



Establishment of In Vivo and In Vitro Setups to Study the Effects of Electric Field on Tumor Growth

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Establishment of In Vivo and In Vitro Setups to Study the Effects of Electric Field on Tumor Growth
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Cover: Picture shows the mouse injected with Glioma G1261 cells under anaesthesia

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*I dedicate this little effort of mine to my parents for
their never ending moral support, encouragement,
love and prayers.*

ABSTRACT

Glioblastoma Multiforme (GBM) is one of the most aggressive types of brain tumors claiming 18000 victims each year in USA. The median survival rate for patients is less than three months[7]. The severity of the disease has attracted the researchers' interest for a long time. The current treatment modalities for cancer include radiotherapy, chemotherapy and surgery. These treatments are palliative in nature and focus more on increasing the life span of the patient, rather than curing the disease.

Recently a group of scientist, Yoram Palti, of the technion Israel institute of technology, and his colleagues have introduced a unique and new approach for the treatment of cancer. They demonstrated through experiments (in vitro and in vivo) that the low intensity 1- 2 V/cm fields of intermediate frequency can inhibit tumor growth [1, 6, 8]. According to Palti's research, the intermediate frequency fields exert dielectrophoretic forces on the cell organelles and ions inside the dividing cells. The right intensity and direction of this force can slow down the division process and destroy dividing cells. On the other hand, these fields are passive to quiescent cells [1]. The small clinical trial was also conducted by the same group and their results show the tendency of the fields to prolong the mean survival time of the patients with Glioblastoma Multiforme [1]. However there are not many published studies to confirm the theory underlying the inhibiting mechanism.

The aim of this master thesis was to develop in vivo and in vitro setups to run the experiments cells in culture and a mouse model to verify the inhibiting effects of the fields and to gain an insight into the mechanism of interaction of fields with cells. The setups developed provide experiment design and protocols, the optimum initial values of the cells, software, equipment, electrode design and optimum condition for carrying out studies on murine GL261 cell lining in vitro and in a C57BL/6 mouse model in vivo. The results obtained show the tendency of the field to inhibit tumor growth. However, the number of experiments conducted is not enough statistically to conclude if there are effects of the intermediate frequency fields on tumor cells.

Keywords: Glioblastoma, Tumor treating fields, intermediate frequency, cancer

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ABBREVIATIONS

GBM	G lioblastoma M ultiforme
DMEM	D ulbecco's M odified E agle M edium
FBS	F etal B ovine S erum
TTfields	T umor T reating F ields
PBS	P hosphate B uffered S aline
GFP	G reen F luorescence P rotein

1. INTRODUCTION

The term tumor is defined as an abnormal mass of tissues and can exist in benign or malignant form. The malignant tumors are called cancerous because they can invade the surrounding tissues and destroy them. On the other hand, benign tumors might grow in size but they do not spread in the body, which simply means they do not cause cancer. Thus the term 'cancer' is assigned to the group of diseases in which abnormal cells divide in uncontrolled manner and are able to invade other tissues. There are different types of cancers and they are named according to the type of organ or tissue they occur in.

Brain tumors include all types of tumors in cranium or central spinal canal. Tumors that begin in the brain are called primary brain tumors and are named according to the type of cells, or part of brain in which they originate. The most common type of primary brain tumors are 'gliomas' because they originate from glial cells. The secondary brain tumors are the result of cancer cells originating from other parts of the body and spread into brains. The World Health Organization categorizes primary brain tumors by cell origin and histological appearance; from the least aggressive (benign) to most aggressive (malignant). The World Health Organization assigned a grade to gliomas, grade I (least malignant) to grade IV (most malignant).

Glioblastoma Multiforme

The occurrence of primary brain tumors has increased dramatically from the last few decades. Glioblastoma multiforme (GBM), a grade IV glioma is one of the most common and malignant primary brain tumor. Despite advances in standard therapy including radiation and chemotherapy, the prognosis for patients with GBM remains poor. Symptoms of GBM depend on size, rate of growth and location of the tumor [7]. According to National Cancer Institute statistics 13,070 death cases were reported in USA due to brain tumors in 2008 alone. In adults, glioblastoma is the most frequent malignant brain tumor [9]. Although GBM occurs in the patients of all ages, the incidence is highest in the elderly and it is also more common in men. Ionizing radiation is the most effective therapy used for the treatment of glioblastoma multiforme. Patients have median survival rate of less than 12 months because of resistance to radiation therapy and other treatments [10].

Intermediate Frequency Fields as a Potential Cancer Treatment

Human body is an electrical system with every organ having its own set of electrical signals. The internal electric system of the body reacts differently with the externally applied electric field. Very low frequency fields in the range of 1 Hz to 1000 Hz have several biological effects on the body. Some of them are stimulants for excitable tissues, bone growth, fracture healing and nerve stimulation etc. The stimulatory effects tend to diminish very fast as the frequency is increased above 1000 Hz. The higher frequencies more than MHz have dominant thermal heating in the cells. The intermediate frequency range between 1 kHz to 1 MHz was believed to have no significant biological effect other than particle alignment and cell rotation. However for the first time in 2004 Yoram Palti, of the Technion Israel Institute

of technology, and his colleagues demonstrated that these fields can inhibit tumor growth. They have termed them as tumor treating fields [6].

Interaction of Intermediate Frequency Fields with Cells

Cells consist of ions, polar molecules, cell membrane and organelles. When a non uniform alternating electric field is applied to the ions and polar molecules, they move in the direction of the applied field; a process known as dielectrophoresis[11]. When the field is uniform, the ions and polar molecules only oscillate.

According to Palti's theory, when the electric potential of alternating direction (ac field) is applied across the living tissue, it generates an electric field in the tissue. The field lines formed have a specific distribution inside and around the cell. This distribution of field lines in the tissue depends upon several factors including, geometry of the cell, relative conductivities of the intracellular fluid, intercellular fluid, cell membrane, direction of the applied field, strength and frequency of the applied field etc. To estimate the distribution of field inside the cell, the dielectric properties of different system components are ignored, provided the applied field is of relatively low frequency. The various components of living tissue are modelled with relative impedance or conductivity. The intracellular and intercellular fluid is of low impedance as compared to the cell membrane which has higher impedance. Therefore, when the electric field is applied only a few field lines enter the cell membrane. These field lines enter the cell membrane from the side close to the electrodes applying the field. Inside the cell, the field lines are uniform and they leave from the opposite end of the cell [11-12]. The distribution inside the non dividing cell is shown in the figure below

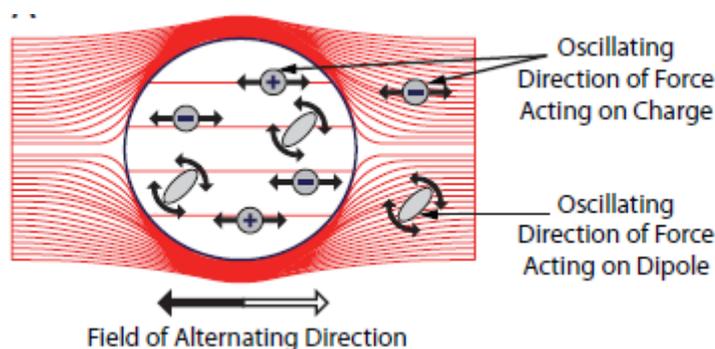


Figure 1: The field distribution in the quiescent cell
(Figure adapted from [1])

The alternating field inside the cell is uniform; therefore the net force on ions, organelles and polar molecules is zero. The only effect they experience is oscillations and particle alignment. In case of dividing cell, the effect of field is different. To understand the effect on division process, a brief overview of mitotic division is explained below. The process of mitosis is divided into six main stages. In the first stage interphase, the chromosomal DNA replicates. In the next stage prophase, the DNA forms into chromosomes. The nuclear membrane and nucleolus disappears and the centrioles move to the opposite poles of the cell. In metaphase, the spindle fibres align the chromosomes on the equatorial plate. In anaphase, the paired chromosomes are disassociated, the spindle fibre elongates and the sister chromosomes are moved to the poles of the cell. By the end of the anaphase both end of cells have complete organelles for separate cells. In telophase, the nuclear membrane is formed into two distinct

cell bodies, formed at opposite poles. In the last stage cytokinesis, the daughter cell splits apart. Furrow forms in the centre and the cell is pinched in two daughter cells [13]

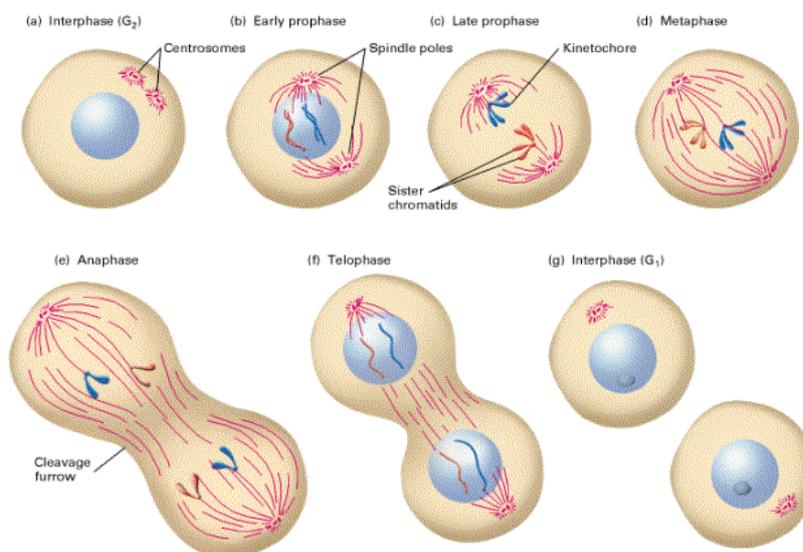


Figure 2: Steps in the Mitotic cell division [2]

In the beginning of cell division, the field inside the cell remains uniform. However after the mitotic spindle formation and in late anaphase and telophase when the cell can be more easily distinguishable in two daughter cells, the field distribution inside the cell is no longer uniform, as suggested by Palti. Rather the field is quite unique and different. The cell at this stage can be considered to be consisting of two sub cells connected by Cytoplasm Bridge. The field lines enter the cell from the first sub cell through the part of the membrane close to the current source (electrode) but do not leave directly from the other sub cell. The field lines converge on the neck connecting the two sub cells. The density of the field increases at the neck, and then the field lines enter the other sub cell and diverge again like the first sub cell. Finally the field lines depart from the second sub cell through the cell membrane close to the current sink (the second electrode)[12, 14]

The distribution of the field line in case of non uniform field in dividing cell is shown in the figure below

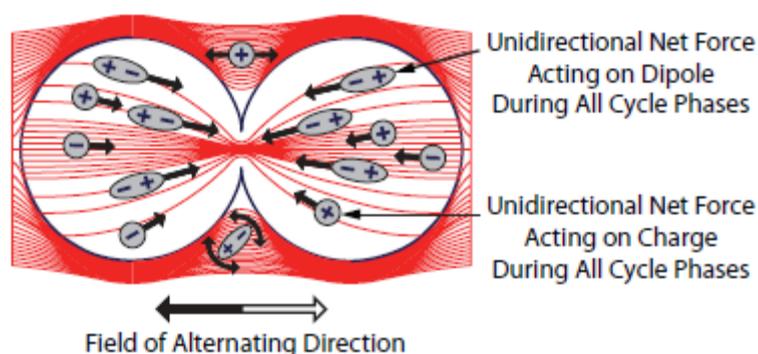


Figure 3: The Field distribution in the Dividing Cell. (Figure adapted from [1])

When the ions, polar molecules and organelles are placed under the converging or diverging field, the electric force acts on them and under dielectrophoresis forces, they are pushed in the direction of converging field. In case of dividing cells, the dielectrophoretic fields are directed towards the cytoplasm bridge connecting the two daughter cells. The intracellular organelles are forced towards the cytoplasm bridge. This increases the pressure in the bridge area and disturbs the division process of the cells.

The researchers suggests that this increased pressure on the cell membrane connecting the two daughter cells, affects the cell division process in the following two ways

- Slowing down the cell division
- Destroying the cell during cell division

In vitro studies done by the Yoram Palti and his colleagues show that the cells that usually take one hour to complete the mitosis were still in division phase after three hours as illustrated in the Figure 4 [6] . Most of the cells disintegrate or explode in the process of mitosis as shown in the figure below

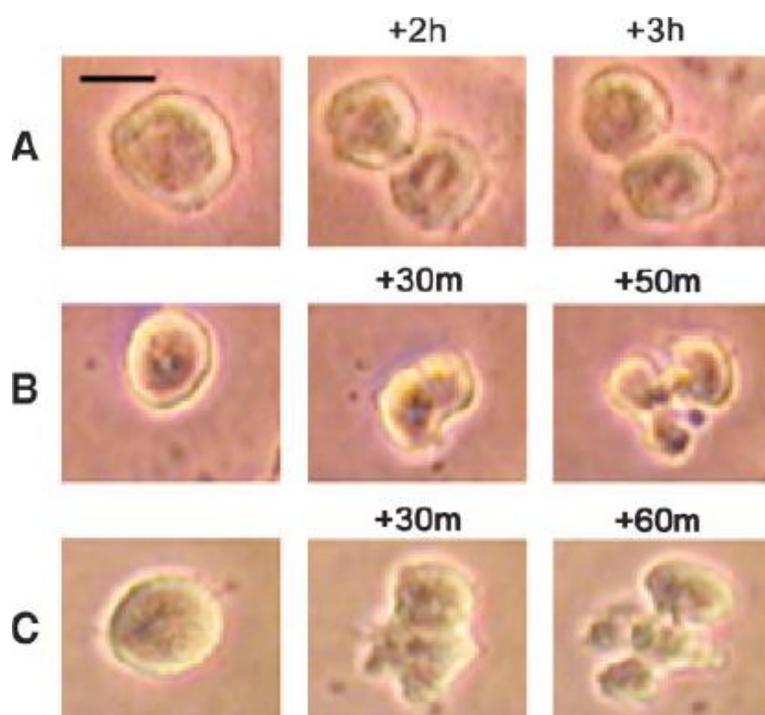


Figure 4: Time lapse Microphotography of tumor cells exposed to TTfields A) slowed down cell division process. B) and C) Cell disintegration during the division process (Figure Adapted from [6])

The field lines only affect the division process if the applied field is parallel to the axis of the bridge. If the fields are orthogonal to the cell division axis then they will have no effect on the cells

The aim of this master thesis is to develop in vivo and in vitro setup to run the experiments on, cell in culture and mice model to verify the inhibiting effect of the fields and to gain an insight of the mechanism of interaction of fields with cells. In the next section of

this thesis 'Material and Methods' is discussed. The Material and Methods is divided into three sections, first section describes system requirements, second section discusses the work on cell in culture on GI 261 cell lining and third section explains in vitro setup. After that results of in vitro and in vivo studies are presented in the report followed by the conclusion and future work

2. MATERIALS AND METHODS

2.1. System Description

2.1.1. System Components

The required setup should be capable of delivering 1-5 V per cm on tumor area at the frequencies of 100 kHz to 500 kHz. The setup is divided into three main components

1. Electrodes
2. TTfield generator
3. Tumor model

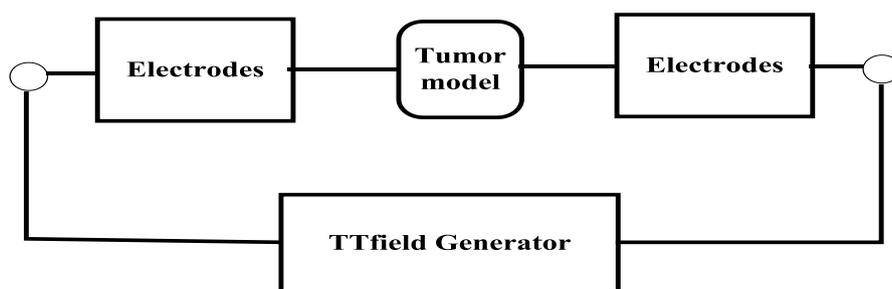


Figure 5: Block Diagram of the system to apply TTFields on Tumor model

Each component is explained below

2.1.1.1. Electrodes

The field is applied to the tumor model through the electrodes. The electrodes are in contact with the skin or tumor over a long duration of time. The conductive electrodes when exposed to the skin for a long time can cause some serious side effects like local damage to skin. Therefore, insulated electrodes are needed to avoid the metal contact with the living tissue.

The electrodes used in this study are Teflon coated copper wires, CU515 [15]. Teflon coating is used because it doesn't contaminate living tissues and has high breakdown voltage to avoid any risk of leakage current going into the tissues. The electrodes are delivering the field through capacitive coupling and are modelled with the capacitors.

2.1.1.2. TT Field generator

The TTfield generator consists of two parts as shown in the Figure 6

- Oscillator
- Amplifier

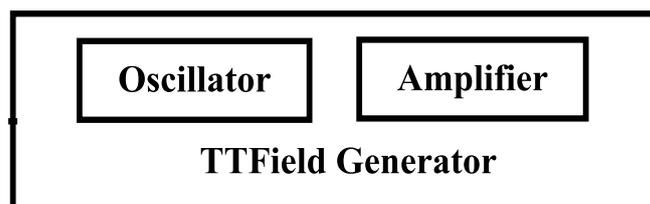


Figure 6: Block diagram of TTField generator

To generate the TTfields an oscillator capable of generating the frequency of 100 kHz to 500 kHz is required. The function generator FG1201B is used for this purpose.

The next step is to amplify the generated frequency to the level required to supply 1-5 V/cm at the tumor site. The wires used are Teflon coated and have very high impedance as compared to the impedance of the tumor or animal tissue. Therefore, most of the voltage applied will drop across the wires. To maintain the 1-5 V at the tumor the required voltage is approximately in the range of 250 to 350 volts.

The amplifiers commonly used and available in the market are in the range of 1 Hz to 1 kHz. The required range of the frequency is not commonly used in simple electronics; therefore it is not easily available. However the Piezo Amplifier A303 fulfils the requirement. The piezo amplifier is capable of generating ± 200 V (400 V ptp) at frequencies in the range 0 to 450 KHz [16]

2.1.1.3. Tumor Model

The tumor model, available in the lab and used in this thesis is murine Glioma GL 261 cell lining. The tumor is modelled with resistance because in this frequency range (100 kHz – 500kHz) the dielectric properties of animal tissue are negligible. The resistivity of the tumor at intermediate frequencies is taken as 0.43 S/m [17]. The approximated resistance of the tumor cells seen by the system is calculated for estimating the required voltage.

2.1.2. Circuit Representation of the System

The electric circuit of the problem with implanted wires, where the wires are in direct contact with the tumor cells is shown in the figure below.

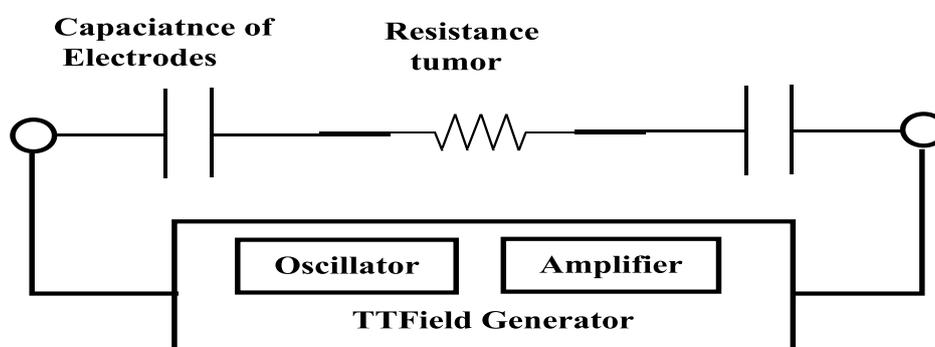


Figure 7: circuit representation of the system applying TT Fields on the tumour model

The values of each component in the circuit are calculated as follow

Impedance of the copper wires

The capacitance of the copper wires is calculated from the dielectric properties of the Teflon as follow

$$C = \epsilon_0 \epsilon_r \frac{A}{d}$$

Where d is the insulation thickness

A is the surface area of the wire

Epsilon r is the dielectric constant of Teflon and is 2.5

d = insulation thickness = 0.04 mm

$$A = 2\pi r h$$

Where r is the radius of the copper wire

h is the length of the copper wire exposed to the tumor = 10 mm

Diameter of the copper wire is 0.25 mm

Radius of the copper wire r is 0.25 mm/2 = 0.125 mm

Area is calculated as

$$A = 2\pi r h$$

$$A = 2 * \pi * 0.125 \text{ mm} * 10 \text{ mm}$$

$$A = 7.85 * 10^{-6} \text{ m}$$

Putting the value of area in equation 1

$$C = 2.5 * 8.85 * 10^{-12} * \frac{7.85 * 10^{-6}}{0.04 \text{ mm}}$$

$$C = 4.3 \text{ pF}$$

Impedance of the the copper wire X_c at the frequency 200 kHz is calculated as follow

$$X_c = \frac{1}{2\pi f C}$$

$$X_c = \frac{1}{2\pi * 200 \text{ K} * 4.3 \text{ p}}$$

$$X_c = 185 \text{ K } \Omega$$

Resistance of the tumor

Resistance of the tumor is estimated by using the resistivity of the tumor tissue.

$$R = \rho \frac{L}{A}$$

where L is the length of the wire exposed to the tumor, A is the area of the insulated wire calculated as follow

$$A = 2\pi r h$$

where r is the surface radius of the insulated wire, h is the length of the wire in contact with the tumor. The diameter of the wire including the insulation is 0.33 mm.

$$A = 2\pi * \frac{0.33 \text{ mm}}{2} * 10 \text{ mm}$$

$$A = 10.362 \text{ mm}^2$$

ρ is the resistivity of the tumor tissue.

$$\rho = \frac{1}{\sigma}$$

It is assumed that the area between the electrodes is filled with the tumor. σ is the conductivity of the tumor tissue and is given as 0.43 S/m [17]

Putting the values in the equation for resistance R

$$R = \frac{1}{0.43} * \frac{10 \text{ mm}}{10.362 \text{ mm}^2}$$

$$R = 2.5 \text{ K}\Omega$$

Voltage required at amplifier

The voltage required by the amplifier to generate 2 V/cm at the tumor is calculated as follows

Voltage drop at the tumor $V_{\text{tumor}} = 2 \text{ V}$

Total voltage required to generate 2V at tumor = V_{tot}

$$V_{\text{tot}} = \frac{\text{Total Impedance of the system}}{\text{Resistance of the tumor}} * V_{\text{tumor}}$$

The major voltage drop is at the insulation of the copper wires, therefore the total impedance is taken to be the impedance of the copper wires

$$V_{\text{tot}} = \frac{370}{2.5} * 2$$

$$V_{\text{tot}} = 300 \text{ Volts}$$

The voltage required at the amplifier is 300 Volts.

2.2. In Vitro study

This section describes the work done on cell culture to analyze the effect of intermediate frequency electric fields on murine Glioma GL 261 cell lining.

2.2.1. Experimental Setup

The method of choice is to apply the field on the cells in a way that they are directly in contact with the wire/electrode. The option of having field generated remotely and directed towards cells is more complicated to implement, because power consumption in this case is very high as a result of air being a good insulator. The wires are Teflon coated therefore do not contaminate the medium and can be directly in contact with it.

Many chambers, wells and dishes are available to culture the cells. However, the petri dishes of approximately 8 cm² size are chosen for this experiment. The main issue considered in choosing the culture plate is the ease of implantation of wires.

Setting up the Wires in the dish

In setting the wires in the culture dishes following few points are considered

- Contamination of cells
- Contamination of culture dishes while implanting wires
- Evaporation of medium

Contamination of cells is avoided by having copper wires insulated with Teflon, sterilized with 70% ethanol and making sure that no part of copper is exposed to the medium.

The culture dishes used are sterilized and are not recommended to be taken out of hood for implantation of wires. Therefore methods like soldering that cannot be done inside the clean hood are avoided. The transparent scotch tape was used to fix the wires inside the petri dishes and the dishes after implantation of the wires were covered with lids to avoid the contamination or evaporation of the medium.



Figure 8: Petri dish with wires attached to its bottom

Connecting the petri dishes with TTfield generator

The end of the wires were connected with TT field generator. The dishes are placed inside the 5% CO₂ humidified incubator to maintain a constant temperature of 37 degree celsius. The TTfield generator that is oscillator and amplifier were kept outside the incubator. The cables were passed in the incubator through the ventilation holes present on the sides of incubator.

Estimation of exposure period of cells to TTfields

The exposure time of cells was decided based on their doubling life (duration of cell division process). Theoretically cells are destroyed or affected when they are undergoing the division process. Therefore, the exposure time chosen should allow enough division cycles for TTfields to destroy the cells. The exposure time chosen was 72 hrs. The cells were exposed to the TTfields after 24 hrs of culturing them in petri dishes so that the cells have enough time to settle in the petri dish before their exposure to TTfields.

Estimation of initial number of cells

The initial number of cells was estimated on the basis that the cells are not too crowded in a given experiment duration. When the cells are highly confluent the division process is compromised and it might be slowed down. This slowing can occur because of their numbers. However it can introduce error in the results if regarded as the effect of TTfields.

The initial volume was estimated by culturing 10,000, 15,000 and 20,000 cells per cm² for 4 days and observing the cell numbers by the end of the duration. The chosen quantity for these experiments based on above observation was 15,000 cells per cm²

Preparation of the Culture Medium

The medium used for cell culturing was prepared as follow according to the instructions of the lab technician Ann Marie from the department of neurology in the University of Gothenburg

DMEM	88%
FBS	10%
PEST	1%
L- glutamine	1 %

Each culture dish was filled with 3 ml of the prepared medium.

Extraction of cells from flask

Following procedure was carried out under the supervision of Ann Marie. Cells were kept in the 75 cm² flasks and were extracted for use by the following protocol. The medium of the flask was discarded and the flask was washed with 3 ml of PBS. This is done to ensure the complete removal of the medium from the flask. The presence of any residual medium slows the extraction process. Then 3 ml of 0.25% EDTA trypsin was added in the flasks. Glioma cells adhere to the surface of the flask. The trypsin is the enzyme that is used to loosen the cells from the bottom of the flask. After adding the trypsin, flasks were kept in 5% CO₂ humidified incubator at 37 degree Celsius for 3 minutes. After this the flasks were stirred thoroughly to ensure complete removal of cells from the bottom of flask. Then 3000 µl of prepared culture medium was added to the flask to stop the reaction of trypsin with the cells. Long exposure of the cells to trypsin can damage the cells. The flask was then emptied in 15 ml test tubes and centrifuged for 4 min at 1000 rcf. This is done to get the cells settled at the

bottom of the tube in the pellet. The medium in the tube was drained away carefully so that the cells in the pellet are not lost.

The cells were then diluted according to the need. The cells were calculated manually with cell counting chamber and the required quantity was extracted for the experiment.

Cell Counting

Cells were counted manually with the cell counting chamber. In cell counting chamber the number of cells per unit volume is counted. The concentration of the cells is considered homogeneous; therefore cells in a small volume are counted and the rest is approximated to have same concentration. The cell counting chamber and its different components are shown in the figure below

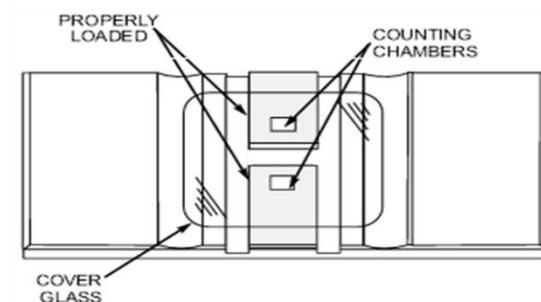


Figure 9: Cell counting chamber and its components

2.2.2. Experiment Setup with Petri Dishes

The experiments were conducted in two sets (n=2). There were two control groups and one treatment group. One control group had exactly same setting as with the treatment group but wires were not connected with the electric current. The other control group had standard settings that are petri dishes without any implantation of wires. This was done to compare the cell growth in actual and also to observe any adverse effects from the wires implantation

- CW Treatment group with electric fields applied
- W Control group with wires but no field applied
- NW Control group with standard dishes without any wires

The block diagram of the setup is shown below.

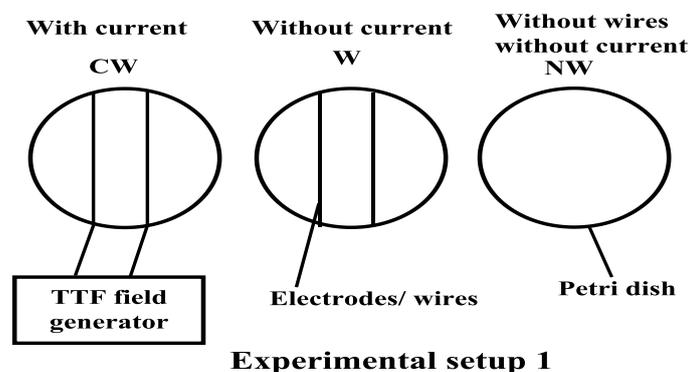


Figure 10: Block Diagram of Experiment setup with Petri Dishes

In each culture dish 15000 * 8 cells were cultured with 3 ml of the prepared culture medium. The dishes are kept for 24 hrs in 5 % CO₂ humidified incubator and after 24 hrs the TTfield generator was switched on. The cells were kept under observation for 72 hrs. The figure from the lab experiments is shown below



Figure 11: Experiment setup with Petri dishes

After the observation period the cells were extracted by the same protocol explained above and counted in the cell counting chamber.

Problems in experiment setup with petri dishes

The first problem encountered in the experiment was drying of the medium in the plates with the wires, that is two groups one of the control group and the treatment group. It was observed that medium was dried in these two groups within four days of experiment. A possible explanation for the evaporation of medium is the increase in air gap between the plates and their covers because of wire insertion. This problem was overcome by having the water filled container in the incubator that increased the humidity in the incubator, hence preventing the medium from drying.

The other problem was that the results of the experiments when repeated several times were not consistent with each other. The treatment group demonstrated the slowing down of division in some experiments but for some others, it was completely negligible. One of the possible reasons for the ineffectiveness of some experiments can be attributed to the fact that the wires were fixed in the center, but the cells are concentrated in the full petri dish and can settle anywhere. In some cases the cells between the wires might be more than the cells in other experiments. The inconsistency in the results can be related to the change in the amount of cells exposed to the TTfields. To overcome this problem the new setup was designed which is explained as follows.

2.2.3. Experiment Setup with Cover Slips

The main objective of this setup was to have cells only between the wires, so that the number of cells exposed to the fields in all groups remains constant. The new approach was to culture the cells on the cover slips, and place the cover slips between the wires in the petri dishes.

Culturing the cells on cover slips

To culture the known amount of cells on the cover slips, the round cover slips of approximately 1 cm radius, as shown in the figure below, were placed in one well of the 24 well plate.



Figure 12: Cover slips used in the experiments

The calculated amount of cells was then added to the well along with the 0.5 ml of the prepared medium. The size of the well is similar to the cover slip so that the cover slip covers the bottom of the well. The 24 well dish was kept in 5% CO₂ humidified incubator for 24 hrs so that the cell can settle on the cover slips.



Figure 13: The Cover slips in 24 well dish. The procedure to culture Cells on Cover slips

After the cells were cultured on the cover slips, they were removed from the wells with pointed forceps and were placed in petri dishes between the wires. The dishes were prepared with the same protocol as explained above in the previous setup with 3 ml of the prepared medium. The rest of the settings, the control group and treatment group were similar with the previous setup. The culture dishes were kept under the TTfields for 72 hrs in the incubator. The block diagram of the setup is shown below

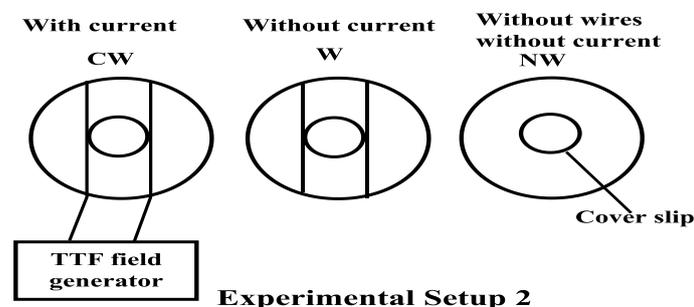


Figure 14: Experiment setup with cover slips in petri dishes

Counting of cells in Cover slips

The cells cultured on cover slips were few in numbers. Manual counting of cells with cell chamber requires extraction of the cells and concentrating them on the pellet as explained before. The cells on cover slips cannot be extracted through this process as the pellet was very small and hence cannot be separated from the medium. Therefore for counting purpose, the cover slips were mounted on microscope slides and the cells were counted directly under the microscope.

Mounting of the cover slips

The coverslips were first washed with PBS twice to wash away the medium. They were then kept in the formaldehyde solution for 5 mins for fixation. After that the cover slips were mounted on microscope slides using dapi as the mounting medium. The slide with cover slip mounted on it is shown in the figure below.



Figure 15: Cover slip mounted on the microscope slide

2.2.4. Results Acquisition

The cells are counted manually with stereo investigator and automatically with ScanR.

Stereo investigator

The workstation of the stereo microscope is shown in the figure below



Figure 16: Stereo Investigator workstation [3]

The cells were analyzed under the fluorescence blue light, a 5 flouro cube and the nucleus of each cell was marked. For counting the total number of cells on each cover slip, the boundary of the whole cover slip was detected and marked. Then the scans of each part of the cover slip were made serially and cells on each scan are counted as shown in the figure below.

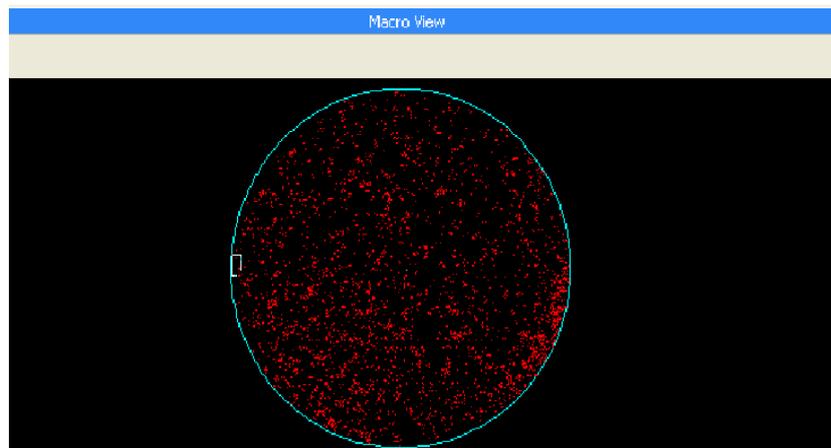


Figure 17: The macroview of the Cell counting through Stereo Microscope with each cell nuclei marked with red colour

The counting of cells in this way is very accurate, because on each scan, cells are counted manually and analyzed individually, therefore reducing the error rate. However the whole process is very time consuming; each cover slip takes 4 to 5 hours. Therefore the automated scanning and counting by ScanR is recommended and is explained below.

ScanR

ScanR is a modular microscope-based imaging platform designed for fully automated image and data analysis of biological samples [5] as shown in the figure below



Figure 18: ScanR Workstation [5]

ScanR is based on two main parts.

- Acquisition software
- Analysis software

ScanR Acquisition software

The images of the cover slips were taken through scanR screening station with the scanR acquisition software. In this software, the radius, thickness and other dimensions of the cover slip were fed into. The three points for detecting the boundaries of the coverslip were also entered. After adjusting the required focus (required lens), it was set for automatic scanning of the entire cover slip. The scans were saved in separate folders with the setting information file that can be read by the scanR analysis software.

ScanR Analysis software

ScanR Analysis Software is designed for the automated analysis of the images that are acquired through scanR screening station and scanR acquisition software.

The image in scanR analysis software is shown in the figure below

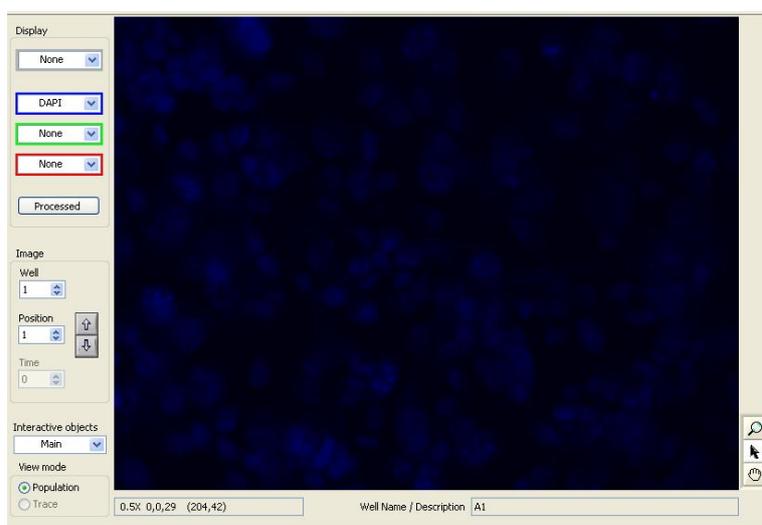


Figure 19: Display window for scans in ScanR

The software has two main modules with different image analysis techniques to facilitate the detection of the cells.

- Intensity
- Edge

Intensity module

In intensity module, the intensity threshold is set to distinguish between the boundary and the background. The intensity based identification is extremely efficient if the cells are not very confluent. However if the cells are close to each other and the difference in intensity between two cells and background is not too high, then the cells are difficult to be distinguished from one another. The results in this case are not very accurate because if the cells are in clusters, then they can be identified as a single cell.

Edge module

In edge module the cells are detected by identifying the boundaries. The boundaries are detected by passing the image through high pass filters or applying other imaging techniques.

The detected boundaries are then connected to form close contours which are identified as cells.

In this module, the clipping knob selects the clipping threshold between cells and the background noise. The minimum and maximum cell size is also given so that the closing cell contour remains in the boundary. The minimum closure quality is also adjusted depending upon the quality of the images.

All the parameters were adjusted to optimize the cell counting results. However in adjusting these parameters there is a trade off between over estimating the cells or underestimating them. The optimized selection means when the number of detected cells is closest to the actual amount of cells. These settings were needed to be modified for each cover slip to get better results.

In our case the counting gives better results with the edge module as compared to the intensity module.

The figure from scanR analysis software demonstrating the detection by the edge module is shown below

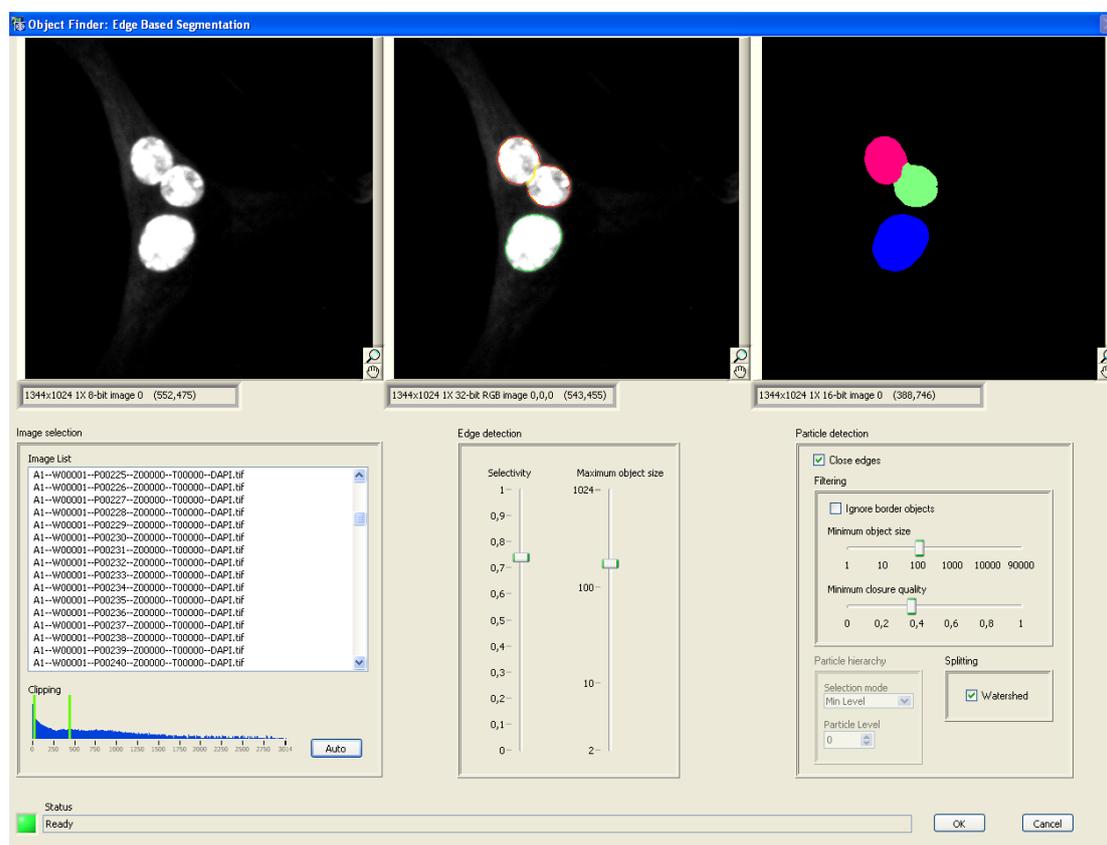


Figure 20: ScanR Analysis Software with different selecting knobs in Edge Module are displayed

The cells in the scan shown above in the Figure 20 are more distinguishable by edge module as compared to the intensity module as the cell boundaries are overlapping each other and the difference in background and cell boundary is not very clear.

The counting results using scanR analysis give only up to 5 % error and are better when compared with manual counting results. The scanning and analysing still takes 2 to 3 hrs, however this procedure is automated.

2.3. In Vivo study

In this section the effect of TTfields was studied in vivo on Mouse model C57BL/6. The experiments on mice were conducted in EBM lab.

2.3.1. EBM – Laboratory for Experimental Biomedicine

All the experiments were carried out in the EBM LAB facility in Gothenburg University. The preliminary one day workshop was attended to obtain work permission with animals in the EBM lab. The experiments were conducted on animals with the ethical permission of National Board of Laboratory Animals under the Swedish Ministry of Agriculture [18]. The ethical permission registration number is 8/2010

2.3.2. External Electrodes/ Internal Electrodes

For treatment of glioblastoma brain tumor, one approach is to apply the electrodes, externally around the skull and over the tumor for a long duration of time. However, for this study the wires are implanted subcutaneously to study the tumor. The choice of using internal electrodes is justified below.

- Lack of availability of the equipment

In case of electrodes placed externally and to establish the field within the range of 5 V per cm in the tumor growth area, the required voltage at the power amplifier, with the dielectric material of strength around 2 to 3, is very high and is not available in the market. Another way is to use the dielectric material with high dielectric constant (2000 – 3000). Such electrodes are also not easily available in market and have to be manufactured individually. Due to time constraints during the study, that approach was not used.

- The direct effect of field is not very apparent

The second reason for using internal electrodes is the fact that to understand the underlying theory of inhibition effect, the field needs to be focused only on the tumor without affecting other areas so that only a small area is under study. This minimizes the parameters that are changing.

- Fixing external electrodes on mouse

Fixing electrodes of wide area outside the mouse is quite difficult because mice tend to bite away anything on their skin. This problem is easier to manage when internal electrodes are used.

2.3.3. Experimental setup

The targeted setup was to use the system which is capable of generating the required field, on the tumor model inside the mouse through implanted wires. The block diagram of the setup is shown below

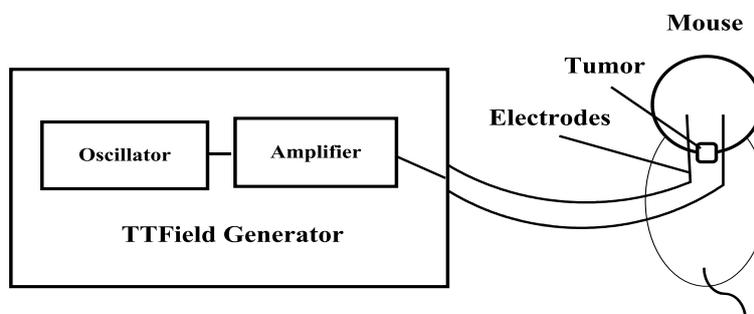


Figure 21: Experiment setup for in Vitro study on mouse model

Setting up the wires in animal cages

After the implantation of the wires, the mice were placed back in their cages where they could move freely. They were kept under observation for 7 days. Special care was taken to adjust the wires in the cage in such a way as to allow their free movement.

The cables used inside the cage for supplying electric field to the electrodes should meet the following requirements.

- Adjustment of electrodes in the cage must allow the movement of animals in the cage.
- Hard to break and capable of twisting without breaking
- Cannot be broken easily with mice bite.
- Can be easily adjusted or fixed on the mice
- Light weight so that they do not restrict mouse movement

To meet the above requirements many alternatives were tried. The Teflon insulated copper wires when used alone can break easily if twisted few times with the mouse movement in the cage.

The spiral wire as shown in the figure below is a good option because it can allow free movement of mouse in the cage, and is not very easily breakable. However it is not light weight and cannot be easily fixed on the mouse.



Figure 22: Spiral cable

After trying many options, the most practical solution that fulfilled all the above mentioned requirements was to place the wires inside tygon rubber tubing. Tygon R-3603 laboratory and

vacuum tubing is crack resistant and easily sterilized [4]. It is transparent so any damage to the wire inside it can be easily detected. It is also very light in weight, flexible, hard to break, and can be stitched or sutured to the mice skin for stability.

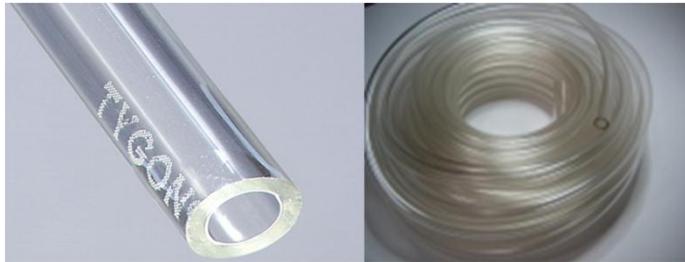


Figure 23: Tygon Rubber Tubing [4]

Growing tumor in animal model

Growing tumor in the flank area is a best approach in most of the experiments [19]. Some of the reasons for using this method are the availability of enough nutrient supply for tumor growth and also the availability of a wide area for the growth before the outer skin starts to ulcerate. However in this thesis the chosen site was the back of the neck. The back of the neck does not have any advantage over the flanks concerning the blood supply, but in our case the main issue was implantation of the wires at the tumor site. If the wires are implanted at the flank area the mouse can easily take it out, whereas the mouse cannot reach the back of its neck, therefore it was chosen as the site for tumor growth.

Initial number of cells required for tumor growth

1 million GL261 cells should be enough to grow a tumor in the flanks of a mouse [19], however the same amount of cells were not sufficient for the back of the neck. The experiments were repeated with several initial volumes ranging from 800 thousand to 20 million. The minimum number of cells required to grow a tumor in the neck area is suggested to be above 5 million cells.

Other modifications to facilitate the tumor growth

The following changes in the normal protocol were made in the process of growing tumor in the mouse model

1. The culture medium used in the injections was modified. In the preparation of the medium, PEST and FBS were omitted [19].
2. The cells were cultured and prepared for injections before they became confluent. The cells must be sub confluent around 75 %.
3. Very concentrated cells were injected, that is 10-20 million cells in 0.1 to 0.2 ml of medium

Design of electrodes

The electrodes consist of 1 cm exposed copper wire covered with Teflon. The rest of the wire was covered with tygon tubing. The tip of the wire was insulated with plastic to avoid the current leakage into the body of the animal. One end of the electrode without tygon covering was implanted in the mouse and the other end was connected with the amplifier. The electrode is shown in the figure below.



Figure 24: The Electrode used in the experiment

Preparation of injections

Targeted injection quantity was 15 million cells in 200 micro litres of medium. The cells were cultured in 75 cm² flasks. Each flask had approximately 5 to 6 million cells. Culture medium with modification was prepared as follows

DMEM	90%
FBS	10%

For preparation of injections the cells were first extracted from the flask with trypsin solution following the same protocol explained earlier. After the cells were extracted they were washed with PBS solution and were centrifuged again for 4 min at 1500 rcf . The cells get settled in the pellet. The PBS was then removed and the modified medium was added according to the requirement. After that the cells are passed through the cell strainer. The prepared injection solution was kept in dry ice until injected inside the mouse.

Injecting the cells

The mouse was given anaesthesia (isoflurane) to avoid any disruption in the location or quantity of cells injected due to mouse movement. The needle was inserted approximately 1 cm below the targeted area. The targeted area was reached subcutaneously skin and then the cells were injected very slowly (approximately in one minute). After injection the needle was tilted 45 degrees and retracted.

If the needle is inserted directly on the targeted area or if the volume of the injections is high then some part of the injections come out with the needle. This introduces the error in the initial count of the cells injected.



Figure 25: Mouse injected with tumor cells under the anaesthesia

Implantation of the wires

For implantation procedure the mouse was kept on operation board under the effect of anaesthesia with the isoflurane. A small incision was made with the needle or scissor at the back of the neck around the tumor. The wires were then inserted in the incision, pushed forward and adjusted from outside manually to be placed around the tumor. After adjusting the wires the incision was closed with suturing. The tip of the wire with plastic coating was also sutured so that the wire remains at its position during the observation period.



Figure 26: The mouse implanted with the electrodes

Observation period

Wires were implanted 7 days after injection and field was applied for one week. The time period to wait before implantation of the wires depends on the amount of cells injected. If the quantity of cells injected is very high, around 20 million, then the fields can be applied after 3 or 4 days



Figure 27: The mice under observation in the cages with the TTfields applied in the EBM lab

Each mouse was kept in a separate cage to avoid any damage from other mice. They were given free access to food and water and the TTfields were also monitored frequently during the observation period. The lab cages with TTfields applied to the mice are shown in the Figure 27.

2.3.4. Perfusion for histological analysis/ Fixation

The animals were sacrificed after seven days of observation period. A sample of the tumor was taken after perfusing the animals. Samples were taken by fixation of the animal with the process of perfusion fixation through heart via the vascular system. The fixative (paraformaldehyde) was injected into the heart from where it spreads into the body via blood flow and the cells are fixated before they die to maintain the original morphology of the cells. The perfusion pump was setup with the perfusion needle at one end of the tubing. The other end of the tubing is switched between two solutions.

- i. 0.9 % of saline solution to wash out the blood
- ii. 4% Paraformaldehyde for fixing the animal tissue

The forceps, scissors, scalpel of appropriate sizes etc were kept at the side of the operating table. The setup is shown in the figure

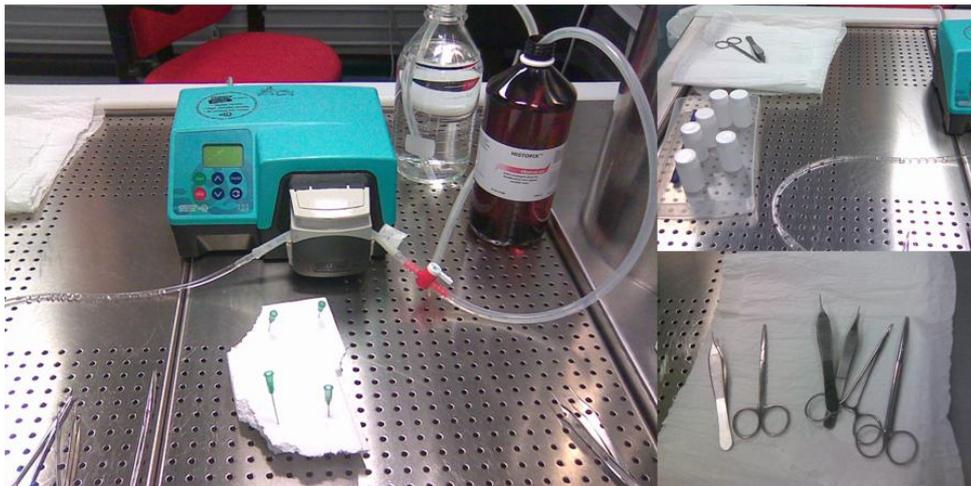


Figure 28: Perfusion operating table with the required apparatus

The animal was given anaesthesia by injecting 0.1-0.4 micro litre of tiopental. After the animal was completely unconscious it was firmly held on the operating board with the back placed on the board. The limbs were held tightly with tape or needles so that the animal doesn't move while perfusion. The thoracic cavity of the animal was opened and the heart was exposed. The heart was held with the forceps and the perfusion needle was entered in the left ventricle and moved up very carefully about 5 mm to reach the aorta. The needle if extended too far can rupture the walls; hence compromising the flow of fixative.

After the needle was adjusted in the right place, the tab was slowly released to allow the 10% saline solution into the body. The atrium was ruptured with the needle and it was made sure that the solution is flowing freely. The perfusion pump was adjusted on 10 to 12 rpm.

The solution was allowed to pass until the clear solution appeared from right ventricle of the heart; colour of the liver changed and the blood was completely washed away. After that, the

formaldehyde solution was allowed to enter the body at the rate of 10 to 12 rpm. To get a satisfying preservation of the tissue approximately a volume of 100ml of 4% paraformaldehyde is needed. A good fixation is also depended on the time the tissue is exposed to the fixative.

After the perfusion the tumor area was dissected out and kept in a separate tube in formaldehyde solution.

After 24 hours the formaldehyde solution was replaced with 30% sucrose in 0,1M phosphate buffer solution and the samples are kept in it for 5 days to allow the specimen to completely infiltrated with the cryoprotectant solution before preparing them for analysis under the microscope.

The samples taken were then cut with a sharp knife with sliding microtome (Leica) and mounted on the slides for analysis under stereology microscope. The G1 261 cells express Green fluorescent protein (GFP) and can be seen under the microscope without requiring any additional staining.

3. RESULTS AND DISCUSSION

3.1. In Vitro Study

In vitro study experiments are conducted in two setups, one where the cells are cultured in petri dishes and the other where the cells are cultured on the cover slips and placed between the wires in petri dishes. The results from both of the setups are explained below

3.1.1. Experimental Results with Petri Dishes

The 1.5×10^4 cells per cm^2 , 12×10^4 in one culture dish were exposed to the TTfields for 72 hrs. The results from the experiments conducted at two different instants are shown in the graphs below

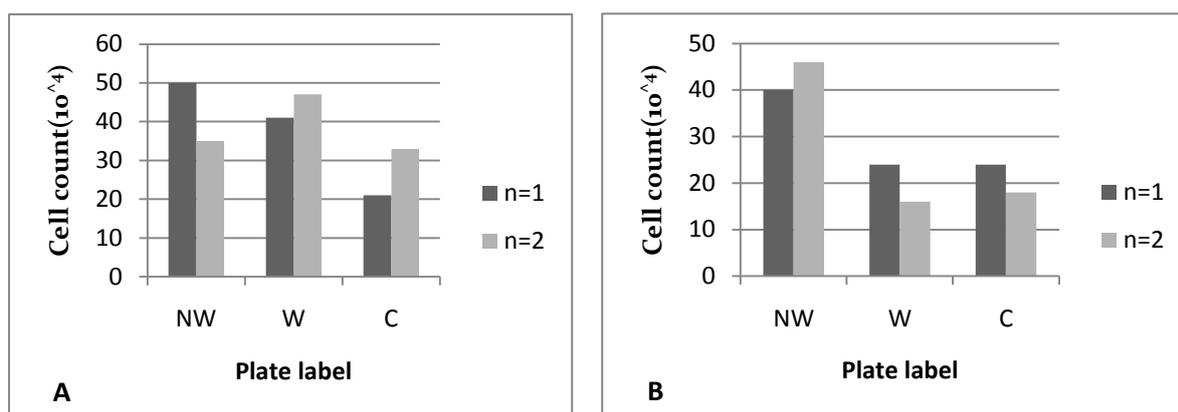


Figure 29: The comparison of cell counts in control and treatment group in the experiments where cells were cultured on petri dishes. Initial number of cells used are 12×10^4

The results from Figure 29 A show the considerable difference between the treatment group and the control group. However the difference between the two plates in one group is also not negligible. This makes it difficult to detect whether the effect is due to the fields applied or is it only the error rate between different culture plates.

The results for the same experiment repeated again with same initial number of cells and the observatory period are shown in Figure 29 B. In this case the difference between W labelled plates and C labelled, that is the control group dish with wires and the treatment group dish with wires supplied with fields is not much. However, the difference with in each plate is very high. The results of experiment 2 are not consistent with those of experiment 1.

The initial number of cells used for both the experiments were kept constant. However the total numbers of cells after the observatory time were not the same in both experiments. This can be attributed to the fact that the cells are affected by the environment they were in before

the start of the experiment. That is, if the cells are too confluent in the chamber then their division rate is reduced, whereas when the cells in chamber have more place to grow and sufficient nutrients then they grow very fast. Now if the cells are taken out from the chamber at a time when they are very confluent then their growing rate can differ from other cells taken from the chamber at the time when the cells are relatively sub confluent.

From the above observations it seems necessary to compare the results of one experiment with in it, rather than comparing the experiments conducted at different time instants even if the conditions in both cases are kept constant.

The inhibiting effect seen in experiment 1 was not seen consistently, in the experiments that were performed later with the same settings. One possible explanation for this can be the fact that the cells under the influence of TTfields are only those that are between the wires in the petri dishes. That number is not kept constant in all the experiments, as only the total number of the cells in each petri dish is controlled. The way the cells settle in dish is not controlled. So in each experiment the exposed cells are varying. To overcome this problem experiments were repeated again with the new setup where the cells were grown on cover slips and those cover slips were placed between the wires

3.1.2. Result of Experiments with Cells Cultured on Cover Slips

The experiments were conducted again with modified setup. The exposure period of the cells to the fields was kept the same, i.e 72 hours. The experiments were conducted with different initial volume range of 10,000 to 15,000. The results for the initial volume of 10,000 and 12,000 are shown in Figure 30 A and B respectively. The cells above 13000 become very confluent and the counting of the cells becomes very difficult. Therefore the recommended volume to use in these experiments is in the range of 10,000 to 13,000. The cells in these cases are counted manually with stereolgy microscope and automatically through scanR . The counting results for scanR are also compared with manual counting. If the analysis software's settings are properly optimized and the initial volume of cells used is in the given range (10,000 to 13,000) so that the cells after the observation period are not very crowded, then the ScanR analysis software counts the cells very efficiently with the error rate measured for these experiments less than 5%.

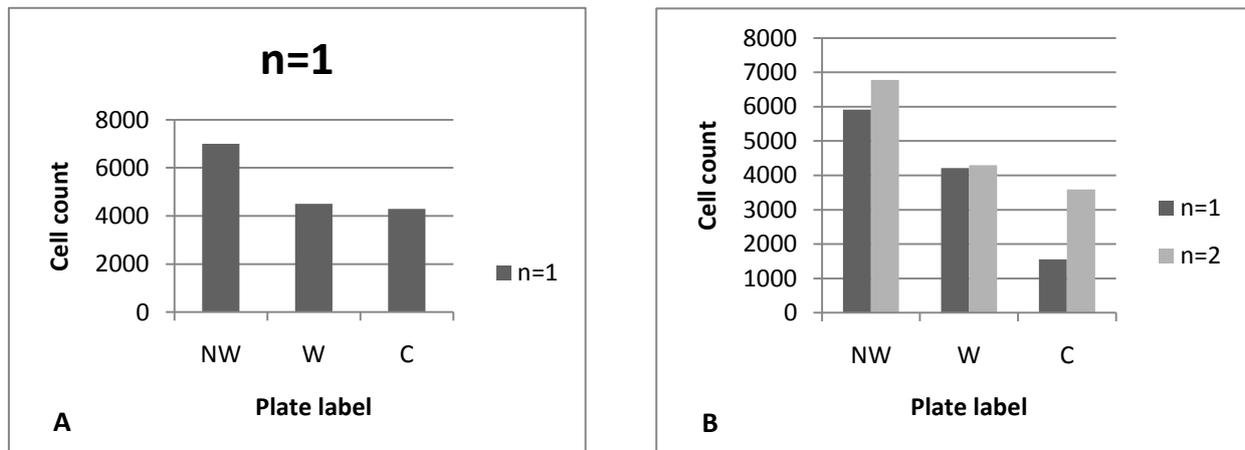


Figure 30: The comparison of cell counts in control and treatment group in the experiments where cells were cultured on cover slips and placed in the petri dishes between the wires A) Initial number of cells used are 10,000 B) Initial number of cells used are 12,000

Both experimental setups showed a slight inhibiting effect of TTfields. However, the statistics are not enough to conclude any effect with certainty.

3.2. In Vivo Study

In vivo, the tumor growth is approximated with the size of the tumor in each animal. For more quick measurements of the tumor volume, tumour in each case is approximated with the cuboid or sphere depending upon the shape of each tumour.

In case, the tumor shape is similar to cuboid, length, width and height is measured and radius in case of sphere is measured with a vernier calliper. The volume of the tumor is then calculated to compare the sizes in different animals.

The experiment was conducted on 6 animals with three animals implanted with wires and three animals without wire, with the same number of initial cells injected. The wires were implanted 7 days after the injection and the animals were exposed to tumor fields for 7 days. The results of the experiment are shown in the Figure 31.

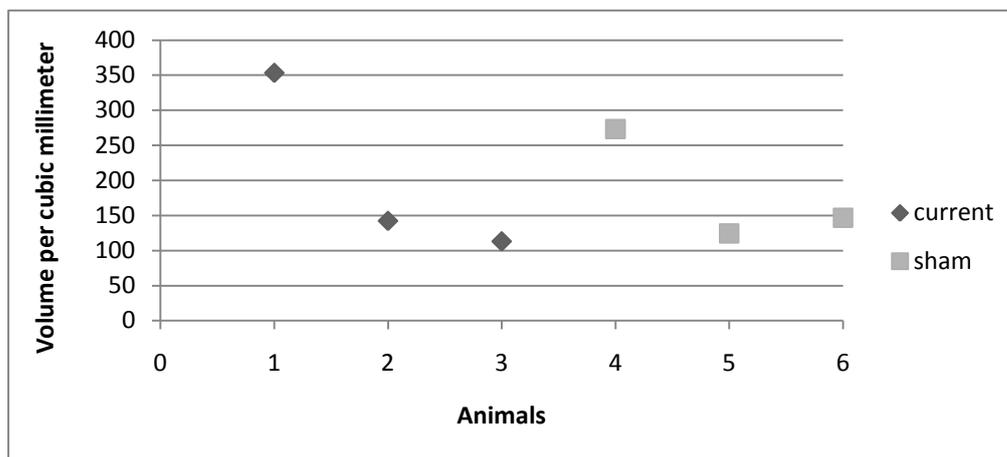


Figure 31: The results of experiment on mice with three mice in control group and three in treatment group. The volume of the tumor is plotted against each animal

The results did not show any significant difference between treatment group and control group. One possible reason might be the fact that the wires were not implanted very accurately around the tumor, as the tumor size at the time of implantation, was not big enough to be detected from outside the skin.

In the second experiment the wires were implanted more carefully by estimating the tumor location from outside the skin and securing the wires firmly around the tumor. The results are shown in the figure below. Nine animals were injected with 8 million G1 261 cells. One animal was sacrificed at the time of implantation of wires to make sure the tumor is growing in the animals. Four animals were implanted with wires and TT fields were applied for 7 days after the injection of cells. Rest of the animals were in control group. The results from the experiments are shown in the figure below

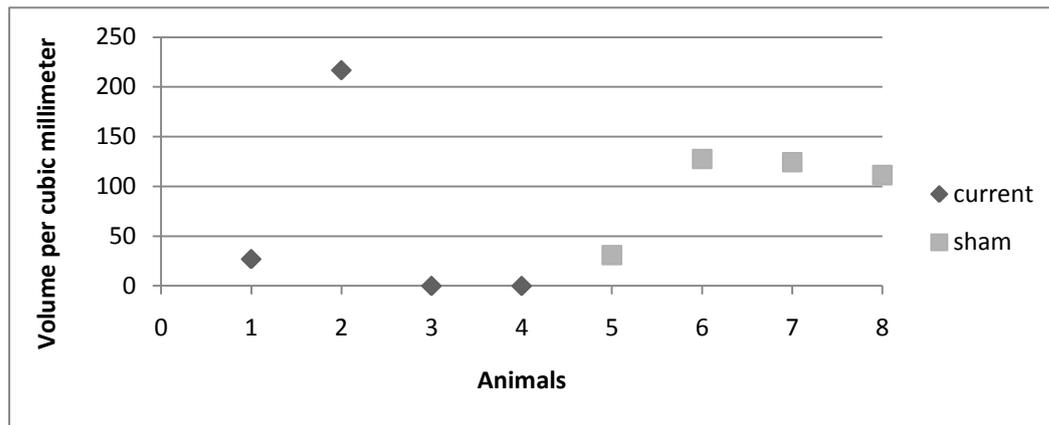


Figure 32: The results of experiment on mice with four mice in control group and four in treatment group. The volume of the tumor is plotted against each animal

The results in this case are better than the previous results. Two animals showed no tumor. However, it is not certain whether the tumor did not grow in them at all or if it was cured already by the fields. In one animal from the treatment group the tumor size was bigger than the average tumor size in control group.

The above results imply that it is very important to have the initial size of the tumor measured in each animal. All the animals were injected with the same number of cells but tumor size in each animal varied. The suggested option is to have an MRI scan of each animal to detect the exact location and size of the tumor before the implantation of wires.

The other observed difference was the morphology and site of tumor growth in each animal. In some animals the tumor was harder than others. The different tumor growth in animals is shown in the figure below.



Figure 33: The different morphologies of tumor growth observed in mice

The cells were injected subcutaneously, however the tumor growth observed was subcutaneous, intradermal and mixed as shown in the Figure 33.

The difference in morphology and histological analysis of tumor was not studied in this research. However, this can be one of the effects of the TTfields.

4. CONCLUSION AND FUTURE WORK

In this thesis, a setup to study the effects of intermediate frequency electric fields is developed. The design and specification of the required equipment like electrodes, amplifiers, oscilloscopes etc is suggested. The parameters like voltage, impedance and initial volume of cells required are calculated. The optimal software ScanR to use with the experiment is also studied and specific modules that can be better in studying the results are suggested.

The overall platform to obtain an in depth understanding of the underlying phenomena of interaction of the TTfields with tumor is provided. However, because of time constraints, enough experiments were not conducted to establish any conclusion about the inhibiting effect of TTfields. The results described in this thesis show tendency of the fields in inhibiting tumor growth but the statistics are not enough to state any effect of the tumor treating fields with certainty.

In future it is proposed that the experiments should be conducted on a bigger scale. In vitro, the culture dishes are suggested to be in the range of 40 to 45 dishes per set. The variation within each culture dish can be reduced and the effect of the fields can be studied more closely.

In vivo, the number of animals used can be increased to 30. Also, it is suggested to use more sophisticated methods like MRI, to estimate the initial volume of the tumor. The morphology of the observed tumor in animals was different. This can also be studied in detail to observe the relation of morphology with the growth of the tumor and with the exposure of the field.

The experiments conducted in this thesis are using the single frequency of 200 kHz. The frequency used can be varied from 100 kHz to 500 kHz to observe if there is any optimal frequency that inhibits tumor growth more than the other frequency. The voltage applied at the tumor model can also be varied to study the specific relation between the tumor growth and the intensity of the field applied.

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