. Microglial cells are resident CNS parenchymal immune cells that are derived from progenitors in the yolk sac and enter the brain during embryonic development. These cells are involved in regulating neuronal development, innate immune response, and wound healing, and can act as antigen-presenting cells in adaptive immunity (7).

3.1.4. Pericytes

The surface of the endothelium is incompletely surrounded by a cellular layer of pericytes. Pericytes play important roles in regulating angiogenesis, deposition of ECM, wound healing, regulating immune cell infiltration, and regulation of blood flow in response to neural activity, and reports suggest that they can also be multipotent stem cells of the CNS (8). Both pericytes and astrocytes play a very important role in the functioning of the BBB. Pericytes control key neurovascular functions that are necessary for proper neural structure and function (9). The pericytes cover the capillary wall and maintain direct contact with the ECs. They also control the number of ECs and microvessel architecture given the fact that pericytes inhibit EC proliferation. Astrocytes play a role in the induction and maintenance of long-term barriers; and can release chemical factors that modulate endothelial permeability (10).

3.1.5. Astrocytes

Finally, astrocytes, a major glial cell type in the CNS, extend cellular processes that ensheathe the blood vessels as well as neuronal synapses and nodes of Ranvier (11).

All this structure helps to manage the microenvironment, regulates the entry of nutrients, the exit of the waste, and regulates homeostasis keeping out ions and molecules. This precise control of CNS homeostasis allows for proper neuronal function and protects the neural tissue from toxins and pathogens, and alterations of these barrier properties are an important component of the pathology and progression of different neurological diseases (2).

3.1.6. Function and dysfunction of the BBB

The BBB is a dynamic multicellular interface that regulates the transport of molecules between the blood circulation and the brain parenchyma. CNS endothelial cells have TJ and unusually low levels of vesicle trafficking that limit transcellular transport or transcytosis. There are two types of transcytosis: through specific receptors and adsorptive transcytosis (12). Transport across brain endothelial cells can be divided into three distinct processes: internalization, sorting, and exocytosis (13). Despite its remarkable sealing properties, the BBB is selectively permeable to key nutrients such as glucose, iron, and lipoproteins, which cross the BBB via specific molecular transport systems (14).

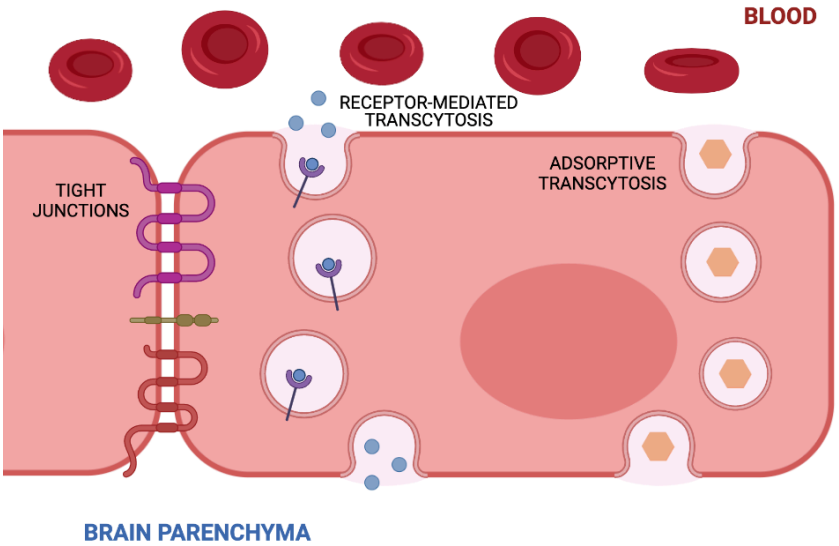

Fig. 2 | BBB transcellular transport. Two types of transcytosis: on the left, receptor-mediated transcytosis; on the right, adsorptive transcytosis (12) Between both cells different tight junctions are located: occludin (purple), claudin (red) and JAM (green). Detailed explanation in tight junction section. Made with BioRender.

The correct function and homeostasis of all the aforementioned components is essential for brain health, as breaching the barrier can lead to neurological diseases such as encephalitis, multiple sclerosis, brain traumas, Alzheimer's disease, epilepsy, strokes, and tumors. In addition, BBB disruption has been observed in a series of other neurological diseases including amyotrophic lateral sclerosis (ALS), epilepsy, edema, PD, as well as systemic diseases, such as liver failure (11). It is clear that there is BBB dysfunction in many different neurological diseases in a wide variety of species, which indicates that this is an evolutionarily conserved and important feature of these diseases. A critical question moving forward is to understand which aspects of this BBB dysfunction are related to healing and which aspects are pathological (2). To treat these diseases, it must be remembered that the BBB renders the brain impervious to most therapies, which creates a bottleneck in drug development. (15). Thus, it is necessary to understand the functioning of the barrier to develop methods that bypass the BBB for the administration of drugs.

3.2. Models

To facilitate neurobiological research and develop new drugs for neurological diseases, different barrier models have been developed, which are compared in Table 1.

Table 1| Five different types of BBB models in vitro with their characteristics (16).

In vitro BBB models	Other cell types	Steady TEER value	Migration assays	Technical requirements	Cost
MONOLAYER MODELS	No	Low to moderate	Yes	Low	Low
COCULTURE MODELS	Yes	Moderate to high	Yes	Moderate	Low to moderate
CONE-PLATE APPARATUS	No	-	No	Low to moderate	Low
DYNAMIC *IN VITRO* BBB MODEL	Yes	High	No	High	High
MICROFLUIDIC-BASED MODELS	Yes	Moderate to high	Yes	Moderate	Yes

The BBB microfluidic model allows for the application of dynamic flow and physiological shear stress and uses co-culture of endothelial cells and astrocytes with direct cell-cell contacts to accurately model the BBB environment. Additionally, electrodes for TEER measurements can be included (this parameter will be explained in more detail in the following sections) (17). There are different types of microfluidic-based models as those mentioned in the following paper (18). Similarly, the dynamic *in vitro* model is based on a three-dimensional hollow fiber culture apparatus, tested in co-culture with endothelial cells and astrocytes, under pulsatile flow to mimic intraluminal blood flow. This method achieved TEER values of $2900 \Omega \cdot cm^2$ (19). However, this method has higher technical requirements and cost than the smaller, cheaper microfluidic models.

The cone-plate apparatus is a model of monoculture. It is based on a rotating cone that generates a shear force, which is transmitted to the endothelial monolayer through the medium. Nevertheless, because the shear stress is not uniformly distributed along the radius of the plates, the endothelial monolayer receives a different shear stress depending on its location on the plates (16).

The BBB co-culture model, especially with astrocytes (20), is suitable for migration assays but is more difficult to maintain than monolayer cultures, even though both are static models that do not require application of shear stress.

The monolayer model is the simplest and cheapest *in vitro* model of the BBB. It is based on the use of a monolayer of ECs cultured in the transwell insert (Fig. 3). The insert mimics the blood (luminal) side, while the well that the insert sits in, mimics the parenchymal (abluminal) side. The support of the microporous membrane (0.4 - 8 µm pores) of the transwell allows the exchange of small molecules and growth factors secreted by cells but prevents the migration of cells between the two compartments. In the case of this work, we have used mouse brain microvascular endothelial cells, bEnd.3 (21), and two types of primary human brain microvascular endothelial cells (HBMEC) (22).

3.3. Transwell model

As it is mentioned before, the Transwell System is essentially a side-by-side vertical diffusion system comprising a microporous semi-permeable membrane that

separates the vascular and parenchymal lateral compartments and is used to enable drug transport studies.

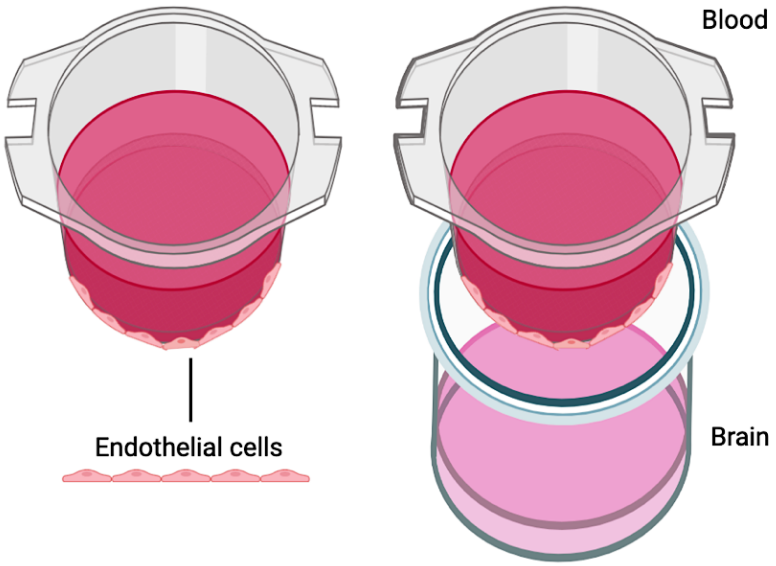

Fig. 3 | Transwell model in monolayer of ECs. Schematic of our model. The well simulates the brain parenchyma while the apical part of the transwell simulates the blood side. Endothelial cells form the monolayer at the bottom of the transwells as is shown in the figure. Made with BioRender.

This device is indicated for the study of the permeability through the BBB and allows the coculture of ECs and other cells associated with CNS. The ease of establishing cultures, moderate scalability, and low cost make this apparatus desirable for use in various research settings, including basic and translational studies. Transwell systems are ideal for linear kinetic transport studies due to the fixed volumes of each compartment. Cells can be separated and harvested for further study (proteomic and genomic analysis) and are available in a variety of pore sizes and with different membranes to meet various experimental requirements. However, there are substantial limitations inherent to these platforms that must be considered. For example, the lack of a three-dimensional structure present in vivo; the lack of endothelial exposure to physiological shear stress limits the differentiation of the endothelium into a BBB phenotype (or the maintenance of BBB properties in fully differentiated cells).

The result is that cells can exhibit reduced polarized transport, limited expression of specific efflux systems, and (in most cases) relatively low transendothelial electrical resistance (TEER) compared to BBB *in vivo* (23).

3.4. Barrier parameters

3.4.1. Tight junctions

An important component of the BBB is the specialized physical barrier composed of TJ present along the apical side of ECs. These cell-to-cell adhesion structures act as gatekeepers of the paracellular pathway by regulating the passive diffusion of molecules and ions in the brain (24).

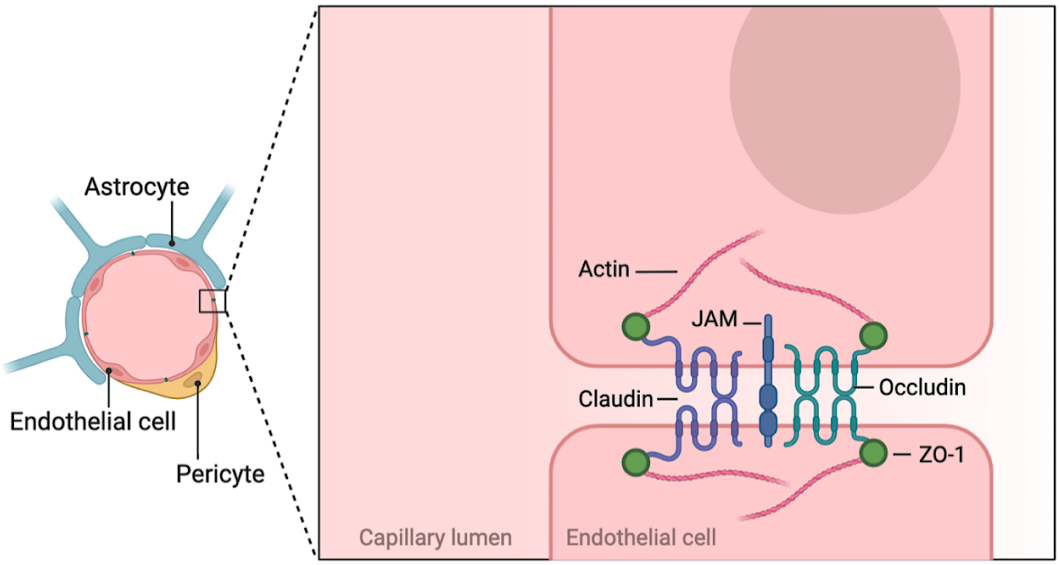

Fig. 4 | TJ location in BBB and their morphologies. The disposition of ZO-1 and actin with respect to the cell and their interaction is displayed. As in Figure 2, claudin, occluding and JAM are shown. Made with BioRender.

Many molecular constituents of TJs have been identified and characterized including claudins, occluding and ZO-1. Furthermore, junctional adhesion molecules (JAMs) are transmembrane proteins within TJs and have been shown to be key to the integrity of the BBB (25). The majority of signaling pathways involved in the regulation of TJ have in common the modulation of cytoskeletal elements that can define the characteristics of the BBB. Furthermore, the interference between the tightly bound components and the cadherin-catenin system suggests a close functional interdependence of the two cell-cell contact systems (26). Zonula occludens protein-1 (ZO-1) is part of the binding complexes and is used in this project to detect tight junctions in the immunocytochemistry assay. Polarized endothelium controls transcellular permeability through the specific expression of transporters and receptor proteins on the apical side of the membrane. In the case of disease pathologies, TJs are compromised, leading to loss of barrier selectivity in the transcellular and paracellular pathways (13).

TJ are composed of more than 40 proteins that are either transmembrane proteins or cytoplasmic actin-binding proteins (27). Therefore, cells with TJ show a cortical localization of actin fibers, which can be stained to confirm the presence of these cell-cell adhesions without the need for specific tight junction protein staining. For this purpose, Phalloidin-TRITC, a fluorescent derivative of phalloidin possessing a high affinity to filamentous actin, has been used in this project to visualize actin in mammalian cells.

3.4.2. Confluence

High confluence is required for TJ to form, as they can only form in directly adjacent cells. Besides, the main objective in IBEC's project is to study PrP^c expression and transcellular transport in the BBB, and cell-cell interaction has proven to be essential for PrP^c junctional expression in previous studies (28). For this reason, cultures must be grown to 100% confluence when cultured in the transwell system.

In transwells, the growth of the seeded cells cannot be followed under an inverted microscope, because the porous membrane makes it difficult to visualize cells in bright field. Even for "transparent" transwell materials (such as PET), confluence cannot be monitored accurately, as in practice these membranes are still not as transparent as standard cell culture vessels. Therefore, culture confluence was controlled by immunocytochemical studies with CellMask™ Green staining, which is a plasma membrane dye.

3.4.3. Lucifer Yellow permeability assays

Lucifer yellow (LY) is a small, hydrophilic molecule that moves across the BBB through passive paracellular diffusion. Therefore, this molecule acts as a marker for the establishment of proper TJ, which are related to low levels of paracellular transport. In permeability assays, the fluorescence of LY can be determined at given time points by measuring fluorescence intensities in both the donor and receiver chambers of the transwell model. The excitation and emission wavelengths are 438 and 535 nm, respectively (29).

To identify suitable cell lines to be used in the BBB transport assays, the cell lines were evaluated for barrier tightness and permeability. The different monoculture models used in this project were tested for their barrier integrity by evaluating permeability to LY among other factors (21).

3.4.4. TEER

The transendothelial electrical resistance (TEER) is an extensively recognized quantitative technique to measure the integrity of tight junction dynamics in cell culture models of endothelial monolayers. The TEER value is an indicator of the integrity of the cell monolayer after it is evaluated for drug transport (eg, with permeability or cytochemical assays). TEER measurements are performed in real time without damaging cells and are generally based on ohmic resistance measurement or impedance measurement over a wide spectrum of frequencies. Some of the barrier models that have been extensively characterized using TEER include the BBB, gastrointestinal tract, and pulmonary models. (29)

Described values

Studies have been described where the TEER value in a monoculture of HBMEC reached values of 100-120 $\Omega \cdot cm^2$ (22). It must be noted that on the first day, an extraordinarily high TEER value was reported; 60 $\Omega \cdot cm^2$. In our experience, it is improbable that confluence was reached at day 1 considering the number of cells seeded, and so the use of older equipment (EVOM epithelial voltohmmeter instead of EVOM2) must be considered as a possible source of background resistance. However, in another paper(30), TEER values in HBMEC were in the range of 40 $\Omega \cdot cm^2$. In this comparative study, the EVOM was also used, albeit with a different electrode (EndOhm) that could account for these lower TEER values.

The TEER value in a monoculture of bEnd.3 reached values of 40 $\Omega \cdot cm^2$. The bEnd.3 cells were cultured for 6 days and TEER was determined using an EVOM with Endohm™ chambers (31). Moreover, in another study, bEnd.3 cells reach values of 40 $\Omega \cdot cm^2$ on day 6 and TEER was measured using an EVOM2 (1)

Factors affecting TEER measurements

There are two critical factors to consider when measuring TEER. The first one is the temperature. TEER measurements have been shown to be temperature dependent (29), and for this reason, 15 minutes before taking the measurements, the transwells were taken out of the incubator while the electrode was being sterilized.

Another determining factor is the location of the electrode. TEER readings with chopstick electrodes are highly dependent on electrode positions, and careful handling of the electrodes is required when inserting them into the well to avoid any

disturbance to the cells. The uniformity of the current density generated by the electrodes across the cell layer has a significant effect on TEER measurements. Therefore, keeping it straight and trying not to move it while stabilizing are factors that can change the value of the measurement.

Finally, other factors to consider when cultivating cells to measure TEER are cell passage number, culture medium pH, and maintaining well volume constant throughout the experiment.

4. Hypothesis and Objective

An *in vitro* monolayer blood brain barrier model can be constructed to study transendothelial transport of brain endothelial cells.

The main objective of this project is to test different ECs and culture methods to determine which ones allow us to obtain the best blood-brain monolayer model in a transwell system. To compare the different cell lines and conditions, we will evaluate the following parameters: TEER measurements, permeability to LY and immunocytochemical parameters (cell confluence, cell morphology, and tight junction staining).

5. Materials and methods

Table 2 | Materials used in the project and their corresponding reference numbers.

MATERIALS	SOURCE
LUCIFER YELLOW	SIGMA L0144
TRANSWELLS 24W	Corning™, 353104
TRANSWELLS 12W	Corning™ 3401
CELL MASK	HCS CellMask™ Stains INVITROGEN (Green stain) H32714
PHALLOIDIN-TRITC	SIGMA MFCD00278840
HOECHST	SIGMA - Bisbenzimide H 33342
BEND.3 MEDIUM	DMEM (41966 Life T) - Lonza 10% FBS (10500-064 Life T) + 1% P/S 100x (15140-122 Life T) + 1% glutamine 200mM (25030-024 Life T)

ECGS (ENDOTHELIAL CELL GROWTH SUPPLEMENT)	HBMEC cells: Merck 02-102 / bEnd.3 cells: SIGMA E2759
CACO-2 MEDIUM	DMEM (11960 Life T) - Lonza 10% FBS (10500-064 Life T) + 1% P/S 100x (15140-122 Life T) + 2% glutamine 200mM (25030-024 Life T) + 1X Non-essential amino acids
HBMEC CELL SYSTEMS MEDIUM	RPMI 1640 Media - Thermofisher
HBMEC SCIENCELL MEDIUM	EGMTM -2 MV (Microvascular Endothelial Cell Growth Medium-2) BulletKitTM – Lonza CC-3202
FIBRONECTIN	Fibronectin bovine plasma - SIGMA/F4759
TEER EQUIPMENT	EVOM2 with STX2 chopstick electrode - WPI

5.1. Cultures

Cells were seeded in T75 flasks, and cell passages were performed 2 times per week or every two or three days depending on the growth rate, in a laminar flow cabinet. Cells were cultured according to their respective technical sheets (see annex). Total confluence cannot be reached during subculturing of the cells, as this poses a risk of detachment of the EC monolayer. The culture in transwells was performed according to the following parameters:

Table 3 | ECs growth conditions followed in transwells.

CELL TYPE	GROWTH TIME	CULTURE MEDIUM	SUPPLEMENT	COATING
bEnd.3	7-9 days	DMEM (41966 Life T)	FBS, P/S, Glutamina ECGS	Matrigel
HBMEC CELL SYSTEMS	7 days	RPMI 1640 Media	-	-
HBMEC SCIENCELL	7-10 days	EGMTM-2MV	BulletKitTM	Fibronectin
CACO-2	22 days	DMEM (11960 Life T)	FBS, P/S, Glutamine, non-essential aa	Collagen

5.2. Lucifer Yellow

Materials required for the assay are transwells assay plates, RINGER-HEPES buffer, 0.1 mg/ml Lucifer-Yellow solution (50 μM), 96-well plate, and fluorescent multi-well plate reader.

To perform the LY Assay firstly, the remaining fluid from the apical and basal wells is aspirated. Before that, 500 ml of RINGER-HEPES is added to the apical wells to clean the culture medium. Also, 1500 μl of RINGER-HEPES is added to all wells required for the assay. Once ready, the transwells are transferred to the first well and 500 μl of LY Solution (50 μM) is added into the apical part. The plate is incubated at 37 ° C for 15 minutes. After that, transwells are transferred to the next and they are incubated at 37ºC for 15 minutes, this last step is repeated two more times. After 60 minutes, 150 μl of the basal wells is transferred to a 96-well plate and the fluorescence is read on a spectrofluorometer with excitation at 485 nm and emission at 535 nm.

For the standard line, also measure the fluorescence for the RINGER-HEPES buffer (blank) and the serial dilution of LY solution.

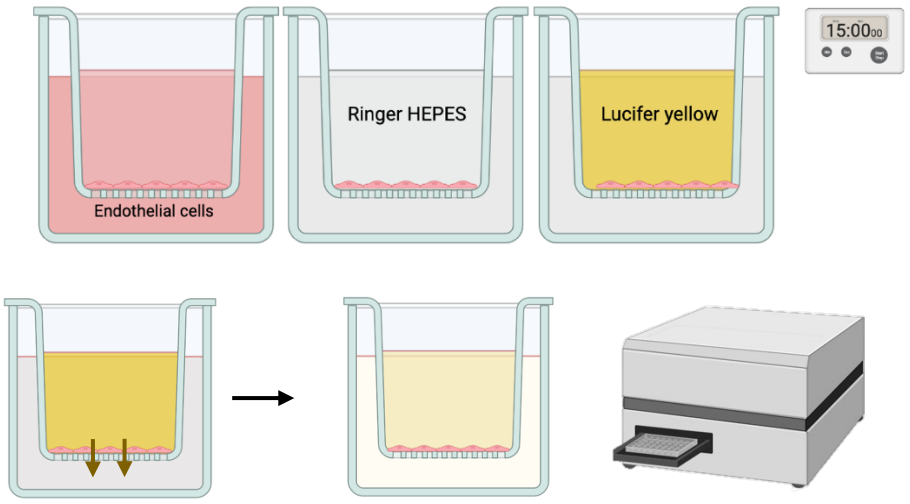

Fig. 5 | LY protocol for ECs monoculture seeded in transwells. It shows how LY solution diffuse through the transwell porus. In the bottom corner, a plate lector, which is necessary to read fluorescence, is displayed. Made with BioRender.

5.3. TEER

The equipment used to measure TEER was the EVOM2 equipment with STX2 electrodes. Before starting to measure, the electrodes are sterilized by immersion in 70%

ethanol for 15 minutes. At the same time, the plate with cells is taken out of the incubator to equilibrate to room temperature. The electrodes are balanced for a few seconds in MilliQ water and then inserted into the Transwell System, so that the shorter electrode is immersed within the culture medium of the apical well, without touching the cell monolayer, and the longer arm is placed through the lateral hole of the transwell. As it is a very sensitive method, three measurements were taken for each transwell, in a total of four transwells per condition.

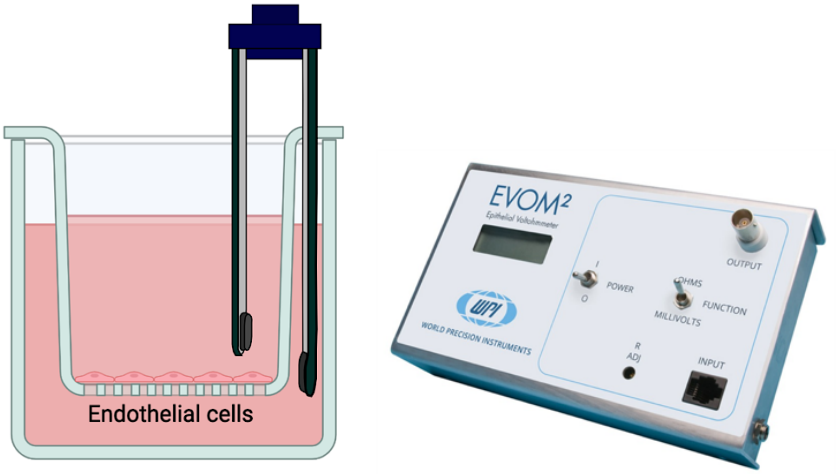

Fig. 6 | TEER measurement with STX2 electrodes in a transwell (EVOM-2 equipment). Correct position of the electrodes is presented, which is critical to ensure accurate measurements. Electrodes connect via a cable with EVOM-2. Made with BioRender and Illustrator.

5.4. Immunocytochemistry

Two types of immunocytochemistry were executed: with and without antibody. The basic protocol was based on fixation, permeabilization, blocking (antibody protocol) and incubations. The first step is fixation. It is performed by extracting the medium from the basal part of the transwell, and without removing the apical medium, adding one volume of 4% PFA (giving a final concentration of 2% PFA) to the apical part for 5 minutes. Next, the mixture is extracted from the apical part and one volume of PFA 4% is added and incubated for 15 minutes at room temperature. After the fixation step, washes are carried out with 1X PBS and cells are permeabilized with PBS-Triton X-100 0.1% for 15 minutes. The protocol then changes depending on whether antibodies are used. If antibodies are used, a blocking step is performed with 10% FBS in PBS-gelatin with 0,1% Triton X-100. In all cases, Phalloidin 1:500 and Hoeschst 1: 200 are used. An incubation step is carried out at 4ºC (primary antibody) or at room temperature (secondary antibody) and shaking it. If we use secondary

antibody with fluorophore, from the addition of the antibody we work in the dark. More specific explanations can be found in the annex.

5.5. Statistic test

The statistical tests have been performed with the GraphPad Prism program.

ANOVA test was used to defined different results of TEER and LY in more than two culture conditions or to help to choose the best cell line between more than two experiments. The t-student test is also used in this project to compare two groups of different conditions or two cell lines in one single experiment. Bar charts have SEM bars (Standard Error of the Mean) that quantifies the precision of the mean.

The TEER scatter charts have been made with Excel and they also show error bars, in this case, SED, Standard Error of a Difference between 2 means.

6. Results

Chronological summary

The project started with bEnd.3 mouse cells, as our colleague G. Battaglia (1) had recently studied the BBB with this type of ECs. The results that were obtained were similar to those published for this cell line, but we found they were not ideal in terms of tight junction formation (also coherent with published data). Later, in an attempt to improve the model and upgrade to human cells, we contacted another colleague at IBEC, Silvia Muro, who works with nanoparticle transport across the BBB (32). Her group kindly gave us a vial of human brain microvascular ECs (HBMEC, Cell Systems), which are widely used in BBB models (22) However, after culturing them in our lab, we suspected that the cells were not in their correct state, since the results were very distant from the bEnd.3 cell line. Regardless, we decided to continue our research with human microvascular cells, and the laboratory acquired the HBMEC from Sciencell. In this way, we could be sure to generate reliable cell stocks from a commercial vial.

In parallel, as a barrier control, we used the CACO-2 colon cancer cell line, as these cells have a very characteristic honeycomb structure with TJ, a very high TEER value (≥1000 Ω·cm2), and a very low permeability value, which is the ideal situation in terms of barrier formation

6.1. bEnd.3

Five experiments were performed in 12-wells Transwells. The first and second experiment were centered on comparing cells seeded with Matrigel 1:48 coating or without any coating. As shown in Figure 4, TEER was higher with the Matrigel coating, reaching values of 30 $\Omega \cdot cm^2$.

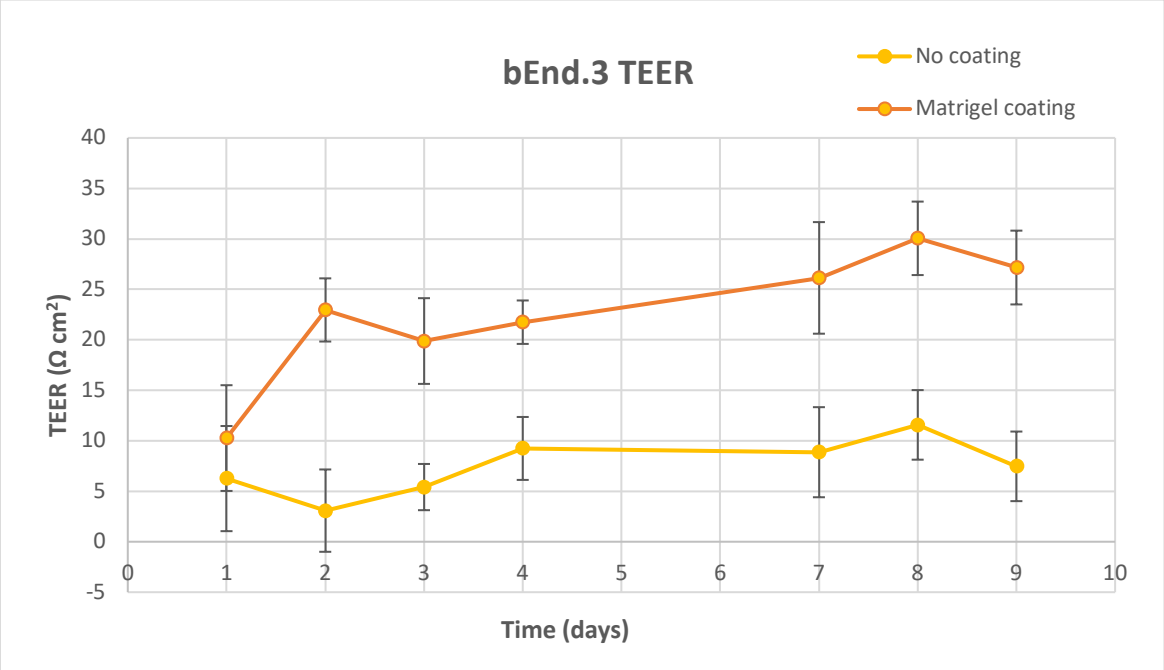

Fig. 7 | bEnd.3 TEER values in 7 days. Experiment 1. TEER stabilizes at day 8 and achieved values of 30 Ω·cm2 in coating transwells condition (orange).

From then on, it was decided to continue with the Matrigel coating due to its positive effect on the TEER. It must be noted that, in these first experiments, the cell stock was found to be contaminated with *Mycoplasma*, and so the next experiments were performed with newer, non-contaminated cells.

Owing to the lack of contamination, from the third and fourth experiment, better results of LY and TEER began to be obtained. The permeability results of experiment 3 were 30.22 $\pm$ 4.85·10^{-6} cm/s. However, in the fourth experiment, the best results were obtained by adding endothelial cell growth supplement (ECGS). In this case, the results were: without ECGS 13,19 $\pm$ 1,57 ·10^{-6} cm/s and with ECGS 8,75 $\pm$ 2,06 ·10^{-6} cm/s.

- *LY results are expressed with their standard error.*

In Figure 8, we show the best TEER results we obtained from experiment 4, which were around 35-40 $\Omega \cdot cm^2$ for both medium compositions:

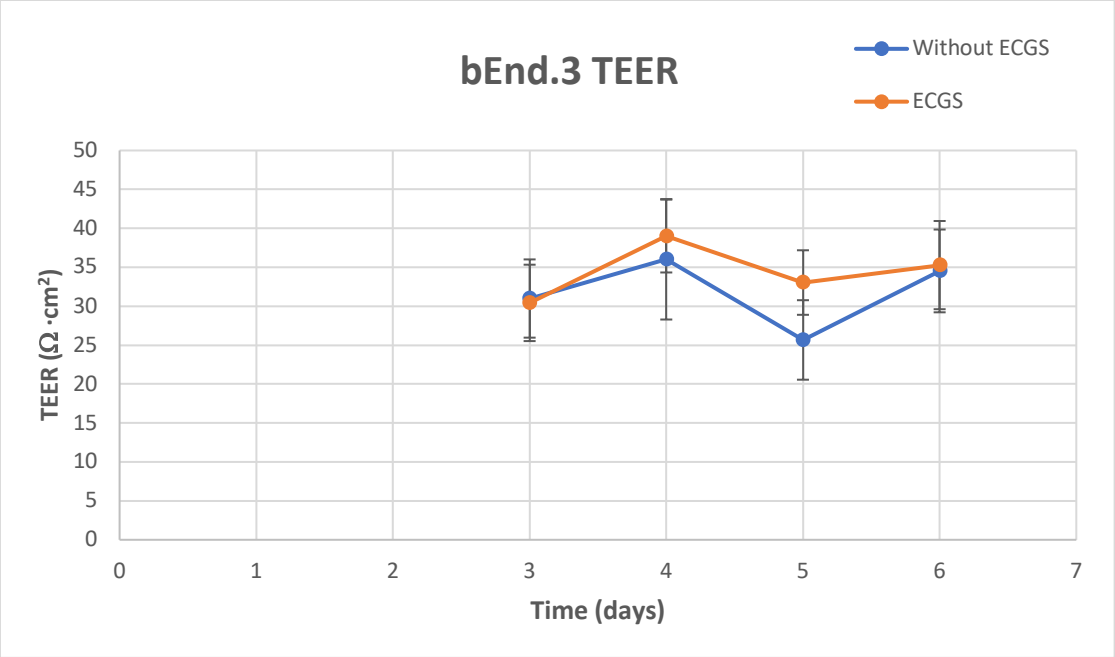

Fig. 8 | bEnd.3 TEER values from day 3 to day 6. Experiment 4. Comparison between cells with ECGS and cells without ECGS. Supplemented bEnd.3 obtained higher values than non-supplemented cells.

And in the fifth experiment, only immunocytochemistry was performed to follow the formation of the barrier from day 3, day 6 and day 9. (Fig. 9) The formation of the barrier does not follow a polygonal morphology as expected for these cells. Even so, on day 9, the bEnd.3 cells seeded without ECGS begin to form more organized shapes. Clusters of cells with polygonal shapes can be observed. Despite this fact, it has been observed that from day 6 to day 8 the TEER has already reached its highest value. Therefore, the BBB should not be in its best condition of integrity as of day 9.

Although TEER measurement should not affect cell culture, it is necessary to note that TEER was not measured in this culture, which could be a reason for the formation of these polygonal shapes not seen in previous cultures, if we hypothesize that the process of measuring the TEER can negatively affect barrier integrity.

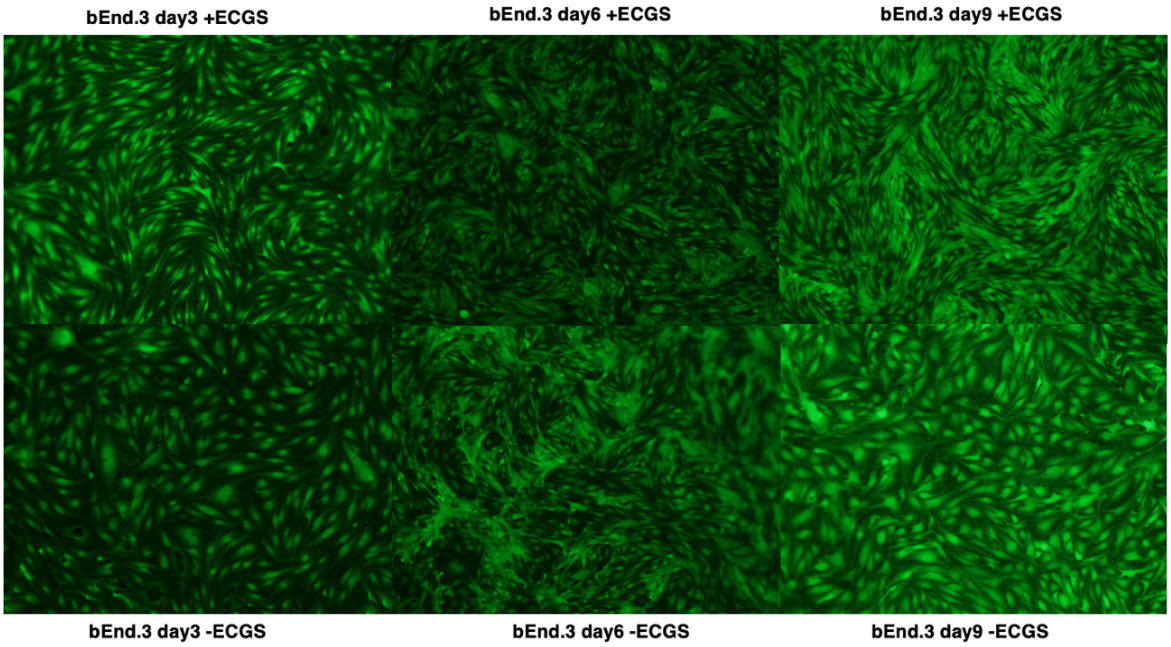

Fig. 9 | CellMask™ Green staining in bEnd.3 cells at different days. Experiment 5. Culture confluence control. From the right to the left, day 3 cells, day 6 cells and day 9 cells culture. In the first line cells supplemented with ECGS are displayed while in the second line non-supplemented cells are shown. Images were taken with fluorescence microscope and 10x objective.

In the Figure, cell growth from day 3 to day 9 is shown. Due to fact that the CellMask™ stains the entire cell membrane, we can get an idea of the number of cells per day of culture. In the images on day 3, many gaps are seen without being covered by cells, while on day 6 these holes are beginning to be covered, and on 9 we can already see a uniform layer of cells. Besides, more cells can be seen in ECGS-containing cultures.

6.2. HBMEC Cell Systems

Two experiments were performed in 24-well Transwells. Both showed high permeability values in the permeability assay, $83{,}47 \pm 16{,}68 \cdot 10^{-6}$ cm/s and $91{,}72 \pm 17{,}86 \cdot 10^{-6}$ cm/s. In the same way, when TEER was measured for 7 days, only a value of 11,6 $\Omega \cdot cm^2$ was achieved. In addition to this, cell morphology and confluence were poor, which indicated that the cells were not healthy and did not behave as an endothelial barrier. Therefore, new endothelial cells were purchased from Sciencell.

6.3. HBMEC Sciencell

The first experiment was to test different conditions of culture. We grew the cells under four conditions. Two different coatings were tested: matrigel 1:48 and fibronectin

2μg/cm^2; (30)and they were cultured in two different media: complete and depleted. Depleted medium is EGM^{TM} -2 MV without hEGF, VEGF and IGF – it has been reported that when these growth factors are eliminated, the formation of TJ is favored. (33)All conditions were seeded in 24-wells transwells for higher throughput, and only fibronectin-depleted was seeded in 12-wells transwells. Cells were maintained for 7 days. The following results were obtained:

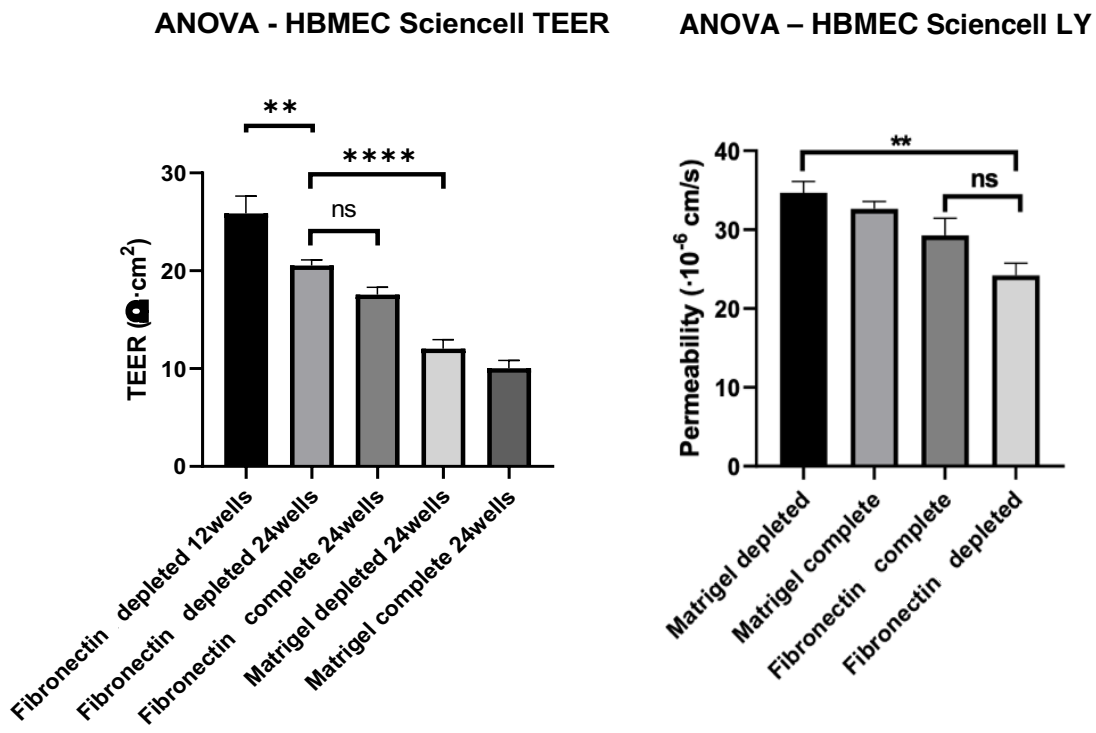

Fig. 10 | ANOVA test for HBMEC Sciencell. Experiment 1. On the left side, TEER comparision graph between all different conditions (coating and medium). On the right side, LY comparision graph between cells seeded in 0,3cm^2 transwells in all different conditions. All results were obtained at day 7. Significance is indicated with (*) the more asterisks, the more significant the difference. Quite the opposite, if "ns" is shown, it means that there is no-significant difference. SEM bars are presented. Made with GraphPad Prism.

In the TEER bar graph, you see the comparison of all the conditions mentioned above is shown. The highest values are for the fibronectin coating, and there is no significant difference between the two media. Fibronectin depleted in the 12-well condition has the highest TEER value of the experiment, which is significantly different from that of the 24-well condition. In addition, it is easier to measure TEER in 12-well transwells because the larger volume of medium allows for more stable TEER readings. For this reason, 12-well transwells were chosen for the following experiments.

In the LY bar graph, only the 24-well Transwell conditions are compared. This is because we realized that the surface-to-volume ratio of the two types of transwell is not equal, and therefore are not comparable in terms of permeability results (see annex). Here, it is confirmed that there is a significant difference between the two coatings, and no significant difference between the two media. Additionally, lower permeability results correlate with higher TEER values for all the tested conditions.

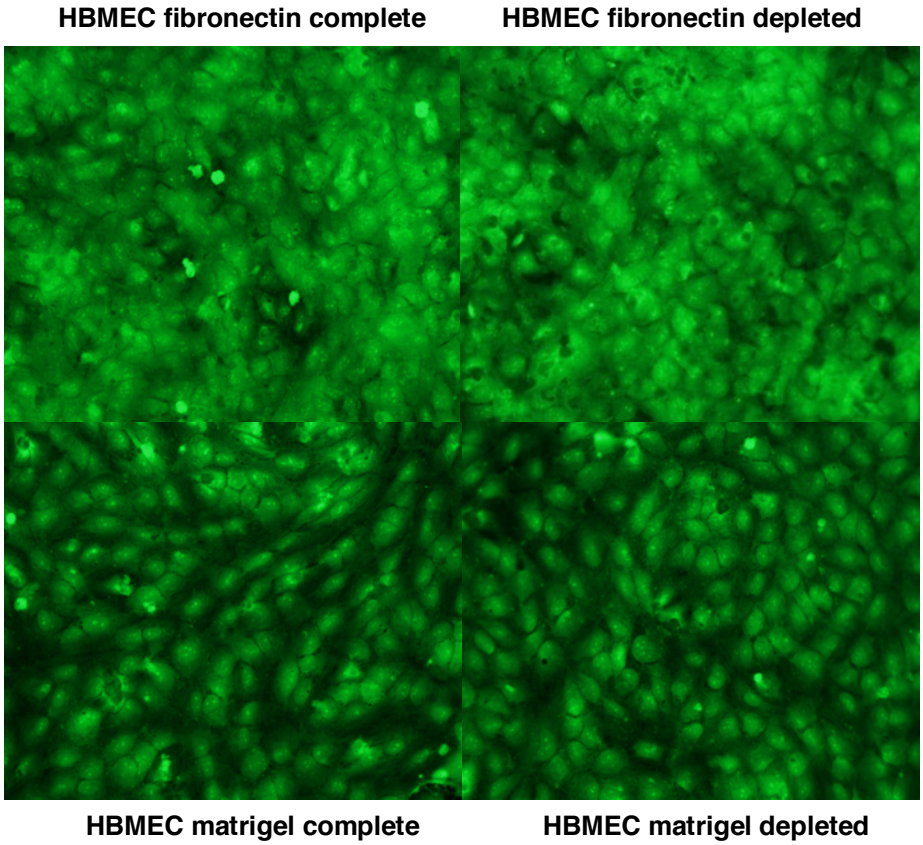

Fig. 11 | CellMask™ Green staining in HBMEC Sciencell cells at day 7. Experiment 1. In the top line, fibronectin coated cells, in the bottom line, Matrigel coated cells. Left column images show cells seeded with complete medium, while right column images show cells seeded with depleted medium. Images were taken with fluorescence microscope and 20x objective.

Here we can see the immunocytochemistry with CellMask, which corroborates the difference already mentioned. Visually, we can see a difference between coatings but not between culture media. In the case of fibronectin, cells are more closely packed and have a more uniform membrane staning, while more gaps are seen with Matrigel.

In the second experiment, cells were seeded in 12-well transwells with fibronectin and depleted medium. In this case, cells were cultured for a longer time in order to test if TEER values improved after Day 7. In addition to this, the fibronectin coating was

incubated for a longer time (1h 30min), since in the first experiment it had been the minimum recommended time (30 min).

In LY assay, better results were obtained compared to the first experiment, $28{,}77 \cdot 10^{-6} \pm 7{,}15$ cm/s. The lower the value, the less permeable is the barrier.

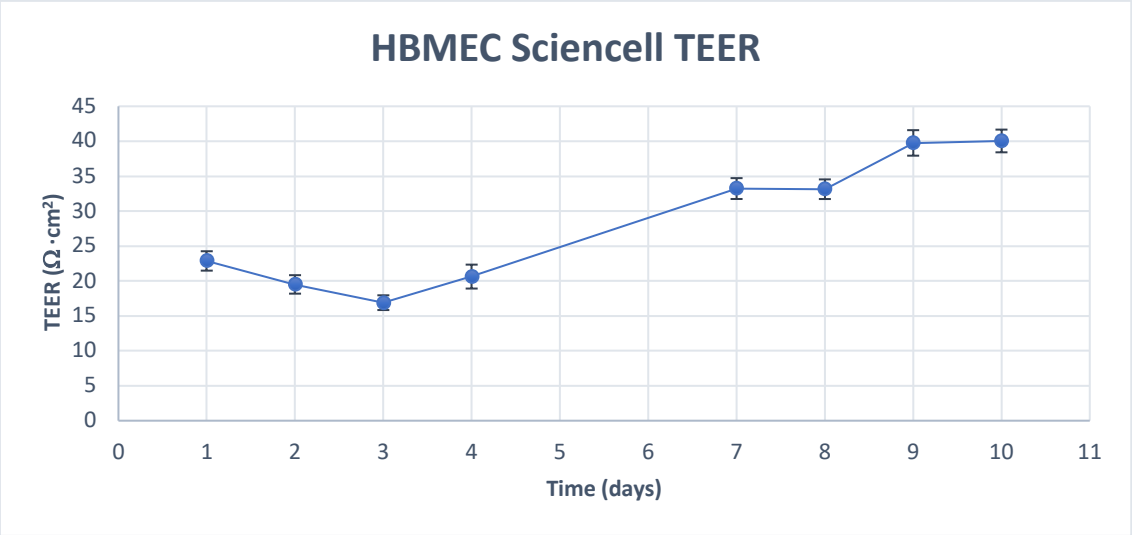

Fig. 12 | HBMEC Sciencell TEER values measured from day 1 to day10. Experiment 2. Highest TEER value in whole project, 40Ω·cm2, was reached in day 10.

TEER was measured periodically. It was seen that TEER continued to rise from day 7, so that the cells grew until day 10. The values of the first experiment were overcome and were equal to the results of the bEnd.3 experiments.

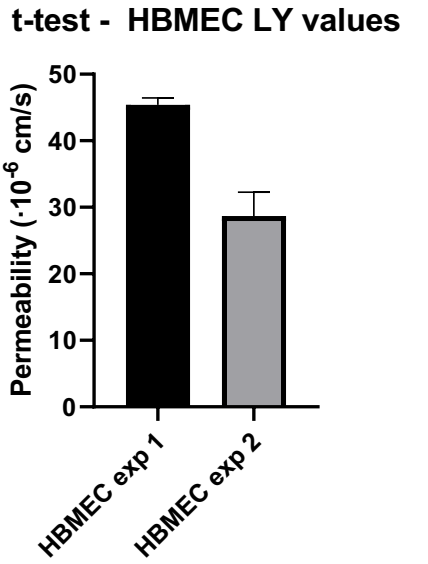

Fig. 13 | t-student Test about the LY results. Both HBMEC Sciencell experiments are compared with SEM bars. Made with GraphPad Prism.

In the bar graph above, a comparison is made between the acquired values of the same condition: fibronectin depleted in 12-well Transwell. The difference between the two cultures is the time of coating with fibronectin. Furthermore, experiment 2 was kept until day 10, while experiment 1 was only kept until day 7.

In experiment 1 a TEER value of the range of 25 $\Omega \cdot cm^2$ was obtained, while in experiment 2 a TEER of the range of 40 $\Omega \cdot cm^2$ was obtained. Therefore, we can confirm that the values of experiment 2 are improved compared to those of experiment 1. For this reason, the experiments will continue with the conditions of experiment 2.

6.4. PrP expression in bEnd.3 and HBMEC

In parallel, we performed a Western Blot (Figure 10) to verify that the ECs expressed PrP, which is a protein of interest for the project. Although the image is not too clear, it was confirmed that HBMEC Sciencell, HBMEC Cell Systems and bEnd.3 express PrP protein. Different molecular weight of the bands between cells is explained by differences in glycosylation patterns of PrP isoforms.

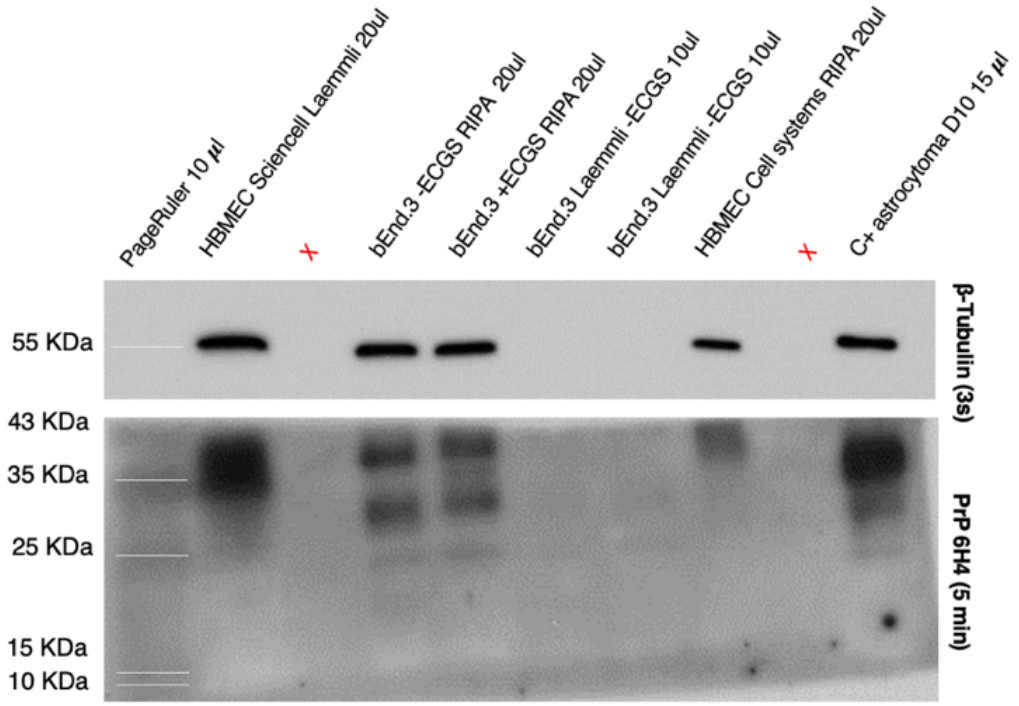

Fig. 14 | PrP Western Blot comparing bEnd.3, HBMEC CellSytem and HBMEC Sciencell. PrP expression is confirmed in all cells as 35kDa, 25kDa and 15 KDa band are exhibited.

6.5. CACO-2

CACO-2 cells were cultured for 22 days in 12-well transwells with collagen coating. These are cells of the gastrointestinal tract, for which the culture and manipulation protocols are widely known. The stabilized value of TEER was 2797,95 $\Omega \cdot cm^2$ (Fig. 11), while the LY permeability value was 0,46 $\pm$ 0,44 $\cdot 10^{-6}$ cm/s. The obtained results are correct according to published data on this cell type and confirm a robust barrier formation. Further comparative analysis of cell morphology with ECs is shown in the discussion.

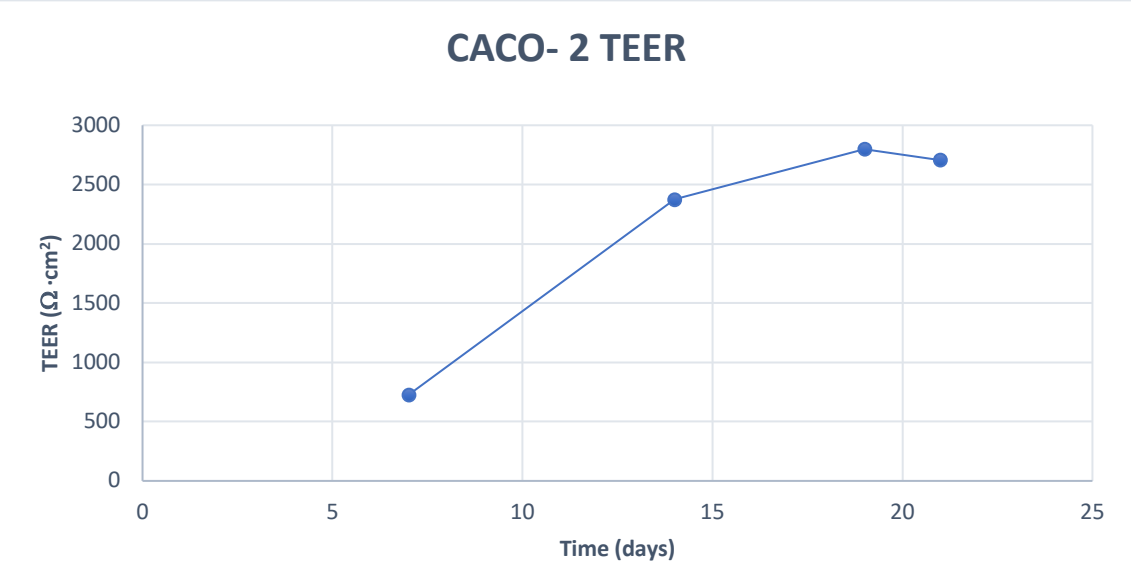

Fig. 15 | CACO-2 TEER values in 22 days experiment. CACO-2 epithelial cells derive from human colorectal adenocarcinoma and the barrier they form has a considerably higher transendothelial resistance than the BBB.

7. Discussion

Results compared between HBMEC Sciencell and HBMEC Cell Systems cells are shown below in Fig.12.

7.1. Comparison between Cell Systems HBMEC and Sciencell HBMEC

These results are from experiment 1 in HBMEC Sciencell and experiment 2 in HBMEC Cell Systems (Fig. 16). Sciencell HBMEC is seen to have much more favorable results.

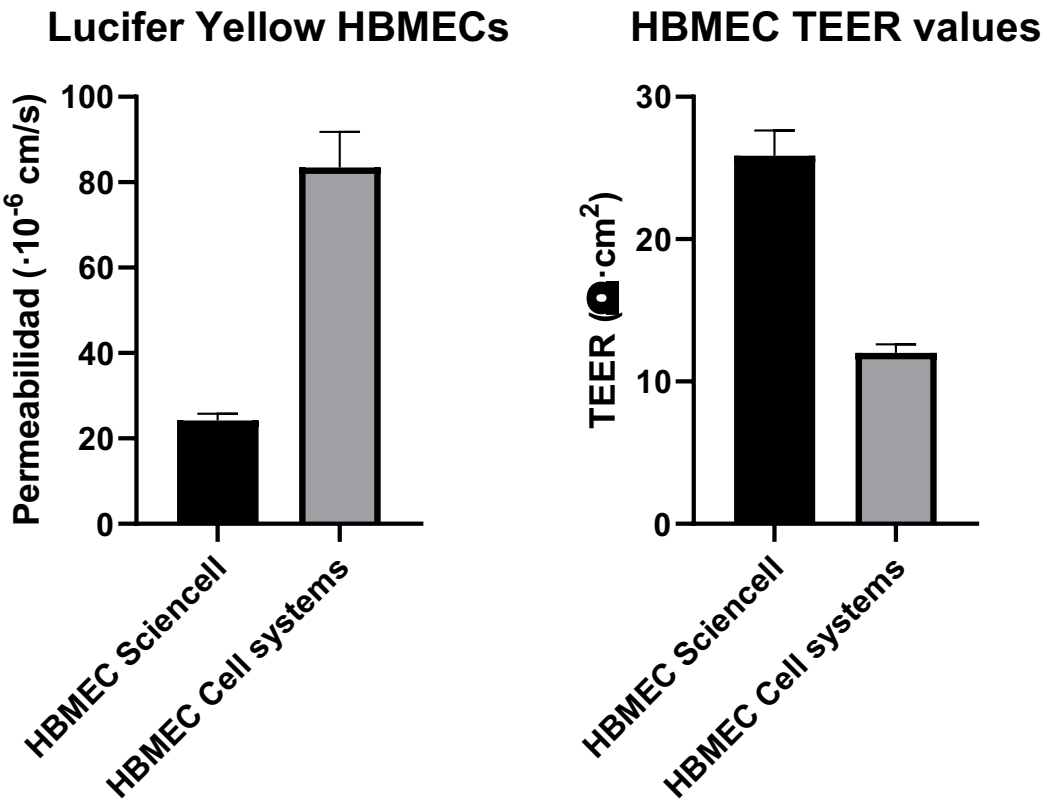

Fig. 16 | t- student Test about the LY and TEER results. Both HBMEC Sciencell cells (experiment 1) and HBMEC Cell Systems cells (experiment 2) are compared with SEM bars. There is a significant difference between the two types of cells. The HBMEC Sciencell are established as the top cell line. It is still believed that the Cell Systems vial was not in correct condition. Made with GraphPad Prism.

As mentioned in the Cell Systems results section, we suspected that the vial of cells was not in its correct state due to the lack of formation of a confluent cell monolayer and irregular cell morphology, which explains poor results in terms of permeability and TEER. For all these reasons, Sciencell's HBMEC cells are the ones we chose to continue with experiments.

From now on, the discussion will compare only Sciencell HBMEC and bEnd.3.

7.2. Comparison between bEnd.3 and Sciencell HBMEC

The best results obtained at the beginning with the bEnd.3 cell line (experiment 4) and the current results of the Sciencell HBMEC will be compared using the ANOVA test.

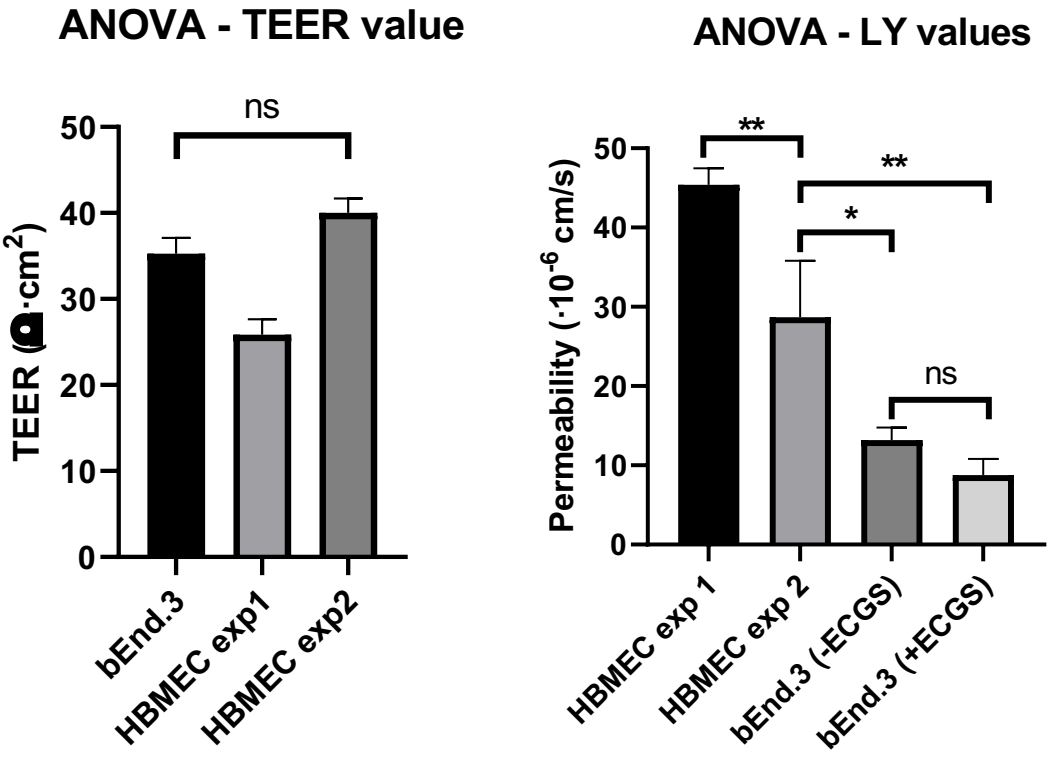

Fig. 17 | ANOVA test for HBMEC Sciencell and bEnd.3. Best results of both cell lines are compared in these bar graphs. On the left side, TEER comparison graph between optimal conditioned cells. On the right side, LY provides the same comparison as the TEER graph. Significance is indicated with (*) the more asterisks, the more significant the difference. If, on the contrary, "ns" is shown, it means that there is no significant difference. SEM bars are displayed. Made with GraphPad Prism.

On the one hand, the TEER values show that the differences between experiment 2 with HBMEC and bEnd.3 (in this case, the best value was obtained by supplementing with ECGS), are not significant. In addition, both have reached the values described in scientific literature. However, in the study of LY values, there is no significant difference between bEnd.3 conditions (with ECGS or without ECGS). Nevertheless, there is a significant difference between the HBMEC and the bEnd.3. Thus, the most optimized value of LY has not been obtained with HBMEC. We can affirm that now, the barrier composed of bEnd.3 cells is the most impermeable compared with the barrier composed of HBMEC Sciencell cells. However, these cells show more promise in terms of tight junction formation and expression of PrP in a junctional localization, as shown in the immunocytochemical staining of actin, which is discussed next.

If the polygonal shape of ECs were discussed, we could see that CACO-2 has a very characteristic honeycomb shape with marked cortical actin staining. Sciencell HBMECs resemble this shape most accurately, while Cell Systems HBMEC did not show uniform

cell morphology. In comparison, in bEnd.3 (although in the last experiments some clusters with polygonal shapes could be observed) a shape as ordered as in the cultures of the HBMEC Sciencell was not achieved, and cells were more fusiform than polygonal.

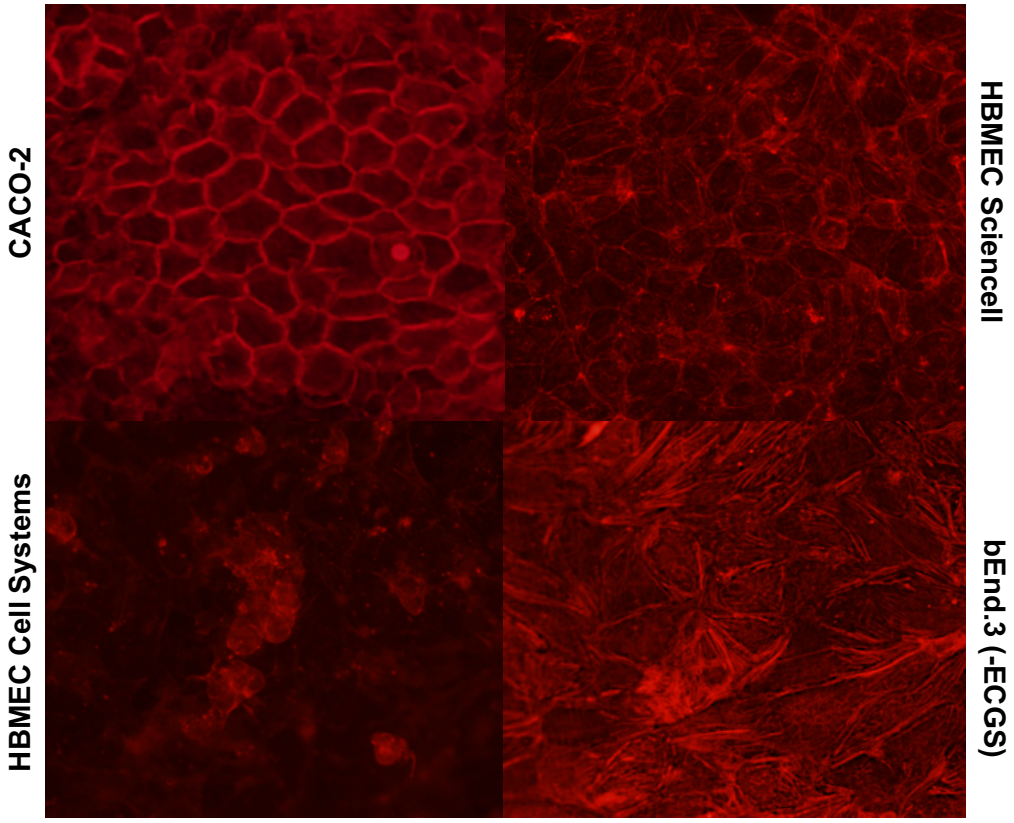

Fig. 18 | Phalloidin-TRITC staning in endothelial cell cultures. Three endothelial types of cells compared with CACO-2 cells. Honeycomb form is achieved in HBMEC Sciencell. Images were taken with fluorescence microscope and 20x objective.

8. Conclusion

- The HBMEC Sciencell monolayer model has been optimized, improving the fibronectin coating, and lengthening the growth period. The last experiment has shown optimal TEER values, and of the same order as the bEnd.3. Still, LY permeability values should be improved, because lower permeability values have been reported for HBMEC (30).

- Although the bEnd.3 data are favorable, both in LY and in the TEER results, the studies will continue with HBMEC. The reason is that, as the study will be conducted for human PrP, working with human cells will approximate the model to *in vivo* conditions.

- A decision has been made to continue working with 12-well transwells as it facilitates TEER measurements and thus has an advantage over 24-well transwells. In addition, there is no difference in growth, or confluence, between the different diameters of the transwell.

9. Self-assessment

First of all, I would like to express my satisfaction with my first contact with research and science on a professional level. From the first moment, they put a lot of confidence in me and let me develop my skills in the laboratory.

During my stay in the project, I have had to use all the tools acquired during my degree in Biotechnology. I have been able to take advantage of the knowledge in Biochemical and Molecular Biology Techniques to carry out biochemical protocols. I have also used the knowledge in Cell Cultures to understand the maintenance conditions of the different cell lines involved in this project. Finally, I have also used my knowledge in Tissue Engineering to understand at a global level the formation of the monolayer and the three necessary points to carry out any biological construction.

My lab skills have increased day by day, and I have ended up working smoothly, with comfort and determination. In addition, my knowledge in neuroscience has expanded remarkably. By doing this work, I have learned to use computer tools such as Illustrator, Graphpad, and microscopy programs, such as CellSens to acquire the photographs of the cells in staining.

Finally, I would like to express my gratitude to the entire IBEC team that, despite the sanitary conditions with which we lived in during my 5 months of stay, they have always made it easier for me to carry out the practices on a face-to-face manner. Providing safety material and free antigen tests. It was a great pleasure to participate in your research.

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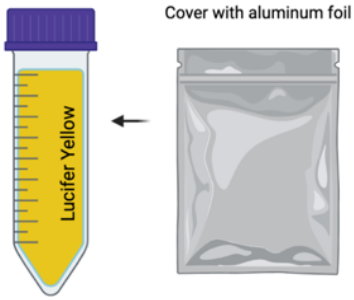
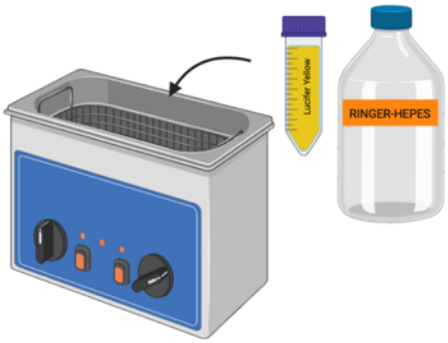
Annexes

i. Lucifer Yellow protocol

1. Prepare LY solution 50µM



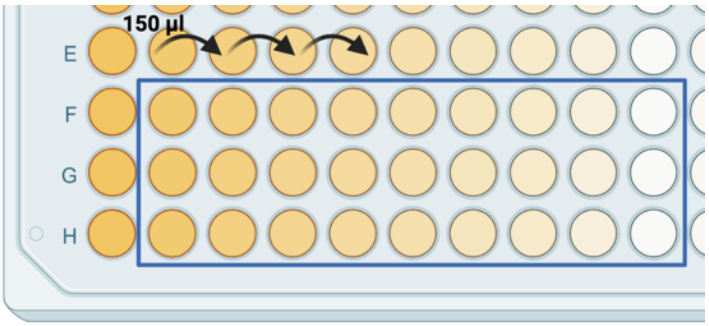
2. Prepare 37ºC bath and put in RINGER-HEPES and LY solution. Make all plates ready for the assay. Fill them with RINGER-HEPES (1,5ml in 12 wells transwells or 0,9ml in 24 wells transwells). Keep them in the incubator.



3. Remove ECs from incubator. Remove endothelial media. Wash ECs with RINGER-HEPES and remove it.
4. Put transwells in first plate line (previously filled with RINGER-HEPES). Add LY in transwells (0,5ml in 12 wells transwells; 0,3ml in 24 wells transwells). Keep them in the incubator for 15 minutes.

5. After 15 minutes, change the transwells to the following well

6. Again, keep them in the incubator for 15 minutes. Repeat for two times
7. Prepare the standard line in a 96-well plate. Add 300µl of LY solution in first column and 150µl of RINGER-HEPES in the following line. Dilute by taking 150µl of LY and mixing in the next well. Take 150µl of the second well and dilute as before. Leave the last column without LY for blank.



8. At the end of the 60 minutes, fill the wells of the 96-well plate with the basal solutions of the assay
9. Go to plate reader and read at a wavelength of 438-535 nM
10. Fill the Excel:

    a. As LY concentration is known, calculate standard line.
    b. Use the slope of the line to know basal (B) sample concentration

$$Fluorescence = m \cdot concentration$$

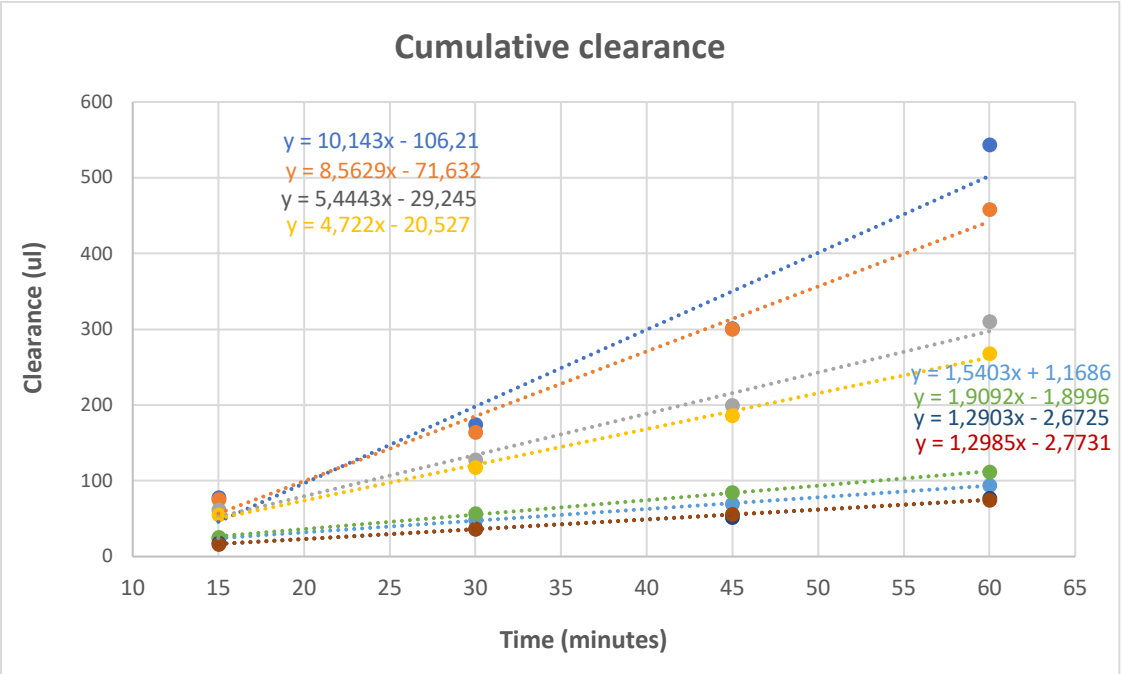
    c. Calculate apical (A) sample concentration at each timepoint as below:

$$CA_{15}=(CA_0*VA-CB_{15}*VB)/VA$$
$$CA_{30}=(CA_{15}*VA-CB_{30}*VB)/VA$$

    d. Calculate clearance:

$$Clearance\ (\mu l) = \frac{[C]B \cdot VB}{[C]A}$$

    e. Calculate cumulative clearance by adding clearance from previous time points to the current time point.
    f. Represent cumulative clearance vs. time in a graph



g. Calculate permeability

CELLS 1		
PSF	7,21805	µl/min
PST	1,5403	µl/min
PSE	1,95816343	µl/min
PE (Pse/cm^2)	0,00174836	cm/min
Pex·10^{-3}	1,748360205	cm/min
Pex10^{-6}	**29,13933676**	**cm/s**

Psf: no-cell slope line average

Pst: cell 1 slope line

$$PSe: \frac{1}{\frac{1}{Pst} - \frac{1}{Psf}}$$

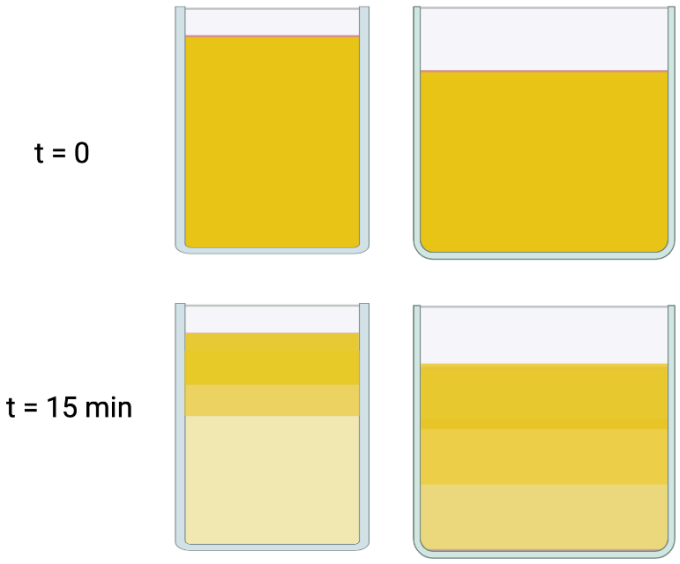
$$Pe: \frac{PSe}{(\text{Transwell diameter} :1000)}$$

Effect of surface-to-volume ratio in LY assays

As mentioned in the results section, it was realized after performing the permeability assays that different transwell sizes have different surface-to-volume ratios. In the case of the 12-well transwell, the surface is 1,12 cm2 and the volume of LY solution is 0,5ml. In the 24-well transwell, the surface is 0,3cm2 and the volume of LY solution is 0,3ml. This gives us a surface-to-volume ratio of 2,24 cm2/ml and 1cm2/ml, respectively.

When we take into account the fact that the assay is performed without agitation, it must be considered that during incubation, a gradient of the LY is formed over time. In transwells with a higher surface-to-volume ratio, this gradient is not as pronounced, and diffusion of the compound occurs at a faster rate. This is due to the fact that the osmotic pressure is higher when the local concentration of LY is higher directly next to the membrane, which is the case for the larger transwells.

For this reason, permeability results obtained in different surface-to-volume ratios are not comparable. A solution to this could be either to maintain the surface-to-volume ratio constant, or to use a shaker during the assay. This last option was not considered due to lack of space in the incubator to introduce a shaker, as well as possible disturbances to other experiments in the incubator.



ii. Immunostaining transwells

These first three steps are carried out in a fume hood.

1. First, extract half of the culture medium from the transwell with a micropipette. Add the same amount of PFA 4% for 5 minutes at room temperature. Holding them with forceps, invert the transwells on a 50 ml Falcon to eliminate the mixture of medium and 4% PFA. To wash the wells, use a plastic Pasteur pipette to absorb the liquid.

2. Add 300 μl to 500 μl of PFA 4% on the wall of the transwell to avoid detaching cells, for 15 min at room temperature.

3. Invert over 50 ml Falcon (for PFA residues) to remove PFA. Then wash with PBS for 5 minutes and repeat 3 times. The PBS is added through the wall and is removed by an inversion process. It is important to wash the wells well to remove any PFA that may have fallen.

    - Once washed, they can be stored in 1X PBS in the refrigerator and closed with parafilm. The process can be resumed the next day, if it is required.

This step can already be done outside the fume hood.

4. Permeabilize for 15 minutes with PBS-Triton 0.1% under stirring and room temperature. Meanwhile, thaw PBS-gelatin and FBS necessary to prepare the Blocking Buffer and the Antibody Buffer.

5. Wash with PBS 3X5 minutes while shaking.

    - If the immunocytochemistry is performed without antibodies, the next step would be to add the stains in a solution with 1X PBS, and incubate for 1 hour with shaking, in the dark and at room temperature. Add Phalloidin (1: 500), Hoechst (1: 200), CellMask (1:5000).

6. Add blocking buffer: PBS-gelatin + 10% FBS + 0.1% Triton. Incubate at least 1h while shaking at room temperature. Add 500 ul to the top, or more if it is feared that it may drop down during incubation.
7. Remove blocking buffer and add 1ary Antibody dissolved in: PBS-gelatin + 5% FBS + 0.1% Triton. Add 500ul above. Alternatively, trim the membrane

before adding the antibody to allow incubation with less antibody volume. Leave O / N in cold room.

8. Wash with PBS 4X5 min * while shaking

Fluorophores are sensitive to light. Cover the plates with aluminum foil.

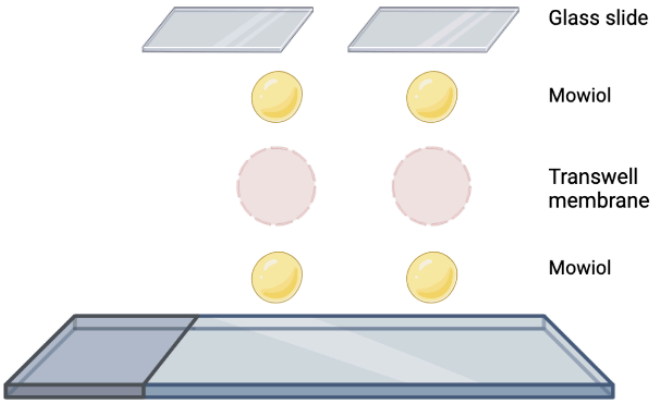
9. 2ary antibody (1: 500) dissolved in: PBS-gelatin + 5% FBS + 0.1% Triton. Add 500μl above. Alternatively, trim the membrane before adding the antibody to allow incubation with less antibody volume. Leave at least 1h, better 1h30-2h, stirring at room temperature. In this step you can also add Phalloidin (1: 500) and / or Hoechst (1: 200) in the same solution.

10. Wash with PBS 4X5 min * while shaking.

11. Trim membrane if was not done before. Cut along the edge with a type 11 scalpel (pointed) and leave in PBS on a 60mm plate.

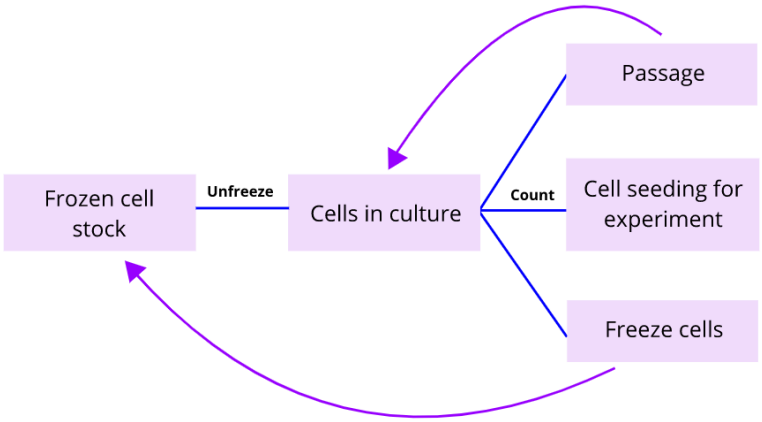
12. Observe in BX61 with immersion objective directly in the Petri dish

13. Mount in Mowiol on microscope slides:



- Dry the membranes before
- Use 20-100 μl of mowiol (according to membrane size)
- Be careful do not form bubbles
- Do not move the membrane once in the microscope slide

iii. Cell maintenance protocol



Medium changes

Approximately, it is done every 2-3 days as long as they have not achieved the confluence for cell passaging. Depending on the cell line, they consume the medium more or less quickly. Look at the color, if it is very orange, it is time to change it.

Process

1. Remove cells from the incubator, check confluence and medium color
2. Heating culture medium
3. If you cannot heat everything several times, separate the appropriate amount (13ml / flask T75)
4. Aspirate culture medium (glass pasteur pipette)
5. Add new culture medium (13 ml / flask T75)
6. Put in the incubator.

Passages

When they reach maximum confluence

• In endothelial cell lines 80-90%

Objective

Spread the cells over more surface area to reduce confluence and allow them to continue dividing.

Process

1. Heating materials in the bathroom
    - Sterile PBS
    - Trypsin

- Culture medium (ensure that it contains at least 10% FBS, and if not prepare medium with 10% FBS)

SOLUTION	VOLUME
PBS	7-8ml/flask T75
Trypsin	2ml/flask T75
Culture medium	8ml for each flask to inactivate trypsin + 13ml for each new flask

2. When they are warm, remove the cells from the incubator, and insert them together with:
   - 10ml serological pipettes (4 or +)
   - Falcon tubes (enough to collect cells from X number of flasks)
   - PBS
   - Trypsin
3. Aspirate the culture medium (Pasteur glass)
4. Add PBS through the side wall
5. Aspirate PBS
6. Add trypsin with a serological pipet to avoid contaminating the trypsin with P1000 pipettes.
7. Put cells in the incubator for 2-5 min (depends on the line). Do not exceed 5 min. Go checking in the microscope if they detach, and give light taps to help detach them.
8. While the cells are in the incubator, save PBS and trypsin, and pour the culture medium into the laminar flow cabinet.
9. When they are detached, quickly inactivate the trypsin with 8ml culture medium.
10. With the same pipette of the culture medium, preferably 10ml, collect medium with cells and let it go again on the wall where the cells were, to finish detaching them. Repeat 3-5 times (depending on how detached they were)
11. Put all the medium in a falcon of those that you had entered the laminar flow cabinet
12. Centrifuge the falcon tubes at 800rpm for 5min and with the possibility of doing it cold to further reduce trypsin activity.
13. Aspirate medium with glass Pasteur
14. Resuspend in 1ml medium with P1000

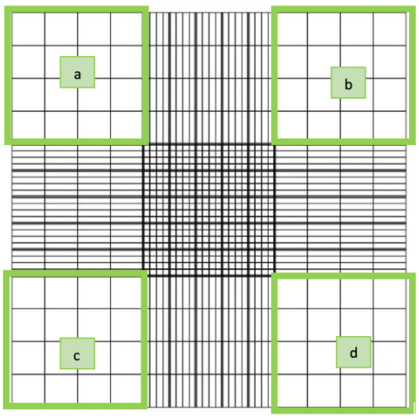
15. Have flasks prepared with new culture medium.
16. Put the adequate amount to make the dilution that corresponds to the cell line in each new flask.
17. Distribute the cells by carefully turning the flasks
18. Label (pass, dilution), put in incubator

Cell count

It is important for most experiments to have a defined number of cells in each replicate. The Neubauer chamber is used by taking a 10µl aliquot of the cell suspension that is generated by making a passage.

Process:

1. Enter the Neubauer chamber into the laminar flow cabinet (put in a corner without sterilization so as not to get it wet). A square cover is put on it.
2. Make sure the suspension is homogeneous (invert the tube ~ 3 times to homogenize)
3. Quickly (so that the cells do not sediment again) take 10µl
4. Holding the cover with your finger, put the tip on the edge of the cover, inclined approx. 45º.
5. Let go of the aliquot of cells to fill the space between the covers and the chamber
6. Focus the microscope at 10x (the standard), for example while centrifuging cells from the passage.
7. Count number of cells seen in the 4 grids shown in green:



Record individually the number of each green grid (a, b, c, d)

8. Take the mean of a,b,c,d → (a+b+c+d)/4 = mean

9. Conversion factor to know the total number of cells in Falcon where the aliquot was taken:

Total number cells in Falcon = mean · 1/0,1μl ·1000ul/1ml ·V Falcon

Total number cells in Falcon = mean · 10.000 · V Falcon

How to seed the desired number of cells

Know how many cells you want to have in each replicate (Nrep) and have an idea of how much cell volume it is reasonable to seed (X μl). You want to have a cell solution with the appropriate concentration to seed X μl for each replicate.

Total number cells in Falcon / V = Nrep / X μl

Isolate V and resuspend the cells in that volume after centrifugation.
Finally, seed the X μl on the plate for each replicate.