Fabrication and characterization of a blood-brain barrier on-a-chip for electrical characterization of cells

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Abstract

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The blood-brain barrier (BBB) is crucial to maintain brain homeostasis and prevent toxic substances from entering the brain. Endothelial cells (EC) are essential for the BBB and in this thesis two different BBB-on-chips were designed for electrical characterization of immortalized mouse EC (bEnd3). Indium-Tin-Oxide (ITO) coated glass slides were etched, creating ITO electrodes with increasing distance between them. The glass slides were attached to a 3D-printed plastic well with UV-glue.

The second prototype was an extension of the first prototype with a copper printed circuit board (PCB) attached to the ITO glass slides using silver epoxy to connect the ITO electrodes to the copper electrodes. The aim with these two chips was to create chips with transparent electrodes for live imaging of the cells with an optical microscope.

The chips were characterized with scanning electron microscopy (SEM) and a profilometer before seeding the cells inside the well. The absolute impedance was measured across two parallel electrodes at a time. The impedance was plotted against the distance between the electrodes. The method used is called transmission line measurements (TLM) and is used to extract the sheet impedance between the electrodes to evaluate the barrier tightness of the cells.

Only one chip from each prototype remained intact after the fabrication and sterilization, making it difficult to draw conclusions from the impedance measurements. However, based on the two chips, the TLM for the first prototype followed a linear trend with a high R-square value whereas, the second prototype showed large variations, causing the R-square value to decrease.
**Tillverkning och karakterisering av blod- och hjärnbarriären på chip för elektrisk karakterisering av celler**


I det här projektet framställdes två olika chip för att undersöka bildandet av proteiner (zonula occludens) mellan endotelceller. Båda chippen bestod av glassskivor (38x40 mm) belagda med tunnfilmer av Indium-Tin-Oxide (ITO). Ett mönster etsades i tunnfilmen för att skapa 2 mm breda elektroder av ITO med ett ökat avstånd (0,1;0,5;1;1,5 och 2 mm) mellan elektroderna. En pulsad laserstråle användes för att etsa mönstret. Glasskivan fästes sedan fast i en 3D-printad brunn med UV-lim. Det som utmärker chipen i det här projektet jämfört med redan existerande chip är att elektroderna är transparenta vilket möjliggör avbildning av cellerna med ett optiskt mikroskop utan att avbryta experimentet.


Till varje chip tillsattes 20 000 celler/cm² innanför den 3D printed plasten. Cellerna växte på glasskivan med ITO elektroder. Impedansen mättes över två parallella elektroder åt gången genom att variera frekvensen från 100 Hz – 1 MHz. Mätningarna gjordes mellan alla elektroder för att erhålla en linjär så kallad transmissionslinje med impedans som funktion av avstånd mellan elektroderna. Den erhållna grafens lutning användes för att extrahera cellernas och cellmediets impedans som funktion av tid.

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List of abbreviations

AC – Alternating current
BBB – Blood-brain barrier
CNS – Central nervous system
D – Distance between electrodes
EC – Endothelial/epithelial cells
I – Current
ITO – Indium Tin Oxide
PCB – Printed circuit board
R – Resistance
SEM – Scanning electron microscopy
TEER – Trans-epithelial/endothelial electrical resistance
TLM – Transmission line measurements
W – Width of electrode
X – Reactance
Z – Impedance
Zsheet – Sheet impedance
Zcontact – Contact impedance
1 Introduction

Annually, 115 million animals are used for animal trials and biomedical purposes. Animal trials are both expensive and might be considered unethical. Moreover, as animals are not analogous to humans, certain metabolisms in animals might not necessarily correspond to metabolisms in humans. Both animal phenotype and physiology differ from that of humans. Although animal trials show a certain rate of success, there are several cases of failure in clinical trials, which indicate a substantial difference between animals and humans.

When testing pharmaceuticals, the process always starts in a laboratory in order to eliminate ineffective pharmaceuticals that, in any case, would not have yielded positive results when tested later on animals. The tests in laboratories without living animals are referred to as in vitro. All tests performed on living animals are referred to as in vivo. By improving in vitro models, it is possible to reduce the number of animals subjected to testing.

The ethical guidelines established for animal testing are summed up in the so called 3R-principle: reduce, replace and refine. The improving of in vitro models focuses on the two first aspects, as it aims to replace animal trials with in vitro trials, thus reducing the number of animal trials.

However, the in vitro process presents its own problems. When seeding cells in a conventional and a static manner on petri dishes, they usually lose their physiological function and become less viable after some time. Therefore, models that mimic the cells’ native environment (the human organs) are imperative in maintaining cell function and morphology, i.e. their structure. The cells in blood vessels are continuously supplied with oxygen and nutrients, and they are mechanically stimulated by shear stresses from blood flow. To achieve a successful cell culture in vitro, the conditions need to simulate the conditions in the body.

A relatively recent field within in vitro studies is organs-on-a-chip. An organ-on-a-chip is a small device that is used to simulate the conditions in the body for cell seeding. By using chips with microfluidics, that is, chips that have interconnected channels allowing fluids to pass the cells and continuously provide them with oxygen and nutrients, it has been possible to achieve promising results in culturing cells. In addition to providing the cells with oxygen and nutrients, the cells are subjected to shear stress, which further simulates the conditions, which exist in human organs. The most famous example of this is a lung on a chip, first developed in Harvard. By mimicking the conditions in the lung, they managed to seed epithelial cells with similar morphology and function as the epithelial cells in the lung. Other organs-on-chips have been developed, such as liver, gut and kidney. Organs-on-chips are mainly meant to mimic a functional unit of a human organ.

So, by augmenting the research already carried out in this field, it is expected to further reduce and replace animal trials, which will contribute to cutting costs for experiment, as well as reducing the possible moral issues with animal testing.

In this report, a simplified blood-brain barrier (BBB)-on-a-chip will be evaluated. The BBB is crucial in maintaining brain homeostasis and in preventing toxins from entering the central nervous system (CNS - nerves in the brain and the spinal cord). The BBB impedes and blocks harmful molecules in the blood from accessing the CNS. A central part in the BBB is played by the endothelial/epithelial cells (ECs). Between these cells, there are intercellular proteins (tight junctions) that prevent most large molecules from entering the CNS. The number of tight junctions is related to the barrier tightness of the BBB.

When developing drugs, it is essential to know if a drug will be able to cross the BBB or not. In some cases, the target is in the brain, making it imperative that the drug will be able to pass the BBB. However,
in other cases, the target is not in the brain, but somewhere else in the body, which increases the risk of side-effects, should the drug pass the BBB.\textsuperscript{14}

To study drug permeability in humans \textit{in vivo} is impractical and sometimes even impossible, making it desirable to mimic the BBB \textit{in vitro}, on a chip.\textsuperscript{15}

\section*{1.1 Aim and scope}

The aim of this project is to fabricate and characterize a BBB-on-a-chip with transparent electrodes, instead of conventional non-transparent electrodes, facilitating imaging of the cells with optical microscopy. The chips will be used to evaluate the formation of tight junctions between endothelial cells. The formation of tight junctions will be assessed with impedance measurements. The impedance of the cells will be extracted from the total impedance using transmission line measurements.

\section*{2 Theoretical background}

\subsection*{2.1 Physiology of the blood-brain barrier}

The blood vessels in the brain differ from those in the rest of the body in that they are less permeable. The less permeable vessels act as a barrier between the blood and the brain and together they make up the BBB. ECs make up the walls in the vessels and the limited permeability is the result of tight junctions formed between them.\textsuperscript{16} Tight junctions are complexes of several proteins, all needed to create a functional barrier between the brain and the body.\textsuperscript{17} The neurons in the brain need to be protected from many substances normally present in the blood that are neurotoxic. The toxic substances are hindered from entering the brain by the BBB. Furthermore, the neurons use chemical and electrical signals to communicate which makes it crucial to regulate ion concentration to avoid interference with the signals. Hence, the local environment of the neurons has to be strictly regulated by the BBB, see Figure 1.\textsuperscript{18}

Small non-polar molecules such as O\textsubscript{2} and CO\textsubscript{2} are able to cross the tight junctions between ECs, however, large or polar molecules need to be actively transported into the brain. Glucose and amino acids that are crucial for normal brain function, are transported via integrated transport proteins.\textsuperscript{19} The transport is selective and regulated by the concentration of solute in the CNS. Larger molecules such as proteins or peptides enter the brain through strictly regulated receptor-mediated transcytosis. The molecules enter vesicles formed in the membrane of ECs, where they are further transported to the other side of the endothelium, i.e. the CNS.\textsuperscript{12}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Transcellular pathways of polar and non-polar molecules across endothelial cells in the BBB.\textsuperscript{12}}
\end{figure}
2.2 Electrical characterization of cells

There are multiple methods used to characterize cells, each of them has their own advantages and limitations that needs to be considered. Staining of cells is a common way of characterizing cells, however, it’s an invasive process for most stains and the characterization can only be carried out at a distinct time point during an experiment since the cells needs to be fixated. The advantage is that clear images of the cells or specific parts of the cells are obtained.\textsuperscript{20}

In this project, electrical characterization will be used to characterize the EC, or more specifically, the tight junctions formed between them. Electrical characterization is a non-invasive method used to study cells in real-time. The measurements can be carried out for several days without disrupting the experiment.\textsuperscript{21}

Electrical characterization of cells is done by culturing cells on top of or in between electrodes and measuring the disturbance of electron flow over time. A low current runs between two or more electrodes placed close to the cells.\textsuperscript{22} As the cells start to proliferate, spread, adhere to the surface of the electrodes or form new tight junctions the current will decrease due to an increase in resistance. The change in resistance is measured and interpreted, providing information about the cells.\textsuperscript{23}

Impedance measurements are more accurate than pure resistance measurements since they include both resistance and reactance. The only difference in the experimental setup is that an impedance analyzer is used instead of a resistance meter. Reactance is a contribution to the resistance arising from magnetic and electric fields interacting with the electrons. Reactance is an imaginary number that can be either positive or negative. The resistance arises from collisions of the charged particles with the internal structure.\textsuperscript{24}

In systems with direct current, the resistance is sufficient to describe the voltage drop. The resistance is obtained by measuring the difference in voltage (U) in the system and converting it to resistance (R) using Ohm’s law:\textsuperscript{25}

\[ U = I \times R \]  \hspace{1cm} (1)

\( I \) is the constant current used. However, using alternating currents (AC) a shift between the voltage phase and current phase gives rise to a complex term describing the reactance (\( X \)), in addition to the resistance. The shift (\( \varphi \)) is caused by alternating current flow through inductors and capacitors and is described by:\textsuperscript{25}

\[ \varphi = \arctan \frac{X}{R} \]  \hspace{1cm} (2)

The combination of resistance and reactance is described by the impedance (\( Z \)):\textsuperscript{26}

\[ Z = R + iX \]  \hspace{1cm} (3)

If the phase shift is zero, the contribution to the impedance is described by the resistance only.\textsuperscript{27} As previously mentioned, the impedance can be used to assess the formation of tight junctions formed between the EC. In this project, the impedance will be analyzed with transmission line measurements (TLM). TLM is a common method used to determine the sheet impedance of semiconductors. The sheet
impedance is specific for the material and is independent of the spacings between the electrodes. To our knowledge, the method has previously not been implemented on cells.

The principle behind TLM is to measure the total impedance between parallel electrodes placed with increasing distance between them. Using equation 4, the total impedance (Zt) is plotted as a function of the distance (d) between the electrodes. The graph obtained is used to extract the sheet impedance (Zsheet) between the electrodes.

A voltage is applied across two adjacent electrodes at a time and the total impedance across them is measured. The impedance (Zt) is measured across two electrodes at a time and plotted against the distance between the electrodes (d). The result is a linear graph (Figure 2) from which the sheet impedance (Zsheet) is extracted, see equation 4:

\[ Z_t = 2Z_{contact} + \frac{Z_{sheet}}{W}d \]  

\[ W \] is the width of the electrodes and \( Z_e \) is the contact impedance. Figure 3 illustrates the width of the electrodes and the distance between the electrodes. In this thesis, the sheet impedance of a semiconductor will be replaced by the impedance of the cells and a contribution from the cell media. After the tight junctions are formed, they will be disrupted in order to compare the difference in impedance with and without the tight junctions. In this way, the contribution of the tight junctions to the total value of the impedance is obtained.

**Figure 2.** Illustration of a graph obtained from equation 4. The y-axis is the total impedance and the x-axis is the distance between the electrodes.

**Figure 3.** Schematic illustration of the increasing spacings (d) between the electrodes used in TLM. \( W \) is the width of the electrodes. The green parts are the electrodes and the white parts are the spacings in between.
2.3 Trans-endothelial electrical measurements

When culturing ECs in vitro, tight junctions are formed after a few days and increase in number with time until a peak is reached. One efficient way to estimate the number of tight junctions formed, is to measure the electrical impedance over the cells with trans-epithelial/endothelial electrical resistance (TEER) measurements. 32

2.4 Previous measurements of cell impedance – ECIS

The impedance of ECs has previously been studied with Electric Cell-substrate Impedance Sensing (ECIS). ECIS is used to analyze the formation of tight junctions between cells as well as cell proliferation, cell adhesion and cell motility. The measurements are done in real-time, making it possible to follow cell behavior over time. The method is mainly based on the kinetics of cell behavior. 33

In ECIS, cells are seeded on top of gold-electrodes in the bottom of cell dishes. Over time, the cells start to proliferate and adhere to the surface of the electrodes and tight junctions are subsequently formed between the cells (Figure 4). The formation of tight junctions increases the impedance of the cells and is measured. 34

![Figure 4. A schematic of the electron flow and how it is impeded by the cells seeded on top of the gold electrodes.]

2.5 Fabrication of chips

In this project, indium tin oxide (ITO) coated glass was used as electrode. ITO is an optically transparent and conductive material used to coat non-conductive substrates, such as glass. The advantage of an optically transparent electrode is that it enables studies with an optical microscope. 36 Wet chemical etching with hydrochloric acid is one method used to etch patterns on ITO coatings. 37 However, pulsed laser etching is less time-consuming than wet chemical etching. The limitation of pulsed laser etching is that the glass substrate might be damaged in the process, creating a rough and uneven surface. Moreover, the surface might be contaminated with debris from ITO residues. 38 In this thesis, pulsed laser etching will be used to etch out stripes of ITO which are used as electrodes with increasing distance between them.
2.6 Calcium switch

Endothelial cells in the body depend on calcium for the formation of cell junctions. If the calcium is removed the junctions will disrupt. This was noticed in an *in vitro* study with endothelial cells from rabbits. Two hours after replacing the cell media with calcium free media, the TEER value decreased by roughly 35%. Adding calcium, the TEER value increased, implying that the junctions were formed again.\textsuperscript{39}
3 Materials and methods

3.1 Fabrication of chips

3.1.1 Prototype 1

ITO-coated glass substrates (38x40 mm) were patterned with pulsed laser to create stripes of parallel electrodes with increasing distance between them. The thickness of the ITO coating was 400 nm. In Figure 5, the white stripes correspond to the electrodes and the black areas were etched away, creating spacings between the electrodes. The electrodes were 2x30 mm and the spacings from left to right were 0.1, 0.5, 1, 1.5 and 2 mm in width and 30 mm in height. The desired pattern of ITO was created in the laser software (XS Designer 1.5.1) and is shown in Figure 5.

![Figure 5. Laser design used to pattern ITO from coated glass substrate.](image)

The following parameters were used to pattern ITO:

- Power: 80%
- Wavelength: 635 nm
- Number of passes with laser: 1
- Frequency: 30 kHz
- Velocity: 250 mm/s
- Line density: 0.01 mm

A 3D-printer (Dimension Elite, Protech) was used to create wells from acrylonitrile butadiene styrene (ABS) plastic. The design was drawn in AutoCAD (see Figure 6). The inner dimensions of the wells were 10x25x5.5 mm and the outer dimensions were 20x35x5.5 mm.

![Figure 6. Design drawn in AutoCAD used to 3D print well.](image)

The well was attached to the ITO-coated glass substrate using UV glue (Norland optical adhesive 81, Norland products inc.), see Figure 7 for a schematic illustration. Glue was distributed in the bottom of the 3D-printed part and the glass was placed on top. A UV lamp (UV LED Curing Lamp, 100 W, wavelength: 365 nm) was held above the sample for 6 seconds to cure the glue. UV-glue was also used to coat the ABS-plastic well to avoid leakage of cell media through the large pores of the ABS-plastic.  

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3.2 Prototype 2

3.2.1 Printed circuit board

A copper laminated polymer sheet was milled and cut with a printed circuit board (PCB) milling machine (LPKF ProtoMat s100, Laser & Electronics AG). The design was drawn in CAD by Sofia Johansson and is shown in Figure 8. The milling motor operated at a speed of 100 000 rpm with a lateral resolution of 0.25 µm. The purple area in Figure 8 correspond to copper whereas the white area is where the copper has been removed. The red lines in the drawing is where the PCB has been cut out from a larger board.

3.2.2 Soldering of pins

Pins (Vertical Pin Header 8POS M20-9990845, Harwin) were soldered onto the copper electrodes using a soldering pen (JonDeTech). Soldering wire (Solder Wire, Ag60/Sn40/PBTin, Multicore Solder) was melted against the soldering pen and fixed the pins to the copper electrodes on the PCB. The thickness of the soldering wire was 0.9 mm.

3.2.3 Silver epoxy

Silver epoxy (CircuitWorks Conductive Epoxy, Chemtronics) was used to connect the ITO electrode stripes to the copper electrodes on the PCB. The epoxy was a combination of two components, an adhesive and a hardener mixed in 1:1 proportion. The mixture was applied with a toothpick to each of the copper electrodes and the ITO coated glass substrate was immediately attached, aligning the ITO pattern with the copper electrodes. Once assembled, the epoxy was left to cure overnight in a fume hood.
3.2.4 Sterilization of chips

The first prototype was sterilized by leaving the chips in isopropanol for two hours. Afterwards, the chips were soaked in water and rinsed after 10 min. The procedure was repeated three times.

The first batch of the second prototype was left in isopropanol for two hours and rinsed three times with autoclaved water in the same way as for the first prototype. The second batch was sterilized by placing the chips in a petri dish filled with isopropanol overnight. The chips were then left to dry in a fume hood and rinsed with autoclaved water. Some of the chips were autoclaved (CertoClav EL Sterilizer, CertoClav) at 120 °C.

3.3 Characterization of chips

3.3.1 Profilometer

A profilometer (Veeko Dektak 150) was used to characterize the surface profile of the patterned ITO. The stylus radius was 12.5 µm and the force used for the measurements was 5 mg.

3.3.2 SEM

Scanning electron microscopy (SEM, Zeiss 1550) was used to image the surface of the ITO glass substrate after etching it with pulsed laser. A secondary electron detector was used to image the surface. The acceleration voltage was maximum 10 kV.

3.4 Cell culture

The cells used in this project were immortalized mouse brain endothelial cells (bEnd3). The cell media (Dulbecco’s Modified Eagle Medium: high glucose, no glutamine) was supplemented with 10 % FBS (Fetal Bovine Serum, Gibco), 1 % PS (Penicillin-Streptomycin, Gibco) and glutamine (GlutaMAX™). 50,000 cells in 1 ml of cell media was added to each chip. The cell density was 20,000 cells/cm².

3.4.1 Staining of cells

The cells in one of the PCB chips were stained with green fluorescent dye (CellTracker™ Green CMFDA Dye, Thermo Fisher). 4 µl of a stock solution (10 mM stain in DMSO) were diluted with 4 ml cell media to get a concentration of 10 µM. The mixture was added to the cells in a cell culture flask and incubated for 40 min. The cells were rinsed with 10 ml of PBS. PBS was removed and replaced with cell media. The cells were trypsinized and 50,000 stained cells were then added to the chip (20,000 cells/cm²). Images were taken 2 and 4 days after seeding the cells with a fluorescent microscope.

3.4.2 Optical microscopy images of cells

An optical microscope (Olympus AX70) with a CCD camera was used to image the cells on the ITO coated glass substrate. It was also used to image the cells stained with green fluorescent dye. UV-light was used for the cells with fluorescent staining.
3.4.3 Calcium switch

The tight junctions were disrupted by replacing the cell media with one without calcium (Dulbecco's Modified Eagle Medium: high glucose, no glutamine, no calcium, Thermo Fisher). The medium was supplemented with 1 mM sodium pyruvate, 4 mM L-glutamine, 10% PBS and 1% Penicillin Streptomycin. The supplements were purchased from Thermo Fisher Scientific.

3.4.4 Impedance measurements of cells

In the first prototype, with only ITO electrodes and a well, the impedance was measured across two parallel ITO electrodes at a time. A 4-point probe station was used for the first measurements. Two of the probes were placed outside the well in contact with an ITO electrode and the other two were placed on the opposite side of the well on the adjacent electrode. The position of the probes was adjusted in different directions by screws. The probes were connected to an impedance analyzer (MFIA Impedance Analyzer Precision LCR Meter, 500 kHz/5 MHz, Zürich Instruments) that measured the impedance over a range of frequencies (100 Hz – 1 MHz) with AC voltage (50 mV). The procedure was repeated across each spacing between the electrodes.

A second manual method with only two probes to measure the impedance was evaluated. Two probes were manually held in contact with two parallel ITO-electrodes, otherwise the measurements were carried out in the same way as the first measurements.

For the second prototype with an attached copper PCB with soldered pins on the sides, the pins were directly connected to wires that in turn were connected to an impedance analyzer. The impedance measurements were carried out under the same conditions as the previous measurements.

3.5 Biocompatibility studies of chips

The different components of the chips were tested, ensuring that they wouldn’t be toxic to the cells. This was done by seeding cells (bEnd3) in three different 6-well plates with ABS-plastic rings, ITO covered glass pieces and UV-glue in the bottom of the wells. The different materials were all sterilized by leaving them in isopropanol for two hours and then rinse with autoclaved water for 10 min three times. The control in all experiments was cells seeded in a bare well plate. To each of the wells 60,000 cells in 3 ml cell media were added, i.e. 6, 250 cells/cm². The well plates were left in an incubator at 37°C and 5% CO₂. Images of the cells were taken with an optical microscope on day 0, 2 and 4.

4 Results and discussion

4.1 Fabrication of chips

Two different prototypes of a BBB-on-a-chip were prepared for impedance measurements of endothelial cells (bEnd3), both prototypes are seen in Figure 9. The first prototype consisted of an ITO-patterned glass slide with a 3D-printed ABS-plastic well attached to it. The cells were seeded inside the ABS-plastic well attached to the glass substrate with ITO electrodes. The impedance measurements were carried out across two parallel electrodes outside the well by placing two probes in contact with the ITO electrodes.

The second prototype was based on the same principle as the first prototype but with a copper PCB attached to the ITO electrodes using silver epoxy to attach it. The impedance measurements were carried out by connecting wires to the pins on the sides.
4.1.1 ITO

The first glass slides that were patterned with laser had a rough surface, making it impossible to see through the surface in a light optical microscope. This was due to the laser being focused incorrectly. Cells seeded on the glass slide wouldn’t grow where the laser had etched the ITO since the surface was too rough. However, the cells grew on the ITO, indicating that there was nothing wrong with the cells. The focus of the laser was later shifted to decrease the roughness of the spacings.

4.1.2 Silver epoxy

The silver epoxy was smeared out as the ITO glass slide was attached to the copper PCB which made it crucial to have an appropriate amount of epoxy on each electrode, preventing short circuits between the electrodes. However, too little epoxy increases the impedance since the contact area between the ITO electrodes and the copper electrodes is decreased. Moreover, the risk that the ITO glass slide would detach increases if the amount of epoxy is reduced. Variations between the electrodes were detected most likely due to the different amount of epoxy.

4.1.3 Soldering of pins

Soldering of pins on the copper PCB was challenging since a slight slip would cause the copper electrodes to short circuit. Moreover, the amount of soldering wire was difficult to control. Some of the silver pins were loosened after attaching the wires which most likely affected the measurements.

4.1.4 Sterilization

The chips with copper PCB that were sterilized for 2 hours in isopropanol were intact, whereas for most of the chips that were sterilized overnight the PCB detached from the ITO glass slide. ITO has a hydrophobic surface, making it less prone to attach to the silver epoxy which most likely is the reason that the epoxy detached from the ITO surface and not from the copper electrodes. Moreover, the copper surface became slightly discolored after the sterilization overnight, indicating that the surface was not completely unaffected after sterilization.

Sterilization by autoclaving increased the impedance of the electrodes of the chips in the second prototype. Most likely due to the silver epoxy detaching slightly from the ITO. It is not surprising that
the silver epoxy was negatively affected by the process since the maximum operating temperature for the silver epoxy is 100 °C according to the datasheet and the autoclave runs at 120 °C at elevated pressure. To overcome this problem, another conductive epoxy could be used.

The first prototype chips sterilized in isopropanol did not seem to be negatively affected by the sterilization process.

### 4.2 Characterization of chips

#### 4.2.1 Profilometer

A profilometer was used to measure the surface profile of the ITO patterned glass slides. The stylus was swept over the largest spacing of 2 mm between the ITO electrodes to analyze the surface profile after etching with pulsed laser.

The laser etched approximately 100-200 nm of the ITO coating that was 400 nm thick, creating a spacing between the electrodes. Hence, the ITO coating wasn’t completely removed after etching the spacings. Even so, the electrodes were not short circuiting since the laser etched the edges of the electrodes twice. Valleys were formed at the edges of the electrodes, isolating the electrodes from the spacings. They were approximately 500 nm deep. In Figure 10 the valleys are seen at approximately 450 µm and 2, 280 µm, the spacing is seen between these two values. The ITO that was etched away from the spacing was redeposited outside the edges. Close to the valleys there are peaks of redeposited ITO debris that subside further away from the valleys.

![Figure 10. Sweep with profilometer over 2 mm spacing of ITO patterned glass slide.](image)

#### 4.2.2 SEM

SEM was used to image the surface of the patterned ITO glass slides. ITO is darker in the image compared to the exposed glass since it is conductive and does not charge. The first SEM image was taken between a spacing and the ITO electrode. The tracks from the pulsed laser are seen as parallel stripes to the left in Figure 11. The stripes seem to end closer to the edge between the etched part and ITO, probably because the information is lost in the valley. The width of lost information is approximately 100 µm which corresponds to the width of the valley in Figure 10. Valleys aren’t possible
to see in SEM if the sample isn’t tilted since the electrons are scattered away from the detector. A trench is seen between the etched part and the ITO, it’s probably the steepest part of the valley. The unetched ITO surface is seen to the right in the same figure. The ITO surface is much smoother than the etched surface.

The second SEM image (Figure 12) shows a corner between the etched part and the ITO surface. The tracks of the laser are seen to the right in Figure 12. Scratches in all directions are seen over the ITO coated surface, however, they are not observed where the ITO is etched away, implying that they are shallow. Furthermore, they are not seen close to the trenches since the redeposited ITO covers them. The ITO glass slides were stacked on top of each other without a protective layer in between which might have caused damage to the surface.

The ITO is shown at a higher magnification in Figure 13. The scale of 200 nm indicates that the sputtered surface is rather even and smooth.

Figure 11. SEM image of ITO coated glass slide after laser patterning. To the left, the laser has etched away the ITO and to the right the ITO is intact.
Figure 12. SEM image of top left corner of etched 1 mm spacing. Tracks of the laser are seen to the right where the ITO has been etched. To the left, the unetched ITO is seen. The frame surrounding the ITO pattern is seen in the top of the image. The substrate is tilted 45°.

Figure 13. SEM image of sputtered ITO surface. The sample is tilted 45°.
4.3 Characterization of cells

4.3.1 Optical microscopy images of cells

An optical microscope was used to analyze the cell growth on ITO inside the well. The main purpose was to assess if the cells preferred one surface over the other. Surprisingly, the cells spread evenly on both the ITO and the glass, where the ITO had been etched away, see Figure 14. Moreover, the cell morphology didn’t differ between the two surfaces. According to previous studies the cell proliferation is affected by surface roughness,\textsuperscript{40} which is why a difference in cell growth was expected for the different surfaces. Moreover, the cells wouldn’t grow on the etched part on a previous design due to a rough surface. The difference in roughness between the etched part and the ITO is most likely not large enough to affect the cell growth in the current design. A homogeneous cell growth is desirable for more reliable results when measuring the impedance.

The cells in the second prototype were stained with green fluorescent dye. Images of the cells were taken after 2 days, see Figure 15. The cells were already confluent after 2 days. As seen in Figure 15, the cells are stretched out and protrusions are formed, indicating healthy cells. Furthermore, the nuclei are also elongated and appear healthy.

![Figure 14. Optical microscopy picture of an ITO glass slide covered with cells.](image)
4.3.2 Biocompatibility of chip

Biocompatibility studies were performed to assess the biocompatibility of the different components of the chip. All components in contact with the cells must be biocompatible for the cells to differentiate and proliferate. The biocompatibility was evaluated by comparing proliferation, cell morphology and cell density in the samples to that of the control instantly after seeding and 2 and 4 days after seeding. Right after seeding the cells are round and have not attached to the surface. The results are presented in Figure 16 where the columns represent each day and the rows from top to bottom represent the different materials ABS-plastic, ITO glass slide, UV-glue and the control.

The cells cultured inside the ABS-plastic rings appear to have a slightly higher cell density after 2 days compared to the other samples. This is most likely due to the smaller surface area available for the cells to grow in since the ring occupy a part of the well. Moreover, there might be some variations in cell density between the different samples. The cells are confluent already after 2 days, indicating that there are no adverse effects of the ABS-plastic compared to the control. Compared to the control, the cells show similar morphology and proliferation. The cells are not really in direct contact with the plastic when they are seeded in the chips since the plastic is covered with a layer of UV-glue.

The cells seeded in chips are mainly in contact with the ITO glass slide, making it imperative to compare cell growth on ITO to cell growth in a well plate. The ITO glass pieces did not cover the entire bottom of the well, making it difficult to seed the cells evenly over the entire surface available. Even so, the cell density does not noticeably differ from that of the control. The cells spread over the ITO surface and exhibit similar morphology to the cells grown on well plates.

The cells seeded on UV-glue are most similar to the cells of the control for all time points. In the chips, the cells are not grown directly on UV-glue but the cell media is in contact with the glue. Hence, it is important that the UV-glue does not leach out any toxic substances into the cell media, causing cell death or change in behavior. No indications of toxicity can be seen. The proliferation and differentiation of the cells match those of the control as well as the cell morphology.
Figure 16. Comparison of cell growth for bEnd3 cells cultured in contact with ABS-plastic, UV-glue, ITO and a control, after 0, 2 and 4 days.
4.4 Impedance measurements

4.4.1 First prototype

The first measurements with a 4-point probe station were challenging since the electrodes were transparent and it was difficult to see when the probes were in contact with the ITO. It was easy to scratch the surface of the ITO without noticing. Moreover, it was difficult to assure that the 4-probes were in contact with the surface at the same time since the slightest tilt would cause the probe to lose contact. Two of the probes were removed to facilitate the measurements. However, the fact that the probes were scratching the conductive surface remained and after a few measurements the contact was lost, causing the impedance value to increase. The disadvantage of using only 2 probes instead of 4 was that the impedance between the probe and the ITO electrode is now included in the measured total impedance which is why the 4-point probe station was the first approach.

A second approach to measure the impedance of the cells was attempted. In this approach, the probes were thicker and held by hand towards the ITO surface. Thicker probes are less susceptible to variations on the surface since a larger area is in contact with the ITO surface. However, since the probes were handheld, a steady hand was required in order to obtain a stable value. Once again, the probes scratched the ITO surface and the contact was lost, giving unreliable measurements. Only one chip out of five lasted for five measurements.

4.4.2 Second prototype

The PCB was attached to the glass substrate to protect the ITO surface and to allow measurements on the copper instead of directly on the ITO coating, which damaged the coating. The pins attached to the extended copper electrodes were connected to wires that were connected to the impedance analyzer. The measurements were much less time consuming and less prone to variations since the probes were not held manually as they were in the first prototype. However, as mentioned earlier the silver epoxy detached from the ITO during sterilization, destroying the chips. The fabrication of the PCB chips was time consuming and there was not enough time to make more of them, only one spare chip remained that could be prepared and used for the impedance measurements. The silver epoxy was sealed and protected by applying UV-glue around the edges of the glass slide which made it possible to sterilize the last chip in the autoclave. In order to draw any statistically relevant conclusions from the impedance measurements more experiments are required since only one chip was tested.

4.4.3 Absolute impedance

The absolute impedance was measured across two electrodes at a time over a range of frequencies (10^2-10^6 Hz). The impedance at 10^4 Hz was plotted against the distance between the electrodes and is shown in Figure 17 for both prototypes at day 1. The resulting linear plots are called transmission lines from which the sheet impedance and contact impedance are extracted, see Figure 2. Trendlines with R-square values were added to the plots to estimate the linearity of the plots. As seen in Figure 17, the plot for prototype 1 follows a linear trend with a high R-square value whereas the impedance for prototype 2 deviates to a larger extent from the trendline. This difference in linearity between prototype 1 and 2 is more pronounced for the subsequent days, see Appendix A. The fluctuating values for prototype 2 most likely causes the following results to be unreliable since they are based on the plots in Figure 17.

The second prototype has more components contributing to the impedance such as the silver epoxy, the soldered pins and the wire connected to the impedance analyzer which probably cause variations over
the different spacings. The overall values are also higher than for the first prototype, indicating that the components added to the first prototype increases the impedance which is not surprising.

![Figure 17](image.png)

**Figure 17.** The total impedance measured at $10^4$ Hz as a function of distance between the electrodes for prototype 1 (left) and prototype 2 (right).

The contribution of the cells and the tight junctions to the total impedance is obtained by subtracting the measured impedance for the cell media only from the total impedance. However, the measurements for the pure cell media for prototype 2 fluctuated to the extent that subtracting the values from the total impedance would give misleading plots. Hence, the results are presented separately. The total impedance for pure cell media is shown in **Figure 18**.

![Figure 18](image.png)

**Figure 18.** Measured impedance of cell media without cells as a function of distance between electrodes for prototype 1 (left) and prototype 2 (right).

### 4.4.4 Sheet impedance

The sheet impedance is extracted from the slope of the transmission lines in **Figure 17**. The unit of the sheet impedance is $\Omega$ per arbitrary area unit (□) and is thereby a material constant and not dependent of the spacings between electrodes. In the case of this project the sheet impedance is the impedance of tight junctions, cells and cell media in the chip. The sheet impedance is expected to increase as the cells start to proliferate and form tight junctions. In **Figure 19**, no increase of the sheet impedance is seen over time for prototype 1, the impedance appears rather constant. The sheet impedance for prototype 2 varies
randomly between 50 and 400 $\Omega/\square$ over time. No increase due to formation of tight junctions is seen in neither one of the prototypes. However, only one chip from each prototype was tested so there is not enough data to discard the prototypes.

Figure 19. Sheet impedance of the first prototype with cells at the frequency $10^4$ Hz as a function of the number of days for prototype 1 (left) and prototype 2 (right).

4.4.5 Contact impedance

The contact impedance is the impedance at the interface of the electrodes and the cells. It is dependent of the area of the electrodes, that are all the same size in this case. The contact impedance is expected to increase as the cells start to proliferate and cover the electrodes. In Figure 17, the intercept with the y-axis is two times the contact impedance. The contact impedance is plotted as a function of days in Figure 20 for both prototypes. For the first prototype the contact impedance is constant over time and much lower than for the second prototype. The second prototype shows a peak in contact impedance at day 3, however, the peak is not pronounced and random fluctuations in the measurements cannot be excluded given that the transmission line in Figure 17 does not follow a linear trend.

Figure 20. Contact impedance as a function of days after seeding the cells for prototype 1 (left) and prototype 2 (right).
4.4.6 Calcium switch

After measuring the impedance of the second chip for 4 days, the cell media was replaced with cell media without Ca\(^{2+}\). 2 hours later the impedance was measured and plotted against the spacings in Figure 21. Comparing the impedance after 4 days with and without Ca\(^{2+}\) the difference seems to be random between the two measurements. The impedance is expected to be lower without Ca\(^{2+}\) since the tight junctions should be disrupted causing the impedance to decrease, however, the impedance is even higher for the third and fourth spacings without Ca\(^{2+}\) as compared to the impedance measured before removing the Ca\(^{2+}\). This improbable outcome implies that the variations within the same sample are large and the measurements are untrustworthy. It is not possible to assert whether or not tight junctions were formed between the cells.

Figure 21. The absolute impedance with cells at 10\(^4\) Hz as a function of the distance between the electrodes at day 4, left figure. In the right figure the cell media was replaced with Ca\(^{2+}\)-free media.
5 Conclusions

Two prototypes of BBB-on-chips were fabricated and characterized with SEM and a profilometer. The chips were used to measure the change in impedance over time as endothelial cells (bEnd3) started to proliferate and form tight junctions.

The first prototype was easy to fabricate and reproducible. The impedance was however difficult to measure without damaging the surface of the ITO electrodes. The sputtered thin film of ITO was more delicate than expected. The impedance was measured by manually holding probes against the ITO, causing the electrodes to eventually lose contact due to scratches on the surface. Furthermore, the transparent electrodes made it difficult to see if the probes were in contact with the surface or not. Holding the probes manually caused variations in the measurements since it was impossible to hold the probes completely still during each measurement.

The second prototype was more time consuming to fabricate and required more skills. The silver epoxy was difficult to apply in the correct amount since it dried fast and the consistency was viscous. Soldering of pins was also challenging due to the risk of short circuiting the electrodes. However, measuring the impedance was easier since the wires were directly connected to the pins so there was no need to hold the probes manually. Even so, the measurements varied more than for the first prototype, most likely due to the silver epoxy and the soldered pins. The contact area of the silver epoxy varied between the electrodes as well as the quality of the soldering of pins which might have caused the variations.

The impedance measurements were carried out with only one chip of each prototype which is not enough to draw statistically significant conclusions. The experiments must be repeated with more chips to ensure that variations are not random.

The cells appeared healthy with elongated protrusions and even spreading over the ITO surface.

The components of the chips did not show any signs of toxicity in biocompatibility studies. The cells in contact with ABS-plastic, ITO and UV-glue did not differ from the well-plate control.
6 Acknowledgements

I would like to thank my supervisors Sofia Johansson and Gemma Mestres for introducing me to this project and guiding me through it. They were both there for me at any time I needed them.

I would also like to thank the whole EMBLA group for encouraging me throughout the project and always coming up with new ideas. I learned a lot about different instruments and techniques. Thank you for always taking your time to teach me!

I would like to thank Maria Tenje for an inspiring lecture that arouse my interest in the topic organs-on-a-chip. She is really an inspiration in many ways.
7 References


8 Appendix A: Impedance measurements

Figure A1-A3 show the absolute impedance measured as a function of distance between the electrodes for prototype 1. The graphs follow a linear trend with high R-square values. Figures A.4-A.6 show the same graphs for prototype 2, however, they are not as linear as prototype 2 and the R-square values are low in comparison to prototype 1.

Figure A.1. Absolute impedance measured 3 days after cell seeding as a function of distance between electrodes.

Figure A.2. Absolute impedance measured 5 days after cell seeding as a function of distance between electrodes.
Figure A.3. Absolute impedance measured 7 days after cell seeding as a function of distance between electrodes.

Figure A.4. Absolute impedance measured 2 days after cell seeding as a function of distance between electrodes.
Figure A.5. Absolute impedance measured 3 days after cell seeding as a function of distance between electrodes.

Figure A.6. Absolute impedance measured 4 days after cell seeding as a function of distance between electrodes.