Electrical and microfluidic technologies for organs-on-chips

Mimicking blood-brain barrier and gut tissues

Marinke van der Helm

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Graduation committee

Chairman & secretary:

Prof. dr. P.M.G. Apers University of Twente

Supervisors:

Prof. dr. ir. A. van den Berg University of Twente

Prof. dr. J.C.T. Eijkel University of Twente

Co-supervisor:

Dr. ir. L.I. Segerink University of Twente

Referees:

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Dr. O.Y.F. Henry Wyss Institute at Harvard University

Members:

Prof. dr. ir. P.H. Veltink University of Twente

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Prof. dr. E.M.J. Verpoorte Rijksuniversiteit Groningen

Prof. dr. P. Renaud École Polytechnique Fédérale de Lausanne

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ELECTRICAL AND MICROFLUIDIC
TECHNOLOGIES FOR ORGANS-ON-CHIPS
MIMICKING BLOOD-BRAIN BARRIER
AND GUT TISSUES

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by

Marieke Willemijn van der Helm
born on 21 June 1991 in Apeldoorn

This dissertation has been approved by:

Prof. dr. ir. A. van den Berg	<i>Supervisor</i>
Prof. dr. J.C.T. Eijkel	<i>Supervisor</i>
Dr. ir. L.I. Segerink	<i>Co-supervisor</i>

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Introduction & outline

The goal of the research presented in this thesis is to develop new technologies for organs-on-chips to enable direct measurements of cell layer functions and to move towards high-throughput. In this introduction, a brief description is included of the tissues that were mimicked in the organs-on-chips described in this thesis. Next, conventional *in vitro* setups for mimicking these tissues are discussed as well as the advantages of organs-on-chips over these conventional *in vitro* models. Then, the most important tests of tissue function are described. Subsequently, the larger framework for the research described in this thesis is sketched and lastly an outline of the thesis is given.

1.1. The promise of organs-on-chips

Between 2000 and 2010, the average cost of the development of a new drug in the United States was estimated to be as high as \$2.6 billion, while the decade before the costs were estimated to be \$1.0 billion [1]. This increase in costs is mostly arising from the high costs of clinical studies and the large number of drugs failing in these clinical trials [1, 2], as only 12% of drugs entering clinical trials are eventually approved by the Food and Drug Administration for clinical use in humans [1]. To bring down the costs and speed up the drug development process, it would therefore be very beneficial to make the preclinical tests more predictive of drug success or failure in clinical trials. Moreover, more predictive preclinical models can also serve as research tools for the discovery of new drug targets and the study of disease mechanisms, as well as application in personalized or precision medicine.

In preclinical tests drug candidates are tested on cell cultures in a lab (*in vitro*) and afterwards in animals (*in vivo*). Besides being costly, labor-intensive and ethically contentious [3], animal models often lack predictive value [4, 5], as is also evidenced by the large percentage of 88% of drugs eventually failing in clinical trials, as mentioned before [1]. This lack of predictive value can be partly attributed to poorly conducted animal experiments [5-8], but also to the differences in (patho)physiology between humans and (genetically modified) animals [6-8]. For example, species-to-species variations occur in the expression profiles of transporter proteins [9-11] and in the electrophysiology of the heart muscle [12]. To overcome these species-specific problems, the use of human tissues in a realistic *in vitro* environment is of great interest. However, the most commonly used *in vitro* models are too simplified to faithfully replicate the micro-environment of a tissue and therefore cells cultured in these models tend to lose their tissue-specific physiology [13]. For this reason, these models also lack predictive value.

The recently introduced field of organs-on-chips has the potential to address these shortcomings [13, 14]. These organ-on-chip models, which contain micrometer-sized, fluid-filled channels in which human cells can be cultured, provide opportunities for engineering a controlled culture environment that resembles the microenvironment of a certain organ by tuning mechanical, biochemical and geometrical aspects [13]. More physiological behavior is expected from such a combination of cells and engineering, resulting in better predictive value [14]. In addition, organs-on-chips can be engineered in such a way that they allow direct measurements of organ functions as well as pharmacokinetics and pharmacodynamics [14]. Furthermore, as all *in vitro* models, organs-on-chips hold the potential of parallelization and high-throughput screening. The goal of organs-on-chips is to mimic functional units of a certain organ rather than complete organs, in order to arrive at realistic but simple *in vitro* models [13, 14]. Such a functional

unit can comprise one or more tissue types, depending on the organ function that needs to be mimicked. This thesis will focus on barrier-forming tissues, of which the main function is to provide a barrier between the different fluid compartments in the body.

The goal of this thesis is to develop new technologies for organs-on-chips of barrier-forming tissues to enable direct measurements of barrier functions and to move towards high-throughput assays. In the next sections of this introduction, the barrier tissues that were mimicked in the organs-on-chips described in this thesis are briefly discussed. Next, conventional *in vitro* setups for mimicking these tissues are discussed as well as the advantages of organs-on-chips over these conventional *in vitro* models. Then, the most important tests of barrier function in organs-on-chips are described. Subsequently, the larger framework for the research described in this thesis is sketched and lastly the outline of this thesis is given.

1.2. Blood-brain barrier and gut tissues

There are many barrier tissues of interest for drug development studies, studies of disease mechanisms and precision medicine using organs-on-chips. The two barrier tissues that are elaborated upon in this thesis will be introduced here: the blood-brain barrier (BBB) and the gut.

1.2.1. Blood-brain barrier

The BBB, comprised of specialized endothelial cells, is the barrier between blood and brain fluids, protecting the brain from harmful influences from the blood and regulating the composition of brain fluids for optimal neuronal function [15, 16]. While blood vessels in the rest of the body allow the exchange of fluids and solutes between blood and interstitial fluids, the key for proper functioning of the BBB is to prevent such free exchange of nutrients, waste products and other agents. In general, transport between blood and surrounding tissues occurs through gaps in the endothelium (paracellular) or through the endothelial cells (transcellular). In brain capillaries, however, tight junction protein complexes between adjacent endothelial cells block paracellular transport of most molecules and thus ensure the establishment of a physical barrier [15]. By specialized transporter pathways, the BBB also regulates transcellular transport and limits which substances can move from the blood to the brain (from the apical to the basal side of the barrier) and which substances can move from the brain into the blood [15, 16]. In close association to the endothelium, other cell types can be found such as pericytes, astrocytes, microglia and neurons, as well as the basal lamina enclosing the blood vessel. Together, these form the neurovascular unit (NVU), which is shown schematically in **Figure 1-1**.

The BBB is of great interest in drug development studies, as it is notoriously difficult to overcome this barrier and to enable drug delivery into the brain [17]. In addition,

Figure 1-1 | Schematic representation of the BBB anatomy. The brain-specific endothelial cells, tightly linked by tight junction protein complexes, form a physical barrier against paracellular transport. Pericytes and smooth muscle cells, enclosed by the basal lamina, as well as astrocytes, microglia and neurons are in close association with the BBB, together constituting the NVU. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience [20], copyright 2006.

impaired BBB function is often associated with brain diseases, such as Alzheimer's disease, stroke and Parkinson's disease, although its role in pathology often is poorly understood [18-20].

1.2.2. Gut epithelium

Gut epithelium lines the small intestine and colon and forms the barrier between the gut lumen and the blood, protecting the body from harmful agents in the digestive tract and regulating the uptake of nutrients from ingested food [21]. Like the BBB endothelium, gut epithelium obtains its barrier function from the presence of tight junction protein complexes between adjacent cells. Furthermore, this epithelial layer displays distinct differentiation into finger-like projections called villi, thus increasing the surface area through which absorption can take place. At the base of these villi, in crypts, epithelial stem cells can be found while mature cells cover the villi towards the tip [22]. Mucus is excreted by specialized epithelial cells to cover the epithelium, protecting it from the acidic environment and digestive enzymes inside the gut lumen [21]. Digested food moves through the gut by peristaltic motions [21]. The gut is inhabited by microbes that play a role in digestion and, suggested in more recent findings, play a role in homeostasis [23] and prevention of infections by competition with pathogens [21]. The gut tissue organization is schematically depicted in **Figure 1-2**.

The gut plays an important role in the absorption of orally administered drugs and is therefore included in pharmacological characterization of drug candidates. In addi-

Figure 1-2 | Schematic representation of the gut anatomy. The tightly linked epithelial cells form a physical barrier between the lumen and the blood vessels and lymphatics. The villi, finger-like protrusions, are covered in mucus. Epithelial stem cells reside in crypts at the base of these villi. The lumen is inhabited by bacteria. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology [23], copyright 2010.

tion, the study of the complex interactions between the gut tissue and gut microbes may help the understanding and treatment of inflammatory bowel diseases [24] and inflammation-promoted colorectal cancer [23].

1.3. *In vitro* models of barrier tissues

Generally, *in vitro* models of barrier tissues such as the BBB and gut contain two compartments separated by a porous membrane. The porous membrane acts as a scaffold on which endothelial or epithelial cells can be cultured. This two-compartment configuration allows access to both the apical and basal sides of a barrier tissue, enabling measurements of barrier function and transport. Methods for measuring barrier function will be explained in the next section. The most widely used platform for compartmentalized culture is the Transwell-type well plate. These Transwell systems use an insert with a porous membrane that can be placed inside a classical well. A barrier tissue can be cultured on the apical side of the membrane, while closely associated cells, such as astrocytes for the BBB, can be cultured in the basal compartment. The general setup of a Transwell culture is displayed in **Figure 1-3**.

Figure 1-3 | Pictures and side-by-side schematic cross sections of *in vitro* culture systems for barrier tissues: Transwell and organ-on-chip. Transwell picture from [25], organ-on-chip picture from [26].

This Transwell-type setup of two compartments separated by a membrane is also employed by many organs-on-chips reported in literature because of the ease of access to both sides of the barrier [27–36]. In these cases, the compartments are formed by microfluidic channels. The general layout of such an organ-on-chip is also displayed schematically in Figure 1-3. The microfluidic channels provide more realistic dimensions and confinement as well as a more physiological cell-to-culture medium ratio to the cells cultured inside the device. Furthermore, a fluid flow of physiological rate can be generated in an organ-on-chip using a pump. This fluid flow exerts physiological shear stress on the cells, mimicking blood flow through brain capillaries or intraluminal fluid flow through the intestines, which has been shown to result in better cell differentiation [14, 37, 38]. As both sides of a barrier tissue can be accessed fluidically, effluents can be extracted continuously from the chip, resulting in better time resolution than static models such as Transwell for monitoring transport across the barrier and analyzing secreted factors. On top of that, while Transwell systems are made of rigid plastics, organs-on-chips can be made of flexible materials. Mechanical deformation of such flexible chips can be used to mimic mechanical stimuli that are normally exerted on tissues *in situ*. For example, stretching of a flexible membrane and the cell layer cultured on it can mimic mechanical movements such as peristalsis in a gut-on-a-chip [29, 38] and breathing in a lung-on-a-chip [28], in both cases aiding differentiation towards functional tissues [14]. Lastly, the *in vitro* co-culture of gut epithelium with its natural microbiome without compromising on gut cell viability is reported to be possible in microfluidic chips [38].

1.4. Measuring barrier function

As the barrier function of the BBB and gut epithelium are of great importance, as was illustrated before, it is crucial to measure the establishment of a proper physical barrier as well as the barrier function in *in vitro* cultures of these tissues. To that end, the expression of barrier-specific proteins, such as tight junction proteins or specialized transporter proteins, can be assessed by various biochemical techniques, such as reverse transcription polymerase chain reaction (RT-PCR), immunofluorescence and Western blotting. However, these techniques are often end-point assays that can only be carried out after terminating the culture period. In addition, they only provide information on the establishment of the anatomy of a proper barrier by the presence of specific ribonucleic acid (RNA) sequences or proteins, and do not provide a direct quantification of barrier functionality.

Alternatively, functional measurements can be carried out by adding labeled solutes (e.g. using fluorescent or radioactive labels) to the apical compartment and measuring the amount of the analyte that has ended up in the basal compartment. These labeled solutes can be inert molecules, to assess paracellular transport through gaps in the barrier and thus to measure the establishment of a physical barrier, or substrates of transporter pathways across the barrier, thus measuring barrier function. In that way, both the quality of the barrier and its barrier-specific transport functions can be assessed. While this method enables researchers to investigate the transport of solutes, this method requires labeled agents and complicated analysis tools and has limited time resolution.

To enable non-invasive, label-free and real-time measurements of barrier function, electrical measurements have been introduced. Among the first researchers to carry out such electrical characterizations of barrier function *in vitro* were Meza et al. in 1980 (kidney epithelium) [39] and Rutten et al. in 1987 (brain endothelium) [40]. At that time it was already recognized that the low ionic conductance (i.e. high electrical resistance) across a cellular barrier is mainly controlled by tight junctions between the cells and thus reflects the transport via the paracellular pathway. This “transendothelial / transepithelial electrical resistance” (TEER, or sometimes TER) is a useful and functional measure of barrier integrity [39, 40]. Since then, TEER measurements have become a widely accepted method for non-invasive and real-time monitoring of barrier tightness [41]. While TEER measurements are limited to assessing the paracellular permeability and are not suitable for measuring barrier-specific transport functions, TEER can be used to quickly evaluate barrier tightness before starting a molecular permeability experiment and to monitor changes in barrier tightness as a result of experimental stimuli [41]. In Table 1-1 the three aforementioned techniques for assessing

Table 1-1 | Comparison of biochemical techniques, molecular permeability and TEER as a measure of barrier function.

	Biochemical techniques	Molecular permeability	TEER
Direct measure of barrier tightness?	No	Yes	Yes
(Direct) measure of barrier specifics? (e.g. transporter proteins)	Yes	Yes	No
Possible during culture period?	No	Yes	Yes
Label-free?	No	No	Yes
Real-time?	No	No	Yes

barrier function are compared side-by-side and in the next section, the general methods used for *in vitro* TEER measurements are described in more detail.

1.4.1. Measuring TEER

Generally, the electrical resistance of a cellular barrier is measured by introducing two electrodes, one on either side of a cellular barrier, and determining the resistance of the path between the electrodes by applying a direct current (DC) signal [41, 42]. The resistance of the cellular barrier is found by subtracting the resistance of a blank (R_{blank} , in Ω , measured before introducing cells) from subsequent measurements (R_{meas} , in Ω). The equivalent circuit corresponding to these DC measurements is illustrated in **Figure 1-4**. Then, to arrive at the TEER in $\Omega \cdot \text{cm}^2$ the found resistance is multiplied with the culture area A (cm^2) that was sampled. Note that multiplication is appropriate here: a larger membrane area will result in a lower resistance (or higher conductance). The thus normalized TEER value is independent of the used porous membrane and culture area and can be compared across platforms. The calculation can be summarized in the following equation:

$$TEER = (R_{meas} - R_{blank}) \cdot A \quad [\Omega \cdot \text{cm}^2] \quad \text{Eq. 1-1}$$

Commercially available systems to measure TEER in Transwell-type culture systems include the EVOM2 Epithelial Voltohmmeter (World Precision Instruments, Sarasota, Florida, USA) [43] and the Millicell ERS-2 Voltohmmeter (Merck Millipore, Darmstadt, Germany) [44]. Generally, the electrodes used by these systems are “chopstick” type electrodes: two sticks with electrode material at the tip that can be inserted on both sides of a cellular barrier. On both sticks there is one silver excitation electrode and one silver/silver chloride (Ag/AgCl) readout electrode. A current is sent through the excitation electrode pair while the resulting potential difference between the readout electrodes is recorded. The Ag/AgCl electrode material is chosen for compatibility with DC measurement conditions [43, 44]. While originally developed for pure DC measurements, these commercial systems now generally use a near-DC signal: a square wave

Figure 1-4 | Simplified equivalent circuits for DC and AC measurements of the tightness of a cell barrier cultured on a porous membrane.

at 12.5 Hz. This square current at low frequency is chosen to prevent polarization at the electrode surface and cell membrane as well as electrochemical reactions, which would occur in pure DC measurements and are harmful for the cell culture [43, 44].

Due to concerns about the positioning stability and the membrane area that is actually sampled by chopstick-type electrodes due to their orientation in the insert and well [45], the Endohm chamber with plate electrodes has been introduced [46]. This chamber contains two round Ag/AgCl readout electrodes surrounded by two annular silver excitation electrodes positioned parallel to the membrane. This parallel and concentric orientation ensures a fixed electrode position with respect to the cellular barrier as well as a more uniform current distribution through the insert, resulting in more accurate TEER measurements and less variation.

Impedance spectroscopy

The DC methods rely on the assumption that the change in measured resistance can be exclusively contributed to biological changes in the barrier, i.e. tight junction formation, rather than changes of non-biological origin, such as differences in medium resistance. In order to eliminate the need for this assumption, impedance spectroscopy can be performed by using an alternating current (AC) signal at various frequencies. The equivalent circuit corresponding to AC measurements is displayed in **Figure 1-4**, next to the equivalent DC circuit. The difference is here that the capacitive behavior of the cellular membrane is included. The barrier function is now characterized by the impedance, which is a complex quantity comprising both resistive and capacitive contributions. Capacitors act as frequency-dependent resistors, with a decreasing impedance at increasing frequency, and generally comprise two conducting parts separated by an electrically insulating material. In these measurements, the capacitive

behavior of the cellular membrane arises from the lipid bilayer, acting as an insulator between the electrically conducting culture medium and cytoplasm [42]. At suitable frequencies, the paracellular resistance is dominant over the cell membrane capacitance and the previously introduced R_{meas} results. At higher frequencies, the impedance of the cell membrane capacitance decreases so that only R_{blank} is probed. In this way, with a single impedance spectroscopy measurement and suitable parameter fitting the TEER can be calculated. Additionally, the measured capacitance provides information about the total surface area and the composition of the cellular membranes [47, 48].

Two commercial systems have been introduced that enable AC TEER measurements in Transwell: the cellZscope (nanoAnalytics GmbH, Münster, Germany) [49, 50] and the ECIS TransFilter system (Applied BioPhysics, Troy, New York, USA) [51]. In these systems, gold or stainless steel electrodes are inserted on both sides of the membrane. An AC signal at a range of frequencies roughly between 1 Hz to 1 MHz is applied between the electrodes and the resulting impedance is measured. From the thus obtained impedance versus frequency data, the resistance of the barrier is derived.

TEER in organs-on-chips

Just as in Transwell systems, in organs-on-chips two electrodes can be introduced on either side of the cellular barrier. This is done either by inserting electrodes in the chip inlets [27-29, 52-55] or by integrating electrodes into the chip during the fabrication process [32-36, 56, 57]. The measurements are conducted analogously to the previously described DC or AC methods for Transwell.

1.5. Framework of the thesis

The research presented in this thesis focuses on the development and characterization of a BBB-on-chip as well as the techniques and theory of TEER measurements in both BBB-on-chip and gut-on-a-chip systems. This work has been carried out at the BIOS Lab on a Chip group, which is part of the MIRA Institute for Biomedical Technology and Technical Medicine and the MESA+ Institute for Nanotechnology, at the University of Twente (UT). Funding was primarily provided by the Strategic Research Orientation Biomedical Microdevices, awarded to L.I. Segerink by the MIRA Institute. The gut-on-a-chip work was carried out in close collaboration with the Wyss Institute for Biologically Inspired Engineering at Harvard University in Boston (USA).

Research into the BBB-on-chip and barrier function in organs-on-chips is broadly embedded in the UT as well as in other institutes in the Netherlands and abroad. Within the BIOS Lab on a Chip group, there are multiple PhD students working on the development and characterization of blood vessels on chips, supported by the VESCEL ERC Advanced Grant to A. van den Berg (grant no. 669768). A recent NWO Building Blocks

of Life grant to Wageningen University & Research in collaboration with the BIOS group will enable additional research into TEER measurements in a gut-on-a-chip. Furthermore, in the Applied Stem Cell Technologies group (UT), a project aiming at replicating the blood-retina barrier is carried out using the chip developed in this thesis. The ZonMW Memorabel grant to K. Broersen of the Nanobiophysics group (UT) aims at developing novel drugs for Alzheimer's disease and delivering them into the brain using newly developed nanocarriers. It is intended to test the transport of these nanocarriers across the BBB in the BBB-on-chip presented in this thesis prior to moving to animal tests. At the Anatomy and Embryology group at the Leiden University Medical Center, V. Orlova is developing protocols to derive brain-specific endothelium from human induced pluripotent stem cells (hiPSC), which can be applied in BBBs-on-chips for precision medicine applications. The contacts in the field of organs-on-chips within the Netherlands are facilitated by the human organ and disease model technologies (hDMT) foundation. At the BUBBL group at Oxford University (UK), M. Aron uses the BBB-on-chip to study ultrasound-mediated BBB disruption for drug delivery applications.

1.6. Thesis outline

To provide background for the development of a BBB-on-chip, in **Chapter 2** an elaborate introduction to the BBB and organs-on-chips is provided, as well as an overview of the BBBs-on-chips that have been published up to this date and future challenges in BBB-on-chip and organ-on-chip development. **Chapter 3** explains the development and characterization of a BBB-on-chip with human brain-derived endothelial cells, of which the TEER is measured with four integrated electrodes. Then, in **Chapter 4** the technology and theory of DC TEER measurements in organs-on-chips is further explored by electrical simulations using a gut-on-a-chip model, emphasizing the effect of a non-uniform current distribution on the measured TEER. **Chapter 5** continues on this by including impedance spectroscopy and a four-terminal sensing structure in the electrical simulations and investigating the effect of villi differentiation on the measured impedance spectra. **Chapter 6** contains the development towards a multiplexed BBB-on-chip, in which up to eight functional BBBs can be cultured and tested simultaneously in different conditions. Lastly, **Chapter 7** concludes this thesis with a summary of the obtained results as well as a list of recommendations for further research.

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Microfluidic organ-on-chip technology for blood-brain barrier research

Organs-on-chips are a new class of microengineered laboratory models that combine several of the advantages of current *in vivo* and *in vitro* models. In this chapter, we summarize the advances that have been made in the development of organ-on-chip models of the blood-brain barrier (BBBs-on-chips) and the challenges that are still ahead.

This chapter is adapted (to include seven recent publications) from:
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2.1. Modeling the blood-brain barrier

2.1.1. Blood-brain barrier structure and function

The blood-brain barrier (BBB) comprises specialized endothelial cells and separates blood from brain interstitial fluids. Together with the choroid plexus which forms the blood-cerebrospinal fluid barrier, and the arachnoid epithelium, this barrier partitions blood and neural tissues in order to provide vital homeostasis in the brain [1, 2]. The BBB serves as a physical and functional barrier which regulates passive and active transport, as well as a metabolic and immunological barrier [1, 2]. The physical barrier is formed by the endothelial cells that are linked by tight junction proteins such as zonula occludens 1 (ZO-1) and claudin. These proteins form complexes that limit permeation of ions and hydrophilic agents via paracellular pathways [1]. The active transport barrier results from the expression of specific membrane transporters and vesicular mechanisms for exchange of specific essential nutrients and waste, and multidrug resistance transporters such as P-glycoprotein (P-gp) that regulate efflux of potentially harmful agents, including lipophilic agents [1, 2]. The metabolic barrier is formed by enzymes that metabolize toxic compounds both intracellularly and extracellularly [1]. As a result of the physical and metabolic barrier, 98% of small-molecule (<400 Da) and 100% of large-molecule drugs cannot cross the BBB [3]. Finally, the immunological barrier results from specialized regulation of the recruitment and transport of leukocytes and innate immune elements by the endothelium [2].

The BBB is part of a larger structure: the neurovascular unit (NVU), consisting of endothelial cells forming the capillary, pericytes, glial cells and neuronal cells, as well as their associated extracellular matrix (ECM) proteins [1]. The NVU anatomy is shown in **Figure 2-1**. The brain capillaries are comprised of tightly linked endothelial cells surrounded by pericytes and a basement membrane (30 to 40 nm thick lamina of a.o. collagen IV, laminin and fibronectin) [2]. The microvessel is also surrounded by astrocytic end-feet and in close contact with microglia and neurons. All these elements have important roles in the formation, maturation and maintenance of the BBB [2, 4].

2.1.2. Current *in vitro* and *in vivo* models

In vivo techniques have provided the most reliable information in BBB research and are still regarded as the gold standard [5]. In pharmaceutical industry drug candidates are normally tested in animals before they are tested in humans. In these models the effects of drugs or treatments at the cellular, tissue, organ and systemic level can be monitored. Moreover, animal models allow the study of pharmacodynamics and pharmacokinetics, as well as of immunological responses. A general advantage of animal models is that they can represent the complexity of the BBB environment [6] and individual diversity also found in humans. However, *in vivo* animal studies are

Figure 2-1 | Anatomy of the NVU. A brain capillary comprised of specialized brain endothelial cells forms the BBB. This capillary is surrounded by basal lamina (basement membrane), pericytes and astrocytic end-feet. Also microglia and neurons are in close contact with the BBB. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, ref. [4], copyright 2006.

costly, labor-intensive and ethically contentious [7]. In addition, the translation of animal models to the human clinic is difficult, evidenced by the statement that more than 80% of candidate drugs that were successfully tested in animal models failed in clinical trials [8, 9]. This is partly caused by poor methodology and regulation of (some) animal experiments [9-12], but also by inadequate reproduction of human pathophysiology by (genetically modified) animals [10-12] and by species-to-species variations in expression profiles of e.g. transporter proteins [13].

As an alternative to animal testing, *in vitro* cell and tissue models are widely adopted and have been improved over the last few decades [14]. Generally, these models consist of cells grown in a controlled environment, making them relatively robust, reproducible, easy to analyze and more fit for high-throughput screening than animal studies [15]. However, these models are often too simple to answer complex research questions. For example, simple Petri dish cultures of brain endothelial cells may be useful to assess cytotoxicity of a drug candidate, but they are not fit for the study of drug

transport through the BBB. To enable drug transportation studies, advances in the culture setup have been made, for example resulting in cell culture on a filter membrane suspended in a well, the so called Transwell setup [16]. This Transwell culture system is now a widely used *in vitro* platform for compartmentalized culturing. It provides a platform for drug studies and allows co-culture of endothelial cells and other cells that are associated with the NVU [17]. In addition, cells from human sources can be used in these models, which will avoid problems in translation of the results to the clinic that arise with *in vivo* animal models. However, these simple cultures still often fail to replicate key features of the BBB, such as shear stress resulting from blood flow and the BBB microenvironment (the NVU), which makes their predictive value for human responses questionable [15].

In summary, *in vivo* animal models are regarded as the gold standard and allow study of cellular, tissue, organ and systemic level functions as well as pharmacodynamics and pharmacokinetics in a complex organism. However, they are costly, laborious, ethically contentious and often lack predictive value. In contrast, current *in vitro* models are more robust, reproducible, easy to analyze and fit for high-throughput than animal models and allow study of human cells and tissues. However, they are often too simplistic to answer complex research questions.

2.1.3. Organs-on-chips

To combine the advantages of *in vivo* and current *in vitro* models of tissues and organs, a new class of *in vitro* models has recently been introduced: organs-on-chips [18]. These so-called chips are microfluidic devices in which tissues can be cultured in an environment that is engineered in such a way that it better replicates the *in vivo* microenvironment of that tissue [15, 19]. This more physiologically relevant microenvironment can be achieved by engineering geometrical, mechanical and biochemical factors from the *in vivo* environment into a microfluidic device [15]. Another advantage of these organ-on-chip platforms is that imaging systems and sensors with real-time readouts can also be integrated [18]. Like in conventional *in vitro* methods, human cells or tissues can be included in organs-on-chips. Furthermore, these devices can be used for personalized (or precision) medicine when cells from a specific donor or group of donors are used. Both healthy and diseased tissues can be mimicked and tested in the same controlled environment. Moreover, organs-on-chips promise to replicate organ-level functions and allow the study of (patho)physiology on a higher level than could be achieved by conventional *in vitro* models. The comparison of organs-on-chips with current *in vivo* and *in vitro* methods is summarized in **Table 2-1**. The first organ-on-chip papers have provided proof-of-principle that better replication of the microenvironment results in more physiological behavior of the tissues inside the organ-on-chip device and thus in better predictive value. Examples are the breathing

Table 2-1 | Comparison of organs-on-chips to current *in vivo* and *in vitro* methods.

	<i>In vivo</i>	<i>In vitro</i>	Organs-on-chips
Human tissue	No	Yes	Yes
Personalized/precision medicine	No	Yes	Yes
Realistic microenvironment	Yes	No	Yes [20, 21]
Control over microenvironment	No	Yes	Yes [20]
Organ-level function	Yes	Limited	Potentially [20, 21, 23]
Real-time readouts	No	Limited	Yes [24]
High-throughput, parallelized testing	No	Yes	Possibly [25-27]
Pharmacodynamics / -kinetics	Yes	No	Potentially [28]

lung-on-a-chip [20], the bacteria-inhabited gut-on-a-chip [21, 22] and the atherosclerosis-on-a-chip [23], which show replication of organ-level functions and physiological responses to stimuli that could not have been studied before. More examples of such organ-on-chip applications are emerging rapidly.

2.1.4. BBB-on-chip models

As shown in the previous paragraphs, the use of microfluidic *in vitro* BBB models can improve BBB modeling by having more realistic dimensions and geometries, and by exposing the endothelium to physiological fluid flow [16]. In addition, in “BBBs-on-chips” not only the expression of specific markers can be tested (e.g. adherens and tight junction proteins), which can provide information on an organ-level function, but one can immediately study functionality: the permeability of the cell barrier. Permeability is now already routinely measured in compartmentalized cultures (e.g. Transwell models), but BBBs-on-chips hold promise to measure more BBB functions directly by incorporating sensors and real-time readouts. An additional example of a BBB function, which cannot be studied in Transwell, is the complex and specialized mechanism of recruitment of leukocytes at the BBB, analogous to the extravasation of leukocytes in the lung-on-a-chip in case of bacterial infection [20]. In addition, the recently discovered glymphatic pathway, which clears solutes from the brain and probably plays a role in neurodegenerative diseases, can be studied for the first time using microfluidic devices in which physiologically relevant blood pressure, intracranial pressure and flows can be applied [29, 30]. When BBBs-on-chips are used to study such a complex biological phenomenon, they will provide deeper understanding of the BBB physiology and answer research questions that could not be answered before [31]. BBBs-on-chips then provide an extra tool for the BBB researchers’ toolbox, next to classic *in vitro* cultures and *in vivo* animal studies. Depending on the research question, the most appropriate model can be chosen.

2.2. Current BBBs-on-chips

To this date, seventeen publications of BBBs or NVUs-on-chips exist, to the best of our knowledge. In this section a summary is provided of all these microfluidic models of the BBB, in order of year of publication. In **Figure 2-2** and **Figure 2-3**, representative images are shown and key features of these models are summarized in **Table 2-2** (2012-2015) and **Table 2-3** (2016-2017). Next to these papers, numerous conference contributions indicate that the field of BBBs-on-chips is quickly moving forward, see for example refs. [32-38].

Booth and Kim

Booth and Kim published about their μ BBB in 2012, which is shown in **Figure 2-2 I** [24], and have also published a follow-up paper in 2014 [28]. Their device consists of poly(dimethylsiloxane) (PDMS) parts with two channels (2 mm (luminal) and 5 mm (basal) wide, 200 μ m deep), that are separated by a porous polycarbonate (PC) membrane (10 μ m thick, 0.4 μ m pores). The PDMS parts are sandwiched between two glass slides with sputtered thin-film silver chloride (AgCl) electrodes in a four-point sensing structure to measure transendothelial electrical resistance (TEER) at near-direct current (DC) conditions. A mouse brain endothelial cell line (bEnd.3) was co-cultured with a murine astrocytic cell line (C8D1A) on the opposite side of the membrane, which was coated with fibronectin. Both channels were perfused at 2.6 μ l/min, which corresponds to a shear on the endothelial cells of approximately 2 mPa (calculated using the method presented in section 2.3.4), which is low compared to the physiologically found shear of 0.3-2 Pa in brain capillaries [39, 40]. The small height-to-width ratio ensured a mostly uniform shear stress across the channel width. TEER measurements yielded values of 180-280 Ω ·cm², indicating the presence of a functional barrier. Apart from measuring the TEER, also permeability measurements of fluorescein isothiocyanate (FITC)-dextrans (4, 20, 70 kDa) and propidium iodide were used to confirm barrier function. Immunofluorescence showed the presence of tight junction protein ZO-1. In addition, the physiological effects of exposure to histamine and high pH were recorded. The TEER was higher and permeability lower inside the μ BBB compared to conventional Transwell models, and co-culture with astrocytes resulted in even more improved barrier functionality. The transient barrier disruption caused by histamine was monitored continuously by measuring TEER [24].

In the second publication the authors further tested this model [28]. For these tests, they co-cultured bEnd.3 cells with the glial cell line C6 (from rat glial tumor) in the two channels coated with collagen IV/fibronectin and polylysine, respectively. The luminal channel width was increased to 4 mm to achieve an even more uniform shear stress across the channel width under dynamic conditions, which was 1.5 Pa at 2 ml/min.

Figure 2-2 | Examples of microfluidic BBB models from literature (2012-2015). Reprinted and adapted with permission from: I Booth [24]; II Yeon [41]; III Griep [42]; IV Achyuta [43]; V Prabhakarpandian [44]; VI Kim [26]; VII Cho [45]; VIII Brown [46]; IX Sellgren [47].

Again, presence of tight junction protein ZO-1 was confirmed with immunofluorescence. Toxicity of seven brain-targeting drugs was assessed by measuring lactate dehydrogenase (LDH) levels and after that the permeability coefficients of subtoxic levels of these drugs were measured in devices with a functional barrier, indicated by a sufficiently high TEER of over $150 \Omega \cdot \text{cm}^2$. The authors showed that the measured permeability coefficients in their model under dynamic conditions correlated well with *in vivo* brain/plasma ratios, demonstrating the potential of this model for the prediction of clearance of drugs by the BBB [28].

Yeon

Also in 2012, Yeon et al. published about their permeability assay system for cerebral microvasculature, shown in Figure 2-2 II [41]. This device, made of PDMS on glass, comprises two channels (25 μm high) connected by microholes (30 μm long, 5 μm high and 3 μm wide). By applying different flow rates in the two channels and thereby generating a pressure difference across the microholes, human umbilical vein endothelial cells (HUVECs) were trapped hydrodynamically in the microholes in close contact to each other. After 23 hours of incubation a barrier was formed. With immunofluorescence the presence of ZO-1 was shown. FITC-labeled dextrans (4, 40, 70 kDa) and various drugs were introduced at the other side of the microholes and the permeability of these agents through the HUVEC layer was assessed with fluorescence microscopy (real-time) and high-performance liquid chromatography (HPLC), respectively. The presence of astrocyte-conditioned medium (ACM) was found to decrease the permeability of the trapped HUVEC layer [41].

Griep

Griep et al. published about their BBB-on-chip in 2013, which is shown in Figure 2-2 III [42]. Their device consists of two PDMS parts with channel imprints (500 μm wide, 100 μm high), glued together with a PC membrane in between (10 μm thick, 0.4 μm pores). Cells from a human cerebral microvascular endothelial cell line (hCMEC/D3) were cultured in the top channel on top of the membrane, which was coated with collagen I. Barrier formation was monitored by determining TEER from impedance spectroscopy measurements with integrated platinum wire electrodes, positioned on either side of the membrane. After two days a steady barrier was achieved and maintained up to seven days with an average TEER ($\pm$ standard error of the mean; SEM) of $37 \Omega \cdot \text{cm}^2 \pm 0.9 \Omega \cdot \text{cm}^2$, which is comparable to the value obtained in the conventional Transwell model ($28 \Omega \cdot \text{cm}^2 \pm 1.3 \Omega \cdot \text{cm}^2$). The expression of tight junction protein ZO-1 was verified with immunofluorescence. In addition, the TEER of this BBB-on-chip increased up to $120 \Omega \cdot \text{cm}^2$ when shear stress was applied (0.58 Pa at 2.5 ml/h flow) using a syringe pump. Upon addition of the inflammatory protein tumor necrosis factor α (TNF- α) the TEER decreased to $12 \Omega \cdot \text{cm}^2$ [42].

Achyuta

Achyuta et al. used a modular approach to create a NVU-on-a-chip, which was also published in 2013 [43]. Their device, shown in **Figure 2-2 IV**, consists of two parts that can be fabricated and used for cell culture separately and are assembled at a later stage. The neural part consists of a 8 mm diameter hole in a 100 μm thick PDMS layer on a cover slip. Freshly isolated E-18 rat cortical cells were cultured in this hole, which was coated with poly-D-lysine, for 10 days. The vascular part is a 10 mm wide, 100 μm high channel in PDMS with posts for support, glued on a PC membrane (7 μm thick, 8 μm pores). After coating with fibronectin, rat brain endothelial cells (RBE₄ cell line) were cultured in this channel for two days under static conditions. After the specified culture periods, the device was assembled and functional tests were conducted. Live/dead staining showed good cell viability for both RBE₄ and E-18 cells. Immunofluorescence showed proper differentiation of the neural culture and good endothelial function, evidenced by excretion of von Willebrand factor (vWF). The presence of tight junction protein ZO-1 was shown with Western blots. Barrier tightness was checked with A₄₈₈-dextran (3 kDa) leakage, perfused at 1 ml/h through the vascular channel and collected in the neuronal part. Less dextran was found in the neural reservoir in devices with a RBE₄ cell layer compared to devices without cells, but more dextran leaked into the reservoir after the cells were exposed to TNF- α . In addition, TNF- α increased the expression of intercellular adhesion molecule 1 (ICAM-1), as expected [43].

Prabhakarpanthian / Deosarkar

Also in 2013, Prabhakarpanthian et al. published on their SyM-BBB which is shown in **Figure 2-2 V** [44]. In a follow-up paper from 2015 Deosarkar and Prabhakarpanthian et al. presented an adapted version of their model for neonatal BBB research, termed B³C [48]. The SyM-BBB device, consisting of a PDMS part with channel structures on a glass slide, is designed to enable simultaneous imaging of the blood compartment (outer ring, 200 μm wide, 100 μm high) and the brain compartment (inner ring). The compartments are connected by micro-gaps (50 μm long, 3 μm wide, 3 μm deep) in the PDMS wall. RBE₄ rat endothelial cells were cultured in the blood compartment, coated with fibronectin, to form a cell layer perpendicular to the gaps. During cell culture, fluidic shear was applied at 0.1 ml/min, corresponding to shear stress of approximately 3 mPa (calculated using the method presented in section 2.3.4), which is also low compared to the physiologically found shear of 0.3-2 Pa in capillaries [39, 40]. ACM could be added to the brain compartment, which promoted tight junction formation. Barrier permeability was measured with FITC-labeled dextran (3-5 kDa) and the activity of the P-gp efflux transporter was assessed using rhodamine 123 with and without the transport inhibitor verapamil. In addition, the expression of P-gp and the tight junction proteins ZO-1 and claudin-1 was checked using Western blots. In the presence of ACM the barrier permeability was decreased, the efflux activity was increased and the

expression of tight junction proteins and P-gp was increased in these devices compared to devices without ACM and to conventional Transwells [44].

In the B³C chip adapted for neonatal BBB research presented in the follow-up paper from 2015 by Deosarkar et al. [48], the shape of the channels was changed to circular, but the side-by-side orientation of the vascular channel and the brain compartment was retained. Primary neonatal rat brain capillary endothelial cells were cultured in the fibronectin-coated vascular channel under 0.01 $\mu\text{l}/\text{min}$ flow using a syringe pump, resulting in a shear stress of 0.38 mPa. For co-culture conditions, primary rat astrocytes were seeded in the brain compartment, which was also coated with fibronectin. When astrocytes were present, the ZO-1 expression, as shown by immunofluorescence, was increased as well as the electrical resistance (which was not normalized to area to obtain the TEER), and the permeability for 40 kDa dextran-Texas red was decreased. Also astrocytic protrusions into the microgaps were seen, leading to cell-cell contact between endothelial cells and astrocytes. The permeability coefficient of 40 kDa dextran of the BBB inside the B³C device was more comparable to the *in vivo* BBB permeability, measured through a cranial window in 2-weeks old anesthetized rats, than the BBB in Transwells. Furthermore, neonatal endothelial cells showed weaker ZO-1 expression than adult cells, but in the presence of ACM they showed a bigger decrease in permeability and increase in electrical resistance than adult endothelial cells [48].

Kim

In 2015, Kim et al. reported a collagen-based three-dimensional (3D) model of brain vasculature, shown in **Figure 2-2 VI** [26]. Their device is comprised of tubes in a collagen I gel (235–360 μm diameter), resulting from pouring collagen I around microneedles in a 3D-printed frame to which fluidic connectors can be coupled. After the microneedles were removed, the resulting tubes were coated with fibronectin. Endothelial cells from the bEnd.3 mouse cell line were cultured in these tubes to replicate the BBB. Immunofluorescence showed intact vessels after 14 days with ZO-1 expression. FITC-labeled dextran (40 kDa) was introduced to the tubes and under static conditions the transendothelial permeability was monitored with fluorescence images taken at certain time intervals, showing an intact cell layer after seven days. Using a mathematical model they were able to derive permeability coefficients from these images. Upon exposure to mannitol, barrier disruption was seen in the permeability measurements as expected. Long-term recovery of the barrier function was also shown after mannitol was removed [26].

Cho

Also in 2015, Cho et al. published on their 3D BBB model, which is shown in **Figure 2-2 VII** [45]. Their device consists of a PDMS part with channel imprints on a glass-bottomed well plate. An acrylic well plate with reservoirs for culture medium was glued

to the top of the device. In the PDMS there are an endothelial channel and a brain channel (both 50 μm high) and an array of small perpendicular side channels (5 μm high) connecting the two main channels. After coating the channels with poly-D-lysine, the channels were filled with a collagen I gel which was replaced again by cell culturing medium in the endothelial chambers, resulting in a thin collagen gel on the walls. RBE₄ rat endothelial cells were seeded in the device and allowed to attach to both the top and bottom surface. Medium was refreshed every day by adding 100 μl fresh medium to one reservoir and removing the same amount of old medium from the other reservoir. After obtaining a monolayer (2-3 days) and showing expression of ZO-1 and VE-cadherin by immunofluorescence, the barrier function was tested by adding 40 kDa dextran-FITC to the endothelial chamber and following the increase in fluorescence in the side channels in time. It took significantly longer for the gradient to reach saturation in a device with RBE₄ cells (seven minutes) than in a device without cells (four minutes). The transmigration of neutrophils across the endothelial barrier and through the side channels was recorded. Upon addition of a chemoattractant (interleukin 8) to the brain channel more neutrophils transmigrated than when no chemoattractant was added. Next, neuroinflammation was mimicked by exposing the BBB to TNF- α . From the cytokine release profile it was concluded that the treatment had elicited an inflammatory effect on the BBB model. In addition, the ZO-1 expression was also shown to decrease. Lastly, they used their platform to study ischemia by exposing the endothelium to low oxygen and low glucose (anaerobic gas and Dulbecco's Modified Eagle Medium (DMEM) without glucose) and subsequently allowing reoxygenation under normal conditions. Formation of reactive oxygen species and activation of Rho kinase as a result of oxidative stress was confirmed, as well as a decrease in ZO-1 expression. Upon addition of antioxidants to counter the reactive oxygen species, the ZO-1 expression level slightly increased after three hours but decreased again after six hours [45].

Brown

Brown et al. published on the NVU chip in 2015, which is shown in **Figure 2-2 VIII** [46]. Their chip consists of three PDMS layers: a vascular chamber with inlet channels (100 μm high and 6.2 mm wide), a brain chamber (4.75 mm wide and 6.2 mm long, 500 μm deep) and a layer with brain perfusion channels (several parallel channels, 100 μm high). The vascular and brain chambers are separated by a PC membrane (0.2 μm pores). Prior to cell seeding the NVU devices were coated with laminin. Primary human brain-derived microvascular endothelial cells (hBMVEC) were cultured on the membrane in the vascular chamber, which was held upside down and under a constant flow of 2 $\mu\text{l}/\text{min}$. This corresponds to a shear stress of 2 mPa (calculated using the method presented in section 2.3.4), which is also low compared to the physiologically found shear of 0.3-2 Pa in capillaries [39, 40]. After 12 days the device was flipped right-

side up and pericytes and astrocytes were loaded in the brain chamber. After two days of culture under flow, the brain chamber was filled with a collagen I matrix with suspended human induced pluripotent stem cell (hiPSC)-derived neurons. The gel was allowed to set for two hours and subsequently the device was perfused for three days before testing the BBB. The cells remained viable up to 21 days, as was shown with live/dead staining (>80% cell viability). Using immunofluorescence staining the presence of tight junctions (ZO-1) was shown and also the percentage of actin filaments that were aligned with the flow direction was quantified. The cells significantly blocked diffusion of FITC-dextran (10 and 70 kDa) from the vascular chamber to the brain perfusion channels, but the permeability was shown to increase in the presence of glutamate, which is known to disrupt tight junctions. In addition, the active transport of ascorbate and its function of reducing permeability was demonstrated. Using a four-point impedance sensing method the TEER was measured. Measurements showed an increase in TEER during the 12-day culture period of the endothelial cells. The authors reported resistance values of 30000-33000 Ω/cm^2 for devices with endothelial cells and 7500 Ω/cm^2 for empty devices, which corresponds to a TEER of 1950-2210 $\Omega\cdot\text{cm}^2$ for a membrane area of 4.75-6.2 mm^2 when the resistance of the empty device is subtracted. Exposing the devices to 33°C ("cold shock") resulted in a significant decrease in TEER [46].

Sellgren

Sellgren et al. published in 2015 on the microfluidic NVU model of which an image is shown in Figure 2-2 IX [47]. Their chip comprises two PDMS parts with channel imprints with a polytetrafluoroethylene (PTFE) or polyester (PET) membrane in between (0.4 μm pores and 40 or 10 μm thick, respectively). The vascular channel was 10 mm long, 1 mm wide and 150 μm high, while the basolateral compartment was 150-300 μm high. Astrocytes from the murine C8D1A cell line were suspended in a collagen hydrogel and loaded in the basolateral compartment. After coating with collagen IV-fibronectin (PET) or collagen I (PTFE), cells from the bEnd.3 cell line were cultured on the membrane in the vascular channel under a fluid flow of 120 $\mu\text{l}/\text{min}$, resulting in a physiologically relevant shear stress of 0.5 Pa. Both membranes were transparent and allowed monitoring of monolayer formation with phase contrast microscopy. After a monolayer was obtained, the collagen gel containing the astrocytes was flushed out and the endothelial barrier function was tested by adding 70 kDa FITC-dextran to the vascular channel and collecting medium from the basolateral channel every 30 minutes. The apparent permeability coefficient of a device with bEnd.3 cells was significantly lower than for a device without cells. The PTFE membrane was able to support monolayer survival under physiological shear stress and immunofluorescence showed claudin-5 expression, while the cells did not show claudin-5 on the PET membrane and were peeled off at the same flow rate. Therefore, it was concluded that PTFE

membranes are more suitable to support cell attachment and relevant shear stress than PET [47].

Walter

In 2016, Walter et al. published about their barrier-on-a-chip device, which is shown in **Figure 2-3 I** [49]. Their device was used to recreate the BBB, of which the results are summarized here, as well as intestinal and lung epithelial barriers. Their device consists of two PDMS parts with channels (both 200 μm wide and 200 μm high), that are separated by a porous PET membrane (23 μm thick, 0.45 μm pores), fixated with a silicone sealant. The PDMS parts are sandwiched between two glass slides with sputter-coated 25 nm thick transparent gold electrodes and fixated with a silicon sealant. The electrodes are positioned in a four-point sensing structure to measure TEER at near-DC conditions. Two PDMS blocks with reservoirs are plasma-bonded to the top glass slide. Prior to use the blood channel was coated with collagen I and the brain compartment with collagen IV. Two different cell models were used: hCMEC/D3 cells and primary rat endothelial cells co-cultured with primary astrocytes and pericytes. The endothelial cells were cultured on top of the membrane in the top channel. If present, the pericytes were cultured on the bottom of the membrane and the astrocytes on the bottom of the bottom channel. The cells were maintained under static conditions for three days, after which a peristaltic pump provided dynamic culture conditions at low shear stress (reported to be 0.15 dyn, but 0.15 $\text{dyn}/\text{cm}^2 = 15 \text{ mPa}$ was meant). Barrier properties were induced with lithium chloride and tightened with a cyclic adenosine monophosphate (cAMP) derivate and a phosphodiesterase inhibitor (RO). The TEER of hCMEC/D3 barriers was (mean $\pm$ standard deviation) $19 \pm 2.8 \Omega \cdot \text{cm}^2$ under static conditions and increased to $29 \pm 7.2 \Omega \cdot \text{cm}^2$ under dynamic conditions. The latter value is higher than the TEER measured in Transwell system ($28 \pm 3.5 \Omega \cdot \text{cm}^2$). The apparent permeability was measured statically by determining the concentration in the brain compartment at different time intervals. The apparent permeability coefficient for FITC-dextran (4.4 kDa) and Evans blue-albumin (67 kDa) was lower for dynamic conditions than for static conditions, but the permeability for sodium fluorescein (376 Da) did not change significantly. Confocal microscopy confirmed the presence of tight junction protein ZO-1 and adherens junction protein β -catenin. The TEER of the primary rat BBB in the device was $114 \pm 38 \Omega \cdot \text{cm}^2$ for both static and dynamic culture conditions, which was lower than the TEER on culture inserts ($173 \pm 22 \Omega \cdot \text{cm}^2$). The apparent permeability coefficient for fluorescein was lower under static conditions than under dynamic conditions. The permeability for the other two tracers did not differ significantly between these conditions. These cells expressed ZO-1 and β -catenin more strongly than hCMEC/D3 cells [49].

Figure 2-3 | Examples of microfluidic BBB models from literature (2016-2017). Reprinted and adapted with permission from: I Walter [49]; II Herland [50]; III Xu [27]; IV J.D. Wang [51]; V Kilic [52]; VI Y.I. Wang [53]; VII Partyka [54]; VIII Adriani [55].

Herland

The 3D BBB-on-chip of Herland et al., published in 2016, is shown in **Figure 2-3 II** [50]. Their device consists of a single PDMS layer with a channel of 1 mm width and 1 mm height, bonded onto PDMS-coated glass. The channel was filled with 5 mg/ml collagen I and subsequently medium was flown through the viscous collagen solution prior to gelation to form a hollow lumen of 600-800 μm diameter ("viscous fingering"). Primary human brain endothelial cells were introduced and lined the lumen. They were allowed to form a monolayer and cAMP and RO were added to the culture medium to tighten the resulting barrier. For co-cultures, primary human astrocytes were mixed in the gel and pericytes were seeded in the lumen prior to endothelial cell introduction. As evidenced by immunofluorescence, the endothelial cells showed ZO-1 (tight junctions) and VE-cadherin (adherens junctions) expression and also secreted collagen IV along the cell-gel interface. Using Alexa 488-labeled dextran (3 kDa), time-lapse microscopy and image analysis the apparent permeability coefficients of BBBs with and without co-cultured cells could be quantified. The presence of astrocytes or pericytes resulted in lower permeability coefficients, indicating improvement of barrier function. Furthermore, the same cultures in Transwell plates gave significantly higher permeability coefficients, indicating that the microenvironment in the 3D BBB chip positively influenced the barrier function. Lastly, they measured the cytokine release profiles upon exposure to TNF- α to mimic neuroinflammation. The chip cultures showed more physiologically relevant changes in cytokine release than Transwell cultures (normalized to culture area) [50].

Xu

Also in 2016 Xu et al. published on their BBB model, shown in **Figure 2-3 III** [27]. Their device consists of 16 functional units each containing one fluidic vascular compartment (400 μm wide and 200 μm high in PDMS) with four gel compartments (70 μm high) representing brain ECM. The 16 functional units are selectively addressable by a PDMS control layer with glass-filled channels and valves. The gel compartments were filled with 6 mg/ml collagen I gel. Primary rat astrocytes were cultured on top of this gel and allowed to attach. After that, primary rat brain microvascular endothelial cells were introduced and cultured on top of the astrocytes. Cells were cultured under 0.01 Pa shear stress. Using immunofluorescence, the endothelial cells were found to express tight junction proteins ZO-1 and claudin-5, adherens junction protein VE-cadherin and transporters P-gp and glucose transporter 1 (Glut-1). Flow and co-cultured astrocytes increased the expression of these proteins. Furthermore, the intensity of sodium fluorescein (376 Da), introduced in the vascular compartment, was measured in the gel to assess the effects of flow and astrocytes on the BBB permeability. Both again had a positive influence on the barrier tightness. In addition, simple TEER measurements with electrodes inserted in the vascular and gel inlets, showed the same trend and resulted

in TEER values of up to $1298 \Omega \cdot \text{cm}^2$ for co-culture under flow. To test the physiology of the BBB, different cancer cell lines were introduced to the vascular compartment to test if they crossed the BBB, corresponding to the formation of brain metastases. Also, a brain cancer cell line was added to the gel compartment to see if it metastasized to the blood. The authors argued that physiologically relevant results were seen. Lastly, they demonstrated the applicability of their device for drug screening by testing whether chemotherapeutic agents were able to cross the BBB and induce cell death in glioblastoma cells. A lipophilic agent clinically used for treatment of glioblastoma induced most cell death [27].

J.D. Wang

In 2016, J.D. Wang et al. published on their microfluidic model of the BBB **Figure 2-3 IV** [51]. Their device comprises two PDMS parts with channel imprints (2 mm wide and 200 μm high), glued onto each other with a PDMS/toluene mixture with a porous PET membrane (0.4 μm pores, 10 μm thickness) in between. Ag/AgCl electrodes for impedance spectroscopy-based TEER measurements are embedded in 500 μm x 500 μm side channels during fabrication. Prior to cell seeding, the channels were coated with fibronectin. bEnd.3 mouse brain endothelial cells were cultured on top of the membrane in the top channel. For a biculture model, immortalized mouse pericytes were cultured on the bottom side of the membrane by flipping the device upside-down. For a triculture model, astrocytes were added to the bottom of the bottom channel after 24 hours of culture. Using a live/dead assay it was shown that all three cell types stayed viable for up to 21 days. The average TEER after three days of bEnd.3 barriers in this device was $140 \Omega \cdot \text{cm}^2$, increasing to $257 \Omega \cdot \text{cm}^2$ in biculture and $283 \Omega \cdot \text{cm}^2$ in triculture. In addition, the permeability for [^{14}C]-mannitol and [^{14}C]-urea was seen to decrease in time and was lower for co-culture than monoculture, while consistently lower for triculture than for biculture. Based on these permeability coefficients also the average radius of tight junctions was computed for all three types of cultures. It is proposed that close contact to pericytes and soluble factors excreted by astrocytes mediate this increase in TEER and decrease in permeability. P-gp function was assessed by measuring the basal-to-luminal and luminal-to-basal permeability of the P-gp substrate [^3H]-dexamethasone and determining the associated efflux ratio. The efflux ratio increased in time and was consistently larger for triculture than for biculture, indicating increasing P-gp function [51].

Kilic

Kilic's publication from 2016 describes a brain-on-a-chip model with a neuronal-glial cell population and brain endothelium, shown in **Figure 2-3 V** [52]. The device comprises a top vascular channel and a bottom brain channel in PDMS (both 5 mm wide and 300 μm high) separated by a porous PDMS membrane (10 μm thick, 5 μm diameter

pores) bonded to glass. To prevent membrane collapse, the top channel had support pillars. While their publication focuses mainly on the differentiation of pluripotent human cells towards neuronal and glial cells, here only the results related to the BBB in their chip are summarized. After differentiation and maturation of a neuronal-glial cell population in the bottom channel, immortalized human brain microvascular endothelial cells were introduced in the top channel. On coverslips these endothelial cells expressed ZO-1 and platelet endothelial cell adhesion molecule (PECAM). In devices without brain cells these endothelial cells formed a barrier with a TEER of 20-90 $\Omega \cdot \text{cm}^2$, as measured with a multimeter and inserted Ag/AgCl wire electrodes, indicating formation of a relatively tight barrier. A physiological response (nitric oxide production) was obtained upon exposure to vascular endothelial growth factor (VEGF) [52].

Y.I. Wang

In 2017, Y.I. Wang et al. published on their BBB-on-chip, shown in **Figure 2-3 VI** [53]. Their 3D-printed modular device comprises (from bottom to top) a perfusion layer with medium perfusion channels and bottom electrodes, a porous PC membrane (0.4 μm pores) as cell support held in place by silicone gaskets, a chamber layer with medium reservoirs and a lid layer with top electrodes. Before assembly, rat astrocytes and hiPSC-derived brain microvascular endothelial cells were cultured on the membrane (after coating with collagen IV and fibronectin for endothelial cells). Then the membrane was sandwiched between the chamber and perfusion layers with the endothelial cells facing the perfusion layer (0.66 mm high). Using a rocking platform the endothelial cells were exposed to gravity-driven flow at rates based on *in vivo* residence times and low wall shear stress. After assembly and some days to allow stabilization, the TEER of co-cultures plateaued around 3000 $\Omega \cdot \text{cm}^2$, in contrast to 370 $\Omega \cdot \text{cm}^2$ for monocultures of endothelial cells, as measured with four integrated Ag/AgCl or silver electrodes in a four-point format at near-DC conditions. Immunofluorescence confirmed the expression of tight junction proteins claudin-5 and ZO-1. Furthermore, the permeability coefficients for FITC-dextran (4, 20 and 70 kDa) and small molecule drugs (caffeine, cimetidine and doxorubicin) were determined and were mostly consistent with *in vivo* findings. Doxorubicin additionally compromised BBB integrity as measured by a decrease in TEER upon exposure [53].

Partyka

Partyka et al. reported their BBB-on-chip, shown in **Figure 2-3 VII**, in 2017 [54]. Their device consists of a single PDMS part on glass. The PDMS part contains two parallel channels and a hydrogel reservoir connecting them. The hydrogel reservoir, filled with a mixture of collagen I, hyaluronic acid and Matrigel with embedded normal human astrocytes, contains tubes of 180-220 μm diameter, fabricated using microneedles. One of these tubes is lined with hCMEC/D3 endothelial cells to form a BBB.

Table 2-2 | Summary of key features of the current BBBs-on-chips (2012-2015). “N.A.” indicates that the specified feature has not been measured or reported. EC = endothelial cell.

	Device materials (bottom to top)	Size of blood compartment (width · height)	Membrane, thickness	Biological coating agents	Endothelial cells, co-cultured cells
Booth [24, 28]	Glass with electrodes – PDMS – PC – PDMS – glass with electrodes	2 mm · 200 µm	PC, 10 µm	Fibronectin	bEnd.3 (mouse EC), C8D1A (mouse astrocytes)
				Collagen IV + fibronectin (blood), polylysine (brain)	bEnd.3 (mouse EC), C6 (rat glial cells)
Yeon [41]	Glass – PDMS	25 µm high	Holes in PDMS wall, 30 µm long	N.A.	HUVEC (human EC), ACM
Griep [42]	PDMS – PC – PDMS	500 µm · 100 µm	PC, 10 µm	Collagen I	hCMEC/D3 (human EC)
Achyuta [43]	Glass – PDMS – PC – PDMS	10 mm · 100 µm	PC, 7 µm	Fibronectin (blood), poly-D-lysine (brain)	RBE4 (rat EC), E18 (rat neural cells)
Prabhakar-pandian [44]	Glass – PDMS	200 µm · 100 µm	3 µm holes in PDMS wall, 50 µm long	Fibronectin	RBE4 (rat EC), ACM
Deosarkar [48]					Primary rat EC, ACM, primary rat astrocytes
Kim [26]	Collagen in 3D-printed frame	235-360 µm diameter	No membrane	Collagen I gel + fibronectin	bEnd.3 (mouse EC)
Cho [45]	Glass – PDMS – acryl reservoirs	50 µm high	Side channels, 5 µm high	Poly-D-lysine + collagen I gel	RBE4 (rat EC)
Brown [46]	PDMS – PC – PDMS – PDMS	6.2 mm · 100 µm	PC, thickness not specified	Laminin	hBMVEC (human EC), primary astrocytes, primary pericytes; hiPSC-derived neurons
Sellgren [47]	PDMS – PTFE or PET – PDMS	1 mm · 150 µm	PTFE: 40 µm, PET: 10 µm	Collagen I (PTFE), collagen IV + fibronectin (PET)	bEnd.3 (mouse EC), C8D1A (mouse astrocytes)

^a Physiological shear stress lies between 0.3 and 2 Pa (3-20 dyn/cm²) [39, 40].

Immunofluorescence and transmission electron microscopy (TEM) showed continuous monolayers of ZO-1 expressing endothelium in the tubes as well as well-spread astrocytes in close contact to the endothelium. The cells had excreted basal lamina, as evidenced by immunofluorescence of laminin. Vessel permeability was assessed using 4 kDa FITC-dextran and the permeability coefficient was derived from

Table 2-2 | Continued.

	BBB-relevant protein expression	Barrier permeability tracers	TEER	Physiological test	Shear stress (during culture) ^a
Booth [24, 28]	ZO-1	FITC-dextran (4,20,70 kDa), propidium iodide	180-280 $\Omega \cdot \text{cm}^2$	Exposure to histamine, pH > 10	2 mPa
	ZO-1	Various brain-targeting drugs	150 $\Omega \cdot \text{cm}^2$	N.A.	1.5 Pa
Yeon [41]	ZO-1	FITC-dextran (4,40,70 kDa) and various drugs	N.A.	N.A.	N.A.
Griep [42]	ZO-1	N.A.	37 $\Omega \cdot \text{cm}^2$ (static), 120 $\Omega \cdot \text{cm}^2$ (flow)	Exposure to TNF- α	0.58 Pa
Achyuta [43]	ZO-1, vWF, ICAM-1	A488-dextran (3 kDa)	N.A.	Exposure to TNF- α	N.A.
Prabhakar-pandian [44]	ZO-1, claudin-1, P-gp	FITC-dextran (3-5 kDa)	N.A.	P-gp function with rhodamine 123 / verapamil	3 mPa
Deosarkar [48]	ZO-1	Texas red-dextran (40 kDa)	Only electrical resistance reported	N.A.	0.38 mPa
Kim [26]	ZO-1	FITC-dextran (40 kDa)	N.A.	Exposure to mannitol	N.A.
Cho [45]	ZO-1, VE-cadherin	FITC-dextran (40 kDa)	N.A.	Transmigration of neutrophils, TNF- α , ischemia	N.A.
Brown [46]	ZO-1	FITC-dextran (10, 70 kDa)	1950-2210 $\Omega \cdot \text{cm}^2$ ^b	Glutamate exposure, active transport & barrier tightening with ascorbate, cold shock	2 mPa
Sellgren [47]	Claudin-5	FITC-dextran (70 kDa)	N.A.	N.A.	0.5 Pa

^b These values were derived from the resistance values in Ω/cm^2 reported in the publication, corrected by the resistance of an empty device and the square of the measured area.

time-lapse fluorescence images. They showed that exposure to a shear stress of 0.05 Pa increased ZO-1 expression and decreased permeability, while addition of TNF- α increased the permeability. Also TEER values, that were measured using alternating current (AC) with electrodes inserted into the outlets, were higher for endothelium exposed to flow (up to 1000 $\Omega \cdot \text{cm}^2$) than for static conditions (up to 300 $\Omega \cdot \text{cm}^2$). These

Table 2-3 | Summary of key features of the current BBBs-on-chips (2016-2017). "N.A." indicates that the specified feature has not been measured or reported. EC = endothelial cell.

	Device materials (bottom to top)	Size of blood compartment (width · height)	Membrane, thickness	Biological coating agents	Endothelial cells, co-cultured cells
Walter [49]	Glass/electrodes – PDMS – PET – PDMS – glass/electrodes – PDMS reservoirs	200 μm · 200 μm	PET, 23 μm	Collagen I (blood), collagen IV (brain)	hCMEC/D3 (human EC) or primary rat EC, primary rat astrocytes and primary rat pericytes
Herland [50]	PDMS-coated glass – PDMS	600-800 μm diameter	No membrane	Collagen I	Primary human brain EC, primary human pericytes, primary human astrocytes
Xu [27]	PDMS – PDMS	400 μm · 200 μm	No membrane	Collagen I	Primary rat brain EC, primary rat astrocytes
J.D. Wang [51]	PDMS – PET – PDMS	2 mm · 200 μm	PET, 10 μm	<i>Fibronectin</i>	bEnd.3 (mouse EC), mouse pericytes, C8D1A (mouse astrocytes)
Kilic [52]	Glass – PDMS – PDMS membrane – PDMS	5 mm · 300 μm	PDMS, 10 μm	N.A.	Immortalized human brain endothelium, human teratocarcinoma-derived neuronal-glial cells
Y.I. Wang [53]	3D-print – silicone gasket – PC – silicone gasket – 3D-print	0.66 mm high, 6.5 mm diameter	PC, not specified	Collagen IV + fibronectin	hiPSC-derived brain endothelium, rat astrocytes
Partyka [54]	Gel in PDMS on glass	180-220 μm diameter	No membrane	Collagen I + Matrigel + hyaluronic acid gel	hCMEC/D3 (human EC), primary human astrocytes
Adriani [55]	Glass – PDMS	920 μm · 190 μm	No membrane	Collagen I	hCMEC/D3 (human EC), HUVEC (human EC), primary rat astrocytes, primary rat neurons

^a Physiological shear stress lies between 0.3 and 2 Pa (3-20 dyn/cm²) [39, 40].

TEER values were nearly one order of magnitude higher than previously reported values, which the authors contributed to the presence of the hydrogel. In addition, they subjected their BBB-on-chip to pulsatile flow, mediated by pressure-driven pumps, and showed that this may play a role in transport of molecules in the brain ECM [54].

Adriani

In 2017, Adriani et al. published on their neurovascular chip shown in **Figure 2-3 VIII** [55]. Their device comprises a single layer of PDMS on glass with four parallel channels (190 μm high), separated by pillar structures. The inner two channels (both 580 μm wide) are filled with primary rat astrocytes in a collagen I gel (7 mg/ml) or primary rat neurons (2.5 mg/ml gel). The outer two channels (920 μm wide) are fluidic

Table 2-3 | Continued.

	BBB-relevant protein expression	Barrier permeability tracers	TEER	Physiological test	Shear stress (during culture) ^a
Walter [49]	ZO-1, β -catenin	Fluorescein (376 Da), FITC-dextran (4.4 kDa), Evans blue-albumin (67 kDa)	19-29 $\Omega \cdot \text{cm}^2$ (hCMEC/D3) 114 $\Omega \cdot \text{cm}^2$ (rat EC)	N.A.	15 mPa ^b
Herland [50]	ZO-1, VE-cadherin	A488-dextran (3 kDa)	N.A.	Exposure to TNF- α	N.A.
Xu [27]	ZO-1, claudin-5, VE-cadherin, Glut-1	Sodium fluorescein (376 Da)	200-1298 $\Omega \cdot \text{cm}^2$	Brain metastases extravasation, brain chemotherapy	0.01 Pa
J.D. Wang [51]	N.A.	[¹⁴ C]-mannitol, [¹⁴ C]-urea	140-283 $\Omega \cdot \text{cm}^2$	P-gp function with [³ H]-dexamethasone	N.A.
Kilic [52]	ZO-1, PECAM	N.A.	20-90 $\Omega \cdot \text{cm}^2$	VEGF exposure	N.A.
Y.I. Wang [53]	ZO-1, claudin-5	FITC-dextran (4, 20, 70 kDa), small drug molecules	Around 3000 $\Omega \cdot \text{cm}^2$	Doxorubicin exposure	Intentionally low
Partyka [54]	ZO-1, laminin	FITC-dextran (4 kDa)	300 $\Omega \cdot \text{cm}^2$ (static), 1000 $\Omega \cdot \text{cm}^2$ (flow)	Exposure to TNF- α , pulsatile flow	0.05 Pa
Adriani [55]	ZO-1, VE-cadherin	Oregon green-dextran (10 kDa), Texas red-dextran (70 kDa)	N.A.	Glutamate exposure	N.A.

^b Shear stress was reported in dyn, but dyn/cm² was meant.

channels used to provide neurobasal culture media to the cells. After seven days of culture, the fluidic channel next to the astrocyte channel is coated with collagen and subsequently lined with human endothelial cells (HUVECs or hCMEC/D3 cells) that were maintained in endothelial growth medium 2 (EGM-2). Immunofluorescence confirmed the formation of a monolayer of endothelial cells expressing VE-cadherin and tight junction protein ZO-1. The barrier permeability coefficient of fluorescently labeled dextrans (10 and 70 kDa), as measured with fluorescence microscopy, was lower for hCMEC/D3 than for HUVEC. Co-culture with astrocytes and neurons decreased the permeability coefficient of HUVEC barriers, but slightly increased the permeability of hCMEC/D3 barriers. Neuronal development was confirmed by examining morphology (neurite outgrowth) and functionality (calcium imaging) of the neurons in the 3D

matrix. Furthermore, the availability of the neurotransmitter glutamate at the neurons was decreased in the presence of an endothelial barrier, indicating both functional neurons and endothelial cells in their device [55].

2.3. Standardization challenges

As evidenced by the body of literature summarized in the previous section, significant steps have been taken toward developing physiological BBBs-on-chips. These recently reported chips show promising improvements when compared to conventional Transwell models: the exposure to fluid flow resulted in better barrier function [24, 27, 28, 42, 44, 47, 49, 54] and dynamic drug permeability studies in chips were found to be more predictive than in conventional static models [28, 48].

However, there are still challenges ahead for developing BBB-on-chip models that will become widely available for BBB-related research applications. One of them is to arrive at commonly accepted standards for quantitative evaluation of the functionality of a BBB-on-chip model [6]. In **Figure 2-2** and **Figure 2-3** as well as **Table 2-2** and **Table 2-3** one can clearly see that both the device designs and the readout protocols vary greatly, as well as the used cell types. This shows the versatility of BBBs-on-chips and more generally of organ-on-chip technology, but this also complicates comparison between models. Therefore, in the following sections an overview is provided of aspects that need to be taken into consideration when designing and testing BBBs-on-chips and aspects that require consensus among researchers.

2.3.1. Permeability

As was mentioned in the introduction, the key function of the BBB is to provide homeostasis in the brain, and more specifically to protect the brain from harmful substances in the blood [1]. The performance of BBBs-on-chips should therefore be evaluated by measuring the permeability of the cell barrier. If this permeability is in agreement with physiological levels, then a valuable BBB-on-chip has been obtained which can be used for testing drug candidates. In general, large and hydrophilic molecules, for example dextrans and ions, are physically blocked by the tight junctions between endothelial cells. Ions and essential nutrients such as glucose, amino acids, peptides and hormones are transported actively into the brain by carriers and receptor mediated transport [5]. Small lipophilic molecules (<400-500 Da) can cross the BBB without significant obstruction [3]. However, multidrug resistance transporters regulate efflux of potentially harmful agents, including lipophilic agents [1, 2]. To assess the full barrier function, analytes from all these classes have to be tested in the device: both hydrophilic and lipophilic molecules that are both passively and actively transported or excreted. The determination of barrier permeability is demonstrated in almost all of the currently existing BBB devices (see **Table 2-2** and **Table 2-3**) [24, 26-

28, 41, 43-51, 53-55]. However, it is important to quantify this permeability in such a way that it can be compared to *in vivo* and other *in vitro* data. For passive transport, this can be done by calculating the permeability coefficient of an analyte (cm/s), which is independent of the used analyte concentration, flow rate and device size, and can also be determined *in vivo* [48, 56]. When an analyte is added to the luminal channel under constant flow and transport takes place toward the basal channel through the cell barrier on a membrane, the permeability coefficient can be determined with the following formulas:

$$P_{meas} = \frac{\dot{m}_a}{A \cdot (C_l - C_b)} = \frac{C_b \cdot Q}{A \cdot (C_l - C_b)} \quad [cm/s] \quad \text{Eq. 2-1}$$

In these formulas P_{meas} is the measured (or apparent) permeability coefficient (cm/s), $\dot{m}_a$ is the mass transport rate (mol/s) across the membrane which – when analyzing the sample flowing out of the basal compartment – can be quantified by multiplying the basal concentration C_b (mol/ml) with the applied flow rate in the basal channel Q (ml/s), A is the membrane area through which the transport takes place (cm²) and C_l is the luminal concentration (mol/ml) [48, 57]. The permeability coefficient of the endothelial barrier, P_{endo} , can be calculated from this measured permeability coefficient P_{meas} and the permeability coefficient measured in a device without endothelium P_0 (blank) as follows [24, 28, 48, 57]:

$$\frac{1}{P_{endo}} = \frac{1}{P_{meas}} - \frac{1}{P_0} \quad [s/cm] \quad \text{Eq. 2-2}$$

This endothelial permeability coefficient can then be compared to permeability coefficients found with the same analyte in other platforms. If there are more complex channel geometries the transport of analytes can also be modeled mathematically to arrive at a permeability coefficient [26, 50, 54, 55]. An advantage of measuring the permeability in microfluidic devices over Transwell systems is that the analyte can be supplied to the luminal channel at a constant flow rate and the transported analyte can also be collected from the basal channel with a constant flow rate. In this way, the assumption that the concentration difference across the membrane stays constant throughout the measurement is met, while in static Transwell systems this difference decreases over time. In addition, a promising feature of organs-on-chips over static Transwell systems is the possibility for real-time monitoring of the permeability when on-line detection systems or fluorescence microscopy are used [44, 48].

A problem that can arise when performing permeability measurements in organs-on-chips is the contribution of other modes of transport than only diffusion (or active transport). For example, if there is a pressure difference between the two compartments, convective flow will result through gaps in the cell barrier. In addition, osmotically driven flow can result if there is a difference in solute concentration between the channels. One has to be aware of these phenomena and limit their

contribution in permeability measurements as much as possible, for example by having identical compartments with the same applied pressure and by using the same fluid (culture medium) on both sides of the barrier. Another factor that can influence permeability measurement is the presence of e.g. astrocytes at the basal side of the membrane. These are reported to have a tightening effect on the BBB, but they will also form a physical barrier against diffusion by themselves. So one should always check for the contribution of the presence of extra cells to the total barrier function.

Summarizing, the permeability of the cell layer is a very important readout of BBBs-on-chips. To validate physiological relevance, different analytes that are either passively or actively transported, excreted or metabolized should be tested with a suitable protocol. To enable comparison between platforms, a universal measure that is independent of the microdevice design, such as permeability coefficient, should be reported.

2.3.2. Transendothelial electrical resistance

Next to permeability, TEER is a widely used quantity to assess barrier tightness [1, 58-61]. TEER mostly represents the electrical resistance against paracellular transport: the tighter the cell layer is packed, the less gaps there will be in the cell barrier through which ions and other charged species can move, resulting in a higher resistance [60]. Only when the cell barrier is tight enough and the contribution of paracellular ion transport pathways is low, the ion transport through paracellular channels formed by tight junction proteins and the transcellular transport of ions (via transporters) is measured [61, 62]. Measuring the TEER has the great advantage over the permeability measurements described before that it is a quick, non-invasive and label-free way to assess barrier tightness. In addition, if a suitable electrode material and measurement method are chosen and the measurement electrodes are integrated into a microfluidic BBB-on-chip device, the measurements can be performed in real-time [24, 61].

To be able to compare barrier resistances between different devices and Transwell platforms, the measured resistance of the endothelial barrier (R_{endo} in Ω , if needed corrected for the resistance of e.g. channels and membranes) is normalized by multiplying it with the area through which the resistance has been measured (A in cm^2), resulting in the TEER:

$$TEER = R_{endo} \cdot A \quad [\Omega \cdot \text{cm}^2] \quad \text{Eq. 2-3}$$

Electrically, the inverse of TEER corresponds to the conductance per unit area. Sometimes the resistance is erroneously normalized by dividing it by area, resulting in an error of factor A^2 [16, 46]. In a device with two perpendicular channels separated by a membrane, the area relevant for the TEER is represented by the membrane surface at the channel junction [24, 42]. In such configurations, one has to take into account that microfluidic channels can have a high electrical resistance when they have small

dimensions: the resistance scales inversely to the cross-sectional area of the channel. Therefore, the electrodes need to be positioned smartly and preferably be fixed in place to prevent measurement errors by variations in electrode placement [59]. Another possible issue that was pointed out by Odijk et al. is that the distribution of the electrical current may not be uniform across the membrane interface in microfluidic devices, resulting in an overestimation of the TEER. This can be corrected with the mathematical model presented in this publication [59] and in **Chapter 4** and **5** of this thesis.

Measuring TEER with impedance spectroscopy (using AC) is preferred over measuring the ohmic resistance with DC. Using AC at the proper frequencies prevents electrode and concentration polarization, and other DC-related effects on the cells [60, 61]. Furthermore, measuring the impedance at different AC frequencies gives more information about the cell culture and even enables direct measurement of the TEER without having to correct for the resistance of the device without cells [58].

Next to device characteristics, for which can be compensated mathematically, also other factors will influence electrical resistance measurements. Analogously to the permeability measurements specified above, TEER measurements will be influenced by the presence of co-cultured cells. The presence of extra cells will provide an extra obstacle for ion transport, resulting in a higher resistance than what would result from the tight endothelium alone [50]. In addition, TEER measurements are sensitive to temperature and the ionic composition of the culture medium [39]. These factors have to be kept constant in TEER measurement protocols.

In conclusion, TEER is a very valuable indicator of barrier tightness that can be measured quickly, non-invasively, label-free and real-time. However, how the TEER is measured needs to be well-thought-through to arrive at valid TEER values and to be able to compare between platforms.

2.3.3. Cells

The cells used are important for the physiological relevance of the BBB-on-chip. The more closely the cells mimic the human BBB, the more predictive the model is expected to be. Until now, many of the BBB-on-chip models and other *in vitro* BBB models use cells from animal sources [16, 59]. Using these cells can provide valuable information for validation purposes, because the *in vitro* results can be more easily compared to *in vivo* results from the same species. However, human cells would be the most predictive and would thus be the cells of choice for future drug development applications.

Endothelial cells derived from brain capillaries already have the appropriate expression profile, so these will be the first choice. However, retaining this phenotype *in vitro* after several passages has been challenging [16]. In addition, human brain tissue and therefore primary human brain endothelial cells are scarce [16]. Although challenging

to make, brain-derived endothelial cell lines are more readily available and provide less batch-to-batch difference, but they also lost part of their phenotype (and possibly genotype) during the immortalization process [16]. In contrast, advances have been made in deriving brain-specific endothelial cells from hiPSCs [53, 63, 64]. This development holds great promise for personalized (or precision) medicine, since brain endothelium can be derived from both “healthy” cells and “diseased” cells (e.g. with genetic defects resulting in BBB pathology *in vivo*), as well as from cells originating from different people or populations [8, 65].

As was mentioned in the introduction, next to endothelial cells also other cells from the NVU, such as astrocytes and pericytes, are important for the formation and maintenance of the barrier [2, 4, 66]. The model will be more physiologically relevant if these cell types are included as well and some of the current BBBs-on-chips have already shown an increase in barrier tightness when these cells are included [24, 27, 43, 46, 50, 51, 53]. However, in most of these devices the two cell types are cultured in different channels or chambers, separated by a membrane with a thickness of several micrometers. A thinner membrane allowing cell-cell contact or having no membrane would be more physiological, but also more challenging to fabricate and test reproducibly. For this purpose hydrogel-based devices, such as the ones in refs. [26, 27, 45, 50, 54, 55], are expected to be beneficial, because they allow direct contact between the endothelial cells lining the lumen and the other cells that are cultured in 3D in the surrounding gel. On the other hand, these devices are more difficult to fabricate and it is more difficult to image the cells inside the device and test the permeability and TEER.

To conclude this section, animal cells are widely used and enable easy comparison of *in vitro* results to *in vivo* tests within the same species. However, the use of human cells in BBBs-on-chips would be most informative for drug development studies or studies of human BBB physiology and pathology, although the tissue source has limited availability. Recent advances show that deriving brain endothelium from hiPSCs potentially provides a more accessible source of relevant cells.

2.3.4. Shear stress

Exposing the endothelium to fluid flow and the associated wall shear stress is reported to have positive effects on endothelial differentiation and cell function, which is expected as such shear flow occurs in the natural environment [25, 67-69]. Microfluidic devices are especially suited for incorporation of fluid flow and shear stress, which is difficult in conventional *in vitro* (Transwell) models, thus presenting a real operational advantage of microfluidic models. The positive effect of shear stress on BBB tightness has already been demonstrated in several BBBs-on-chips [24, 27, 28, 42, 54], but shear stress is not yet standardly applied. Furthermore, as was already mentioned before, the wall shear stress is not always of physiological level, which is $0.3\text{--}2\text{ Pa}$ for brain

capillaries [39, 40]. For a channel with a rectangular cross-section and with a steady laminar flow of a Newtonian fluid, the shear stress is calculated as:

$$\tau = \frac{6 \cdot \mu \cdot Q}{w \cdot h^2} \cdot \left(1 + \frac{h}{w}\right) \cdot f^*\left(\frac{h}{w}\right) \quad [Pa] \quad \text{Eq. 2-4}$$

in which τ is the shear stress (Pa), μ is the viscosity of the fluid used in the microfluidic channel (Pa·s), Q is the volumetric flow rate (ml/s), and w and h are the channel width (at the surface of endothelial culture; cm) and height (cm), respectively [70]. The function $f^*(x)$ is an infinite summation series of which the output values for most common input values are listed in ref. [70]. If the channel width is much larger than the channel height ($w \gg h$) and the aspect ratio h/w approaches zero, this equation reduces to:

$$\tau = \frac{6 \cdot \mu \cdot Q}{w \cdot h^2} \quad [Pa] \quad \text{Eq. 2-5}$$

To approximate the viscosity of culture medium the viscosity of water at 37°C can be used, which is 0.7 mPa·s [39].

In a tube with a circular cross-section the wall shear stress will be equal along the entire inner wall because of the cylindrical symmetry. However, inside a rectangular channel the shear stress will not be uniform across the channel width because of the presence of the side walls. Therefore, to achieve a mostly uniform shear stress on all cells across the channel width, the width should be much larger than the height ($w \gg h$), resulting in a flat flow profile. This situation is illustrated in **Figure 2-4** for different aspect ratios (channel height over channel width). The flow profile in a channel with a rectangular cross-section can be approximated with the following equations:

$$\frac{u_{x,y}}{u_{max}} = \left[1 - \left(\frac{2x}{h}\right)^2\right] \left[1 - \left|\frac{2y}{w}\right|^m\right] \quad \text{Eq. 2-6}$$

$$m = \frac{w}{h} \sqrt{2} + 0.89 \frac{h}{w} \quad \text{Eq. 2-7}$$

In these equations $u_{x,y}$ is the fluid velocity at a given position (x, y) inside the channel cross-section, which is scaled to the maximum velocity, u_{max} ; h is the channel height and w is the channel width (with $w > h$) [71]. The resulting flow profile for Prabhakarparandian's chip [44] with $h/w = 100 \mu\text{m} / 200 \mu\text{m} = 0.5$ is shown in **Figure 2-4A**, while the flow profile in Booth's chip [24] with $h/w = 200 \mu\text{m} / 2 \text{ mm} = 0.1$ is shown in **Figure 2-4B**. The lower aspect ratio of the Booth chip results in a much more uniform flow profile across the channel width. In **Figure 2-4C** the flow profiles for more aspect ratios are shown as two-dimensional (2D) projections, clearly demonstrating that a smaller aspect ratio h/w results in a more uniform flow profile. All flow profiles were modeled and displayed using MATLAB R2013a (The MathWorks, Inc.). The result of a non-uniform flow profile is that the cells near the side walls will always experience a lower shear than the cells in the middle of the chip. In addition, this lower flow rate at

A. Prabhakarparandian ($h/w = 0.5$) **B. Booth ($h/w = 0.1$)** **C. Flow profiles at different aspect ratios**

Figure 2-4 | Flow profiles at different channel aspect ratios of rectangular channels. **A.** 3D flow profile inside the BBB chip of Prabhakarparandian [44]. **B.** 3D flow profile inside the BBB chip of Booth [24, 28]. The endothelial cells are cultured on the bottom surface of the depicted channels. **C.** 2D projections of flow profiles at different aspect ratios. All flow profiles were modeled with MATLAB R2013a.

the edges results in longer retention times of paracrine signaling agents and analytes for permeability measurements at the edges. The mechanisms mentioned above can result in differences in cell behavior or measured permeability across the channel width. Moreover, the growth of cells in a channel can influence the flow profile and the associated shear stress. If the cells form a thick layer compared to the channel height, the average shear stress on the cells will be higher compared to the shear stress on an empty surface at the same volumetric flow rate. Epithelia are more likely to pose such problems than endothelia because of their relative thickness.

In conclusion, physiologically relevant shear stress is an important stimulus for endothelial cells. This can be applied easily on cells in a microfluidic device. However, the channel geometry influences the shear stress distribution on the cells. In a rectangular channel the most uniform shear stress is achieved if the channel height is much smaller than the channel width.

2.4. Conclusion

The use of BBBs-on-chips has great potential to further the field of BBB research. In microfluidic platforms the advantages of *in vivo* and *in vitro* models are combined: organ-on-chip technologies enable the study of organ-level function like *in vivo* models, while still being robust, reproducible and easy to analyze like *in vitro* models. There are already a number of reports of BBBs-on-chips in literature that show novel approaches and promising results. These examples already show some benefits of the use of microfluidics for BBB research applications. In addition, organ-on-chip technologies provide flexibility in the design of and control over microenvironments, as well as readout protocols. This enables the development of a wide range of BBB-on-chip models that can each answer specific research questions.

However, to accelerate the development and enable comparison and validation of the BBB-on-chip models, it is beneficial to have some standardization and consensus among researchers. In this chapter four aspects are highlighted. Determining the BBB permeability is an important readout of any BBB model. To enable comparison between platforms, the barrier permeability should be reported as universal values, such as permeability coefficients. Furthermore, TEER is a quick and non-invasive measure of barrier tightness. If correctly measured and calculated, TEER values can also be compared between devices. Brain microvascular endothelial cells from animal sources are more widely available, but human cells are more informative for human BBB research. hiPS cells hold promise as a more accessible source of relevant cells, also suitable for personalized medicine. Lastly, the endothelial cells inside BBBs-on-chips can be exposed to physiologically relevant shear stress. Suitable channel geometries are required to achieve mostly uniform shear stress across the cell barrier.

Next to these considerations for optimal designs and protocols for BBBs-on-chips that were highlighted in this review, there are more microenvironment parameters that will benefit from a more standardized approach. Among these are the choice of chip materials and geometries, and incorporation of biological agents in the microenvironment. To this end, it is beneficial to have multidisciplinary teams developing BBBs-on-chips in order to have both biology and engineering aspects covered [6]. In addition, widespread application of microfluidic BBBs-on-chips also requires cheap fabrication, easy operation and possibility for high-throughput and parallelized models [72]. We are confident that the rapidly emerging field of BBB-on-chip models will have a real impact on biomedical science and drug development in the near future.

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A blood-brain barrier on chip with direct TEER measurements

In organs-on-chips, fluid-filled microchannels pose a high electrical resistance compared to the transendothelial electrical resistance (TEER) to be measured. As a result, small changes in temperature or medium composition that affect the channel resistance strongly affect the apparent TEER. To solve this, we propose a simple and universally applicable method to directly determine the TEER in microfluidic organs-on-chips. Using four electrodes and six different measurement configurations, the TEER can be directly derived independent of channel properties. This method removes large variation of non-biological origin. While its applicability is shown in a blood-brain barrier on chip, this simple and robust method is applicable to any organ-on-chip device with two channels separated by a porous membrane.

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and:

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3.1. Challenges in TEER measurements

Transendothelial or transepithelial electrical resistance (TEER) is a powerful, non-invasive measure of the tightness of a cellular barrier *in vitro*. It is widely used in both conventional Transwell culture systems and in recently developed organs-on-chips to monitor barrier formation and function [1-5]. An introduction to TEER measurements is provided in **Section 1.4 of Chapter 1**. Organs-on-chips are microfluidic devices in which human tissues can be cultured in a controlled environment that is engineered to better mimic the physiologically relevant *in vivo* microenvironment of that specific tissue than standard *in vitro* culture systems [6-8]. Generally, these devices comprise two microfluidic channels that are separated by a porous membrane on which the barrier-forming tissue is cultured. To measure the TEER of a cellular barrier inside such an organ-on-chip device, there have been reports of devices with integrated electrodes on either side of the porous membrane close to the cellular barrier [9-12]. These devices are shown in **Chapter 2** in more detail. However, these electrodes often hamper visual inspection of the cells and need to be fabricated and carefully aligned in a cleanroom environment [9-11]. To solve this, optically transparent electrodes have been reported [12], but these require precise sputtering of a gold layer with only 25 nm thickness, for which specialized cleanroom equipment is needed.

Alternatively, there are reports of electrodes being inserted in the outlets of organs-on-chips, analogous to the common method for the conventional *in vitro* Transwell system. This method relies on standard manufacturing techniques, is easy to perform experimentally and still allows optical access to the cells inside the chip [13-17]. However, the reliability and stability of TEER measurements that are performed with this method are lower, due to the small channel geometries of organ-on-chip systems. To illustrate this, the electrical resistance of an electrolyte, such as cell culture medium, in a microfluidic channel can be approximated with **Eq. 3-1** (assuming uniform current density):

$$R_{ch} = \rho \frac{l_{ch}}{A_{ch}} \quad [\Omega] \quad \text{Eq. 3-1}$$

In this formula, R_{ch} is the electrical resistance of the electrolyte inside a microfluidic channel (Ω), ρ is the specific electrical resistance of the electrolyte ($\Omega \cdot \text{m}$), l_{ch} is the length of the channel (m) and A_{ch} is the cross-sectional area of the channel (m^2). Using this formula it can be easily seen that the small cross-sectional area A_{ch} and the relatively long channel length l_{ch} result in a high resistance R_{ch} for microfluidic channels. Variations in the measured resistance can easily arise from differences in electrode position when the electrodes are reinserted, resulting in a different channel length l_{ch} . In addition, area A_{ch} can change in a part of the channel when air bubbles or other non-conducting inhomogeneities are present. Furthermore, the specific electrical resistance

ρ is a material property of the electrolyte, depending on electrolyte composition and temperature. Therefore, measuring at e.g. different temperatures or at a higher ion concentration as a result of evaporation results in variations in ρ and thus in the measured resistance. In conclusion, all these variations add up to the measured TEER and can therefore wrongly suggest that the tightness of the cellular barrier changes, while changes of non-biological origin are the actual cause. Therefore, l_{ch} , A_{ch} and ρ need to be tightly controlled to achieve reliable resistance measurements using the conventional methods. Lack of control and standardization can be a cause of the large variation in TEER reported for the same cell type in the standard Transwell system [3]. In addition, TEER measurements were conducted in only ten out of the seventeen organs-on-chips summarized in **Chapter 2**, illustrating the challenge of incorporating TEER measurements into organs-on-chips.

To overcome this, we present a simple and universally applicable method to directly determine the TEER in microfluidic organs-on-chips without the need for integrated electrodes close to the cellular barrier. Using four electrodes inserted into the two channels – two on each side of the membrane – and six measurements we can directly determine the TEER. To illustrate the applicability of this method, we mimic the blood-brain barrier (BBB) inside our microfluidic organ-on-chip device using a human microvascular cerebral endothelial cell line (hCMEC/D3). The BBB is primarily formed by a tight monolayer of endothelial cells, which are linked to each other by tight junction proteins (e.g. zonula occludens 1 proteins; ZO-1). The BBB regulates transport of molecules and cells between blood and brain, providing an optimal environment for neuronal function [18], and BBB dysfunction is involved in various neurological disorders [19]. Our presented method is applicable to any organ-on-chip device with two channels that are separated by a porous membrane, making standardized TEER measurements more easily available for organ-on-chip applications.

3.2. Experimental

To complement the descriptions of the materials and methods described in this section, the protocols used for the experiments are provided in **Appendix A.1**. A technical discussion of these protocols is included in **Appendix A.2**.

3.2.1. Chip fabrication

Polydimethyl(siloxane) (PDMS) base agent and curing agent were mixed in a 10:1 weight ratio (Sylgard 184 Silicone elastomer kit, Dow Corning, Midland, MI, USA). After degassing, this mixture was poured onto a silicon wafer patterned with the negative photoresist SU-8 and cured for four hours at 60°C. The PDMS with channel imprints was cut into top and bottom parts, and four inlets (1-1.2 mm diameter) were punched into the top parts. Dust was removed using Scotch tape (3M). Leakage-free bonding of

the two PDMS parts with a membrane in between was achieved by using a PDMS/toluene mortar at 5:3 weight ratio (toluene from Merck, Germany) [11, 20]. This mixture was spin-coated onto a glass coverslip (1500 rpm for 60 s, ramped at 1000 rpm/s; Spin 150, Polos, the Netherlands) and a thin layer was transferred to both PDMS halves using an ink roller. A porous membrane of $\sim 9 \text{ mm}^2$, cut from polycarbonate (PC) membranes with $0.4 \text{ }\mu\text{m}$ pores (Transwell culture inserts, Corning Incorporated) was placed in the center of the bottom halve. The top part was aligned and gently placed on the bottom part without applying pressure, after which the chips were baked for three hours at 60°C . Lastly, four platinum wires (200 μm diameter; Alfa Aesar, Karlsruhe, Germany) were inserted into the electrode channels of the chip and fixed using Norland Optical Adhesive 81 (NOA81, Cranbury, NJ, USA), by applying a drop of NOA at the channel's entrance and curing it with ultraviolet (UV) light (365 nm for 5 s at 350 mW/cm^2) as soon as the channel was filled by capillary forces. The electrodes were inserted about 1 mm into the microchannel to ensure the exposure of a sufficiently large surface area to culture medium, which resulted in a large enough electrical double layer capacitance to minimally influence the TEER readout (see Figure 3-2E and F). The fully assembled chip was placed on a plastic dish and fixed with a two-component epoxy adhesive (Loctite M-31 CL Hysol, Henkel) to prevent the electrodes from being pulled out of the chip during measurements. To cure the NOA and epoxy adhesive the chips were baked for two hours at 60°C . The chip design and assembled chip are shown in Figure 3-1.

3.2.2. Cell culture and staining

The cells used in the BBB chip are immortalized human cerebral microvascular endothelial cells (hCMEC/D3 cell line, kindly provided by Dr. P.-O. Couraud, INSERM, Paris, France) [21]. hCMEC/D3 cells were cultured with endothelial growth medium 2 (EGM-2: EBM-2 with EGM-2 SingleQuots, Lonza) in T75 culture flasks (Greiner Bio-One), coated with $20 \text{ }\mu\text{g/ml}$ human plasma fibronectin (Gibco) in phosphate-buffered saline (PBS; Sigma) for >30 minutes at 37°C . The cells were incubated at 37°C in humidified air with 5% CO_2 . Medium was refreshed every 2-3 days and confluent flasks were subcultured or used for experiments (passages 28-33).

Prior to cell seeding, the microfluidic chips were rinsed with PBS and coated with fibronectin ($20 \text{ }\mu\text{g/ml}$) for three hours at 37°C . After flushing any air bubbles out, the chips were filled with EGM-2 and incubated for two hours. hCMEC/D3 cells were obtained from a confluent flask using 0.05% trypsin-EDTA (Gibco), suspended in fresh EGM-2 at $5 \cdot 10^6$ cells/ml (corresponding to $2 \cdot 10^5$ cells/ cm^2) and pipetted into the top channel of a microfluidic chip. After one hour of static incubation non-attached cells were washed away with EGM-2 and medium-filled pipette tips were inserted as reservoirs in all inlets. Medium was refreshed twice daily by inserting new medium-filled pipette tips in the inlets, replacing the medium inside the chip by gravity-driven flow.

Figure 3-1 | Design and assembly of the organ-on-chip device with integrated electrodes. **A.** Exploded view of the microfluidic chip, consisting of a top PDMS part with the top channel (TC), a membrane (M) and a bottom PDMS part with the bottom channel (BC). Four platinum wire electrodes (E1-4) are inserted and fixed in the side channels. In the top channel and on top of the membrane, hCMEC/D₃ brain endothelial cells were cultured to replicate the BBB. **B.** Assembled chip, fixed to a plastic dish.

At the end of an experiment the channels were washed with PBS and the cells were fixed with 3.7% paraformaldehyde (Sigma Aldrich) in PBS for 30 minutes at room temperature. The fixative was washed away with PBS and the cells were shortly incubated in permeabilization and blocking buffer (PBB), which consisted of 0.1% (v/v) Triton X-100 (Sigma Aldrich) and 1% (m/m) bovine serum albumin (BSA; Sigma) in PBS. Then the cells were incubated for two hours at room temperature with mouse anti-human ZO-1 immunoglobulin G (IgG; 5 μg/ml in PBB; BD Transduction Laboratories). After washing three times with PBS, the cells were incubated for one hour at room temperature with goat anti-mouse IgG conjugated to Alexa Fluor 488 (5 μg/ml in PBB; Invitrogen). After flushing three times with PBS, the nuclei were stained with NucBlue (1 drop per 500 μl PBB; Ready Probes reagent, Molecular Probes, Life Technologies) for 30 minutes at room temperature, after which the channels were flushed three times with PBS. Chips were opened by removing the top part and electrodes, while keeping the membrane fixed to the bottom part. The bottom part was placed upside-down in PBS on a glass cover slip. The cells were imaged with phase contrast and fluorescence microscopy using the EVOS FL Cell Imaging System (Life Technologies; green filters (ex 470/22, em 510/42) for ZO-1 and blue filters (ex 357/44, em 447/60) for NucBlue).

3.2.3. TEER measurements

The TEER measurement setup consisted of a lock-in amplifier with a probe cable circuit (schematically shown in **Figure A-1** in **Appendix A.3**), operated by a LabVIEW programme (designed, manufactured and programmed in-house; now available from LocSense, the Netherlands). Proper functioning of this lock-in setup was verified with two commercial machines (SP-300 potentiostat, Bio-Logic Science Instruments; and

Impedance/Gain-Phase analyser, type 4194A, Hewlett Packard; data not shown). Impedance spectra were recorded using an alternating current (AC) signal with 0.15 or 0.3 V_{RMS}, scanning from 200 Hz to 1 MHz. Each of the 100 logarithmically spaced measurement points was the average of 50 periods. Measurement errors due to changes in temperature and medium conductivity are minimized by refreshing the medium inside the chip and allowing it to reach room temperature prior to each measurement, according to established TEER protocols commonly used in literature.

Typical impedance spectra

Typical impedance spectra are shown in **Figure 3-2A**, modeled for a chip without cells (solid line) and through a cellular barrier (dashed line) according to the equivalent AC circuit presented in **Figure 1-4** in **Chapter 1**. Four main regions can be identified, each dominated by a specific electrical component. Below approximately 1 kHz, the double layer capacitance at the electrode-culture medium interface dominates, characterized by a negative slope for impedance magnitude and a phase shift approaching -90°. The frequency at which the double layer capacitance dominates, depends on the electrode area exposed to culture medium. The resistive plateau above 1 kHz (without cells) or 100 kHz (with cells) with a phase shift close to 0°, corresponds to the resistance of the culture medium inside the microfluidic channels, depending on channel length and cross-sectional area, and inside the membrane, depending on the porosity and thickness. When measuring through a cellular barrier, an extra resistive plateau between 1 and 10 kHz is seen as well as a local maximum in the phase diagram. This region is of critical importance for the determination of the TEER as a clear increase in impedance results when cells are present in the measured path, and is therefore termed the “region of interest”. The extra slope between 10 and 100 kHz corresponds to the cell barrier capacitance, which arises from the electrically insulating lipid bilayer membranes and is dependent on the total area of the cell layer [22]. The boundaries of these regions as well as the impedance magnitudes depend on the system being studied and change with, among other parameters, channel dimensions, culture medium conductivity, electrode position and cell type. For further reading about the theory and practice of electrical impedance spectroscopy on barrier-forming tissues the review article by Benson et al. is recommended [23].

Measured resistance and TEER derived from spectra

From the measured impedance spectra the resistances between the electrodes were determined in the “region of interest”: from the resistive plateau at 10 kHz. In this way, the resistance between two electrodes was determined for all six combinations, denoted as $R_{i \rightarrow j}$ with i and j referring to the electrodes as numbered in **Figure 3-1** and **Figure 3-2**. Since each measured resistance is the sum of the resistors in its path, as is

shown in the equivalent resistive circuit presented in **Figure 3-2B**, a system of six equations and five unknowns results. From this system of equations the values of all five resistors can be determined using Gaussian elimination, including the resistance of the cellular barrier and membrane (R_m). The two solutions per unknown that resulted from these six equations with five unknowns were summed to obtain one solution that includes as many measurements as possible. The obtained TEER ($\Omega \cdot \text{cm}^2$) is determined by normalizing R_m to the culture area A_{cult} (cm^2). Thus **Eq. 3-2** (**Figure 3-2G**) was used to calculate the TEER:

$$TEER = A_{cult} \cdot R_m = A_{cult} \cdot \frac{1}{4} (R_{1 \rightarrow 2} + R_{1 \rightarrow 4} + R_{2 \rightarrow 3} + R_{3 \rightarrow 4} - 2R_{1 \rightarrow 3} - 2R_{2 \rightarrow 4}) \quad [\Omega \cdot \text{cm}^2] \quad \text{Eq. 3-2}$$

Finally, the TEER of the cellular barrier is determined by subtracting the TEER of the system prior to cell seeding ($t = 0$) from all subsequent measurements.

The typical values for these resistances can be estimated using **Eq. 3-1** presented in the introduction. A channel of cross-sectional area $A_{ch} = 500 \mu\text{m} \cdot 375 \mu\text{m} = 1.9 \cdot 10^{-7} \text{m}^2$ with two electrodes spaced by $l_{ch} = 7 \text{mm}$, filled with culture medium with a specific conductivity of approximately $\sigma = 1.6 \text{S/m}$ and thus a resistivity of $\rho = 1.6^{-1} \Omega \cdot \text{m} = 0.625 \text{m}$, has an electrical resistance of $R_{ch} = 2.3 \cdot 10^{-4} \Omega$. In the same device, the resistance of a cell barrier with a TEER of $30 \Omega \cdot \text{cm}^2$ (a typical value for the hCMC/D3 cell line [24]), on a culture area of $A_{ch} = 500 \mu\text{m} \cdot 500 \mu\text{m} = 2.5 \cdot 10^{-3} \text{cm}^2$ will have a resistance of $R_{barrier} = 12 \text{k}\Omega$. This shows that the expected channel resistance is of the same order of magnitude or even larger than the expected cellular barrier resistance, which corresponds to our experimental results shown in **Figure 3-2E** and **F**. This effect is even more pronounced for longer channels with smaller cross-sectional areas.

Note that the effect of (especially long and shallow) channel geometries on the current distribution along the cellular barrier should be taken into account and, if needed, mathematically corrected when measuring TEER, as is discussed in **Chapter 4** and **5**. In our device the length and height of the culture area are comparatively small ($500 \mu\text{m}$ and $375 \mu\text{m}$, respectively) at the interface of the two channels, resulting in a local channel resistance of $R_{ch} = 1.6 \text{k}\Omega$ and a barrier resistance of $R_{barrier} = 12 \text{k}\Omega$, as was estimated before. Because of the relatively large difference between these resistances, we can assume that the current distribution is uniform across the cellular barrier. Therefore, it was not required to mathematically correct the TEER with respect to the current distribution in our device.

3.3. Results & discussion

Microfluidic chips contain small volumes and, as a result, they have high internal electrical resistances in comparison to the resistance of interest, the TEER. This means that very small variations in e.g. temperature and medium solute concentrations that

Figure 3-2 | Representative impedance data and determination of TEER. **A.** Schematic impedance spectrum showing impedance magnitude (Ω) and phase shift ($^\circ$) versus frequency (Hz), typical for electrical impedance spectroscopy on chips without cells (solid line) and with cells (dashed line). There are four main regions in the impedance spectra measured with cells, dominated by: the double layer capacitance at the electrodes, the culture medium resistance, the cell barrier resistance (if present) and the cell membrane capacitance (if present). The "region of interest" indicates where the contribution of the cell layer can be quantified (red arrow). **B.** Equivalent resistive circuit of the chip, showing the top channel resistors R_1 and R_3 , the bottom channel resistors R_2 and R_4 and the membrane and endothelial cell (EC) barrier resistor R_m . **C.** Schematic cross section showing the endothelial cells cultured in the top channel. **D.** Schematic top view of the BBB chip showing the configuration of the electrodes and the culture area A_{cult} of 0.25 mm^2 through which the impedance is measured. **E.** Representative impedance spectra of a blank chip filled with cell culture medium. **F.** Representative impedance spectra of a chip in which hCMEC/D3 brain endothelial cells were cultured for three days. **G.** Formula to calculate TEER from the measured resistances between all six combinations of four electrodes (Eq. 3-2).

affect the inherent resistance of the system (see **Figure A-2** and **Figure A-3** in **Appendix A.4**) can change the apparent TEER. To explore this effect, we performed an experiment in which we filled devices with EGM-2 cell culture medium and monitored the resistance during 15 days ($n = 3$). We subtracted the first measurement from all subsequent measurements, expecting to find a horizontal line at 0 k Ω for these blank chips. However, in the representative data of **Figure 3-3A** it can be seen that there is a big spread in the measured impedances due to the large variations in resistance inherent to microfluidic systems, even though we used established TEER protocols to minimize the effect of changes in temperature and medium conductivity. When we used these six measurements between the electrode pairs to directly determine the membrane resistance R_m , as is shown in **Figure 3-3B**, the variation is greatly reduced. The same is seen for all three chips, of which all data is shown in **Figure A-4A** and **B** in **Appendix A.5**. Therefore, we concluded that our four-electrode method enables the direct isolation of the membrane resistance, regardless of variations in the system.

Next, we performed an experiment in which we cultured hCMEC/D3 cerebral endothelial cells inside chips and monitored barrier formation with TEER measurements during three days ($n = 4$). We subtracted a blank measurement (before adding the cells) from all subsequent measurements, expecting to find the increase resulting from biological changes in the barrier tightness. However, as can be seen in the representative data of **Figure 3-3C**, different resistances resulted per electrode pair. With our four-electrode method we were able to directly determine the resistance of the membrane plus cell barrier from these six measurements, as is shown in **Figure 3-3D**. The same is seen for all four chips, of which all data is shown in **Figure A-4C** and **D** in **Appendix A.5**. In conclusion, our newly developed method makes sure that the resistance of the cellular barrier and membrane are isolated from the system, diminishing the influence of variance of non-biological origin.

Next, the TEER was monitored of four BBBs-on-chips, as shown in **Figure 3-4A**. The average TEER of 22 $\Omega \cdot \text{cm}^2$ in the plateau from day 1-3 and the biological variation (standard error of the mean (SEM) = 1.3 $\Omega \cdot \text{cm}^2$) was close to other TEER values reported for hCMEC/D3 cells [24]. Microscopic inspection of the cells after terminating the experiment showed that there was a continuous cell layer (representative image shown in **Figure 3-4B**) and the cells expressed tight junction proteins (**Figure 3-4C**), contributing to this increased TEER. For barrier-forming tissues, one may expect higher TEER values and more ZO-1 expression. While the hCMEC/D3 cell line is a comparatively reproducible source of human endothelial cells that is often used in literature, it is known to poorly develop tight junctions [1]. In our study, however, we preferred biological relevance over the barrier formation capabilities and chose to work with this human cell line. As a next step we want to expose the hCMEC/D3 cells to shear stress and co-culture them with BBB-associated cells to increase barrier formation [24].

Figure 3-3 | **A.** Measurements of the membrane resistance in a blank chip (filled with EGM-2 culture medium). Measured impedance at 10 kHz for each electrode pair. Dashed lines correspond to measurements only through the microfluidic channels, solid lines indicate that the membrane was included in the measured path. The first measurement (at 0 days) was subtracted from all subsequent measurements. A horizontal line at 0 k Ω was expected, but large variation is observed. For easy comparison the resistance is also displayed in units of TEER ($\Omega \cdot \text{cm}^2$). **B.** The calculated membrane resistance R_m has much less variation than the measured resistances. The first measurement was subtracted from all subsequent measurements. **C.** TEER measurements in a chip with hCMEC/D3 cells. Measured impedance at 10 kHz for each electrode pair. The blank measurement (at 0 days, before adding cells) was subtracted from all subsequent measurements. There is large variation between the electrode pairs. For easy comparison the resistance is also displayed in units of TEER ($\Omega \cdot \text{cm}^2$). **D.** By calculating R_m the resistance of the membrane and cells is isolated. The first measurement was subtracted from all subsequent measurements.

3.4. Conclusion

TEER is a non-invasive indicator of the tightness of a cellular barrier that is widely used in conventional Transwell systems as well as in organs-on-chips. Electrodes are conveniently inserted in the chip's outlets because integrated electrodes close to the cellular barrier in organs-on-chips require specialized cleanroom processes and, more importantly, often hamper visual inspection of the cells inside the device. Unfortunately,

Figure 3-4 | A. Measured TEER values of four BBBs-on-chips during three days compared to blank chips. SEM = standard error of the mean. **B.** Phase contrast and fluorescence microscopy of NucBlue stained nuclei confirmed the presence of a continuous monolayer of endothelial cells inside the chips. Due to the high contrast of the PC membrane, the cells growing on the membrane cannot be viewed using phase contrast microscopy, but fluorescently stained nuclei confirmed the presence of a continuous monolayer of endothelial cells. The inset shows where the images were taken in the chip. **C.** Fluorescence microscopy confirmed the presence of tight junction protein ZO-1.

conventional TEER measurements are often prone to variation and imprecision due to the high resistance of the fluid-filled microfluidic channels. Therefore, the apparent TEER is greatly influenced by any change in channel resistance due to non-biological factors e.g. changes in temperature, medium conductance, electrode position and the presence of air bubbles. This makes it difficult to draw quantitative conclusions and compare results.

In this chapter we showed that the TEER in organs-on-chips can be directly determined with four electrodes and six measurements – independent of changes in non-biological factors in the system. With less than four electrodes the TEER cannot be determined directly, because there are more unknowns than measurement possibilities. This method is suitable for any chip with two channels in each of which two electrodes

can be inserted, while still being compatible with the simple measurement setup that is used for conventional two-electrode TEER measurements. In conclusion, our newly developed method to determine TEER with four electrodes without the need for separate measurements of empty chips is expected to be easily incorporated in existing organ-on-chip systems and would provide more useful and meaningful TEER measurements in organ-on-chip applications.

3.5. References

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Direct current transepithelial electrical resistance in a gut-on-a-chip

In literature highly conflicting values are reported for transepithelial or transendothelial electrical resistance (TEER) measurements of barrier tissues, even when identical cell lines and experimental setups are used. The differences are even more pronounced when comparing measurements in microfluidic organ-on-chip systems to those obtained in conventional Transwell. The goal of this chapter is therefore to enhance the reliability of TEER measurements in organs-on-chips, focusing on the often-used direct current TEER measurements, by introducing electrical simulations to correct for the effects of device geometry on the measurements. The model is validated by comparing the corrected TEER values obtained in a microfluidic gut-on-a-chip to a conventional Transwell culture. Moreover, we show that small gaps in cell coverage are sufficient to cause a significant drop in TEER.

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4.1. Variations in TEER measurements

Transepithelial or transendothelial electrical resistance (TEER) is a widely used parameter to characterize the quality of the barrier function of epithelial or endothelial cell monolayers. In principle, measuring TEER across cellular barriers is a non-destructive, label-free method, providing real-time information on barrier quality. Therefore, it is an very suitable and relatively low-cost method to monitor cell growth in organ-on-a-chip microfluidic systems [1]. The aim of this chapter is to consider issues that arise when TEER is measured in microfluidic systems, particularly when the results of these measurements are compared to values found in conventional monolayer or bi-layer culture systems, such as Transwell culture devices.

A schematic model of a typical tissue barrier consisting of a culture of epithelial or endothelial cells on a porous support is shown in **Figure 4-1**. In principle, two pathways exist for ion transport across the cell monolayer. The first is the transcellular pathway, which includes lipophilic, receptor-mediated, adsorptive and protein transport. The second is the paracellular route that involves transport through cell junctions and the intercellular space [2]. A simple equivalent circuit model can be made of these pathways across a barrier, as shown in **Figure 4-1**, in which the transported ions and other charged molecules are the charge carriers. Only resistive properties are taken into account in this equivalent circuit, as we only consider non-frequency dependent behavior in this chapter. The transcellular pathway R_{trc} , is the sum of the apical cell membrane resistance (R_a) and the basolateral cell membrane resistance (R_b). R_{pc} depicts the paracellular pathway and is equal to the sum of the tight junction resistance (R_{tj}) and the intercellular resistance (R_{ic}). In this framework, the total TEER is the equivalent resistance of R_{trc} and R_{pc} in parallel:

$$T_{TEER} = R_{trc} // R_{pc} = \frac{(R_a + R_b) \cdot (R_{tj} + R_{ic})}{R_a + R_b + R_{tj} + R_{ic}} \quad [\Omega] \quad \text{Eq. 4-1}$$

Typically, the paracellular pathway is dominant over the transcellular pathway in the overall TEER, especially at the beginning of the barrier culture when adherens junctions or tight junctions between the cells have not yet fully formed ($R_{pc} > R_{trc}$). Additionally, an extra parallel pathway through a gap resistance (R_{gap}) is indicated by the dashed line in the figure, representing partial cell coverage of the cell support. This pathway will be discussed in more detail in the next sections. Finally, the porous membrane on which the cells are cultured can be modeled by a resistor in series with the circuit presented in **Figure 4-1**.

Please note that this model is only valid for the transfer of direct current (DC) signals. DC is here defined as measurements using a constant current or potential, i.e. a signal with a frequency of 0 Hz. However, this simplified model still applies for alternating current (AC) measurements at low frequencies, which are used by commercial

Figure 4-1 | Endothelial cell layer forming a barrier on a porous membrane (image based on Abbott et al. [2]). A simplified circuit representing the transcellular and paracellular pathways is shown. Also, an optional parallel pathway, resulting from gap resistance R_{gap} , is indicated by a dashed line.

systems to measure TEER, such as the EVOM2 (World Precision Instruments, Inc.) or the Millicell ERS-2 (Millipore) instruments. These systems use a near-DC current (12.5 Hz square wave) of 10 μ A and a four-terminal measurement method with either silver/silver chloride (Ag/AgCl) chopstick electrodes or special chambers with patterned Ag/AgCl electrodes, which facilitate measurements of TEER in Transwell culture inserts. A summary of average TEER values measured in Transwell devices for various cell types is shown in **Figure 4-2**. A detailed overview of this literature is included in **Table C-1** in **Appendix C.2**.

There are various factors that influence TEER, including the physical support that is used for cell culture [3] and temperature [4, 5], as well as the material, quality and surface state of the electrodes. It is clear from the data in **Figure 4-2** and **Table C-1** that TEER values fluctuate significantly for various cell types. Moreover, even TEER values for the same cell type vary greatly in different studies. Generally, the values reported for co-cultures are higher than those for monocultures. Judging from these data, it is clear that TEER measurements show large variance, raising the question whether it is a suitable method to quantitatively compare specific barrier tightness in a reproducible and standardized manner.

In literature, values for TEER in microfluidic chips are often different from those measured in Transwell systems using the same cell types [6-10]. In this chapter, we show that these differences can result from specific measurement-related effects in microfluidic systems, rather than having a biological origin. TEER values measured in microfluidic devices can also vary greatly, and we demonstrate that this can be caused by small variations in cell confluency, which has a great impact on the measured TEER value. We focus on determining TEER by DC methods, as it is the main measurement

Figure 4-2 | TEER values and standard deviations for various cell types measured using DC techniques. Light-colored bars indicate monocultures, while the dark-colored bars indicate co-cultures. **Human cells:** BMEC = human brain microvascular endothelial cells [12–21]; hCMEC/D3 = human cerebral microvascular endothelial cells [20, 22, 23]; HUVEC = human umbilical vein endothelial cells [12, 24–26]; ECV304 = endothelial cells differentiated from HUVECs [20, 27, 28]; Caco-2 = human colorectal adenocarcinoma cells [29–37]. **Murine cells:** bEnd.3 = murine brain endothelial cells [20, 26, 38–42]; BMEC = murine brain microvascular endothelial cells [25, 40, 43–52]; RBEC = rat brain endothelial cells [24, 46, 53–56]. **Porcine cells:** BMEC = porcine brain microvascular endothelial cells [57–63]. **Bovine cells:** BMEC = bovine brain microvascular endothelial cells [64–66]; BCEC = bovine brain capillary endothelial cells [67–70]. For the exact cell types used in co-cultures and further details, see **Table C-1** in **Appendix C.2**.

method used in most past reports quantifying TEER values (**Table C-1**). In addition, we use the previously reported gut-on-a-chip [10, 11] as a model organ-on-chip system to show how TEER measurements can differ when carried out in Transwell inserts versus microfluidic chips.

4.2. Theory

4.2.1. TEER in a microfluidic chip

To understand the theory behind TEER measurement in a microfluidic system, consider the simplified geometry of a typical organ-on-a-chip, which consists of two parallel channels separated by a membrane with pores (**Figure 4-3A**). In an equivalent circuit model of this fluidic chip (**Figure 4-3B**), inlet and outlet channels l_a to l_d are depicted by resistors R_a to R_d . The parts of channels $a-d$ and $b-c$ that are connected by the membrane are indicated by l_{mem} (the length of the membrane). The sum of resistors R_{T1} to R_{Tn-1} is equivalent to the resistance (reciprocal of the conductance) of the top

Figure 4-3 | **A.** Schematic chip layout, showing the top channel (blue), the bottom channel (red) and the pores in the membrane between those channels (pink). **B.** Equivalent circuit of the chip, showing a network of resistors representing the top channel (R_{Tx}), bottom channel (R_{Bx}), membrane (R_{Mx}) and inlet ports (R_a and R_d for top channel, R_b and R_c for bottom channel). The subscript x with a value between 1 and n denotes the x^{th} resistor.

channel $a-d$ over the length depicted by l_{mem} . Similarly, the sum of R_{B1} to R_{Bn-1} is equivalent to the resistance of the bottom channel $b-c$ over length l_{mem} . Note that $R_{T1} = R_{T2} = R_{T3} = R_{Tx}$, with x between 1 and $n-1$. This also holds for all resistances R_{Bx} . As the top channel and bottom channel have identical dimensions, the actual value of each resistor R_{Tx} or R_{Bx} , now named R_x , can be calculated using the following equation:

$$R_x = \frac{l_{mem}}{n \cdot w_{ch} \cdot h_{ch} \cdot \kappa} \quad [\Omega] \quad \text{Eq. 4-2}$$

In this equation l_{mem} is the length of the channel above the membrane (m), w_{ch} and h_{ch} are the width and height of the channel (m), n is the number of resistors included to model the channel (typically 1000 or more for accurate results), and κ is the conductivity (S/m) of the cell culture media inside the chip. The inlet and outlet resistors can be calculated using the same equation, by replacing l_{mem} with the length of the specific channel portion (e.g. l_a). For the membrane resistors (including the cellular barrier resistance) R_{M1} to R_{Mn} , the value can be calculated by:

$$R_{Mx} = \frac{h_{mem}}{P \cdot w_{ch} \cdot \frac{l_{mem}}{n} \cdot \kappa} + \frac{R_{TEER}}{w_{ch} \cdot \frac{l_{mem}}{n}} \quad [\Omega] \quad \text{Eq. 4-3}$$

In this equation h_{mem} is equal to the membrane thickness (m), P the porosity of the membrane (%) and R_{TEER} the TEER resistance ($\Omega \cdot \text{cm}^2$). The first term in Eq. 4-3 describes the resistance due to the cell support (e.g. a porous polyester (PET) or polycarbonate (PC) membrane), while the second term describes the resistance of the cell barrier. Please note that R_{TEER} would be the TEER measured in bulk systems, like Transwell inserts, where no significant potential differences can occur in the bulk of the liquid above and below the cell barrier.

The actual potential drop and current distribution over the membrane can be calculated using either a numerical or analytical approach. In the first approach, the equivalent model is approximated by a finite number of n resistors. Using Ohm's law and Kirchhoff's current law, the network can be solved, as is explained in **Appendix B**. This numerical approach is verified using an analytical approach, which shows good agreement for TEER resistances above $50 \Omega \cdot \text{cm}^2$. The analytical model is difficult to solve for lower TEER values and does not allow the study of current distribution over the membrane. Therefore, only data generated with the numerical approach are shown in this chapter, while the analytical approach and the comparison of the two methods is included in **Appendix C.1**.

The numerical approach can be used to show what happens when a DC TEER measurement is performed in the gut-on-a-chip. Experimentally, one would first measure an empty device from a to b , thus determining the value of the equivalent resistor corresponding to R_a , R_b , R_T , R_B and R_M . Then, one would grow a layer of cells on the membrane, effectively increasing R_M . One would then measure the resistance of the device with cells ($R_{(a \rightarrow b), \text{cells}}$) and subtract the measurement from the empty device ($R_{(a \rightarrow b), \text{blank}}$), and multiply the resulting value with the total membrane area for normalization. This yields the apparent TEER ($R_{app, TEER}$) of the cell layer in this device:

$$R_{app, TEER} = (R_{(a \rightarrow b), \text{cells}} - R_{(a \rightarrow b), \text{blank}}) \cdot w_{ch} \cdot l_{mem} \quad [\Omega \cdot \text{cm}^2] \quad \text{Eq. 4-4}$$

Most papers report this apparent TEER as their measured TEER values in organ-on-a-chip systems.

4.2.2. Effect of cell coverage of the supporting substrate

The effect of cell monolayer coverage of the support membrane on the total resistance can be studied using the extended circuit configuration shown in **Figure 4-1**. This extended circuit comprises a resistor describing a possible gap in the cell layer, parallel to the transcellular and paracellular pathways (dashed line and R_{gap} in **Figure 4-1**). The resistance of this gap is equal to the membrane resistance only, as described by the first term of Eq. 4-3, with n being equal to 1:

$$R_{gap} = \frac{h_{mem}}{P \cdot w_{ch} \cdot l_{mem} \cdot \kappa} \quad [\Omega] \quad \text{Eq. 4-5}$$

Figure 4-4 | Gut-on-a-chip. **A.** Schematic cross-section of the microfluidic device showing the top channel lined by gut epithelium, cultured on a porous membrane, the bottom channel and the vacuum chambers used to apply mechanical strain to the cultured cells. **B.** Photograph of the gut-on-a-chip connected to a syringe pump and a vacuum controller for actuation. For visualization, the top and bottom channels were filled with a blue and red dye, respectively. Adapted from [10] with permission of The Royal Society of Chemistry.

Because R_{gap} is placed parallel with respect to the cell layer resistance R_M (with $n = 1$), it is possible to calculate the total resistance R_{tot} for Transwell systems as a function of cell coverage c varying between $c = 0$ (no coverage) and $c = 1$ (total cell confluence):

$$R_{tot} = \left(\frac{c}{R_M} + \frac{1-c}{R_{gap}} \right)^{-1} \quad [\Omega] \quad \text{Eq. 4-6}$$

Using Eq. 4-6 the effect of cell coverage on the total resistance was modeled for the PET Transwell setup used in this chapter. Note that in this equation R_M is simplified to a single resistor ($n = 1$). This is only allowed if the potential is evenly distributed above and below the cell barrier, which is assumed to be the case in the Transwell culture device.

4.3. Experimental

4.3.1. Device geometry

To measure TEER under microfluidic conditions, on-chip measurements were performed using the previously reported gut-on-a-chip model that is shown in Figure 4-4 [10, 11]. Briefly, the microfluidic device was fabricated using two patterned poly(dimethylsiloxane) (PDMS; Sylgard, Dow Corning) parts bonded together with a PDMS membrane in between. The patterned parts contain the geometries of the top and bottom microchannels (150 μm high and 1000 μm wide) as well as the vacuum chambers. The PDMS membrane is 30 μm thick and contains 10 μm diameter pores with 25 μm pitch in a hexagonal pattern. Gut epithelium is cultured in the top channel on top of the porous membrane. Fluid flow is applied through the access ports and mechanical strain

Table 4-1 | Parameters used to model the gut-on-a-chip, obtained from two earlier publications [10, 11].

Parameter	Description	Value
w_{ch}	Channel width	1000 μm
h_{ch}	Channel height (both top and bottom)	150 μm
l_{mem}	Channel length at porous membrane	1 cm
l_a	Channel length at inlet ports	5 mm
$R_a = R_b = R_c = R_d$	Channel resistance at inlet ports	20 k Ω
$R_T = R_B$	Channel resistance at porous membrane	39 k Ω
P	Membrane porosity	13%
h_{mem}	Membrane thickness	30 μm
κ	Conductivity of culture medium	1.67 S/m

is applied by evacuating the vacuum chambers, thereby stretching the membrane. Important geometric and electric parameters that were used for the model described in the previous section, are listed in **Table 4-1**.

4.3.2. Cell culture

Human intestinal epithelial Caco-2 cells (BBE clone) were obtained from the Harvard Digestive Disease Center, and routinely grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) containing 4.5 g/L glucose and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, supplemented with 20% fetal bovine serum (FBS; Gibco), 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco).

Cells were either seeded in Transwell inserts or in the microfluidic gut-on-a-chip devices, and maintained at 37°C in a humidified incubator under 5% CO₂ in air. For Transwell experiments, Caco-2 cells were seeded at a density of $1.5 \cdot 10^5$ cells/cm² on porous PET Transwell membrane inserts (0.33 cm², 0.4 μm pores; Corning, Tewksbury, MA, USA) that were pre-coated with a mixture of type I collagen (rat tail, 50 $\mu\text{g}/\text{ml}$; Gibco) and Matrigel (300 $\mu\text{g}/\text{ml}$; BD Biosciences, Bedford, MA, USA) in serum-free DMEM for two hours. Culture medium was refreshed every other day on both the apical and basolateral side of a Transwell chamber.

For experiments in the gut-on-a-chip, the devices were prepared by flowing 70% (v/v) ethanol through the channels for sterilization, drying the entire system in a 60°C oven overnight, and then immediately exposing them to ultraviolet (UV) light and ozone (UVO Cleaner 342, Jelight Company Inc., Irvine, CA, USA) for 40 minutes to activate the surface of the microchannels. The gut-on-a-chip devices were then coated with the same mixture of collagen I and Matrigel as used in the Transwell cultures. Caco-2 cells were seeded into the top microchannel, as illustrated schematically in **Figure 4-4**,

at $1.5 \cdot 10^5$ cells/cm² and allowed to attach under static conditions. After one hour, culture medium was perfused through the upper channel at 30 μ l/h (fluid shear stress of 2 mPa or 0.02 dyne/cm²) for one day. Subsequently, medium was flowed through both the upper and lower channels at the same rate, and vacuum-driven stretching motions (10% in cell strain, at a frequency of 0.15 Hz) were applied through hollow vacuum chambers to induce peristalsis-like mechanical deformations of the cells using a pneumatic controller (FX5K Tension; Flexcell International Corporation, Hillsborough, NC).

4.3.3. TEER measurements

In Transwell cultures, TEER resistance values were measured with a Millicell ERS meter (Millipore, Bedford, MA) and chopstick-like electrodes with silver (Ag) and silver/silver chloride (Ag/AgCl) pellet electrodes. TEER values were determined by subtracting the baseline resistance value of coated inserts without cells and multiplying with the total membrane culture area of 0.33 cm².

The TEER of a Caco-2 monolayer in the gut-on-a-chip microdevice was measured using a multimeter (87V Industrial Multimeter, Fluke Corporation, Everett, WA, USA) coupled to Ag/AgCl electrodes (200 μ m in diameter; A-M Systems, Inc., Sequim, WA, USA). The electrodes were inserted in the tubing of the inlet of the top channel of the microdevice (*a* in **Figure 4-3**) and the outlet of the bottom channel of the microdevice (*b*). Again, the baseline resistance value measured in the absence of cells was subtracted from values of devices with a cell monolayer and the thus obtained measured resistance was multiplied with the total membrane culture area of 0.1 cm².

4.4. Results & discussion

4.4.1. TEER model

The theoretical model of the gut-on-a-chip device can be used to calculate what the apparent TEER of a measurement would be if cell layers with different TEERs were growing in the device. **Figure 4-5** shows the apparent TEER of the gut-on-a-chip as calculated using the numerical approach and Eq. 4-4, compared to the actual TEER value used as input in the model (Transwell TEER or R_{TEER} in Eq. 4-3). The apparent gut-on-a-chip TEER value is calculated from the potential drop between ports *a-b*, and an arbitrary current of 1 A, which is applied as boundary condition between ports *a-b*, normalized to the total membrane area. Interestingly, the apparent TEER value calculated for the gut-on-a-chip is higher than the actual R_{TEER} (Transwell TEER) used as input in the model. For example, the apparent TEER value for a measurement in the gut-on-a-chip geometry would be 1550 $\Omega \cdot \text{cm}^2$ if the actual (Transwell) TEER was only 1000 $\Omega \cdot \text{cm}^2$, given uniform cell coverage. The differences are even more dominant for the lower range of actual (Transwell) TEER values. It is worth noting that the effect

Figure 4-5 | Apparent TEER resulting from electrical simulations with the gut-on-a-chip geometry versus the actual (Transwell) TEER value as used as input in the model. The dashed line is a guide for the eye, showing $y = x$, to emphasize the deviation from the case that the whole membrane contributes equally to the measured resistance.

would be even more pronounced in microdevices with longer channels or smaller channel heights.

The reason for this theoretical deviation between apparent TEER and actual TEER in a microdevice with a gut-on-a-chip geometry becomes clear when studying the local current distribution in the device. **Figure 4-6** shows the normalized current distribution through the membrane area for the gut-on-a-chip geometry and low ($1 \Omega \cdot \text{cm}^2$) and high ($1001 \Omega \cdot \text{cm}^2$) TEER values. At low TEER, any potential difference between top and bottom channel is almost immediately equalized at the beginning and end of the channel. With increasing TEER, more current flows through the entire membrane although still most current flows through the first and last part of the membrane.

Properly conducted Transwell TEER measurements are usually optimized by aiming at an equal current density through the entire membrane. In any case, conductivity of the culture medium is such that the bulk liquid in Transwell systems ensures an almost equal potential drop over the entire membrane. In microfluidic chips, this is clearly not always the case, as is illustrated by **Figure 4-6**. This is because the conductance of fluid-filled microfluidic channels is easily many orders of magnitude lower compared to the bulk in Transwell systems. As a result, only part of the membrane is conducting current, giving rise to a higher apparent TEER in these chip systems than in a Transwell system with the same membrane area.

One of the ways to overcome this issue would be to integrate electrodes inside the top and bottom channel to ensure an equal potential drop over the entire membrane.

Figure 4-6 | A. Artist impression of the current (red line) flowing through the membrane and cell barrier, from port a to b. The current density is depicted as a gradient from black to green (high to low). **B.** Calculated current distribution through the membrane over the length of the channel (x) for low ($1 \, \Omega \cdot \text{cm}^2$) and high ($1001 \, \Omega \cdot \text{cm}^2$) TEER values using the geometry of the gut-on-a-chip.

Major drawbacks of that approach are that the electrodes will block the field of view and the device fabrication will be more complicated and costly. Moreover, the electrodes are not immediately compatible with systems such as the gut-on-a-chip and lung-on-a-chip devices [9], where mechanical deformations such as stretching are required.

Alternatively, the theoretical model described in this chapter can be used to convert the apparent TEER that is measured in a microfluidic chip to a TEER that can be compared to Transwell data using a simulated conversion graph, as shown in **Figure 4-5**.

Figure 4-7 | TEER measurements for the gut-on-a-chip (dark blue circles) and Transwell (red squares) using human intestinal epithelial Caco-2 cells. The corrected gut-on-a-chip line (light blue triangles), is calculated using the translational graph shown in **Figure 4-5**, which is only valid in the monolayer regime. Error bars denote standard deviation; for chip measurements: $n = 7$, for Transwell measurements: $n = 12$.

While this graph corresponds to the specific geometry of the gut-on-a-chip used for this study, such conversion graphs can easily be calculated for other geometries as well.

4.4.2. TEER measurements in Transwell *versus* gut-on-a-chip

Analysis of the same human intestinal epithelial Caco-2 cells grown in both the gut-on-a-chip and Transwell inserts revealed that the absolute TEER values measured in the gut-on-a-chip are consistently higher than those measured in the Transwell cultures (**Figure 4-7**). The TEER measurements initially follow a similar trend in both systems. After approximately 72 hours TEER values in the gut-on-a-chip measurements keep increasing and become higher than the TEER values measured in Transwell. This is most likely a result of spontaneous three-dimensional (3D) villus morphogenesis from a two-dimensional (2D) monolayer of Caco-2 cells, as is evidenced by **Figure 4-8**, when the cells experience flow and peristalsis-like motions in the gut-on-a-chip, starting after about three days of culture [10, 11]. The gut-on-a-chip displays intestinal villi and crypt characteristics with physiological growth up to several hundreds of microns in height, and increased expression of intestine-specific functions including mucus production, as was shown in an earlier publication [10]. In contrast, Caco-2 cells retain a polarized, but flat monolayer morphology under conventional static culture conditions (**Figure 4-8A**), which is observed during even up to two months of culture. Thus, the increased TEER profile after 72 hours may be attributed to enhanced intestinal differentiation.

Figure 4-8 | Differential interference contrast (DIC) microscopy images of Caco-2 cell morphology. **A.** A monolayer of Caco-2 cells grown in a static Transwell for 3 weeks. **B.** A monolayer of Caco-2 cells cultured in a gut-on-a-chip for 24 hours. The array of small white circles shows the pores visible beneath the Caco-2 cell monolayer. **C.** Villi formed in a gut-on-a-chip device after ~100 hours. All scalebars are 50 μm .

4.4.3. Model and experimental agreement

Up to 60 hours, the model seems to predict the differences between the microfluidic chip and the Transwell quite accurately. Microscope observations confirm that after 72 hours the cells cultured in the gut-on-a-chip transform from a planar cell monolayer (Figure 4-8B) into 3D villi-like structures (Figure 4-8C). Because the height of the villi reaches $\sim 120\ \mu\text{m}$, the accessible space in the upper microchannel (with a height of $150\ \mu\text{m}$, see Table 4-1) above the villi progressively decreases, which proportionally increases the resistance R_T in the top channel. This would account for the progressive increase in measured resistance at later time points in the gut-on-a-chip. As the current model does not take this effect into account, the experimental TEER was not converted to actual TEER at times beyond 72 hours.

4.4.4. Effects of poor cell coverage when measuring TEER

In principle, the TEER parameter describes the quality of the barrier function of a cell layer. In our model, as shown in **Figure 4-1** and **Eq. 4-6**, we discriminate between a pathway through the intact cell monolayer and a pathway through a gap in the monolayer. **Figure 4-9** shows the impact of a gap in the monolayer due to partial cell coverage. As can be seen from this figure, even a minor defect in monolayer confluency will have a major impact on the measured TEER. For example, assuming a cellular barrier with $1000 \Omega \cdot \text{cm}^2$ cultured on the PET Transwell membrane used in this chapter, at 95.5% cell coverage the measured TEER value will be 80% lower than the TEER of a culture with full cell coverage. This effect is even more pronounced for higher TEER and lower membrane resistance. We suspect that this is a major reason for the large variations observed in TEER measurements in literature (see **Figure 4-2** and **Table C-1** in **Appendix C.2**). Even monolayers that show the expression of tight junctional proteins using fluorescent staining can have low TEER if small gaps are present (e.g. at the edge of a Transwell insert or a microfluidic channel). The effects of cell coverage are of particular importance in microfluidic systems because of the relatively small surface area as well as the decreased effective surface area due to the unequal current distribution effect described in the previous sections.

4.4.5. DC versus AC TEER measurements

In this chapter and earlier papers, the TEER in the gut-on-a-chip is measured using a multimeter [9, 10]. Special care needs to be taken if a multimeter is used to determine the resistance, as the applied voltage to the electrodes can vary significantly among manufacturers, models and also between various resistance measurement ranges. The used voltage can influence the determination of TEER or in the worst case deplete the Ag/AgCl electrodes quickly, leading to measurement errors and cell toxicity due to the release of silver ions [71]. Moreover, common Transwell TEER measurement devices such as the Millicell ERS device are not suited for the gut-on-a-chip. The fixed current of $10 \mu\text{A}$ used by the Millicell device results in potentials that exceed the maximum measurable potential of 200 mV due to the high resistance of the microchannels. For reproducibility, each TEER measurement in the gut-on-a-chip was carried out with a freshly prepared pair of Ag/AgCl electrodes as prolonged use of a single electrode would lead to undesired changes in resistance due to changes in the electrode surface. In both chip and Transwell the TEER measurement is very sensitive to temperature variations.

Given the downsides of these simple DC-based systems, more complex impedance-based systems have been developed for measuring TEER [72]. The company Flocel Inc. is offering an AC measurement system, but it is tailored towards special proprietary cartridges used for cell culture and is therefore not easily transferable to Transwell or

Figure 4-9 | Calculated relative Transwell TEER, as a percentage of the input TEER of the cell layer, as a function of cell coverage. A large drop in measured TEER results from a small drop in cell coverage.

organs-on-chips systems. To our knowledge, the only AC systems commercially available to determine TEER in Transwell are the cellZscope system [72] (nanoAnalytics GmbH, Germany) and the ECIS TransFilter system (Applied BioPhysics, USA).

While the model presented here is tailored for DC measurements, in principle it can be extended to include the cell membrane capacitance and the double layer capacitance at the electrode surface. Ultimately we expect that measurements using AC will allow direct determination of TEER by comparing the impedance at two distinctively different frequencies, eliminating the need for measuring blanks. This will be elaborated upon in **Chapter 5**. Moreover, Ag/AgCl electrodes would be no longer required as measuring with AC enables the use of other (inert) electrode materials such as platinum.

4.5. Conclusion

TEER measurements show large variations in literature, not only between various cell lines but also between different studies with the same cell lines. It has also been difficult to compare results obtained with different culture systems, such as Transwell culture inserts *versus* microfluidic organs-on-chips. Our results show that when measuring TEER in organs-on-chips, the confined environment of microfluidic channels results in higher values compared to Transwell. Our analysis also revealed that this seemingly higher TEER has a geometrical origin, rather than a biological one: it arises from the non-uniform distribution of the current through the membrane and cell barrier. Importantly, TEER measurements obtained in microfluidic systems can be compared to

those obtained in Transwell systems using the theoretical model and comparison method we presented here. The cell coverage over the supporting substrate is also an important factor. Only the slightest gap (4.5%) can reduce the measured TEER significantly (80%). Even if fluorescent staining indicates the presence of a good barrier with tight junctions, TEER values might be lower if a small gap is present somewhere in the cell monolayer. We suspect that small defects in cell coverage are the main cause for large variations in measurements reported in literature.

4.6. References

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Impedance spectroscopy and electrical simulations of a gut-on-a-chip

In this chapter, the previously reported gut-on-a-chip and electrical cell layer measurement methods are advanced to include impedance spectroscopy and four-terminal sensing with integrated electrodes for more sensitive measurements of cell layer resistance. Furthermore, the electrical simulations were expanded to provide more insight into these measurements and aid interpretation by visualizing the potential and sensitivity distributions inside the chip. Using these simulations the cell layer resistance of flat gut epithelium was normalized to transepithelial electrical resistance, enabling comparison between different organs-on-chips and to conventional Transwell cultures. Furthermore, the effect of villi formation on the impedance spectra was studied, resulting in the identification of cell layer capacitance as a promising predictor of the state of villi differentiation.

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M.W. van der Helm, O.F.Y. Henry, A. Bein, T. Hamkins-Indik, M.J. Cronce, W.D. Leineweber, M. Odijk, A.D. van der Meer, J.C.T. Eijkel, D.E. Ingber, A. van den Berg & L.I. Segerink (2015). Impedance spectroscopy and electrical simulations reveal both transepithelial barrier function and tissue structure by non-invasive sensing in organs-on-chips. *In preparation*

5.1. Revealing cell layer characteristics

Organs-on-chips are microfluidic devices in which living cells are cultured and subjected to engineered conditions that replicate key aspects of the microenvironment of a real human organ or tissue [1, 2]. These *in vitro* cell culture models are designed to be more physiologically relevant than conventional *in vitro* models and are therefore expected to be of great value for drug development, disease modelling and precision medicine. Of special interest are barrier-forming tissues, such as gut epithelium, lung epithelium and blood-brain barrier (BBB) endothelium, which are of vital importance for protection and proper functioning of the associated organs [3]. Barrier tissues are studied widely for drug delivery applications and understanding their pathological states in relation to various diseases [3]. Examples of cellular barriers in organs-on-chips are the alveoli-on-a-chip [4], airway-on-a-chip [5], gut-on-a-chip [6, 7] and BBB-on-a-chip [8, 9].

In organ-on-chip applications, epithelial or endothelial layers are generally cultured on a porous membrane suspended between two microfluidic channels, representing the luminal and abluminal compartments (see also **Section 1.3 in Chapter 1**). A frequently used method to examine the barrier properties of such functional tissue is the assessment of transepithelial or transendothelial electrical resistance (TEER). TEER measurements are non-invasive, label-free and can be carried out continuously to closely monitor barrier formation and differentiation as well as barrier disruption upon exposure to various stimuli [3, 10, 11]. This ability for fast and continuous measurements stands in contrast to permeability measurements using tracer molecules, which generally require longer protocols and analysis times, and biological end-point assays such as immunostaining and gene expression profiling, which can only be performed after termination of the culture period. The TEER of barrier-forming tissues in organs-on-chips is generally measured using electrodes that are inserted or integrated into the chip.

When setting up TEER measurements in organs-on-chips, there are three main aspects to consider: the electrical measurement protocol, the sensitivity distribution along the cell layer and the electrode placement in the chip. In the next section, these aspects are considered for a new gut-on-a-chip as a model organ-on-chip system. This gut-on-a-chip is based on **Chapter 4** and previously reported literature [6, 7], and comprises two channels with a porous membrane in between on which villi-forming gut epithelium is cultured. Ultimately, we combine four-terminal impedance spectroscopy with electrical simulations to reveal cell layer characteristics in the gut-on-a-chip, as is elaborated upon in the results section. The presented methods can be adapted to fit any organ-on-chip device and measurement protocol to provide insight in tissue barrier function, aiming at broad application in the field of organs-on-chips to study the characteristics of functional tissues inside the devices.

5.2. Considerations for TEER measurements

5.2.1. Electrical measurement protocol

Thus far, most TEER measurements in organs-on-chips have been carried out using direct current (DC) or square-wave currents at low frequency, measuring the ohmic resistance of a cell layer, as is also done by the traditional commercial systems used for Transwell TEER measurements [4, 6, 7, 12-19]. However, the use of alternating currents (AC) to perform electrical impedance spectroscopy is preferred because it results in more reliable determination of the cell layer resistance as there is no need for measuring blanks and error-prone correction of the measured ohmic resistance [11, 20]. In addition, the adverse effects of electrode polarization are eliminated [20] and more biocompatible electrode materials than silver/silver chloride can be used. Furthermore, impedance spectroscopy provides additional information about the capacitance of the cell layer [10, 11]. This capacitance arises from capacitive charging at the electrically insulating lipid bilayer membranes separating the extracellular and intracellular conducting media and is dependent on the total area and the composition of the cell membranes [20, 21]. It is thus expected to indicate an increase in surface area of the cell layer when it forms wrinkles or villi. Although impedance spectroscopy has already been used in organs-on-chips [8, 9, 22-24], we are the first to exploit the data of the entire frequency domain to assess tissue function and structure. In this chapter, we present measurements of both the cell layer resistance and capacitance of a gut-on-a-chip and show what information can be obtained from both quantities.

A simple equivalent circuit of AC measurements of a cell layer is shown in **Figure 1-4** in **Chapter 1** and comprises the cell layer resistance parallel to the cell layer capacitance, which are in series with the resistance of the culture medium and membrane. In two-terminal impedance spectroscopy, where the measured signal is the potential difference between the excitation electrodes, the double layer capacitance at the interface between the electrode and culture medium is added in series as well [3, 10]. This double layer capacitance is often prominent in on-chip electrical measurements [8, 9], because its influence on impedance spectra increases with decreasing electrode size. However, when a four-terminal sensing method is used with separate electrode pairs for excitation and readout of the measured potential, the double layer capacitance minimally affects the measurement [25]. Furthermore, the contributions of contact and lead resistances of the electric circuitry outside of the chip are also eliminated from the measured resistance in four-terminal sensing. This results in more sensitive measurements of the electrical properties of the cell layer [26]. Therefore, in this chapter, we focus on four electrodes and a four-terminal sensing structure.

5.2.2. Sensitivity distribution

Generally, methods to convert measured cell layer resistance (in Ω) to TEER (in $\Omega \cdot \text{cm}^2$) assume that the entire cell layer contributes equally to this measured resistance and use the total culture area for normalization. However, electric fields and current densities develop in three-dimensional (3D) space, so that different volume elements contribute unequally to the final measured resistance [27]. In addition, in four-terminal measurements the electric field arising between the excitation electrodes can locally be in line with, opposite to or orthogonal to the electric field between the readout electrodes, resulting in local differences in contribution to the total measured resistance. To understand this, calculating the sensitivity distribution inside a chip is very insightful: the higher the value of the sensitivity coefficient in a volume element, the more a change in the resistivity of this volume element will affect the total measured resistance. In this chapter we follow the formulation of Grimnes and Martinsen [27] to calculate the sensitivity coefficient S :

$$S = \frac{\mathbf{J}_1 \cdot \mathbf{J}_2}{i} \quad \text{Eq. 5-1}$$

In this equation, $\mathbf{J}_1$ is the current density vector (A/m^2) in each volume element when a current i (A) is injected between the two excitation electrodes. $\mathbf{J}_2$ is the current density when i is injected between the two readout electrodes [27] (following Geselowitz's reciprocity theorem [28]). A negative sensitivity means that an increase in the resistivity of this volume element will result in, maybe surprisingly but not counterintuitively, a lower total measured resistance. More information and theory on the sensitivity distribution is included in **Appendix D.1**.

As is indicated, the sensitivity distribution is a useful tool to demonstrate which volume elements contribute most to the measured impedance. Especially in organs-on-chips, a uniform sensitivity distribution along the entire cell layer is seldom achieved because of inherently small channel dimensions. As a result, the electrical resistance of the channels approaches the order of magnitude of the cell layer resistance to be measured [7, 9, 25]. The adverse effect of non-uniform sensitivity distributions on the interpretation and quantification of TEER data has been recognized and described by several authors [7, 10, 11, 25]. Srinivasan et al. advised to perform electrical simulations and modeling while designing a chip to arrive at uniform current density [11]. Odijk et al. provided such electrical simulations for long microfluidic channels with two electrodes outside of the culture area and showed the non-uniform current distribution in the membrane during DC TEER measurements [7]. Yeste et al. also simulated DC TEER measurements in both Transwell and microfluidic chips with several integrated electrode configurations and a four-terminal measurement setup, and used the sensitivity distribution to visualize the actual contribution of various parts of the cell culture to the total measured resistance [25]. Additionally, both Odijk et al. and Yeste et al. used their

simulations to correct the measured cell layer resistance to arrive at a TEER that can be compared across devices [7, 25]. Here we expand the electrical simulations of Odijk et al. and Yeste et al. to include four-terminal impedance spectroscopy and to visualize the sensitivity distributions for our measurements as well as to model the formation of villi in the gut-on-a-chip and assess their effect on the impedance spectra. In addition, with these simulations we can convert the cell layer resistance to TEER by normalizing to the actual probed area of the cell layer rather than the total membrane area.

5.2.3. Electrode placement

Conveniently, TEER measurements can be conducted using electrodes inserted into the inlets of an organ-on-chip [4, 6, 16, 19]. However, it is extremely challenging to position the electrodes in exactly the same way in subsequent measurements, giving rise to uncertainty in TEER quantification. Therefore, integration of electrodes into the chip is preferred and shown to be possible using both cleanroom and non-cleanroom processes. These electrodes, however, block sight on the cell culture when they are integrated directly above and below the cell layer [8, 12, 13, 22, 23]. To overcome these limitations, electrodes can be fixed outside of the culture area [15, 29] or be made of thin, see-through layers of gold [5, 14].

In the gut-on-a-chip reported here, a four-terminal configuration was adopted using a very thin (nanometer-scale) semi-transparent layer of gold integrated directly above and below the cell culture [5]. In order to minimize the distortion for optical microscopy, relatively small electrode areas (1 mm^2 each) were used. This was possible because four-terminal impedance spectroscopy eliminated the influence of the double layer capacitance while electrical simulations helped interpretation of the resulting impedance spectra and quantification of TEER and cell layer capacitance in spite of unequal sensitivity distributions.

5.3. Experimental

The design of the gut-on-a-chip was previously reported by Henry et al. [5]. The use of this chip, including the culture of villi-forming gut epithelium and the impedance spectroscopy protocol, is based on **Chapter 4** and other previous reports [5-7].

5.3.1. Chip fabrication

The gut-on-a-chip comprises five layers, as is shown in **Figure 5-1A** and **B**. The polycarbonate (PC) substrates (1 mm thickness) that were used to form the top and bottom parts of the microfluidic chips, were cut to 25 mm by 45 mm rectangles. The PC substrates were sonicated in isopropanol for five minutes and blow dried in a stream of compressed air. Cleaned substrates were subsequently exposed to an oxygen plasma for two minutes (20 SCCM, 100 W) and put in direct contact with a shadow mask that

defined the electrode geometries. Thin metal layers of titanium (3 nm, 0.2 nm/s), gold (20 nm, 0.1 nm/s) and titanium (1 nm, 0.2 nm/s) were sequentially deposited onto the substrates by e-beam evaporation (Denton Vacuum LLC, USA), which resulted in semi-transparent gold electrodes.

For the 1 mm high top channel and 0.2 mm high bottom channel, poly(dimethylsiloxane) (PDMS) channel layers were laser cut from PDMS (Sylgard® 184, Dow Corning) films spin coated onto acrylic discs (1 mm: two spin coating cycles at 200 rpm for 60 s, cured at 60°C; 0.2 mm: one spin coating cycle at 200 rpm for 120 s, cured at 60°C). Cured PDMS layers were covered with low-tack tape (3M, Scotch® Magic tape) prior to laser cutting the desired channel geometries. Bonding of the PDMS layers to the patterned PC substrates were achieved using silane chemistry [5]. All parts were first activated in an oxygen plasma (15 SCCM, 100 W, 30 s). PC substrates and PDMS layers were immediately immersed for 20 minutes in aqueous solutions of 1% (v/v) (3-glycidyloxypropyl) trimethoxysilane and 5% (v/v) (3-aminopropyl) triethoxysilane (APTES), respectively. All parts were subsequently rinsed in double distilled water and dried in a stream of compressed air. PC top and bottom substrates were aligned and pressed against the 1 mm and 0.2 mm thick laser cut PDMS layers, respectively, and kept in an oven overnight at 60°C. Finally, the resulting PC/PDMS parts and a permeable PDMS membrane (50 µm thick with 7 µm diameter pores in a hexagonal pattern with 40 µm center-to-center spacing) were activated in an oxygen plasma (15 SCCM, 100 W, 30 s) before being aligned and bonded together as previously described [5].

The fabrication as reported for the four-electrode chip of **Figure 5-1A** was adapted slightly to fabricate the six-electrode chip of **Figure 5-6A**: the shadow mask determining the electrode geometries was changed as well as the thickness of the top PDMS layer (0.8 mm).

5.3.2. Chip preparation and cell seeding

The human adenocarcinoma cell line Caco-2 (Caco-2 BBE, Harvard Digestive Disease Center) was maintained and passaged in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). Before seeding Caco-2 cells in the chips, the channels were pre-treated with 1% (v/v) (3-aminopropyl) trimethoxysilane (Sigma) in phosphate-buffered saline (PBS; Gibco), flushed with PBS and ethanol, and then coated with rat-tail collagen type I (30 µg/ml; Gibco) and Matrigel (100 µg/ml; BD Biosciences, Bedford, MA). Caco-2 cells were seeded at a concentration of $2 \cdot 10^6$ cells/ml and were allowed to attach overnight. When chips were cultured statically, medium was refreshed once per day by flushing with a pipette. When chips were cultured under flow, the inlets of both top channel and bottom channel were connected to a syringe pump

Figure 5-1 | Gut-on-a-chip design, operation principles and the equivalent electrical circuit used for simulations. **A.** Microfluidic gut-on-a-chip device. Reproduced from ref. [5] with permission of The Royal Society of Chemistry. **B.** Exploded view of the chip, showing two PDMS parts with laser-cut channels (1 mm wide) separated by a porous PDMS membrane, sandwiched between two PC substrates with integrated semi-transparent gold electrodes. **C.** Side view of the main channel. Caco-2 cells are cultured on the membrane as a flat monolayer (when cultured statically) or in a villi structure (when cultured under flow). During four-terminal impedance spectroscopy, a current is applied between the excitation electrodes (green, solid arrow) and through the bare membrane, flat monolayer or villi, while the resulting potential difference between the readout electrodes is measured (purple, dashed arrow). **D.** Conversion of the side view to an electrical circuit of m by n nodes connected by resistive and/or capacitive elements representing the culture medium, porous membrane, cell layer and electrodes.

and medium was perfused at 30 $\mu\text{l/h}$, resulting in a shear stress of 41 μPa on the gut epithelium according to Eq. 2-4 in Chapter 2 [30].

5.3.3. Four-terminal impedance spectroscopy

Impedance spectroscopy measurements were carried out daily using an Autolab PGStat12 (Metrohm Autlab B.V.) over a period of 12 days. Electrochemical impedance spectroscopy in galvanostatic mode using a four-terminal setup was used to excite the cell culture with an AC current of 10 μA at 50 frequencies, sweeping from 100 kHz down to 10 Hz, and to record the potential difference between the readout electrodes. Microfluidic chips were transferred from the incubator onto an aluminum plate kept at 37°C to reduce the effect of temperature drift during measurements.

A schematic of the measurement is presented in Figure 5-1C, in which the excitation and readout electrode pairs are indicated. The excitation electrode in the top PC layer was connected to the counter electrode of the Autolab probe and the bottom excitation electrode to the working electrode. The readout electrodes were connected to the reference electrode (top) and the sensing electrode (bottom). Impedance spectroscopy was repeated three times in a row and the resulting cell layer resistances were averaged over these technical triplicates.

5.3.4. Confocal microscopy and image analysis

To study villi formation in time, a subset of chips was fixed and stained on day 3, 6, 8, 10 and 12, followed by confocal microscopy. Image processing was performed with Imaris (Bitplane) to convert the confocal Z-stack to reconstructed 3D surfaces and top-view height maps. The villi area ratio was determined using the image processing package Fiji [31] available for ImageJ, where the 3D surface area is used to calculate the blanket surface to 2D surface area.

5.4. Electrical simulations

For the electrical simulations, the volume inside the chip was divided into small volumes, termed “model elements”, that are electrically connected and have the electrical properties (e.g. resistance or capacitance) of the material they represent. The chip structure yields model elements with the electrical properties corresponding to culture medium, electrodes, porous membrane or cell layer, as is shown schematically in Figure 5-1D. The chip design allowed for the 3D chip volume to be reduced to a two-dimensional (2D) network of horizontally and vertically connected resistors and capacitors to decrease computation time. A network of 2000 by 144 nodes was sufficient to arrive at convergence (Appendix D.2). The four types of model elements are elaborated upon next, after which the simulation of galvanostatic impedance spectroscopy

is explained as well as visualizing the potential and sensitivity distributions arising during impedance spectroscopy.

5.4.1. Model elements

Culture medium element

A culture medium element describes the resistance of a confined rectangular cuboid volume of culture medium inside the microfluidic channel. As current can travel in all directions through the medium-filled channels with isotropic resistance, there are nodes where current can enter and exit both on the horizontal and vertical planes of the cuboid volume. Its resistance is calculated using the following equations:

$$R_x = \frac{l_{ch}/n}{\kappa \cdot w_{ch} \cdot h_{tot}/m} \quad [\Omega] \quad \text{Eq. 5-2}$$

$$R_y = \frac{h_{tot}/m}{\kappa \cdot w_{ch} \cdot l_{ch}/n} \quad [\Omega] \quad \text{Eq. 5-3}$$

In these equations, R_x is the total horizontal resistance of a medium element (Ω), R_y is the total vertical resistance of a medium element (Ω), l_{ch} is the modeled channel length (m), w_{ch} is the channel width (m), h_{tot} is the total channel height (m), κ is the specific conductivity of culture medium (S/m), m is the number of nodes along the channel height and n is the number of nodes along the channel length. The values used for these parameters are listed in **Table 5-1**.

Membrane element

A membrane element describes the electric properties of the culture medium inside the pores of a confined piece of the membrane, acting as parallel anisotropic conductors with a vertical orientation. As current can only pass in vertical direction, this element has only two vertically oriented nodes and its resistance can be calculated as follows:

$$R_{mem} = \frac{d_{mem}}{P \cdot \kappa \cdot w_{ch} \cdot l_{ch}/n} \quad [\Omega] \quad \text{Eq. 5-4}$$

In this equation, R_{mem} is the resistance of the piece of membrane (Ω), d_{mem} is the membrane thickness (m) and P is the membrane porosity (%), defined as the ratio between the volume of open space and the total membrane volume. By using the porosity rather than modelling individual pores, we assume that the membrane is uniform. As the volume of this element depends on the membrane thickness instead of on the number of elements m , it may represent a different volume than the culture medium element, giving rise to an adaptive mesh.

Cell layer element

The cell layer element is modeled as a circuit of a resistor, representing ionic transport through the cell layer, in parallel with a capacitor, representing the cell membrane

Table 5-1 | Input parameters for electrical simulations of the gut-on-a-chip.

Parameter	Description	Value
w_{ch}	Width of channel	1000 μm
$h_{ch,t}$	Height of top channel	1000 μm
$h_{ch,b}$	Height of bottom channel	200 μm
l_{ch}	Length of overlapping channels	16.7 mm
κ	Conductivity of culture medium [7]	1.67 S/m
d_{mem}	Thickness of membrane	50 μm
P	Porosity of membrane	2.1%
C_{cell}	Approximated cell membrane capacitance [32]	4 $\mu\text{F}/\text{cm}^2$
C_{dl}	Approximated double layer capacitance [33]	20 $\mu\text{F}/\text{cm}^2$
l_{el}	Length of the electrodes in the channel	1 mm
d_{el}	Distance between two coplanar electrodes	1 mm
i_{in}	Input current, applied between the excitation electrodes	10 μA
f	Frequency range of applied sinusoidal current	10 Hz - 100 kHz
$TEER$	Range of possible TEER values of Caco-2 monolayer [7]	0 - 1000 $\Omega \cdot \text{cm}^2$

capacitance. As we neglect the contribution of longitudinal current through the cell layer, this element has either two vertically or two horizontally oriented nodes and has an anisotropic impedance, depending on the approximated orientation of the cell layer. The impedance of a defined area of the cell layer is:

$$Z_{Ty} = \frac{1}{1/R_{Ty} + j \cdot \omega \cdot C_{Ty}} = \left(\frac{w_{ch} \cdot l_{ch} / n}{TEER} + j \cdot 2\pi f \cdot C_{cell} \cdot w_{ch} \cdot l_{ch} / n \right)^{-1} \quad [\Omega] \quad \text{Eq. 5-5}$$

$$Z_{Tx} = \frac{1}{1/R_{Tx} + j \cdot \omega \cdot C_{Tx}} = \left(\frac{w_{ch} \cdot h_{tot} / m}{TEER} + j \cdot 2\pi f \cdot C_{cell} \cdot w_{ch} \cdot h_{tot} / m \right)^{-1} \quad [\Omega] \quad \text{Eq. 5-6}$$

In these equations, Z_{Ty} is the impedance of a vertical cell layer element (Ω), R_{Ty} is the resistance of the cell layer element (Ω) derived from the input $TEER$ ($\Omega \cdot \text{cm}^2$) divided by the area of the cell layer element (cm^2); C_{Ty} is the capacitance of the cell layer element (F) derived from the typical cell membrane capacitance C_{cell} (F/ cm^2) multiplied by the area of the cell layer element (cm^2); j is the imaginary unit ($\sqrt{-1}$), $\omega = 2\pi f$ is the angular frequency of the applied sinusoidal current (rad/s), which is calculated from the input frequencies f (Hz) of the AC signal applied to the excitation electrodes. The impedance of a horizontal cell layer element, Z_{Tx} , is calculated in a similar way. When a cell layer is cultured directly on top of the membrane, their combined impedance is the sum of the cell layer impedance and the membrane resistance in series:

$$Z_{T+mem} = Z_{Ty} + R_{mem} \quad [\Omega] \quad \text{Eq. 5-7}$$

As the gut epithelium inside microfluidic chips can form villi [6, 34], this is also included in the model. This is achieved by replacing the vertical and horizontal culture medium

elements that are in the location of the sinusoidally modeled villi with cell layer elements.

Electrode

The electrode element describes the double layer capacitance at the interface of the electrode and culture medium for a specified area. Its impedance is:

$$Z_{el} = \frac{1}{j \cdot 2\pi f \cdot (C_{dl} \cdot w_{ch} \cdot l_{ch} / n)} \quad [\Omega] \quad \text{Eq. 5-8}$$

In this equation, Z_{el} is the electrode element impedance and C_{dl} is the typical double layer capacitance of the gold electrode in contact with culture medium (F/cm²).

5.4.2. Simulation of potential and sensitivity distributions

Identically to the experimental setup, an input current is applied to the node corresponding to one of the excitation electrodes while the node of the other excitation electrode is connected to ground. The readout electrode pair is allowed to have a floating potential but no current. Then, using Ohm's law and Kirchhoff's current law, a system of linear equations is derived for the network of nodes connected by the model elements described in the previous section. Using this system of equations, the known input current and the impedances of the model elements, the potential in each node is calculated. The applied boundary conditions are identical to the experimentally used input current and frequency. This method was proposed by Odijk et al. [7] and the principles are explained in detail in **Appendix B**. In this chapter, the simulations were optimized for sparse matrices to reduce memory load. The resulting potentials in each node are visualized to provide insight in the developed electric field inside the chip. Subsequently, using these potentials and the impedance between the nodes, the current distribution through the cell layer is calculated. This is the current density vector $\mathbf{J}_1$ described in **Eq. 5-1** and **Appendix D.1**, as the area through which current passes is defined by the area of the cell layer element. According to the reciprocity theorem, $\mathbf{J}_2$ is obtained by applying the excitation current to the readout electrodes. Using these two current density vectors and the input current i_{in} the sensitivity distribution through the cell layer is calculated. Lastly, the sensitivity is normalized by multiplying it with the squared number of cell layer elements n . With this normalized sensitivity distribution S_n , as calculated in **Eq. 5-9**, insight is provided into the contributions of each part of the cell layer to the total measured resistance.

$$S_n = \frac{\mathbf{J}_1 \cdot \mathbf{J}_2}{i_{in}^2} n^2 \quad \text{Eq. 5-9}$$

From the potential difference between the readout electrodes, the impedance is calculated analogously to the experimentally measured impedance. This output impedance is checked for convergence as described in **Appendix D.2** to arrive at a stable solution.

The simulations were designed and implemented in Matlab R2016b (The MathWorks, Inc.).

5.4.3. Simulation of galvanostatic impedance spectroscopy

To mimic impedance spectroscopy measurements in the gut-on-a-chip, the input current magnitude i_{in} and frequencies were taken equal to the experimental values (see **Table 5-1**). The measured impedance is derived from the simulations by taking the potential difference between the two readout electrode nodes and dividing it by the input current. After simulation for multiple frequencies, the resulting impedances are collected in an impedance spectrum. The cell layer resistance R_{cell} is derived from a spectrum by subtracting the minimum impedance magnitude (at 100 kHz) from the maximum impedance magnitude (at 10 Hz) (both Ω):

$$R_{cell} = |Z_{max}| - |Z_{min}| \quad [\Omega] \quad \text{Eq. 5-10}$$

The cell layer capacitance is derived by finding the frequency at which the impedance magnitude is half of the maximum impedance magnitude (logarithmically interpolated). This frequency f_{RC} (Hz) is inversely proportional to the RC time constant τ (s). Thus, the capacitance can be derived by:

$$C_{cell} = \frac{\tau}{|Z_{max}|} = \frac{1}{|Z_{max}| \cdot 2\pi f_{RC}} \quad [F] \quad \text{Eq. 5-11}$$

This method for determining the cell layer resistance and capacitance relies on the assumption that the shape of the impedance spectrum corresponds to the equivalent circuit of a resistor and capacitor in parallel (cell layer), in series with another resistor (culture medium and membrane). It is also used for extracting the cell layer resistance and capacitance from experimental data.

5.5. Results & discussion

5.5.1. Impedance spectroscopy measurements in the gut-on-a-chip

Caco-2 human gut epithelial cells were cultured on top of the membrane for up to 12 days, both statically to achieve monolayer formation as well as under continuous flow of 30 $\mu\text{l/h}$ (dynamically, corresponding to shear stress of 41 μPa) to trigger villi differentiation [6, 34]. During the culture period, impedance spectra were recorded daily using integrated semi-transparent gold electrodes, which also allowed inspection of the cells cultured behind or in front of them (**Appendix D.3**). The integration of these electrodes was conveniently realized using direct metal patterning of PC substrates via a shadow mask, thereby avoiding cumbersome photolithographic steps typically used in microfabrication. The electrical measurements conveniently enabled the continuous monitoring of cell layer formation and maturation *in situ*. The thus obtained impedance spectra of the static and dynamic cultures (**Figure 5-2A** and **B**, respectively) have the

Figure 5-2 | Experimental data obtained during the 12-day culture period of Caco-2 gut epithelial cells inside two microfluidic devices. **A-B.** Impedance spectra showing the development of a statically cultured gut epithelial barrier (**A**) and a gut epithelial barrier cultured under flow (dynamically) (**B**) from day 1 to 9 (day 0 is before introducing cells). The negative slopes between 100 Hz and 10 kHz result from the cell layer capacitance, while the plateaus at low frequencies (left, <100 Hz) correspond to the total resistance of the cell layer and chip, and the plateaus at high frequencies (right, >10 kHz) correspond to the resistance of the chip alone. **C.** Measured cell layer resistance versus culture time. The resistance of the statically cultured cell layer increased in time up to a plateau, while the resistance of the cell layer cultured under flow decreased after day 6. **D.** Measured cell layer capacitance versus time of culture. The capacitance of the static cell layer reached a plateau after day 7, while the capacitance of the dynamic cell layer further increased.

characteristic shape expected for cell layers [10, 35]. The cell layer resistance and capacitance could be derived directly from these spectra, resulting in Figure 5-2C and D. In this way, we were able to distinguish the contributions of the cell layer to the total measured impedance from the contributions of the rest of the chip. This effectively eliminates the need for measuring blanks and clearly poses an advantage over conventional single-frequency TEER measurements [11, 20]. Although precautions were taken, e.g. to minimize temperature fluctuations, variability in the blank chip resistance can be recognized in these experimental spectra (around 100 kHz), which illustrates the advantage of impedance spectroscopy over single-frequency TEER measurements, in which any variability in blank resistance directly influences the determination of the cell layer resistance.

During static culture, the cell layer resistance increased in time (**Figure 5-2C**), as is expected from tight junction formation and barrier maturation. The cell layer capacitance increased up to day 7 and then reached a plateau (**Figure 5-2D**), indicating that after that period the cell membranes undergo minimal change in terms of total area or composition [20, 21]. In contrast, during dynamic culture the cell layer resistance decreased after day 6 while the cell layer capacitance increased. This coincided with the formation of villi, as observed by microscopy. Therefore, it is hypothesized that this characteristic decrease in resistance and increase in capacitance provides a tool for monitoring villi formation without the need for microscopy.

5.5.2. Normalizing cell layer resistance to TEER

To mimic the development of a statically cultured cell layer, a set of electrical simulations with input TEERs increasing from $0\ \Omega\cdot\text{cm}^2$ (no cell layer) to $1000\ \Omega\cdot\text{cm}^2$ (fully developed cell layer [7]) were performed, while keeping all other parameters constant. The resulting impedance spectra (**Figure 5-4A**) resembled the experimental spectra (**Figure 5-2A**) and were characterized by an increase in measured cell layer resistance. Potential distributions at three different input TEER values and three different frequencies (**Figure 5-3A**) provide insight into the measurements displayed in **Figure 5-2**. As there are no equipotential planes above and below the cell layer, the potential difference between the readout electrodes is smaller than the potential difference between the excitation electrodes, resulting in an underestimated impedance of the chip. In addition, the potential distribution depends on both the input TEER and frequency, even resulting in changed electrode polarities of the readout electrodes at low input TEER (top) and high frequency (bottom) as is more often seen in four-terminal impedance spectroscopy [27]. In **Appendix D.4** this is further illustrated and it becomes clear that the simulations are valuable aids for data interpretation. Furthermore, the phase spectra corresponding to these impedance spectra are shown there, which show a -180° phase shift at high frequencies. An explanation is included, showing that the cause is changing electrode polarity resulting from chip and electrode geometry [27].

Additionally, because of these non-uniform potential distributions, the cell layer also contributes non-uniformly to the measured cell layer resistance. This is evident in the normalized sensitivity distributions through the cell layer (**Figure 5-3B and C**), calculated using the current distributions derived from the potential distributions. Generally, a higher sensitivity indicates that a change in resistivity of the associated model element will affect the total measured resistance more than a change in resistivity of a less sensitively probed model element [27, 28]. Especially at low input TEER (**Figure 5-3B**) and high frequency (**Figure 5-3C**) the center part of the cell layer contributes much more to the measured resistance than the outer parts. This is a result of the

Figure 5-3 | Potential and sensitivity distributions corresponding to increasing input TEER values and increasing frequencies. **A.** Potential distributions at different input TEER values and frequencies. The color scale (indicating linearly increasing potential from blue to yellow) and equipotential lines (black) show that there are no equipotential planes above and below the cell layer. In addition, the electrode polarity changes at low TEER and high frequency. **B-C.** Sensitivity distributions through the membrane with cell layer at different input TEER values (**B**) and different frequencies (**C**), of which none matched with a uniform contribution of the entire cell layer (normalized sensitivity of 1, red dashed lines).

Figure 5-4 | Simulated impedance spectra with increasing input TEER values to mimic the development of a statically cultured cell layer and normalization to TEER. **A.** Simulated impedance spectra at five input TEER values, corresponding well to the experimental impedance spectra of the statically cultured gut-on-a-chip (Figure 5-2A). **B.** Calibration curve relating cell layer resistance to actual TEER values. For easy interpretation, a linear guide for the eye is added to show the deviation from the case that the effective area is equal to the actual cell layer area (red dashed line). **C.** Experimental cell layer resistance of the development of the statically cultured gut-on-a-chip, normalized to TEER values. They are in good agreement with the average TEER $\pm$ standard deviation of Transwell cultures of Caco-2 cells (Appendix D.5). **D.** The actual probed area (effective area) versus cell layer resistance. The guide for the eye (red dashed line) shows the actual membrane area, emphasizing the deviation.

resistance of the microchannels approaching the cell layer impedance [7, 9]. At increasing input TEER and decreasing frequency, the cell layer impedance becomes more dominant over the microchannel resistance and a larger part of the cell layer contributes to the measured impedance. However, at none of the simulated conditions the cell layer is uniformly probed during impedance spectroscopy measurements. It becomes clear that finding the appropriate area to normalize the measured cell layer resistance (Ω) to TEER ($\Omega \cdot \text{cm}^2$) is not trivial, even though finding this normalized TEER is essential for enabling comparison across devices. This underlines the need for electrical simulations to find the normalized TEER [7, 10, 11, 25].

To enable normalization, the cell layer resistance derived from simulated impedance spectra was plotted against the input TEER, resulting in a calibration curve (Figure

5-4B). For further insight, **Figure 5-4D** shows the effective area that was actually probed during the measurement for each cell layer resistance. Using the calibration curve, the experimentally determined cell layer resistance values of the statically cultured gut-on-a-chip were normalized to TEER values (**Figure 5-4C**). After a week of cell layer maturation, the static on-chip TEER values are in good agreement with TEER measured in standard Transwells (average $\pm$ standard deviation of $736 \pm 120 \Omega \cdot \text{cm}^2$, measured in three wells at four time points after a culture period of six weeks; see **Appendix D.5**).

5.5.3. Exploring effect of villi on impedance spectra

To assess the effect of villi formation on the impedance spectra, a set of electrical simulations with different degrees of villi formation were performed, while keeping all other parameters constant and the input TEER at $750 \Omega \cdot \text{cm}^2$. The villi architecture was approximated with a sine function with a period and height range corresponding to confocal slices of the dynamically cultured gut-on-a-chip (**Appendix D.6**). The state of villi formation is reported as “villi area ratio”, defined as the total surface area of the villi-structured gut epithelium divided by the total surface area of the porous membrane (**Figure 5-5B** inset). In addition to TEER and frequency, as was seen before, villi area ratio also influenced the potential distribution in the chip (**Figure 5-5A**). The resulting impedance spectra (**Figure 5-5B**) predicted well the distinctive developments in experimental spectra of the dynamically cultured gut-on-a-chip (**Figure 5-2B-D**): the villi formation gave rise to a decreased cell layer resistance and an increased cell layer capacitance (**Figure 5-5C**). This is explained as both the resistance and capacitance are dependent on the total area of the cell layer: a larger (corrugated) cell layer area results in more parallel resistors and capacitors, thus in a smaller total resistance and a larger total capacitance. The decrease in cell layer capacitance at villi area ratios around 4 and higher is expected to be due to limitations of the readout method of the spectra rather than reflecting the true cell layer capacitance. As the impedance spectra indicate a further increase in cell layer capacitance, the apparent decrease in **Figure 5-5C** should be considered in that context. Due to the non-trivial relation between cell layer resistance and TEER (**Figure 5-5C**) as well as the limitations of sinusoidally modeled villi and 2D simulations, we were not able to quantify the TEER of the corrugated monolayer. Nevertheless, the increasing capacitance that was seen in the experimental data is a direct result from the villi formation, as is evidenced by these simulations.

5.5.4. Cell layer capacitance as indicator of villi differentiation

For these results a six-electrode chip with a different top channel height was used (**Figure 5-6A** and **B**). The six electrodes were again used in a four-terminal configuration

Figure 5-5 | Electrical simulations with increasing villi area ratio to assess the influence of villi formation on impedance spectra. **A.** Potential distributions at different villi area ratios and frequencies. The color scale (indicating linearly increasing potential from blue to yellow) and equipotential lines (black) show that there are no equipotential planes above and below the cell layer. **B.** Simulated impedance spectra at four villi area ratios, corresponding well to the experimental impedance spectra of the dynamically cultured gut-on-a-chip (**Figure 5-2B**). The inset shows how the villi area ratio is determined. **C.** Relation between simulated cell layer resistance (open blue squares) and capacitance (orange squares) and villi area ratio, showing that the experimentally observed decrease in cell layer resistance and increase in capacitance resulted from villi formation.

A. Six-electrode chip **B. Exploded view** **C. Measured impedance spectra** **D. Reconstructed villi surfaces**

Figure 5-6 | Chip design and experimental data obtained from Caco-2 gut epithelium cultured dynamically inside a six-electrode device. **A.** Six-electrode gut-on-a-chip device mounted in a chip holder. **B.** Exploded view of chip and holder, showing two PDMS parts (200 μm and 800 μm high) with laser-cut channels (1 mm wide) separated by a porous PDMS membrane, sandwiched between two PC substrates with integrated semi-transparent gold electrodes (each 1 mm wide). Electrical interfacing is facilitated with the printed circuit board of the chip holder. **C.** Impedance spectra obtained in these dynamically cultured guts-on-chips during the 12-day culture period show the same characteristics as **Figure 5-2B**. Day 1 is before introducing fluid flow. The inset shows the used four-terminal electrode configuration. **D.** Villi surfaces reconstructed from confocal images of five microfluidic devices fixed on five different days, showing an increase in number and height of villi. The images were edited for optimal visibility of the villi. Scalebar is 100 μm .

Figure 5-7 | The measured cell layer capacitance predicts the villi area ratio as villi differentiation parameter for nine chips on five days during a 12-day culture period. Of each chip a height map with color scale is shown below the graph. Scalebar is 100 μm .

for impedance spectroscopy, as is shown in the inset of **Figure 5-6C**. The electrical simulations were adapted to fit this chip design and in **Appendix D.7** the simulation results for this chip can be found. Analogously to **Figure 5-3**, **Figure 5-4** and **Figure 5-5**, the potential distributions and sensitivity distributions were visualized for the six-electrode chip and a calibration curve was derived to enable normalization of monolayer resistance to TEER. These results illustrate the adaptability of the simulations to fit any chip design and electrode configuration.

During a 12-day culture period, impedance spectra were recorded daily (**Figure 5-6C**) and every 2-3 days a subset of chips was fixed and stained. Confocal imaging followed by image analysis (Imaris, Bitplane) resulted in reconstructed villi surfaces throughout the culture period (**Figure 5-6D**). From these reconstructed surfaces the villi area ratio was determined (following the same definition as **Figure 5-5B** inset). In addition, the cell layer capacitance was derived from the impedance spectra recorded on the last day of culture for each chip. An increase in capacitance is accompanied by an increase in villi area ratio (**Figure 5-7**). Therefore, we concluded that cell layer capacitance has potential to predict the degree of villi differentiation without the need for microscopy.

5.6. Conclusion

In this work, we have demonstrated novel methods for combining impedance spectroscopy with electrical simulations to reveal cell layer characteristics in a gut-on-a-chip. Impedance spectroscopy allowed the immediate determination of the measured cell layer resistance, eliminating the need for measuring blanks. Furthermore, a four-terminal sensing approach allowed us to record changes in the electrical properties of the cell layer without being influenced by the double layer capacitance at the electrode-electrolyte interface or the contact and lead resistances. Electrical simulations provided insight in the measurements, for example by explaining the -180° phase shifts seen in experimental and simulated impedance spectra. Furthermore, simulations enabled normalization of measured cell layer resistance to TEER values by determining the effective area that contributed to that measured resistance. This normalization allows comparison of TEER values across platforms. Furthermore, these simulations provided insight in the influence of villi formation on impedance spectra and we showed that cell layer capacitance is a promising predictor of the state of villi formation.

As we have shown, the electrical simulations can be adapted to fit other electrode configurations and channel sizes. In addition, also other chip designs, different measurement methods and 3D geometries can be adopted, providing general methods for broad application in the field of organs-on-chips to reveal cell layer characteristics of functional tissues cultured inside the devices. Furthermore, this method can help deciding on the best electrode locations and chip designs to arrive at uniform electric fields. Alternatively, electrode configurations can also be designed to achieve local probing of cell layers [27], comparable to multi-electrode arrays in which a cell layer that is cultured directly on the electrode-integrated substrate can be locally probed [10, 36, 37].

5.7. References

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Towards a multiplexed organ-on-chip

To increase the throughput of organ-on-chip research a multiplexed chip with eight parallel channels was designed. Via a common access port, all eight channels can be addressed at once for cell seeding and parallelized culture under identical environmental conditions, without the need for extra pipetting steps. Via eight separate access ports, the channels are individually addressable, allowing eight different culture conditions in a single chip with simple actuation. Preliminary results illustrate the applicability of the parallelized culture and the eight different conditions in a one-layer device. Furthermore, the feasibility of creating a two-layer device using the same chip design was demonstrated as well as the applicability of this device for culture of blood-brain barrier-specific endothelial cells. Lastly, as this device is designed to allow functional readouts of barrier properties, the operation principles of permeability and transendothelial electrical resistance measurements are explained.

6.1. Chips for multiplexed cell culture

The previous chapters have described the development and use of a blood-brain barrier (BBB)-on-chip and a gut-on-chip device, with a focus on enabling measurements of barrier function using transendothelial or transepithelial electrical resistance (TEER). This chapter will focus on increasing the throughput of organ-on-chip research, moving towards a multiplexed organ-on-chip device. Such multiplexed devices hold promise to increase throughput by allowing parallel cell cultures in a single microfluidic chip with the same ease-of-handling as chips with a single culture. Furthermore, while the tissues inside a single device are subjected to the same environmental conditions during the culture period, different experimental stimuli can be applied to the separate tissues in the multiplexed chip and functional readouts can be compared, analogously to standard well plates. This is expected to improve reproducibility and next to saving fabrication time, also experimental time is expected to be reduced by including multiple functional tissues in single chips.

In the next section, the multiplexed chips for cell culture that are available in literature are discussed, followed by a list of requirements for our multiplexed organ-on-chip. Then the chip design is discussed and proof-of-concept experiments and results are described. This chapter ends with a conclusion on these first steps towards a multiplexed organ-on-chip device and recommendations for further research, including suggestions for the incorporation of functional readouts in the multiplexed chip.

6.1.1. Current multiplexed cell culture chips

In literature, there are several reports of multiplexed microfluidic chips used for the parallel culture of cell monolayers. The simplest designs contain multiple channels that branch from a single common inlet and outlet, enabling parallel cultures of cell monolayers inside a single chip [1-3]. An example is shown in **Figure 6-1 I**. However, as these channels are not individually addressable, only a single experimental condition can be tested per chip. Also, the effluents of each channel cannot be analyzed separately, as they are collected in a single outlet.

Alternatively, there are reports of chips with parallel culture chambers and an integrated gradient generator to enable on-chip creation of multiple experimental conditions with increasing concentrations of a reagent of interest [4-8]. Although useful, these chips are limited to a range of concentrations of a single reagent, not allowing parallel testing of multiple reagents, as can be seen in **Figure 6-1 IV**.

To enable the creation of multiple conditions using multiple reagents in a single chip, there have been reports of microfluidic chips with multiple channels for cell culturing, each with their own inlet and outlet (**Figure 6-1 II**). Via these separate inlets, the parallel cultures can be subjected to different conditions and via the separate outlets the effluents can be collected for analysis [9-12]. While these chips may save fabrication

Figure 6-1 | Examples of multiplexed microfluidic chips used for the parallel culture of cell monolayers, reprinted and adapted with permission from the indicated references. **I.** Chip with parallel channels with a common inlet and outlet [2]. **II.** Chip with parallel channels with separate inlets and outlets [9]. **III.** Two-layer device with parallel channels for multiple shear stresses and one collection channel [16]. **IV.** Parallel culture chambers with a common inlet and outlet and a gradient generator [4]. **V.** Individually addressable parallel culture chambers with complex actuation [13]. **VI.** Chip with parallel microchamber arrays and on-chip reservoirs [17].

time, these chips do not save experimental time or improve ease of actuation, as each channel requires separate pipetting or pumping actions. Chips with interconnected culture chambers that allow the parallel exposure to multiple reagents are also reported, but these generally have complex actuation protocols with valves and many pumping lines [13-15], as can be seen in Figure 6-1 V.

A simpler solution for parallelization was provided by having eight cell culture arrays, comprised of five microchambers for cell culture, each connected to one of eight separate medium reservoirs and to a single waste chamber, as is shown in **Figure 6-1 VI** [17]. By pressurizing all eight reservoirs with a single pump, eight different conditions are applied to five parallel cultures each in a single chip. In contrast, with a single pumping action through the common waste chamber, cells are introduced simultaneously in all culture chambers. However, this chip has a large footprint ($\pm 18 \text{ cm}^2$) due to the on-chip reservoirs. In addition, converting this design into a two-layer device in which barrier functions can be tested, is expected to be challenging.

Lastly, there is one multiplexed BBB-on-chip device with two layers reported, shown in **Figure 6-1 III**. The first layer contains four parallel channels for cell culture that are separated by a porous membrane from the second layer with a single channel [16]. The four parallel channels can be simultaneously addressed via a common inlet and separately analyzed by their four separate outlets. This chip was specifically developed for testing of four different shear stresses in a single chip, with high channel width-to-height ratios (ranging from 3.7 to 15.7) for uniform shear stress across the channel width. However, while integrated electrodes allowed parallel TEER measurements to assess barrier function, the single collection channel limits the applicability of parallel permeability testing as the reagents passing from the apical side to the basal side of the four parallel cell barriers will mix.

6.1.2. Design requirements

From the currently available multiplexed chips in combination with previous experiences in BBB chip design and use, a list of requirements is derived. The multiplexed organ-on-chip device should:

- Allow a minimum of eight parallel monolayer cultures in a single microfluidic chip, that are subjected to identical environmental conditions during culture, but allow application of different experimental stimuli on each parallel culture;
- Have simple actuation for ease-of-handling during culture and experiments;
- Have a fabrication protocol with no more steps than a chip of similar design without parallel channels;
- Allow individual collection and analysis of effluents from each parallel culture;
- Allow functional readouts of barrier function and co-culture by creation of a two-layer device;
- Have a small footprint (maximum size 25 mm by 75 mm) to fit on microscope slides for (automated) imaging;
- Allow optical access to all parallel cultures for microscopic analysis during and after the culture period;

- o Have a uniform flow profile across the channel width to subject all cells to the same shear stress.

6.2. Multiplexed chip design

The design of the multiplexed organ-on-chip is displayed in **Figure 6-2A**. This chip has eight parallel channels, each branching from a common access port but having a separate access port as well. Via the common access port, all channels can be simultaneously addressed for e.g. introducing cells and replacing culture medium inside the chip, as is schematically shown in **Figure 6-2E**. Via the eight separate access ports, the channels can be individually addressed and eight different conditions can be created in the same chip by pulling fluids from eight separate reservoirs through the channels towards the common access port using a single syringe pump as is shown in **Figure 6-2E**. By inverting the flow, the effluents from each channel can be collected separately at the eight access ports and analyzed independently. For proof-of-concept experiments, a one-layer device can be fabricated, as is shown schematically in **Figure 6-2C**. A two-layer device can be easily created by bonding two identical chips together, of which one is inverted, and aligning the eight channels, as is shown in **Figure 6-2D**. The circles in the design of **Figure 6-2A** marking the access ports in both the top part and the aligned bottom part enable easy punching of all access holes. By having both eight apical channels and eight corresponding basal channels, the function of each barrier can be individually assessed using TEER or permeability measurements. The chip is designed to fit on a standard microscope slide of 25 mm width, making it compatible with microscopy readouts.

To allow even distribution of flow through the channels, all branches are designed to be equal in length. The width of the parallel channels is 500 μm , while the width of the channels after each junction increases consecutively to 630 μm , 794 μm and 1000 μm . Furthermore, the channel height of 50 μm ensures a uniform flow profile across the channel width, as is shown in **Figure 6-2B**, made following the method of **Section 2.3.4 of Chapter 2** [18]. To achieve a relevant shear stress τ of approximately 0.5 Pa [19, 20] on the endothelial cells cultured inside the parallel channels of this device, a volumetric flow rate of 1.2 $\mu\text{l/s}$ would be required at the common access port according to **Eq. 2-4 in Chapter 2** [21], corresponding to 0.15 $\mu\text{l/s}$ in each of the eight channels. As flows are combined at each junction, the shear stress increases to 0.8 Pa (at a channel width of 630 μm , with factor f^* linearly interpolated), 1.3 Pa (for 794 μm) and 2 Pa (for 1000 μm). Although the shear stress is thus increasing from the separate access ports to the common access port, the shear stress values are still within the physiological range of 0.3-2 Pa for brain capillaries mentioned in literature [19, 20].

Figure 6-2 | **A.** Design of multiplexed chip with eight parallel channels having eight separate access ports, equidistantly spaced from the common access port. **B.** Illustration of flow profile inside the parallel channels, modeled with MATLAB R2016b (The MathWorks, Inc.) [18]. **C.** Schematic representation of the one-layer device, comprised of one layer with channel imprints bonded onto a glass microscope slide. **D.** Two-layer device design. Two identical PDMS parts, of which one inverted (blue), can be bonded together with a membrane (grey) in between. **E.** Operation principles. From the common access port, all channels can be addressed at once. From each separate access port, all channels can be addressed individually.

6.2.1. Estimating risk of cross-talk

Since the eight parallel channels are not completely separated fluidically, there is a risk of cross-talk between the conditions in adjacent channels. There are two possible contributors: diffusion and advection of solutes into the adjacent channel. The risks of cross-talk by these two contributors are estimated in the following sections.

Contribution of diffusion

To estimate the risk of contamination of adjacent channels by diffusion during an experiment, the Péclet number is calculated for typical experimental parameters. The

Péclet number is defined as the ratio of the rate of advective transport of a certain solute and the rate of diffusion perpendicular to that flow, resulting in the following equation:

$$Pe = \frac{u \cdot l}{D} \quad [] \quad \text{Eq. 6-1}$$

In this equation, Pe is the dimensionless Péclet number, u is the flow velocity (m/s), l is the characteristic length of diffusion (m) and D is the diffusion coefficient of the solute (m²/s).

For a conservative estimation, a characteristic length of $l = 315 \mu\text{m}$ is taken, which is half of the width of the channel after the junction. This is conservative because solutes that have diffused over this length do not yet reach the parallel part of the channels and have not yet contaminated the culture that is analyzed. For the first experiments a volumetric flow rate of $20 \mu\text{l/min}$ was used at the common access port. In one of the eight channels with $50 \mu\text{m}$ height and $315 \mu\text{m}$ width this results in a linear flow rate of $u = 2.6 \text{ mm/s}$. Lastly, as diffusion coefficient $D = 1 \cdot 10^{-9} \text{ m}^2/\text{s}$ is taken, which reflects the order of magnitude of the diffusion coefficient of small molecules in water. For these parameters, the resulting Péclet number is $Pe = 8 \cdot 10^2$. This indicates that advective flow is dominant over diffusion and that the flow rate can be decreased by a factor of ~ 800 before diffusion starts to play a significant role. Due to the conservatively chosen characteristic length and diffusion coefficient, in practice even lower flow rates can be acceptable. Concluding, diffusion of solutes into adjacent channels forms is negligible at the flow rate of the first experiments.

Contribution of advective flow

The chip is designed to have flow equally split at each junction, under the condition that the fluid reservoirs at the eight separate access ports are identical. In that case, all fluid will move to the common access port and there will be no cross-talk between conditions. However, when the fluid reservoirs are not identical, for example because of a difference in fluid height, fluid can flow into adjacent channels. To estimate the risk of this advective flow during an experiment, the following equation can be used:

$$\Delta P = Q \cdot R_h = Q \cdot \frac{12 \cdot \mu \cdot L}{w \cdot h^3} \quad [\text{Pa}] \quad \text{Eq. 6-2}$$

In this equation, ΔP is the pressure difference (Pa) between two ends of a channel with hydrodynamic resistance R_h (Pa·s/m³) through which a fluid flows at a rate of Q (m³/s). The hydrodynamic resistance is dependent on the dynamic viscosity μ of the fluid (Pa·s) and the channel length L (m), width w (m) and height h (m), where $w \gg h$ [22].

When in experiments fluid is pulled from the common access port at a volumetric flow rate of $20 \mu\text{l/min}$, this should result in an equal flow of $2.5 \mu\text{l/min}$ in each of the eight parallel channels. However, when the fluid levels in the reservoirs at the eight separate access ports is unequal, there are pressure differences between the channels

giving rise to flow in a different direction (towards the reservoir with the lowest fluid level). To result in a net flow directed towards the common access port in spite of any differences between the reservoirs, the maximum pressure difference allowed between two adjacent reservoirs can be determined. In that case, we assume that all fluid flows directly from a channel into a neighboring channel with a lower fluid level at a flow rate of $Q = 2.5 \mu\text{l/min}$ and with viscosity $\mu = 0.7 \text{ mPa}\cdot\text{s}$ [19]. The distance between two adjacent access ports through the channels is approximately $L = 25 \text{ mm}$ (measured through the channel centers) and the channel width and height are $w = 500 \mu\text{m}$ and $h = 50 \mu\text{m}$, respectively. Then, the pressure difference giving rise to this flow rate is calculated to be $\Delta P = 1.4 \cdot 10^2 \text{ Pa}$. This pressure difference corresponds to a height difference of $h = 14 \text{ mm}$, using the equation $P = \rho \cdot g \cdot h$ (with $\rho = 1 \cdot 10^3 \text{ kg/m}^3$ as density of water and $g = 1 \cdot 10^{-1} \text{ m/s}^2$ as gravitational acceleration). Height differences of this relatively small order of magnitude could practically arise when no attention is paid to equalizing the fluid levels inside the reservoirs. Therefore, the risk of advective flow has to be taken into account when designing experiments. In conclusion, while diffusion forms a negligible risk for cross-talk between conditions, the risk of advective flow, e.g. as a result of different fluid heights in reservoirs, needs to be minimized to prevent cross-talk, especially when lower flow rates are required.

6.3. Experimental

6.3.1. Chip fabrication

The multiplexed design presented in **Figure 6-2A** was translated to a photolithographically patterned mold by spin-coating a $50 \mu\text{m}$ thick layer of the negative photoresist SU-8 on top of a silicon wafer and selectively exposing the SU-8 to ultraviolet (UV) light through a photomask patterned with the chip designs. Next, poly(dimethylsiloxane) (PDMS) base agent and curing agent (Sylgard 184 Silicone elastomer kit, Dow Corning) were mixed at a 10:1 weight ratio and after desiccation the mixture was poured onto the mold. After baking at 60°C for at least four hours, the PDMS slab was removed from the mold and cut into separate chip parts. Access holes were punched with a 1 mm diameter biopsy punch (Integra Miltex).

One-layer device

For proof-of-concept experiments, one-layer devices were used comprising a chip part and a glass microscope slide. Before bonding, dust was removed from both the chip surface and the glass slide using Scotch tape. Next, these surfaces were treated with oxygen plasma for 45 s at 500 mTorr (PDC-001 Plasma Cleaner, Harrick). The activated surfaces of the PDMS parts were carefully pressed onto the glass microscope slides and baked again at 60°C for at least one hour.

Two-layer device

To move towards a multiplexed BBB-on-chip, two-layer chips were fabricated. As is shown in **Figure 6-2D**, a two-layer device can be fabricated using two identical PDMS parts with channel imprints, of which one is inverted. These two parts were glued together with a porous membrane in between, analogously to the BBB chip described in **Chapter 3**. As following the same protocol for leakage-free bonding resulted in mortar blocking the channels, the viscosity of the mortar was lowered by increasing the volume ratio toluene/PDMS from 3:5 to 2:1. Then, a layer of $\sim 1\ \mu\text{m}$ thickness instead of $\sim 3\ \mu\text{m}$ results after spin-coating [23]. Furthermore, the PDMS parts were placed directly on top of the spin-coated mortar instead of relying on transferring mortar with an ink roller to prevent it from being pushed into the channels. Because of the thinner layer of mortar transferred onto the chip, stamping the edges of the polycarbonate (PC) membrane in the mortar is even more important for proper membrane enclosure. In addition, alignment of all eight channels of both chip parts proved to be more challenging, but was achieved successfully by using an x-y-z translation table fitted to a microscope. Lastly, the chips were baked for six hours.

6.3.2. Cell culture

The cells cultured in the multiplexed chip are immortalized human cerebral microvascular endothelial cells (hCMEC/D3 cell line, kindly provided by Dr. P.-O. Couraud, INSERM, Paris, France) [24]. These cells were cultured with endothelial growth medium 2 (EGM-2: EBM-2 with EGM-2 SingleQuots, Lonza) in collagen I-coated T75 culture flasks (CELLCOAT, Greiner Bio-One). The cells were incubated at 37°C in humidified air with 5% CO_2 . Medium was refreshed every 2-3 days and confluent flasks were subcultured or used for experiments (passages 28-33).

On-chip culture

Prior to cell seeding, the microfluidic chips were exposed to oxygen plasma to ensure complete filling of the channels and to promote cell attachment. Subsequently, the chips were rinsed with phosphate-buffered saline (PBS; Sigma) and coated with fibronectin ($40\ \mu\text{g}/\text{ml}$) for two hours at 37°C . After flushing out any air bubbles, the chips were filled with EGM-2 and stored at room temperature until cell seeding. Next, hCMEC/D3 cells were collected from a confluent flask using 0.05% trypsin-EDTA (Gibco) and suspended in fresh EGM-2 at $20 \cdot 10^6$ cells/ml (corresponding to $1 \cdot 10^5$ cells/ cm^2 in the channels). The cell suspension was pipetted into all eight channels simultaneously with a single pipetting action through the common access port. After one hour of static incubation at 37°C and 5% CO_2 , non-attached cells were washed away with EGM-2. Medium was refreshed twice daily by inserting medium-filled pipette tips in the common access ports of each device, replacing the medium inside the chip by gravity-driven flow.

Live/dead staining

To assess the viability of the cells cultured inside the multiplexed chip, live/dead staining was performed using a solution containing 1 μ l calcein-AM, 4 μ l ethidium homodimer-1 (EthD-1) (Live/Dead Viability/Cytotoxicity Kit for mammalian cells, Invitrogen) and 1 drop NucBlue (Ready Probes reagent, Molecular Probes, Life Technologies) in 60 μ l PBS after flushing the channels two times with PBS. Introduction of the reagents and flushing is performed with single pipetting actions through the common access port. After incubation for 30 minutes at 37°C and 5% CO₂, fluorescence microscopy was performed using the EVOS FL Cell Imaging System (Life Technologies; green filters (ex 470/22, em 510/42) for calcein (product of enzymatic cleavage of calcein-AM by living cells; ex 495 nm, em 515 nm), red filters (ex 531/40, em 593/40) for ethidium homodimer-1 (cannot cross intact cell membranes; ex 528, em 617 in presence of DNA) and blue filters (ex 357/44, em 447/60) for NucBlue). The contrast of the resulting images was equally enhanced and subsequently merged in ImageJ (Wayne Rasband, NIH, version 1.51j8).

6.3.3. Proof-of-concept individually addressable channels

For all experiments showing the ability to individually address the channels, the common access port of a one-layer chip was connected with tubing (Tygon) and blunt needles (Metcal) to a 5 ml syringe (BD). The syringe was inserted in a syringe pump (Harvard PHD 2000 Programmable Syringe Pump), set at a flow rate of 20 μ l/min in withdrawal-mode.

Multiple dyes

To illustrate the possibility of creating eight different conditions in a single chip, food dyes (JO-LA red, blue, yellow and green, Bharco Foods) were introduced in each channel of a chip without cells. To that end, the chip was connected to the syringe pump as described before. To prevent air from entering the channels, all separate access ports were covered with PBS. Then, pipette tip reservoirs filled with different dyes were inserted into the eight access ports, resulting in dye being pulled through the channels. When the pipette tips empty, they can be refilled by pipetting extra fluid on top of the fluid present in the pipette tip reservoir.

Selective trypsin-mediated detachment

To show that there is no cross-talk between the channels, trypsin was introduced to every other channel of a chip in which cells were cultured for three days. To that end, the chip was connected to the syringe pump as described before and the separate access ports were covered with PBS. Pipette tip reservoirs with either trypsin or EGM-2 were inserted into the separate access ports and these solutions were pulled through for 30 minutes at a flow rate of 20 μ l/min ($\tau = 0.14$ Pa according to Eq. 2-4). Then,

Figure 6-3 | Result of creating eight different conditions in a single chip. Dyes are pulled through the channels from eight separate pipette tip reservoirs using a single syringe pump, withdrawing at a rate of 20 µL/min. No cross-talk is seen between the eight channels.

EGM-2 was added to all channels and flow was applied for another 15 minutes to ensure all trypsin was removed from the chip to stop the trypsinization. After stopping the flow and disconnecting the tubing, a live/dead staining was performed as is described previously, to show the effect of trypsin on the cells in each channel.

6.3.4. Cell culture in two-layer device

To show the applicability of the two-layer device for cell culture, cells were introduced into the top channel of a device following the method described before. The formation of a monolayer of hCMEC/D3 cells was monitored using phase contrast microscopy (EVOS FL Cell Imaging System, Life Technologies). After four days the viability of the BBB cultured inside the device was assessed with a live/dead staining, performed as described before.

6.4. Results & discussion

6.4.1. Multiple dyes

In Figure 6-3 the result of the proof-of-concept experiment with dyes is displayed, showing that the eight channels are individually addressable and that this method can be used to create different conditions in adjacent channels. It can be seen that there is no cross-talk between the channels; only where the channels meet, the dyes come together and flow laminarly to the common access port, while slight diffusive mixing occurs.

This working principle relies on the identically designed channels, the eight pipette tip reservoirs in the separate access ports and the pump withdrawing fluid from the common access port. As was calculated in **Section 6.2.1**, a difference in fluid level in the reservoirs can result in cross-talk between the channels. As long as care is taken that there are minimal differences in fluid levels between the reservoirs (less than the order of magnitude of the estimated 14 mm), there will be no cross-talk. This was successfully achieved, as evidenced by **Figure 6-3**, by adding the same amount of fluid to each pipette tip reservoir.

6.4.2. On-chip cell culture and trypsin exposure

Successful seeding and culturing of hCMEC/D3 endothelial cells was achieved in a multiplexed device, as can be seen in **Figure 6-4A**. Equal seeding density was achieved in every channel with a single pipetting action through the common access port, which resulted in visually equal monolayers in all channels after three days of culturing.

Then, the common access port was connected to a pump and in every second channel a pipette tip reservoir with trypsin was introduced, while EGM-2-filled pipette tip reservoirs were inserted in the other channels. After 30 minutes, all cells had detached and were flushed out of the trypsin channels, while the cells in the EGM-2 channels remained viable and unharmed (**Figure 6-4B**). The fact that no cross-talk occurred between adjacent channels was especially well visible where the channels meet (**Figure 6-5**). There cells were flushed out from the trypsin channel and from half of the combined channel, while the cells in the EGM-2 channel and the other half of the combined channel remained unaffected. This shows that this chip is suitable for creating eight independent conditions for the parallel cultures.

Although for these two proof-of-concept experiments the volume available in the pipette tip reservoirs was sufficient or only needed small refills, longer exposure times might be needed for other experiments. To achieve that, the flow rate can be lowered, keeping in mind that the risk of cross-talk by advection then increases. At lower flow rates a height difference smaller than ~14 mm can already result in advection into adjacent channels. Alternatively, larger reservoirs can be used on-chip or off-chip. Also in that case, it is very important that the larger reservoirs are designed properly, by using the same tube lengths and reservoir heights for each reservoir, as to avoid pressure differences between the reservoirs and the associated uneven flow. An added advantage of larger and wider reservoirs is that the applied flow affects the fluid height less than for narrow pipette tip reservoirs.

6.4.3. Two-layer multiplexed device

Two-layer multiplexed devices with appropriately enclosed membranes resulted from following the fabrication protocol described for the BBB chip in **Chapter 3** with the

Figure 6-4 | A. After three days of culture, very similar endothelial monolayers were observed in the eight parallel channels of a multiplexed chip. The endothelial cells were introduced into the chip with a single pipetting action through the common access port. The inset shows where the images were approximately taken. **B.** The channels were individually addressed with trypsin or EGM-2 culture medium. Cells were flushed out of the trypsin channels after 30 minutes exposure, while the cells in the EGM-2 culture medium channels remained live and unaffected.

Figure 6-5 | Where two channels meet, the effect of exposure to trypsin or EGM-2 is clearly visible. Cells are flushed out of the trypsin channel, while the cells inside the EGM-2 channel remained unaffected. Partly detached cells in the middle of the channel, visible as brighter green, indicate the transition between the two flows. The inset indicates where the image was approximately taken.

multiplexed PDMS chip parts. The only changes needed in the fabrication protocol were increasing the volume ratio toluene/PDMS of the mortar, performing direct contact transfer of mortar onto the chip parts, stamping the membrane edges into the mortar and baking for six hours.

When hCMEC/D₃ cells were cultured inside this two-layer device, monolayers were formed in each channel, as can be seen by phase-contrast microscopy on the PDMS surface during the culture period (**Figure 6-6A**). Live/dead staining at the end of the culture period revealed monolayers on the PC membrane as well (**Figure 6-6B**). The live/dead staining also showed that the cells were viable inside the two-layer multiplexed chip. However, it can also be seen that the channel walls are not entirely straight, indicating that mortar has entered the channels on top of the membrane. Although this will decrease the surface area through which transport can take place between the apical and basal channels, it is expected to have a negligible influence on the functionality of the multiplexed BBB device. Otherwise, this can be addressed by further decreasing the mortar viscosity, provided that the membrane is enclosed successfully, and improving the transfer of mortar onto the PDMS parts or by increasing the channel height.

From this experiment it was concluded that the two-layer multiplexed device is applicable for cell culture and BBB replication. Next, enabling measurements of the barrier function of the eight separate BBBs is of interest. To that end, conceptual methods for permeability and TEER measurements are explained at the end of this chapter.

Figure 6-6 | Cell culture in the two-layer multiplexed BBB device. **A.** Formation of a monolayer of hCMEC/D3 cells is seen in the multiplexed BBB device during the culture period. Due to membrane opacity, phase-contrast microscopy was only possible on PDMS surfaces. The remaining six channels had similar results. Scalebar is 200 μm . **B.** Live/dead staining of hCMEC/D3 cells at the end of the culture period revealed viable cells (green) on top of the PC membrane. However, the absence of entirely straight channel walls indicates that some mortar has entered the channels. Scalebar is 200 μm .

Subsequently, the eight BBBs can be subjected to eight different conditions and their effect on the barrier tightness can be assessed in a single chip.

6.5. Conclusion

In this chapter, proof-of-concept is provided for a multiplexed organ-on-chip. In a device comprised of a single PDMS layer with channel imprints bonded to a glass microscope slide, it was shown experimentally that in one chip eight different conditions can be created, while all channels can still to be addressed at once as well. To that end, the multiplexed chip design has eight parallel channels, each equidistantly branched from a common access port, while each channel has their own separate access port as well. Via the common access port, parallelized culture under identical environmental conditions was achieved without the need for extra pipetting steps. Via the eight separate access ports, the channels were individually addressed using a single pump, allowing eight different culture conditions in a single chip with simple actuation.

Also, these separate access ports allow the separate collection of effluents from each culture when they are required for the individual study of the function of each culture.

In addition, the feasibility of creating a two-layer device using the same PDMS parts was demonstrated as well as the applicability of this device for culture of BBB-specific endothelial cells. The number of steps in the fabrication protocol of the two-layer device is the same as for the fabrication of the original BBB chip, with alignment as only point of extra attention.

The small footprint of the multiplexed chip of 25 mm by 28 mm allowed up to two chips to be fitted on a microscope slide for easy imaging. In addition, the transparent PDMS and glass allow full optical access to all parallel cultures in the one-layer device. The two-layer device allows fluorescence-aided visualization of the cells cultured on the PC membrane. Furthermore, although not fully explored in this work, the small height-to-width ratio of the channels gives rise to a mostly uniform flow profile across the channel width. Because of the equidistant branching, physiologically relevant shear stress can be theoretically applied on all channels with a single pump. Lastly, although not experimentally shown, the two sets of channels in the two-layer device in principle allow co-culture of BBB endothelium with brain-specific cells and allow functional readouts of barrier properties, as is conceptually explained in the next section. In conclusion, the presented multiplexed device has met or has the possibility to meet all requirements presented in the introduction.

6.6. Outlook

As is stated before, the cell culture experiments in two-layer devices showed that viable hCMEC/D3 monolayers could be seeded and maintained in the top channel and on top of the membrane. In addition, in one-layer chips it was shown that the chip design allowed the creation of eight independent conditions using the separate access ports. A next step would be to measure the barrier function of the eight parallel BBBs and test the effect of different conditions on the barrier tightness. The conceptual methods of for this will be explained here.

6.6.1. Permeability measurements

As a functional test of barrier tightness, the molecular permeability can be determined, as was explained in **Section 2.3.1** of **Chapter 2**. In the multiplexed device, the permeability can be determined following the principle depicted in **Figure 6-7A**. Via the common access port of the top part, functioning as apical compartment in which brain endothelium is cultured, culture medium with a (fluorescent) tracer can be introduced using a syringe pump. In addition, via the common access port of the bottom part, functioning as basal compartment, blank culture medium can be introduced at a steady flow rate using a second syringe (on the same syringe pump). The effluents from each

Figure 6-7 | A. Operation principle of permeability measurements in the two-layer multiplexed device, using only two syringes in a single syringe pump. Medium with a tracer or blank medium is introduced via the common access port and samples with transported tracer are collected at each separate access port of the bottom channel. **B.** Multiplexed chip design with electrode channels. To facilitate insertion and fixation of the electrodes (200 μm diameter wires), the electrode channels are 250 μm high. **C.** A membrane that only has pores in the center prevents crosstalk between intersecting fluidic and electrode channels of the apical (pink) and basal (blue) layers and thus facilitates TEER measurements in the two-layer multiplexed device.

separate bottom access port can be collected and analyzed to determine the amount of tracer that has crossed the cellular barrier. Using Eq. 2-1 and Eq. 2-2 the permeability coefficient can be determined for all eight BBBs. In addition, also when different conditions are created by pulling different solutions from reservoirs at the eight separate access ports, the permeability can be measured. In that case, a tracer added to all reservoirs results in the same gradient across the cultured BBBs as in the previously described method, so that the permeability coefficients can be determined.

Potential challenges

Important for permeability measurements is that the single pumping actuation results in the same, known flow rate in every parallel channel. While a slight difference in flow rate does negligibly change the concentration of solutes available to the cells and thus would not be problematic in the experiments performed in this chapter, for permeability measurements controlling the flow rate is very important to arrive at reliable results. To that end, the chip is designed to have flow equally split at each junction, but any fabrication flaws or experimental problems can change that. For example, debris or air bubbles inside the chip can increase the resistance of one or more channels, thus resulting in less flow through these channels and more flow through the remaining channels. In addition, as was explained in Section 6.2.1, differences in fluid height in the reservoirs can change the flow rate through the associated channels. Controlling these potential sources of uneven flow is expected to be challenging, but required for reliable permeability measurements.

Another challenge is expected to arise from transmembrane pressure gradients during permeability measurements. By applying flow to both the common access ports,

the flow in the channel above the membrane will be in the opposite direction of the flow below the membrane. In addition, there will be a pressure drop of $5 \cdot 10^1$ Pa along the channel, as calculated using Eq. 6-2 (with an assumed flow rate of $Q = 20 \mu\text{l/min}$ and channel length at the overlapping part of $L = 9.4 \text{ mm}$). Resulting from this, and assuming the two channels are identical, there will be a pressure difference between the two channels of $5 \cdot 10^1$ Pa where they first meet, linearly decreasing to $-5 \cdot 10^1$ Pa where they part. Using Eq. 6-3 (valid for cylinders [22]) for the PC membrane with $1 \cdot 10^8$ pores per cm^2 , thus $2 \cdot 10^6$ pores between the two channels along the positive gradient, with thickness $L = 10 \mu\text{m}$ and pore diameter $d = 0.4 \mu\text{m}$, the resulting flow rate is roughly $0.3 \mu\text{l/min}$, or about 2% of the applied flow rate. The advection resulting from these pressure differences is therefore expected to influence the permeability measurements. Decreasing the number and size of the pores in the membrane as well as decreasing the length of the parts through which transport of solutes and fluids can take place, is expected to reduce this effect.

$$\Delta P = Q \cdot R_h = Q \cdot \frac{128 \cdot \mu \cdot L}{\pi \cdot d^4} \quad [\text{Pa}] \quad \text{Eq. 6-3}$$

6.6.2. TEER measurements

To enable TEER measurements as non-invasive and quick measurements of barrier function, directly compatible with different conditions in each channel, the mold of the multiplexed chip can be redesigned to include electrode channels. Electrodes can be integrated in these channels following the same fixation protocol as with the BBB chip described in **Chapter 3** and **Appendix A.1**. To that end, the channels should be high enough to allow wire electrodes to be inserted and fixated, as is indicated in the chip design with electrode channels displayed in **Figure 6-7B**. The assembly principle is shown in **Figure 6-7C**. To prevent the glue used to fixate the top electrodes from entering the bottom channel, a membrane with pores only in the culture area is required. The first steps towards developing such membranes were promising and the fabrication principles are described in the next section.

When both the electrodes and the selectively porous PDMS membrane are incorporated in the multiplexed device, the TEER of a cell layer cultured on top of the membrane can be measured in each channel to determine the barrier function of each separate BBB. As the culture channels are long and shallow, there will be a non-uniform sensitivity distribution along the channel length and the measured “apparent TEER” has to be corrected mathematically following the models described in **Chapter 4** and **5** to arrive at the true TEER value that can be compared across platforms.

Selectively porous membrane

To restrict the transport of electric current to the straight parallel channel parts, a membrane with pores only in that area has to be fabricated (as illustrated in **Figure 6-7C**).

Two different approaches resulted in advances towards the fabrication of PDMS membranes in which pores can be selectively introduced in a specific section. The first approach was based on the fabrication process of thin PDMS membranes [25], in which we successfully introduced pores using sacrificial columns [26]. The second approach was based on a different publication about thin PDMS membranes [27], in which we introduced pores locally by etching [28]. Both fabrication processes can also be adapted to arrive at thinner membranes with larger pores than the previously used PC membranes, to enable direct cell-cell contact between the brain endothelium cultured on the membrane in the apical compartment and any co-cultured cells in the basal compartment. In addition, an added advantage of these PDMS membranes is that they are transparent and can be bonded to the PDMS parts more easily using oxygen plasma instead of mortar.

6.7. References

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Summary & outlook

In this chapter, the main results and conclusions presented in this thesis are summarized. In addition, suggestions for further research are presented, of which preliminary results for the incorporation of brain-specific astrocytes in the blood-brain barrier on chip are described in more detail.

7.1. Summary

There is a need for more predictive models for studying disease fundamentals as well as for preclinical research in order to reduce costs of drug development and promote identification of new drug targets. To address this need, organs-on-chips have been introduced recently. These organ-on-chips, which contain micrometer-sized, fluid-filled channels in which human cells can be cultured, provide opportunities for engineering a controlled culture environment that resembles the microenvironment of a certain organ by tuning mechanical, biochemical and geometrical aspects. Cells cultured in such physiological devices are expected to display more physiological behavior, thus improving the predictive value of such *in vitro* models when compared to classical well plate cultures. Moreover, *in vitro* platforms allow the use of human cells, thus overcoming the species-to-species variations often hampering translation of *in vivo* results to human clinical trials.

While organs-on-chips hold promise to refine current *in vitro* methods and in part replace animal testing, there still are challenges ahead before these new models become widely available. The work described in this thesis was therefore aimed at developing new technologies for organ-on-chip applications, focused on blood-brain barrier (BBB) and gut tissues, which are both barrier-forming tissues. In **Chapter 2** several challenges for barrier tissues-on-chips were identified, among which are lack of standardization of transendothelial / transepithelial electrical resistance (TEER) measurements and limited throughput. In **Chapter 3-5** we discussed several ways to arrive at predictive TEER measurements in organs-on-chips, while in **Chapter 6** steps towards multiplexed chips with increased throughput were reported. The main results and conclusions of these chapters are summarized next.

In **Chapter 3** it was identified that measuring TEER is challenging in organs-on-chips due to the micrometer-sized channels filled with culture medium that are often present between the electrodes and the membrane area with the cellular barrier to be measured. These fluid-filled channels have an electrical resistance that is often comparable to the barrier resistance. As a result, small changes in temperature or culture medium composition that affect the channel resistance, strongly affect the apparent TEER. To solve this, we proposed a simple and universally applicable method to directly determine the TEER in microfluidic organs-on-chips using electrodes that are integrated into both the apical and basal channels outside of the culture area, without the need for a cleanroom environment. Using four electrodes and six different measurement configurations, the TEER was directly derived independent of channel resistance properties. Therefore, this method removed large variation of non-biological origin, thus effectively isolating the TEER. While its applicability is shown in a BBB-on-chip, this simple

and robust method is also applicable to any other organ-on-chip device with two channels separated by a porous membrane.

Another problem that can arise when performing TEER measurements in organs-on-chips is addressed in **Chapter 4**. During direct current (DC) TEER measurements, often there is a non-uniform current distribution through cellular barriers and membranes due to channel geometries, which is especially the case when there is a long and narrow membrane area with microfluidic channels on either side and electrodes inserted in the access ports. The current distribution through this membrane is non-uniform so that different areas of the membrane contribute differently to the total measured resistance. When the total membrane area is then used for normalization, the TEER is overestimated. Therefore, electrical simulations were presented to correct for the effects of device geometry on the measurements. TEER measurements were simulated in a gut-on-a-chip with long and shallow channels, in which two electrodes were inserted in the access ports for DC-based TEER measurements. The model is validated by comparing the corrected TEER values obtained in a microfluidic gut-on-a-chip to a Transwell culture system. After this correction, the gut tissue cultured on chip showed TEER values very similar to the gut tissue cultured in Transwell systems. Furthermore, insight was provided into a possible origin of the large variation in *in vitro* TEER measurements seen in literature, which even occurred when identical cell lines and experimental setups were used. A simulation of Transwell TEER measurements showed that small gaps in cell coverage (0.4% of total area) were sufficient to cause a significant drop in measured TEER (80%).

Chapter 5 continues on the work presented in **Chapter 4** by expanding the electrical simulations to include four-terminal TEER measurements using alternating current (AC) signals. Furthermore, simulations were performed for different configurations of multiple electrodes integrated in the microfluidic channels of an organ-on-chip. A gut-on-a-chip model with six small integrated but transparent gold electrodes and a four-terminal AC measurement protocol was used to validate these simulations. The simulations showed that a non-uniform sensitivity distribution arose from the used electrode configuration, meaning that different membrane areas contributed differently to the measured resistance. The experimentally obtained barrier resistance measured with four-terminal impedance spectroscopy was normalized using the electrical simulations to arrive at a TEER that can be compared across platforms. In addition, the effect of villi differentiation of gut tissue on the measured impedance spectra was correctly predicted using the electrical simulations. The measured decrease in barrier resistance and increase in cellular capacitance resulted from the increase in total surface area due to villi formation. In this way, impedance spectroscopy provided a tool for monitoring villi differentiation without the need for microscopy.

Lastly, **Chapter 6** showed the first results of a multiplexed organ-on-chip to increase throughput. This chip comprised eight parallel channels that can be addressed at once via a common access port for cell seeding and parallelized culture under identical environmental conditions, without the need for extra pipetting steps. Via eight separate access ports, the channels were individually addressable, allowing the creation of eight different culture conditions in a single chip with a single syringe pump for simple actuation. Preliminary results illustrate the applicability of the parallelized culture and the eight different conditions in a one-layer device. Furthermore, the feasibility of creating a two-layer device using the same chip design was demonstrated, as well as the applicability of this device for culture of BBB-specific endothelial cells. Lastly, as this device was designed to allow functional readouts of barrier properties, the operation principles and feasibility of permeability and TEER measurements were explored.

7.2. Outlook

7.2.1. Improving physiological relevance

While the BBBs-on-chips reported in **Chapter 3** and **Chapter 6** as well as some of the recently published BBBs-on-chips [1-3] only make use of endothelial cells to replicate the BBB, there are many cellular and molecular constituents of the neurovascular unit (NVU) that influence the formation and maintenance of the BBB, as was shown in **Figure 1-1** of **Chapter 1**. Among these constituents is the brain-specific cell type of astrocytes, which are reported to induce BBB-specific characteristics in endothelial cells *in vivo*, to which they extend their cellular processes [4]. Also *in vitro*, astrocytes have been reported to enhance barrier tightness, improve the expression of polarized transporter proteins and specialized enzymes [4] as well as improve the physiological response of endothelium to biochemical stimuli [5]. In return, the presence of brain endothelium is reported to influence the differentiation of astrocytes [4]. Additionally, the astrocyte culture environment greatly influences the morphology and function of astrocytes [5, 6]. Therefore, introducing astrocytes in a suitable culture environment into the BBB-on-chip is expected to improve the physiological relevance of the model. The methods and first results of the incorporation of astrocytes in a three-dimensional (3D) culture environment into the BBB-on-chip presented in **Chapter 3** are shown in **Appendix E**. To create such an environment mimicking brain tissue, hydrogels were chosen to provide structural support for the culture of astrocytes. The successful incorporation and fixation of this cell-laden hydrogel in a microfluidic poly(dimethylsiloxane) (PDMS) channel is shown in **Appendix E** and in **Figure 7-1**. It is expected that the hydrogels with astrocytes can be introduced into the BBB-on-chip device in co-culture with the brain endothelium following the same methods and that the hydrogel is compatible with TEER measurements due to its high water content.

Figure 7-1 | Astrocytes cultured inside a collagen I hydrogel in a microfluidic channel. Green shows actin filaments and blue shows nuclei, imaged with EVOS FL Cell Imaging System (Life Technologies). Scalebar is 100 μm . Experimental details are included in Appendix E.

In addition to introducing astrocytes or other cellular and molecular constituents of the NVU, the physiological relevance of the BBB-on-chip can also be improved by subjecting the brain endothelium to the shear stress that is normally applied by blood and blood cells flowing through brain capillaries. This wall shear stress is reported to improve endothelial differentiation and cell function [7-10]. In addition, fluid flow is necessary for certain physiological processes such as the recruitment of leukocytes at the BBB [11, 12]. A relevant fluid flow can be applied to the BBB devices presented in Chapter 3 and 6 using a volume-driven or pressure-driven pump. The channel dimensions of the multiplexed BBB device were chosen such that a uniform shear stress results along the channel width. For both chips, however, recirculation of medium is advised, as the volumetric flow rates needed for a relevant shear stress of 0.5 Pa [13, 14] are 30 ml/h for the original BBB-on-chip and 4 ml/h for the multiplexed BBB-on-chip according to Eq. 2-4 and Eq. 2-5 in Chapter 2 [15].

In general, for improving physiological relevance, the trade-off between model complexity and predictive value should always be considered. If a simple model is sufficient to answer a research question, there is no need for more complex models with extra components. If, however, the model is too simple for the research question to be answered, then inclusion of more physiological factors is required [16].

7.2.2. Permeability measurements

Within the ZonMW Memorabel grant mentioned in Chapter 1, novel nanocarriers are being developed for the transport of Alzheimer medication across the BBB [17]. To test

the efficiency of the transport of these nanocarriers in the BBB-on-chip presented in **Chapter 3**, protocols for performing permeability measurements need to be developed. In these chips, flow can be applied through both the apical and basal channels and (fluorescently) labeled nanocarriers can be added to the apical fluid reservoir. By providing flow in both channels, a stable gradient is expected to arise across the cellular barrier, resulting in barrier-mediated transport from the apical to basal channel. To determine the rate of transport or permeability coefficients, the effluents can then be collected and analyzed, or analyzed on-line using *in situ* fluorescence microscopy [18, 19]. A low flow rate can help providing enough residence time at the cellular barrier and membrane so that a detectable amount of nanocarriers can move to the basal channel. To unravel the mechanism of transport, various inhibitors of known transport pathways can be added to identify which influence the rate of transport. Furthermore, microscopy can help identify the localization of the nanocarriers inside the cells during transport. At present, these permeability measurements have only been tried in Transwell plates to address difficulties with the detection of the fluorescently labeled nanocarriers and their interactions with human brain endothelial cells (hCMEC/D3 cell line) before introducing these particles into the microfluidic BBB-on-chip.

7.2.3. Device materials

The material that has been used for the BBB-on-chip and gut-on-a-chip devices in this thesis is the elastomer PDMS. This material is often chosen for organs-on-chip applications due to its biocompatibility, impermeability to water, permeability to gas, optical transparency, flexibility and rapid prototyping potential [20]. While the impermeability to water makes PDMS devices suitable for enclosing aqueous solutions and creating culture environments for (human) cells, hydrophobic molecules are readily absorbed by PDMS [21]. Unfortunately, many drug candidates with BBB transport capabilities are small hydrophobic molecules as they can pass through the barrier without the aid of transporter proteins [22]. Also, small hydrophobic tracer molecules, such as radio-labeled verapamil, are used as to assess the presence and function of efflux transporters such as P-glycoprotein (P-gp) in the BBB *in vivo* [23, 24]. The hydrophobic properties of PDMS thus makes testing promising hydrophobic drug candidates and possible tracers for determining the *in vivo* barrier function challenging. This is illustrated in **Figure 7-2**, where the absorption of verapamil by a piece of PDMS is shown (experimental details are mentioned in the caption). Therefore, research is conducted into exploring the application of different materials in organs-on-chips, for example polyurethanes [25] or cyclic olefin copolymers [26, 27], or into applying surface coatings to prevent absorption of small hydrophobic molecules [21]. Then, organs-on-chips would become available for testing a host of promising drug candidates and tracers.

Figure 7-2 | Absorption of verapamil from 400 µl solution (1 µg/ml) by a piece of approximately 150 mm² PDMS. PDMS pieces were incubated at room temperature in verapamil solutions in separate containers. At four time points one or two pieces of PDMS were removed from their verapamil solutions, which were subsequently stored at -20°C. The concentrations of these solutions (circles) were determined with liquid chromatography-mass spectrometry (LC-MS) at the University Medical Center of Groningen using calibration samples. The average per time point and the starting concentration are represented with a horizontal line segment. It becomes clear that the presence of PDMS lowers the amount of verapamil available in solution.

Furthermore, the organs-on-chips presented in this thesis have either a polycarbonate (PC) or PDMS membrane to act as a support for cell culture between the apical and basal channels. In that way, this membrane mimics the function of the basement membrane by separating apical and basal compartments and providing support. However, the composition as well as the thickness, stiffness and porosity of the basement membrane are very different from PC or PDMS membranes [28]. Different materials with physical properties closer to basement membranes can be chosen to arrive at a biomimetic membrane. Alternatively, a removable membrane can be employed as a temporary culture support, that can ideally be removed after the endothelium or epithelium has deposited their own basement membrane.

7.2.4. Applications of blood-brain barrier on chip devices

The microfluidic BBB device presented in Chapter 3 has been used in different projects. Firstly, it is used as a platform to study BBB disruption by ultrasound and microbubbles, eventually aiming at targeted drug delivery into the brain (courtesy of M.M. Aron, BUBBL group, Oxford University) [29]. The BBB device allows the ultrasound-mediated cavitation of microbubbles and monitoring of the resulting acoustic emissions. Furthermore, with simultaneously conducted TEER measurements the barrier disruption and recovery is monitored. Lastly, as this device also allows simultaneous fluorescence microscopy readouts next to acoustic and TEER readouts, this platform has the potential

to allow the study of the mechanisms behind ultrasound-mediated BBB disruption as well as the screening of novel cavitation agents [29].

Secondly, this microfluidic device has been used to mimic the blood-retina barrier (courtesy of Y.B. Arik, AST, University of Twente) [30]. To that end, human retinal pigment epithelium is cultured on top of the membrane and a vascular network is mimicked in the bottom channel. The aim of this model is to replicate age-related macular degeneration, which results from leaky blood vessels in the retina, to test new therapeutic strategies. This macular degeneration disease model on chip is aimed to address the shortcomings of current animal models of the disease, which include species-to-species differences as well as the late onset and long progression of the disease [30].

Next to these two applications that are currently being explored, we see opportunities in the use of human induced pluripotent stem cells (hiPSCs) in the BBB-on-chip [31]. Compared to freshly isolated primary human brain endothelial cells, hiPSC-derived brain endothelial cells are expected to be more readily available and have a more stable phenotype. Compared to brain-specific endothelial cell lines such as hCMEC/D3, which was used in this thesis, hiPSC-derived brain endothelial cells are expected to give rise to better barrier function and more physiologically relevant TEER values by improved tight junction expression [32]. Up until now most studies using hiPSC-derived brain endothelium have used Transwell systems for assessing BBB function [31, 33-35]. However, culturing these cells inside BBBs-on-chips can help to study the BBB function in the presence of on-line TEER measurements and under shear stress to study any flow-related effects. Furthermore, disease-specific brain endothelium can be obtained using hiPSC technology, for example by deriving iPSCs from a patient with Huntington's disease [34], to study any defects in the endothelial cells and the accompanying BBB dysfunction related to Huntington's and other neurodegenerative diseases. This can help understanding disease mechanisms and identifying new drug targets as well as moving towards personalized medicine.

7.2.5. TEER measurements and simulations

Chapters 3-5 all showed the importance of understanding TEER measurements in microfluidic devices to arrive at predictive TEER values that can be compared across platforms. In Chapter 3 we presented a method for isolating the TEER from six impedance measurements using four electrodes in a BBB-on-chip. The advantage of this method is that any changes in the electrical resistance of the microfluidic channels, which often have a resistance comparable to the TEER to be measured, have a minimized effect on the measured TEER. This electrode configuration and measurement method has recently been applied in a BBB-on-chip developed at Lund University (Sweden) to enable TEER measurements with four integrated electrodes [36].

Furthermore, in **Chapter 4** and **5** we have provided methods to simulate TEER measurements in any device following any measurement protocol, to provide other researchers with insight into these measurements and with a tool to mathematically correct their TEERs to arrive at values that can be compared across platforms. Lastly, in **Chapter 5** we showed that by performing impedance spectroscopy the barrier capacitance can be measured, which is a good indicator of villi differentiation because it reflects the increase in total surface area of the gut tissue. We hope that impedance spectroscopy and electrical simulations become widely used to improve the predictive value of TEER measurements in organs-on-chips.

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Supplementary information, figures and tables

A. Supplementary information Chapter 3

A.1. Protocols for fabrication and use of a BBB-on-chip

A video of these protocols can be found in Van der Helm et al. [1] and by scanning the QR-code. The protocols below are adapted from this publication.

- 1 Fabrication of the organ-on-chip device
 - 1.1 Make a poly(dimethylsiloxane) (PDMS) replica of a mold with the channel designs, fabricated using standard photolithography techniques, and obtain the PDMS parts of the microfluidic chip.
 - 1.1.1 Weigh approximately 27 g PDMS base agent and 2.7 g curing agent; the mass ratio needs to be 10:1. This is enough for a 3 mm thick PDMS slab on a mold with 130 mm (5 inch) diameter. Mix these components thoroughly.
 - 1.1.2 Degas the mixture in a desiccator for approximately 45 minutes to remove air bubbles.
 - 1.1.3 Meanwhile, prepare the mold for the liquid PDMS mixture by sticking clear tape around the mold or place the mold in a suitable wafer holder.
 - 1.1.4 Pour the degassed PDMS mixture onto the mold. If any air bubbles remained in the PDMS mixture or at the mold surface, degas it again for approximately 30 minutes.
 - 1.1.5 Cure the PDMS mixture in an oven at 60°C for four hours. Allow to cool down.
 - 1.1.6 In a cross-flow hood, pull the cured PDMS from the mold; the mold can be reused immediately to make more PDMS replicas.
 - 1.1.7 Cut the PDMS replica into separate top and bottom chip parts using cutting lines in the PDMS.
 - 1.1.8 Punch four holes in the top parts using a sharp biopsy punch with 1-1.2 mm diameter to form inlets and outlets. Punch from inside to outside to prevent PDMS debris from collecting in the chip. Cover the chip parts with clear tape to protect them against dust.
 - 1.1.9 Cut polycarbonate (PC) membranes from Transwell inserts into squares of approximately 3 mm by 3 mm.

- 1.2 Assemble a porous membrane leakage-free in between two PDMS parts in order to assemble a two-layer device interfaced with a porous membrane.

Note: This protocol is adapted from Griep et al. [2] and Chueh et al. [3].

- 1.2.1 Prepare a PDMS/toluene mortar using 0.7 g of PDMS base agent, 0.07 g of curing agent and 540 μ l of toluene, resulting in a 5:3 weight ratio. Vortex the mortar thoroughly.
- 1.2.2 Spin-coat 200 μ l of mortar onto a glass coverslip at 1500 rpm for 60 s (ramped at 1000 rpm/s) to acquire a thin, uniform layer of mortar.
- 1.2.3 Transfer a thin layer of mortar from the coverslip onto a bottom part and a top part of the chip with an ink roller. Put the bottom part in an oven-safe dish.
- 1.2.4 With a set of tweezers, dip the edges of a membrane into the spin-coated mortar and place it carefully in the middle of the bottom part.
- 1.2.5 Carefully place the top part on the bottom part while paying attention to the alignment.

Note: Do not exert pressure onto the chip and do not slide the top part over the bottom part to prevent mortar from entering the channels and clogging the membrane.
- 1.2.6 Cover the chip inlets with clear tape to prevent dust from entering the chip and bake them at 60°C for three hours.

- 1.3 Integrate electrodes into the side channels.

Note: This protocol is adapted from Griep et al. [2] and Douville et al. [4].

- 1.3.1 Cut platinum wire into pieces of approximately 2 cm. Clean by submerging them in acetone for 30 minutes. Rinse with water and ethanol and allow to dry.
- 1.3.2 In a cross-flow hood, put a chip on a plastic dish. Insert four platinum wires into the electrode channels of a chip using a pair of tweezers and bend them down onto the plastic dish to enable fixation to the dish in a subsequent step. Insert the wires 0.7–1 mm into the culture channel, past the T-shaped channel junction.
- 1.3.3 Apply a drop of ultraviolet light (UV)-curable glue at the electrode channel entrance and allow the glue to fill the channel around the electrode by capillary forces.
- 1.3.4 Switch on UV and cure the glue when it reaches the end of the electrode channel.

Caution: Do not look into the UV light source as this may harm your eyes.
- 1.3.5 Fixate the four integrated electrodes to the plastic dish with a two-component epoxy adhesive. This allows easier handling of the electrodes during measurements without the risk of pulling the electrodes from the chip.
- 1.3.6 Cover the chips with clear tape and bake them at 60°C for two hours. Allow to cool down and store dust-free until use. In this way they can be safely stored for up to a month.

- 2 On-chip culture of brain-derived endothelial cells
 - 2.1 Coat the chips to promote cell attachment.
 - 2.1.1 Fill both channels with phosphate-buffered saline (PBS) to wet them before introducing reagents. Check under a microscope if there are any air bubbles in the channels. If so, remove them by flushing with extra PBS.
 - 2.1.2 Fill both channels with 30 μl of 20 $\mu\text{g}/\text{ml}$ human fibronectin in PBS. Incubate at 37°C for three hours. Check for air bubbles and, if needed, flush the channels with PBS and refill with fibronectin solution.
 - 2.1.3 Flush the chips with endothelial growth medium 2 (EGM-2) and incubate them at 37°C and 5% CO_2 in an incubator for two hours.
 - 2.1.4 Measure the transendothelial electrical resistance (TEER) of the blank chips as described in **step 3** of this protocol to ensure that all of the electrodes are in direct contact with fluid in the channels. In empty chips, typical TEER values are 0.1 $\Omega\cdot\text{cm}^2$.
 - 2.2 Seed cells into the top channel.
 - 2.2.1 Remove the culture medium from a culture flask (75 cm^2 culture area) with a confluent monolayer of human cerebral microvascular endothelial cells (hCMEC/D3).
 - 2.2.2 Wash the cells with PBS. Add 2 ml of 0.05% trypsin-EDTA and incubate at 37°C and 5% CO_2 for 2-5 minutes until the cells have detached from the culture flask.
 - 2.2.3 Deactivate the trypsin with culture medium supplemented with 20% fetal bovine serum (FBS). Count the cells and calculate the total number of cells in suspension.
 - 2.2.4 Meanwhile, centrifuge the hCMEC/D3 cells at 390 $\times g$ for five minutes and remove supernatant.
 - 2.2.5 Resuspend the cell pellet in the appropriate volume of EGM-2 to arrive at a concentration of $5\cdot 10^6$ cells/ml. This results in a seeding density of $2\cdot 10^5$ cells/ cm^2 in the chip.
 - 2.2.6 Slowly pipette 30 μl of the well-mixed cell suspension into the top channel and remove the pipette from the inlet in a fluent motion while still exerting pressure.
 - 2.2.7 Check the seeding density under a microscope. A uniform distribution of cells throughout the top channel should be achieved.
 - 2.2.8 Incubate the chips at 37°C and 5% CO_2 for at least one hour. Flush out any non-attached cells with EGM-2.

2.3 Maintain the on-chip endothelial culture.

2.3.1 Twice per day, insert culture medium-filled pipette tips as reservoirs in the inlets and replace the medium inside the chip by gravity-driven flow.

Note: To avoid air bubbles from entering the microchannels and destroying the cell layer, make sure to make fluid-fluid contact between the pipette tip and the fluid on top of the chip before inserting the tip into an inlet. This can be facilitated by adding a small drop at the in/outlet prior to pipette insertion.

2.3.2 Also add pipette tip reservoirs in the outlets to prevent the channels from drying.

2.3.3 Incubate the chips at 37°C and 5% CO₂.

3 On-chip TEER measurements

3.1 Set up the TEER measurement apparatus, consisting of a lock-in amplifier and a probe cable circuit as was specified in Van der Helm et al. [5]. Alternatively, potentiostats or impedance analyzers are also suitable for these impedance-based TEER measurements.

3.2 Take the chip from the incubator and allow it to reach room temperature for at least 10 minutes. Remove any fluid from the plastic dish around the electrodes to prevent electrode bridging outside of the chip.

3.3 Take the impedance spectrum from 200 Hz to 1 MHz for each combination of two electrodes, resulting in 6 impedance spectra per chip in only 5-10 minutes. Check if the impedance spectra have the expected magnitudes and shapes to validate the TEER measurement, as is described in the **Section 3.2.3 of Chapter 3**.

3.4 Put the chip back in the incubator at 37°C and 5% CO₂ after finishing the measurement.

3.5 Process the obtained impedance spectra to arrive at a normalized TEER value.

3.5.1 Get the measured resistance $R_{i \rightarrow j}$ between electrodes i and j , as denoted in **Figure 3-2B and D**, from the resistive plateau at 10 kHz in the corresponding impedance spectra.

3.5.2 Calculate the TEER using the six measured resistances $R_{i \rightarrow j}$ corresponding to one chip at one time point by using the equation of **Figure 3-2G [5]**:

$$TEER = A_{cult} \cdot \frac{1}{4} (R_{1 \rightarrow 2} + R_{1 \rightarrow 4} + R_{2 \rightarrow 3} + R_{3 \rightarrow 4} - 2R_{1 \rightarrow 3} - 2R_{2 \rightarrow 4}) \quad [\Omega \cdot \text{cm}^2]$$

In this equation, A_{cult} is the culture area through which the resistance was measured, R_m is the resistance of the cellular barrier and the membrane, $R_{1 \rightarrow 2}$, $R_{1 \rightarrow 4}$, $R_{2 \rightarrow 3}$ and $R_{3 \rightarrow 4}$ are the resistance values measured through the cellular barrier and $R_{1 \rightarrow 3}$ and $R_{2 \rightarrow 4}$ are the resistance values measured in the straight channels [5]. The equation is derived from the equivalent circuit illustrated in **Figure 3-2B**.

3.5.3 Subtract the blank TEER from the TEER at all time points to obtain the development of the TEER in time.

- 4 Cell morphology and tight junction expression by fluorescence staining
- 4.1 Prepare the reagents for fluorescence staining.
 - 4.1.1 Dilute 37% formaldehyde at a 1:10 volume ratio in PBS to obtain a 3.7% formaldehyde solution.
 - 4.1.2 Prepare the permeabilization and blocking buffer (PBB) by adding 0.1% (v/v) Triton X-100 and 10 mg/ml bovine serum albumin (BSA) to PBS.
 - 4.1.3 Prepare the primary antibody solution by diluting mouse anti-human zonula occludens 1 (ZO-1) immunoglobulin G (IgG) to 5 µg/ml in PBB.
 - 4.1.4 Prepare the secondary antibody solution by diluting goat anti-mouse IgG Alexa Fluor 488 to 5 µg/ml in PBB.
 - 4.1.5 Prepare the nuclear staining solution by adding 1 drop of NucBlue reagent to 500 µl PBB.
- 4.2 Flush both channels twice with PBS.
- 4.3 Add the 3.7% formaldehyde solution into both channels and incubate at room temperature for 30 minutes.
- 4.4 Wash away the fixative by flushing both channels twice with PBS.
- 4.5 Add PBB to both channels and incubate at room temperature to permeabilize the cell membranes and block any nonspecific protein binding sites.
- 4.6 Add the primary antibody solution and incubate at room temperature for two hours.
- 4.7 Wash the cells three times with PBS.
- 4.8 Add the secondary antibody solution and incubate at room temperature for one hour.

Note: To prevent photobleaching of the fluorophores, protect the chips from light from this step onwards.
- 4.9 Wash the cells three times with PBS.
- 4.10 Add the nuclear staining solution and incubate at room temperature for 30 minutes.
- 4.11 Wash the cells three times with PBS.
- 4.12 Store the chips at 4°C until imaging, in a closed Petri dish to prevent drying and protected from light.
- 4.13 Image the cells inside the channel under a fluorescence microscope with suitable light source and filters.
- 4.14 For imaging at high magnification, retrieve the bottom part with the membrane and cells attached by removing the top part and electrodes. Put the bottom part upside-down on a coverslip in a drop of PBS and store at 4°C, in a wet environment to prevent drying and protected from light.

5 Tables of materials and equipment

Name of Material	Company	Catalog no	Comments/Description
Poly(dimethylsiloxane) (PDMS)	Dow Corning	1673921	Base agent and curing agent, Sylgard 184 Silicone elastomer kit
Scotch Magic tape	3M		
Biopsy punch	Integra Miltex	33-31AA-P/25	1.0-1.2 mm diameter
Polycarbonate (PC) membrane, 0.4 μ m pore size	Corning	3401	Cut from Transwell culture inserts
Toluene	Sigma-Aldrich	244511	
Platinum wire	Alfa Aesar	10287	200 μ m diameter
Norland Optical Adhesive 81	Norland Products	NOA 81	UV-curable adhesive
Epoxy adhesive	Henkel	30673	Loctite M-31 CL Hysol
hCMEC/D3 cells	-	-	Human cerebral microvascular endothelial cell line, kindly provided by Dr. P.-O. Couraud, INSERM, Paris, France
Phosphate-buffered saline (PBS)	Sigma	P4417	
Human plasma fibronectin, 20 μ g/ml	Gibco	33016015	Diluted in PBS
Endothelial growth medium 2 (EGM-2)	Lonza	CC-3162	Endothelial basal medium-2 (EBM-2) supplemented with EGM-2 SingleQuots
Trypsin-EDTA, 0.05%	Gibco	15400-054	
Fetal bovine serum (FBS)	Gibco	26140-079	

Name of Equipment	Company	Catalog no	Comments/Description
Oven	Binder	9010-0190	
Spin coater: Spin 150	Polos	SPIN150-NPP	
UV light source	Manufactured in-house	-	365 nm for 5 s at 350 mW/cm ²
Allegra X-12R Centrifuge	Beckman Coulter	-	
Incubator	Binder	CB E2 150	
Boxense	LocSense	-	Lock-in amplifier with probe cable circuit, specialized for on-chip TEER measurements

A.2. Technical discussion

In **Chapter 3**, the engineering of an organ-on-chip device and the direct determination of the transendothelial electrical resistance (TEER) of a cellular barrier cultured in the device were presented. The presented method of integrating electrodes without clean-room equipment and the direct TEER determination using four electrodes is applicable to any organ-on-chip device with two microfluidic compartments. The chip layout and geometry can be adapted to fit the requirements of the envisioned experiments, as long as the four electrodes are separated in two compartments. The four electrodes can even be conveniently inserted in the inlets of existing chips, provided that they are fixated in place for the duration of the six measurements. The leakage-free bonding method can be optimized for different membranes and channel geometries by changing the PDMS/toluene ratio. A higher toluene content results in a thinner spin-coated layer of mortar [3] and may be more suitable for shallower and narrower channels in the PDMS parts. A lower toluene content results in a thicker mortar layer [3] and may be more suitable to enclose thicker membranes between the PDMS parts.

As can be seen in the schematic impedance spectra in **Figure 3-2A** and the experimental spectra in **Figure 3-2E** and **F**, the impedance measurements are influenced by the double layer capacitance at the electrode-medium interface. Due to the small size of the electrodes inside the microchannels, the double layer capacitance can dominate over the resistive plateau of the cellular barrier in impedance spectra, complicating quantification of the TEER. To overcome this, the electrodes can be inserted further into the culture channels before fixation. This will increase the surface area of the electrode exposed to the culture medium and with that the double layer capacitance will increase as well. This results in a shift of the capacitive slope to lower frequencies, so that the resistive plateau of the cellular barrier can be more easily recognized and quantified. Although the resistance of the measured path between two electrodes will become smaller, this will not influence the TEER quantification following the presented method.

While measuring through the cellular barrier, it is possible that the extra resistive plateau cannot be recognized. This can be the result of a fluidic connection between the two channels, for example if the membrane is poorly enclosed by the mortar, leading to a measured path around the cellular barrier. In addition, there can be electrical bridging outside the chip if the electrodes are connected by a droplet of culture medium. This is generally combined with a lower measured impedance and can be solved by removing this bridge of culture medium. Lastly, if there is no resistive plateau and the measured impedance is orders of magnitude higher than expected, there may be a loose connection in the electrical wiring or at the power source.

In the future, the physiological relevance of the current blood-brain barrier (BBB)-on-chip can be increased by exposing the endothelial cells to shear stress at physiological levels, which is reported to promote BBB differentiation and increase barrier tightness and is hard to achieve in conventional *in vitro* models [6]. In addition, the bottom channel of the presented device provides a suitable compartment for brain-derived cells to be co-cultured with the endothelium. This is also expected to increase the barrier function and also enables the study of the complex interactions between relevant cell types under pathological conditions [6].

A.3. Probe cable circuit

Figure A-1 | Probe cable used in combination with lock-in amplifier in the TEER measurement setup. The probe resistance was $10 \text{ k}\Omega$, optimized for use in our BBB-on-chip. The impedance between electrode i and j can be calculated using the probe resistance, the applied voltage U_{in} and the measured voltage response U_{out} .

A.4. Influence of environmental parameters on measured impedance

Figure A-2 | A. Influence of temperature on the measured impedance in a chip. A chip was filled with EGM-2 cell culture medium and taped to a microscope heating stage (Leica MA TS). The impedance spectra were measured for each electrode pair at different temperatures after allowing the system 10-15 minutes to equilibrate to that temperature. The resulting impedances at 10 kHz are plotted here (colored lines), showing a clear decrease in resistance as a result of increasing temperature. The calculated R_m (black) shows an 11-fold decrease in sensitivity for temperature, from $-244 \text{ } \Omega/^{\circ}\text{C}$ to $-21 \text{ } \Omega/^{\circ}\text{C}$ (linearly approximated). **B.** Influence of ion concentration on the measured impedance in a chip. A chip was filled with KCl solutions with concentrations around 0.11 M, which has the same conductivity as EGM-2 culture medium (1.3 S/m at 20°C , as measured with the Mettler Toledo SevenMulti Conductivity meter). For each measurement, the chip was flushed thoroughly and taped to a microscope heating stage set at 25°C (Leica MA TS). After allowing the system 5-10 minutes to equilibrate the impedance spectra were measured for each electrode pair at the increasing ion concentrations. The resulting impedances at 10 kHz are plotted here (colored lines), showing a clear decrease in resistance as a result of increasing ion concentration. The calculated R_m (black) shows a 5-fold decrease in sensitivity for ion concentration, from $-139 \text{ } \Omega/\text{mM}$ to $-29 \text{ } \Omega/\text{mM}$ (linearly approximated).

Figure A-3 | Influence of CO₂ concentration on the measured impedance in a well. A well in a 12-wells plate was filled with 2 ml EGM-2 culture medium and left for 22 hours in an incubator (37°C , 5% CO₂) to equilibrate. Then the plate was exposed to normal atmosphere and impedance spectra were recorded during 90 minutes on a microscope heating stage set to 37°C (Leica MA TS). There was a $\pm 60\%$ increase in impedance at 10 kHz between 0 min and 90 min, indicating that there was a decrease in amount of ions resulting from the lower CO₂ concentration.

A.5. Reducing variation by using six measurements

Figure A-4 | A. Measurements of the membrane resistance in three blank chips (filled with EGM-2 culture medium). Measured impedances at 10 kHz for each electrode pair. Dashed lines correspond to measurements only through the microfluidic channels, solid lines indicate that the membrane was included in the measured path. The first measurement (at 0 days) was subtracted from all subsequent measurements. A horizontal line at 0 k Ω was expected, but large variation is observed in all three chips. For easy comparison the resistance is also displayed in units of TEER ($\Omega \cdot \text{cm}^2$). **B.** The calculated membrane resistance R_m has much less variation than the measured resistances. The first measurement was subtracted from all subsequent measurements.

C. TEER measurements in four chips with hCMEC/D3 cells. Measured impedances at 10 kHz for each electrode pair. The blank measurement (at 0 days, before adding cells) was subtracted from all subsequent measurements. There is large variation between the electrode pairs. For easy comparison the resistance is also displayed in units of TEER ($\Omega \cdot \text{cm}^2$). **D.** By calculating R_m the resistance of the membrane and cells of each chip is isolated. The first measurement was subtracted from all subsequent measurements.

A.6. References

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B. Solving (large) electrical networks

Suppose there is a network with 9 nodes (labeled **A-I**) oriented in a 3-by-3 configuration, all connected horizontally and vertically by resistors (**Figure B-1**). At node **A** an input current i_{in} is applied. Node **I** is connected to ground, serving as reference node with a potential of 0 V. Furthermore, all horizontal resistors are equal: $R_1 = R_2 = R_6 = R_7 = R_{11} = R_{12} = R_h$. Also, all vertical resistors are equal: $R_3 = R_4 = R_5 = R_8 = R_9 = R_{10} = R_v$. The directions of the currents through all resistors are marked in the image.

Figure B-1 | Network consisting of 9 nodes, all connected horizontally and vertically by resistors.

Kirchhoff's current law states that the sum of the currents in each node should equal 0 A. So for all nodes this results in:

$$\begin{aligned}
 \text{A: } & i_{in} + i_3 - i_1 = 0 \\
 \text{B: } & i_1 + i_2 - i_4 = 0 \\
 \text{C: } & i_5 - i_2 = 0 \\
 \text{D: } & i_6 - i_3 - i_8 = 0 \\
 \text{E: } & i_4 + i_9 - i_6 - i_7 = 0 \\
 \text{F: } & i_7 - i_5 - i_{10} = 0 \\
 \text{G: } & i_8 - i_{11} = 0 \\
 \text{H: } & i_{11} + i_{12} - i_9 = 0 \\
 \text{I: } & i_{10} - i_{12} - i_{in} = 0
 \end{aligned}$$

Eq. B-1

When the branch currents are expressed in node voltages and resistances following Ohm's law:

$$\begin{aligned}
 \text{A: } i_{in} + \frac{u_D - u_A}{R_3} - \frac{u_A - u_B}{R_1} &= 0 \\
 \text{B: } \frac{u_A - u_B}{R_1} + \frac{u_C - u_B}{R_2} - \frac{u_B - u_E}{R_4} &= 0 \\
 \text{C: } \frac{u_F - u_C}{R_5} - \frac{u_C - u_B}{R_2} &= 0 \\
 \text{D: } \frac{u_E - u_D}{R_6} - \frac{u_D - u_A}{R_3} - \frac{u_D - u_G}{R_8} &= 0 \\
 \text{E: } \frac{u_B - u_E}{R_4} + \frac{u_H - u_E}{R_9} - \frac{u_E - u_D}{R_6} - \frac{u_E - u_I}{R_7} &= 0 \\
 \text{F: } \frac{u_E - u_F}{R_7} - \frac{u_F - u_C}{R_5} - \frac{u_F - u_I}{R_{10}} &= 0 \\
 \text{G: } \frac{u_D - u_G}{R_8} - \frac{u_G - u_H}{R_{11}} &= 0 \\
 \text{H: } \frac{u_G - u_H}{R_{11}} + \frac{u_I - u_H}{R_{12}} - \frac{u_H - u_E}{R_9} &= 0 \\
 \text{I: } \frac{u_F - u_I}{R_{10}} - \frac{u_I - u_H}{R_{12}} - i_{in} &= 0
 \end{aligned}$$

Eq. B-2

Rewriting the equations and filling in R_h and R_v yields:

$$\begin{aligned}
 \text{A: } i_{in} &= u_A \left(\frac{1}{R_1} + \frac{1}{R_3} \right) - \frac{u_D}{R_3} - \frac{u_B}{R_1} = \\
 &\quad u_A \left(\frac{1}{R_h} + \frac{1}{R_v} \right) - \frac{u_B}{R_h} - \frac{u_D}{R_v} \\
 \text{B: } 0 &= u_B \left(\frac{1}{R_1} + \frac{1}{R_2} + \frac{1}{R_4} \right) - \frac{u_A}{R_1} - \frac{u_C}{R_2} - \frac{u_E}{R_4} = \\
 &\quad u_B \left(\frac{2}{R_h} + \frac{1}{R_v} \right) - \frac{u_A}{R_h} - \frac{u_C}{R_h} - \frac{u_E}{R_v} \\
 \text{C: } 0 &= u_C \left(\frac{1}{R_5} + \frac{1}{R_2} \right) - \frac{u_F}{R_5} - \frac{u_B}{R_2} = \\
 &\quad u_C \left(\frac{1}{R_v} + \frac{1}{R_h} \right) - \frac{u_F}{R_v} - \frac{u_B}{R_h} \\
 \text{D: } 0 &= u_D \left(\frac{1}{R_6} + \frac{1}{R_3} + \frac{1}{R_8} \right) - \frac{u_E}{R_6} - \frac{u_A}{R_3} - \frac{u_G}{R_8} = \\
 &\quad u_D \left(\frac{1}{R_h} + \frac{2}{R_v} \right) - \frac{u_E}{R_h} - \frac{u_A}{R_v} - \frac{u_G}{R_v} \\
 \text{E: } 0 &= u_E \left(\frac{1}{R_4} + \frac{1}{R_9} + \frac{1}{R_6} + \frac{1}{R_7} \right) - \frac{u_B}{R_4} - \frac{u_H}{R_9} - \frac{u_D}{R_6} - \frac{u_F}{R_7} = \\
 &\quad u_E \left(\frac{2}{R_v} + \frac{2}{R_h} \right) - \frac{u_B}{R_v} - \frac{u_H}{R_v} - \frac{u_D}{R_h} - \frac{u_F}{R_h} \\
 \text{F: } 0 &= u_F \left(\frac{1}{R_7} + \frac{1}{R_5} + \frac{1}{R_{10}} \right) - \frac{u_E}{R_7} - \frac{u_C}{R_5} - \frac{u_I}{R_{10}} = \\
 &\quad u_F \left(\frac{1}{R_h} + \frac{2}{R_v} \right) - \frac{u_E}{R_h} - \frac{u_C}{R_v} - \frac{u_I}{R_v} \\
 \text{G: } 0 &= u_G \left(\frac{1}{R_8} + \frac{1}{R_{11}} \right) - \frac{u_D}{R_8} - \frac{u_H}{R_{11}} = \\
 &\quad u_G \left(\frac{1}{R_v} + \frac{1}{R_h} \right) - \frac{u_D}{R_v} - \frac{u_H}{R_h} \\
 \text{H: } 0 &= u_H \left(\frac{1}{R_{11}} + \frac{1}{R_{12}} + \frac{1}{R_9} \right) - \frac{u_G}{R_{11}} - \frac{u_I}{R_{12}} - \frac{u_E}{R_9} = \\
 &\quad u_H \left(\frac{2}{R_h} + \frac{1}{R_v} \right) - \frac{u_G}{R_h} - \frac{u_I}{R_h} - \frac{u_E}{R_v} \\
 \text{I: } -i_{in} &= u_I \left(\frac{1}{R_{10}} + \frac{1}{R_{12}} \right) - \frac{u_F}{R_{10}} - \frac{u_H}{R_{12}} = \\
 &\quad u_I \left(\frac{1}{R_v} + \frac{1}{R_h} \right) - \frac{u_F}{R_v} - \frac{u_H}{R_h}
 \end{aligned}$$

Eq. B-3

The resulting system of equations can also be rewritten in matrix form:

$$\begin{bmatrix} \frac{1}{R_h} + \frac{1}{R_v} & -\frac{1}{R_h} & 0 & -\frac{1}{R_v} & 0 & 0 & 0 & 0 & 0 \\ -\frac{1}{R_h} & \frac{2}{R_h} + \frac{1}{R_v} & -\frac{1}{R_h} & 0 & -\frac{1}{R_v} & 0 & 0 & 0 & 0 \\ 0 & -\frac{1}{R_h} & \frac{1}{R_h} + \frac{1}{R_v} & 0 & 0 & -\frac{1}{R_v} & 0 & 0 & 0 \\ -\frac{1}{R_v} & 0 & 0 & \frac{1}{R_h} + \frac{2}{R_v} & -\frac{1}{R_h} & 0 & -\frac{1}{R_v} & 0 & 0 \\ 0 & -\frac{1}{R_v} & 0 & -\frac{1}{R_h} & \frac{2}{R_h} + \frac{2}{R_v} & -\frac{1}{R_h} & 0 & -\frac{1}{R_v} & 0 \\ 0 & 0 & -\frac{1}{R_v} & 0 & -\frac{1}{R_h} & \frac{1}{R_h} + \frac{2}{R_v} & 0 & 0 & -\frac{1}{R_v} \\ 0 & 0 & 0 & -\frac{1}{R_v} & 0 & 0 & \frac{1}{R_h} + \frac{1}{R_v} & -\frac{1}{R_h} & 0 \\ 0 & 0 & 0 & 0 & -\frac{1}{R_v} & 0 & -\frac{1}{R_h} & \frac{2}{R_h} + \frac{1}{R_v} & -\frac{1}{R_h} \\ 0 & 0 & 0 & 0 & 0 & -\frac{1}{R_v} & 0 & -\frac{1}{R_h} & \frac{1}{R_h} + \frac{1}{R_v} \end{bmatrix} \begin{bmatrix} u_A \\ u_B \\ u_C \\ u_D \\ u_E \\ u_F \\ u_G \\ u_H \\ u_I \end{bmatrix} = \begin{bmatrix} i_{in} \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix} \quad \text{Eq. B-4}$$

As node **I** is the reference node, $u_I = 0$ can be filled in and the rightmost column of the matrix can be omitted. Then, to prevent having an overdetermined system, the equation corresponding to reference node **I** (bottom row) can also be omitted in this matrix:

$$\begin{bmatrix} \frac{1}{R_h} + \frac{1}{R_v} & -\frac{1}{R_h} & 0 & -\frac{1}{R_v} & 0 & 0 & 0 & 0 & 0 \\ -\frac{1}{R_h} & \frac{2}{R_h} + \frac{1}{R_v} & -\frac{1}{R_h} & 0 & -\frac{1}{R_v} & 0 & 0 & 0 & 0 \\ 0 & -\frac{1}{R_h} & \frac{1}{R_h} + \frac{1}{R_v} & 0 & 0 & -\frac{1}{R_v} & 0 & 0 & 0 \\ -\frac{1}{R_v} & 0 & 0 & \frac{1}{R_h} + \frac{2}{R_v} & -\frac{1}{R_h} & 0 & -\frac{1}{R_v} & 0 & 0 \\ 0 & -\frac{1}{R_v} & 0 & -\frac{1}{R_h} & \frac{2}{R_h} + \frac{2}{R_v} & -\frac{1}{R_h} & 0 & -\frac{1}{R_v} & 0 \\ 0 & 0 & -\frac{1}{R_v} & 0 & -\frac{1}{R_h} & \frac{1}{R_h} + \frac{2}{R_v} & 0 & 0 & 0 \\ 0 & 0 & 0 & -\frac{1}{R_v} & 0 & 0 & \frac{1}{R_h} + \frac{1}{R_v} & -\frac{1}{R_h} & 0 \\ 0 & 0 & 0 & 0 & -\frac{1}{R_v} & 0 & -\frac{1}{R_h} & \frac{2}{R_h} + \frac{1}{R_v} & -\frac{1}{R_h} \end{bmatrix} \begin{bmatrix} u_A \\ u_B \\ u_C \\ u_D \\ u_E \\ u_F \\ u_G \\ u_H \end{bmatrix} = \begin{bmatrix} i_{in} \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix} \quad \text{Eq. B-5}$$

This can be summarized as:

$$\mathbf{G} \cdot \mathbf{U} = \mathbf{I} \quad \text{Eq. B-6}$$

Here $\mathbf{G}$ is a matrix containing the conductivities (which is the inverse of resistance) between all nodes, $\mathbf{U}$ is a vector containing the potentials in these nodes and $\mathbf{I}$ is a vector with the currents imposed onto these nodes.

To directly produce such a matrix describing the system of equations of any (large) electrical network, the following patterns or rules can be followed, as recognized in this matrix:

- o The matrix eventually has an equal number of rows and columns, both equaling the total number of nodes minus 1.

- When two nodes i and j are connected, in the matrix at place $\mathbf{G}(i, j)$ and $\mathbf{G}(j, i)$ the negative conductance between the nodes is filled in: $\mathbf{G}(i, j) = \mathbf{G}(j, i) = -1/R_{ij}$. If two nodes i and k are not connected, the conductivity between these nodes is 0: $\mathbf{G}(i, k) = \mathbf{G}(k, i) = 0$. The matrix is thus symmetrical in the main diagonal. In addition, capacitors can also be described in such a conductance matrix as their conductance is $G_C = j\omega C$.
- On the main diagonal the negative sum of each row is inserted: $\mathbf{G}(i, i) = -\mathbf{G}(i, j) - \mathbf{G}(i, k) - \dots$

C. Supplementary information Chapter 4

C.1. Numerical and analytical approach to solving large networks

The equivalent circuit model, as depicted in **Figure 4-3** can be solved using either a numerical or analytical approach. Both of these approaches are described here, including their benefits and drawbacks.

Numerical approach

To calculate the equivalent resistance of the membrane area of the chip, indicated by l_{mem} (so excluding the inlet and outlet channel resistance, as is depicted in **Figure 4-3**), the membrane can be represented by a finite number of n resistors and $2n$ nodes. Following the theory of solving electrical networks explained in **Appendix B**, a system of equations can be derived describing the conductivities between the nodes and as boundary conditions the applied current to each node.

To enable comparison with the analytical approach, transepithelial electrical resistance (TEER) measurements were simulated using an applied current i_{in} to the nodes at port a and b (**Figure 4-3**) according to the experimental setup and various TEERs in the system of equations. These input TEERs are the actual TEER values. From the resulting known conductivities and currents in the system of equations, the potential in each node can be calculated using Ohm's law:

$$\mathbf{U} = \mathbf{I} \cdot \mathbf{G}^{-1} \quad \text{Eq. C-1}$$

Then, the equivalent resistance of the membrane, as would have been measured experimentally, is calculated by dividing the potential drop between the nodes corresponding to port a and b , by the applied current i_{in} . This overall resistance is normalized to the surface area of the membrane to obtain the apparent TEER.

The amount of membrane resistors n is varied until n is sufficiently large so that the results converge to a stable value. Typically that occurs at $n = 1000$ for the chip geometry discussed in **Chapter 4**.

Analytical approach

Instead of using a numerical approximation of n resistors for the membrane, it is also possible to solve this model analytically by taking the limit for n to infinity. To that end,

we redraw **Figure 4-3** as is shown in **Figure C-1**. We define the resistors in this ladder network as follows:

$$\begin{aligned} R_m &= R_M \cdot n \\ R_t &= \frac{R_T}{n} \\ R_b &= \frac{R_B}{n} \end{aligned} \quad \text{Eq. C-2}$$

Figure C-1 | Equivalent circuit model used in the analytical approach.

The currents and voltages at the first step of the ladder, as denoted with a single accent ('), can be described by the known input voltages and currents:

$$\begin{aligned} u'_a &= u_a - i_a \cdot R_t \\ u'_c &= u_c - i_c \cdot R_b \end{aligned} \quad \text{Eq. C-3}$$

$$\begin{aligned} i'_a &= i_a - i_m = i_a - \frac{u'_a - u'_c}{R_m} = i_a - \frac{u_a - i_a \cdot R_t - u_c + i_c \cdot R_b}{R_m} \\ &= i_a \left(1 + \frac{R_t}{R_m} \right) - i_c \frac{R_b}{R_m} - u_a \frac{1}{R_m} + u_c \frac{1}{R_m} \\ i'_c &= i_c + i_m = i_c + \frac{u'_a - u'_c}{R_m} = i_c + \frac{u_a - i_a \cdot R_t - u_c + i_c \cdot R_b}{R_m} \\ &= -i_a \frac{R_t}{R_m} + i_c \left(1 + \frac{R_b}{R_m} \right) + u_a \frac{1}{R_m} - u_c \frac{1}{R_m} \end{aligned} \quad \text{Eq. C-4}$$

These equations can be rewritten in matrix form:

$$\begin{bmatrix} i'_a \\ i'_c \\ u'_a \\ u'_c \end{bmatrix} = \begin{bmatrix} 1 + \frac{R_t}{R_m} & -\frac{R_b}{R_m} & -\frac{1}{R_m} & \frac{1}{R_m} \\ -\frac{R_t}{R_m} & 1 + \frac{R_b}{R_m} & \frac{1}{R_m} & -\frac{1}{R_m} \\ -R_t & 0 & 1 & 0 \\ 0 & -R_b & 0 & 1 \end{bmatrix} \cdot \begin{bmatrix} i_a \\ i_c \\ u_a \\ u_c \end{bmatrix} \quad \text{Eq. C-5}$$

Because every step of the ladder is identical, the currents and voltages at the second step of the ladder (denoted with double accent '') will be:

$$\begin{aligned}
 \begin{bmatrix} i_a'' \\ i_c'' \\ u_a'' \\ u_c'' \end{bmatrix} &= \begin{bmatrix} 1 + \frac{R_t}{R_m} & -\frac{R_b}{R_m} & -\frac{1}{R_m} & \frac{1}{R_m} \\ -\frac{R_t}{R_m} & 1 + \frac{R_b}{R_m} & \frac{1}{R_m} & -\frac{1}{R_m} \\ -R_t & 0 & 1 & 0 \\ 0 & -R_b & 0 & 1 \end{bmatrix} \cdot \begin{bmatrix} i_a' \\ i_c' \\ u_a' \\ u_c' \end{bmatrix} \\
 &= \begin{bmatrix} 1 + \frac{R_t}{R_m} & -\frac{R_b}{R_m} & -\frac{1}{R_m} & \frac{1}{R_m} \\ -\frac{R_t}{R_m} & 1 + \frac{R_b}{R_m} & \frac{1}{R_m} & -\frac{1}{R_m} \\ -R_t & 0 & 1 & 0 \\ 0 & -R_b & 0 & 1 \end{bmatrix}^2 \cdot \begin{bmatrix} i_a \\ i_c \\ u_a \\ u_c \end{bmatrix}
 \end{aligned}
 \tag{Eq. C-6}$$

Analogously, for the n^{th} step of the ladder, the following is true:

$$\begin{bmatrix} i_{a,n} \\ i_{c,n} \\ u_{a,n} \\ u_{c,n} \end{bmatrix} = \begin{bmatrix} 1 + \frac{R_t}{R_m} & -\frac{R_b}{R_m} & -\frac{1}{R_m} & \frac{1}{R_m} \\ -\frac{R_t}{R_m} & 1 + \frac{R_b}{R_m} & \frac{1}{R_m} & -\frac{1}{R_m} \\ -R_t & 0 & 1 & 0 \\ 0 & -R_b & 0 & 1 \end{bmatrix}^n \cdot \begin{bmatrix} i_a \\ i_c \\ u_a \\ u_c \end{bmatrix}
 \tag{Eq. C-7}$$

Isolating identity matrix $\mathbf{I}$ yields:

$$\begin{bmatrix} i_{a,n} \\ i_{c,n} \\ u_{a,n} \\ u_{c,n} \end{bmatrix} = \left(\begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} + \begin{bmatrix} \frac{R_t}{R_m} & -\frac{R_b}{R_m} & -\frac{1}{R_m} & \frac{1}{R_m} \\ -\frac{R_t}{R_m} & \frac{R_b}{R_m} & \frac{1}{R_m} & -\frac{1}{R_m} \\ -R_t & 0 & 0 & 0 \\ 0 & -R_b & 0 & 0 \end{bmatrix} \right)^n \cdot \begin{bmatrix} i_a \\ i_c \\ u_a \\ u_c \end{bmatrix}
 \tag{Eq. C-8}$$

Filling in the expressions of R_m , R_t and R_b from Eq. C-2 yields:

$$\begin{aligned}
 \begin{bmatrix} i_{a,n} \\ i_{c,n} \\ u_{a,n} \\ u_{c,n} \end{bmatrix} &= \left(\mathbf{I} + \begin{bmatrix} \frac{R_T}{n^2 \cdot R_M} & -\frac{R_B}{n^2 \cdot R_M} & -\frac{1}{n \cdot R_M} & \frac{1}{n \cdot R_M} \\ -\frac{R_T}{n^2 \cdot R_M} & \frac{R_B}{n^2 \cdot R_M} & \frac{1}{n \cdot R_M} & -\frac{1}{n \cdot R_M} \\ -\frac{R_T}{n} & 0 & 0 & 0 \\ 0 & -\frac{R_B}{n} & 0 & 0 \end{bmatrix} \right)^n \cdot \begin{bmatrix} i_a \\ i_c \\ u_a \\ u_c \end{bmatrix} \\
 &= \left(\mathbf{I} + \frac{1}{n} \cdot \begin{bmatrix} \frac{R_T}{n \cdot R_M} & -\frac{R_B}{n \cdot R_M} & -\frac{1}{R_M} & \frac{1}{R_M} \\ -\frac{R_T}{n \cdot R_M} & \frac{R_B}{n \cdot R_M} & \frac{1}{R_M} & -\frac{1}{R_M} \\ -R_T & 0 & 0 & 0 \\ 0 & -R_B & 0 & 0 \end{bmatrix} \right)^n \cdot \begin{bmatrix} i_a \\ i_c \\ u_a \\ u_c \end{bmatrix}
 \end{aligned}
 \tag{Eq. C-9}$$

Then, the limit for $n \rightarrow \infty$ can be taken to arrive at the analytical solution for this ladder network:

$$\begin{bmatrix} i_{a,\infty} \\ i_{c,\infty} \\ u_{a,\infty} \\ u_{c,\infty} \end{bmatrix} = \lim_{n \rightarrow \infty} \left(\mathbf{I} + \frac{1}{n} \begin{bmatrix} \frac{R_T}{n \cdot R_M} & -\frac{R_B}{n \cdot R_M} & -\frac{1}{R_M} & \frac{1}{R_M} \\ -\frac{R_T}{n \cdot R_M} & \frac{R_B}{n \cdot R_M} & \frac{1}{R_M} & -\frac{1}{R_M} \\ -R_T & 0 & 0 & 0 \\ 0 & -R_B & 0 & 0 \end{bmatrix} \right)^n \cdot \begin{bmatrix} i_a \\ i_c \\ u_a \\ u_c \end{bmatrix} \quad \text{Eq. C-10}$$

To calculate this limit, the following standard limit is applied:

$$e^A = \lim_{n \rightarrow \infty} \left(1 + \frac{1}{n} A \right)^n$$

$$\exp(A) = \lim_{n \rightarrow \infty} \left(\mathbf{I} + \frac{1}{n} \mathbf{A} \right)^n \quad \text{Eq. C-11}$$

Furthermore, it can be noted that $i_{a,\infty} = i_a$, and $i_{c,\infty} = i_b$, and $u_{a,\infty} = u_d$ and $u_{c,\infty} = u_b$ (see **Figure C-1**). Thus, the solution can be simplified to:

$$\begin{bmatrix} i_d \\ i_b \\ u_d \\ u_b \end{bmatrix} = \exp \left(\begin{bmatrix} 0 & 0 & -\frac{1}{R_M} & \frac{1}{R_M} \\ 0 & 0 & \frac{1}{R_M} & -\frac{1}{R_M} \\ -R_T & 0 & 0 & 0 \\ 0 & -R_B & 0 & 0 \end{bmatrix} \right) \cdot \begin{bmatrix} i_a \\ i_c \\ u_a \\ u_c \end{bmatrix} \quad \text{Eq. C-12}$$

Note that $\lim_{n \rightarrow \infty} \left(\frac{1}{n} \right) = 0$ is taken for the $\frac{1}{n}$ terms remaining inside the matrix.

To compare this method to the numerical approach, TEER measurements were simulated by introducing the applied currents and resistances in **Eq. C-12**. Subsequently, the four unknown potentials were calculated from this system of equations. Again, the equivalent resistance of the membrane is calculated by dividing the potential drop between the nodes corresponding to port *a* and *b*, by the applied current i_{in} . This overall resistance is normalized to the surface area of the membrane to obtain the apparent TEER corresponding to the actual input TEER used in this analytical approach.

Comparison and conclusion

The graph in **Figure C-2** summarizes the simulation results of both the numerical approach (solid blue line) and the analytical approach (dashed orange line) for varying input TEER values, as is described above. As can be seen in the graph, both methods yield similar results for the range of input TEERs (actual TEERs) shown. Note that a drawback of the analytical approach is the difficulty in solving the complex matrix equation for small TEER values. Therefore, the results at TEERs close to zero are inaccurate, as is evidenced by the horizontal line at the x-axis. Furthermore, an added advantage of the numerical approach is that the potential and current distributions in the membrane can be visualized. Therefore, we have chosen to include only the results from the numerical approach in **Chapter 4**.

Figure C-2 | Measured TEER as a function of actual TEER solved using the analytical model (dashed orange line) compared to the Kirchhoff matrix model (solid blue). The dashed black line is a guide for the eye, indicating $y=x$ and thus the case that the whole membrane area contributes equally to the measured resistance.

C.2. Overview of TEER measurements in literature

Table C-1 | Overview of literature reporting TEER measurements. **Human cells:** BMEC = human brain microvascular endothelial cells; hCMEC/D3 = human cerebral microvascular endothelial cell line; HUVEC = human umbilical vein endothelial cells; ECV304 = endothelial cells differentiated from HUVECs; Caco-2 = human colorectal adenocarcinoma cells. **Murine cells:** bEnd.3 = murine brain endothelial cells; BMEC = murine brain microvascular endothelial cells; RBEC = rat brain endothelial cells. **Porcine cells:** BMEC = porcine brain microvascular endothelial cells. **Bovine cells:** BMEC = bovine brain microvascular endothelial cells; BCEC = bovine brain capillary endothelial cells. See list of references in **Chapter 4** for full bibliographic details.

				TEER (Ω·cm ²)	Support/pore size	Remarks		
Human	BMEC	monoculture	Man	2008	<i>J. Clin. Dev. Immunol.</i>	100	not mentioned	
			Wong	2004	<i>Exp. Neurol.</i>	200	membrane of purified collagen (Cellagen discs)	
			Lin	2013	<i>Brain Res.</i>	225	0.4 μm and 8 μm	
			Shimizu	2012	<i>Neurochem. Res.</i>	18	0.4 μm	
			Thomsen	2013	<i>ACS Chem. Neurosci.</i>	43	Transwell, size not mentioned	
			Mishiro	2012	<i>Neuroscience</i>	140	0.4 μm	
			Wassmer	2006	<i>Infect. Immun.</i>	128	3 μm	
			Lippman	2012	<i>Nat. Biotechnol.</i>	200	0.4 μm	
			Chaitanya	2011	<i>J. Neuroinflammation</i>	245	8 μm PET Transwell inserts (Falcon)	
			Kuo	2011	<i>Colloids Surf., B</i>	175	1 μm	
	co-cult.	Lippmann	2012	<i>Nat. Biotechnol.</i>	1450	0.4 μm	Astrocytes	
		Chaitanya	2011	<i>J. Neuroinflammation</i>	232	8 μm PET Transwell inserts (Falcon)	HFA	
		Kuo	2011	<i>Colloids Surf., B</i>	230	1 μm		
	hCMEC/D3	mono	Vu	2009	<i>Eukaryot. Cell</i>	60	8 μm	
			Cucullo	2008	<i>J. Cereb. Blood Flow Metab.</i>	65	0.4 μm	
			Chaitanya	2011	<i>J. Neuroinflammation</i>	297	8 μm PET Transwell inserts (Falcon)	
		co	Cucullo	2008	<i>J. Cereb. Blood Flow Metab.</i>	69	0.4 μm	HA
			Chaitanya	2011	<i>J. Neuroinflammation</i>	176	8 μm PET Transwell inserts (Falcon)	HFA
	HUVEC	monocult.	Yamada	2014	<i>Microvasc. Res.</i>	12	0.4 μm	
			Xie	2005	<i>J. Control. Release</i>	25	8 μm	
			Man	2008	<i>J. Clin. Dev. Immunol.</i>	80	not mentioned	
			Seok	2013	<i>Arch. Pharm. Res.</i>	87	Transwell, size not mentioned	
		co	Seok	2013	<i>Arch. Pharm. Res.</i>	207	Transwell, size not mentioned	C6
	ECV304	mono	Easton	2002	<i>Brain Res.</i>	70	0.4 μm	
			Neuhaus	2011	<i>Brain Res.</i>	120	1 μm	
			Chaitanya	2011	<i>J. Neuroinflammation</i>	353	8 μm PET Transwell inserts (Falcon)	
		co	Easton	2002	<i>Brain Res.</i>	225	0.4 μm	C6
			Chaitanya	2011	<i>J. Neuroinflammation</i>	327	8 μm PET Transwell inserts (Falcon)	HFA

Human	Caco-2	monoculture	Kotzé	1999	<i>J. Pharm. Sci.</i>	250	Transwell, size not mentioned	
			Tsuzuki	2007	<i>Lipids</i>	330	0.4 µm	
			Gusti	2014	<i>Physiol. Rep.</i>	400	3 µm	
			Rodriguez-Gaztelumendi	2011	<i>Toxicol. Lett.</i>	700	0.4 µm	
			Smith	2004	<i>Pharm. Res.</i>	800	0.4 µm	
			Kotzé	1998	<i>J. Control. Release</i>	900	Transwell, size not mentioned	
			Jevprasesphant	2003	<i>Pharm. Res.</i>	900	Transwell, size not mentioned	
			El-Sayed	2002	<i>J. Control. Release</i>	1000	3 µm	
			Borchard	1996	<i>J. Control. Release</i>	1300	Transwell, size not mentioned	
Murine	bEnd.3	monoculture	Li	2010	<i>Ann. Biomed. Eng.</i>	20	0.4 µm	
			Booth	2012	<i>Lab Chip</i>	25	PC, pore size not mentioned	
			Wuest	2013	<i>J. Neurosci. Met</i>	30	0.4, 1, 3 and 8 µm	
			Hue	2013	<i>J. Neurotrauma</i>	30	0.4 µm	
			Chaitanya	2011	<i>J. Neuroinflammation</i>	276	8 µm PET Transwell inserts (Falcon)	
			Inamura	2013	<i>Neurochem. Res.</i>	22	0.4 µm	
			Seok	2013	<i>Arch. Pharm. Res.</i>	45	Transwell, size not mentioned	
		co-culture	Li	2010	<i>Ann. Biomed. Eng.</i>	30	0.4 µm	Astrocytes
			Booth	2012	<i>Lab Chip</i>	250	PC, pore size not mentioned	C8D1A (mouse)
			Chaitanya	2011	<i>J. Neuroinflammation</i>	208	8 µm PET Transwell inserts (Falcon)	HFA
			Seok	2013	<i>Arch. Pharm. Res.</i>	84	Transwell, size not mentioned	C6
	BMEC	monoculture	Fleegal-DeMotta	2009	<i>J. Cereb. Blood Flow Metab.</i>	1	0.45 µm	
			Calabria	2006	<i>J. Neurochem.</i>	40	0.4 µm Transwell-Clear filter	
			Demeuse	2002	<i>J. Neurosci. Methods</i>	50	1 µm	
			Dohgu	2011	<i>J. Neuroinflammation</i>	65	0.4 µm	
			Weidenfeller	2007	<i>J. Neurochem.</i>	75	0.4 µm	
			Shayan	2011	<i>Eur. J. Pharm. Sci.</i>	130	0.4 µm	
			Wuest	2013	<i>J. Neurosci. Methods</i>	150	0.4 µm	
			Honda	2006	<i>Cell. Mol. Neurobiol.</i>	158	0.45 µm	
			Xie	2005	<i>J. Control. Release</i>	200	8 µm	
			Jiang	2012	<i>Int. J. Alzheimers Dis.</i>	300	0.4 µm	
			Zhou	2013	<i>Biomed. Environ. Sci.</i>	125	1 µm	
			Zhu	2012	<i>J. Ethnopharmacol.</i>	40	8 µm	
		co-culture	Calabria	2006	<i>J. Neurochem.</i>	200	0.4 µm Transwell-Clear filter	Doping puro-mycin+HC
			Demeuse	2002	<i>J. Neurosci. Methods</i>	425	1 µm	Astrocytes
			Weidenfeller	2007	<i>J. Neurochem.</i>	110	0.4 µm	NPC
			Shayan	2011	<i>Eur. J. Pharm. Sci.</i>	190	0.4 µm	Astrocytes
			Wuest	2013	<i>J. Neurosci. Methods</i>	200	0.4 µm	Astrocytes
			Zhou	2013	<i>Biomed. Environ. Sci.</i>	350	1 µm	Astroglial cells

Species	Cultures	Authors	Year	Journal	Cell number	Media	Cell type	
Murine	RBEC	monoculture	Hayashi	2004	<i>Regul. Pept.</i>	8	0.4 μm	
			Nakagawa	2009	<i>Neurochem. Int.</i>	100	Transwell, size not mentioned	
			Dohgu	2011	<i>Microvasc. Res.</i>	150	0.4 μm	
			Yamada	2014	<i>Microvasc. Res.</i>	175	0.4 μm	
			Toyoda	2013	<i>Cell. Mol. Neurobiol.</i>	281	8 μm and some 0.4 μm	
			De Vries	1996	<i>J. Neuroimmunol.</i>	150	0.4 μm	
		co-culture	Hayashi	2004	<i>Regul. Pept.</i>	100	0.4 μm	Pericytes
			Nakagawa	2009	<i>Neurochem. Int.</i>	200	Transwell, size not mentioned	Astrocytes
			Dohgu	2011	<i>Microvasc. Res.</i>	280	0.4 μm	Pericytes
			Toyoda	2013	<i>Cell. Mol. Neurobiol.</i>	329	8 μm and some 0.4 μm	Pericytes / astrocytes
Porcine	BMEC	monoculture	Smith	2007	<i>J. Drug Target.</i>	80	0.4 μm	
			Kuhlmann	2009	<i>Neurosci. Lett.</i>	218	0.4 μm	
			Franke	2000	<i>Brain Res. Brain Res. Protoc.</i>	400	0.4 μm	
			Franke	1999	<i>Brain Res.</i>	500	0.4 μm	
			Zhang	2006	<i>Drug Metab. Dispos.</i>	550	0.4 μm	
			Patabendige	2013	<i>Brain Res.</i>	595	0.4 μm	
			Neuhaus	2008	<i>J. Pharm. Sci.</i>	149	1 μm	
		co-culture	Smith	2007	<i>J. Drug Target.</i>	834	0.4 μm	C6
			Kuhlmann	2009	<i>Neurosci. Lett.</i>	278	0.4 μm	Astrocytes
			Franke	1999	<i>Brain Res.</i>	800	0.4 μm	Serum-free
Patabendige	2013	<i>Brain Res.</i>	779	0.4 μm	Astrocytes			
Bovine	BMEC	mono	Colgan	2008	<i>Brain Res.</i>	30	0.4 μm	
			Rubin	1991	<i>J. Cell Biol.</i>	61	0.4 μm	
			Haorah	2005	<i>Alcohol. Clin. Exp. Res.</i>	250	0.4 μm	
		co	Colgan	2008	<i>Brain Res.</i>	45	0.4 μm	C6
			Rubin	1991	<i>J. Cell Biol.</i>	115	0.4 μm	Astrocytes
	BCEC	mono	Dehouck	1990	<i>J. Neurochem.</i>	416	0.4 μm	
			Gaillard	2001	<i>Eur. J. Pharm. Sci.</i>	131	0.4 μm	
			Salmeri	2013	<i>Cell. Microbiol.</i>	70	0.4 μm, 3 μm	
			Boveri	2005	<i>Glia</i>	150	0.4 μm	
			Dehouck	1990	<i>J. Neurochem.</i>	661	0.4 μm	Astrocytes
co	Gaillard	2001	<i>Eur. J. Pharm. Sci.</i>	857	0.4 μm			
	Salmeri	2013	<i>Cell. Microbiol.</i>	267	0.4 μm, 3 μm	Pericytes		

D. Supplementary information Chapter 5

D.1. Sensitivity distribution theory

In four-terminal resistance measurements, electric fields and current densities develop in 3D space, resulting in different contributions of separate volume elements to the final measured resistance [1]. Theory for resistance extraction from such four-terminal measurements was developed by Geselowitz [2], and was recently summarized by Wang et al. [3]. Geselowitz's approach starts by mapping the pattern of current flow between the current carrying electrodes. At every location in the measured volume, the current flow is described by the current density $\mathbf{J}$ (A/m²): a vector quantity with a magnitude and direction. The local current density gives rise to a local electrical field, which is also a vector quantity, dependent on the local electrical resistivity. Geselowitz shows that this generated field is picked up the strongest by the readout electrodes, when this field is exactly in line with the field that would exist when the same current was sent through the readout electrodes instead of through the excitation electrodes (shown schematically in **Figure D-1**). The interchangeability of the excitation and readout electrode pairs is Geselowitz's reciprocity theorem. On the basis of this theorem, later authors calculated a sensitivity coefficient for every small volume element, quantifying the extent to which it contributes to the total measured impedance [1, 4]. As this sensitivity coefficient is thus based on the extent to which the generated and measured fields of vectors line up, it contains the dot product of two vectors. Grimnes and Martinsen [1] calculated the sensitivity coefficient based on the dot product of the current densities, while, as another option, Metherall et al. [4] had used the dot product of the developed electric fields in earlier work. In this chapter we follow the formulation of Grimnes et al. for the sensitivity coefficient S :

$$S = \frac{\mathbf{J}_1 \cdot \mathbf{J}_2}{i^2} \quad \text{Eq. D-1}$$

In this equation, $\mathbf{J}_1$ is the current density vector (A/m²) in each volume element when a current i (A) is injected between the two excitation electrodes. $\mathbf{J}_2$ is the current density when i is injected between the two readout electrodes [1]. The higher the value for the sensitivity coefficient, the more a change in the resistivity of this volume element will affect the total resistance. A negative sensitivity means that an increase in the resistivity of this volume element will, maybe surprisingly but not counterintuitively, result in a lower total measured resistance.

Figure D-1 | Schematic illustration of the sensitivity distribution of four-terminal impedance sensing in a uniformly conductive cylinder. The electrical field arising when current is applied between the excitation electrodes is superimposed on the electric field arising when the same current is applied between the readout electrodes (following the reciprocity theorem). Where the fields align, the resulting sensitivity is high; where the fields are perpendicular the resulting sensitivity is 0; and where the fields oppose each other the resulting sensitivity is negative. Adapted from Kauppinen et al. [5].

Geselowitz [2], Metherall [4] and Grimnes [1] et al. only considered resistive behavior in the measured volume, and not the case where (some of) the charge is capacitively stored in the measured volume. Capacitive charging, however, will occur across a cell layer in organs-on-chips during impedance spectroscopy because of the capacitive behavior of cell membranes [6, 7]. The sensitivity coefficient equally applies to such a case, as the current densities in Eq. D-1 can be written as the sum of a conductive current density σE , and a capacitive charging current density $\epsilon dE/dt$:

$$S = \frac{J_1 J_2}{i^2} = \frac{(\sigma E_1 + \epsilon \frac{dE_1}{dt}) \cdot (\sigma E_2 + \epsilon \frac{dE_2}{dt})}{i^2} \quad \text{Eq. D-2}$$

In this equation, E is the electric field vector (V/m), σ is the conductivity (S/m) and ϵ is the permittivity (F/m). With increasing frequency, dE/dt increases and thereby the magnitude of the contribution of the capacitive charging current to the total current increases; in impedance spectroscopy this is observed by a decrease of impedance with increasing frequency. Therefore, the sensitivity coefficient distribution within the measured volume was derived from the finite-element model with both resistive and capacitive elements presented in Chapter 5.

D.2. Convergence of four and six-electrode simulations

Figure D-2 | Investigating the convergence of the output parameter R_{meas} (resistance between readout electrodes resulting from input current and associated electrode potentials) in the four-electrode chip of **Figure 5-1A**. Electrical simulations were performed iteratively, doubling the total number of nodes each time. Top: values of output parameter R_{meas} . Bottom: change (in %) of the current simulation compared to the previous simulation. At approximately 2000 by 144 nodes (~290000, red dashed line) the change between subsequent simulations reaches 0.5%, leading to the conclusion that this is a sufficient amount of nodes to faithfully model the TEER measurements in the gut-on-a-chip. As input parameters a flat monolayer (**A**) or a monolayer with 250 μm high villi (**B**) was used, both with a TEER of 1000 $\Omega\cdot\text{cm}^2$, subjected to an AC current of 100 Hz.

Figure D-3 | Investigating the convergence of the output parameter R_{meas} in the six-electrode chip of **Figure 5-6A**. For subsequent simulations networks of 2000 by 120 nodes (~240000, red dashed line) were used. As input parameters a flat monolayer (**A**) or a monolayer with 250 μm high villi (**B**) was used, both with a TEER of 1000 $\Omega\cdot\text{cm}^2$, subjected to an AC current of 100 Hz.

D.3. Semi-transparent gold electrodes

Figure D-4 | The semi-transparent gold electrodes on the poly-carbonate (PC) substrates of the gut-on-a-chip device allow microscopic inspection of the Caco-2 cells cultured between them.

D.4. Changing electrode polarization and -180° phase shift

Figure D-5 | Impedance spectra from Figure 5-2, Figure 5-4 and Figure 5-5 accompanied by their phase spectra. It can be seen that both the experimental measurements and the simulations of an electrical network consisting of only resistors and capacitors give rise to approximately -180° phase shifts. This can be explained by changes in the general direction of the current, which is illustrated using the potential distributions at a TEER of $1000 \Omega \cdot \text{cm}^2$ that were also shown in Figure 5-3. When going from 10 Hz to 100 kHz, the polarity of the readout electrodes shifts -180° due to channel geometry and electrode configuration: the potential of the bottom readout electrode becomes larger than the potential of the top readout electrode, effectively swapping the terminals. This effect has also been described by Grimes and Martinsen [1]. Unfortunately, this effect renders this data unsuitable for general impedance fitting methods.

D.5. TEER of gut epithelium cultured in Transwell

Table D-1 | TEER of Caco-2 monolayers in Transwell for comparison with TEER in static chips. TEER values were measured at four time points in three wells with Caco-2 barriers, which were allowed to mature for six weeks. The average TEER $\pm$ standard deviation for these twelve measurements was $736 \pm 120 \Omega \text{ cm}^2$. Analogously to the chips, TEER was measured using electrical impedance spectroscopy by connecting the same Autolab PGStat12 to the probe cables of an Endohm chamber.

6 weeks +	0 h	1 h	2 h	24 h
Transwell 1	766 $\Omega \cdot \text{cm}^2$	865 $\Omega \cdot \text{cm}^2$	832 $\Omega \cdot \text{cm}^2$	613 $\Omega \cdot \text{cm}^2$
Transwell 2	813 $\Omega \cdot \text{cm}^2$	846 $\Omega \cdot \text{cm}^2$	822 $\Omega \cdot \text{cm}^2$	662 $\Omega \cdot \text{cm}^2$
Transwell 3	792 $\Omega \cdot \text{cm}^2$	676 $\Omega \cdot \text{cm}^2$	679 $\Omega \cdot \text{cm}^2$	460 $\Omega \cdot \text{cm}^2$
Average				736 $\Omega \cdot \text{cm}^2$
Standard deviation				120 $\Omega \cdot \text{cm}^2$

D.6. Modeling villi architecture

Figure D-6 | Confocal images of a Caco-2 cell layer cultured under flow for 9 days revealed an approximated villi height of 150 μm to 250 μm and an average inter-villi spacing of 167 μm . Therefore, the sinusoidally modeled villi have a period of 167 μm and a height range of 0 μm (flat monolayer) up to 300 μm (differentiated villi). Blue shows nuclei, green shows actin filaments, scalebar is 100 μm .

D.7. Six-electrode chip simulations

Figure D-7 | Potential and sensitivity distributions resulting from electrical simulations with different input TEERs to model static barrier development in the six-electrode chip. Note in **A.** that also in this chip electrode polarity inverts at low input TEER or high frequency.

Figure D-8 | Impedance spectra and calibration curve resulting from electrical simulations with different input TEERs to model static barrier development in the six-electrode chip. Note in **A.** that there is a -180° phase shift seen in the impedance spectra, corresponding to the changing polarity in **Figure D-7A.**

Figure D-9 | Electrical simulations with increasing villi area ratio to assess the influence of villi formation on impedance spectra.

D.8. References

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E. Introducing astrocytes into the blood-brain barrier on chip

The experiment and results described in this appendix are the first steps towards incorporating astrocytes into the blood-brain barrier (BBB)-on-chip presented in **Chapter 3**. To create a physiological three-dimensional (3D) culture environment mimicking brain tissue, hydrogels were chosen to provide structural support for the culture of astrocytes. In this appendix, the incorporation and fixation of a cell-laden hydrogel in a microfluidic poly(dimethylsiloxane) (PDMS) channel is shown. Different surface treatments were tried for optimal gel fixation and the distribution of astrocytes throughout the gel was studied.

E.1. Experimental

E.1.1. Chip fabrication

A single chip layer of the BBB chip presented in **Chapter 3**, without the electrode channels, was used as a one-layer PDMS device with a single channel. To that end, the PDMS layer was bonded to a PDMS-coated glass cover slip, resulting in microfluidic channels of 500 μm width, 300 μm height and 13 mm length with four PDMS surfaces. The only difference with the BBB chip is then the absence of the polycarbonate (PC) membrane and electrodes.

First, PDMS base agent and curing agent (Sylgard 184 Silicone elastomer kit, Dow Corning) were mixed at a weight ratio of 10:1. After degassing, this mixture was poured onto a patterned silicon wafer and cured for four hours at 60°C. The cured PDMS was peeled off the patterned wafer and the resulting PDMS slab with channel imprints was cut into separate chip parts. Two access ports were punched in each part using a biopsy punch (1 mm diameter; Integra Miltex). Glass cover slips of 24 mm by 40 mm (Menzel-Gläser) were coated with a thin layer of PDMS by spin-coating 200 μl degassed PDMS mixture at 6000 rpm for three minutes (Spin 150, Polos, the Netherlands) and baking at 60°C for two hours. Finally, the PDMS chip parts and PDMS-coated glass cover slips were exposed to oxygen plasma (PDC-001 Plasma Cleaner, Harrick) and put into contact with each other to achieve bonding. The chips were kept in a Petri dish and stored at room temperature for at least 24 hours.

E.1.2. Surface treatments

Normally, PDMS has a hydrophobic surface that is unsuitable for the attachment of hydrophilic polymers such as collagen I [1]. Therefore, to ensure proper fixation of an astrocyte-laden hydrogel in a PDMS channel, a surface treatment is required. There are different surface treatments reported in literature, of which three are tested here. The first surface treatment tested is plasma oxidation to temporarily modify the surface with reactive hydroxide groups for protein attachment [2], the second is plasma oxidation followed by charge-mediated adsorption of poly-lysine with which proteins can interact [2] and the third is a silane-based chemical modification providing reactive sites for the covalent binding of proteins [3].

Plasma oxidation

To oxidize the PDMS surfaces of microfluidic channels, providing a reactive and hydrophilic surface to which hydrogels can attach [2], assembled chips were exposed to oxygen plasma for 40 s at 50 W (CUTE, Femto Science). Plasma-oxidized chips were either stored at 4°C for three hours prior to introducing the hydrogel (termed "Plasma oxidation I"), or they were filled immediately after the treatment ("Plasma oxidation II").

Poly-lysine coating

To coat the PDMS surface with the positively charged polymer poly-lysine by adsorption [2], following a protocol described in literature [4], plasma oxidation was performed as is described before. Immediately afterwards, the channel was filled with 1 mg/ml poly-lysine (left enantiomer, ScienCell Research Laboratories) in sterile deionized water and incubated for two hours at room temperature. Then, the channel was rinsed with sterile deionized water, aspirated and kept at room temperature shortly until the hydrogel was introduced.

APTES+GA treatment

To chemically modify the PDMS surface with (3-aminopropyl)triethoxysilane (APTES) and glutaraldehyde (GA), which can subsequently bond covalently to the amine groups of proteins [3], plasma oxidation was performed as is described before. Immediately afterwards, chips were submerged in a solution of 3% (v/v) APTES (Sigma) in water and incubated for 30 minutes. To ensure proper filling of the channels, the APTES solution was also pipetted directly into the channels. Next, channels were thoroughly rinsed with 100% ethanol (Boom) and subsequently chips were submerged in ethanol for 30 minutes. Then, chips were removed from the ethanol and channels rinsed once with fresh ethanol and subsequently dried with compressed air. The channels were filled with 20% (v/v) GA (Sigma) in phosphate-buffered saline (PBS) and submerged in this solution for 30 minutes. Lastly, channels were rinsed with MilliQ water and dried with

compressed air, followed by overnight baking at 60°C. Prior to introducing the hydrogel, chips were kept at 4°C.

No treatment

For comparison purposes, channels without any surface treatments were also included in this experiment. Prior to adding the collagen I gel, the channels were washed three times with sterile deionized water and kept at 4°C.

E.1.3. Cell culturing

Primary human astrocytes (ScienCell Research Laboratories) were cultured in T75 culture flasks (Greiner Bio-One) coated with 2 µg/cm² poly-lysine, using astrocyte medium (AM), supplemented with 2% fetal bovine serum (FBS), 1% astrocyte growth supplement and 1% penicillin/streptomycin solution (Gibco). The cells were incubated at 37°C in humidified air with 5% CO₂. Medium was refreshed every 2-4 days and confluent flasks were subcultured or used for experiments (up to passage 7).

For experiments, astrocytes were collected from a confluent flask using 0.05% trypsin-EDTA (Gibco). After centrifuging, the cell pellet was suspended in fresh AM to be added to a hydrogel, of which the preparation steps are described next.

E.1.4. Hydrogel

In the human body, extracellular matrix (ECM) is composed of highly hydrated polymer networks with various constituents. One of the most abundant proteins in ECM is collagen I. Although it is not naturally present in the brain, it is used often to mimic brain ECM *in vitro* [5-11] as it can provide the structural support and stiffness that is normally provided by the high cell content of brain (approximately 80% of the total brain volume) [12]. Therefore, collagen I was chosen as starting material. After proof-of-principle, the hydrogel components can be tuned to provide a more physiological environment, for example by including a polymer that is abundantly present in brain ECM such as hyaluronic acid [13], which has also been used before for *in vitro* modeling of brain ECM [11, 12].

Preparation and seeding

To test the robustness of the surface treatments, a hydrogel of 3 mg/ml collagen I (from rat tail, 9.66 mg/ml stock solution; Corning) and astrocytes at 5.5·10⁶ cells/ml was desired. This hydrogel has low collagen concentration and high cell content when compared to the vendor's recommendations and other studies (e.g. 3·10⁶ cells/ml in 5 mg/ml collagen I gel [8] or 0.6·10⁶ cells/ml in 7 mg/ml collagen I gel [10]), which results more readily in detachment and compaction of the gel from the chip walls [10]. Therefore, the surface treatment that results in the best-attached gel in this experiment is

expected to be suitable for subsequent experiments with different polymer concentrations and cell contents.

To prevent premature gelling, the collagen I as well as mixtures with collagen were kept on ice and the microfluidic chips were pre-cooled to 4°C. To prepare 300 µl hydrogel containing 3 mg/ml collagen I and $5.5 \cdot 10^6$ astrocytes/ml, first 10 µl 10x PBS with phenol red (Sigma) and 2.1 µl sodium hydroxide (NaOH; Sigma) solution (1 M) were added to an Eppendorf tube and put on ice, according to the vendor's recommendations. Once a neutral pH is reached, the phenol red-containing hydrogel turns pink and in that way it serves as a control. Then, 195 µl of astrocyte suspension ($8.5 \cdot 10^6$ cells/ml in AM) was added. Lastly, 93 µl collagen I from a stock solution of 9.66 mg/ml was added and mixed thoroughly but carefully, avoiding the introduction of air bubbles. The resulting hydrogel mixture was kept on ice during the seeding process. To seed the cell-laden hydrogel in microfluidic chips, 30 µl of the mixture was slowly pipetted in a microfluidic channel and incubated for 30 minutes in a closed Petri dish. Then, a pipette tip with 150 µl AM was inserted into the inlet and a pipette tip with 100 µl AM in the outlet, creating slow, gravity-driven flow to refresh the medium inside the chip.

E.1.5. Staining and imaging

During the experiment, the astrocytes as well as the hydrogel integrity were monitored using phase contrast microscopy (EVOS FL Cell Imaging System, Life Technologies). At the end of the experiment the channels were washed with PBS by inserting a pipette tip with 100 µl PBS in the inlet and incubating for 30-60 minutes. Then, the cells were fixed by inserting a pipette tip with 100 µl of 3.7% paraformaldehyde (Sigma Aldrich) in PBS and incubating for 60 minutes at room temperature. The fixative was washed away by incubating three times with PBS-filled pipette tips for 30-60 minutes. Chips were stored at 4°C in a wetted Petri dish until staining. Then, a solution was prepared with NucBlue and ActinGreen (both 1 drop per 500 µl; Ready Probes reagent, Molecular Probes, Life Technologies) as well as 0.1% (v/v) Triton X-100 (Sigma Aldrich) in PBS. A pipette tip with 70 µl of this solution was put in the inlet and incubated at room temperature for one hour. After that, the staining solution was washed away with PBS-filled pipette tips that were incubated at room temperature for 20 minutes each. The cells were imaged with phase contrast microscopy using the EVOS FL Cell Imaging System (Life Technologies) and confocal microscopy using the Nikon A1 Confocal Laser Microscope (excitation lasers at 405 nm and 488 nm). The stack of confocal images was processed using ImageJ (Wayne Rasband, NIH, version 1.51j8).

Figure E-1 | Phase-contrast microscopy images of microfluidic channels that were subjected to different surface treatments and incubated with a collagen I hydrogel and astrocytes for 24 hours. Only the APTES+GA surface treatment resulted in a fully attached gel, even close to the access hole and in the channel corner, while the gels in the other channels had detached and compacted. Scale bar is 500 μm .

E.2. Results & discussion

E.2.1. Fixation of cell-laden hydrogel in microfluidic channel

An astrocyte-laden hydrogel was successfully assembled and introduced without any air bubbles into the single-channel chips that were subjected to different surface treatments. There were two plasma-treated chips stored at 4°C (Plasma oxidation I), one plasma-treated chip that was immediately filled after (Plasma oxidation II), one poly-lysine-coated chip, one APTES+GA-treated chip and two untreated chips. After 24 hours of incubation it could be seen with phase-contrast microscopy that the hydrogel remained fully attached only to the channel walls of the chip that was treated with APTES+GA, as can be seen in **Figure E-1**. The hydrogels in the other chips had partly or fully detached from the channel walls and compacted. Detachment was most prominent at the access ports and in the channel corners, but even those areas remained unaffected in the APTES+GA-treated chip. As the collagen I concentration was low and the cell concentration high, resulting in a weak hydrogel that readily compacts [10], it is expected that this surface treatment can also successfully fixate hydrogels with different, more physiological polymer and cell contents. In addition, it is expected that this surface treatment allows the fixated hydrogel to better withstand forces exerted onto the channel by culture medium-filled pipette tips during long-term culture experiments. Furthermore, because GA readily reacts with amine groups, any protein-based hydrogel or modified polymer can be fixated in the chip using the APTES+GA surface

Figure E-2 | Confocal microscopy of the APTES+GA-treated chip filled with astrocytes distributed in a collagen I gel. The nuclei were stained with NucBlue (blue) and actin filaments with ActinGreen (green). **A.** Maximum intensity projection of the full stack of images, showing the distribution of cells in x-y direction. **B.** Reconstructed side view showing the distribution of cells in z direction. **C.** Confocal images at different heights in the channel (indicated in the image), each showing a similar number of cells. The detected intensity of the fluorescent dyes decreases with increasing height. Scalebar is 100 µm for all images.

treatment. Additionally, as it is reported that plasma-activated PC can react with APTES as well [14, 15], it is expected that this surface treatment is also suited for the fixation of hydrogel to the PC membrane in the BBB-on-chip device.

E.2.2. Distribution of astrocytes in hydrogel

Besides proper attachment, a good distribution of cells throughout the gel is also required. To check this, the cells inside the gel were fluorescently stained and images were taken throughout the height of the APTES+GA-treated and hydrogel-filled channel using confocal microscopy. In **Figure E-2C** images taken at different heights are

displayed, each showing a similar number of cells. **Figure E-2A** shows a maximum intensity projection (by ImageJ), showing well-distributed cells throughout the channel width and length. In addition, a star-shaped morphology can be seen: rounded cell bodies with long and thin processes extended through the gel [16]. This indicates that the cells remained live and viable in the collagen I gel during the incubation period and that replacing the medium using a small gravity-driven flow through the hydrogel was successful. Lastly, the reconstructed side view in **Figure E-2B** (using ImageJ) shows a good distribution of cells throughout the channel height. It can be seen that the fluorescence intensity of the images at the bottom is higher than at the top, which is commonly seen in confocal microscopy and results from absorption and scattering of the excitation laser light and the emitted photons. Because of this decreasing intensity, we were not able to image the top part of the channel (approximately last 50 μm). However, as the cell density is very similar in these separate slices, we expect that the cell density in the part of the channel that could not be imaged is also similar.

E.3. References

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List of abbreviations

(h)iPSC	(Human) induced pluripotent stem cell
2D	Two-dimensional
3D	Three-dimensional
AC	Alternating current
ACM	Astrocyte-conditioned medium
Ag/AgCl	Silver/silver chloride
AgCl	Silver chloride
AM	Astrocyte medium
APTES	(3-Aminopropyl)triethoxysilane
BBB	Blood-brain barrier
BBB-on-chip	Blood-brain barrier-on-chip
bEnd.3	Murine brain endothelial cell line
BSA	Bovine serum albumin
C8D1A	Murine astrocytic cell line
cAMP	Cyclic adenosine monophosphate
DC	Direct current
DIC	Differential interference contrast (microscopy)
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGM-2	Endothelial growth medium 2
ex/em	Excitation / emission wavelength
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GA	Glutaraldehyde
Glut-1	Glucose transporter 1
hCMEC/D3	Human cerebral microvascular endothelial cell line
HPLC	High-performance liquid chromatography
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule 1
IgG	Immunoglobulin G
LC-MS	Liquid chromatography-mass spectrometry
LDH	Lactate dehydrogenase
m/m	Mass per mass ratio

NOA	Norland Optical Adhesive
NVU	Neurovascular unit
PBB	Permeabilization and blocking buffer
PBS	Phosphate-buffered saline
PC	Polycarbonate
PDMS	Poly(dimethylsiloxane)
PECAM	Platelet endothelial cell adhesion molecule
PET	Polyester (polyethylene terephthalate)
P-gp	P-glycoprotein
PTFE	Polytetrafluoroethylene
RBE ₄	Rat brain endothelial cell line
RNA	Ribonucleic acid
RO	Phosphodiesterase inhibitor
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of the mean
TEER	Transendothelial / transepithelial electrical resistance
TEM	Transmission electron microscopy
TNF- α	Tumor necrosis factor α
UV	Ultraviolet
v/v	Volume per volume ratio
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor
ZO-1	Zonula occludens 1 (tight junction protein)

Samenvatting

Elektrische en microfluidische technologieën voor organen-op-een-chip om de bloed-hersenbarrière en het darmweefsel na te bootsen

Voor geneesmiddelenonderzoek worden vaak proefdieren gebruikt. Helaas reageren deze dieren vaak anders op nieuwe medicijnen dan mensen, waardoor er veel kosten gemaakt worden voor de ontwikkeling van niet-werkzame medicijnen en veelbelovende nieuwe medicijnen misschien zelfs over het hoofd worden gezien. Daarom zijn er recent “organen-op-een-chip” ontwikkeld: kweekkamers op microschaal (ook wel microfluidische kanalen genoemd) waarin menselijk orgaanweefsel gekweekt kan worden. Verschillende technologieën maken het mogelijk om deze kweekkamers zo te maken dat die erg lijken op de natuurlijke omgeving van een weefsel. Wanneer dit weefsel in zulke passende kweekkamers gekweekt wordt, zal dit weefsel zich ook natuurlijker gaan gedragen. Op deze manier kunnen organen-op-een-chip onderzoek naar nieuwe geneesmiddelen versnellen en betrouwbaarder maken. Maar voor het zover is, zijn er nog genoeg uitdagingen, waarvan enkele in dit proefschrift onderzocht zijn.

Dit proefschrift beschrijft de ontwikkeling van nieuwe technologieën voor organen-op-een-chip, met de focus op toepassingen voor onderzoek naar de bloed-hersenbarrière en de darmen. Voor beide weefsels is het erg belangrijk dat ze een barrière vormen enerzijds tussen de bloedsomloop en de hersenen en anderzijds tussen de bloedsomloop en de darminhoud, zodat precies gereguleerd kan worden welke (voedings)stoffen uitgewisseld worden tussen deze weefsels. Wanneer deze barrierefunctie echter verstoord is, wordt de hele orgaanfunctie verstoord. Ook vormen beide weefsels een uitdaging in medicijnonderzoek, enerzijds bij medicijnafgifte in de hersenen en anderzijds bij medicijnopname uit voedsel.

In organen-op-een-chip kan deze barrierefunctie elektrisch gemeten worden, met als uitkomst de transendothele (of transepithelale) elektrische weerstand (“TEER”). Helaas werd bij deze metingen vaak geen rekening gehouden met de weerstand van de kweekkamers zelf. We hebben laten zien dat een verandering in de weerstand van de microfluidische kanalen in de chip, bijvoorbeeld door een temperatuurschommeling of een luchtbel, al snel grote gevolgen heeft op de gemeten TEER. Om dit te voorkomen, hebben we een nieuwe manier van meten ontwikkeld, waarbij gebruik gemaakt wordt van vier elektrodes en zes wiskundige vergelijkingen om de TEER direct uit de gemeten

weerstanden te isoleren, onafhankelijk van veranderingen in weerstand van de kweekkamer. Deze methode is eenvoudig toepasbaar in andere organen-op-een-chip, om zo betrouwbaardere metingen van de barrièrefunctie van allerlei weefsels te kunnen krijgen.

Een volgende uitdaging was dat veel organen-op-een-chip last hebben van een ongelijke verdeling van stroom tijdens elektrische metingen. Dit komt ook doordat de weerstand van de kweekkamer dicht bij de weerstand van het orgaanweefsel ligt, waardoor niet de TEER van het hele orgaanweefsel gemeten kan worden. Wij hebben met behulp van elektrische simulaties een methode ontwikkeld om de TEER toch te kunnen normaliseren – ondanks de ongelijke stroomverdeling – zodat deze alsnog vergeleken kan worden met de TEER die gemeten is in andere systemen. Daarnaast konden we aantonen dat het met de elektrische metingen mogelijk is om de vorming van darmvlokken te volgen. Wanneer darmvlokken ontstaan, neemt het totale oppervlak van darmweefsel gekweekt in een orgaan-op-chip toe. Dit resulteert weer in karakteristieke meetresultaten. Met behulp van elektrische simulaties konden we deze experimentele resultaten verklaren. Dergelijke elektrische simulaties kunnen ook aangepast worden voor eenvoudige toepassing in andere organen-op-een-chip.

Tot slot hebben we de eerste stappen gezet in de richting van het opschalen van organen-op-een-chip. Daarvoor is een chip ontwikkeld met acht kweekkamers, die elk een eigen ingang hebben maar ook via een netwerk van microkanalen verbonden zijn met één gemeenschappelijke ingang. We hebben laten zien dat via de gemeenschappelijke ingang stoffen tegelijkertijd verdeeld kunnen worden over alle kamertjes, bijvoorbeeld gedurende de kweekperiode. Daarnaast konden we via de acht aparte ingangen ook verschillende stoffen toevoegen aan de acht kweekkamers, wat de mogelijkheid geeft om tegelijkertijd acht nieuwe medicijnen te testen.

Scientific output

Peer-reviewed journal publications

M.W. van der Helm, O.Y.F. Henry, A. Bein, T. Hamkins-Indik, M.J. Cnonce, W.D. Leineweber, M. Odijk, A.D. van der Meer, J.C.T. Eijkel, D.E.I. Ingber, A. van den Berg & L.I. Segerink (2017). Impedance spectroscopy and electrical simulations reveal both transepithelial barrier function and tissue structure by non-invasive sensing in organs-on-chips. *In preparation*.

Y.B. Arik, **M.W. van der Helm**, M. Odijk, L.I. Segerink, R. Passier, A. van den Berg & A.D. van der Meer (2017). Barriers-on-chips: measurement of barrier function of tissues in organs-on-chips. *In preparation*.

H. Le-The, J.T. Loessberg-Zahl, M.P. Tibbe, M.A. Palma do Carmo, **M.W. van der Helm**, J.G. Bomer, A.M. Leferink, L.I. Segerink, A. van den Berg & J.C.T. Eijkel (2017). Large-scale fabrication of free-standing and sub- μm PDMS through-hole membranes. *Submitted*.

M.W. van der Helm, M. Odijk, J.-P. Frimat, A.D. van der Meer, J.C.T. Eijkel, A. van den Berg & L.I. Segerink (2017). Fabrication and validation of an organ-on-chip system with integrated electrodes to directly quantify transendothelial electrical resistance. *Journal of Visualized Experiments*, (127), e56334.

M.W. van der Helm, M. Odijk, J.-P. Frimat, A.D. van der Meer, J.C.T. Eijkel, A. van den Berg & L.I. Segerink (2016). Direct quantification of transendothelial electrical resistance in organs-on-chips. *Biosensors and Bioelectronics*, 85, 924-929.

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M. Odijk, A.D. van der Meer, D. Levner, H.J. Kim, **M.W. van der Helm**, L.I. Segerink, J.-P. Frimat, G.A. Hamilton, D.E. Ingber & A. van den Berg (2015). Measuring direct current trans-epithelial electrical resistance in organ-on-a-chip microsystems. *Lab on a Chip*, 15(3), 745-752.

Conference contributions

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H. Le-The, M.A. Palma do Carmo, **M.W. van der Helm**, J.G. Bomer, L.I. Segerink, A. van den Berg & J.C.T. Eijkel. Large-area fabrication of sub- μm PDMS through-hole membranes. *21st International Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS)*, 22-26 October 2017, Savannah, Georgia, USA (poster).

M.M. Aron, L. Barnsley, S. Shrivastava, **M.W. van der Helm**, L.I. Segerink & E.P. Stride. Ultrasound-mediated blood-brain barrier disruption: Correlation with acoustic emissions. *Acoustics: 173rd Meeting of the Acoustical Society of America*, 25-29 June 2017, Boston, USA (poster). Published in *The Journal of the Acoustical Society of America*, 141(5): 3460.

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Student Fellowship

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Young Scientist Award for the Best Poster Presentation

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Other

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Chapter 3

The BBB chip was designed by dr. Jean-Philippe Frimat. Johan Bomer has performed all cleanroom-related work for mold fabrication. Dr. ir. Bjorn de Wagenaar has supplied several tools and tricks for chip fabrication. The lock-in amplifier setup for impedance spectroscopy was designed and fabricated by dr. ir. Mathieu Odijk and dr. ir. Dirk-Jan van den Broek. Anouk Paulissen is gratefully acknowledged for excellent filmography.

Chapter 4

This chapter resulted from a collaboration with the Wyss Institute for Biologically Inspired Engineering at Harvard University, Boston, USA. Dr. ir. Mathieu Odijk coordinated this collaboration and wrote the manuscript. Experiments were carried out by

dr. Hyun Jung Kim, dr. Andries van der Meer and Tiama Hamkins-Indik at the Wyss Institute. Electrical simulations of the chip (numerical approach) were carried out by dr. ir. Mathieu Odijk, the analytical approach was established by dr. Daniel Levner. The literature research of **Figure 4-2** and **Appendix C.2** was performed by dr. Jean-Philippe Frimat. Dr. ir. Bjorn de Wagenaar assisted with the artwork of **Figure 4-1**. Special thanks go out to prof. dr. Don Ingber for his thorough revisions. Lastly, dr. Julia Mikhal helped with the elaborated explanation of the analytical approach in this thesis.

Chapter 5

This chapter resulted from the continuation of the collaboration started in **Chapter 4**. Experiments and image analyses were coordinated by dr. Olivier Henry at the Wyss Institute and conducted by dr. Olivier Henry, dr. Amir Bein, Tiama Hamkins-Indik, Michael Cronce and William Leineweber.

Chapter 6

The multiplexed chip was designed and fabricated by Marciano Palma do Carmo (master student Biomedical Engineering, University of Lisbon, Portugal) in our group. He also carried out the experiments. Cleanroom processes were greatly assisted by Johan Bomer, Hai Le-The and dr. Wesley van den Beld. Thomas Burgers (bachelor student Biomedical Engineering, University of Twente, the Netherlands; co-supervised by Martijn Tibbe) contributed to the protocols for two-layer devices.

Chapter 7

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About the author

Marinke van der Helm was born on 21 June 1991 in Apeldoorn, the Netherlands. After finishing primary school she attended the Carolus Clusius College in Zwolle. There she completed her pre-scientific education, containing courses in chemistry, physics, mathematics, biology, Latin and Greek.

After her first year of studying Biomedical Engineering at the University of Twente she obtained the *Jong Talent Aanmoedigingsprijs* from the Royal Holland Society of Sciences and Humanities for extraordinary study results. In 2012 she both completed the extracurricular Honours Programme and obtained her Bachelor's degree (BSc, *cum laude*) in Biomedical Engineering. She continued her Master's degree Biomedical Engineering specializing in Molecular, Cellular and Tissue Engineering at the University of Twente. As part of her Master's curriculum she spent three months at the Wyss Institute at Harvard University (Boston, USA) in the group of prof. dr. D.E. Ingber, MD, under supervision of dr. A.D. van der Meer. In 2014 she completed her Master's curriculum (MSc, *cum laude*) with a research assignment at the BIOS Lab on a Chip group at the University of Twente under supervision of dr. ir. L.I. Segerink, prof. dr. J.C.T. Eijkel and prof. dr. A. van den Berg.

She continued her research as PhD candidate in the field of organs-on-chips, specifically focusing on a blood-brain barrier-on-chip and technology-driven organ-on-chip development. During her PhD she supervised multiple students from different nationalities, of whom work is incorporated in this thesis. She was involved in teaching several courses, including electrical circuitry (IEEE, bachelor module Electrical Engineering) and cell culture on chip (Lab on a Chip, bachelor module Electrical Engineering and Biomedical Engineering). Next to that she tutored dozens of Biomedical Engineering students. Furthermore, she won two poster prizes (INVITROM Symposium, *Young Scientist Award for the Best Poster Presentation* and hDMT International Organs-on-Chips Symposium, *Best Poster Award*) and was selected to visit the Global Young Scientists Summit in Singapore, where she was tutored by several Nobel prize laureates.

Today, 19 January 2018, she hopes to be granted her doctorate by her promotor prof. dr. ir. A. van den Berg and prof. dr. J.C.T. Eijkel and co-promotor dr. ir. L.I. Segerink based on her work as presented in this thesis.

At the time of writing, she is continuing her career at the Erasmus Medical Center (Rotterdam) and Groene Hart Ziekenhuis (Gouda) as medical specialist in training to become a clinical chemist, with special interest in lab-on-a-chip systems and point-of-care diagnostics.