INTRODUCTION TO THE BLOOD-BRAIN BARRIER

# IMPLEMENTING A GREEN FLUORESCENT PROTEIN TAG COUPLED TO ZONULA OCCLUDENS-1 IN BRAIN ENDOTHELIAL CELLS.

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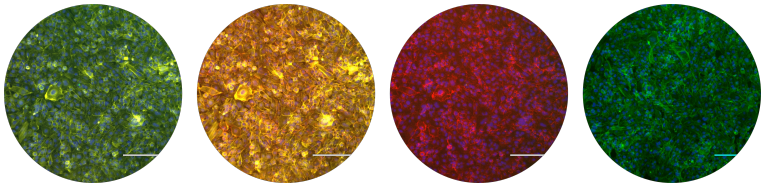

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## ABSTRACT

De bloed-brein barrière (BBB) speelt een cruciale rol bij de bescherming van het brein tegen ziekteverwekkers. Echter kunnen door deze rol de medicijnen het brein ook niet bereiken. Dit komt door de tight-junctions tussen de endotheelcellen van de BBB. In dit onderzoek worden hCMEC/D3 endotheelcellen getransfecteerd met een plasmide, die een GFP-tag bevat en deze bindt aan het ZO-1 eiwit van de tight-junctions. Er is gevonden dat deze cellen getransfecteerd kunnen worden met Lipofectamine 3000 en Lipofectamine LTX in Heparine-vrij medium. Andere transfectie reagentia zoals DharmaFECT, ViaFECT en Lipofectamine 2000 werkten niet op de hCMEC/D3 cellen. Ook werkte Opti-MEM en EGM-2 niet als geschikt medium voor alle transfectie reagentia. Vervolgens is er een kill curve gedaan met G418 selectief antibioticum en dit gaf geschikte concentraties G418 selectieve antibioticum. Deze concentraties zijn vervolgens toegepast op het genereren van een stabiele cellijn. Wanneer dit gelukt is, kunnen de endotheelcellen van de BBB live gemonitord worden en kan het mechanisme van het openbreken van de BBB beter onderzocht worden.

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# Chapter 1

# Introduction

## 1.1 Background information

The blood-brain barrier (BBB) is of vital importance for the protection of the human brain, as it prevents toxins from entering into the central nervous system. However, it also poses some significant problem with delivering therapeutics to the brain, because therapeutics also can not pass this barrier[1]. Therefore, different methods are created to let the therapeutics pass the BBB, including opening up the BBB or decreasing the molar mass of the therapeutics[2, 3]. This is of particular interest with diseases such as Alzheimer's disease, which are localized in the brain, regarding that the brain can not be reached by therapeutics[4]. Around 285.000 people in the Netherlands have dementia, from which an estimated 200.000 have Alzheimer's disease[5]. Due to these high numbers, Alzheimer's disease is creating an enormous economical, public and social problem. Therefore, research in this field is of the utmost importance. Opening of the BBB can possibly help with numerous diseases where therapeutics needs to pass through the BBB, these are mostly neurodegenerative diseases such as Alzheimer's disease, but also Parkinson's disease and Huntington's disease[4].

The BBB is an important feature of the microvasculature of the central nervous system(CNS), which prevents the toxins from entering into the CNS. The BBB is made of a neurovascular unit, which contains different types of cells such as endothelial cells, mural cells, astrocytes, pericytes, the basement membranes and immune cells, shown in figure 1.1 [1, 6].

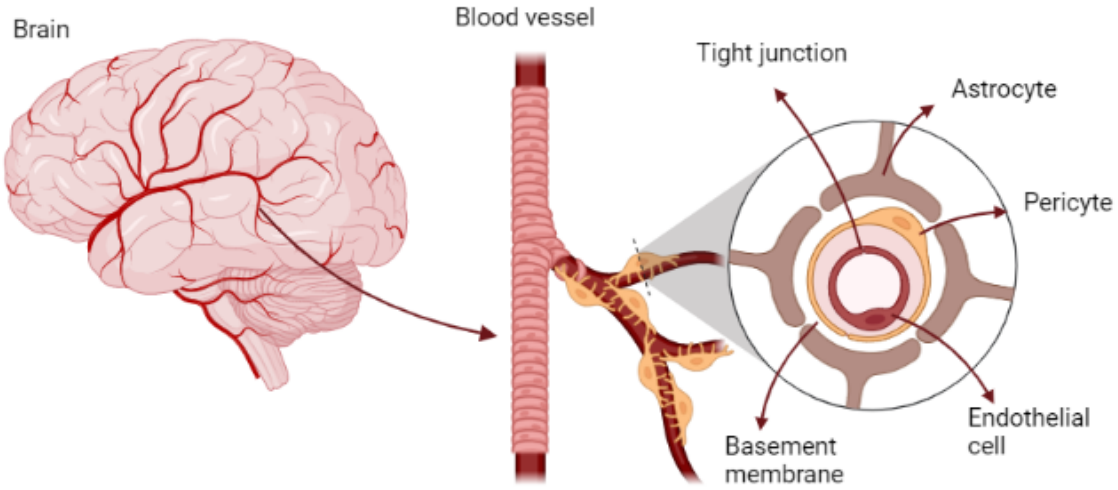


**Figure 1.1:** The Blood-Brain Barrier. From a macro view is zoomed in into the cross section of the blood vessel of the brain. Different cell types such as Astrocytes, Pericytes and Endothelial cells are shown. Also the tight-junction between the endothelial cell and the basement membrane can be seen. (Created with BioRender)

Endothelial cells (ECs), which form the blood vessels, are important for opening the BBB[7]. The ECs are normally semi-permeable and therefore provide the exchange of different solutes[7, 8]. An exception for this

exchange is for the blood vessels that enter the CNS [7, 8]. Here, the exchange is limited to solutes with lipophilic molecules that are under 400 Da [9]. This limitation of the paracellular flux of solutes is mainly caused by the tight junctions (TJs) of the ECs [9].

The ECs are connected with each other through these TJs, these cellular connections in the TJs occur because transmembrane molecules interact with cytoplasmic adaptors to form a linkage with the cytoskeleton[1]. A few of these transmembrane molecules are occludins, claudins and junction adhesion molecules[10].

Zonula occludens (ZO) proteins are a type of cytoplasmic adaptors and these proteins belong to the membrane-associated guanylate kinase family[11, 12]. These proteins are scaffolding proteins that link the cytoskeleton to the transmembrane molecules in the TJs[11]. There are three different types of ZO proteins, respectively 1, 2 and 3, of which ZO-1 was the first one discovered. Research states that the cellular permeability can be increased for larger solutes due to depletion of the ZO-1[13]. Therefore, the ZO-1 protein can indeed form a linkage between the actin cytoskeleton and the transmembrane molecules, which plays a role in the cellular permeability between the ECs[13].

The hCMEC/D3 cell line is a brain microvascular endothelial cell line, which is positive for different TJ proteins and scaffolding proteins including ZO-1[14]. Besides that, the hCMEC/D3 cell line is a very stable cell line, which grows easily and is transferable, while maintaining the BBB phenotype[15]. Therefore this cell line is suitable to study the TJs of the BBB[15].

Since opening up the BBB can help therapeutics pass through the BBB to the brain, different methods are being developed to achieve this opening with among others using a permeability enhancer, nanoparticles or active transporters.[16–19]. An other method is Focused Ultrasound (FUS), which can be used in combination with microbubbles that are injected intravenous[6]. Through the oscillation of the microbubble, the BBB can be opened. This method is currently the only non-invasive method to transiently open the BBB for therapeutics to pass to the brain[6]. The liquid pressure of the ultrasound and the size of the microbubble are important parameters[3]. When the pressure is too low, the BBB will not open and when the pressure is too high there can be tissue damage as a result [3].

Visibility of the ECs can help to understand the mechanism of opening the BBB and to determine if the BBB is disrupted and the therapeutics should be able to pass through the BBB. To see live if how the opening of the BBB has occurred with for example FUS, a fluorescent ZO-1 protein tag can be transfected into the ECs with a plasmid. Plasmids are build up from DNA and can contain different regions inserted by humans such as the Origin of Replication , an antibiotic resistance gene, an inserted gene with restriction sites and a promoter site[20].

A method for plasmid transfection makes use of liposomes, for example Lipofectamine[21]. There are different types of Lipofectamine or other transfection reagents and they contain cationic lipids that form liposomes, which help transfection of the plasmid into the cell[21–23]. The positive charges of the cationic lipids form electrostatic interactions with the negatively charged DNA of the plasmids[22]. Once the DNA plasmids form an interaction with the liposomes, they are also protected against nuclease activity, which can break down the DNA[22].

When the cells are transfected there normally is a transient transfection of a few days, but also a stable cell line can be generated. This happens when the transfected DNA is integrated into the nuclear genome[24]. To obtain this, the cells are treated with an selective antibiotic for which the DNA plasmid is resistant and only the cells that are transfected stay alive[24]. These cells can grow again and eventually the DNA will be integrated into the nuclear genome[24]. The used plasmid is resistant to G418 selective antibiotic.

Monitoring the fluorescent TJs gives useful information about visualizing endothelial cells, which helps to understand the mechanism behind opening of the BBB. Additionally, live monitoring with an fluorescent microscope is useful to understand and study different methods of opening the BBB, such as FUS. Therefore, the

aim of this study is to insert a fluorescent protein tag to the ZO-1 in the hCMEC/D3 cells through transfection of a plasmid with a suitable transfection reagent.

## 1.2 OUTLINE

Chapter two explains the used materials and methods, here there will be a part about the cell maintenance of the hCMEC/D3 cells, the different transfection experiments, the cell staining, kill curve and the generation of a stable cell line. In chapter three, the results of the transfection-, cell staining-, kill curve- and stable cell line generation experiments are shown and explained. In chapter four, the results are examined and conclusions are made about the suitability of the transfection reagents in combination with medium. Also explanations for the results and recommendations for future research are given. Chapter six is giving a short overview of the conclusions which can be drawn from the results. Finally, chapter seven contains the appendix with additional figures from the experiments.

# Chapter 2

# Materials and Method

## 2.1 Materials

The used cell line is the hCMEC/D3(Merck, Germany)cell line. The used types of Lipofectamine are Lipofectamine 3000 with the P3000 reagent(Invitrogen, US), Lipofectamine LTX with the PLUS Reagent(Invitrogen, US) and Lipofectamine 2000(Invitrogen, US). Besides Lipofectamine, also DharmaFECT(Horizon, England) and ViaFECT(Promega, US) are used. The cell culture medium used, is Endothelial cell Growth Medium 2 (EGM-2)(PromoCell, Germany). For transfection also Opti-MEM(Gibco, US) and EGM-2 without Heparin is used as medium.

## 2.2 Cell culture

First the culture ware was coated with a 100$\mu$g/ml collagen solution including Phosphate Buffered Saline (PBS) (Gibco, US) and Collagen-I from a rat tail(Corning, US). After the coating, the cells were seeded with 10 ml EGM-2 into the new T75 flask to reach a confluency of 90% in three days and they were incubated at 37°C with 5% CO2. This step needed to be performed every three or four days to keep the cells alive. The cells were seeded to reach a 70-90% confluency a day after seeding in a 48-wells plate for experiments. Only for the stable cell line generation, a 12-wells plate was used. The hCMEC/D3 cells were used between passage 30 and 35.

## 2.3 Transient transfection

The hCMEC/D3 cells were transfected with plasmid DNA(Addgene, US, sequence number 140271) containing a GFP-tag to target the ZO-1 protein. This plasmid DNA is raised in E-coli bacteria and isolated with the PureYield Plasmid Midiprep system(Promega, US). Before the transfection the cells had to be confluent around 70-90%. For the transfection, five different transfection reagents were used, which had a common protocol, shown in figure 2.1. First the amounts of plasmid DNA and transfection reagents were added to two eppendorf tubes diluted in Opti-MEM, EGM-2 or EGM-2 without Heparin, depending on the experiment(Table 2.1). Then these tubes were added in a 1:1 ratio together and incubated at room temperature. Two or three days after transfection the results were imaged with the EVOS FL microscope(Life Technologies, US).

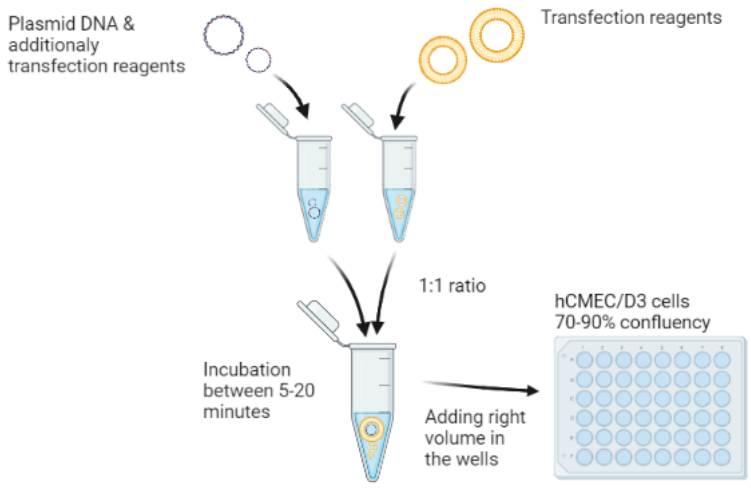


**Figure 2.1:** Visualisation of the transfection protocol. First the plasmid DNA with additional transfection reagents were added together in an eppendorf tube. In the other eppendorf tube the main transfection reagent was added. Both were diluted in Opti-MEM, EGM-2 or EGM-2 without Heparin, depending on the experiment. This was added together with a 1:1 ratio and incubated at room temperature. The incubation time is dependent on the type of transfection reagent, which differs between 5 and 20 minutes. Finally, the DNA-lipid complex is added to the cells. The total volume was kept the same. (Created with BioRender)

Six different transfection experiments were tested and optimized. In these experiments Lipofectamine 3000, Lipofectamine LTX, Lipofectamine 2000, ViaFECT and DharmaFECT were examined with different concentrations following table 2.1. All these concentrations were tried with Opti-MEM, but only the highest concentrations of each transfection reagent were tried in EGM-2 and EGM-2 without Heparin. Negative controls were performed with cells in solely medium and medium with transfection reagent without the plasmid DNA.

**Table 2.1:** The used tranfection reagents with their incubation time, added amount of DNA-lipid complex and added amount of transfection reagent, additional transfection reagent and DNA in this DNA-lipid complex.*DharmaFECT has also a five minutes incubation time before the two eppendorf tubes are added together.

| Transfection reagent | incubation time(min) | DNA-lipid complex($\mu$l) | transfection reagent($\mu$l) | Additional transfection reagent($\mu$l) | DNA(ng) |
|---|---|---|---|---|---|
| Lipofectamine LTX | 5 | 20 | 0,4/0,7/1 | PLUS reagent: 200 | 200 |
| Lipofectamine 3000 | 10-15 | 20 | 0,3/ 0,6 | P300 reagent: 400 | 200 |
| Lipofectamine 2000 | 5 | 20 | 0,4/0,7/1 | - | 200 |
| ViaFECT | 5-20 | 20 | 0,3/0,6/1,2 | - | 200 |
| DharmaFECT | 20* | 50 | 0,1/0,55/1 | - | 500 |

## 2.4 CELL STAINING

A cell staining was performed to examine if the ZO-1 protein are visible with the EVOS FL microscope. Besides ZO-1 a positive control of VE-Cadherin staining was used. Also the nucleus and actin filaments were stained to visualise the cells, information is shown in table 2.2. To fixate the cells 4% Formaldehyde(Sigma-Aldrich, US) diluted in Milli-Q(Merck, Germany) was used and this was incubated for at least 15 minutes at room temperature. After the cells were washed three times with PBS, they were blocked for at least one hour with a blocking solution containing PBS with 5% BSA(Sigma-Aldrich, US) and 0,1% Triton X100(Sigma-Aldrich, US) at room temperature. After blocking, the cells were washed again three times with PBS and the primary antibodies were added for ZO-1 and VE-Cadherin.

These primary antibodies were incubated overnight at 4 °C and were washed away three times with PBS. After this the secondary antibodies were added and they were incubated for at least 30 minutes at room temperature. After this incubation, the cells were washed again three times with PBS. After washing, a Phalloidin and DAPI staining were done separately, washing with PBS three times in between. The DAPI was incubated for 5 minutes at room temperature and the Phallodin was incubated for 20 minutes at room temperature. The cells were washed again three times with PBS and the cells were imaged with the EVOS microscope in PBS.

**Table 2.2:** The used antibodies for the staining with their Vendor, catalog number, host, reactivity, dilution and staining.

| Antibody | Vendor | Catalog number | Host | Reactivity | Dilution | Staining |
|---|---|---|---|---|---|---|
| ZO-1 | BD Bioscience | 610967 | Mouse | Human | 1:100 | Tight-junctions |
| VE-Cadherin | RD Systems | AF938 | Goat | Human | 1:100 | Adherens junctions |
| Phalloidin Alexa Fluor 633 | Molecular Probes | A22284 | - | - | 1:80 | Actin filaments |
| DAPI | Molecular Probes | D1306 | - | - | 1:4000 | Nucleus |
| Alexa Fluor 568 | Thermo Fisher | A11057 | Donkey | Goat | 1:100 | Secondary antibody |
| Alexa Fluor 488 Ready probe | Invitrogen | R37120 | Goat | Mouse | 2 droplet/ml | Secondary antibody |

## 2.5 KILL CURVE

A kill curve was generated to choose the right concentration of G418 selective antibiotic(Sigma life sciences, US), in order to generate a stable cell line. A range of different concentrations of G418 selective antibiotic were tested on the hCMEC/D3 cells, these concentrations of G418 are 0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 $\mu$l G418 per ml EGM-2. The selective medium was changed every two or three days. Pictures were taken on day three and seven with a Eclipse TS100(Nikon, US) to determine the low, middle and high concentration of G418 selective antibiotic.

## 2.6 STABLE CELL LINE GENERATION

The aim is to generate a cell line in which the DNA from the plasmid is build into the cell's own DNA. Therefore all cells that do not contain the plasmid DNA are killed with the G418 selective antibiotic, since these cells are not resistant. First the transfection was performed, for this each well contained Lipofectamine LTX, which was 6,25 $\mu$l in each of the six wells from the 12 wells plate. Each well also contained 1,25 $\mu$l PLUS reagent and 1,25 $\mu$g plasmid DNA. Lipofectamine LTX is chosen as tranfection reagent, because it showed a higher transfection efficiency in previous experiments. The transfected cells were incubated for two days at 37 °C with 5% CO2.

The protocol of Mirus Bio LLC(US) is used[25], where a low, middle and high concentration of G418 selective antibiotic are chosen from the results of the kill curve. The low concentration is the concentration which showed low visible toxicity on the seventh day of the kill curve, this was 50 $\mu$l/ml. The middle concentration is the concentration at which all cells are death on the seventh day of the kill curve, this was 300 $\mu$l/ml. The highest concentration is the concentration at which the first toxicity is visible at day three of the kill curve, this is 400 $\mu$l/ml. All these conditions were used in the experiment with the middle and high concentration in duplicate. Also a control without selective antibiotic was performed. These concentrations were added on the third day after transfection and the selective medium was changed every two to three days. After ten days the cells could grow in normal EGM-2.

# Chapter 3

# Results

## 3.1 TRANSFECTION

To obtain transient transfection the cells were transfected with different transfection reagents, shown in table 2.1. All the condition were similar but only the media changed. No fluorescently tranfected cells were observed in both Opti-Mem and EGM-2 with Heparin after transfection with Lipofectamine LTX(Fig. 3.1A & 3.1B). However, fluorescently transfected cells were observed in Heparin-free EGM-2 after transfection with Lipofectamine LTX(Fig. 3.1C).

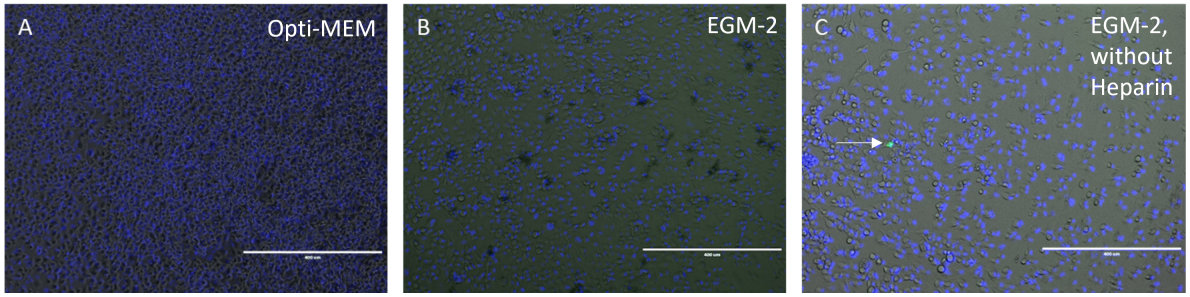


**Figure 3.1:** Transfection with Lipofectamine LTX in Opti-MEM, EGM-2 and EGM-2 without Heparin. Transfection was performed in Opti-MEM with 50.000 cells per well (A) EGM-2 with 60.000 cells per well(B), EGM-2 without Heparin with 60.000 cells per well(C). There is a GFP-signal in the transfection with EGM-2 without Heparin, the other media gave no GFP-signal. The GFP-signal is visible with the white arrow. The magnification used is 10x and the scalebar is 400 $\mu$m.

Also Lipofectamine 3000, Lipofectamine 2000, ViaFECT and DharmaFECT transfection reagents were used in Heparin-free medium. It can be seen that besides Lipofectamine LTX, Lipofectamine 3000 also shows fluorescently transfected cells(Fig. 3.2A & 3.1B). Other transfection reagents such as Lipofectamine 2000, DharmaFECT and ViaFECT showed no fluorescently transfected cells(Fig. 3.2C, 3.2D & 3.2E). Regarding the control condition, it can be seen that there are less cells when the DNA-lipid complex is added to the cells(Fig. 3.2F). In addition, no transfection reagent did not work in Opti-MEM or EGM-2(Appendix figure 6.1 & 6.2).

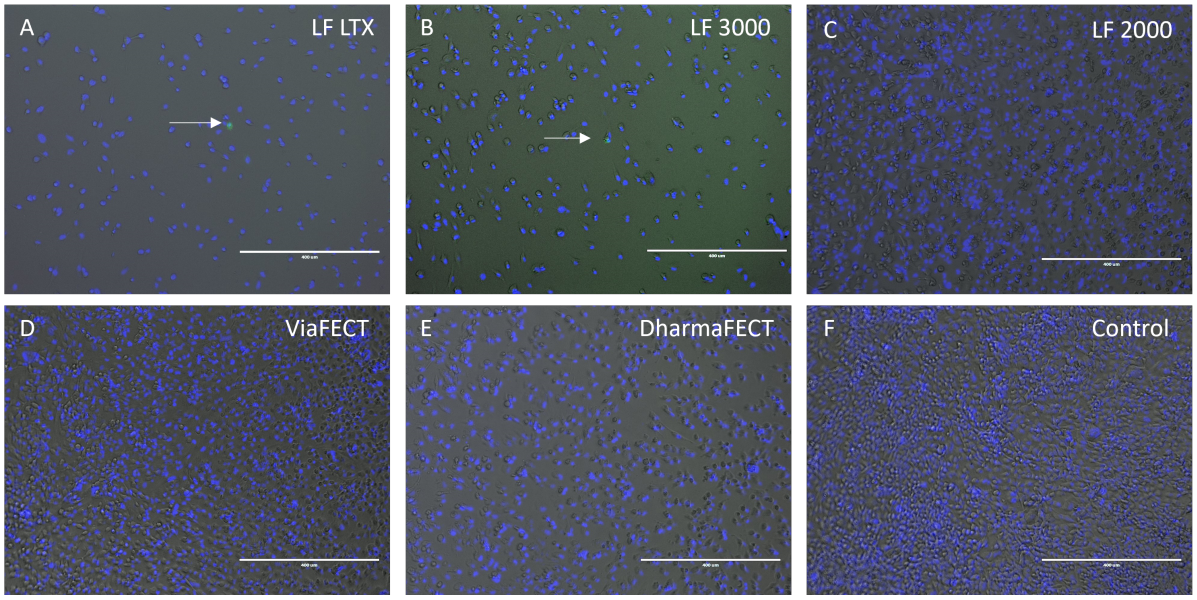


**Figure 3.2:** Transfection with DharmaFECT, ViaFECT, Lipofectamine- 3000, LTX and 2000. All the transfection experiments were done with the use of EGM-2 without Heparin and all wells contained 60.000 cells. Transfection was performed with Lipofectamine LTX (A), Lipofectamine 3000(B), Lipofectamine 2000(C), with ViaFECT(D), DharmaFECT 1(E). Also a control with cells without transfection reagent was performed(F). In transfection with Lipofectamine LTX and Lipofectamine 3000 GFP-signal is visible, all the other conditions did not contain visible GFP-signal. The GFP-signal is visible with the white arrow. All pictures are taken with a 10x magnification and the scalebar is 400 $\mu$m.

## 3.2 CELL STAINING

The cells were stained to examine if the ZO-1 protein is visible with the EVOS FL microscope and to compare localization of GFP-ZO-1 in transfected cells. It can be seen that the ZO-1 signal is visible at the cell membranes, but the signal is low.(Fig 3.3A). The control VE-Cadherin, which should be positive in endothelial cells, can also be seen in red at the cell membranes with a higher signal(Fig. 3.3B). Also, the actin filaments are stained with a magenta colour to show the cell structure, which can be seen at the location of the cytoskeleton inside the cell with a high signal(Fig. 3.3C).

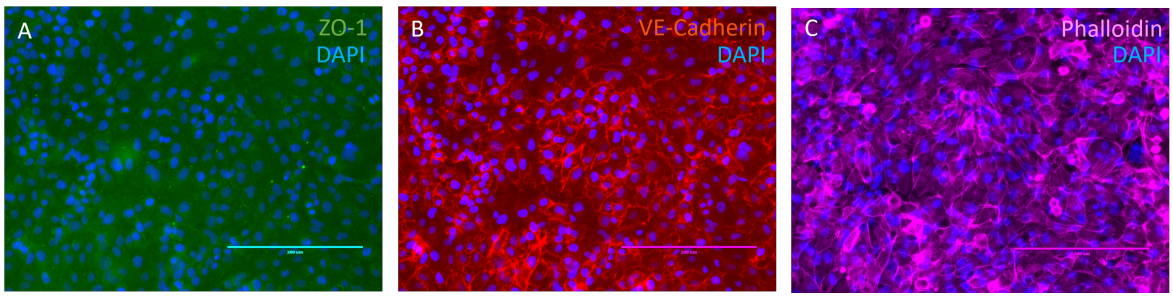


**Figure 3.3:** A staining of the hCMEC/D3 cells from the same sample. The cell stainings that are performed are a ZO-1(green) staining and the nucleus stained with DAPI(blue)(A), a VE-Cadherin(red) staining and the nucleus stained with DAPI(blue)(B), a actin filament(magenta) staining with Phalloidin and the nucleus stained with DAPI(blue)(C). A 20x magnification is used and the scalebar is 200 $\mu$m

## 3.3 KILL CURVE

To obtain a stable cell line, a kill curve had to be made to choose the correct concentrations for the selective medium. Toxicity can seen at day seven with the 50 $\mu$l/ml G418 and there are less cells than at day three(Fig 3.4A, 3.4B & 6.4). The first toxicity at day three was visible at a concentration of 300 $\mu$l/ml, where the cells are already less confluent(Fig. 3.4C, 3.4D & 6.3. Finally, all the cells seemed to be dead with 400 $\mu$l/ml on day 7(Fig.3.4E 3.4F & 6.4.

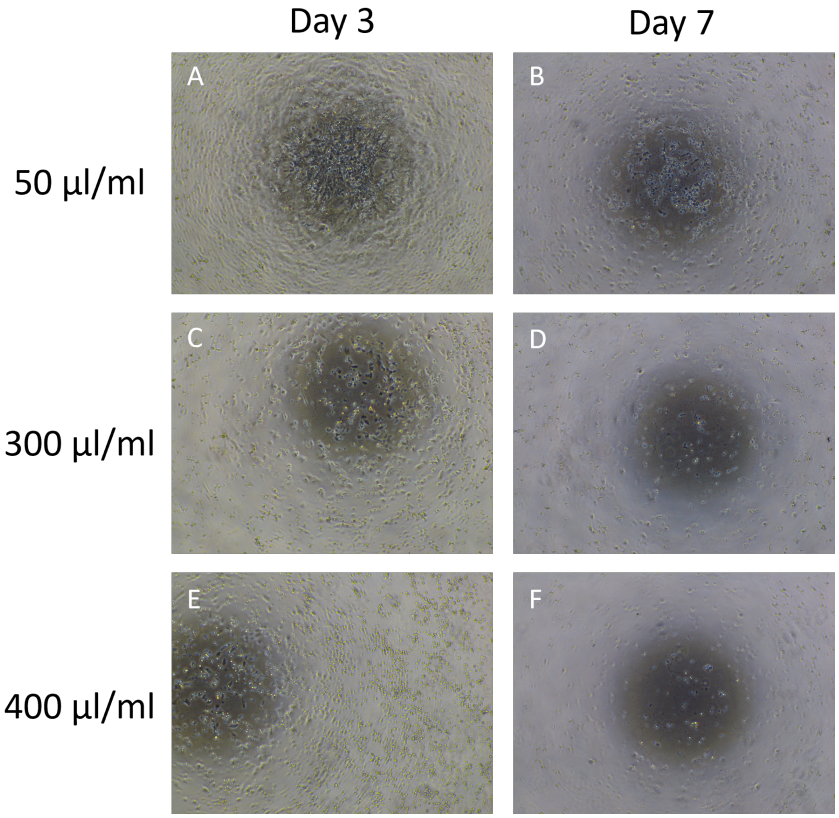


**Figure 3.4:** Kill curve of hCMEC/D3 cells with EGM-2 containing different concentrations of G418 selective antibiotic. Shown is day three with 50 $\mu$l/ml G418 selective antibiotic, there is no sign of toxicity (A). Shown is day seven with 50 $\mu$l/ml G418 selective antibiotic. Sign of toxicity is visible and cell death is around 30 %(B). Shown is day three with 300 $\mu$l/ml G418 selective antibiotic, there is sign of toxicity and cell death is around 50% (C). Shown is day seven with 300 $\mu$l/ml G418 selective antibiotic, there is sign of toxicity and cell death is around 95 %(D). Shown is day three with 400 $\mu$l/ml G418 selective antibiotic, there is sign of toxicity and cell death is around 65 %(E). Shown is day seven with 400$\mu$l/ml G418 selective antibiotic, there is sign of toxicity and cell death is around 100 %(F). Pictures are taken at 4x magnification with a Eclipse TS100 Nikon microscope.

The percentage of dead cells is estimated at day three and seven with sign of visible toxicity(Table 3.1). The sign of toxicity is positive when it is visible that dead cells are present or less cells than the control. For 50 $\mu$l/ml at day three the percentage dead cells is 0%, there is no sign of toxicity, and at they seven this percentage is 70% and there is a visible sign of toxicity. For 300 $\mu$l/ml at day three 50 % and at day seven 95% are dead cells, both show visible toxicity. For 400 $\mu$l/ml at day three 65% and day seven 100% are dead cells, both show visible toxicity. This estimation is made from the results of figure 3.4. The showed concentration are the low, middle and high concentrations of G418 selective antibiotic in EGM-2.

**Table 3.1:** The estimated toxicity in percentage on day three and day seven. The concentrations of G418 selective antibiotic are 50 $\mu$l/ml, 300 $\mu$l/ml and 400 $\mu$l/ml in EGM-2. The sign of toxicity is indicated with a positive (+) or negative (-) sign. The pictures taken with the Eclipse TS100 Nikon can be seen in figure 3.4

| Concentration G418 ($\mu$l/ml) | Percentage dead cells Day 3 | Percentage dead cells Day 7 | Sign of toxicity Day 3 | Sign of toxicity Day 7 |
|---|---|---|---|---|
| 50 | 0% | 30% | - | + |
| 300 | 50% | 95% | + | + |
| 400 | 65% | 100% | + | + |

## 3.4 STABLE CELL LINE

Two days after incubation from the transfection a visible GFP-signal was present(Fig. 3.5A). Around 5% green fluorescent cells are visible at a 10x magnification. The cells are round and a lot of cell death was seen in the EGM-2 without Heparin.

The cells are better visualized after zoomin in to a 40x magnification at day three after transfection to examine the localization of the fluorescence. A few fluorescently transfected cells can be seen(Fig. 3.5B). Whereas, the transfected cells show fluorescence located in the cytosol, but also in the nucleus of the cell, the ZO-1 staining showed expression at the cell membrane(Fig. 3.5B & 3.3).

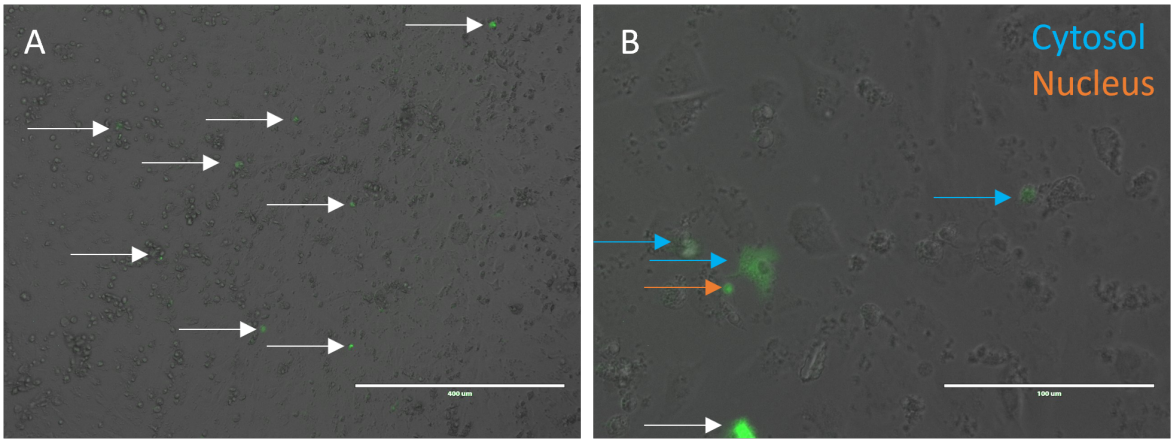


**Figure 3.5:** Transfection of the stable cell line generation. An overlay of the transfected cells for the stable cell line on day two after transfection. The white arrows indicate the transfected cells. A magnification of 10x is used and the scalebar is 400 $\mu$m(A). An overlay of the transfected cells for the stable cell line on day three after transfection. The arrows indicate the transfected cells and the colour the localization of the signal. The orange arrow means the localization in the nucleus, the blue arrow means the localization is in the cytosol and the white arrow means the localization is not known. A magnification of 40x is used and the scale bar is 100 $\mu$m(B)

# Chapter 4

# Discussion

The aim was to transfect the hCMEC/D3 cells to get a step closer to live monitoring the mechanism behind the opening of the BBB. Monitoring this mechanism can help with visualising the ECs in different studies for delivering therapeutics to the brain such as FUS. First, transfection needed to be optimized for hCMEC/D3 before creating a stable cell line. Therefore, different transfection experiments were done with different transfection reagents and media. The type of media used during the transfection was important, as Opti-MEM and EGM-2 with Heparin gave negative results whereas EGM-2 without heparin showed positive transfection.

## 4.1 Effects of transfection medium

Opti-MEM is a serum-free medium, where EGM-2 does contain serum. Serum changes the size and the surface properties of the lipid complex and therefore inhibits the plasmid DNA delivery into the cell [26]. Serum also contains Heparin, which plays another role in inhibiting the transfection efficiency[26]. As a matter of fact, Heparin is a negatively charged glycosaminoglycan(GAG) and proteoglycans are build up from multiple GAG-chains, which consist of polysaccharides[27]. Interesting is that proteoglycans are a part of the extra cellular matrix and play a role in the endocytosis pathway for cationic polymers, lipids and polypeptides[28, 29]. Therefore the difference in transfection efficiency between cell lines is possibly caused by these proteoglycans[27]. In addition to this, Heparin interferes with the DNA-lipid complex resulting in early release of DNA. [26, 30]. This early release is possibly before the DNA-lipid complexes reaches the cells and therefore the transfection is not successful. This early release could also be the case with the transfection of Lipofectamine 3000 and Lipofectamine LTX in Heparin-containing media. However, Heparin can also improve cell viability with 15 to 20% without a significant decrease in transfection efficiency if added 2 hours after the transfection[31]. This mechanism is still unknown, but it is suggested that Heparin helps the recovery of the cell membrane. Future research can examine if adding Heparin helps to reduce the cell toxicity and therefore results in more viable transfected cells. A suggestion is that, it is also possible that the negatively charged Heparin binds to the positively charged liposomes and therefore inhibits the binding to the negatively charged cell surface proteoglycans[27]. Another suggestion is that Heparin, as GAG, cross-links proteoglycans from different cells and therefore makes them unavailable for the DNA-lipid complex to bind to[27].

## 4.2 Visualization

For generating a useful stable cell line, a requirement is that the GFP-signal is well visible under the fluorescence microscope. Therefore the cells could be live monitored. From the results of the cell staining can be concluded that the VE-Cadherin gave a high signal, but the ZO-1 staining gave a low signal. In contrast with the results from the transfection, where can be seen that the signal of the ZO-1 visualisation was higher and had another localization in the cell. Despite the fluorescent signal from the cell staining being at the cell membrane, the signal from the transfection was more in the cytosol or the nucleus of the cell. To see where this difference is coming from, a suggestion is to examine the signals with both the fluorescence microscope and the confocal microscope. In this way, it can be examined if the quality of the fluorescence microscope can be the cause of the low signal of the ZO-1 staining. Additionally, with the confocal microscope, the localization of both signals can be examined with higher quality imaging.

## 4.3 Stable cell line

Next, the kill curve was performed in order to know which concentrations of G418 selective antibiotic were needed for the generation of the stable cell line. From the results, the low, middle and high concentrations of

G418 selective antibiotic were chosen. These were necessary to kill the cells that do not contain the resistance gene. The results seemed sufficient to kill the cells in the generation of the stable cell line, but this is not validated yet. The stable cell line generation was performed, in order to gather cells which have the target gene transfected in their nuclear genome and therefore should give a longer ZO-1-GFP-signal. All the cells should contain the GFP-tag and the same goes for their progeny. It was also noted that with the generation of the stable cell line more surface area was sufficient for transfection, since the transfection with a higher surface area has a higher transfection efficiency. The generation of the stable cell line is still in process and the future will tell if it was successful.

## 4.4 CELL VIABILITY

Something that was noticed with the transfection was that the fluorescence already decreased after the second day of transfection. A possible explanation can be that the cells which showed a green fluorescent signal on day two, had just died and were washed away when changing the medium. From the negative controls containing the transfection reagent, but without the plasmid DNA, it can be concluded that the transfection reagent was not the cause. Since the cells showed no difference with the control which contained only medium (data not shown). Furthermore, from the results, it can be seen that the cells were less confluent and showed a round morphology when they were transfected. Therefore, it can be concluded that the DNA-lipid complex has a negative impact on the cells, confirmed by other literature[32]. However, the research from the group of Masotti et al. and C.Madeira et al. state that the cell viability decreases when the lipid/DNA ratio increases [33, 34]. As suggested above, Heparin addition 2 hours after transfection can possibly reduce cell toxicity in the cells [31]. Also, higher cell viability is desired, because when there are more viable cells obtained, higher transfection efficiency can be obtained.

## 4.5 OUTLOOK

Other transfection experiments by Okura et al. in hCMEC/D3 cells showed a higher transfection with the transfection of siRNA with Lipofectamine 2000 and Lipofectamine RNAiMAX[35]. The difference with this experiment is that siRNA is used instead of plasmid DNA. Also, other types of transfection methods into hCMEC/D3 cells can be used, such as a nucleofection or lentiviral transduction[36–38]. A Nucleofector(Lonza) transfers the gene of interest directly into the nucleus by applying electrical pulses[39]. In research with hMSC cells the nucleofection gave a higher transfection efficiency, but also a lower cell viability and recovery rate regarding transfection with cationic liposomes[34, 40]. As well, Lentiviruses transport their genetic material into the host cell through membrane fusion and let the host cell amplify their genetic material[41].

Recommendations for future research are to examine other transfection methods, such as nucleofection and lentiviral transduction, and to optimize the transfection with Lipofectamine LTX or Lipofectamine 3000. Optimization can be done by changing the concentrations of Lipofectamine, plasmid DNA or changing the cell density[42]. A start was made for this optimization when the transfection initially did not seem to work yet. Since there is transfection obtained, it can be optimized now for a higher transfection efficiency. Also using another transfection method such as nucleofection or lentiviral transfection could possibly help achieving a higher transfection efficiency.

For live monitoring of the ECs from the BBB and research in this field, a stable cell line should be generated with a GFP-tag onto the ZO-1 protein. The fluorescently tagged ECs can be used for live examining if and how the BBB breaks open. When the BBB opens this creates an opportunity for therapeutics to pass through the BBB. Opening up the BBB can be done with methods such as FUS, which uses a microbubble and ultrasound to temporarily open the BBB[6]. Current research does not only examine opening the BBB but also lets the therapeutics pass through the BBB with, among others, nanoparticles, active transporters in the BBB or a brain permeability enhancer[16–19].

FUS in combination with a microbubble shows promising results in different studies. So showed several

animal studies that it is possible to reduce the A$\beta$ plaques, which play a role in causing Alzheimer's disease, with FUS and a microbubble in combination with therapeutics such as a GSK-3 inhibitor or intravenously immunoglobulin[43, 44]. Also, FUS with a microbubble showed in a mouse model to help deliver glial cell line-derived neurotropic factor passed the BBB, which transduces the dopaminergic neurons to regenerate Dopamine and therefore slowing down the progression of Parkinson's disease[45]. Moreover, a rat model showed that the delivery of siRNA-Htt with FUS and a microbubble through the BBB resulted in a decrease of the Htt protein, which plays a role in Huntington's disease[46]. However, a problem within animal studies is that only 1/10 of the successful animal trials resulted in a successful clinical trial, therefore a representative in vitro model is desired[47]. Furthermore, animal trials stir up a lot of ethical discussion about animal well-being. A solution for a better model can be to make use of an organ-on-chip model of the BBB[48]. Organ-on-chip models of the BBB are out of the scope of this thesis, but hopefully this research helped to get a step closer to improve the visibility of the ECs with a fluorescence microscope. This visualisation can potentially be used in an organ-on-chip model in the future.

The transfection of the hCMEC/D3, which was aimed for, was successful with a low transfection efficiency using Lipofectamine LTX and Lipofectamine 3000 in EGM-2 without Heparin. The next step is to obtain a stable cell line with a ZO-1-GFP tag, therefore more research to optimize the transfection and cell viability is necessary. When a stable cell line is obtained, it can be an addition to research of the BBB and hopefully, one day a solution can be found for diseases where therapeutics need to pass the BBB such as Alzheimer's-, Parkinson's- and Huntington's disease.

# Chapter 5

# Conclusion

From the cell staining it can be concluded that the cells contain the VE-Cadherin and ZO-1 proteins. However, the ZO-1 gives a very poor signal. Useful is to determine if it is because of the quality from the EVOS microscope, this can be examined with a confocal microscope.

A low, middle and high concentration of G418 selective medium were obtained from the kill curve and these concentrations seemed sufficient in the stable cell line generation. However, this research is still going on and future will tell if the generation of the stable cell line worked.

It is known that different cells react differently to different transfection reagents. This study shows that tranfection did not work in both in EGM-2 and Opti-MEM. This study also showed that transfection with Lipofectamine LTX and Lipofectamine 3000 in EGM-2 without Heparin works for hCMEC/D3 cells and the DharmaFECT, ViaFECT and Lipofectamine 2000 transfection reagents did not work.

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# Chapter 6

# Appendix

## 6.1 Results

### 6.1.1 Transfection

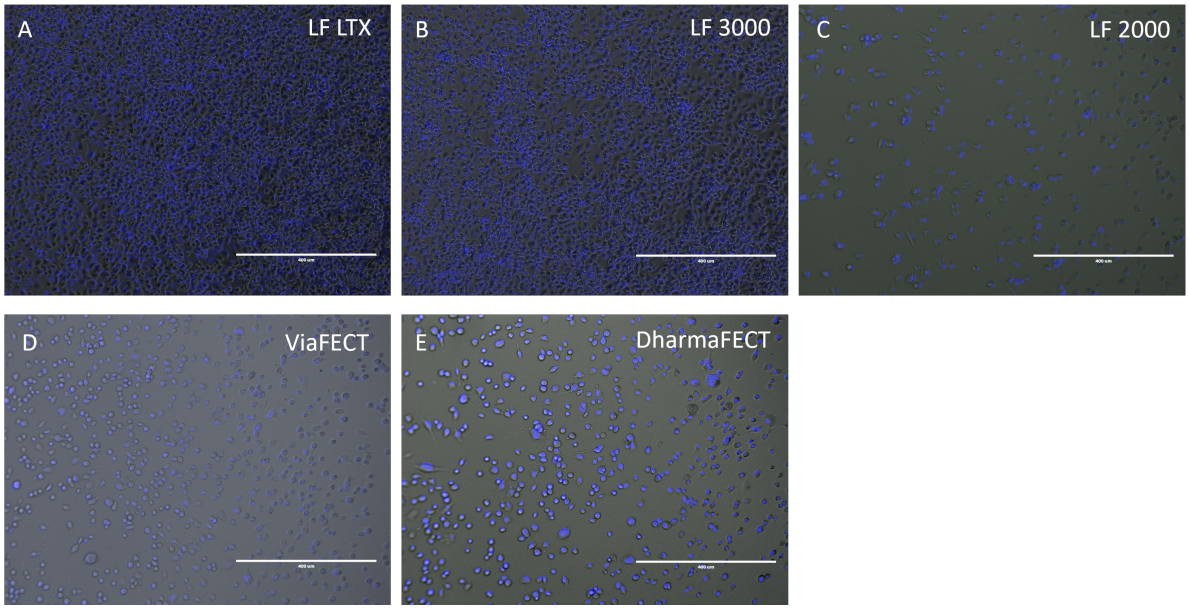


**Figure 6.1:** Transfection with DharmaFECT, ViaFECT, Lipofectamine- 3000, LTX and 2000 in Opti-MEM. All wells contained 60.000 cells. Transfection was performed with Lipofectamine LTX (A), Lipofectamine 3000(B), Lipofectamine 2000(C), with ViaFECT(D), DharmaFECT 1(E). All the conditions did not contain visible GFP-signal. All pictures are taken with a 10x magnification and the scalebar is 400 $\mu$m.

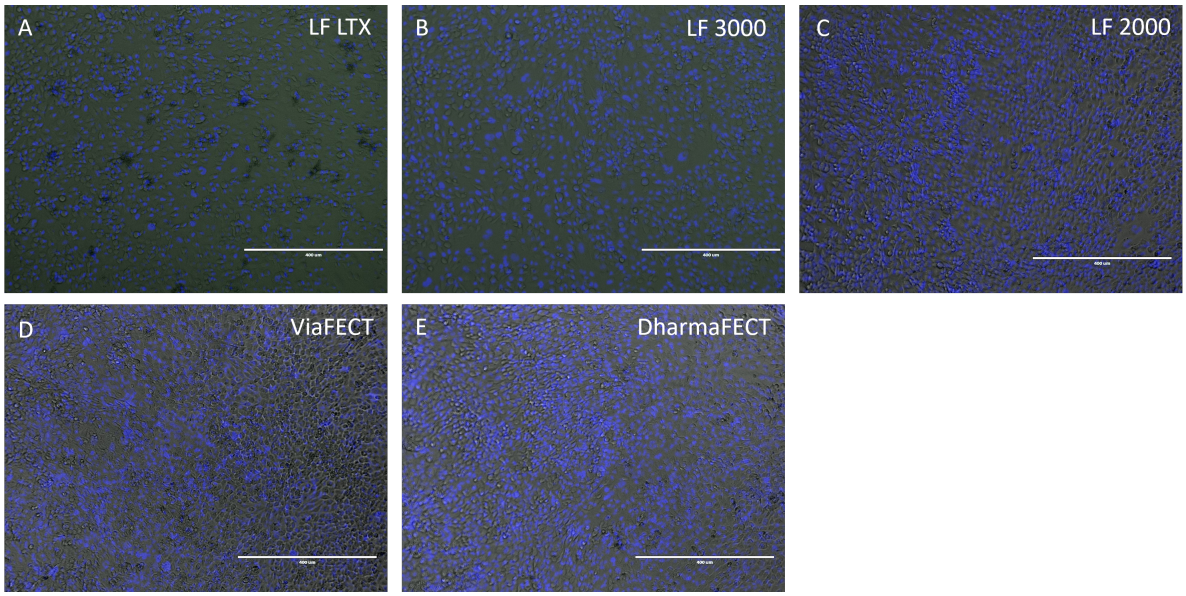


**Figure 6.2:** Transfection with DharmaFECT, ViaFECT, Lipofectamine- 3000, LTX and 2000 in EGM-2. All wells contained 60.000 cells. Transfection was performed with Lipofectamine LTX (A), Lipofectamine 3000(B), Lipofectamine 2000(C), with ViaFECT(D), DharmaFECT 1(E). All the conditions did not contain visible-GFP signal. All pictures are taken with a 10x magnification and the scalebar is 400 $\mu$m.

### 6.1.2 Kill curve

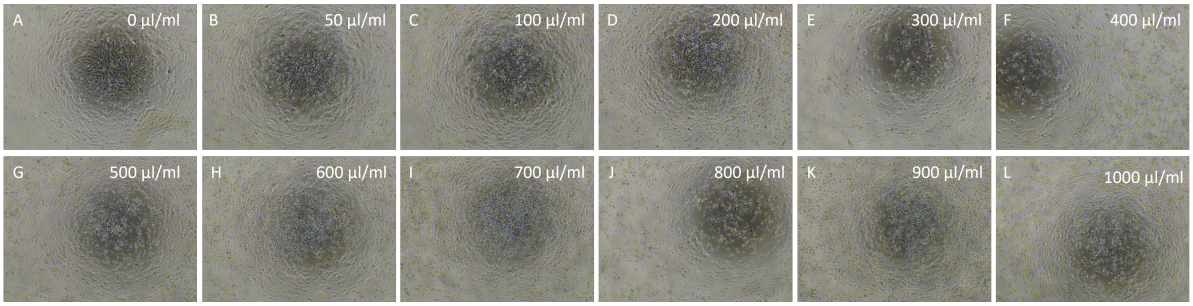


**Figure 6.3:** Day three of the kill curve with 60.000 cells per well. The conditions contain medium without G418 selective antibiotic(A), with 50 $\mu$l/ml G418 selective antibiotic(B),with 100 $\mu$l/ml G418 selective antibiotic(C), with 200 $\mu$l/ml G418 selective antibiotic(D), with 300 $\mu$l/ml G418 selective antibiotic(E), with 400 $\mu$l/ml G418 selective antibiotic(F), with 500 $\mu$l/ml G418 selective antibiotic(G), with 600 $\mu$l/ml G418 selective antibiotic(H), with 700 $\mu$l/ml G418 selective antibiotic(I), with 800 $\mu$l/ml G418 selective antibiotic(J) with 900 $\mu$l/ml G418 selective antibiotic(K), with 1000 $\mu$l/ml G418 selective antibiotic(L). The pictures are taken at a 4x magnification with a Eclipse TS100 Nikon microscope.

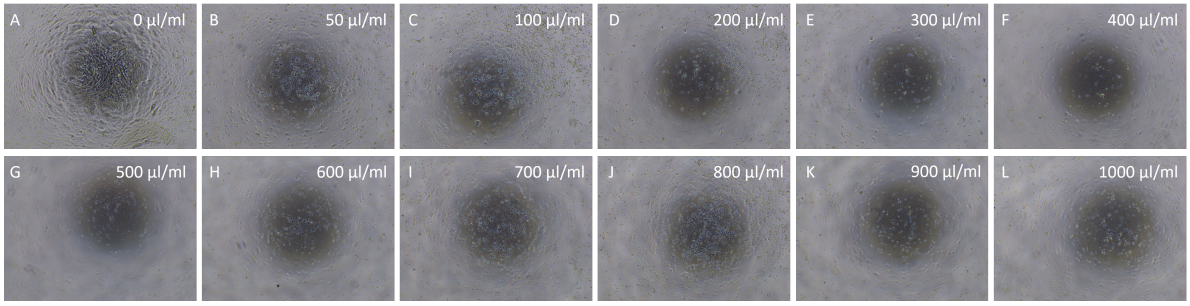


**Figure 6.4:** Day seven of the kill curve with 60.000 cells per well. The conditions contain medium without G418 selective antibiotic(A), with 50 $\mu$l/ml G418 selective antibiotic(B),with 100 $\mu$l/ml G418 selective antibiotic(C), with 200 $\mu$l/ml G418 selective antibiotic(D), with 300 $\mu$l/ml G418 selective antibiotic(E), with 400 $\mu$l/ml G418 selective antibiotic(F), with 500 $\mu$l/ml G418 selective antibiotic(G), with 600 $\mu$l/ml G418 selective antibiotic(H), with 700 $\mu$l/ml G418 selective antibiotic(I), with 800 $\mu$l/ml G418 selective antibiotic(J) with 900 $\mu$l/ml G418 selective antibiotic(K), with 1000 $\mu$l/ml G418 selective antibiotic(L). The pictures are taken at a 4x magnification with a Eclipse TS100 Nikon microscope.