BIOMEDICAL ENGINEERING MSc PROGRAM
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**Application Grade Thesis**

# Tumour Treating Fields: A study on using uninsulated electrodes for the purpose of inhibiting the growth of cancer cells.

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# Abstract

Glioblastoma is a highly aggressive and challenging brain tumor, necessitating the exploration of innovative treatment strategies. This study investigated the impact of low-intensity, intermediate-frequency alternating electric fields (TTFields) on the growth of SF-268 human brain glioblastoma/astrocytoma cell lines. The research methodology involved exposing the cells to TTFields using conductive electrodes, in contrast to the more common approach of using insulated electrodes and evaluated their viability using the MTT assay. Initially, the experimental process yielded ambiguous results, prompting adjustments to the delivery method of TTFields. Adjustments in exposure time and resting periods demonstrated initial promise but the lack of sustained significant differences suggested the need for further optimization. Fine tuning the parameters of the delivery method and quantitative assay provided sufficient evidence that an inhibitory effect due to TTFields exposure was taking place. These findings contribute to the understanding of TTFields as a potential therapeutic approach for glioblastoma and emphasize the need for further investigation of alternative approaches for their effective delivery. Future research should focus on exploring TTFields potential through novel treatment strategies, while elucidating the underlying molecular mechanisms to enhance their therapeutic efficacy. Ultimately, the goal is to develop effective and safe treatment modalities for improving patient outcomes in the management of glioblastoma and other brain tumors.

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# Chapter 1: Introduction

In the last few decades, extraordinary advances in the medical and pharmaceutical fields have been achieved in the frontier of cancer studies. However, cancer still accounts for a large number in the mortality rate of patients worldwide. It is estimated that high grade glioma represents the most common and aggressive brain tumour in adults, while also causing significant mortality and morbidity, although relatively rare compared to other cancer types. Classical techniques for prognosis and treatment are steadily being optimized in the scope of better results in the overall survival rate of cancer patients. Despite this, there is still a substantial number of methodologies and treatment options, that require the patients to undergo painful/ stressful procedures that may consecutively induce various health risks or trauma. Moreover, the efficacy of these treatment plans is rather insufficient in many cases, as they reside in outdated modalities and lack in innovation and personalized approaches.

In the recent years new modalities are beginning to emerge. Utilizing the rising, accumulating knowledge in biophysics and cell biology, cancer studies today provide insight about tumour development and treatment resistance to standard therapies and aim to the development of novel approaches for cancer treatment. This dissertation discusses the development of one of those novel approaches for cancer treatment, Tumour treating fields.

Tumour treating fields, are a recently developed cancer treatment modality that utilizes alternating electric fields of low-intensity and intermediate frequency. They have been found to have various effects on living tissues, which are frequency-dependant. In vitro experiments have shown that alternating electric fields of the aforementioned specifications, can achieve a profound inhibitory effect on the growth rate of a wide range of human and rodent tumour cell lines and malignant tumours in animals. This inhibitory effect is non-thermal and it specifically affects mitotic cells, while healthy quiescent cells remain intact.

Accumulated data from preclinical and clinical studies, supports their effectiveness as a single agent therapy in recurrent glioblastoma or as a supplementary therapy along with standard chemo-radiation. This led to the approval of therapeutic use of Tumour Treating Fields by the US Food and Drug Administration (FDA) in a clinical setting. For the purpose of therapeutic use, low-intensity, intermediate-frequency (100–300 kHz), alternating electric fields, are usually delivered by means of insulated electrodes, directly on the shaved scalp using a conductive hydrogel. These electrodes are placed after head mapping with MR imaging and the patient is required to wear a battery-operated system for at least 18 hours a day, for an extended period of up to 4 weeks.

Despite the amount of undergoing research regarding Tumour Treating Fields, the exact mechanism of action is not fully understood yet. Currently, there are two proposed models for the acting mechanism. The first and most common, is the reduce/arrest of cell

proliferation of malignant tumours. The second and less accepted is the complete degradation of cells undergoing division.

In this dissertation, the focus will lie on the first mode of action regarding the delay of tumour growth of glioma cells, while implementing a less common methodology for the delivery of Tumour Treating Fields. In contrast to the majority of studies, the exposure setup will be comprised of uninsulated electrodes and the exposure time will be highly decreased. This technique shows great potential for the therapeutic use of Tumour Treating Fields as it can improve the quality of the treatment, as well as the quality of the patient's life, taking into consideration the possible long term side effects caused by the prolonged exposure time of the treatment.

# Chapter 2: State-of-the-art

Research for therapeutic applications of low-frequency sinusoidal and pulsed electric and magnetic fields dates back to the late nineties, even though bioelectric properties of tissues and cells have been in the scope of science long before the turn of the century. It was during the sixties and seventies that scientists begun incorporating medical applications of bioelectric impedance measurements specifically for apnoea monitoring and venous thrombus detection. The use of externally applied electrodes was also common for stimulation of internal organs and in medical practise specifically for cardiac peace-making. However, it was during the early two thousands where the concept of delivering low intensity, intermediate frequency, alternating electric fields through insulated electrodes for medical purposes was conceived and thus gave rise to the science of tumour treating fields.

It had been known already, that alternating electric fields display a wide variety of effects when applied to living tissues which are almost exclusively dependant on their frequency.

In the case of very low frequencies, which are considered to range up to 1KHz, electric fields stimulate excitable tissues through membrane depolarization. This was the premise of medical applications that were dominant in the sixties and in the examples that were mentioned before. Other examples of studies using low-frequency electric fields, suggest prolific effects such as stimulating bone growth and accelerated fracture healing. Interestingly enough, as the frequency of the electric field increases above 1 kHz, the stimulatory effect diminishes.

At high frequencies (above several MHz), the tissue displays a very different effect. First and foremost, due to dielectric loss, the heating of the tissue becomes more and more evident as frequency and intensity increases. This phenomenon finds use in treatment modalities such as diathermy and radio frequency tumour ablation. However, for medical purposes, electric fields with high frequencies must be used very cautiously and for a limited exposure time, due to the possible side effects and the increased stress that is being caused to patients.

Intermediate frequencies that range from tens of kHz up to 1MHz, have been thought to have no biological effect on tissues, especially in low intensities. It has been reported that electric fields of these specifications alternate too fast for causing stimulation of any excitable tissues, while the dielectric loss, hence the heating of the tissue is nearly negligible. However, since the early two thousands, it has been found that fine-tuned alternating electric fields of intermediate-frequency (100-300kHz) and low intensities (1-

3V/cm), delivered through insulated electrodes, cause proliferation arrest in cancer cells. Moreover, in vivo experiments showed promising leads for reduced tumour growth in animal models, without any significant side-effects.

In the latter aforementioned specifications tumour treating fields, have been shown to have two core anti-mitotic mechanisms which evidently prolong the cell cycle and may eventually cause cell cycle arrest or arguably cell death. Both mechanisms affect the mitotic process of dividing cells, either by disrupting the formation of molecular machinery necessary for the last phases of mitosis, or by defecting the telophase/ cytokinesis part of mitosis, through a phenomenon called dielectrophoresis.

The first mechanism of action of this anti-mitotic effect, impairs the formation/organization of the mitotic spindle. This, critical for mitosis molecular construct, is comprised of polymerized microtubules, specifically α-tubulin and β-tubulin dimers. Microtubules are essentially in a dynamic state of polymerization and depolymerization as they also take part in the cytoskeleton remodeling during mitosis. Being polar molecules, tubulin α and β, are inherently affected by electric fields and their orientation. Due to this fact, in the presence of tumor treating fields, it has been shown that actively dividing cells, display a decreased ratio of polymerized and total tubulin. These findings showcase the interference of TTFields in the canonical formation of the mitotic spindle, which can potentially result in abnormal chromosome formation and even cell death in cases where the mitosis is exceedingly abnormal.

The second mechanism by which tumour treating fields affect cell division is by dielectrophoresis. This phenomenon occurs during the later stages of mitosis, when the cell membrane begins to constrict in order to pull the two sets of chromosomes and produce the daughter cells. When an electric field is applied to non-dividing cells, this field is usually uniform due to the circular shape of the cell (Fig. 1A). However, in an hourglass shaped cell going through cytokinesis, the electric field is non-uniform and the electrical forces are very high in the centre of this narrow cell junction (Fig. 1B). This effect induces motion of polar biomolecules and cell dipoles, moving them close to the furrow mitotic region, which can potentially lead to structural disorder and may even cause cell apoptosis. Studies have shown that electric forces of the applied electric field are greater in cells that have a narrower mitotic furrow, hence they are more sensitive to TTFields. More importantly, this effect only takes place when the mitotic furrow is aligned and parallel to the electric field which explains why tumour growth is not completely disrupted.

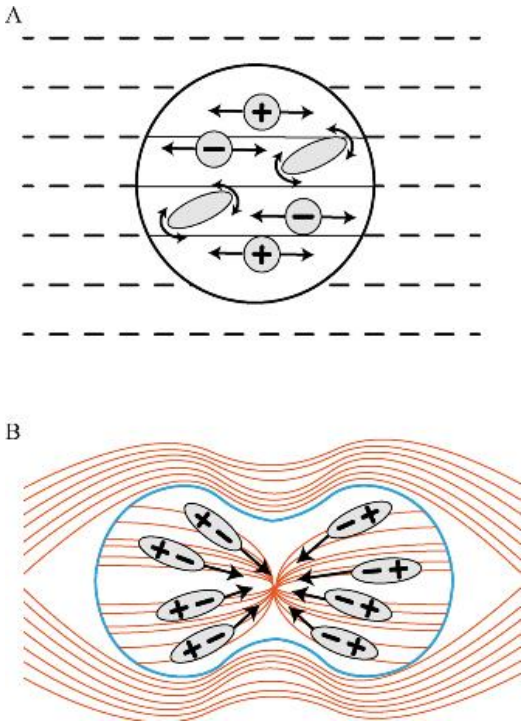


*Figure 1: Electric field distribution in and around A. Non-dividing cells and B. Dividing Cells. Chengke Luo (M.D.), et al.,*

According to the literature, both effects are demonstrated when tumour treating fields are applied for extended amounts of time, to cells undergoing mitosis that is oriented roughly along the field direction. However recent findings suggest that tumour treating fields exhibit non-mitotic effects that may also affect tumour growth. One of these effects regards the efficiency of DNA damage repair capacity of TTF treated cells. Results from gene expression analysis and ingenuity pathway analysis, showcase higher DNA damage and replication stress and significant downregulation of tumour suppression genes, involved in DNA double breaks repair.

Although extensive research has brought to light many biological effects of TTFields, either by in vitro or in vivo studies which will be discussed later, there is still room for development regarding the complete picture of the acting mechanism.

The pioneering study which proposed the two core mechanisms of action, happened in 2004. It specifically reported that TTFields can disrupt cancer cell replication of human and rodent cell lines, using insulated electrodes as a means of delivery. More importantly, this process doesn't affect quiescent cells while also doesn't produce significant tissue heating. Researchers subjected 11 different types of cells to TTFields, namely human melanoma, glioma, prostate and breast cell lines and also mouse melanoma, adenocarcinoma and rat glioma cell lines. They used completely insulated wires for delivering TTFields which were fixed in culture dishes, at a distance of 1mm of each other. A few thousands of cells were plated between the electrodes and after they had been

attached to the plate surface, culture media was added and the dishes were transferred to an incubator (Fig. 2A). After an incubation period of 24h, the cells were exposed to TTFields continuously for up to 72h.

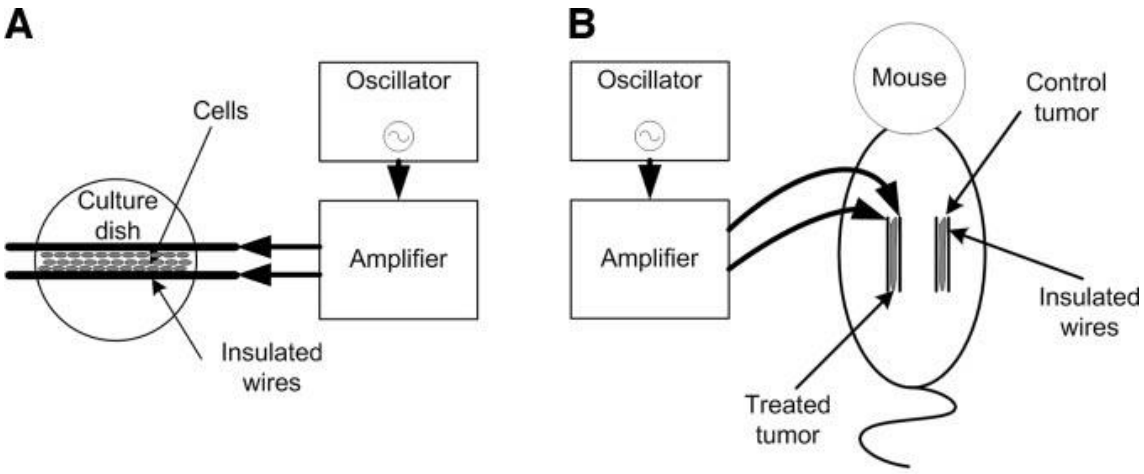


*Figure 2: Schematic representation of experimental setup A. In Vitro and B. In Vivo. Kirson et al., 2004*

The main findings from this study came from the comparison of the ratio of the decrease in growth rate of treated cells to that of untreated cells. In all of 11 cell lines the ratio was greater than 0, which indicated an inhibition in the growth rate. Results were collected using colorimetric measurements and a standard cell proliferation kit. Furthermore, researchers tested the effect of TTFields on non-replicating cultures and found no significant difference between control populations and treated cells. They also tested time, intensity and frequency dependence of the effect of TTFields on malignant melanoma and glioma proliferation. Regarding exposure time dependence, cells that were treated for at least 24h throughout the experiment, were found to have significantly larger doubling times after terminating the process. Specifically untreated cells had a doubling time of roughly 24h, whereas TTFields treated cells had nearly doubled time of replication and overall a decreased growth rate for up to 48h after treatment. In respect to the intensity dependence, they found that the inhibitory effect of TTFields is becoming more profound as intensity increases, to a certain threshold where complete proliferation arrest is achieved. They also showed that this threshold may vary between certain cell lines, as it did between mouse melanoma and rat glioma. Finally, they also tested the dependency of frequency to the effect of TTFields, which was hypothesized to have a strong correlation to the inhibition of mitosis. This was evident due to the dependence of cell membrane electric impedance on frequency, hence the penetrating ability of the field. The results were very interesting as they showed a substantial difference in optimal frequencies of TTFields for the two cell lines tested. It was the first indication that frequency is, if not the most important, one of the the most important parameters to consider in order to maximize the inhibitory effect of tumour treating fields. It was shown much later in another study, that the frequency variable is

directly associated with the size of the cell, through which came the explanation for the significant variations in optimal frequencies between cell lines.

Another very important parameter, that researchers put into consideration has to do with the orientation of the electric field. As a matter of fact, at any point in space, an electric field has a defined orientation that corresponds to the direction of the force it exerts on polar elements. Taking that into account, along with the structural differences of quiescent and dividing cells, with the later exhibiting an hourglass shape during cytokinesis compared to a spheroid shape, researchers hypothesized that electric field forces would have maximal effect when the cells are oriented along the lines of force of the field. To test this hypothesis, they used four melanoma cultures that were fixed and stained with toluidine blue after treatment with TTFields. They then grouped these cultures according to their cleavage axis orientation, relative to the electric field direction and counted the number of damaged and live mitotic cells. They ended up with four groups of mitotic cells, whose cleavage axis corresponded to four different angles formed in relation to the electric field, 0°, 45° (two sectors 45° and 135°) and 90°.

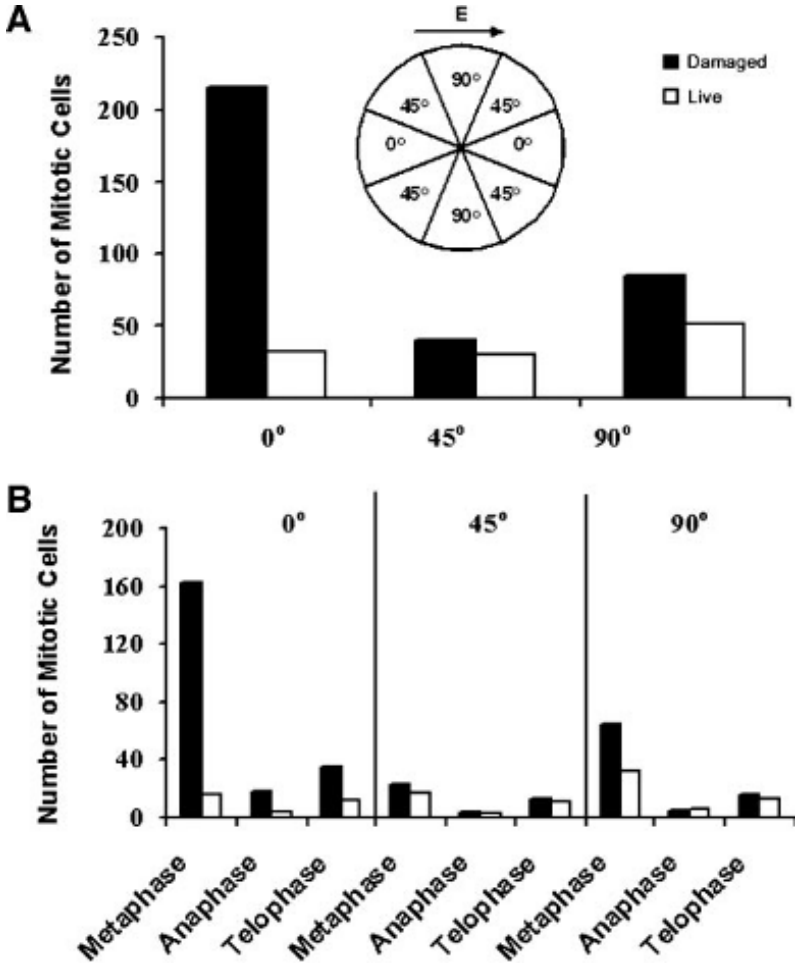


*Figure 3: Dependency of TTFields-induced cellular damage on the orientation axis of cell division relative to field direction.*

Their results are presented in Figure 3. In Figure 3A it is evident that live cells numbers are spread equally across all sectors, whereas the damaged cells that had their division axis parallel to the direction of the field were significantly higher in number. Furthermore, researchers also divided the groups of cells in accordance with their stage of mitosis in the time of fixation. As Fig. 3B suggests, the majority of damaged cells had their axis of division oriented along the field, however the group that had the higher percentage of damaged cells was the one that its cells were in the stage of metaphase.

Finally, regarding the in-vitro experiments of this particular study, researchers tested whether TTFields actively affect actin filaments, which are considered to be polar molecules, along with microtubule formation during mitosis. Through staining with monoclonal antibodies against actin filaments and microtubules and then using fluorescence microscopy, they concluded that TTF treated cells had abnormal microtubule behaviour, hence mitotic spindle formation as expected, but there was no significant difference between stained actin filaments in control and TTF treated cultures.

After the promising results from in vitro experiments, researchers proceeded to perform in vivo experiments as well, testing the newfound tumour treating fields on live mice. They used mice that had been inoculated with malignant melanoma and adenocarcinoma cells and in the same manner, TTFs delivery was performed using insulated wires which had been intradermally placed to the back of each mouse (Fig. 2B). A second set of electrodes that was not connected to a voltage amplifier, served as a control. Treatment lasted for 3-6 days continuously beginning 1 day after cell line inoculation. Results showed that intermediate frequency (100-200 kHz), low intensity (<2V/cm) TTFields, displayed an inhibition effect on malignant melanoma growth compared to non-treated control tumours. Treated tumours were found to be significantly smaller in both malignant melanoma and adenocarcinoma carrying mice, however in the case of melanoma the results were more substantial. Furthermore, after treatment the tumours and surrounding tissues were fixed and stained in order to be analysed histopathologically. The analysis showed that adjacent to the tumour tissues, were not damaged or affected by any means from the TTF treatment.

In summary, this study laid the groundwork for using alternating electric fields, as a treatment method for cancer both in vivo and in vitro. The researchers tackled many aspects of this novel approach on disrupting tumour growth and presented a plethora of results and suggestions regarding their optimal use. More specifically they addressed time, frequency, intensity and field orientation dependencies, while also suggesting two core mechanisms of action of tumour treating fields. They also tested the effects on various types of cancerous cell lines and also displayed that non dividing cells were unaffected.

Having successfully shown that low intensity, intermediate frequency, electric fields inhibit cancerous cell growth in vitro and in vivo, following the lines of the aforementioned very important study, the same team of scientists went on to extend their findings to additional cell lines and animal tumour models.

Specifically, researchers carried out experiments on human breast carcinoma; MDA-MB-231, and human non-small-cell lung carcinoma (H1299) and to animal tumor models (intradermal B16F1 melanoma and intracranial F-98 glioma) using external insulated electrodes. Their findings can be summarized as follows.

Firstly, they exhibited once more, the frequency dependency of the inhibitory effect of TTFields. The TTFields inhibitory efficacy vs. frequency was studied on mice inoculated with B16F1 melanoma. 26 live mice were treated for 5 days by TTFields of different frequencies. Using a frequency of 100kHz, treated tumor size was 50-70% that of control tumors. This was in alignment with their original study which supports the conclusion that this was the optimum frequency for the specific cell type. In contrary rats bearing intracerebral glioma were completely unaffected by 100 kHz TTFields, whereas 200 kHz showed substantial inhibition of tumor growth. In subsequent experiments, it was shown that optimal TTFields frequency is inversely related to cell size (Figure 4) in a way consistent to the diameter variability of the different cell types studied.

Secondly, the results of the previous study combined with the findings of experiments on live mice and rats from this particular study, led to the initiation of a pilot clinical trial of the effects of TTFields in 10 patients with recurrent glioblastoma (GBM). The results of the clinical trial suggested that time to disease progression and overall survival time were both increased in relation to historical control patients. In addition, researchers examined the safety profile for TTFields treatment. Their findings showed no altercations directly linked to the method of treatment, except some minor cases of contact dermatitis that was easily treated with steroid cream application and periodic relocation of the electrodes.

This goes to show, that TTFields can efficiently arrest the proliferation of a wide range of tumour cells in vivo, while also considering safety measures and the quality of life of the patients.

From that point on, several pilot studies and clinical trials begun to expand the idea of using TTFields as an anti-mitotic treatment for GBM (Glioblastoma), experimenting with proper position of the electrodes, construction material selection for safer treatment and of course responsible patient candidate selection for the trials. Some studies paired the use of TTFields as a treatment method with conventional methods such as chemotherapy, where the use of alternating electric fields acted as a supplementary treatment option for live animal models that were already treated with the most common chemo-therapy agents such as paclitaxel, doxorubicin and cyclophosphamide. Dose response curves were established for the combination of the two therapeutic methods and a shift to the left was reported. This shift indicated that lower doses of the chemotherapy agents achieved the same growth inhibition in the presence of TTFs. Additionally, the combination of the two methods achieved a greater inhibitory effect than each of the two methods applied by itself. Researchers tested the adjoined treatment method for statistical significance and proved that TTFields mediate an enhanced sensitivity to the conventional method of chemotherapy.

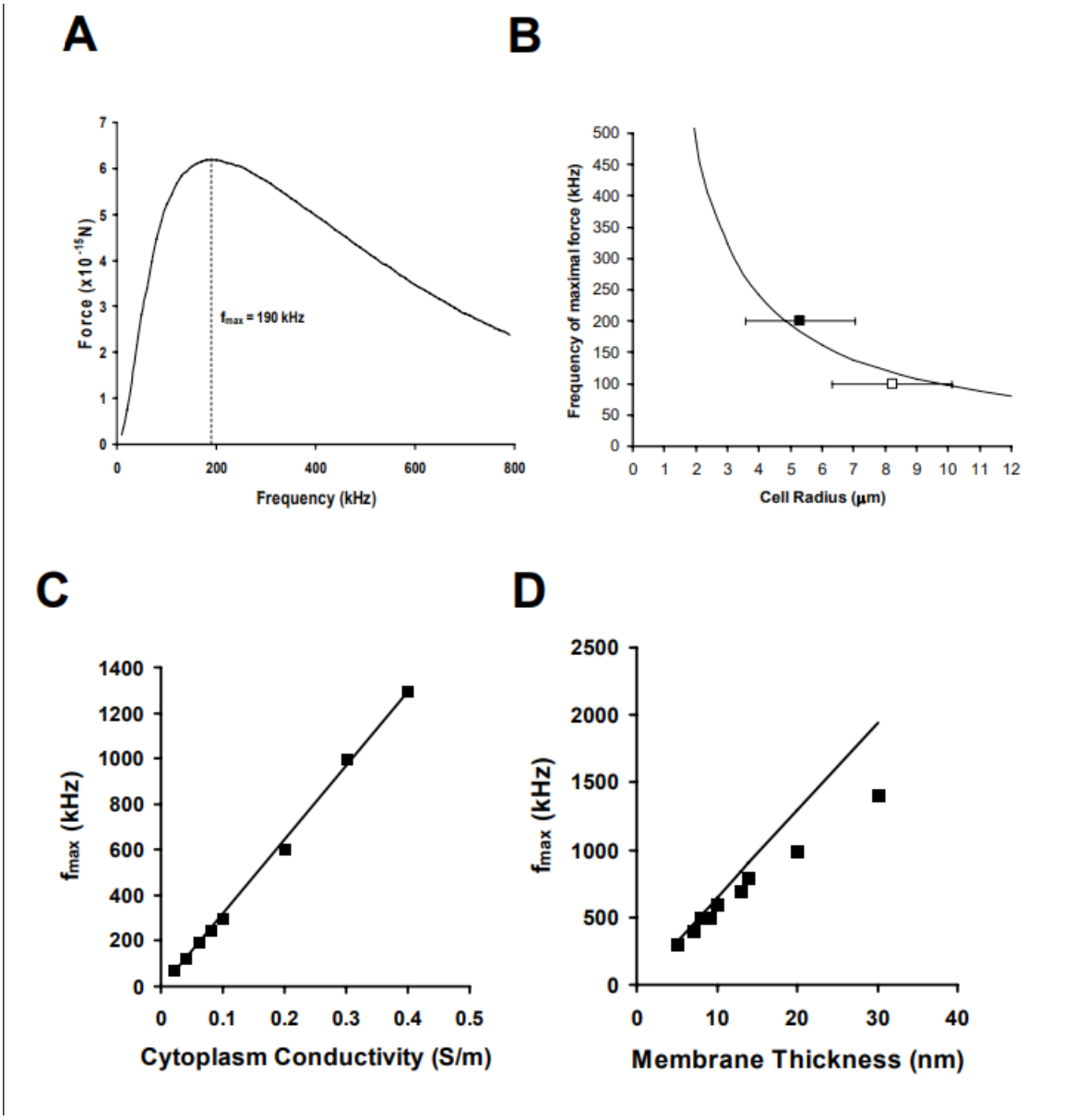


*Figure 4: Calculation of the force on a 1 μm polarizable spherical particle in a dividing cell as function of cell radius, membrane thickness and cytoplasm conductivity*

After a few years, a system called Optune was developed by Novocure as a treatment option for recurrent GBM in adults. The medical device was approved by the FDA in 2011. The system contains several transducer arrays (electrodes), that are applied directly to the shaved scalp and a battery pack that can be carried in a bag. Patients are required to wear the device for a minimum of 18 hours daily, for as long as the treatment continues. With the development of Optune, more clinical studies begun to launch in the following years further exploring the potential of TTFields against GBM, non-small lung cancer brain metastases and accompanied with conventional treatment option such as chemo-radiation with TMZ. A very important trial was completed in 2014 where 695 patients were selected to undergo treatment with TTFs along with chemo-radiation. Patients treated with Optune and TMZ

(210 patients) had statistically significant improvements in median progression-free survival (7.1 vs 4.0 months, p = 0.001) and overall survival (19.6 vs 16.6 months, p = 0.034) compared with patients treated with TMZ alone (105 patients). These data support the use of TTFs combined with TMZ in the treatment of newly diagnosed GBM.

However, as mentioned in the introduction of this dissertation, the majority if not the entirety, of the studies that put TTFields under the microscope, are more or less similar in the way that the TTFields are delivered to the patient or the cell culture. The means of delivery consists of a set of insulated electrodes that are either dipped into the culture media in the case of in vitro experiments, or directly placed on the scalp or region of interest for the in vivo studies.

One of the few exceptions that are available in the literature, is a study that took place in 2021 where the use of uninsulated electrodes as a means of delivery for low-intensity, intermediate frequency alternating electric fields was carried out by researchers at the university of Alexandria. Their research lied on the fact that conductive electrodes have the advantage of conducting electrical energy more rapidly compared to insulated electrodes, which could potentially greatly minimize the exposure time of TTFields. The experimental setup involved applying TTFs through a pair of plate silver/silver chloride electrodes (Fig.5) connected to a function generator. The researchers measured the electric field, current, and temperature during the exposure. For the purpose of the experiments, human lung cancer cells (A549 cell line) were cultured and exposed to TTFs for different durations.

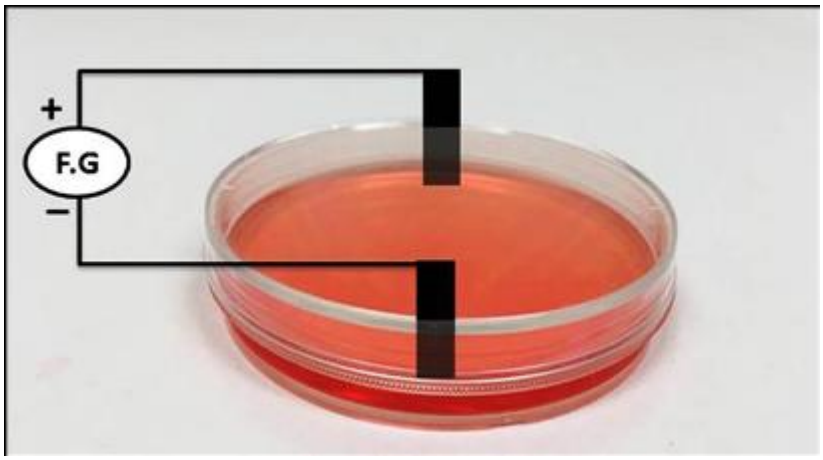


*Figure 5: Schematic diagram of the TTFs-uninsulated electrodes exposure system setup.*

The effects of TTFs delivered through conductive electrodes were assessed through various assays. The colony-forming assay was used to evaluate the proliferation capacity of A549 cells, and the results demonstrated that TTFs inhibited cell proliferation in a time-dependent manner (Fig.6). Annexin V/propidium iodide staining was employed to assess cell death, and flow cytometry analysis revealed the induction of cell death by TTFs-conductive electrodes.

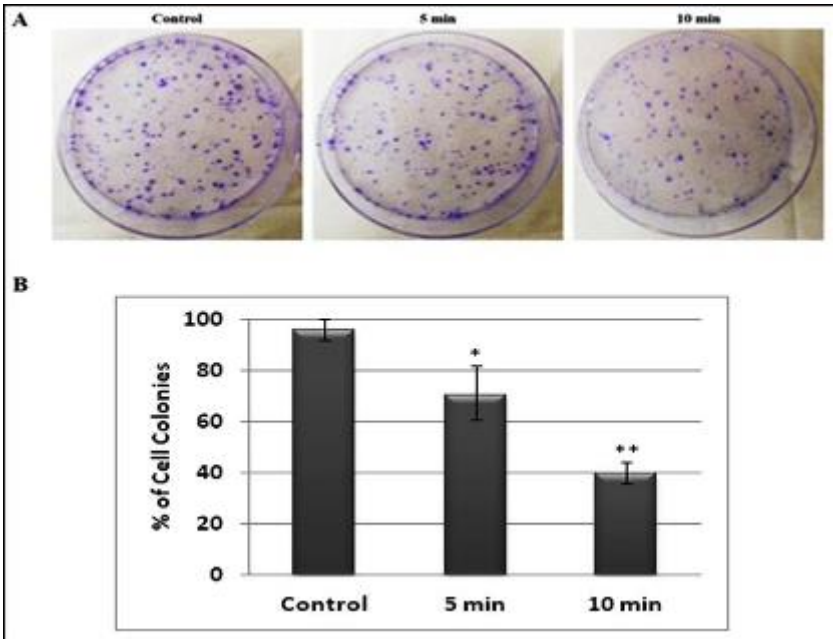


*Figure 6: (A) Colony formation results in A549 cultured cells after TTFields exposure for different durations (0, 5, and 10 min). (B) Colony count for the colony formation assay.*

The researchers also examined oxidative stress markers by measuring the total antioxidant capacity and glutathione levels in the cell lysates after TTFs exposure. The results showed that no significant changes in the total antioxidant capacity were observed after TTF exposure as compared to control cells. Moreover, regarding the temperature measurements, results showed a very slight increase of only 0.7 ± 0.1 ℃ after 40 minutes of exposure.

Overall, the findings suggested that TTFs delivered through conductive electrodes effectively inhibited cell proliferation and induced cell death in lung cancer cells. Further investigation was needed to better understand the potential of this approach for treating lung and other cancer types and optimize its therapeutic effects while minimizing side effects.

This dissertation aims to replicate the results from the experiments of the aforementioned study on a different cell type, while implementing methodologies and knowledge gathered from past studies and literature.

# Chapter 3: Research methodology

For the purpose of the alternating electric fields delivery, the methodologies that were implemented will be analysed below. Any adjustments made to the original experimental process will also be addressed in the discussion section. The effect of low-intensity, intermediate-frequency alternating electric fields exposure was examined on SF-268 human brain glioblastoma/ astrocytoma cell lines.

## In Vitro Experimental Set Up.

At first, cultures were grown in standard culture flasks. Out of approximately 2.8 million cells in 1mL, 9μL were plated onto 6cm dishes, so ~30.000 cells in each of the 6 plates (3 positive controls, 3 negative controls). After proper handling, the cell cultures were incubated for a period of 24hours before treatment in a 5% CO2 humidified incubator held at 36°C. The TTFields were generated by a LAG-53 function generator that was connected in parallel with an oscilloscope in order to monitor the voltage and frequency. Afterwards, a dremel was used to carve proper holes/slits in the lids of two 6cm dishes, so that a pair of 30-mm-long stainless-steel electrodes could be inserted into the slits for each experiment. Before each experiment took place, the lids that were modified, were washed with 70% ethanol and disinfected by 5 minutes of UV exposure. The same process was followed for the stainless-steel electrodes both before the start of the experiment and at the end. The entirety of the experimental process took place in a biosafety cabinet. The experimental setup and circuit diagram can be seen in figure 7.

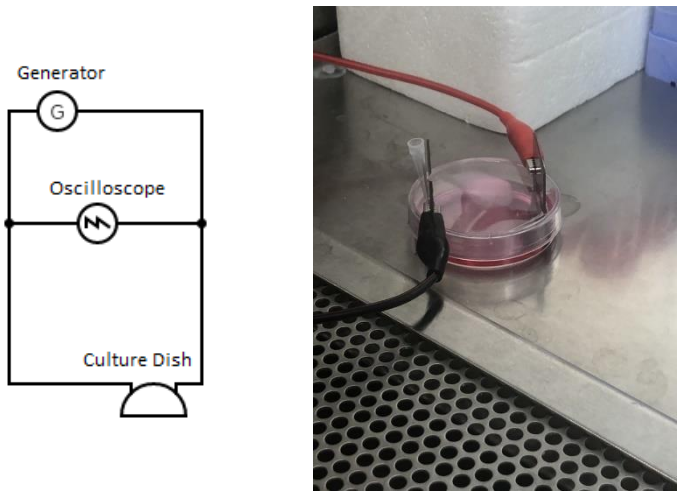


*Figure 7: On the left: Circuit diagram of the TTFields setup using a generator, an oscilloscope and a pair of stainless-steel electrodes. On the right: a picture of the setup during the experiment (a sterile pipette tip is used to prevent the stainless-steel piece from moving).*

Regarding the TTFields, a frequency of 200kHz (sine wave) was used for the experiments on the cell cultures. The voltage was set at 4V Peak-Peak so as to have a field of 1V/cm acting between the electrodes. This was calculated based on the distance (4cm) between the two electrodes that were dipped into the culture media (4mL). The initial exposure for the cultured cells was 15 minutes for the positive control and 15 minutes for the negative control for which the setup was exactly the same, but the function generator was turned off, so no current was passing through the media and a new set of stainless-steel pieces of the exact same size was used. Afterwards, the cell cultures were transferred to the incubator for a rest period of 4 days.

The first adjustment that was made, took under consideration the fact that the direction of the electric field plays a very important role. In order to account for the multiple orientations of the mitotic spindles of each cell, it was decided that a secondary acting field with a different orientation was necessary. For that matter, the exposure time of 15 minutes was divided so as to have a time interval of 7,5 minutes with the initial setup and another 7.5 minutes where the orientation of the field was perpendicular to the initial one. This was performed by rotating only the bottom part of each plate (so the lid remained in the initial position) by approximately 90 degrees after the first 7.5 minutes. The rest of the experimental parameters remained the same.

Then, the next adjustment had to do with the resting period of the treated cells. After the results from the initial experiments that had a resting period of 4 days, it was decided that a different time window between the TTFs exposure and the quantitative assay should also be considered. Thus, it was decided that the resting period of the treated cells should change to 24 hours.

Finally, for the last sets of experiments, it was decided that after growing the cell cultures, the plating should be performed in 6 well culture dishes that had cover slips placed in them. The glass cover slips would then be transferred to the modified 6 cm culture dishes for the purpose of the experiment and the colorimetric assay would also take place in different 6- well culture dishes.

## Colorimetric assay to define cell viability.

In order to assess the results of each set of experiments, MTT assay was performed. The MTT assay is a colorimetric method used to evaluate the metabolic activity of cells. It relies on NAD(P)H-dependent cellular oxidoreductase enzymes that can indicate the number of viable cells in specific conditions. These enzymes have the ability to convert the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into an insoluble purple-colored substance called formazan. Thus, the MTT assay measures cell metabolic activity by assessing the reduction of MTT facilitated by NAD(P)H-dependent enzymes and provides a reliable indicator for cell viability. 1:20 MTT (Thiazolyl Blue Tetrazolium Bromide)

was added to the culture dishes and the cells were transferred to the incubator for a waiting period of 2 hours. The culture media is then removed and 1mL of DMSO (Dimethyl Sulfoxide) is added. Finally, a 1:4 dilution with DMSO is performed and optical density is measured at 550nm. Measured values are then indicative of cell viability, so control cultures were compared to treated cultures and all measurements were normalized against the values from control cultures.

# Chapter 4: Research findings / results

The study investigated the impact of TTFs treatment, using conductive electrodes, on the growth of brain glioblastoma cells. The cells were subjected to TTFields for a duration of 15 minutes in total, followed by fixation and staining. The results obtained from the MTT assay are presented below.

## Preliminary experiments

The results for the initial experimental process were extremely ambiguous. The optical density of treated cells versus the control cultures, showed insignificant differences between the two and in one case the treated cells showed an optical density that was slightly higher than that of the control cells. It was quickly decided that an adjustment to the delivery of the TTFields was necessary.

## Adjusting the exposure time and resting period of SF-268 cells

Figure 8 represents the results of the altered delivery method for delivering alternating electric fields, where after exposing the cells to the TTFields for 7,5 minutes, the bottom part of the culture dish was rotated by 90 degrees in order for the field orientation to be perpendicular to the initial one for the rest 7.5 minutes. In the first two sets of replicates, there is a significant difference between the control and treated cultures, of approximately 15% of viable cells. However, in the 3rd and 4th set, values from optical density measurements are quite indistinguishable. Evidently, data from this specific set of replicates does not support the idea that an inhibitory effect is taking place and sure enough, the criteria for statistical significance $p > 0.05$ (student's t-test) aren't met.

Figure 9 is a representation of the endpoint of the experimental process, where the MTT assay has been completed and the optical measurement of each sample is pending. Moreover, at this point in the study, the resting period of cells has also been modified from 4 days to 24 hours. In this picture, there is a visible difference in the color of the untreated (bottom plates) and treated (top) cultures. Despite the case of the second (from the left) set of replicates, the rest of the three plates with untreated cell cultures appear to have a darker shade of purple in relation to their respective positive controls. This difference in color translates to different concentrations of formazan in each culture dish. Thus, based on the principle of the MTT assay, higher concentration of formazan can be related to higher metabolic activity by the cells, hence it can be deduced that cell viability was higher in untreated cultures.

Nevertheless, it must be mentioned that while in this particular experiment the difference in cell numbers between TTF treated and untreated cell cultures is visible with naked eye, due to the colour of the final product, that was not the case for the majority of the experiments. Figure 10 shows a more common case for the MTT assay where the difference in colour between negative and positive control (top: negative, bottom: positive, for reference) isn't

as clear.

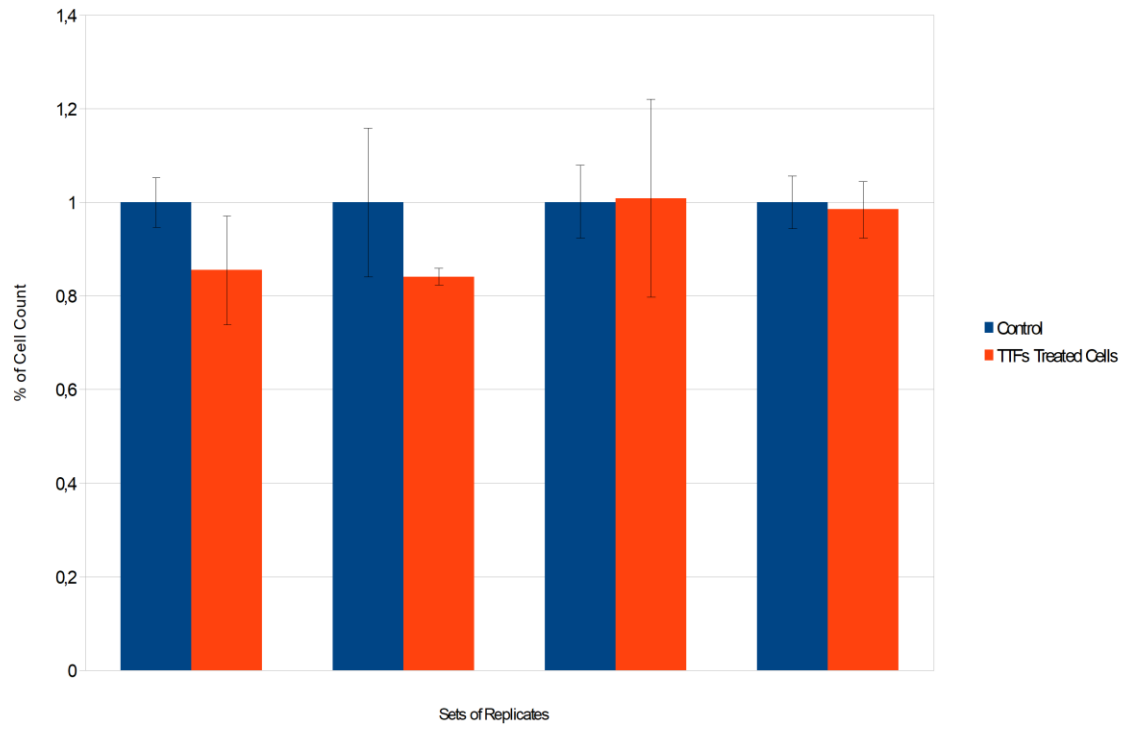


*Figure 8: Optical density results after the adjustment of the delivery method of TTFields. Cell counts from control cultures (blue) are used for normalization, so their values are set to 1, whereas treated cultures (red) had their values normalized in respect to the control values.*

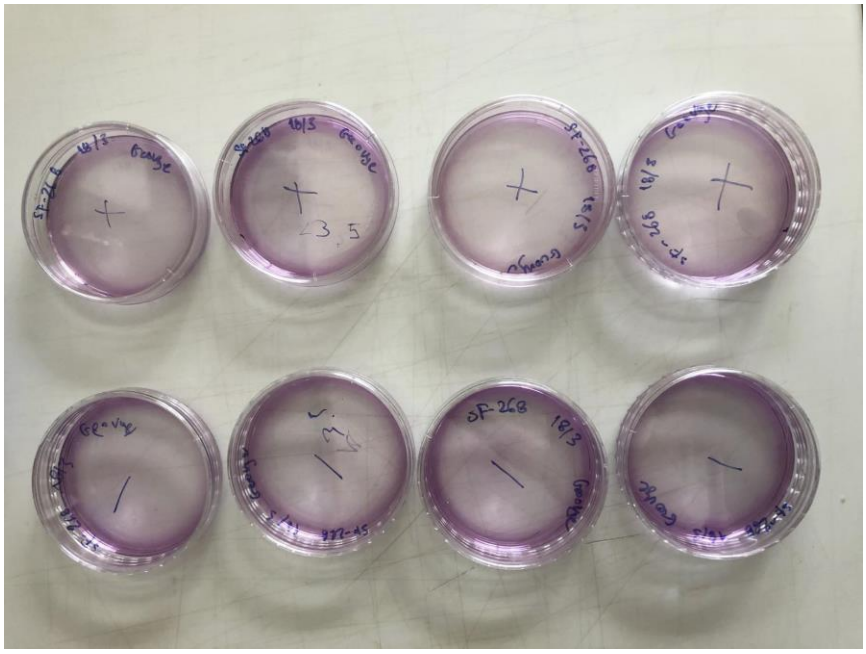

*Figure 9: An image taken after the 2-hours waiting time of the MTT assay and the addition of 1mL of DMSO. Top: 4 positive control plates (TTF treated cells). Bottom: 4 negative control plates (untreated cells).*

*Figure 10: An image that represents the typical final products after the experiment and the MTT assay. Top: Untreated cultures, bottom: treated cultures.*

## Plating cells on glass cover slips

Figure 11 shows the endpoint of the MTT assay just before the addition of 1mL of DMSO. The purple spots that can be seen on top the cover slips represent the stained cell cultures. As discussed in the research methodology section, both the experiment and the colorimetric assay, took place in different plates than the one used for the initial plating. That is the reason for the purple spots being present only on top of the cover slips and not around them. For reference, the three plates at the top are untreated cell cultures and the three plates at the bottom are cells that have been exposed to tumour treating fields. In this particular image a clear difference in the number of stained cultures can be seen in the middle and especially in the far right set of plates. In contrast the first set of plates is rather ambiguous in respect to which plate has the higher cell count and the optical density measurements are required.

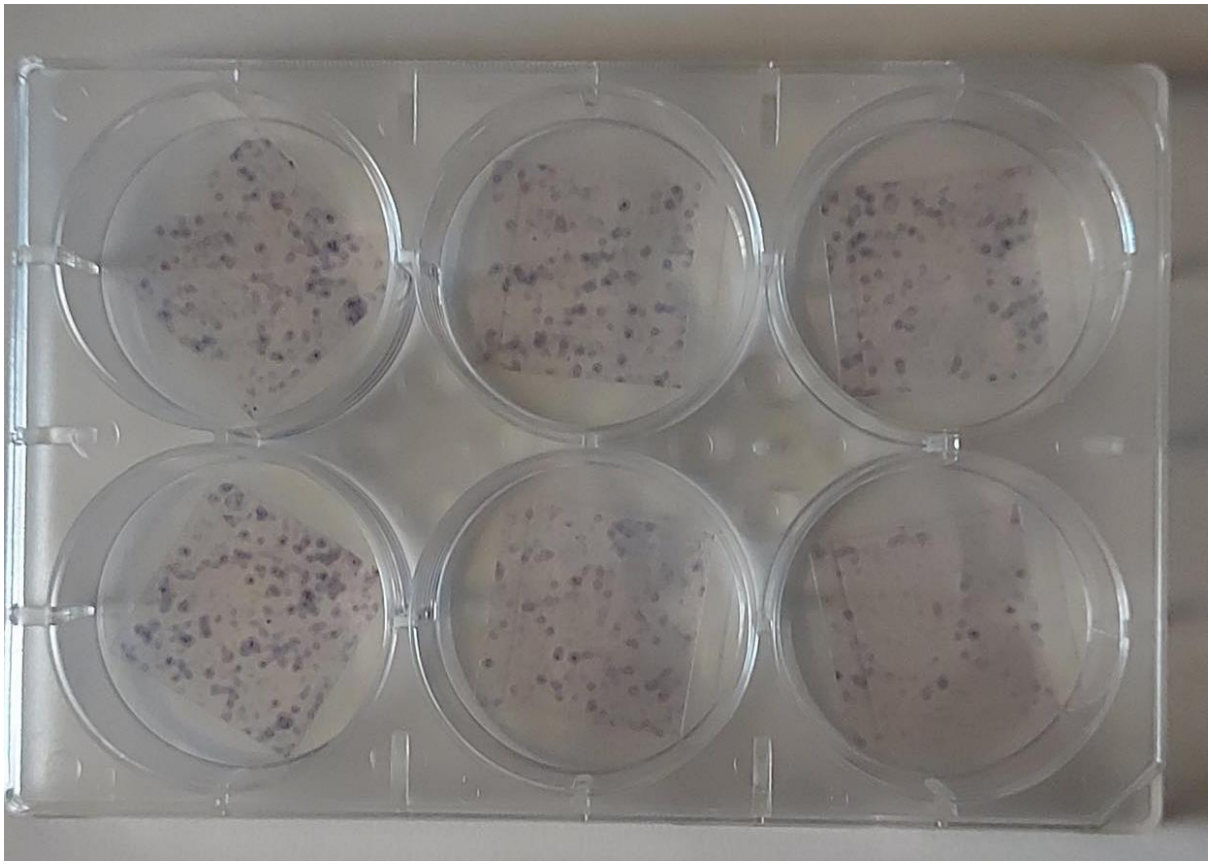

*Figure 11: A 6-well plate that contains stained cultures of SF-268 cell cultures on top of glass cover slips. Top: 3 plates of untreated cells, bottom: 3 plates of treated cells.*

The results from experiments where glass cover slips were used for the plating of SF-268 cells, are depicted in figure 12. This graph, along with figure 8 constitutes to the total number of viable cells that were measured based on the principle of the MTT assay. Each set of replicates represents a sole experiment that tested the efficiency of tumor treating fields on disrupting tumour growth. In each experiment, 3 control cultures that are exposed to TTFields served as positive control and 3 untreated cell cultures (through shutting down the function generator) served as negative control. In total, 12 plates of SF-168 cultures were treated with alternating electric fields for a total of 15 minutes. The results showcase a small but significant difference in viability in relation to the 12 plates of untreated cell cultures. Specifically, a median difference of 16.2% ± 6.5 in viable cells was found between exposed and unexposed cultures ($p<0.05$, student's t-test).

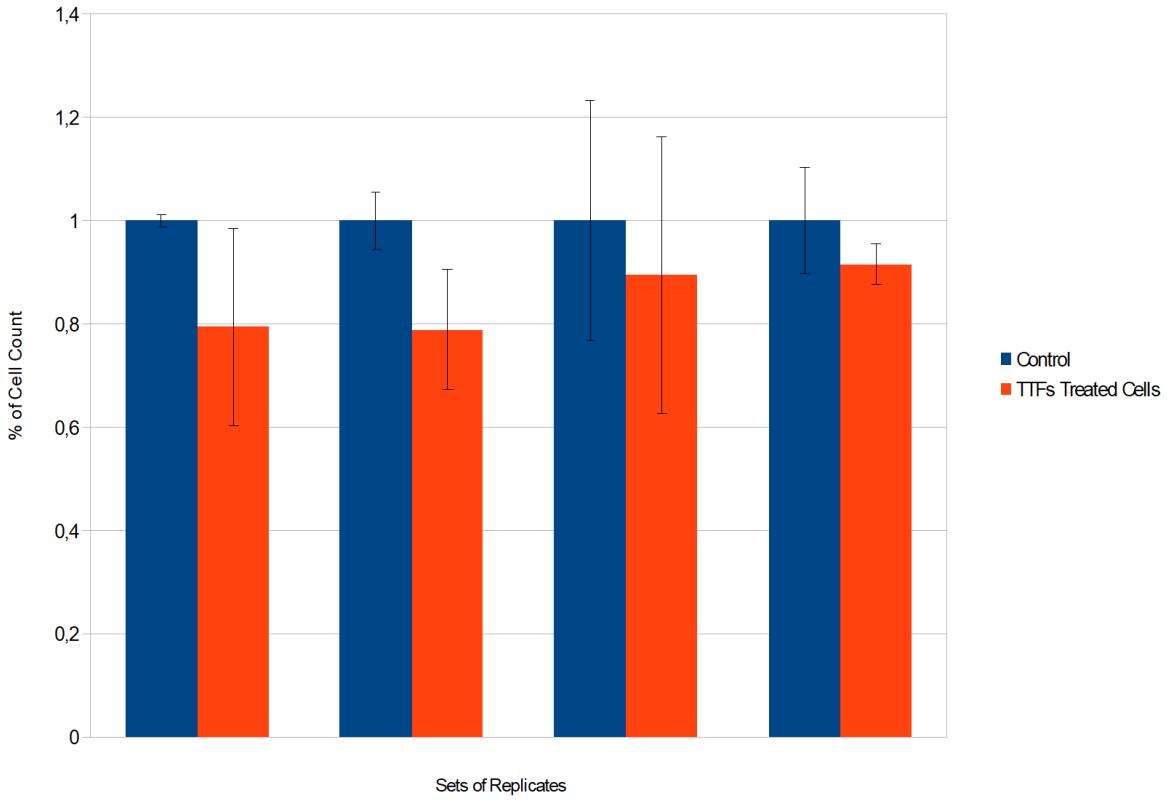


*Figure 12: Optical density results the MTT assay after using a different plating method for the cell cultures.*

# Chapter 5: Discussion and analysis of findings

The present study aimed to investigate the impact of low-intensity, intermediate-frequency alternating electric fields (TTFields) on SF-268 human brain glioblastoma/astrocytoma cell lines using conductive electrodes. The research methodology involved an in vitro experimental setup where the cells were exposed to TTFields for a total duration of 15 minutes. The results obtained from the MTT assay were analyzed to evaluate cell viability and metabolic activity.

The initial experimental process yielded ambiguous results, with insignificant differences in optical density observed between the treated and control cultures. In some cases, the treated cells even showed a slightly higher optical density than the control cells. These findings indicated that the delivery method of TTFields needed adjustments in order to gain meaningful insight to the true effect that was taking place.

At the time of the first experiments, the standardization of the experimental process was still in progress. Numerous factors that could potentially hinder the effective delivery of TTFields, were still being investigated. Namely the output voltage of the function generator, was dropping to less than 1V/cm after each set of replicates, which ultimately affected the validity of the quantitative results. After only two repetitions, it was decided that the experimental process should be more strictly monitored so as to have the exact same conditions for each set of replicates. To ensure this, at the end of each experiment, the function generator was turned off for several minutes, before another set of replicates was exposed to TTFields. This seemed to have fixed the voltage dropping issue, however results from optical measurements were still unclear.

To address this, an adjustment was made in the delivery method and exposure time of TTFields. Results from the aforementioned studies, emphasized the significance of the orientation of the electric field in respect to the axis of the mitotic spindle of dividing cells. Thus, it was decided that in order to enhance the potential effect of the tumour treating fields, the random orientation of the mitotic spindle of each cell needed to be accounted for. For that matter, it was decided that the cells would be exposed to TTFields for 7.5 minutes with the initial field orientation, followed by another 7.5 minutes of exposure with a perpendicular field orientation. In this way, a larger population of cells would be affected by the acting field. This altered delivery method showed the first promising results (Figures 8 & 9).

The comparison between the initial experimental process and the adjusted exposure time sheds light on the importance of optimizing the delivery method of TTFields. The inconsistent results obtained initially, highlight the need for fine-tuning the parameters to ensure consistent and reproducible outcomes. While the adjusted exposure time method showed initial promise, the lack of sustained significant differences suggests that additional adjustments or further investigation was required to enhance the desired inhibitory effect. This aligns with previous studies that have emphasized the importance of optimizing

TTFields parameters like field intensity, frequency or means of delivery, in order to maximize their potential in disrupting tumour growth.

Even though subsequent experiments had mixed results and statistical significance was not achieved, the experimental process was beginning to get standardized.

Further analysis involved the MTT assay, which provided insight into the metabolic activity of the cells. In some cases, the colorimetric assay showed a visible difference in color between the untreated and treated cultures. The untreated cultures exhibited a darker shade of purple, indicating higher metabolic activity and thus cell viability. These results are in line with the well-established principle that the formation of formazan crystals, resulting from the conversion of MTT by mitochondrial enzymes, correlates with viable cell numbers. However, it was noted that the difference in colour between treated and untreated cultures was not always visually apparent, emphasizing the need for quantitative measurements using the mass spectrophotometer.

At this point, it was hypothesized that due to the extended, at the time, resting period of cells, the potential inhibitory effect was not discernible. The 4 days between the exposure to alternating electric fields and the quantitative assay, was a long-enough period for each culture to reach a very high confluency, completely suppressing the desired inhibitory effect. For that reason, the resting period was reduced to 24 hours. This would theoretically improve the chances for detecting any change in viable cell counts between positive and negative controls.

Another adjustment in the research methodology involved plating the cells on glass cover slips, which were then transferred to new culture dishes both for the purpose of the experiment and colorimetric assay. Up until this time, the plating of the cells took place in standard 6cm culture dishes and when it was time for an experiment, the lids of the plates were replaced by the modified lids in order for the electrodes to be inserted in the culture media and for the TTFields to be delivered. However, due to inconsistent results in optical density measurements during the colorimetric assay, it was thought that there is a urgent need for a more strategized approach. When cell plating takes place, it is common practice that the plate is shaken in order for the liquid media that contains the cells to be spread evenly across the surface area of the plate. However, it became clear that due to this practice and the physical aspects of the stainless-steel electrodes and the plate itself, the TTFields were only focused on a specific region of the cell culture located in the center of the culture dish. Any cells that are located in the perimeter of the plate and especially behind the two electrodes, would be completely unaffected by the TTFields due to their distribution (Fig.1 for reference). In that way, when measuring the cell viability of treated cells, the unaffected cells were also included which in return added a significant deviation in the true optical measurement results between treated and untreated cell cultures. To address this issue, it was decided that the cell plating would take place in culture dishes that had class cover slips placed in them. The plating process would remain the same and the cells would spread and grow evenly across the bottom surface of the plate. However, during the experimental process, the cover slips would be removed from their original plates and

placed in new plates for the purpose of treatment with TTFields. At the end of each experiment, the cover slips would be placed back in their original plates. The same protocol was followed for the colorimetric assay as well. After the addition of the MTT and culture staining, the cover slips would be once again removed from their original plates and transferred to new plates for the rest of the process. In this way only cells that were plated on top of the cover slips were being measured and subsequently only actually affected cells were being accounted for. Figure 11 shows the setup just before the optical density measurements; the stained cell cultures are located on top of the glass cover slips which have been removed from their original plates.

As demonstrated in Figure 11, this new setup allowed for a clear difference in the number of stained cultures in some cases. Many sets of replicates from experiments using the final setup, suggested varying cell viability between treated and untreated cell cultures. However, in some cases, the difference in color between treated and untreated cell cultures was not visible with naked eye. Hence, optical density measurements were required to determine viability accurately.

The results obtained from experiments, shown in Figure 12, where cells were plated on glass cover slips showed a small but significant difference in viability compared to untreated cultures. The median difference in viable cells was 16.2% ± 6.5, indicating that TTFields had a measurable and statistically significant, inhibitory effect on cell growth. These findings align with previous research studies that have demonstrated the potential of TTFields as a therapeutic approach for glioblastoma treatment. More importantly, this study highlights the fact that using conductive electrodes for delivering low intensity, intermediate frequency, alternating electric fields can produce an inhibitory effect while greatly minimizing the exposure time to less than 30 minutes.

The comparison between the results obtained using different experimental setups, such as the use of glass cover slips, further underscores the importance of optimizing the methodology. The observed differences from the colorimetric assay and cell counts acquired from optical density results, highlight the potential impact of subtle variations in experimental conditions on the outcomes. These findings reinforce the need for meticulous attention to detail and standardization in experimental procedures to ensure reliable and reproducible results.

# Chapter 6: Conclusion and recommendations

This study aimed to investigate the impact of low-intensity, intermediate-frequency alternating electric fields (TTFields) on SF-268 human brain glioblastoma/astrocytoma cell lines. Specifically, the goal was to reproduce experiments from a study where the research methodology involved an in vitro experimental setup, using conductive electrodes. The analysis of the research findings employed the MTT assay, which evaluated cell viability/ metabolic activity.

Overall, the research findings indicate that the efficacy of TTFields in inhibiting cell growth depends on various factors, including the delivery method, exposure time, resting period and the data acquisition. While the initial experimental process and the adjusted exposure time did not consistently yield significant results, the use of glass cover slips during plating, along with other adjustments in the resting period of cultures, showed promising outcomes. In specific, a 16.2% ± 6.5 difference in cell viability between treated and untreated cultures was found. This small but significant inhibitory effect in tumour growth was the product of 15 minutes of exposure to TTFields.

It is worth noting that this study has several limitations. The in vitro experimental setup may not fully capture the complexities and dynamics of tumour microenvironments in vivo. Thus, the experimental methods used in this study cannot be directly applied in clinical cases, especially when conductive electrodes are being employed. Moreover, the specific SF-268 cell line used in this study may have inherent characteristics that influence its response to TTFields. Additionally, the sample size and number of replicates could be expanded to enhance statistical power and reliability of the results.

In conclusion, the findings of this study contribute to the growing body of research on the application of TTFields for glioblastoma treatment. Further investigations are warranted to optimize the parameters of TTFields delivery and explore their potential clinical applications. Ultimately, the objective is to create therapies that are both effective and safe, aiming to enhance patient outcomes in the treatment of glioblastoma and other brain tumors.

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