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Method development for siRNA silencing in primary hippocampus culture by means of Cellaxess electroporation

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Abstract

Transfection of neurons by electroporation is a technique which enables the study of neuronal function, development and physiology with the aim of developing new drugs to cure different brain disorders. An optimization of the transfection protocol for Cellaxess electroporation with maximal transfection efficiency and maximal cell viability was developed for primary rat hippocampus culture.

Keywords

Rat hippocampal neurons, Cellaxess electroporation, transfection protocol optimization

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Method development for siRNA silencing in primary hippocampus culture by means of Cellaxess electroporation

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Sammanfattning

I Sverige drabbas varje år fler personer av någon form av hjärnsjukdom än av cancer. På grund av antalet personer som lider av hjärnsjukdomar och de höga kostnaderna förknippade med detta, är det viktigt att förstå hur specifika gener påverkar fysiologin, utvecklingen och funktioner av hjärnan och dess byggstenar, nervceller. För den neurologiska forskningen är det viktigt att hitta tekniker för diagnostisering och utvecklande av nya botemedel för olika typer av hjärnsjukdomar.

Transfektion är en metod inom molekylärbiologin som går ut på att föra in främmande molekyler in i celler. Exempel på molekyler som kan introduceras är DNA, RNA och proteiner. Cellaxess® är en transfektionsapparat som använder sig av elektroporation, det vill säga elektriska pulser för att öppna upp porer i membranet som omger en cell. Transfektion är en av flera metoder som möjliggör studier av sambandet mellan gener i nervceller och olika hjärnsjukdomar.

I detta projekt har jag optimerat ett protokoll för att transfektera primära nervceller från fetal råtthippocampus genom elektroporation med Cellaxess®CX3. Optimeringen av transfektionsprotokoll innebar fastställandet av de gynnsammaste förhållandena för att elektroporationen skulle få så hög transfektionseffektivitet och hög cellöverlevnad som möjligt.

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1. ABBREVIATIONS

AD	Alzheimer's disease
AdV	Adenovirus
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
ds	Double-stranded
FHS	Fetal horse serum
GFP	Green fluorescence protein
HSV	Herpes simplex virus
NGF	Nerve growth factor
nt	Nucleotide
PBS	Phosphate buffered saline
PC12	Pheochromocytoma cell line
PEST	Penicillin/streptomycin
PNBM	Primary neuron basal medium
PNGM	Primary neuron growth medium
PTGS	Posttranscriptional gene silencing
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
siRNA	Short interfering RNA
ss	Single-stranded
UTR	Untranslated region

2. INTRODUCTION

Alzheimer's disease (AD), epilepsy and stroke are a few examples among other brain disorders that can affect how persons think, act and behave. One third of the population of Sweden will at some point in their lifetime be affected by a brain disorder. This means that more people suffer from brain disorders compared to those who suffer from cancer (Newsdesk, 2008). The total cost of treating brain disorders every year is 85 billion SEK (Newsdesk, 2008) compared to 29 billion SEK (Cancerorganisationernas publikationer, 2006), which is the total cost of cancer treatment per year. Due to the numerous kinds of brain disorders and the high costs that it consequently means, it is essential to discover how specific genes affect the development and function of neuronal cells. It is hence important as well as challenging to find techniques for diagnosing and monitoring brain disorders and also for developing new drugs. Research of for example the hippocampus area of the brain could give more information of e.g. AD and the hippocampus is consequently an important target for new drug discovery.

Cellectricon AB placed in Mölndal is a spin off company from the Chalmers University. The idea of Cellectricon AB is to combine microsystems and microfluidic with biology to develop new instruments for miniaturized cell-based screening in the biotechnology and biomedical areas. So far Cellectricon AB has developed two platforms; Dynaflow® for ion channel drug discovery and Cellaxess®, transfection instruments based on electroporation technique.

The Cellaxess® transfection technique is an effective method for studying the influence of genes and other molecules in different types of cells. For maximal transfection efficiency and cell viability, every cell type needs an optimized transfection protocol. This master degree work includes optimization of Cellaxess®CX3 transfection parameters of brain neurons from rat hippocampus. After optimization of the transfection parameters, the work also includes development of a gene silencing assay by introduction of small interfering RNA (siRNA) in rat hippocampal neurons. The Cellaxess®CX3 transfection optimization and development of gene silencing assays enables studying of genes involved in e. g. different kinds of brain disorders. Knowledge about brain disorders and other diseases will consequently make it possible for new drug discovery.

2.1 Memory and Hippocampus

The human brain is divided into three parts; the cerebrum, the cerebellum and the brain stem. Cerebrum consists of 4 lobes; the frontal lobe, the parietal lobe, the occipital lobe and the temporal lobe. The temporal lobe controls memory, language and personality. One part of the temporal lobe is hippocampus, which it located in the middle of the brain, see Figure 1. (Stroke Information)

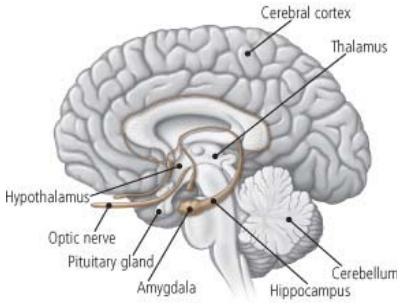


Figure 1. Hippocampus is a part of the temporal lobe and located in the middle of the brain (Figure used with permission from Mike Murphy, Radiokinesis).

There are different types of memories that are divided into the sensory memory, the short term memory, the working memory and the long term memory. In the sensory memory perceptions, that will disappear within a second, are stored. The short term memory let us keep information for less than one minute. The working memory stores information when we are working for example when we are reading or writing. Our long term memory is divided into two areas; nondeclarative and declarative memory. Nondeclarative memory represents learned habits and skilled behavior. Declarative memory is divided into two groups; episodic memory and semantic memory. The semantic memory is associated with cerebral cortex and this memory let us store general knowledge. The episodic memory, which stores and connects events, is associated with the hippocampus region in the brain. Hippocampus is connected to the cerebral cortex and new episodic memories can thereby be stored as semantic memories in the cortex. If hippocampus is damaged it can consequently give a kind of amnesia, a memory loss, of recently stored information. (Sutherland, et al., 2006; Society of Neuroscience, 2006; The Brain from Top to Bottom)

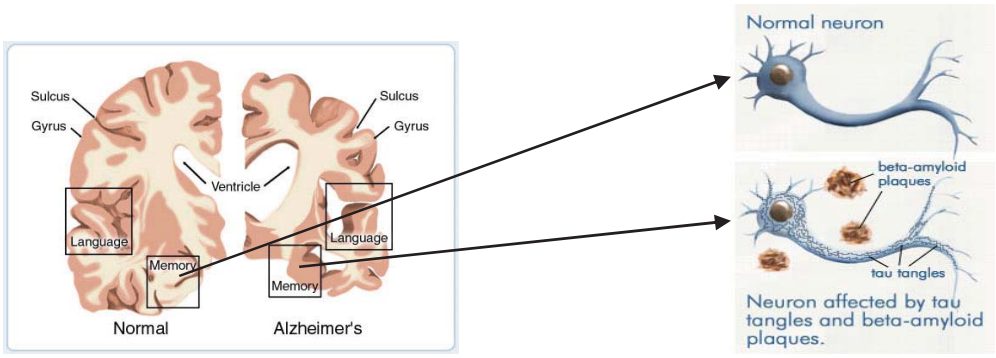
2.2 Alzheimer's Disease

In 1906 Alois Alzheimer was the first person to present a rare degenerative neuronal disease, which today is a famous and more common disease named after him as Alzheimer's disease (AD). AD is the most common type of dementia and those who are affected of the disease are often over the age of 65. Only in Sweden about 96 000 (Pfeizer, Alzheimers.nu) persons

suffer from AD. The increase of people suffering from AD is mainly because of today's longer average length of life. Symptoms of AD are memory loss, difficulties to understand, read, speak and concentrate and problem to recognize the surroundings. Today there is no treatment for the disease and people affected by AD will finally die. Many efforts to understand and cure AD have been made and some knowledge about the disease has been discovered. One discovery is that AD starts in the hippocampus and cerebral cortex areas of the brain. (Society for Neuroscience, 2006; Herz, 2007)

There are two types of AD; Familiar Alzheimer's disease (FAD) or early-onset Alzheimer's disease and late-onset Alzheimer's disease. 10% of the people suffering from AD have been affected by FAD, which can hit people as young as 35 years old. FAD is inherited and caused by mutations in genes on chromosome 1, 14 and 21. Late-onset AD is developed usually after the age of 65. No specific genes that cause the disease have been found, but some genetic factors that can cause a risk to develop late-onset AD have been discovered (American Health Assistance Foundation).

The hippocampus and the cerebral cortex are the first parts of the brain that are affected of AD. AD is believed to be caused by abnormalities of a small fibrillary protein, beta-amyloid, and a protein important for the structure in neurons called tau. The beta-amyloid forms protein plaques around the neurons and these plaques can be formed in 10-15 years before changes of the brain are seen. The beta-amyloid plaques will finally lead to chronic inflammations and significant AD symptoms are noticeable. Modifications of the protein tau will cause fibrillary tangles inside the neurons, which will lead to neuron apoptosis. Beta-amyloid and tau are important for memory and intellectual functions and as they are changed this will lead to neuronal cell death and AD is at that time a fact. The brain will overall shrink and the ventricles will be enlarged, see Figure 2. (Society of Neuroscience, 2006; Baron et al., 2006)



2A **2B**

Figure 2. *Figure 2A:* A cross-section of a normal brain to the left and a brain affected of Alzheimer's disease (AD) to the right (Figure used with permission from American Health Assistance Foundation). AD is caused by degeneration of neurons, which is seen in the picture as shrinkage of brain tissue and enlarged ventricles. *Figure 2B:* A normal neuron on top and a neuron affected of AD at the bottom (Figure used with permission from Regan Carey, About Dementia). Beta-amyloid plaques around the neurons and tau tangles in the cell bodies can be seen in the AD affected neuron.

2.3 Aim of the Project

Every cell type that is going to be transfected needs an optimized protocol containing a detailed instruction of the transfection process. The instruction comprises growing of cells, transfection protocol and information of optimal expected result of the transfection. Some cells, e.g. primary cells, are more complicated and require a longer method development time to reach a good transfection result.

This degree project includes method development for cDNA and siRNA transfection of hippocampus primary culture by Cellaxess® electroporation. The aim is to evaluate the transfection efficiency with the Cellectricon's patented electroporation technique in hippocampus primary culture. Also development of an appropriate assay to show gene silencing by means of siRNA and to document the best silencing level with kept viability will be performed with the hippocampus primary culture. The project also includes transfection of the pheochromocytoma cell line, PC12, which has been isolated from a neuron endocrine tumor in the adrenal gland from rat (Greene & Tischler, 1976). PC12 starts to differentiate to a neuron like phenotype when treated with neuronal growth factor, NGF, and is therefore a good model system for neurobiological and biochemical studies (Greene & Tischler, 1976; Martin & Grishanin, 2003). Lipid transfection with Effectene® Transfection Reagent was also done with both the hippocampus primary culture and the PC12 cell line too compare lipid transfection with Cellaxess® transfection.

3. BACKGROUND

3.1 Hippocampus Primary Culture

Primary cultures consist of cells or tissues that are taken directly from an organism and grown in the first passage. Primary cell cultures are in this manner as close to a real tissue as it is possible to be and the use of primary cells will assure that the specific primary cells behave like the cells in the tissue do. Another reason why primary cells are used is that the cells do not have time to dedifferentiate and can thus retain all the properties as the parent material. (Harrison and Rae, 1997).

Hippocampus contains a majority of neurons, both pyramidal neurons (see Figure 3) and granule neurons, and only few glia cells. This makes embryonic rodent hippocampal neurons to excellent model cells for studies of neuronal development, degeneration and synaptic properties. The main drawbacks are that neurons do not proliferate and only a small amount of hippocampal neurons and other neurons can be obtained from one embryonic rat dissection. Many animals are therefore used every time neuron cells are needed. (Eichenbaum, 2000; Meberg & Miller, 2003; Gärtner et al., 2006)

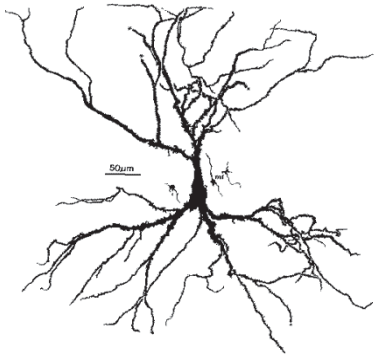


Figure 3. Hippocampal pyramidal neuron (Figure used with permission from David Beeman, Brain, minds and media).

Dissociated primary cell cultures from live embryonic dissections are used as in vitro model systems for studies in the neurobiology area. Fresh cultures prepared directly after the rat dissections do however have some disadvantages; the rat dissections are time consuming and competent laboratory persons are needed to implement the dissection. That is why primary neurons now are commercially available. (Krantis et al., 2003)

The difficulty of studying the signaling process in primary neurons in culture is that the cells are hard to manipulate due to the lack of proliferation and low transfection efficiency both in

vivo and in vitro (Dalby et al., 2003; Huang and Richter, 2007; Teruel et al., 1999; Bergen et al., 2007). Transfection of neurons is difficult mainly due to the multiple biological barriers to cross before getting into the cells and the differentiated neurons are also very sensitive and can easily die (Bergen et al., 2007).

3.2 RNA Interference

Protein translation can be regulated by a gene-silencing phenomenon called RNA interference (RNAi) or posttranscriptional gene silencing (PTGS) (Hammond et al., 2001). RNAi is the ability of double-stranded (ds) RNA to inhibit a single-stranded (ss) target mRNA expression by fully complementary base-pairing. The RNAi takes place when the specifically base-pairing between the dsRNA and the target ss mRNA is recognized and cleaved by nucleases. (Milhavet et al., 2003; Lodish et al., 2003)

It is suggested that RNAi phenomenon evolved in both plant and animal cells as a defense mechanism of unwanted foreign genes such as viruses and other mobile genetic elements. It is clear that RNAi is an important regulator of endogenous gene expression in almost all eukaryotic cells. (Milhavet et al., 2003)

RNAi was discovered when researchers tried to manipulate gene expression in *Caenorhabditis elegans* (Lodish et al., 2003). It was seen that mRNA degradation was induced in presence of ds RNA segments when one of the strands was the same as the mRNA sequence (Lodish et al., 2003). Further studies of RNAi in *C. elegans* and *Arabidopsis thaliana* showed that long dsRNA are processed into short double-stranded segments, 21-23 nucleotides with a 2-nucleotide 3´-overhang and a 5'phosphorylated end of each strand, called short interfering RNA (siRNA) (Milhavet et al., 2003; Lodish et al., 2003). It was discovered that the PTGS was initiated by the cleavage into siRNA, which is performed by an evolutionary conserved RNaseIII-like enzyme, Dicer (Billy et al., 2001; Hannon, 2002). Thereafter the effector step starts by unwinding of the siRNAs and dividing it into a sense and an antisense strand. The antisense strand is then associated with a protein-RNA effector nuclease complex, RNA-induced silencing complex (RISC), which becomes activated (Sverdlov, 2003; Hannon, 2002). The antisense strand is used as a guide strand and together with RISC the complex recognizes and degrades target mRNA (Hammond et al., 2001; Milhavet et al., 2003; Sverdlov, 2003). The mRNA degrading process is illustrated in Figure 4.

Both Dicer and RISC are ATP-dependent (Milhavet et al., 2003). In worms and plants the RNAi is amplified and spread through the organism. The amplification requires a protein called RNA-dependent RNA polymerase (RdRP) (Agami, 2002). The spreading is a phenomenon called transitive RNAi, which researchers suggest is a movement of the silencing signal along a specific gene (Hannon, 2002).

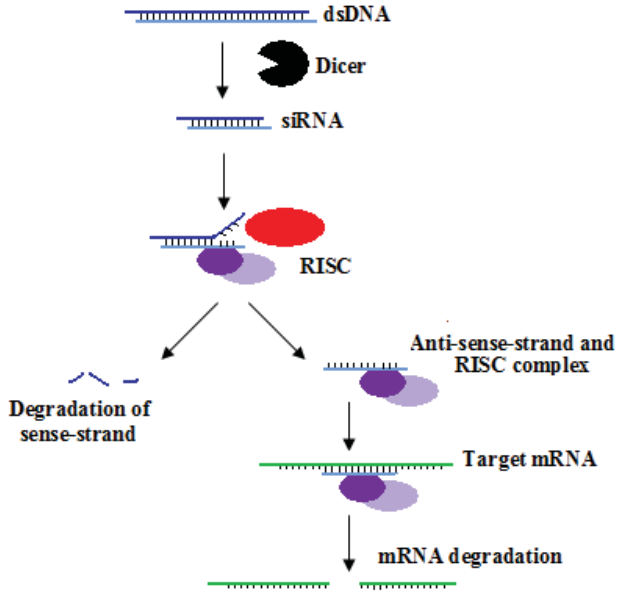


Figure 4. Double stranded RNA is processed into short interfering RNA by Dicer. A complex of the anti-sense strand and RISC is formed. The anti-sense strand and RISC complex can subsequently degrade the target mRNA.

There are many applications of RNAi. RNAi is used for establishing proteins and the signal transduction pathways including the biological responses in the cells, studying genes involved in cell cycle regulation for cancer research, identifying genes involved in developmental processes, studying of macromolecular synthesis and degradation, identifying genes that control cell death and finding genes that could prevent viral invasion and replication. The use of RNAi in gene therapy is a growing method for specific disease treatment. (Milhavet et al., 2003)

3.3 Transfection Methods

Transfection represents introduction of foreign DNA or other molecules into a cell. Transfected DNA can affect the cell to express genes that the cell does not naturally express and transfected RNA can silence genes that naturally are expressed. The ideal transfection method should transfect cells with high efficiency, high viability, be independently of the sizes of constructs, have low toxicity and be easy and safe to carry out. There are many different transfection methods, but no method can be applied to all cell types. Especially

primary cells e. g. neurons are harder to transfect and consequently requires a transfection method which transfect with high efficiency without causing cell death. (Washbourne and McAllister, 2002; Milhavet et al., 2003)

To achieve the most efficient transfection, it is necessary to optimize the transfection parameters; e.g. cell culture conditions, transfection agent, and transfection time for every cell and cell type. (Washbourne and McAllister, 2002; Milhavet et al., 2003)

3.3.1 Viral Methods for Neuron Transfection

Effective and safe gene transfer using viruses requires the right combination of the viral vector, envelope and promoter (Blesch, 2003). Herpes simplex virus (HSV) and adenovirus (AdV) are two examples of viruses that have been used for viral vectors. There are many advantages using viral gene delivery systems for neuron transfection; the vectors have high transfection efficiency, viruses are easy to use in vitro and in vivo and the vector is continuously expressed in the cell (Milhavet et al., 2003; Washbourne and McAllister, 2002). Nevertheless the use of viral vectors have several limitations; the viral vectors can be toxic and time consuming to design, the size of the construct is limited, the viruses can be dangerous to the people working with them and because of all safety requirements the costs are high (Washbourne and McAllister, 2002; Gresh et al., 2003).

3.3.2 Non-Viral Methods for Neuron Transfection

Non-viral transfection methods include chemical, physical and electrical methods. The advantages by using non-viral methods are that they are easier, less toxic and there is no limitation of plasmid size compared to viral gene delivery systems. The main disadvantage, which is general for all of the transfection methods, is reduced transfection efficiency.

Calcium phosphate-mediated co precipitation and liposomes are examples of chemical transfection methods. In calcium phosphate-mediated co precipitation a calcium-phosphate-DNA co precipitates are formed that are taken up by the cells and this method is widely used in dissociated neural cultures. The use of liposomes means DNA surrounded by fusogenic cationic lipids are fused with the cell membrane. Microinjection and biolistics belong to physical transfection methods. Microinjection means that DNA is injected into the nucleus or the cRNA into the cytoplasm and this method is effectively used for transfection of single neurons. Biolistics is a technique where the neurons are hit by gold particles that are coated with DNA and is effective for transfection of tissue slices. Electroporation is an electrical

transfection method and is very good for neuron transfection. (Washbourne and McAllister, 2002)

3.4 Electroporation

Electrofusion and electroporation are two examples of electromanipulation of cells. Both methods use induced electrical potential that reaches a critical threshold for electrical breakdown of the cell membrane, which temporarily reduces the cell membrane integrity and makes it more permeable. Electrofusion can be used if cells are close together and electric pulses are applied, which consequently make the cells to fuse to form hybridomas. Electroporation on the other hand means that the electric pulses open pores in the cell membrane of the cells so that molecules that do not normally pass through the cell membrane can get into the cytoplasm of the cell. The electroporation method can deliver a range of molecules e.g. dyes, drugs, dsDNA, dsRNA and antibodies and allows transfection of single cells, cultured slices and tissues in vivo and in vitro (Golzio et al., 2003). (Washbourne and McAllister, 2002)

Golzio et al. (2003) have a theory based on experiments to understand how the electroporation process works. First electrical pulses permeabilize the cell membrane and plasmids are electrophoretically moved to the cell surface. A complex between the permeabilized part and the plasmid is formed and pulses allow the plasmid to diffuse into the cytoplasm. Some of the plasmids can thereafter get into the nucleus. siRNA only has to enter the cytoplasm and not the nucleus, which make it easier to transfect RNA compared to transfection of DNA. Many parameters affect the opening of the pores in the cell membrane. The permeabilization depends e. g. on the electric field strength, the number of electric pulses and the age, the shape, the size and the orientation of the cells in the electrical field. Reactive oxygen species (ROS) and water that flow into the cell can affect the viability. ROS are dangerous reactive intermediates that arise from normal metabolism and can damage nucleic acids and proteins (Fridovich, 1999).

Teruel and Meyer (1997) showed that the pore formation in the electroporation process depends on the cell composition; plasma membrane, cytoskeleton and local cell adhesion. Their result suggested a two-step process for pore formation. First small pores in the membrane are formed and thereafter small ions can enter, which lead to a partial breakdown

of the membrane potential. The decreasing of membrane potential leads to slower opening of large entry sites. After milliseconds to seconds both small and large pores are resealed.

Electroporation has many advantages including easy handling, fast and safe. There is no limiting size of the plasmid vector, but it has been shown that the efficiency is lower when the plasmid size is larger. The disadvantages are that the whole electroporation mechanism has not yet been discovered, the method lack specificity and it is hard to use in vivo because the electric field has to be applied close to the cells which can lead to cell death. (Golzio et al., 2003; Bergen et al., 2007)

3.5 Cellaxess® Transfection Systems

The developed transfection instrument of Cellectricon AB, Cellaxess®, can be used for introduction of molecules with different sizes; e.g. oligonucleotides, siRNA, drugs, dyes and large plasmids to adherent cells and tissues. The unique approach of the Cellaxess® systems is the ability to transfect adherent cells in cell cultures by using electroporation. Compared to conventional electroporation techniques Cellaxess® system has many advantages e. g. no pre-treatment of cells is needed, there is only a low cell consumption, both pre-mitotic and post-mitotic cells can be transfected and Cellaxess® is easy and fast to use.

The Cellaxess®CX1 and Cellaxess®CX3 electroporation systems were first developed. Cellaxess®CX1 is used for transfection of tissue slices and specific cell culture regions and Cellaxess®CX3 is used for transfection screening of cell cultures in 96-well plates or dishes. Cellectricon AB has also developed a high throughput Cellaxess® system, which is called Cellaxess®HT and used for RNAi screening. Cellaxess®HT is a fully automated robot and it is able to transfect 384-well plates with a maximum throughput of 20 000-50 000 wells each day.

3.5.1 Cellaxess®CX3

Cellaxess®CX3 consists of a Cellaxess Pump Unit, a Cellaxess Pulse Generator, a Cellaxess®CX3 Safety Box, Cellaxess®CX3 Electroporation Head and Cellaxess Commander Software, see Figure 5. Cellaxess®CX3 is able to transfect 3 wells in 96-well plates or a comparable area in a 35 mm culture dish at each electroporation.

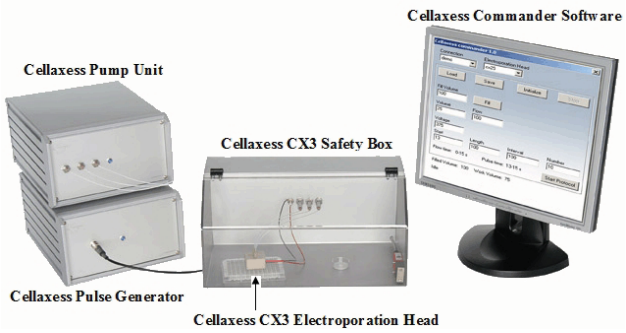


Figure 5. The Cellaxess®CX3 system with a pump unit and pulse generator to the left, a safety box connected to the electroporation head in the middle and the software to the right (Figure used with permission from Cellectricon AB).

3.5.2 Cellaxess®CX3 Electroporation Head

The electroporation head is coupled to the pump unit and the pulse generator via the safety box. The electroporation head consists of 3 hollow capillaries and together the 3 capillaries can transfect 3 wells in a 96-well plate at the same time. The electroporation head stands on 3 legs that make the capillaries stay 70 µm from the bottom of the wells. The electroporation head is shown in Figure 6A and an enlargement of one of the capillaries is shown in Figure 6B. Inside the capillary there is an inner metal electrode and around the capillary there is an outer electrode. The electroporation process starts by filling the capillaries with the transfection molecule. The electroporation head is placed in a 96-well plate with one capillary in one well with seeded cells. The transfection starts by dispensing the transfection molecule and in the same time applying electrical pulses which give rise to an electrical field.

The strength and gradient of the electrical field can be controlled by changing the inner and outer diameter and varied by the distance between the bottom of the plate and tip of the capillary or by changing the applied current. The electrical field is strongest at the inner rim of the capillary and decreases with the distance towards the outer rim, see Figure 6C and 7. The electrical field in the capillary axis direction becomes zero. The strongest electrical field is obtained under the capillary wall and is decreasing with the radius. The result is a doughnut-shaped pattern of electroporated cells. If the current is too low only the cells closest under the inner electrode will be electroporated. If the current is too high it will result in cell death closest to the inner electrode and electroporated cells further out of the radius. The best result is achieved when as many cells as possible are electroporated without causing too much cell death. (Olofsson et al., 2005)

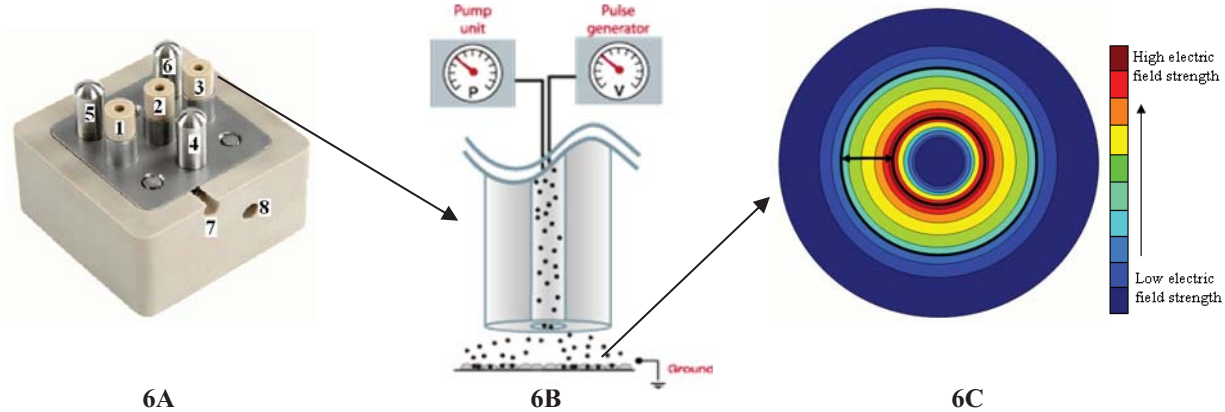


Figure 6. *Figure 6A:* The electroporation head showing the 3 hollow capillaries (1-3) that are supported by the three spacer legs (4-6) that make the capillaries stay 70 µm from the bottom of the well. The head is connected to a negative electrode (7) and a positive electrode (8). *Figure 6B:* Enlargement of one of the capillaries, which shows how the transfection molecules are dispensed and electric pulses are applied to the adherent cells at the bottom of the well. The cells under the capillary that lay between the outer and inner electrode will be transfected. *Figure 6C:* A figure showing the electric field strength which is strongest at the inner rim of the capillary and decreases towards the outer rim of the capillary. The inner black ring represents the inner rim and the outer black ring represents the outer rim. The arrow shows the width of the capillary wall compared to the electric filed strength. (Figure 6A and 6B are used with permission from Cellectricon AB.)

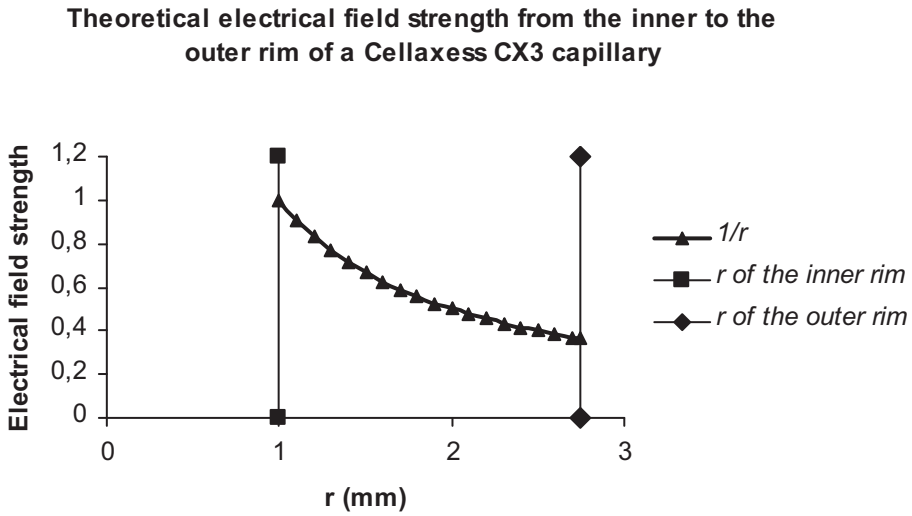


Figure 7. The theoretical electrical field strength of a capillary of Cellaxess®CX3 is highest at the inner rim and decreases towards the outer rim.

For every cell type an optimized transfection protocol have to be designed for maximal transfection efficiency and cell viability. For Cellaxess this means that every parameter that affect the transfection need to be optimized. Some of the parameters that are important to optimize are listed in Figure 8. A too gentle transfection protocol will result in no transfected cells, but the cells are still alive. A too hard transfection protocol will result in transfected cells, but probably also in cell death. An optimized transfection protocol implies that the transfection result in as many transfected cells as possible with maximal cell viability.

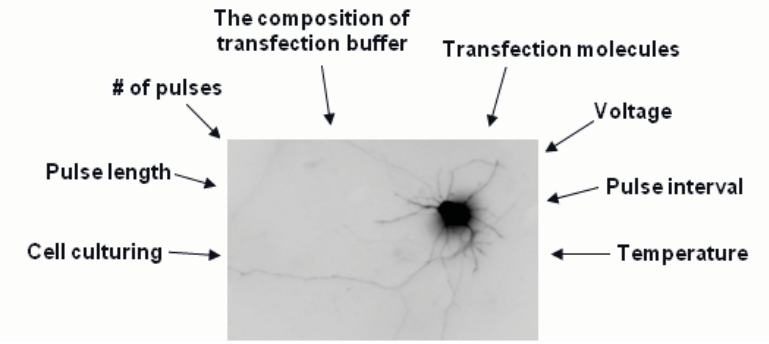


Figure 8. Parameters that affect the electroporation of Cellaxess®CX3. The parameters need to be optimized for every cell type for maximal transfection efficiency and cell viability.

Electroporation Pulse Design

The Cellaxess®CX3 electroporation pulse electroporates with a square pulse which means that the pulse goes from 0 V to the chosen voltage and stays at the same voltage until the pulse is ended and goes down to 0 V again, see Figure 9.

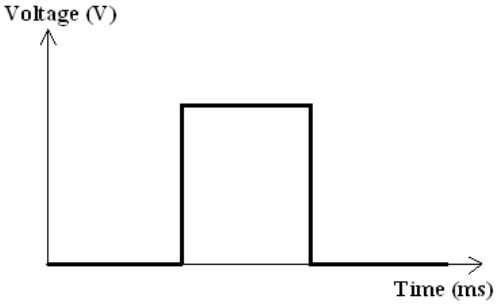


Figure 9. Cellaxess®CX3 electroporates with a square pulse.

For maximal transfection efficiency and cell viability, i.e. the optimal level when as many cells as possible are transfected with minimal cell death, it is important to optimize the transfection protocol. Cellaxess®CX3 variable transfection protocol parameters in the Cellaxess® Commander Software include number of electric pulses, electric pulse length, interval between electric pulses, electrical pulse amplitudes, dispense volume and dispense rate that can be changed for an optimized transfection protocol and electric field strength. For optimization of the transfection protocol, all variable parameters need to be optimized by increasing or decreasing the specific parameter.

3.6 Lipid Transfection

Electroporation was mostly used as transfection method in this project, but also lipid transfection was used. The aim with the lipid transfection was to compare Cellaxess®CX3 electroporation with another transfection method.

Lipid transfection is a method where the transfection molecules are incorporated into lipid vesicles, so called liposomes. Electrostatic interactions between cationic charged lipids and anionic charged DNA are the driving force of forming liposomes. The liposomes can then fuse with the cell membrane and the transfection molecule can enter the cell, see Figure 10. The transfection efficiency depends on the transporting of liposomes to the cells, fusion of the liposome to the cell membrane, the DNA uptake to the cell and nuclear entry of the DNA. Lipid transfection has many advantages; it is a non-immunogenic method and lipids are easy to produce. One drawback is low transfection efficiency. (Ma et al., 2007; Watson et al., 1992)

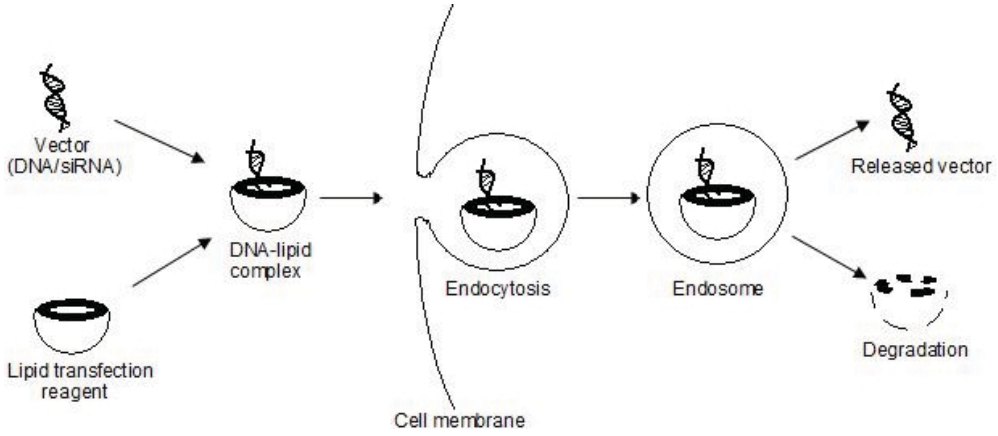


Figure 10. The process of lipid transfection: The DNA is mixed with lipids that together form DNA-lipid complexes. The lipid complexes fuse with the cell membrane by endocytosis and the DNA is released inside the cell.

3.6.1 Effectene® Transfection Reagent

Effectene Transfection Reagent is non-liposomal lipids, which signify that the lipids form a micellar structure around the DNA instead of forming a double layered liposomal structure. Effectene Transfection Reagent is particularly good to use for sensitive cells like primary cells, since it is an effective transfection method with minimal cytotoxicity. DNA is mixed with a chemical, Enhancer, and buffer with the right salt concentrations. The DNA gets condensed and when the cationic lipid is added the lipids will coat the condensed DNA to non-liposomal complexes that are added to the cultured cells. The lipid complexes will fuse with the cell membrane and the DNA can enter the cell. (Qiagen, Effectene® Transfection Reagent Handbook, 2002)

3.7 Electroporation Reporter Molecules

Different kinds of transfection molecules can be used to see successful electroporation and transfection results. One commonly used type of reporter molecule to detect transfection is

plasmids coding for Green Fluorescence Protein (GFP). Another reporter molecule that can be used for evaluation of transfection is dextran molecules, a sugar, coupled to a fluorophore.

3.7.1 GFP

Green Fluorescent Protein (GFP) was discovered in 1962 as the bioluminescence protein aequorin in the jellyfish called *Aequorea victoria*. After purification of aequorin it was shown that the protein absorbs blue light and emits green light. Variants of GFP have been constructed and GFP is today widely used in research as fluorescent reporters. (Bizzarri et al., 2008) GFP-coding plasmids are for example used in the transfection area to show successful transfection. The plasmid must get into the nucleus of the cell to be able to be expressed by the transcription machinery of the cell and only living cells are able to express the GFP. The Nobel price in chemistry in 2008 was given to Osamu Shimomurawho, Martin Chalfie and Roger Y. Tsien who discovered the GFP (Nobelprize.org).

3.7.2 Dextran Alexa Fluor 546

Dextran Alexa Fluor 546 is a sugar molecule with a certain molecule size conjugated to a fluorophore. The size of the sugar molecule can easily be customized and dextran can thus be used as a model molecule for studying how large molecules that are able to enter through the electroporated pores in the cell membrane. Dextran inserted into cells by electroporation stays in the cytosol and can be observed in both dead and living cells.

3.8 Alamar Blue Assay

The Alamar Blue assay is used as a tool for protocol optimization for the Cellaxess® systems. Alamar Blue has both a colorimetric and a reduction-oxidation indicator which consequently both fluoresces and changes color as a result of cell metabolism. In wells with no dead cells the Alamar Blue is metabolized and turns red and in wells with many dead cells the color stays blue due to low metabolic activity. The fluorescence intensity is higher in wells with high cell viability compared to wells with low cell viability. The expected result of Cellectricons viability assay is light intensity linear proportional to the voltage with a negative slope, see Figure 11.

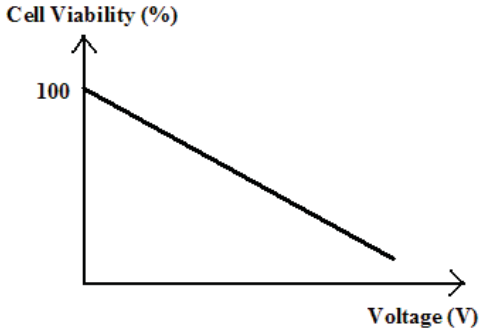


Figure 11. The cell viability 4 hours after Cellaxess® electroporation. The cell viability depends on the used electroporation voltage.

4. MATERIALS AND METHODS

It was important that all cell culture and transfection procedures were performed aseptically by working in a Laminar Flow (LAF) cabinet and e. g. the water, flasks, pipettes, papers and LAF cabinet were cleaned with 70% ethanol and/or autoclaved.

4.1 Cellaxess®CX3- Running Procedure

The transfection procedure with CX3 can be summarized in the following steps:

First the pump system was washed with ethanol and autoclaved distilled water. The transfection agent was diluted in electroporation buffer (cell specific culture medium if nothing else is specified in specific experiment) and filled into the three capillaries. The electroporation head, balanced on the three legs that made the tips of the capillaries be positioned 70 µm above the cells, was placed in the 96-well plate. The transfection agent was dispensed from the capillaries and an electric field was applied. The cells under the capillary wall were electroporated and transfected and the cells in the middle that were not exposed to the field worked as reference cells. After transfection of the first three wells the electroporation head was placed in the next triplet of wells. As all the wells in the 96-well plate were transfected the plate was directly analyzed or incubated in 37°C for 24 h before analyzing. The pump system was washed with autoclaved distilled water and turned off. See Appendix 1 for a more detailed Cellaxess®CX3 running procedure.

4.2 Primary Cells and Cell Lines

Due to the high cost of hippocampal neurons, experiments with the PC12 cell line were also performed. The PC12 cell line is derived from rat adrenal pheochromocytoma tumors and is a useful model system for neurobiological and biochemical studies. Parallel experiments were therefore done with PC12 and hippocampal neurons.

4.3 PC12 Cell Line

A vial with frozen PC12 cells (ATCC, No: CRL-1721) was thawed in a 37˚C water and thereafter centrifuged for 5 min at 1500 rpm. The cell pellet was resuspended in 5 ml growth medium (Ham's F12 containing L-Glutamine and 1% PEST (Penicillin/streptomycin) (PAA, Cat. No: E15-817), 15% Fetal Horse Serum (FHS) and 2,5% Fetal Calf Serum (FCS)) by pipetting up and down. The cell suspension was pipetted into a T-25 flask and incubated at 37˚C and 5% CO_2. The medium was changed every 4-5 days and when the cells were confluent they were splitted into a new passage. The splitting process started by removing the growth medium and the cells were washed in 1 ml Ca^{2+}/Mg^{2+}-free Phosphate buffered saline (PBS). 1 ml accutase was added and the flask was incubated at 37˚C for 5 min. 1 ml culture medium was added and the cell suspension was moved to a 15 ml tube and pipetted up and down. The cells were counted in a Burker chamber **(**Marienfeld Bürker CE) and were thereafter seeded in a new T-25 flask with 5 ml culture medium and/or in 96 well plates coated with collagen (0,05 mg/ml Collagen type I from rat tail Sigma, Cat. No: C 3867). The cells were incubated at 37˚C and 5% CO_2 (Thermo Scientific, Heraeus, BBD 6220).

The PC12 cells that were going to be transfected were usually differentiated for one week before the transfection. Two days after seeding in the 96-well plate the culture medium was removed and the same volume of differentiation medium (50% PC12 growth medium, 50% ml Ham's F12 with L-Glutamine and 1% PEST and 0,1% 0,1 µg/µl Nerve Growth Factor Beta (NGF, rat recombinant, Sigma, Cat. No: N2513)) was added. The differentiation medium was changed every second or third day.

4.4 Rat Hippocampal Neurons

Hippocampal neurons, 1 000 000/vial with 0,25 ml/vial, from Lonza (Cat. No: R-Hi-501, Lot. No: 140205) cryopreserved and freeze-stored in liquid nitrogen for ca 10 months were used for all transfections. Except for neurons the vials also contained glia cells that followed with the neurons after the dissection. Neurons need glia cells to survive and stay healthy. All vials were from the same batch.

The serum-free medium was prepared from the Primary Neuron Growth Medium (PNGM) BulletKit® from Lonza (Cat. No: CC-4461). The kit consisted of 200 ml Primary Neuron Basal Medium (PNBM) (Cat. No: CC-3256, Lot. No: 01117134, Exp. Date: 08-05-06) and PNGM SingleQuots (Cat. No: CC-4462, Lot. No. 08106112, Exp. Date: 08-10-22). The

SingleQuots consisted of 2 ml Penicillin/Streptomycin (Cat. No: PT-4133HH), 2 ml L-glutamine (Cat. No: 4460HH) and 4 ml NSF-1 (Cat. No: CC-4459HH). The SingleQuots were thawed at RT and 1% L-glutamine and 1% penicillin/streptomycin and was added to the PNBM. Before use of the medium 2% NSF-1 was added.

4.4.1 Seeding of Rat Hippocampal Neurons

The culture procedure was performed according to detailed recommendations from the supplier. A vial with 250 µl hippocampus neurons stored in liquid nitrogen was thawed in 37˚C for 1 min. The cells were moved from the vial to a 15-ml Falcon tube and the first 2 ml of 7 ml pre-heated medium was added drop-wise onto the cells to avoid osmotic shock. Additionally 5 ml medium was added carefully onto the cells for a total volume of 7 ml cell suspension. The Falcon tube was inverted two times and 200 µl of the cell suspension was added to each well on a 96-well plate pre-coated with poly-D-lysine. The cells were counted with a microscope (Leica DM/L) in a Burker chamber. The plate was incubated for 4 hours in 37˚C. 165 µl of the medium was removed leaving the cells in little medium on the bottom and 115 µl new pre-heated medium was carefully added to each well. The neurons were incubated in 37˚C and 5% CO_2 for 7 days before the transfection. 50% of the medium was changed five days after seeding.

4.4.2 Immunostaining of Rat Hippocampal Culture

To be able to see which kinds of cells that had been transfected after the transfection, immunostaining of MAP2 and GFAP was done.

The primary antibodies were directed against Microtubule-Associated Protein 2 (MAP2), a protein which exists in the dendrites of neurons or against Glial Fribillary Acidic Protein (GFAP) which exists in glia cells. The immunostaining started by removing the medium and fixing the cells by applying 100 µl 4% paraformaldehyd (PFA) in PBS for 15 minutes in RT. The cells were washed with 100 µl PBS 2x10 minutes and 100 µl of the permeabilization buffer (PBS, 1% normal goat serum (Santa Cruz Biotechnology), 0,25% Triton X-100) was applied and stored at RT for 20 minutes. Primary antibodies was diluted in permeabilization buffer, 1:50 MAP2 mouse IgG1 (Santa Cruz Biotechnology, sc-32791) and 1:400 GFAP rabbit IgG (Sigma, G9269) and 50 µl was added to each well. The plate was incubated at 4°C over night. The wells were washed with 100 µl wash solution (PBS, 0,25% Triton X-100) 2x15 minutes. 50 µl secondary antibodies diluted in permeabilization buffer, 1:100 green fluorescein-conjugated goat anti-mouse IgG1 FITC (Santa Cruz Biotechnology, sc-2078) or

1:400 Rhodoamine Red-X-conjugated goat anti-mouse IgG (Invitrogen, R6393) and 1:400 Rhodamine Red-X-conjugated goat anti-rabbit IgG (Jackson Immunoresearch) was added to the wells and incubated in dark for 1 hour at RT. The wells were thereafter washed with wash solution 2x15 minutes. The fixed and immunostained cells were then stored in 100 µl PBS in 4°C until the analyzing in fluorescence microscope.

The immunostaining protocol included incubation of primary antibodies (MAP2 and GFAP) in 4°C over night. To save time incubation in 37°C for 1 h was tried. The 1 h immunostaining result was the same concerning the fluorescence intensity and specific binding as the over night immunostaining result. The primary antibodies incubation for the specific dilutions of MAP2 and GFAP primary antibodies was thus changed to 1 h in 37°C in the immunostaining protocol.

4.5 Lipid Transfection with Effectene® Transfection Reagent

Lipid transfection was done with Effectene® Transfection Reagent (Qiagen) which is a non-liposomal lipid transfection method. According to the starting points in Qiagen's handbook 0,1 µg DNA, 0,8 µl Enhancer and 29,9 µl Buffer EC was prepared for each well and mixed by vortexing 1 s. The mixture was incubated for 2-5 min and centrifuged for a few seconds. 2,5 µl Effectene® Reagent was mixed with 20 µl Buffer EC per well and was added to the Enhancer mixture. The solution was pipetted up and down 5 times and incubated 5-10 min for transfection-complexes formation. Meanwhile the medium in the wells in the cell plate was removed and new fresh medium was added. The transfection-complexes were added drop-wise onto the cells in the wells. The plate was gently swirled and incubated at 37ºC and 5% CO_2 for 24 h. Different protocols with other amount of Cop-GFP and Cop-GFP/Effectene® Reagent ratio were tested.

4.6 Microscope Evaluation

Most of the cells were stained with Hoechst (Invitrogen, H3569), which binds to genomic material (DNA) and can be used to quantify cell density. The cells transfected with Cop-GFP were put for at least 24 h in incubator at 37°C and 5% CO_2 before analyzing. 24 h incubation was needed for the Cop-GFP-coding plasmids to get into the nucleus of the cells and thereafter be expressed with maximal protein expression. Dextran Alexa Fluor 546 (Invitrogen) only needed to get into the cytoplasm of the electroporated cells and could for that reason directly be observed in a microscope. The Dextran Alexa Fluor 546 electroporated

wells were washed 3 times with PBS and left in PBS for evaluation. The medium in the Cop-GFP transfected wells was changed to PBS or fix buffer (4% PFA and PBS). The hippocampal neurons and glia cells were immunostained to be able to see what type of cell that was transfected. The cells were studied in a fluorescence microscope (Leica) and/or a plate reader (Tecan, Infinite F500).

4.7 Transfection Experiments

4.7.1 Transfection of PC12 Cells

The aim of the experiments with the PC12 cell line was to test how different parameters affected the transfection efficiency of the cells with as low cell death as possible. Previous transfection protocol optimization had been done and the aim was to confirm the protocol and also to test other parameters that could have an influence of the transfection. The parameters that were tested were number of electroporation pulses, transfection voltages, collagen coating vs. no collagen coating of plates, differentiation vs. no differentiation and different transfection molecules. The parameters tested are listed in Table 1. Alamar Blue assays were done to see the cell viability 4 h after transfection. Cellaxess®CX3 transfection of PC12 cells were also compared to lipid transfection of PC12 cells.

Table 1. Parameters tested for optimization of the transfection efficiency and cell viability for the PC12 cells line.

Parameters for PC12 transfection optimization	Tested parameters
#of pulses	3 and 10
Pulse length	5 and 25 ms
Voltage	120, 140, 160 and 180 V
Transfection molecule	GFP and dextran
Collagen coating before seeding of the cells	Yes and no
Differentiation of the cells before transfection	Yes and no

Transfection Protocols

3 different transfection protocols for Cellaxess®CX3 electroporation were used for transfections of the PC12 cell line, see Table 2. 40 ng/µl Cop-GFP (4,5 kDa) was diluted in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and without Phenol Red (PAA, Cat. No: E15-877). After transfection optimization of transfection protocol, other parameters were tested with knowledge from the previous transfection results.

Table 2. 3 different transfection protocols for PC12-cell transfection were tested.

Transfection protocol 1	
Dispense volume	18 µl
Dispense rate	18 µl/min
Dispense duration	0-60 s
Number of pulses	**3**
Pulse interval	10 s
Pulse length	**25 ms**
Electroporation start after dispense	50 s

Transfection protocol 2	
Dispense volume	18 µl
Dispense rate	18 µl/min
Dispense duration	0-60 s
Number of pulses	**3**
Pulse interval	10 s
Pulse length	**5 ms**
Electroporation start after dispense	50 s

Transfection protocol 3	
Dispense volume	18 µl
Dispense rate	18 µl/min
Dispense duration	0-60 s
Number of pulses	**10**
Pulse interval	10 s
Pulse length	**5 ms**
Electroporation start after dispense	50 s

Dextran Alexa Fluor 546 Electroporation

The aim was to compare the efficiency and specificity of different transfection molecules; Cop-GFP and Dextran Alexa Fluor 546. 18 µl 40 ng/µl Cop-GFP (4,5 kDa) and 18 µl 67 µg/µl Dextran Alexa Fluor 546 (10 kDa) both diluted in DMEM with L-glutamine and without Phenol Red was used for transfection of PC12 cells. 3 pulses, 10 s pulse interval, 25 ms pulse length and 120-180 V were used for the transfection. The Cop-GFP-transfected cells were thereafter analyzed 24 h after transfection and Dextran Alexa Fluor 546-transfected cells were analyzed immediately after transfection.

Uncoated Plate and Collagen Coated Plate

The same transfection protocol (25 ms in pulse length, 10 s pulse interval and 3 pulses) with 120-180 V was used for comparison of PC12 cells differentiated at a collagen coated and uncoated plate. 18 µl 40 ng/µl Cop-GFP was used for the transfection.

Viability Assay

PC12 cells were electroporated with 18 µl DMEM with L-glutamine and without Phenol Red to see the cell viability 4 h after electroporation at different voltages (0-280 V). The cells were electroporated with 3 or 10 pulses with 25 ms in pulse length and 10 s pulse interval. After transfection the cells were incubated at 37°C for 2 h. The medium was removed and 100 µl Alamar Blue (10% Alamar Blue (BioSource, DAL1100), 2% FCS and Ham's F12) was

added. The cells were incubated for another 2 h and the light intensity was thereafter analyzed in a plate reader (Tecan, Infinite F500).

Effectene® Transfection Reagent Lipid Transfection

Lipid transfection was tried on both undifferentiated and differentiated PC12 cells. Different transfection protocols were tried with different amounts of Cop-GFP (0,1 µg, 0,05 µg and 0,025 µg per well in a 96-well plate) and different Cop-GFP/Effectene® Reagent ratios (1:25, 1:12,5 and 1:6,25). Two wells of each protocol were transfected. The amounts of Cop-GFP and Effectene® Reagent are shown in Table 3.

Table 3. Lipid transfection of PC12 cells with different amounts of Cop-GFP amount and ratios of Cop-GFP/Effectene® Reagent.

Amount of GFP	Cop-GFP/Effectene® Reagent ratio
0,1 µg	1:25
0,1 µg	1:12,5
0,1 µg	1:6,25
0,05 µg	1:25
0,05 µg	1:12,5
0,05 µg	1:6,25
0,025 µg	1:25
0,025 µg	1:12,5
0,025 µg	1:6,25

4.7.2 Transfection of Rat Hippocampal Neurons

The aim was to optimize the transfection parameters to transfect as many neurons as possible with minimal transfection of glia cells and low cell death. The optimization of hippocampal neuron transfection included testing of parameters for high transfection ratio of neurons with high neuron survival. First different transfection protocols with varied transfection parameters in the Cellaxess® Commander Software were optimized. The transfection protocol parameters included number of electric pulses, electric pulse length, electric pulse interval, voltages, dispense volume and dispense rate. The values of the parameters for the transfection protocol were chosen from knowledge about other transfection optimization protocols of other cells at Cellectricon AB and also by reading articles to see what parameters other people had used for neuron transfection with electroporation. After optimization of the transfection protocol other parameters were tested by using the optimized transfection protocol. The other parameters included incubation time after transfection, electroporation buffers and different levels of endotoxin in the Cop-GFP. All parameters tested are listed in Table 4. Also lipid

transfection with Effectene® Transfection Reagent was used to compare the transfection efficiency with Cellaxess®CX3 electroporation.

Table 4. Parameters tested for optimal transfection efficiency and cell viability.

Parameters for hippocampal neuron transfection optimization	Tested parameters
#of pulses	1 and 2
Pulse length	1, 5 and 25 ms
Voltage	1, 50, 100, 120, 140, 150, 160, 180, 200, 250, 280 and 300 V
Pulse interval	1 and 15 s
Culturing time before transfection	7 and 12 days
Incubation time after transfection	24 and 48 h
Endotoxin reduced Cop-GFP	Yes and no
Electroporation buffer	Primary Neuron Basal Medium and Intracellular buffer

Transfection Protocols

6 different transfection protocols with different voltages (1, 50, 100, 120, 140, 150, 160, 180, 200, 250, 280 and 300 V) were tested, see Table 5. 100 ng/µl Cop-GFP diluted in PNBM was used for transfection. The aim was to try to transfect neurons and also to evaluate the influence of the electroporation in cell viability. In each experiment at least one well with seeded neurons was used as a control and was thus not transfected. The aim of the control was to see if the neurons had survived the freezing and also to see the ratio between hippocampus neurons and glia cells. The transfected neurons were incubated for 24 and thereafter the neurons were fixed and immunostained.

Table 5. Six different hippocampal neuron transfection protocols were tested. The varied parameters were number of pulses, pulse interval and pulse length.

Transfection protocol 1	
Dispense volume	18 µl
Dispense rate	18 µl/min
Dispense duration	0-60 s
Number of pulses	**2**
Pulse interval	**15 s**
Pulse length	**25 ms**
Electroporation start after dispense	50 s

Transfection protocol 2	
Dispense volume	18 µl
Dispense rate	18 µl/min
Dispense duration	0-60 s
Number of pulses	**2**
Pulse interval	**15 s**
Pulse length	**5 ms**
Electroporation start after dispense	50 s

Transfection protocol 3	
Dispense volume	18 µl
Dispense rate	18 µl/min
Dispense duration	0-60 s
Number of pulses	**1**
Pulse interval	**15 s**
Pulse length	**25 ms**
Electroporation start after dispense	50 s

Transfection protocol 4	
Dispense volume	18 µl
Dispense rate	18 µl/min
Dispense duration	0-60 s
Number of pulses	**2**
Pulse interval	**15 s**
Pulse length	**1 ms**
Electroporation start after dispense	50 s

Transfection protocol 5	
Dispense volume	18 µl
Dispense rate	18 µl/min
Dispense duration	0-60 s
Number of pulses	**2**
Pulse interval	**1 s**
Pulse length	**5 ms**
Electroporation start after dispense	50 s

Transfection protocol 6	
Dispense volume	18 µl
Dispense rate	18 µl/min
Dispense duration	0-60 s
Number of pulses	**2**
Pulse interval	**1 s**
Pulse length	**1 ms**
Electroporation start after dispense	50 s

Culturing Time

The transfection efficiency and cell viability of neurons that had been cultured for 7 days and for 12 days was compared. The neurons were transfected with 100 ng/µl Cop-GFP diluted in PNBM, 5 ms pulse length, 15 pulse interval and 2 pulses. From the previous result of the transfection protocol optimization the voltages 120 and 150 V were used.

24 or 48 h Incubation after Cop-GFP Transfection

The influence of the incubation time of transfected neurons was studied by incubation of the plate for 24 h and 48 h before analysing the transfection result. The number of transfected neurons and also the intensity of the GFP expression were compared after the different incubation times. 100 ng/µl Cop-GFP diluted in PNBM, 25 ms pulse length, 2 pulses and 15 s pulse interval at different voltages were used as transfection protocol.

Endotoxin Reduced Cop-GFP Transfection

The Cop-GFP plasmid (pCop-GFP-c, EvrΩgen, Cat. No: FP501) routinely used for transfection validation contained a high content of endotoxin, 40 000 Endotox units (EU)/ml. The Cop-GFP was hence purified in an EndoTrap column from Lonza and the final level of endotoxin after two runs through the column was 43 EU/ml. Transfection of 100 ng/µl Cop-GFP with and without endotoxin was compared. The Cop-GFP was diluted in PNBM. The neurons were transfected with 5 ms pulse length, 15 s pulse interval, 2 pulses and 120 or 150 V.

Electroporation Buffer

The composition of electroporation buffer was tried. Intracellular buffer (ICB) was prepared with the same salt composition (135 mM KCl, 0,1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 5 mM EGTA, pH 7,3) as inside the neurons. Transfection with 100 ng/µl Cop-GFP diluted in ICB was tried instead of dilution in PNBM (e.g. 400 mM KCl, 200 mM $CaCl_2$, 77,30 mM $MgCl_2$, 0,10 mM $Fe(NO_3)_3$*9H2O, 3000 mM NaCl, 125 mM NaH_2PO_4*H2O, 2200 mM $NaHCO_3$, 2600 mM HEPES, pH 7,0-7,5, PAA). The intracellular buffer was made by dissolving salts in distilled water and after that the pH was set to 7,3. The neurons were transfected with 1 or 5 ms pulse length, 15 s pulse interval, 2 pulses and voltages in the range of 120-250 V were tested.

Effectene® Transfection Reagent Lipid Transfection

The aim of the Effectene® Transfection Reagent lipid transfection was to study transfection efficiency and cell viability by using a chemical transfection method and then compare the result with Cellaxess®CX3 transfected neurons. Lipid transfection was performed with 4 different protocols by transfecting with Cop-GFP plasmids with both reduced and unreduced endotoxin. Two wells of each protocol were transfected. The amounts of Cop-GFP and ratios of Cop-GFP and Effectene® Reagent are shown in Table 6.

Table 6. Lipid transfection of hippocampal neurons with different amounts of Cop-GFP with reduced and unreduced endotoxin and different ratios of Cop-GFP/Effectene® reagent.

Amount of Cop-GFP	Cop-GFP/Effectene® Reagent ratio
0,1 µg (reduced endotoxin)	1:25
0,1 µg (reduced endotoxin)	1:12,5
0,5 µg (reduced endotoxin)	1:25
0,5 µg (reduced endotoxin)	1:12,5
0,1 µg	1:25
0,1 µg	1:12,5
0,5 µg	1:25
0,5 µg	1:12,5

5. RESULTS AND DISCUSSION

5.1 PC12 Cell Line

Several experiments were done the first weeks, but did not give any good results because the cells were very sensitive and most of the cells died after electroporation. After a few weeks a bacteria contamination in the seeded PC12 cells (batch 1 from ATCC) was discovered. It was

analyzed as an uncommon bacterium called *Stenotrophomonas maltophilia* (Appendix 2 Figure 14C and 14D). Since attempt of trying to locate the source of the bacteria contamination was done during some weeks, no PC12 experiments could be done during this time. The bacteria source was not identified and everything that was associated with the PC12 cells was thrown away and new NGF etc. was ordered. PC12 cells from a new batch were ordered from ATCC (batch 2). When the PC12 cells from batch 2 were grown, it could be seen that these cells did not morphological look like or behave like the batch 1 PC12 cells. The results in this report only include results from experiments with the batch 2 PC12 cells. Pictures of the morphological difference of the two batches can be seen in Appendix 2 Figure 14 and 14B.

5.1.1 Optimization of transfection parameters

The batch 2 PC12 cells were not hard to transfect and no cell death could be seen after electroporation. One problem was however non reproducible transfection results, the experiments differed even though exactly the same transfection parameters were used. Despite problems with reproducibility an optimized transfection parameters of the batch 2 PC12 cell are listed in Table 7. More detailed results are described in the part 5.1.2-5.1.4.

Table 7. The optimized transfection parameters for batch 2 PC12 cells.

Parameters for PC12 transfection optimization	Tested parameter	Optimized parameter
#of pulses	3 and 10	**3 and 10**
Pulse length	5 and 25 ms	**25 ms**
Voltage	120, 140, 160 and 180 V	**160 V**
Transfection molecule	GFP and dextran	**GFP**
Collagen coating before seeding of the cells	Yes and no	**Not important**
Differentiation of the cells before transfection	Yes and no	**Not important**

5.1.2 Transfection Protocols

Pulse Length and Number of Pulses

3 different transfection protocols testing pulse length and number of pulses were used. 5 ms in pulse length and 3 or 10 pulses resulted in no transfection of cells. 25 ms in pulse length and 3 pulses resulted in transfected cells, but the problem was that a large number of cells fused during electroporation.

Voltage

Voltages between 120-180 V were tried and after analyzing the cells it could be seen that 160 V was the best voltage to use for maximal transfection efficiency (Appendix 2 Figure 15A-15D). It was that transfection with 160 V was the best voltage, since the transfection resulted in a broad ring-formed area of transfected cells.

5.1.3 Electroporation of Dextran

A clear difference of transfected Cop-GFP and Dextran Alexa Fluor 546 could be seen (Appendix 2 Figure 16A-16D). The GFP fluorescent light was more intense than the Dextran Alexa Fluor 546 light and GFP was therefore easier to detect. Dextran Alexa Fluor 546 also showed to be more unspecific and as a result the Dextran Alexa Fluor 546 gave more background noise than the Cop-GFP. The major advantage was that only cells that had survived the Cop-GFP transfection expressed GFP, compared to transfected Dextran Alexa Fluor 546 that could be observed in both dead and living transfected cells.

5.1.4 Uncoated Plate and Collagen Coated Plate

The same transfection protocol with 25 ms pulse length, 10 s interval and 3 pulses were used for PC12 cells seeded in a collagen coated and uncoated plate. No difference in transfection efficiency and cell viability could be seen if the collagen coated plate was compared to the uncoated plate (Appendix 2 Figure 15E-15F).

5.1.5 Viability Assay

The cell viability, 4 h after electroporation, was studied with an Alamar Blue assay. Electroporation protocols were chosen in a broad range of voltages (0-280 V) so that a severe cell death could be expected for the highest voltages.

Mean value of light intensity of three wells (three capillaries) for each voltage was calculated and plotted. Cell viability was plotted for cells electroporated with 3 and 10 pulses, see Figure 12 and 13. No obvious decrease of fluorescence intensity could be seen in the curves. It was concluded that the cells were not sensitive and did not die when transfecting with 3 or 10 electric pulses with 25 ms in pulse length. The scattered values can be a result of a variance in number of cells in the wells. For a better and more obvious cell viability result longer pulses and a higher number of electric pulses were needed.

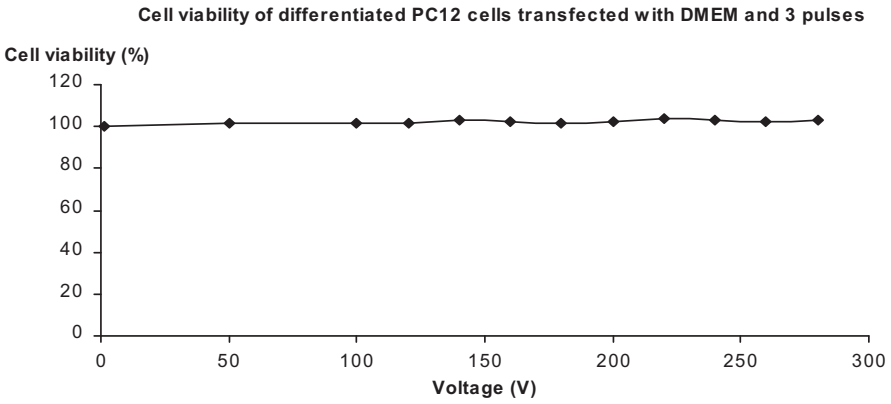


Figure 12. A diagram showing the cell viability of differentiated PC12 cells 4 h after transfection. The cells were transfected with 3 pulses.

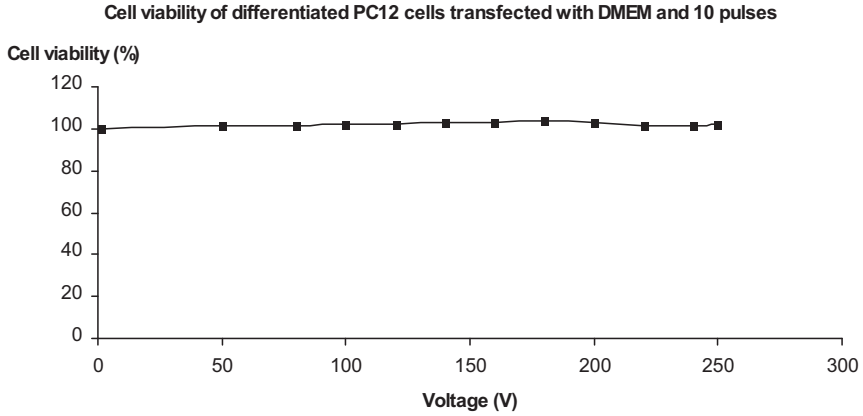


Figure 13. A diagram showing the cell viability of differentiated PC12 cells 4 h after transfection. The cells were transfected with 10 pulses.

5.1.6 Effectene® Transfection Reagent Lipid Transfection

The lipid transfection method showed to be rather efficient at PC12 cells with high cell viability. No difference in transfection efficiency between differentiated and undifferentiated PC12 cells could be seen. Some cell death, but no cell fusion was seen.

There was a clear difference between different amounts of Cop-GFP (Appendix 2 Figure 17A-17E). With 0,1 µg Cop-GFP per well many cells were transfected compared to transfection with 0,05 µg and 0,025 µg Cop-GFP where only a few cells were transfected. Cop-GFP/Effectene® Reagent ratio 1:25, 1:12,5 and 1:6,25 were tested in combination of 0,1 µg Cop-GFP. It could be seen that with high concentration of Effectene® Reagent many cells became transfected but not with optimal cell viability. Cop-GFP/Effectene® Reagent ratio 1:6,25 was too low, only few transfected cells could be observed. Cop-GFP/Effectene® Reagent ratio 1:12,5 in combination with 0,1 µg Cop-GFP per well gave the best transfection result with high cell viability. The results are summarized in Table 8.

Table 8. The best lipid transfection protocol was 0,1 µg Cop-GFP with the Cop-GFP/Effectene® reagent ratio 1:12,5.

Amount of GFP	Cop-GFP/Effectene® reagent ratio	Transfection efficiency	Cell viability
0,1 µg	1:25	+ +	- -
0,1 µg	**1:12,5**	**+**	**+**
0,1 µg	1:6,25	-	+
0,05 µg	1:25	-	+
0,05 µg	1:12,5	-	+
0,05 µg	1:6,25	- -	+ +
0,025 µg	1:25	- -	+ +
0,025 µg	1:12,5	- -	+ +
0,025 µg	1:6,25	- -	+ +

+ + = High
+ = Good
- = Not good
- - = Low

5.2 Rat Hippocampal Neurons

The total number of cells was counted in each vial. The supplier (Lonza) specifies that the number of cells per vial is 1 000 000 cells but control counts showed a larger number of cells in all vials, see Table 9. It is though unclear whether the specified number is for all cells in the vial or only the neurons. Before plating it was very hard to distinguish between different cell types. Another reason for the high number of cells can be that the supplier put a larger number of cells in the vial and after thawing when some cell death occurred the total number alive cells is about 1 000 000 cells per vial.

Table 9. Number of cells in each hippocampus vial.

	# of cells in the Hippocampal vials
1	172 000 cells/well → 6 020 000 cells/vial
2	216 000 cells/well → 7 776 000 cells/vial
3	127 000 cells/well → 4 572 000 cells/vial
4	138 000 cells/well → 4 968 000 cells/vial
5	186 000 cells/well → 6 696 000 cells/vial
6	100 000 cells/well → 3 600 000 cells/vial
7	112 000 cells/well → 4 032 000 cells/vial
8	220 000 cells/well → 7 920 000 cells/vial
9	144 000 cells/well → 5 184 000 cells/vial
10	140 000 cells/well → 5 040 000 cells/vial

In some of the wells dark rings could be observed (Appendix 3 Figure 18C-18D). The rings looked like dead neurons that had been destroyed into small pieces, maybe because of the osmotic pressure when the neurons were thawed.

5.2.1 Cellaxess®CX3 Transfection Optimization

A transfection protocol was optimized before other transfection parameters were tested. The transfection protocol optimization was done by using Cop-GFP diluted in PNBM. The aim was to transfect as many hippocampal neurons as possible with high viability and without transfecting glia cells. After several hippocampal neurons transfection experiments with Cop-GFP transfection diluted in PNBM it was established that the hippocampal neurons were very hard to transfect. A majority of the neurons did not survive the electroporation and only a few neurons and some glia cells were transfected. The best transfection parameters are listed in Table 10. More detailed results are described in part 5.2.5-5.2.8 and 5.2.10.

Table 10. Result of the transfection optimization of the hippocampal neurons.

Parameters for hippocampal neuron transfection optimization	Tested parameters	Optimized parameter
#of pulses	1 and 2	**2**
Pulse length	1, 5 and 25 ms	**1 and 5 ms**
Voltage	1, 50, 100, 120, 140, 150, 160, 180, 200, 250, 280 and 300 V	**200-300 V**
Pulse interval	1 and 15 s	**15 s**
Culturing time before transfection	7 and 12 days	**7-12 days**
Incubation time after transfection	24 and 48 h	**24 h**
Endotoxin reduced Cop-GFP	Yes and no	**Not important**
Electroporation buffer	Primary Neuron Basal Medium and Intracellular buffer	**Intracellular buffer**

5.2.2 Culturing

When the plate was studied in a microscope the first two-three days, dots of different sizes could be observed. The bigger dots showed to be the neurons. After a few days outgrowth from some of the cells could be seen and the outgrowth increased a little bit each day until one week after seeding when it was time to transfect the neurons (Appendix 3 Figure 18A-18B). One vial with neurons was cultured 12 days after seeding. More and longer outgrowths from the neurons and also larger cell bodies could be seen at the 12 days cultured neurons compared to the 7 days cultured neurons (Appendix 3 Figure 19A-19F).

5.2.3 Immunostaining

Immunostaining against MAP2 in the hippocampal neurons and GFAP in glia cells showed to be an excellent evaluation method to facilitate distinction between neurons and glia cells after transfection. The best staining protocol was found to be the use of red secondary antibodies against MAP2, which made it possible easily detect the green GFP-transfected neurons. In the

control well green secondary antibodies against MAP2 and red secondary antibodies against GFAP were used.

5.2.4 Physical Damage

One major problem with Cellaxess®CX3 transfection of neurons was physical damage when the electroporation head was put down in the wells (Appendix 3 Figure 20A-20F). This was discovered by analyzing wells that had been transfected with 1 V (Appendix 3 Figure 21A-21B). There should not be any transfection at voltages as low as 1 V and conclusively the thin black ring that could be seen in the edge of the well was due to physical damage when placing the electroporation head into the wells. If the head was not put down completely horizontal it was hard to avoid physical damage from the edge of the capillaries. The neurons were more sensitive compared to the glia cells and many neurons died because of the physical damage.

The physical damage had two explanations. The first explanation was the µClear 96-well plate which was used for cell culturing. The µClear was too thin compared to other 96-well plates and the electroporation head was too heavy which made the head press down the bottom of the wells which consequently made the capillaries come to close to the cells. The other reason for the physical damage was that the primary cells are more sensitive than e.g. cell lines.

5.2.5 Transfection Protocols

Six different transfection protocols were tested. In all experiments the transfection molecule was Cop-GFP diluted in PNBM electroporation buffer. See Appendix 3 Figure 23A-23F and 24A-24F for pictures of transfected neurons.

Number of Pulses

1 and 2 pulses were compared. Transfection with 1 pulse resulted in transfected glia cells in some of the wells but no transfected neurons. Transfection with 2 pulses showed to be more reproducible and effective. Transfected glia cells and some neurons could be seen in more wells transfected with 2 pulses than transfection with 1 pulse.

Pulse Length

The 1, 5 and 25 ms pulse lengths were compared. The 1 ms pulse was too short and did transfect very few neurons and some glia cells in some wells and 1 ms was not as reproducible as 5 and 25 ms. If 5 and 25 ms pulse length was compared it could be seen that

both pulse lengths resulted in some transfected neurons and many transfected glia cells but 5 ms pulse length resulted in higher cell viability than 25 ms.

Pulse Interval

The 1 s and 15 s pulse intervals were compared and no difference in cell viability could be seen. In those well where 1 s pulse interval was used many transfected glia cells and hardly any transfected neurons could be seen. 15 s resulted in both transfected neurons and glia cells and was therefore used for maximal neuron transfection.

Voltage

Different voltages were compared and transfected neurons could be seen in wells transfected with 150, 180 and 250 V when transfecting with Cop-GFP diluted in PNBM and 5 ms in pulse length, 15 s pulse interval and 2 pulses.

5.2.6 Effects of Transfection after 7 and 12 Days Culturing

7 days and 12 days cultured neurons were transfected with Cop-GFP diluted in PNBM to investigate the influence of the culture time on transfection result. The electroporation protocol was 5 ms in pulse length, 15 s pulse interval and 2 pulses and varying voltages. The transfection efficiency and cell viability showed to be the same with the 7 and 12 days differentiated neurons concerning both number of transfected neurons and glia cells.

5.2.7 24 h or 48 h Incubation after Cop-GFP Transfection

To evaluate the optimal expression time for Cop-GFP in hippocampal neurons, the neurons were studied 24 and 48 hours after transfection. For transfection Cop-GFP was diluted in PNBM and the electroporation protocol was 5 ms pulse length, 2 pulses and 15 s pulse interval. 24 h incubation resulted in some transfected cells that expressed GFP with high intensity. When the same plate was studied after 48 h a decrease in both number of transfected cells and light intensity was clearly visible. When the plate was studied after fixation and compared with 24 and 48 h incubation a break-down of GFP in some of the neurons could be seen. This was seen as small dots of GFP expression along the outgrowths of the neurons (Appendix 3 Figure 21C).

5.2.8 Endotoxin Reduced Cop-GFP

The purification of Cop-GFP was done to investigate if the endotoxin level was important for the transfection efficiency and viability when the neurons were transfected. Cop-GFP with reduced endotoxin level diluted in PNBM was transfected with 5 ms pulse length, 2 pulses

and 15 s pulse interval. No major difference could be seen compared to endotoxin contaminated Cop-GFP, but the low endotoxin Cop-GFP showed slightly more transfected glia cells than the endotoxin containing Cop-GFP. The endotoxin did not show to be a problem when transfecting neurons.

5.2.9 Effectene® Transfection Reagent Lipid Transfection

Neurons transfected with 0,1 µg Cop-GFP having Cop-GFP/Effectene® Reagent ratio 1:25 and 1:12,5 cell death was observed and only few transfected neurons with weak GFP-expression that had an altered morphology were detected (Appendix 3 Figure 22A). In wells transfected with 0,5 µg Cop-GFP having Cop-GFP/Effectene® Reagent ratio 1:25 and 1:12,5 the cell viability was very low. Those neurons that had survived did not look healthy and many of them had lost all outgrowths from the cell body (Appendix 3 Figure 22B). No difference between wells transfected with endotoxin reduced Cop-GFP compared to wells transfected with unreduced endotoxin Cop-GFP was observed. The result of the lipid transfection is shown in Table 11.

Table 11. The lipid transfection of the hippocampal neurons did only transfect few neurons and a majority of the neurons died.

Amount of Cop-GFP	Cop-GFP/Effectene® reagent ratio	Transfection efficiency	Cell viability
0,1 µg (reduced endotoxin)	1:25	-	-
0,1 µg (reduced endotoxin)	1:12,5	-	-
0,5 µg (reduced endotoxin)	1:25	-	- -
0,5 µg (reduced endotoxin)	1:12,5	-	- -
0,1 µg	1:25	-	-
0,1 µg	1:12,5	-	-
0,5 µg	1:25	-	- -
0,5 µg	1:12,5	-	- -

+ + = High
+ = Good
- = Not good
- - = Low

5.2.10 Electroporation Buffer

To investigate the influence of the composition of electroporation buffer, neurons were transfected with Cop-GFP diluted in intracellular buffer (ICB) with other ion concentration content than PNBM. Intracellular buffer had almost the same salt composition and pH as the inside of neuronal cells. Transfection protocol used was 1 or 5 ms in pulse length, 2 pulses and 15 s pulse interval and varying voltages. The transfection resulted in many transfected neurons and also glia cells in wells transfected with all different voltages (Appendix 3 Figure 25A-25F and Figure 26A-26F). The transfected neurons looked healthier and expressed GFP with more intense light than neurons that had been transfected with PNBM. The number of

transfected neurons and glia cells in 120 V and 150 V was less than in wells transfected at higher voltage. If 150 and 250 V was compared it could be seen that in wells transfected with 250 V more neurons than glia cells were transfected and in 150 V transfected wells more transfected glia cells than neurons could be observed. The cell viability was much higher in all transfected wells compared to transfection with PNBM. The wells transfected with 1 ms pulse length and 300 V resulted in many transfected neurons and almost no glia cells could be seen.

5.3 Cellaxess®CX3

One problem with the electroporation head was air bubbles in the capillaries. Bubbles in capillaries can cause a break in the electric circuit from the inner electrode to the outer electrode. This prevents the electric pulse from being applied and the cells are not electroporated and transfected (Appendix 3 Figure 18A-18B). If this happened the electroporation without dispensing was tried again.

6. CONCLUSIONS

6.1 PC12

A clear difference between the batch 1 and batch 2 PC12 cells concerning morphological shape, dividing time, transfection efficiency and cell viability could be seen and it was therefore hard to draw any conclusions between the transfection experiments done with both kinds of cells. But after several questions to the supplier (ATCC), they guarantee that both batches of PC12 are actually the same cell type which can be expected to behave differently depending on culture flask material etc. PC12 are artificial cancer cells and sometimes the cells behave strange and not as expected.

One major problem with both batch 1 and batch 2 PC12 cells was cell fusion after electroporation.

The transfection with Cellaxess®CX3 at the batch 2 closely adherent, epithelial like PC12 cells was not very reproducible and the result varied a lot between experiments. It was hard to predict how the result would be and it was therefore hard to really tell that 160 V was the best voltage to use.

Lipid transfection of batch 2 PC12 cells showed to be efficient if the Effectene® Reagent ratio was high and lipid transfection also resulted in high cell viability.

6.2 Rat Hippocampal Neurons

It has been reported that frozen primary cells are more sensitive and harder to work with than fresh dissociated neurons. But after growing and transfecting these primary rat hippocampal neurons it has been confirmed that the cryopreservation and 10 months freezing period did not show to be any problem concerning growth and staining of the neurons. Pictures of the neurons were compared with pictures of how the neurons were supposed to look like from the supplier and no difference could be seen. Pictures were also sent to the supplier who confirmed our conclusions that the neurons looked like they were expected to look like.

Fresh hippocampal neurons grow and stay healthy for at least 3-4 weeks. Lonza guarantee that the frozen neurons can stay healthy 7 days after seeding, but the experiment testing 12 days growing of the neurons showed more outgrowth and larger cell bodies of the neurons, which points that the frozen neurons can be used even for longer culturing time.

The conclusion about the transfection protocol was that shorter pulses with high voltage seemed to result in the best transfection efficiency and cell viability of neurons when transfecting with Cop-GFP and PNBM.

The endotoxin in the Cop-GFP did not show to have any influence of the transfection efficiency and the cell viability of the neurons. In conclusion hippocampal neuron experiments with Cop-GFP containing endotoxin can therefore be performed.

High amounts of Effectene® Reagent lipids method showed to be lethal for the neurons. For an optimized lipid transfection protocol resulting in higher transfection efficiency and cell viability more experiments with different amount of Cop-GFP and lipid concentrations have to be done.

From the results it could be concluded that neurons are very sensitive to specific ions. If the composition of ions is the same as inside the neurons, the neurons can be transfected with higher cell viability. Transfection with intracellular buffer, which has almost the same salt

composition and pH as inside the neuronal cells, showed to be the key to transfection of neurons by using Cellaxess®CX3 electroporation.

7. FUTURE PERSPECTIVES

7.1 PC12

Further experiments of trying parameters that could have an influence of the transfection process could be done on the batch 2 PC12 cells. More cell viability assays with different transfection protocols have to be tried.

7.2 Rat Hippocampal Neurons

The transfection of frozen hippocampal neurons was higher than expected considering neuron transfection efficiency and viability. Further optimization of the transfection protocols with varied pulse length, number of pulses, pulse interval and voltages has to be performed using hippocampal neurons with Cop-GFP diluted in intracellular buffer for even higher transfection efficiency and cell viability. Other parameters like temperature may also influence the transfection efficiency and need to be further investigated for an optimized transfection protocol. As the transfection efficiency and cell viability is optimized a siRNA assay can be developed.

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10. APPENDIX 1 – CELLAXESS®CX3 RUNNING PROCEDURE

1. The pump and pulse generator were turned on. The computer and Cellaxess Commander Software were started.
2. The connection was set to the right COM-port and Electroporation head was chosen in the Cellaxess Commander Software.
3. The electroporation head was connected to the three tubing and two electrodes.
4. A waste water container was placed below the electroporation head inside the safety box.
5. One container with ethanol and one closed container with sterile water were prepared. The inlet tubing connected to the pump was placed in the ethanol container and 500 µl ethanol was flushed through. The inlet tubing was placed in the sterile water container and 2000 µl water was flushed through. The flushing of 2000 µl water was repeated 2 times.
6. The water drops on the capillaries were shaken of and an air gap was made to avoid carryover between the water and transfection liquid.
7. The transfection reagent was prepared by diluting the vector or siRNA to the right concentration in the right electroporation buffer comprised of cell culture medium without serum and antibiotics. The transfection liquid was transferred to a fill plate and the electroporation head was placed in the fill plate.
8. The capillaries were thereafter filled with the transfection liquid.
9. An electroporation protocol with pre-designed transfection parameters (dispense volume, dispense rate, number of pulses, pulse length and pulse interval) was created or loaded.
10. The 96-well plate was moved from the incubator to the safety box and the capillaries on the electroporation head were carefully positioned in a triplet of wells containing cells. It was checked that the electroporation head was stable on the three legs. The lid of the safety box was thereafter closed and the electroporation was started.
11. When the first electroporation was finished, the electroporation head was taken up from the culture and the head was placed in the next triplet of wells with cell culture.
12. When the electroporation of all wells was finished the system was flushed with 2000 µl sterile water. The inlet tubes were then transferred to the ethanol container and 500 µl ethanol was flushed through. The inlet tubes were again placed in the sterile water

container and 2000 µl sterile water was flushed through the system. The inlet tubes were removed from the water and the system was flushed again with 2000 µl to empty the water.

13. The electroporation head and the fill-plate were sprayed with ethanol and left to dry.

11. APPENDIX 2 - PC12 CELL LINE

Pictures in appendix 2 were made by a digital camera (Hamamatsu CCD camera, C474-295) and edited in Adobe Photoshop CS.

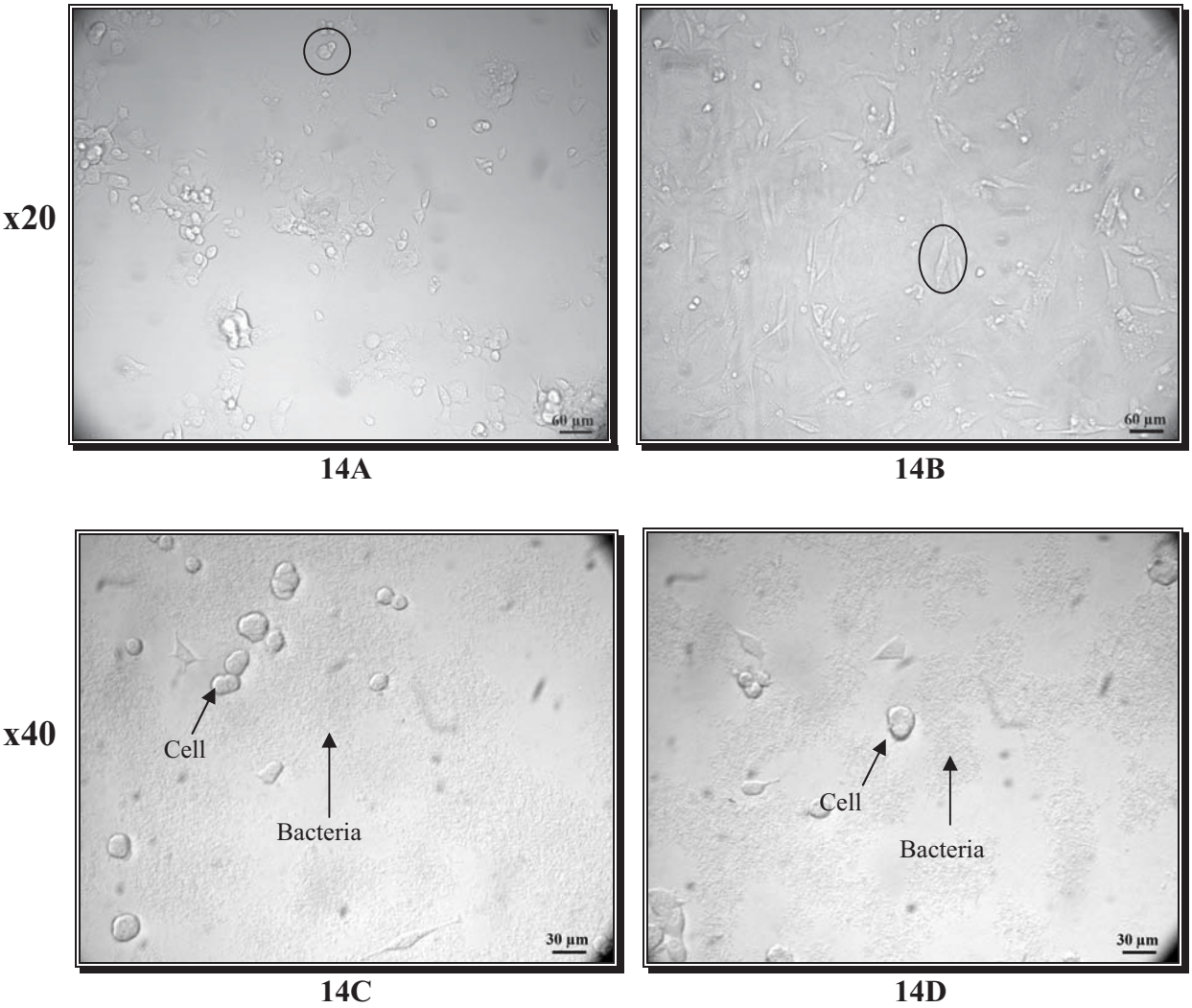

Figure 14. *Figure A:* PC12 cells from batch 1, ATCC. The circle shows a typical, undifferentiated PC12 cell from the batch 1. *Figure B:* PC12 cells from batch 2, a fresh delivered vial from ATCC. The circle shows a typical, undifferentiated PC12 cell from batch 2. A clear morphological difference can be seen between batch 1 and batch 2 PC12 cells. *Figure C and D:* Pictures of the bacteria contamination in PC12 cells from batch 1 three days after seeding in a 96-well plate. The cells are the big rings and the bacteria are seen as tiny dots at the bottom of the well.

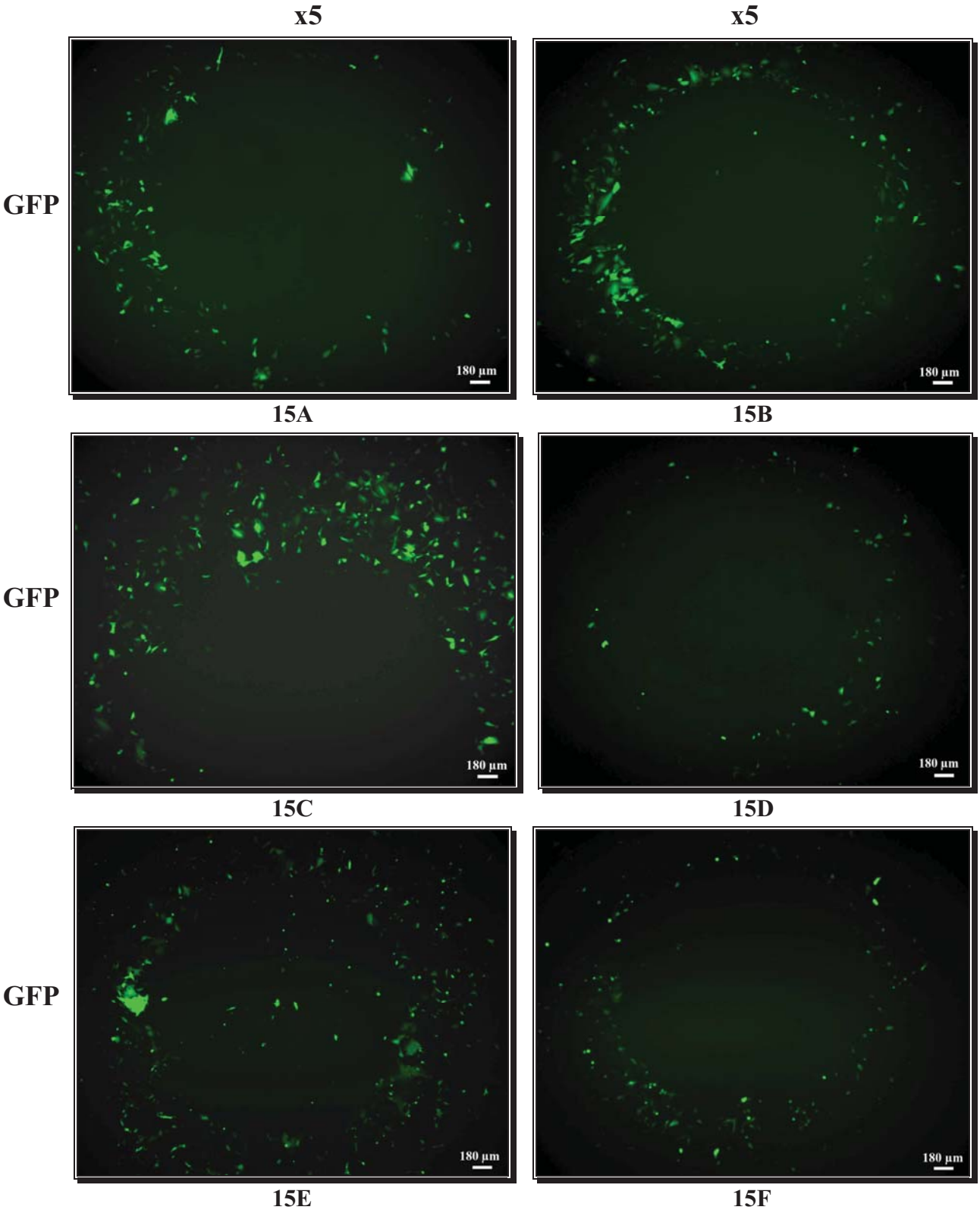

Figure 15. Cop-GFP transfection result of differentiated batch 2 PC12 cells at different voltages. The best voltage was 160 V, which resulted in high transfection efficiency with high cell viability. *Figure A:* Transfection at 120 V, collagen coated plate. *Figure B:* Transfection at 140 V, collagen coated plate. *Figure C:* Transfected at 160 V, collagen coated plate. *Figure D:* Transfection at 180 V, collagen coated plate. *Figure E and F:* Transfection of cells seeded on an uncoated plate. The cells in both pictures have been transfected at 160 V. No cell death could be observed either when transfecting cells on coated or uncoated plate. The major problem was cell fusion which can be seen as big green dots at the pictures. No difference in transfection efficiency or cell viability can be detected in the uncoated plate compared to the coated plate.

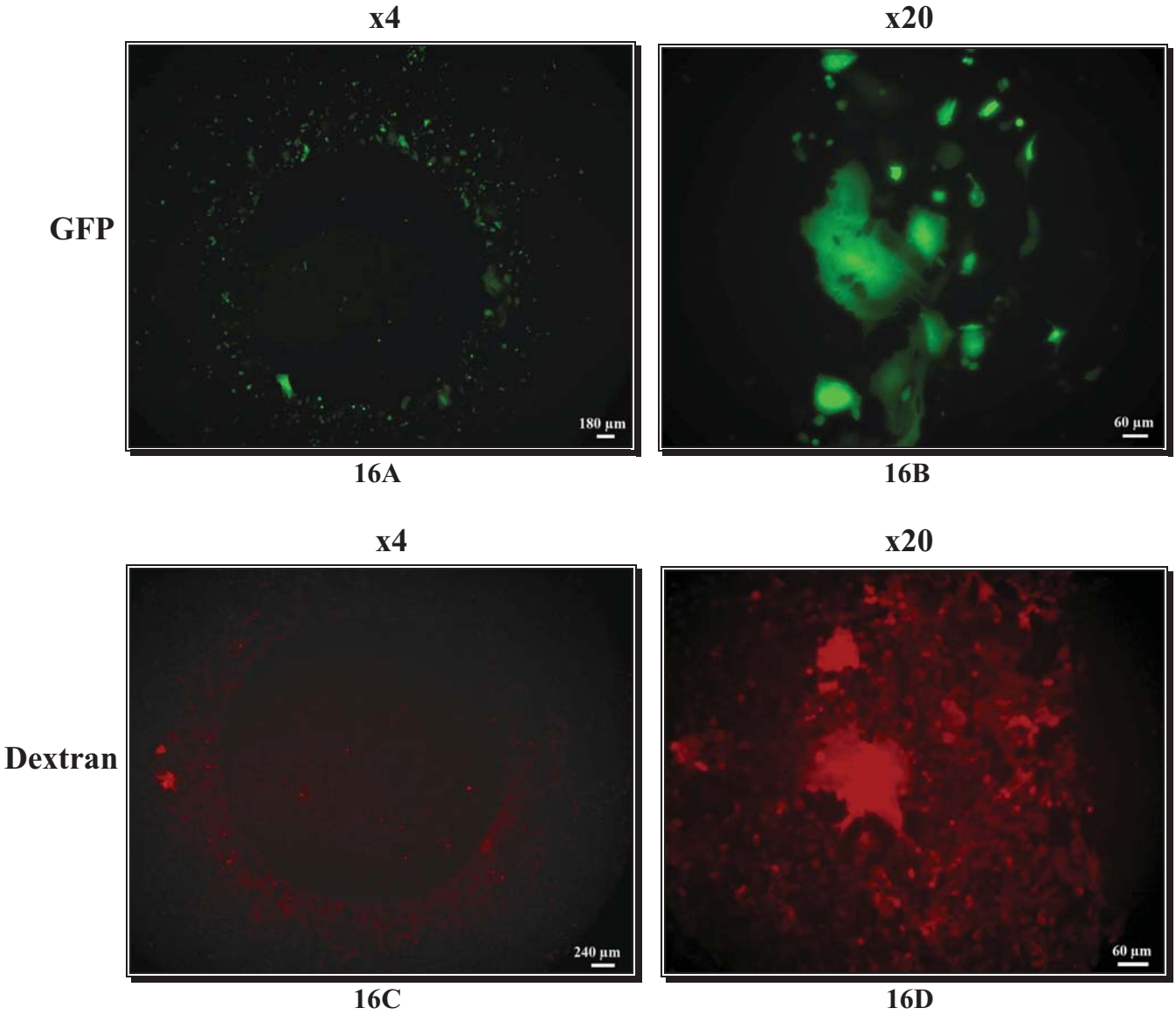

Figure 16. Transfection with Cop-GFP and Dextran Alexa Fluor 546 of batch 2 PC12 cells. The transfection of cells results in a ring-formed structure due to the circular hollowed capillary and the spreading of the electric field. *Figure A and B:* 160 V Cop-GFP transfected cells. *Figure C and D:* 160 V dextran transfected cells. Figure B and D show cell fusion in higher magnification. If the transfection of Cop-GFP and Dextran Alexa Fluor 546 is compared more background noise of dextran can be seen.

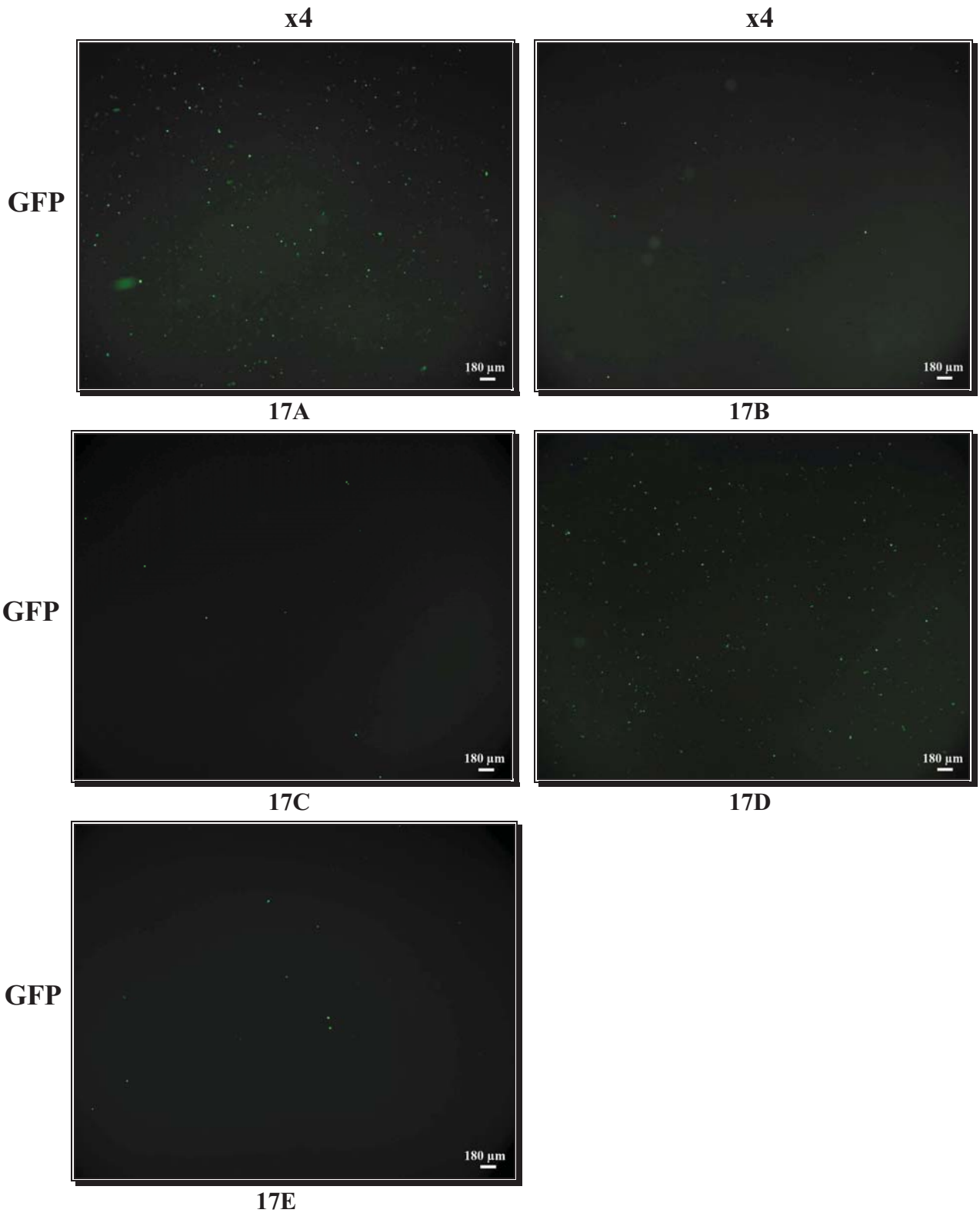

Figure 17. Lipid transfection with Cop-GFP of undifferentiated PC12 cells. *Figure A:* 0,1 µg Cop-GFP/well with Cop-GFP/Effectene® Reagent ratio = 1:25. *Figure B:* 0,05 µg Cop-GFP/well with Cop-GFP/Effectene® Reagent 1:25. *Figure C:* 0,025 µg Cop-GFP/well with Cop-GFP/Effectene® Reagent 1:25. *Figure D:* 0,1 µg Cop-GFP/well with Cop-GFP/Effectene® Reagent 1:12,5. *Figure E:* 0,1 µg Cop-GFP/well with Cop-GFP/Effectene® Reagent 1:6,25. The best transfection result with high cell viability was lipid transfection with 0,1 µg Cop-GFP/well and Cop-GFP/Effectene® Reagent ratio 1:12,5.

12. APPENDIX 3 – RAT HIPPOCAMPUS NEURONS

Pictures in appendix 3 were made by a digital camera (Hamamatsu CCD camera, C474-295) and edited in Adobe Photoshop CS.

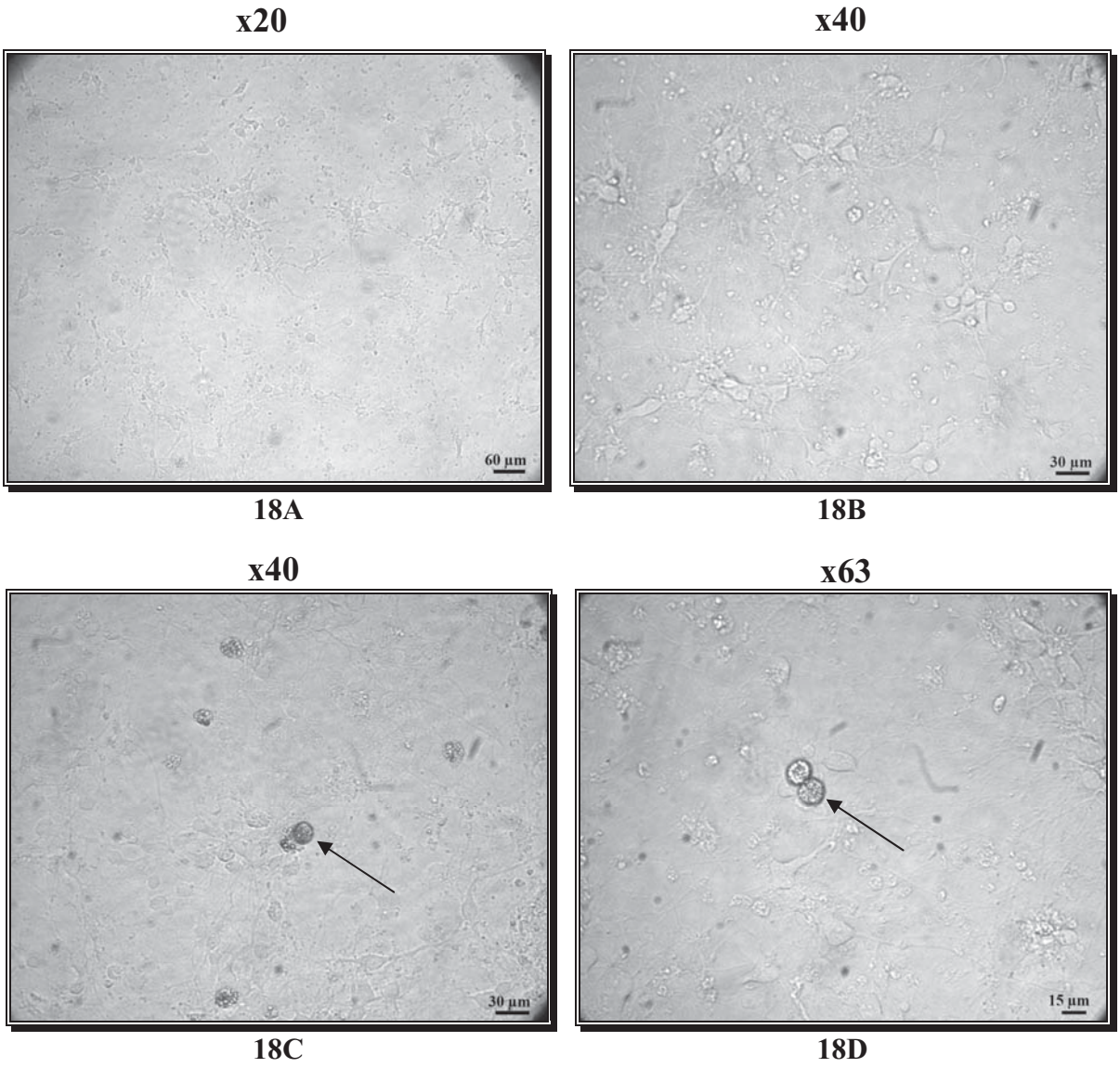

Figure 18. *Figure A and B:* Hippocampal neurons and glia cells in bright field with different magnifications. *Figure C and D:* Bright field pictures of dark rings that looked like damaged cells or bacteria contaminated neurons. After 5 days incubation of the neurons no spread of the dark rings could be seen and it was therefore concluded that the dark rings were a result of cell damage.

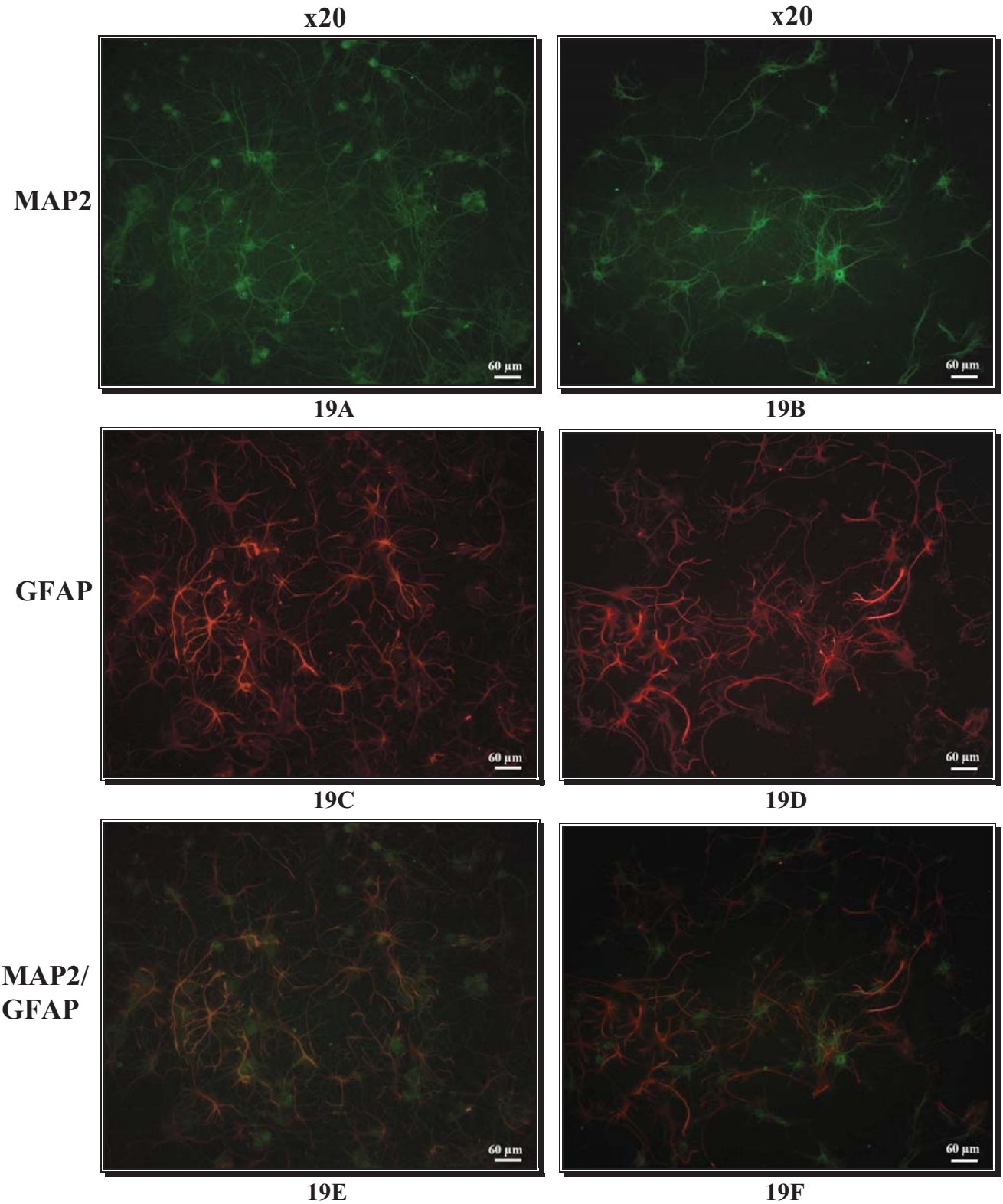

Figure 19. The pictures show immunostaining of MAP2 in hippocampus neurons (green) and GFAP in glia cells (red) with 20 times magnification. *Figure A:* MAP2 immunostained hippocampal neurons differentiated 12 days. *Figure B:* MAP2 immunostained hippocampal neurons differentiated 7 days. *Figure C:* GFAP immunostained glia cells differentiated 12 days. *Figure D:* GFAP immunostained glia cells differentiated 7 days. *Figure E and F:* Superimposed neurons and glia cells pictures from picture A/C and B/D.

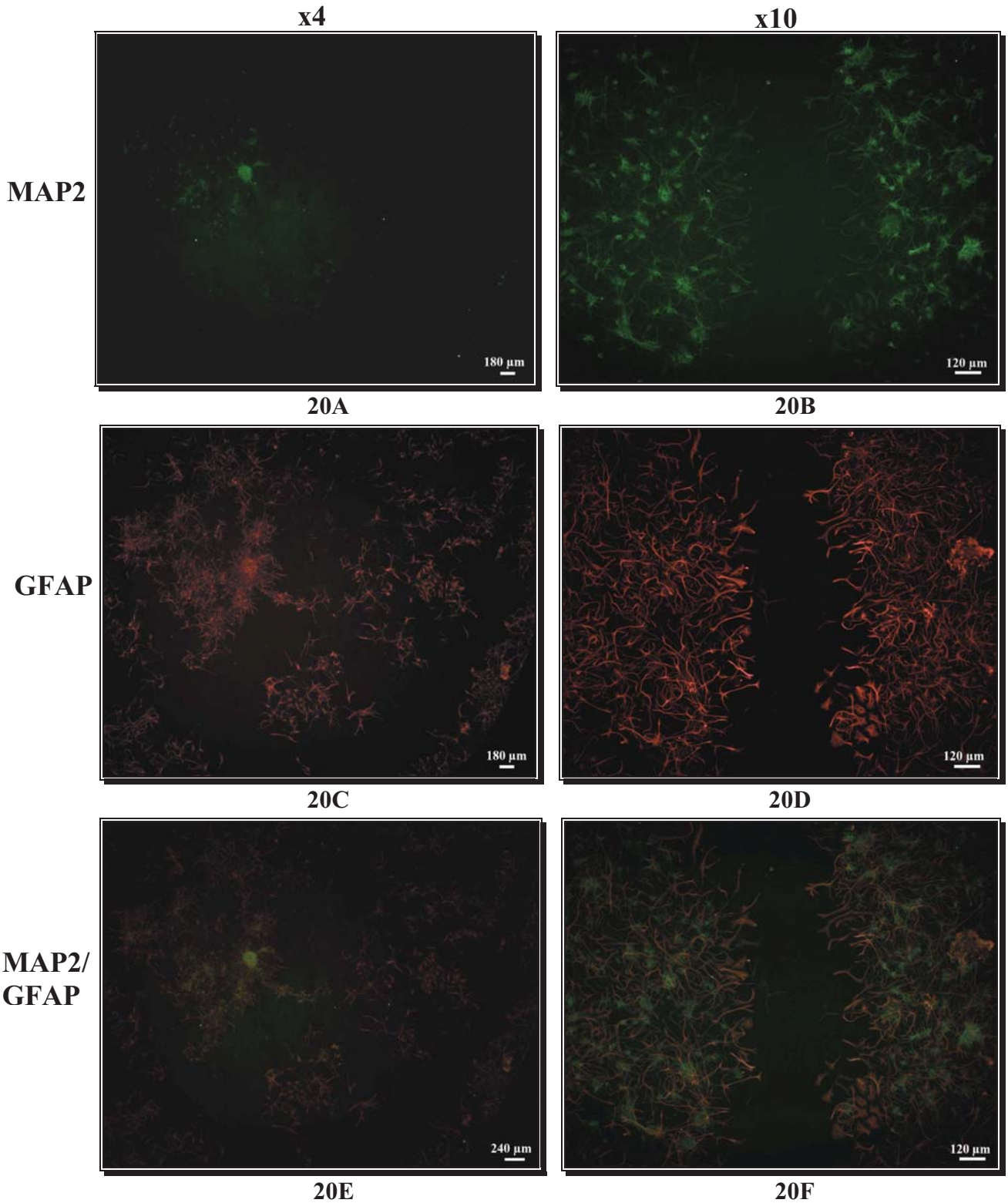

Figure 20. The figures show cells that have been transfected with 2 pulses, 15 s pulse interval and 5 ms pulse length. *Figure A, C and E:* Pictures showing MAP2-stained neurons (green) and GFAP-stained glia cells (red) that have been transfected with 100 V. *Figure B, D and F:* Pictures showing MAP2-stained neurons (green) and GFAP-stained glia cells (red) that have been transfected with 1 V. If Figure A with MAP2-stained neurons is compared to Figure C with GFAP-stained glia cells it can be seen that there are more living glia cells after electroporation. This can clearly be seen in the superimposed picture of MAP2 and GFAP in Figure E. This shows that neurons are more sensitive to the electroporation than the glia cells. The cells in Figure B, D and F have been transfected with 1 V and this means almost no voltage at all and no dead cells should be seen. But when the plates were analyzed a thin black ring almost in the edge of the well could be seen even in 1 V. This can consequently be a result of physical damage when placing the electroporation head in the well.

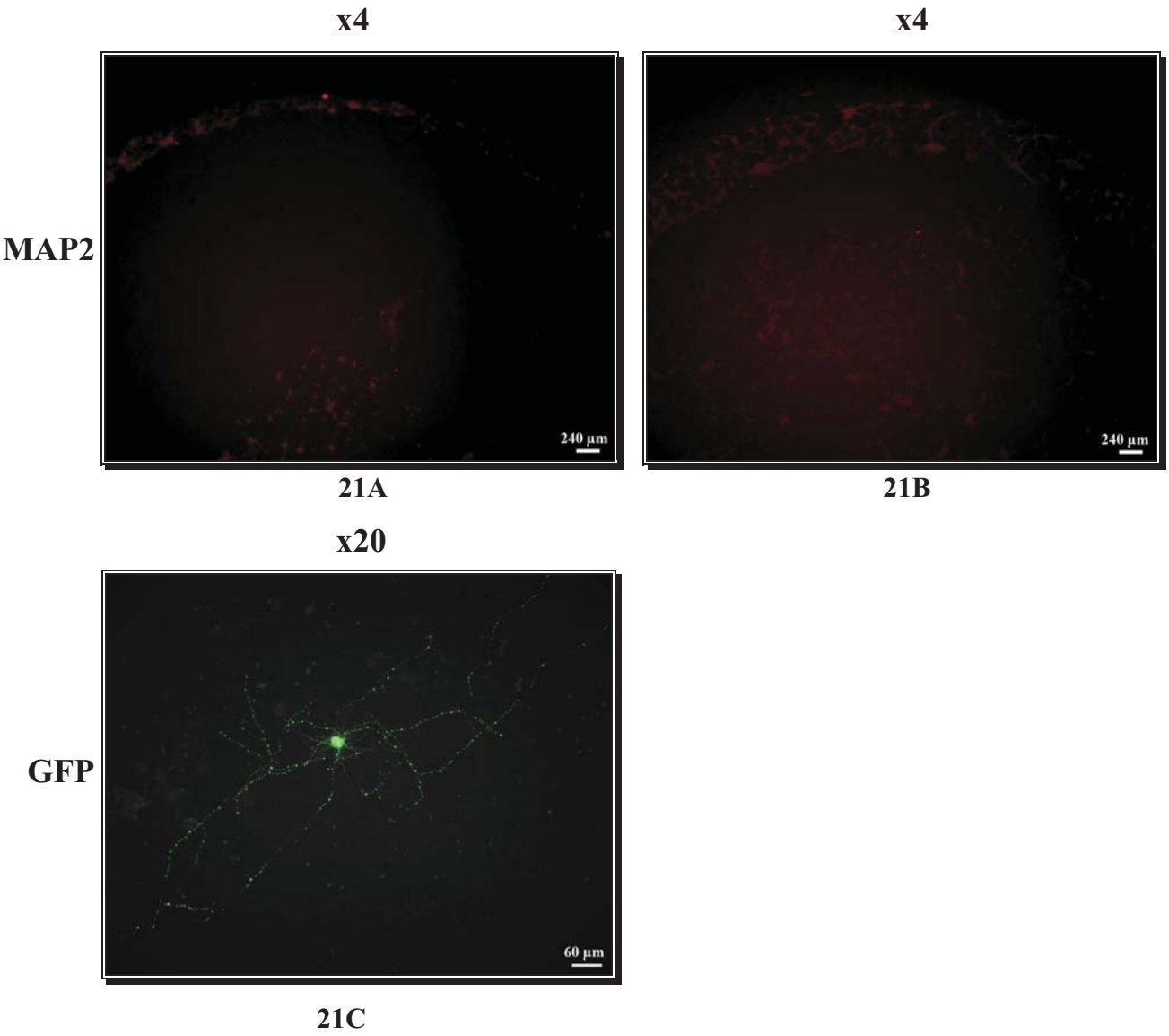

Figure 21. *Figure A and B:* Bubbles in the capillaries resulted in no electroporation. The figures show 2 different wells that have been electroporated with 50 V. Successful electroporation in the figure A but not in the figure B. The black ring in figure B is because of physical damage from the electroporation head. *Figure C:* A neuron or a glia cell after fixation, which had been transfected with 25 ms in pulse length, 2 pulses, 15 s pulse interval and 160 V. A break-down of GFP expression seen like dots of GFP along the outgrowth after fixation can be seen.

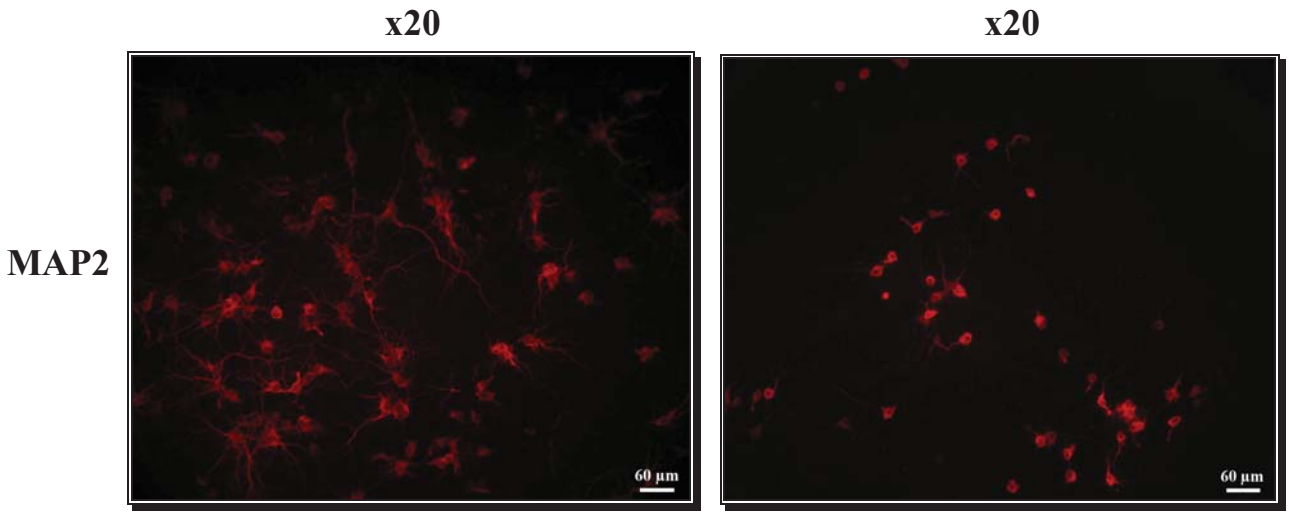

Figure 22. Lipid transfection of neurons resulted in only a few bad looking transfected neurons. The figures show the red MAP2-immunostained neurons. *Figure A:* 0,1µg Cop-GFP/well with Cop-GFP/Effectene® Reagent ratio 1:25.*Figure B:* 0,5 µg Cop-GFP/well with Cop-GFP/Effectene® Reagent ratio 1:25. In figure B more cell death can be seen and the neurons that survived lost almost all outgrowths.

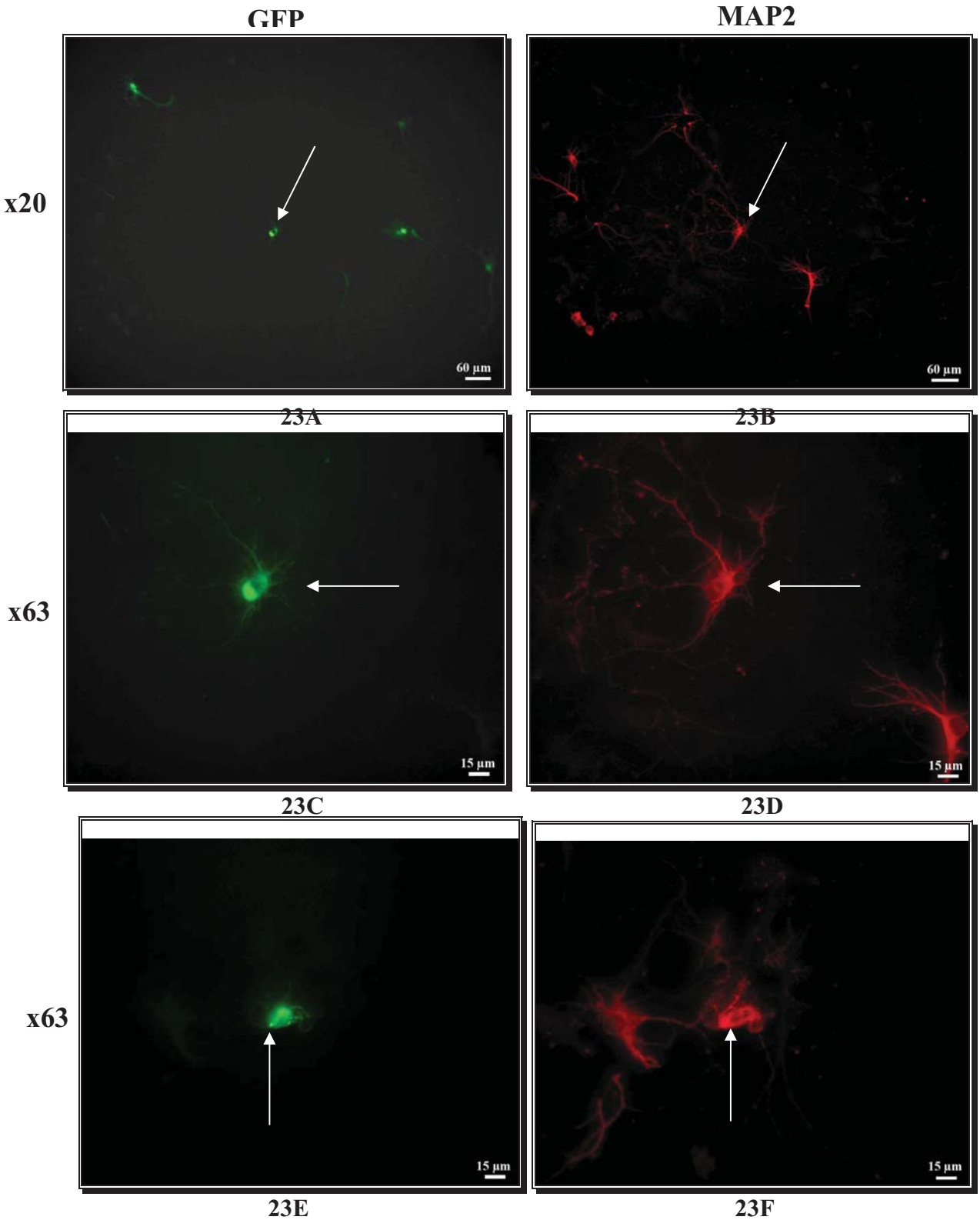

Figure 23. *Figure A, C and E* Transfected neurons expressing GFP that are confirmed by red immunostaining against MAP2 in neurons which is shown in *Figure B, D and F*. Figure A and C shows the same transfected neuron but with different magnifications. The transfected neurons in A, C and E had been transfected with Cop-GFP diluted in Primary Neuron Basal Medium with 150 V, 5 ms in pulse length, 2 pulses and 15 s pulse interval. Figure E and F show an unhealthy neuron.

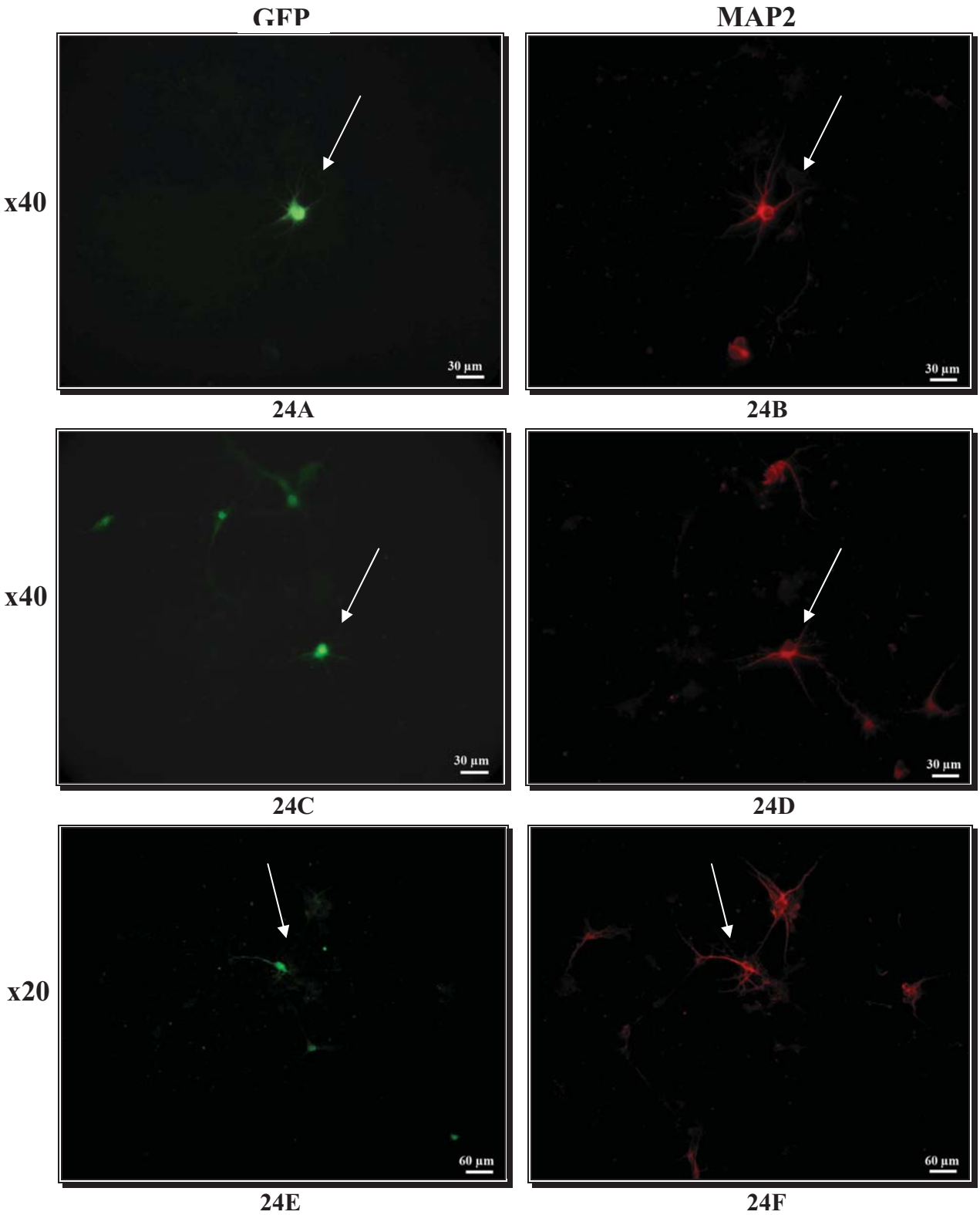

Figure 24. *Figure A, C and E:* Transfected neurons that are confirmed by red immunostaining against MAP2 in neurons which is shown in *Figure B, D and F.* The transfected neurons in A, C and E had been transfected Cop-GFP diluted in Primary Neuron Basal Medium, 150 V, 5 ms in pulse length, 15 s in pulse interval and 2 pulses.

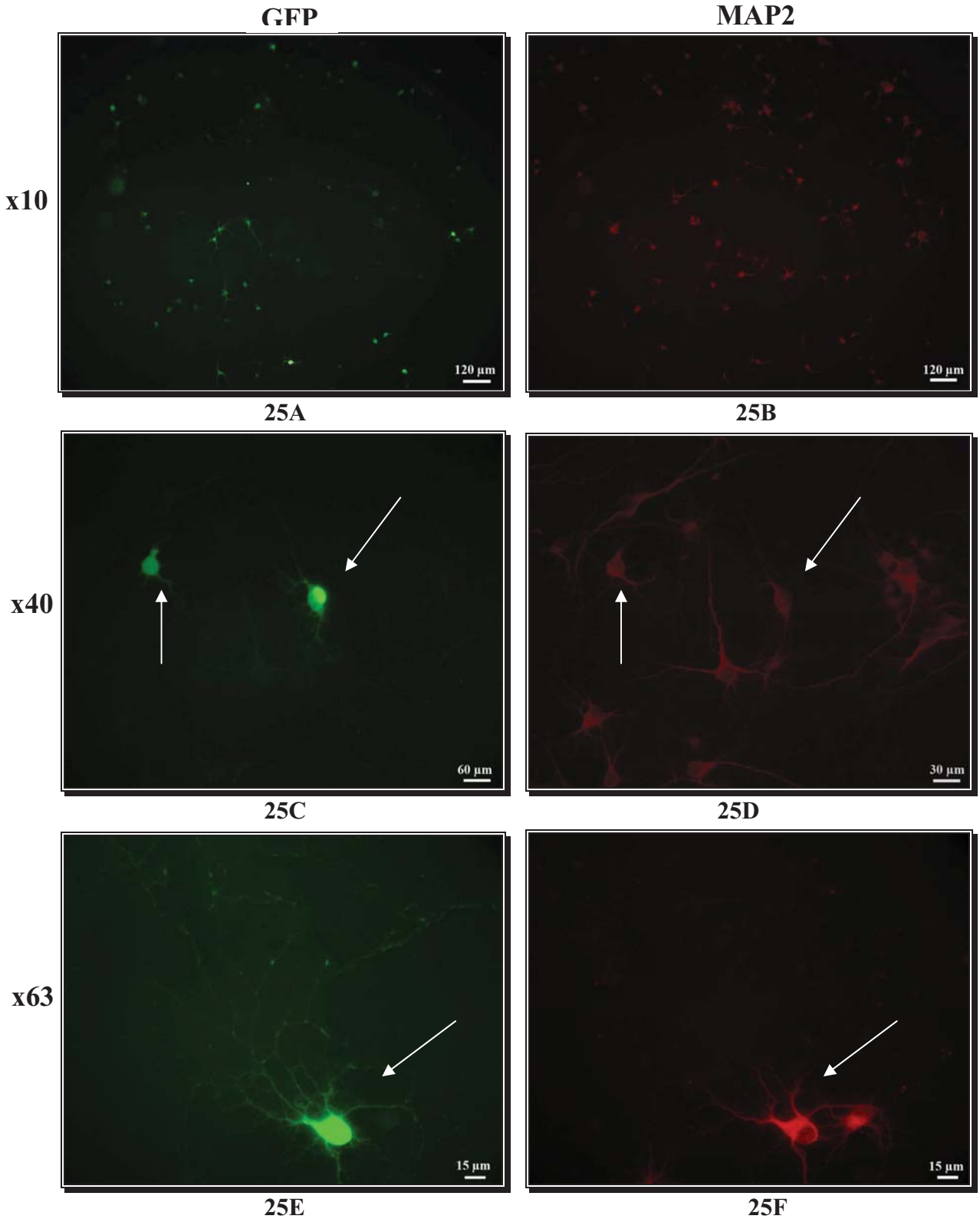

Figure 25. *Figure A, C and E:* Transfected neurons expressing GFP. All neurons had been transfected with GFP diluted in intracellular buffer, 15 s in pulse interval, 5 ms in pulse length and 2 pulses. The neuron in figure A was transfected with 150 V while the neurons in figure C and E were transfected with 250 V. The neurons transfected with intracellular buffer generally looked healthier with more outgrowths than those neurons that had been transfected with primary neuron basal medium. *Figure B, D and F:* Red MAP2 immunostaining of the GFP expressing neurons in Figure A, B and C.

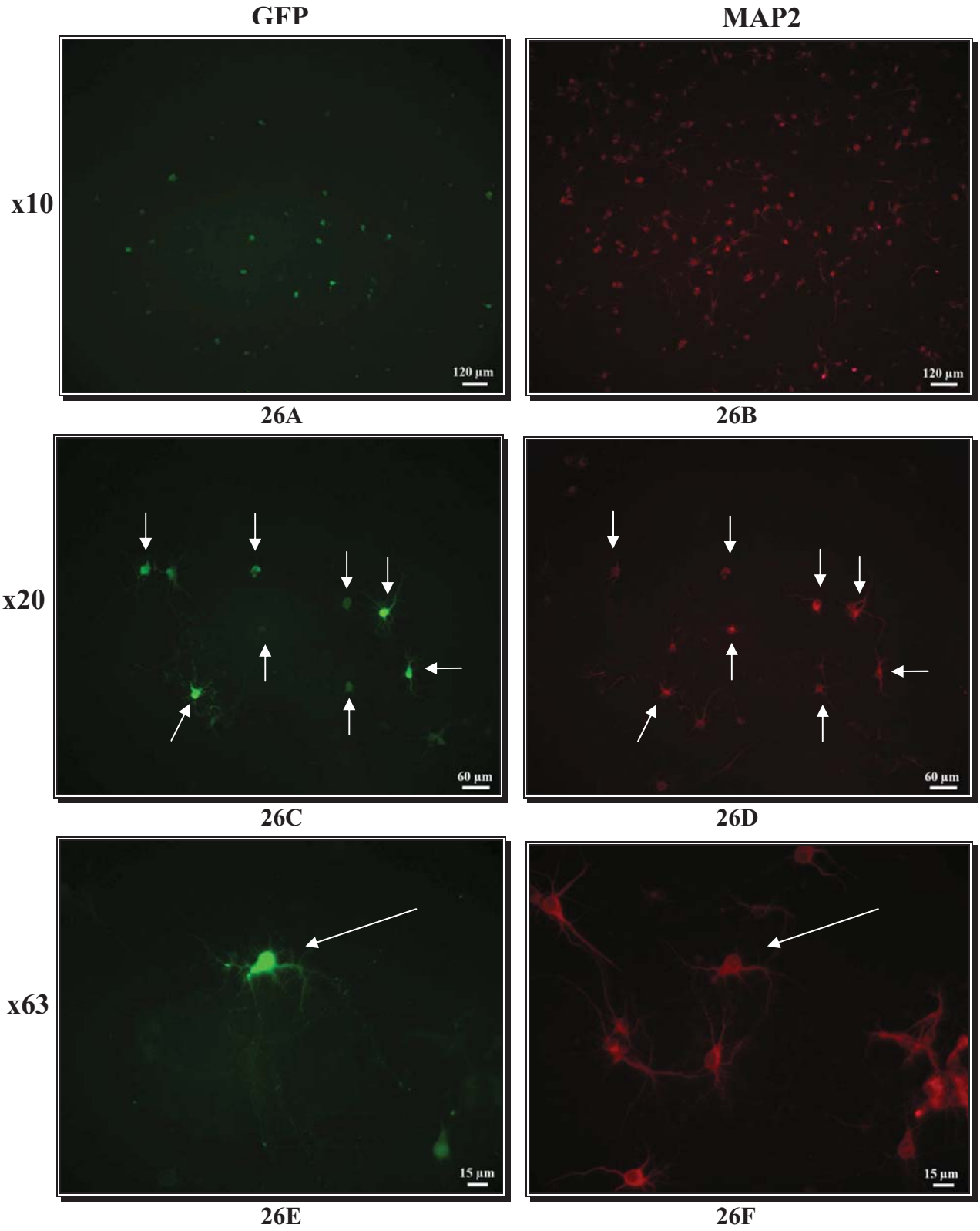

Figure 26. *Figure A, C and E:* Transfected neurons expressing GFP. All neurons had been transfected with Cop-GFP diluted in intracellular buffer, 15 s in pulse interval, 1 or 5 ms in pulse length and 2 pulses. The neurons in figure A was transfected with 300 V and 1 ms. The neurons in figure C were transfected with 250 V and 5 ms. The neuron in figure E was transfected with 200 V and 5 ms. The neurons transfected with intracellular buffer generally looked healthier with more outgrowths than those neurons that had been transfected with primary neuron basal medium. *Figure B, D and F:* Red MAP2 immunostaining of the GFP expressing neurons in Figure A, B and C.