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Methods To Investigate Hyperthermia Induced By Tumor Treating Fields

Ruchi Singh

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**METHODS TO INVESTIGATE HYPERTHERMIA INDUCED BY
TUMOR TREATING FIELDS**

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**METHODS TO INVESTIGATE
HYPERTHERMIA INDUCED BY TUMOR
TREATING FIELDS**

A

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METHODS TO INVESTIGATE HYPERTHERMIA INDUCED BY TUMOR TREATING FIELDS

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Tumor Treating Fields (TTFields) are an antineoplastic treatment delivered via application of alternating electric fields using insulated transducer arrays placed directly on the skin in the region surrounding the tumor. TTF's is a non-invasive application of low-intensity (1-3 V/cm), intermediate-frequency (100-500 kHz) alternating electric fields. The predominant mechanism by which TTFields are thought to kill tumor cells is the disruption of mitosis through the depolymerization of microtubules and interruption of the spindle structure leading to mitotic catastrophe and the formation of non-viable daughter cells.

Tumor Treating fields do not stimulate nerves and muscle because of their high frequency, and do not generate heat because of their low intensity. However, there is no study to support the mechanism of TTF's. It is still under research. There is not much research done on the hyperthermia induced by tumor treating fields. It's still not known whether heat also plays a role in inhibiting cancer growth and killing cancer cells.

Our hypothesis is that the TTF's at 150 kHz frequency induce hyperthermia in pancreatic cancer cells. Two specific aims are set to approach this hypothesis. First specific aim is to design and develop an engineering set up capable of generating frequency with amplitude modulation to induce TTF's (150kHz). And second aim is to perform experiments on panc-1 cells stained with PEG BODIPY dye cultured in a custom designed Delta T dish using picoquant confocal microscope. Lifetime measurement of the dye clearly suggests that temperature does increase in the cells with time.

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LIST OF ABBREVIATIONS

- TTFields:** Tumor treating field
- BODIPY:** Boron-dipyrromethene
- PMT:** Photomultiplier tube
- FI:** Fluorescence intensity
- LVF:** Linear variable filters
- TCE:** Transparent conductive electrode
- LCD:** Liquid crystal display
- LED:** Light emitting diodes
- OLED:** Organic light emitting diodes
- PET:** Polyethylene terephthalate
- ITO:** Indium tin oxide
- PLA:** Polylactic acid
- PDMS:** Polydimethylsiloxane
- EtO:** Ethylene oxide
- FBS:** Fetal bovine serum
- DMEM:** Dulbecco's modified eagle medium
- PENSTRAP:** Penicillin-streptomycin
- PBS:** Phosphate buffered saline
- FLIM:** Fluorescence lifetime imaging microscope

CHAPTER ONE

INTRODUCTION

Biophysical aspects of cancer and electromagnetic field

Six decades ago, Fröhlich formulated the hypothesis of electrical polar coherent vibrations in biological systems due to nonlinear interactions between polarized oscillating fields with metabolic energy supply in the cells [1]. It states that the electromagnetic fields and excitation in the coherent vibration is caused by the changes in electrical charges inside the microtubule. As per this hypothesis, electromagnetic field generated by these coherent vibration plays a significant role in organization, energy supply, transport, signal processing, communication and interactions inside a cell and among cells [2]. Frohlich's mechanism gets disturbed when there is malfunctioning in mitochondria and disintegration in the cytoskeleton which is very likely in cancer cells. Thus, there is reduction in activity and energy production in mitochondria in cancer cells which in turns reduces the excitation of coherent vibrations. Several studies were done on it and leads to the conclusion that there is a difference in coherent vibration between a healthy and a cancer cell. Mechanism behind the cancer metastasis is also assumed to be dependent on these coherent vibrations [3]. Cancer cells change the inner engineering of the cells and their interactions with other cells in tissues which cause disturbances in Frohlich's coherent vibration and of electromagnetic radiation which can be measured in order to differentiate cancer cells from the healthy cells. Properties of cytoskeleton, cytosol and the plasma membrane gets distorted in cancer cells and they also exhibit loss of cohesion, contact inhibition and tendency to separate due to lack of adhesion among them. Fröhlich stated fundamentals of biophysical theory [1-3] and also, he developed an electromagnetic vibration model.

History of electrical and magnetic therapy

Human race curiosity about the magnetic and electrical therapies has been inevitable. It's important to understand the history and development of the therapeutic use of electric and magnetic fields in order to predict the current medical acceptance of the electromagnetic therapy [4]. With the advances in medical breakthroughs in this field, history of magnetic and electrical therapy would act as a guide to proceed in this field.

Both electrical and magnetic therapy has connection with the science of the most ancient times. Human beings have always been very curious about magnets which is clearly seen as many old civilizations are known to use magnets for various purposes. In ancient India, magnets have been extensively used for the well-being of humans [4]. Sushrutha, the famous Indian Physicist used magnets because of its antiseptic properties in his surgeries including cosmetic surgery. Ancient Indian scriptures detailed about the use of lodestone in Indus valley civilization which Sushrutha used as a reference [3, 4]. Magnets were also used to heal wounds during spinal and arthritis treatment. An Indian Scientist, AK Bhattacharya did extensive research on magnetism and how it can be used in medical field for the well-being of human beings. His findings were then later applied in medical therapies and he believed that the polarity is present in each cell of the body which later laid the foundation of many prominent therapies [4, 5].

In history, several civilizations of the ancient times dealt with the science of magnetic and electrical energy. Indus valley, Egyptian civilization have conferred utmost eminence to the use of magnets to contribute towards well-being of humans [6]. Use of the electric and magnetic therapies for healing and treating disease has been known and popular since the time of ancient Indus valley civilization and Greek civilization. But, in each era it was hard to get enough proof and well-known mechanism behind it in order to convince the scientific community [7].

However, the exact time and place for the discovery of electricity and magnetism is not known. But, a very early widely quoted report on the discovery of magnetism states that Pliny the Elder (23-79 AD) mentioned in his book on Natural History that a shepherd named Magnes in Turkey while roving about on Mount Ida noted that the iron nails in his sandals were attracted to the ground. It is this observance which led him discover the magnets originally named as magnesium [8]. The allure of electric and magnetic forces has attracted visionaries for thousands of years. At those time, people were able to discover electric charge by rubbing amber against the fur and it took them more than two millennia to figure out the difference between the electrical and magnetic forces. Many ideas swirled out of the minds of visionaries allured by the electric and magnetic field, for example Greeks used magnetic ring for treating arthritis. Ancient early civilization like Indian, Chinese, Greek used lodestones for medical practices which provided benefits to the patients [7, 8]. Although, there is not much documentation on the mechanism of such therapies in these early civilizations. In the middle ages, it has been used for variety of ailments like epilepsy, diarrhea, baldness, acupuncture, pain, aging, neurological diseases etc.

In 1289, Peter Peregrinus discovered the north and south poles with his experiment with a small needle and lodestone. In the early days, his contribution to this field was very significant. He was the first European to figure out how the magnetic needle can be used to as a compass for navigation purposes. Also, he proposed various ways to utilize magnetic energy by converting it into mechanical energy. In the middle ages, Paracelsus proposed several ways to treat few diseases like epilepsy, diarrhea, and hemorrhage [9].

Franz Anton Mesmer who was an Austrian physician during 1734-1815 came in contact with an astrologer Marian Theresa. She was also a priest who was using magnetized pieces of iron to treat neuropsychiatric patients and achieving success in it [8] [1]. In the year 1777, Mesmer used magnetic therapy to cure a child pianist who had been blind since he was 3 years old. He got

success in reversing his blindness, but it brought imbalance and depression along with it, later it is followed by a seizure which made him again blind. After this incident, he was expelled from medicine field and was bound to leave the city [8]. In mid 1700s Benjamin Franklin got deep into the therapeutic effects of electricity. He tried to treat paralysis using electric shocks. Though, some of his patients got temporary movement and strength in the limbs but it was not successful in the long run as he didn't consider the connection between electricity and magnetism.

Hans Christian Oersted in the early 18th century did some experiments to understand the relationship between electrical energy and magnets. During his experiment, he observed that the wire carrying electricity gets affected by the force exerted on it by a nearby magnet. Also, he found that a wire carrying electricity deflects a compass needle which gave him a better understanding about the electrical and magnetic forces.

It was not until the 19th century, that scientists got the better understanding of electromagnetic field with the Michael Faraday discovery of magnetic field inducing a current in a moving wire. Also, the electromagnetic field equation which is being widely used currently for the calculation in this field is derived by James Clerk Maxwell during this century. In the late 1800s, a Russian scientist came up with this idea that cells have their own energy and cell processes are governed by the flow of negative and positive charges which defines their amplitude. It was after this that the variety of electromagnetic products started occupying the market and got sold. A physician Elisha Perkins from Connecticut, USA developed some magnetic attractors made from metallic alloys which he claimed that can take off the electrical fluid lying at the roof of the suffering [1, 2]. C.J. Thatcher promoted his healing magnetic cap, waistcoat, stocking liners, insoles and claimed that disease occur when the body loses its ability to gain magnetic power from the atmosphere [1]. So, his clothing set would provide complete protection of all the important organs of the body and help restore the magnetic power of the body

[3,4]. Electromagnetic treatment got somewhat accepted in the late 19th century with the discovery of all these products and publication of scientific papers, books in support of its effectiveness, and the exact mechanism behind it was a debatable topic since then. Albert Abrams did a very interesting treatment to a patient in which he tuned his/her organs to certain specific electromagnetic frequencies using his device Dynamizer or Oscilloclast in order to diagnose and treat him/her. He claimed that his device works even at long distances [1,5].

In early 1980's, the first FDA approved pulsed electromagnetic field therapy device was introduced, which became popular in other parts of the world as well. It was used to treat non-union bone fracture [10]. From here on, scientists started expanding their knowledge and understanding of how our physiology gets affected by the electrical and magnetic fields. Since then, lot of PEMF devices came up for various purpose like decrease Migraine Frequency, improve Nerve Function for Diabetics, alleviate the arthritis function, heal damaged nerves etc. In the late 19th and early 20th century, Nikola Tesla came up with his theories about electromagnetism with the understanding of alternating and direct current. He discovered the magnetic loop coil which is still being used in most of magnetic devices [8-10]. With the discovery of the electron, electromagnetism concept got to the atomic level, which gave an understanding that all the matter is electric in nature. Albert Einstein also explained that electricity and magnetism are interrelated, and they are different aspects of the same phenomenon.

Safety issues

A review on the electromagnetic frequency, its origin, background, mechanism, history from 1000 BC to present brings forth that the safety concerns from the EMF has taken a rise in the last 50 years. In the late 1970's and early 1980's several epidemiologic studies suggested that there is link between the EMF exposure and leukemia, other cancers, chromosomal aberrations etc. Recently, National Research Council panel stated that better designed studies support the

view that “no conclusive and consistent evidence” exists to show that EMF exposure causes significant increases in the risks of cancer, neuro behavioral dysfunction, or reproductive dysfunction [6,7]. A convincing mechanism behind the electromagnetic frequency amalgamation with the cells is yet to be known. It’s hard to even predict the safety of such radiation as one study might say that electromagnetic fields are beneficial for a certain disease at certain condition [12, 13] but find no effect in another condition [11]. There are so many variable and assumptions involved that it’s hard to come to any conclusion.

Tumor treating field and its biological mechanism

Cancer is still, leading cause of deaths in united states. In spite of so much research being done in this area in the last 50 years, there is still lot to understand and the exact cause of many cancers is still unknown. Traditional treatment like surgery, chemotherapy and radiation therapy has their own challenges like tolerability and adherence. There is a high need of a different effective approach to cancer treatment [14]. Tumor treating field is a new innovative therapeutic approach, which is being added as a fourth modality to cancer treatment. In this therapy, alternative electric fields having intermediate frequency and low intensity are used non-invasively to reduce the growth of the tumor by interfering with dividing cells. It is developed and pioneered by a global oncology company, Novocure [15]. This unique approach to cancer treatment was the idea of Dr. Yoram Palti who is now a emeritus professor of physiology and biophysics at the Technion Israel Institute of Technology in Haifa, Israel. Around a decade ago, he hypothesized that alternating electric field in a particular frequency range has the ability to disrupt the mitotic cell division in cancer cells leading to their death [14, 15]. He rationalized the frequency range (100-300kHz) for the alternating electric field which when applied to the cancer cells, disrupt the spindle structure and other essential processes in the cells, thus leading to mitotic catastrophe

resulting into apoptotic cell death [14]. Intermediate frequency effects the rapidly dividing cancer cells, sparing the nondividing cells.

Dr. Palti set up a laboratory at his home to test this hypothesis, where he was able to successfully perform this experiment and found that alternating electric fields when applied at a particular frequency which is specific to tumor type, disrupt the cell division which results into cell death, not affecting the healthy cells [15]. Just after the result of this hypothesis, he founded this company Novocure in year 2000 which is the only leading company working on tumor treating fields. Results from previous studies suggests that electric field induce apoptosis in the cells as it interferes with the separation of the chromosomes during the mitotic division [16]. Target of the tumor treating field is proteins, cell structures etc. which are essential for cell cycle, leading to cell cycle arrest resulting into non-viable cells and anti-tumor immune response [14].

Novocure developed a device Optune for GBM patients. It has got the FDA approval owing to its benefit of prolonging the survival for newly diagnosed and recurrent GBM patients [14]. This device has been proved to be safe to use and has been significantly beneficial for the GBM patients. Practically, when cancer patients use Optune device in hospital, it delivers low intensity and intermediate frequency alternating electric fields to the tumor continuously for about 18 hours a day which disrupt the mitosis of rapidly dividing cancer cells and killing them by sparing the healthy cells [15].

The actual biological mechanism of TTFs is that there are three phases of cell cycle i.e metaphase, anaphase, telophase of mitotic cell division during which TTFs are more effective. As the electric field is applied to the cancer cells, there will be back and forth movements in the charged molecules whereas the dipolar molecules will rotate. But, as the frequency of the electric field is increased, it decreases the movement of such molecules which happens in the intermediate frequency range [16]. Since, molecules like tubulin dimers and septins

have high electrical dipole moment, it makes them align with the electric field direction under a uniform field distribution. This alignment of the tubulin dimers and septin in the direction of alternating electric field disrupts the formation of the microtubule and localization of septin fibers during the metaphase of the cycle. And this disruption results into a mitotic catastrophe which gives the possibility of the cell death during the mitotic process [15, 16]. Although, not all cells die during the metaphase of the cell cycle, some might survive and would be able to move to anaphase and telophase. But, dividing cells attains a shape of hourglass as it starts the process of splitting into daughter cells which causes non-uniformity in alternating electric field during these phases. This non-uniform field induces an effect called electrophoresis in which polarized molecules move towards the cleavage furrow, disabling the proper division of the cell resulting into two non-viable daughter cells [14-16]. Thus, TFields has an antimitotic effect which may either result into cell death during the metaphase or to the formation of abnormal daughter cells in anaphase or telophase having uneven number of chromosomes which makes them non-viable.

CHAPTER TWO

CHARACTERIZATION AND MEASUREMENT TECHNIQUES

PEG BODIPY dye

It's a new dye which is developed in a chemistry lab at Rice university and collaborative work is done using this dye in few projects at my lab. Boron dipyrin (BODIPY) are being termed as molecular rotors in previous studies and have been used for sensing the change in viscosity, polarity and temperature. Dye which is used in this hyperthermia study for sensing the change in temperature is basically 1,3,5,7-tetramethyl-8-phenyl-BODIPY modified with a PEG chain. Rice university researchers used the light emitting properties of particular molecules to create a fluorescent nanothermometer. BODIPY is best suited to the task as its fluorescence lasts only a little while inside the cell and the duration depends heavily on changes in both temperature and the viscosity of its environment. But, at high viscosity, which is the environment in typical cells, its fluorescence lifetime depends on temperature alone. It means that at a specific temperature, the light turns off at a particular rate, and that can be seen with a fluorescence lifetime imaging microscope.

In the synthesis of BODIPY dye, first of all p-Iodobenzoyl chloride (1.32 g, 4.95 mmol) was dissolved in 50 mL dry dichloroethane and purged with N₂. Thereafter, 2,4 dimethylpyrrole (1 mL, 9.6 mmol) is distilled was added via syringe and refluxed for 13 h. This is an exothermic reaction, so the mixture is cooled to room temperature before 3.5 ml of triethylamine was added. This solution was then stirred for about 10 mins, then boron trifluoride etherate (5.1 mL, 41 mmol) was added and refluxed for 2 h. The reaction was then cooled, and the solvent was removed under reduced pressure.

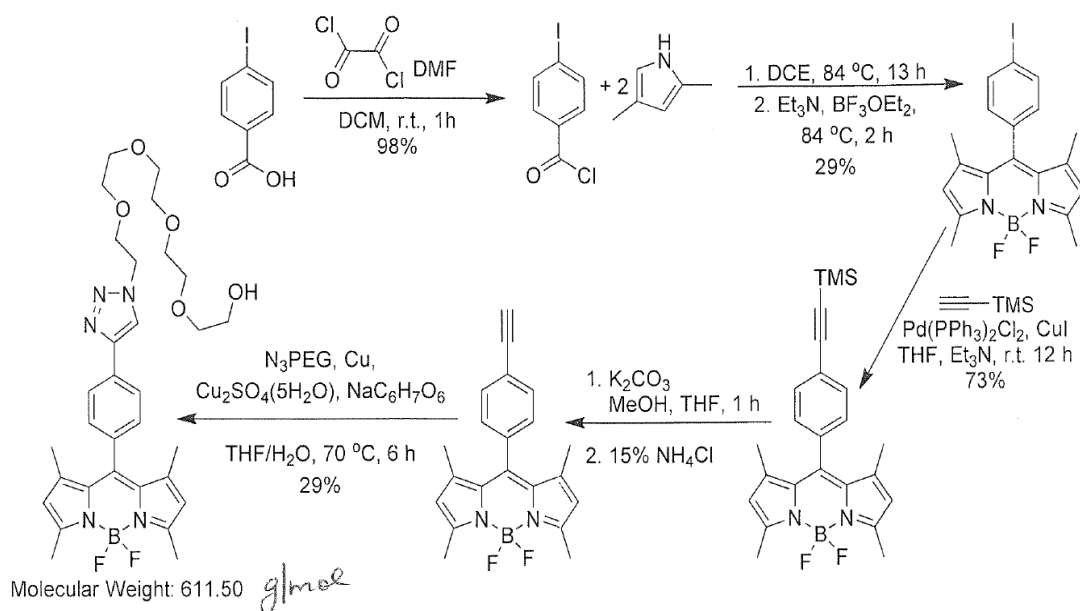


Fig. 2.1 Synthesis of PEG-BODIPY [Meredith W. McDowella, Ashleigh D. Smith McWilliams. 2019. Sensing Intracellular Temperature with a BODIPY Molecular Rotor, *J. Phys. Chem. B* 123, 34, 7282–7289, Used with permission]

An orange solid was purified through a silica column (1-3% EtOAc/hexanes). Yield 0.64 g, 1.4 mmol, 29%. $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.86 (d, 2H, $J=8.4$ Hz) 7.06 (d, 2H, $J=8.8$ Hz) 6.00 (s, 2H) 2.56 (s, 6H) 1.43 (s, 6H). This method for the synthesis of the dye is adapted from the previous paper published on BODIPY dye by Dr. Marti's lab in Rice University [17].

Characteristics like modularity, photostability and biocompatibility makes BODIPY based dyes very useful and profusely used biological dyes. Phenyl ring present on the BODIPY core (meso- or 8 position) is able to rotate and thus it acts molecular rotor which is sensitive to temperature, viscosity and polarity. Methyl groups present on the 1 and 7 positions of the PEG BODIPY dye modulates the free rotation of phenyl group. This dye is biocompatible and can penetrate to cells which can be used to get the internal temperature of cells using fluorescence lifetime Imaging (FLIM).

During the synthesis of dye, all the reactions are oxygen and water sensitive, reason why they are done under N_2 . Fluorescent molecule BODIPY which is a product of second step in the synthesis process is temperature sensitive as its fluorescence decreases with increase in temperature. It also can rotate and doesn't dissolve in water. Copper click reaction is the main step of the synthesis process and its end product is the BODIPY dye. Lifetime of this dye is not affected by the concentration, photo bleaching, path length etc. Single bond rotation of phenyl group is responsible for the change in lifetime due to viscosity to a certain point. With change in viscosity, lifetime increases linearly which means that in water lifetime of the dye is less and lifetime of dye increases in PBS. It decreases in liquid like chloroform and increases in any oil or honey.

Excitation wavelength of this dye is 488 nm and its emission wavelength is 520 nm. Lifetime of BODIPY dye is 3 ns which means that all the dye particles which fluoresces at 488 nm comes back to ground state in 18 ns. But, laser excites the particles of the dye while working on confocal microscopy and 100 ns is the usual wait time to make sure that all the fluoresces particles comes back to the ground state within 100 ns time. In the lab, 370 nm laser is used to calculate the lifetime of the dye while in confocal microscope 498 nm laser is used as it doesn't affect the lifetime measurement of the dye. Only thing changes is just the brightness. According to Caucasian law, no matter what laser is used to excite the dye, lifetime remains the same. Only difference is that at 488 nm, BODIPY dye absorbs the best and thus it emits at its best with high brightness.

During the quantum yield measurements, it was found that its lifetime gets effected by the increase in its non-radiative decay with increase in temperature. It's a perfect nanothermometer for measuring the temperature of the cell for the animal studies as well. Nevertheless, it is to be considered this dye stains the cell not the nucleus of cell. Concentration of dye used in the

experiments was 100 μM which was found to have no toxicity in the cells. Lab experiments suggest that lifetime of this dye found to decrease logarithmically with temperature with a resolution of 0.4 $^{\circ}\text{C}$. Change in temperature depicted by change in lifetime of BODIPY dye is more accurate and precise as the value of lifetime is independent of concentration and photo bleaching.

Nikon confocal microscope

The difference between a confocal microscope and conventional microscope is that the image generated by confocal microscope is very sharp. The way to achieve this is by eliminating most of the light which is not from the microscope's focal place. The image generated is having good contrast in comparison to an image from a conventional microscope. It also provides a thinner cross section of the specimen.

Background

Distinguishing work on confocal microscopy was done by Marvin Minsky in 1955 [18]. His invention would perform a point-by-point image construction by focusing a point of light sequentially across a specimen and then collecting some of the returning rays as shown in fig 2.2. By illuminating a single point at a time, most of the unwanted scattered light was avoided that obscures an image when the entire specimen is illuminated at the same time. To build the image, the specimen was scanned by moving the stage rather than the light rays. The sole reason for this one was to prevent the problem of trying to maintain sensitive alignment of moving optics. Using a 60 Hz solenoid to move the platform vertically and a lower-frequency solenoid to move it horizontally, a frame rate of approximately one image every 10 sec was obtained.

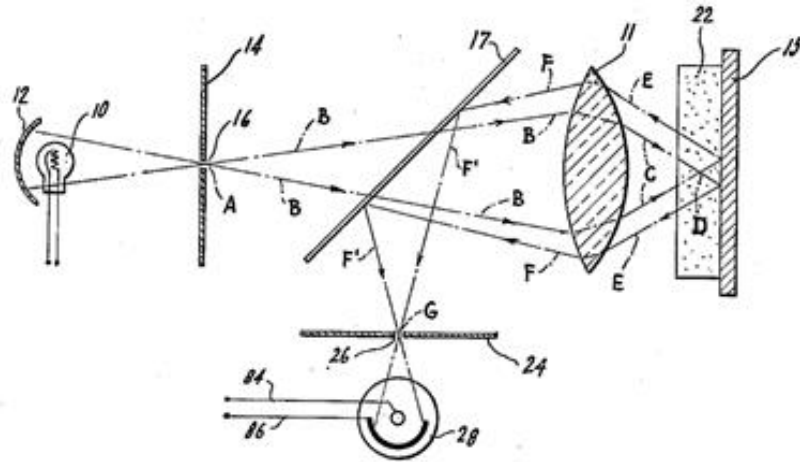


Fig. 2.2 Confocal point sensor principle [*Confocal microscopy, D Semwogerere & ER Weeks, Encyclopedia of Biomaterials and Biomedical Engineering (Taylor & Francis, 2005), Used with permission*]

In today's confocal microscopes the key elements of Minsky's design are already present. These include pinhole apertures and point-by-point illumination of the specimen. The new developments in optics and electronics are present in the latest designs which add improvements in the quality of the image.

Fluorescence and fluorescence microscopy

Fluorescence is the process in which a molecule absorbs the light and then emits light of different color. At ground state the molecules are at their lowest energy state, but they can absorb a photon of light that will increase their energy [19]. This causes an electron to elevate to a discrete singlet excited state. In Fig. 2.3, this is represented by the top black line. Generally, the molecule quickly dissipates some of the absorbed energy through collisions with neighboring molecules causing the electron to drop to a lower energy level. There could be a case in which the neighboring molecules cannot accept the larger energy difference by which the molecule can

come to its ground state. In this scenario, it can undergo spontaneous emissions [20]. Due to spontaneous emission, it loses the remaining energy by emitting light of a longer wavelength.

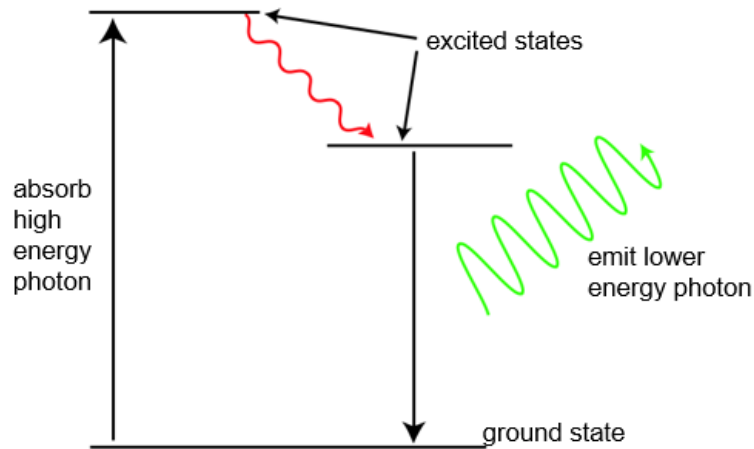


Fig. 2.3 Mechanism of fluorescence [20]

Fluorescent microscope has become very significant in various fields of science where they play major role in the field of biology and biomedical sciences. It has outdated the conventional optical microscope due to its essential attributes such as optical components, filters, shutters, detectors, computer-controlled focus, stage position etc. as shown in figure 2.4. Such characteristics of the modern microscope has enabled digital acquisition of images particularly obtaining the images at the wavelengths which are otherwise visually undetectable.

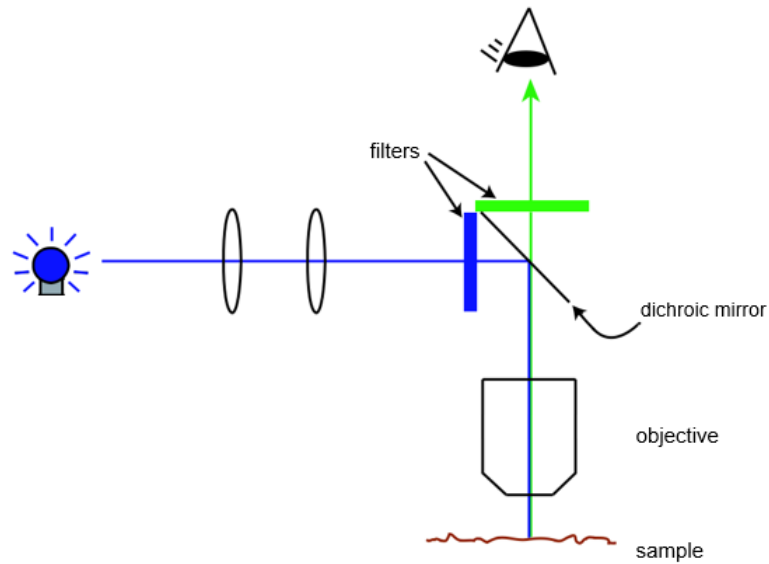


Fig. 2.4 Basic setup of a fluorescence microscope [20]

Confocal microscopy

Confocal microscopy can be understood by imagining a pair of lenses which are focusing light from one lens to another (using their focal point) as shown in Figure 2.5. All the light from the dark point will pass through the pinhole if a screen with a pinhole is placed at the other side of the lens system. Since almost the entire light will get blocked by the screen, it will create an image of the light blue point that is significantly reduced compared to the image of the dark blue point.

The two pinholes of confocal microscope significantly reduce the background haze that is generally created in case of a conventional fluorescence image. The two points, the focal point and the pinhole are known as “conjugate points”. One of the key components in using this technology is to use a light source of high intensity. This is because if enough intensity of light is not used then it will create a noisy image [21]. To create an image in case of confocal microscopy, a detector is attached to a computer. It helps in building the image one pixel at a time.

A confocal microscope is much better in generating images in comparison to other conventional microscopes. The images generated by confocal microscope are sharper and also has the ability to collect data in three dimensions.

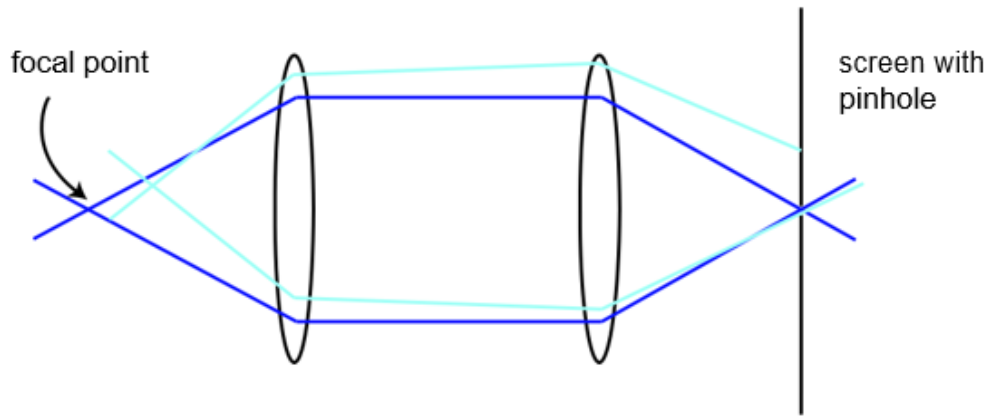


Fig. 2.5 Rejection of light not incident from the focal plane [20]

Microplate reader

A microplate reader is an instrument used to measure chemical, biological or physical reactions, properties and analytes within the well of a microplate. It consists of small wells in which separated reactions take place. The net result of these reactions is to convert the presence of an analyte or the progression of biochemical processes into optical signals as shown in fig 2.6. The MP (micro-plate) reader detects these light signals which are produced by samples which have been pipetted into a microplate [22]. This signal is traced by a detector, usually a photomultiplier tube (PMT). This converts photons into electricity that is then quantified by the microplate reader. This process generates a bunch of numbers which the sample is quantified.

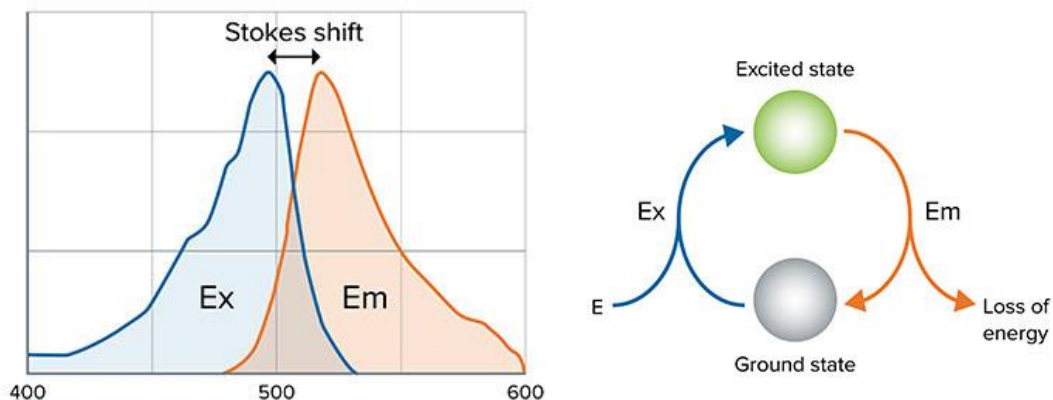


Fig. 2.6 Stokes shift and fluorescence [22]

The optical signal changes during the reaction determines how the samples need to be excited. Depending on the nature of the optical signal changes during a reaction and consequently on the detection mode, samples may need to be excited by light at specific wavelengths. Filters and monochromators are equally employed on the emission/detection side to increase sensitivity.

The optical properties of these samples are the result of a biological, chemical, biochemical or physical reaction. The result of analytic reactions in different optical changes is used for analysis. Absorbance, fluorescence intensity and luminescence are the most popular and most frequently used detection modes in laboratories worldwide [23]. Additionally, advanced modes such as fluorescence polarization, time-resolved fluorescence and alpha screen are also available.

Generally speaking, a xenon flash lamp or LED is used as a light source by microplate reader to excite a fluorophore (fluorescent molecule) at a particular wavelength. The wavelength required to excite the sample can be selected using either a filter of a specific wavelength or a monochromator tuned to the required wavelength.

It can be seen in figure 2.7 that, fluorophore then emits light of a different wavelength, selected by a second filter or monochromator. This emitted fluorescence is detected by a photomultiplier

tube (PMT), and the fluorescence intensity of the sample is expressed as relative fluorescence units.

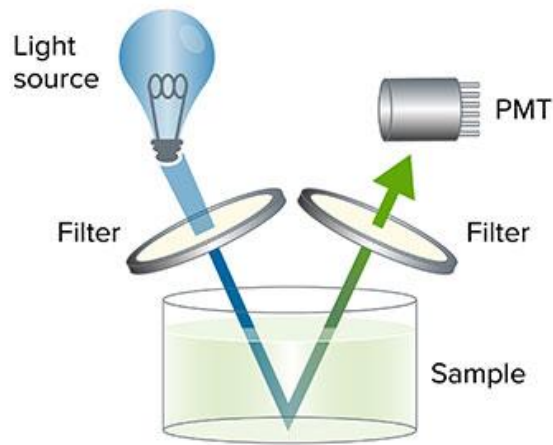


Fig. 2.7 Energy release in ground state [22]

CLARIOstar^{Plus}

These monochromators are based on linear variable filters (LVF), special filters that vary spectral properties over their length, transmitting or blocking specific wavelengths at different positions. LVFs eliminate the need for concave gratings employed in conventional monochromators to separate and mechanically select colored light. A linear variable filters monochromator consists of two aligned linear variable filter slides that separate light into distinct wavelengths and continuously adjustable bandwidths. The CLARIOstarPlus is equipped with two LVF monochromators, one for excitation and one for emission. A very different linear variable dichroic-mirror separates excitation and emission light [24].

CLARIOstarPlus provides support for multiple applications like DNA, RNA, and protein quantification, cell-based assays, enzyme activity and kinetic assays, genotyping reporter gene assays, protein-protein interactions, molecular binding assays. BMG LABTECH's Control Software is using the microplate reader, while data are analyzed with the MARS data analysis software. This two software's are fully compliant with FDA regulation 21 CFR Part 11 and can

be used on multiple PC systems at no extra cost. The control software provides the ability to define protocols and instrument parameters. Finally attempts have been made to simplify and improve assay setup, to have it deployed for filter visualization where in one can witness the integration and luminophores. CLARIOstarPlus shown in fig 2.8 is the one being used for taking measurements during the experiments.



Fig. 2.8 CLARIOstar^{Plus} Apparatus [24]

CHAPTER THREE

DESIGN AND DEVELOPMENT OF DEVICE

Study design

In order to study about the hyperthermia induced by tumor treating fields, first, an engineering set up is needed to induce set of frequencies to panc-1 cells cultured in a cell culture dish. Then, a cell culture device needs to be custom designed in such a way that both top and bottom surface is electrically conductive so that wires can be attached to induce alternating electric field into it. After doing literature study, I grasped few points which needs to be considered while designing this device. Two most important points were: firstly, that such a device should not be toxic to the cells and secondly, this electrically conductive cell culture dish should be reusable after sterilizing it. Based on various factors and limitations, I designed the first preliminary sketch to devise the device as shown in fig. 3.1. After analyzing the requirements of the device, I figured out the various material and parts which would be required to make a prototype of the device.

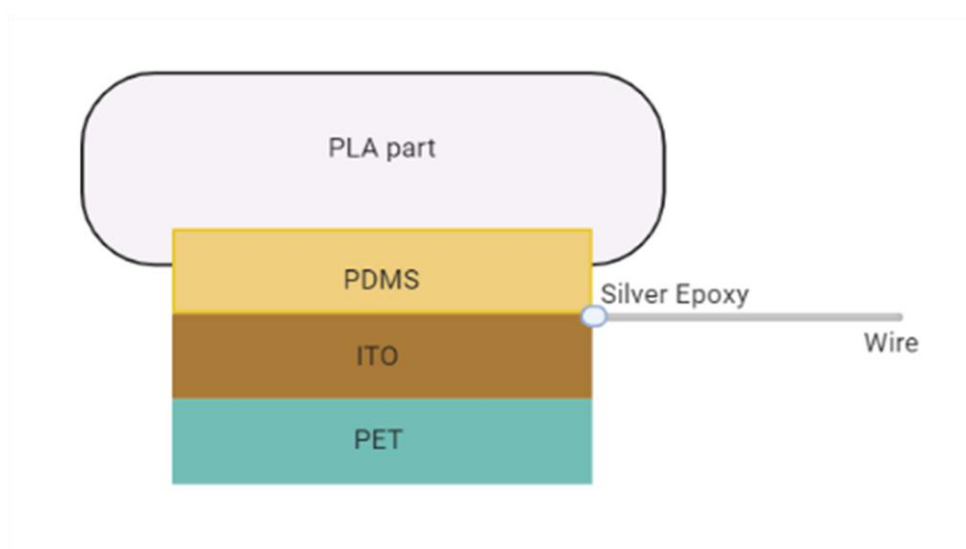


Fig. 3.1 Preliminary sketch of device

Transparent Conductive Electrode (TCE) has become very popular in the electronic industry and has been used extensively in the display devices such as liquid crystal displays (LCDs), solar cells, light emitting diodes (LEDs), organic light emitting diodes (OLEDs) and touch screens. Such transparent electrodes have PET (Polyethylene terephthalate) on one side and ITO (Indium tin oxide) on other side which is electrically conductive in nature. Recently, ITO emerged as an ideal material in electrical devices as a transparent conductive electrode because of its excellent optical and electrical properties. Other properties of ITO like high transparency and low sheet resistance makes it very useful to be used as TCE as in fig 3.2. Conductive electrode is needed to be engraved on the top and bottom side of the culture dish in order to connect it to the frequency generator set up through wires.

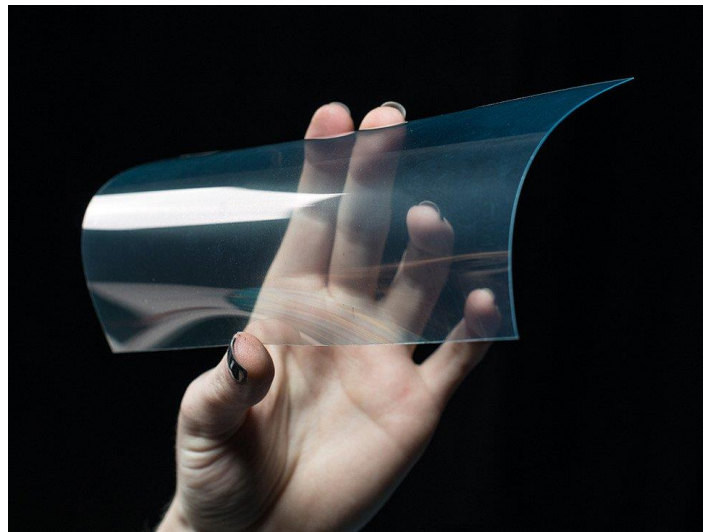


Fig. 3.2 Transparent conductive electrode [25]

Polylactic Acid, shortly named as PLA is an ideal and commonly used plastic filament material in desktop 3D printing for rapid prototyping products for various production or research projects. Basically, monomer of this plastic material is derived from fermented plant starch such as from corn, cassava, sugarcane or sugar beet pulp. PLA is a biodegradable material as renewable resources are used efficiently and economically to produce it. 3D printers like RepRap use PLA

shown in fig 3.3 as a feedstock material for fabrication purpose. I used PLA to 3D print circular grooves called electrode pillar to fit in the electrodes.



Fig. 3.3 PLA 3D printing filament [26]

Polydimethylsiloxane, commonly known as PDMS is an organic polymer based on silicon (polymerized siloxane). It has very unique properties like inert, nontoxic, non-flammable, optically clear, unusual flow properties which makes it being widely used in microfluidic or lab-on-a-chip applications to form devices with defined microstructures. Sylgard 184 is one of the most popular formulations of PDMS used in biological-based research. This is the one, which I used to make the prototype for my experiments, is clearly shown in fig. 3.4. It is transparent encapsulant with good dielectric and flame resistance. It is generally made in 10:1 mix ratio to be used as encapsulant.



Fig. 3.4 PDMS silicone encapsulant elastomer kit [27]

Silver Epoxy Paste is an electronic epoxy adhesive, as shown in fig 3.5 generally used for solderless connections and bonding in electric design due to its good electrical and thermal conductivities. It is a coating formulation based on pure silver which had wide application in flexible optoelectronic devices. Fastest curing times and excellent bond strength is due to its unique combination which makes it popular in screen printing applications. In my experiment, I used it to make a solderless connection on the electrode to connect a wire.



Fig. 3.5 Silver conductive epoxy adhesive [28]

Method development and validation

Preliminary design was created using Autodesk software and all the material to be used were sorted as shown in fig 3.2- fig 3.5. Now, I worked on the process of development of the method to make a workable device and its validation. First, electrode holder design was created using the Autodesk software and 3D printed. These 3D printed electrodes were made up of plastic called PLA. Transparent electrodes were cut in diameter of 2 cm using an electrode cutter. PDMS was prepared by mixing elastomer base and curing agent in 10:1 ratio and spin down at 1200 rpm for 5 minutes. Then, it was used to glue down the electrodes in the electrode holder with ITO on outside so that silver epoxy can be used to connect a wire to the ITO side. Once, all the parts were assembled and glued together, this device was EtO (Ethylene oxide) sterilized in EtO sterilizer as shown in fig. 3.6.



Fig. 3.6 EtO table top sterilizer [29]

Thereafter, panc-1 cells were cultured in this device and it was found that cells do not grow well in it. Either they get infected or do not grow at all. So, this was the problem which needed to be addressed. It was observed that the PDMS placed at the bottom does not cure completely due to less air. So, it might be possible that PET and EtO is reacting with uncured bottom PDMS. I did an experiment to find the root cause of it.

At first, I designed the experiment to test whether the silver epoxy used to connect the wire to electrode was creating problem or not. In order to test it, 6 well plate were taken and electrodes with silver epoxy were fixed down to upper 3 wells using PDMS and in lower three wells, electrodes are glued to the bottom without the silver epoxy. This 6 well plate was kept at room temperature for 48 hours to let the PDMS cure and then EtO sterilized.

Once, its sterilized panc-1 cells were cultured in it and again dead cells were found after 3 days which suggests that it's not silver epoxy but something else which might be toxic to cells. After this experiment, next step was to check whether the air trapped in the PDMS while curing it at room temperature was making some problem to the cell growth. So, an experiment was designed to test it. I prepared two 6 well plate and cured the electrodes to the bottom using PDMS.

Then, one 6 well plate was kept in the desiccator for about 12 hours and other 6 well plate was kept in an oven at 65 °C for 8 hours. After curing and EtO sterilizing the plates, panc-1 cells were cultured in both 6 well plate and observed for about 7 days. Cells cultured in 6 well plate which was put in the desiccator to remove the trapped air were growing well and reached saturation in 5 days. But, it was observed that cells were not attached to the surface rather they were floating in media. While, the cells cultured in other plate were dead after 5 days.

Hydrogels and plasma treated plastics are biocompatible and cells grow on it and stick to it. PDMS and PLA are not cytotoxic to cells. So, cells grow on it which means it does not stop cells to grow. That's why, we used PET surface of the electrode to have cells grow on it. But cells were not adhering to the surface of PET after using desiccator and EtO sterilization which implies that after EtO sterilization, it does not remain the same PET surface. Some reactions might have caused surface of PET unfavorable for growth of cells. It didn't have small surface angle as cells don't adhere to it and get infected eventually. PET surface needs to be well treated to make it favorable for the cells to grow on it. In order to solve this problem, I tried some surface treatment compatible to cells or biocompatible surface coatings like hydrogels etc. I prepared two 6 well plate with electrodes glued to the bottom surface using PDMS and soaked it in ethanol for 24 hours (overnight). Then, it was coated with hydrogel for increasing the adherence of cells with electrode. I tried coating the electrodes with several different wt/volume hydrogel measurement like 0.25 %, 0.50%, 0.75% and 1.0% in PBS and incubated it for 1 hour in an incubator. It is important to sterile filter the hydrogel before making its solution in PBS for coating. Panc-1 cells were seeded after taking it out from the incubator and it was found that 0.50 % wt/volume hydrogel gave the best result as in fig. 3.7. Most of the cells were attached to the hydrogel treated PET surface due to charge interaction. After several experiments and trials, I developed a

methodology to develop a lab made prototype device to do further experiments using confocal microscopy.



Fig. 3.7 Well plate with electrodes at the bottom coated with hydrogel

Prototype

Final version of the device was designed using Autodesk software and 3D printed as shown in fig 3.8. Now, the methodology and right process to make a lab made prototype to culture the panc-1 cells in it for the confocal microscopy experiments was well clear and defined after various validations. First, electrode pillar made up of PLA was designed and 3D printed.



Fig. 3.8 (a) 3D printer (b) Design of top part of electrode pillar (c) Design of bottom part of electrode pillar

Then, PDMS was prepared in a vial by mixing 20ml of sylgard 184 silicone elastomer base and 2 ml of sylgard 184 silicone elastomer curing agent. To mix it well, this vial was spin down at 1200 rpm for 5 minutes followed by vacuum desiccator for 30 minutes to get rid of trapped air. PDMS prepared in this proportion was used to cure the electrodes in 3D printed electrode pillars which were then fixed to the bottom of 6 well plate. Transparent electrode sheets were cut into circular shapes using a cutter and then ITO, PET side resistance was measured using multimeter. ITO has impedance whereas PET does not have any resistance. All parts including 3D printed electrode pillars were ethanol rinsed, wiped and UV sterilized in biosafety cabinet for 24 hours as in fig. 3.9.

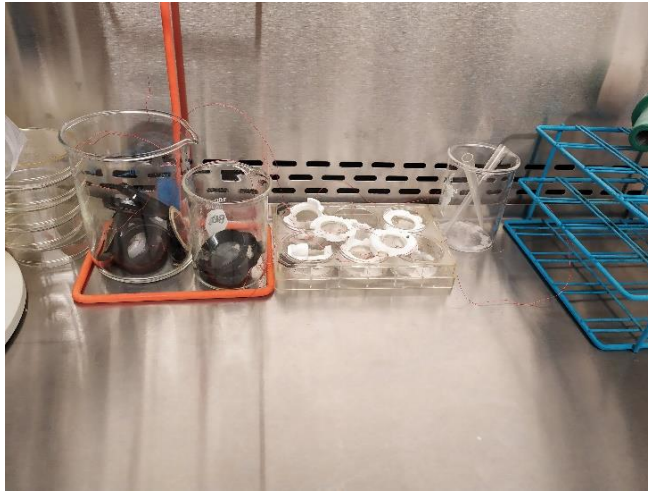


Fig. 3.9 UV sterilization of 3D printed parts in biosafety cabinet

Wire was attached to the ITO side using silver epoxy which was then glued down to the electrode pillar and then cured in the 6 well plate using the prepared PDMS. Then, 1 ml of PDMS was poured in each well of 6 well plate. Circular shape electrodes were ethanol wiped and affixed on the top of PDMS in the 6 well plate followed by placing it in vacuum desiccator for 2 hours. Thereafter, transferred to oven to cure for 1 hour at 65 °C. After curing, it was EtO sterilized for 12 hours. Now, this sterilized device prototype was ready to seed the cells in it. Once it was EtO sterilized, all the work was carried out in biosafety cabinet. 6 well plate was rinsed twice with milli Q sterile water and soaked in it for 3 hours followed by rinsing it with PBS twice and soaked in PBS overnight as in fig. 3.10. After this step, well plate was rinsed with DMEM and UV sterilized for 1 hour before it was seeded with panc-1 cells. After seeding the panc-1 cells, electrode pillar support glued at the bottom was covered with top electrode support with TCE in it connected with a wire. So, there were two wires connected to top and bottom electrode to induce the alternating electric field to the panc-1 cells.

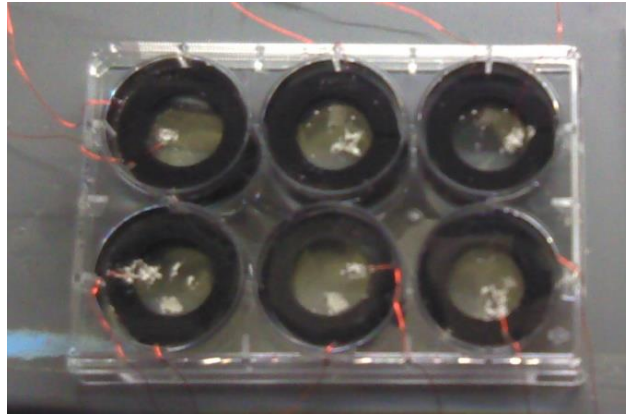


Fig. 3.10 Panc-1 cells seeded in 6 well plate with electrodes fixed in electrode pillars placed at the top and bottom of each well

This prototype was made to do the experiments for inducing the electric field and get the results for live and dead cells. Several experiments were done as explained above in method development and prototype section, before I figured out the right conditions and measurements for inducing the electric fields which effects the panc-1 cells and decrease their numbers by analyzing the live and dead cells numbers.

Experimental trials

Once, lab made prototype was made and tried several times based on various conditions, its customized to a single well prototype. I bought Delta- T culture dish from the company called Biopetechs and attached a wire to the bottom side using the silver epoxy similar to the lab made prototype. Then, in order to induce the electric field from the top, a cylinder shape top electrode support of 2 cm diameter and 1 cm height was 3D printed as in fig. 3.11.



Fig. 3.11 (a) Delta T culture dish (b) Wire attached to the bottom part of culture dish (c) 3D printed top electrode support

Then, a wire was attached to a TCE and glued to this electrode support which was then fixed to the top cover of the Delta T culture dish. This was designed in a way that PET surface of the top part just touches the media in which panc-1 cells were seeded. Utmost care was taken to make sure that the media did not over flow in this culture dish as that could make silver epoxy particles leak in media and be cytotoxic to the cells. Wires, 3D printed electrode support, TCE were rinsed in 200F ethanol twice before they were integrated in the Delta T culture dish inside the biosafety cabinet. Once it's all integrated as one piece, it is UV sterilized for 24 hours inside biosafety cabinet and then panc-1 cells were seeded in it as shown in fig. 3.12.



Fig. 3.12 (a) Electrode support with wire attached electrode fixed to the top cover of Delta T culture dish (b) Lab made device prototype

Few trial experiments were performed in confocal microscope using this prototype device and confronted several problems during these trials. It was found that as top of this lab prototype is not transparent, it made experiments very difficult in the confocal microscope room as top needs to be removed several times during the experiment to make few adjustment which exposes the

cells to the open air and infect the cells. So, there was a need of a transparent top cover for this culture dish so that panc-1 cells can be observed through top light confocal microscope before doing the experiment with the confocal microscope and picoquant software along with the complete electrical set up to induce the TTF's. Other problem was that the wire was connected using silver epoxy which has high probability to leak in media and be toxic to cells. TCE was not completely sealed to the 3D printed electrode support and media was leaked to the other side of the electrode which might interfere with the intensity of the electric field induced. Also, the wetness of the media made the silver epoxy and wire detach from the ITO surface. I figured out that if the wire can be attached to the outer side of the transparent cover, it will solve both problems. There was a high need to have professional construct of this device. So, I worked with a Bioptechs to devise and construct a professional design of this device which I used for further experiments.

Final design

When several prototypes have gone through the trial experiments. Prototype design which was more appropriate for the experiment was selected and 3D printed. Then, I contacted Bioptechs for designing and making more professional version of the device so that precise measurements and experiments can be done.

Professionally designed device received from Bioptechs had the black electrode made up of material known as Black-Ertalyte. O-rings used in it were made up of silver-graphite material and conductive in nature. ITO coated glass attached at the bottom of the dish was 0.175 mm and removable. It had ITO coating on the glass at the bottom of the culture dish whose thickness was 1000 Angstrom as in fig. 3.13. This is where, the wire can be connected. Wire used in this final design of Delta T culture dish was multistrand 26 gauge. There is a Si O ring encapsulated in it as well.

Sterilization of this device was something to be considered carefully. Since, ITO transmits well below 400 nm, thus UV sterilization would work and will not damage the device or its components. ETO sterilization was not recommended at all as some of its part might damage during this sterilization process. Autoclave till 125 °C was recommended and good for sterilizing this device without damaging it as this device is designed in a way where it can be reused for future experiments. This device was fitted into a Biopetechs temperature controller as in fig 3.13 while working on confocal microscope which help to maintain the temperature of the media during the experiment.



Fig. 3.13 Biopetechs temperature controller



Fig. 3.14 (a) Biopetechs device (b) Top part (c) Bottom part

CHAPTER FOUR

EXPERIMENT

Sterilization of Delta-T culture dish

Most effective way of sterilization is the moist heat under pressure which an autoclave does. Steam sterilization inside the autoclave is nontoxic, inexpensive, rapid anti-microbial way of sterilization for laboratory equipment's. Autoclave is a device which uses the heat of the steam to kill all the microorganisms present on the load introduced inside it. It is used in all scientific settings, also in medical industry with some autoclaves having some special cycles, vacuum functions, electric boilers etc. Steam has more latent heat than the water at the same temperature. Hence, it has more energy to break down the proteins inside the cells of microorganisms and coagulate [30]. In order to attain the efficient heat transfer, its necessary to maintain the quality of steam in ratio of 97% steam and 3% moisture to have successful sterilization process. There are few common temperatures which are recommended for steam sterilization such as 250 F, 270 F, 275 F.

Inside the autoclave, items are exposed directly to steam at a particular temperature and pressure for specified amount of time in order to sterilize the items. There are four main parameters to be considered for autoclave steam sterilization which are: Temperature, Pressure, Steam and Time. Pressure is used as a means to obtain specific high temperatures such as 250 F and 270 F for specified amount of time to kill microorganisms. Exposure time inside the autoclave depends on many factors such as type of item, wrapped or unwrapped, sterilizer type etc. [30]. Two main types of steam sterilizers which are widely used are gravity displacement autoclave and high speed pre vacuum sterilizer. These days, disposable test packs are used to enclose the item inside the autoclave and color change of the dot present on the top of the pack indicate whether its sterilized or not [31].

An autoclave is a scientific equipment in which high pressure steam is used to sterilize the items. In the first phase of the cycle, low pressure environment is created by removing the dry air from the chamber. Thereafter, steam is introduced into the chamber through pre purified water source at a pressure above 100 kPa followed by the sterilization cycle. All different kinds of autoclave have one thing common which is a steam chamber as in fig. 4.1 in which steam is generated at the desired temperature for the desired time to attain the sterilization process.

There are the three different phases of sterilization cycle inside the autoclave:-

- **Purge Phase:** In this phase, pressure inside is increased continuously which increases the air temperature to a continuous flow purge.
- **Exposure Phase:** This is the phase when the actual sterilization process takes place by maintaining the desired temperature and pressure for the desired time.
- **Exhaust Phase:** At this phase, slowly pressure is released through an exhaust valve in the chamber which in turn decreases the temperature.



Fig. 4.1 Autoclave Sterilization [32]

In this experiment, Delta T culture dish were sterilized in an autoclave at 270 F for 1 hour. Interster disposable test pack as in fig. 4.2 was being used to enclose the Delta T culture dish inside it and then its inserted inside the steam chamber of autoclave to go through the sterilization

process. And, once the sterilization cycle was completed, the test pack was taken out and the dot present on the top of the pack was observed. If there was a change in the color of the dot present on the top, it means that the item inside the pack was sterilized well. Now, the sterilized culture dish was used to seed the panc-1 cells in it for the further experiments.



Fig. 4.2 Interster disposable test pack [33]

Seed panc-1 cells in Delta T culture dish

In order to seed panc-1 cells in Delta T culture dish, first step was to prepare the DMEM (Dulbecco's Modified Eagle Medium) cell media. It was made by adding FBS (Fetal Bovine Serum) which helps in neutralizing the trypsin and PENSTREP (Pencillin Streptomycin solution) which was an antibiotic preventing any infection in cells. To prepare DMEM media, 10% FBS (50 ml) and 1% PENSTREP (5.5 ml) was added to 500 ml of DMEM media flask. Second step was to seed the panc-1 cells in prepared media to culture the cells which can be further splitted and seeded into the Delta T culture dish. Frozen panc-1 cell line vial stored in nitrogen tank was thawed in water at 37°C for about 2 minutes. Thereafter, this thawed vial was added to 25 ml DMEM flask and then the flask was kept in the incubator for cells to grow. Usually, cell media was refreshed in 2-3 days and cells were split when flask was confluent which was in about 5-6 days. After 3 days of culturing cells, flask was usually less than 50 % confluent, hence

media was changed so that the cells can thrive well in fresh media and the flask was again kept inside the incubator. After 3 days, flask was observed to be about 100% confluent and thus, cells need to be splitted which was the third step. At first, media was removed from the 20 ml flask. Then, 10 ml PBS (Phosphate-buffered saline) was added to the flask as it helps to maintain a constant pH and also removes dead cells. Moreover, the osmolarity and ion concentrations of this solution match those of the human body (isotonic). After a minute, PBS was removed, 3 ml trypsin was added to the flask which was then kept in incubator at 37°C for 1 min. Trypsin was added to detach the cells from the surface just after washing them with PBS. Since, trypsin has an enzyme which works to detach the cells from surface. That's why, media was added after 3-5 minutes of adding the trypsin just to neutralize the trypsin enzyme effect. After 1 min, flask was taken out of the incubator and observed under microscope to make sure that the cells were detaching from the surface. If they were not, then the flask was slowly tapped once or twice to move the cells inside. Once the cells were detached inside the flask, media was added which was twice (6 ml) the amount of trypsin (3 ml). Now, this 9 ml mixture in a vial was spin down at 1200 rpm for about 5 minutes. After 5 minutes, vial was taken out and the cells were collected at the bottom which can be clearly distinguished by its white color. Rest of the solution in the vial which contains the trypsin and media was aspirated out carefully by making sure that it doesn't disturb the cells at the bottom. Then, 4 ml of DMEM media was added to this vial containing the panc-1 cell and its mixed well by aspirating in and out. For splitting the panc-1 cells in the ratio of 1:4, 20 ml DMEM media was taken in a flask. Then, 1 ml out of the 4 ml media containing cells in the vial was aspirated out and mixed in the 20 ml flask. Now, this flask had fewer cells to be cultured for a week in incubator at 37 °C

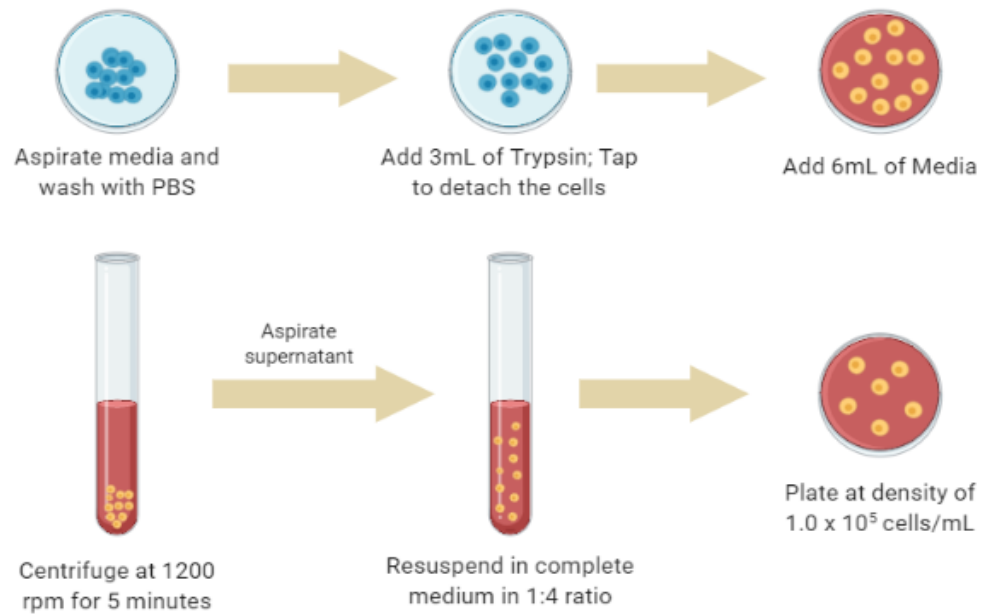


Fig. 4.3 Panc-1 cells splitting process

Cells were counted using Invitrogen automated cell counter as in fig 4.4 and then calculation was done to seed the cells on Delta T culture dish. Cells were plated at density of 1.0×10^5 cells/mL on Biotech Delta T culture dish and allowed to adhere overnight in incubator at 37°C .

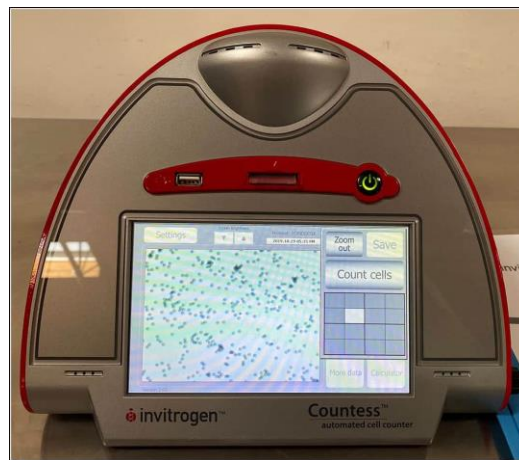


Fig. 4.4 Invitrogen automated cell counter [34]

Stain cells with PEG BODIPY and image under FLIM

Frequency generator device and electrically conductive cell culture dish (Delta T culture dish) engineering set up was devised which was my first specific aim and then second aim was to stain the panc-1 cells with PEG BODIPY dye followed by imaging under FLIM to get the change in lifetime for four different time duration by maintaining the temperature at 37°C. Fig 4.5 shows all the components used in devising the frequency generator device.



Fig. 4.5 (a) Power supply (b) Waveform generator (c) Signal Oscilloscope

All these components were connected through an electronic board and amplitude regulator as shown in fig 4.6 to make an electronic set up to produce different frequencies having the flexibility to alter the amplitude using amplitude regulator. Using this set up, three different frequencies can be induced simultaneously. Software used to change the frequency which needs to be created was Analog Devices, AD9958/59 Evaluation Software.

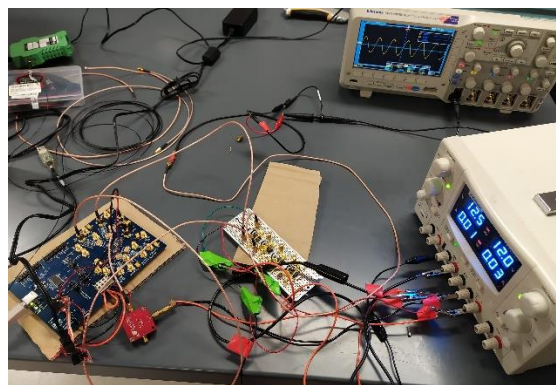


Fig. 4.6 Electronic set up to generate frequency

Once, the electronic set up was configured by me and my labmate Andrew Anderson, electrical field was characterized for panc-1 cells in air as well as in media before preparing for confocal microscopy experiment which is clearly shown in the figure 4.7

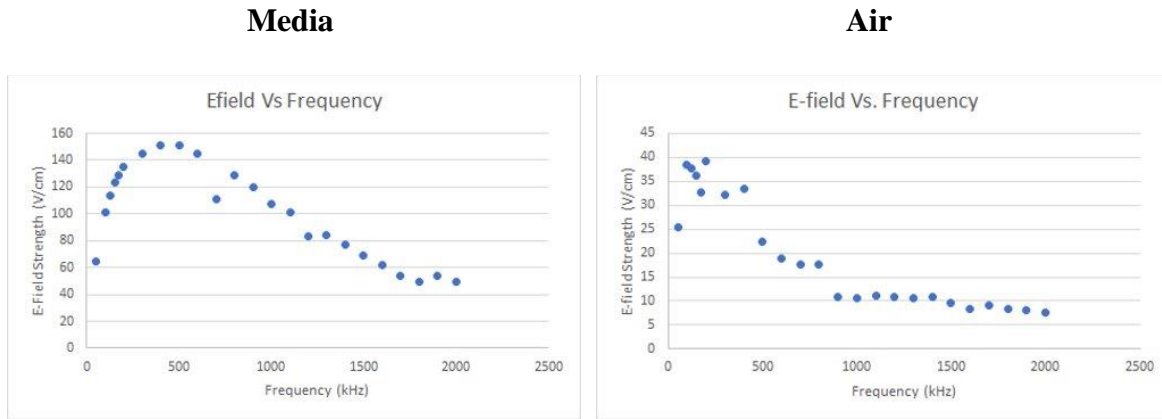


Fig. 4.7 Characterized electric field in air and media

PEG BODIPY dye was synthesized and stored in appropriate conditions for the FLIM experiments. In each experiment, just before staining the panc-1 cells with the dye it is important to check whether dye is still good in terms of its absorbance, fluorescence intensity etc. to stain the cells. So, microplate reader was used to measure the absorbance spectrum of dye in comparison to absorbance spectrum of PBS. Graph shown in fig 4.8 clearly indicates that the PEG BODIPY dye has peak absorbance at 498 nm while PBS doesn't absorb light at any wavelength.

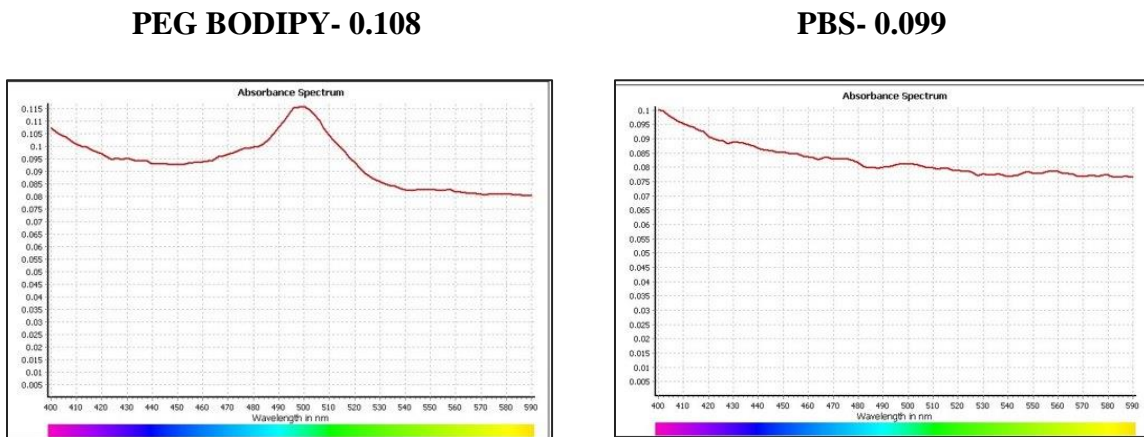


Fig. 4.8 Raw data absorption spectrum from microplate reader

Now, since the dye is good to be used to stain the cells, plated cells in the Delta T culture dish was checked. As, the plated cells adhered to the bottom of the Delta T culture dish after keeping it in the incubator at 37°C for 24 hours. It was taken out of the incubator and prepared for confocal microscopy imaging. Phenol free media and PBS was taken out of the refrigerator and thawed at 37°C. Then, media was aspirated, and cell culture dish was rinsed with 1 ml PBS two times. 500µl of PBS and 500µl of PEG BODIPY dye (100 µM) was pipetted out and poured in the Delta T culture dish which was placed in incubator for 2 hour. Next step was to prepare phenol free media. After 1 hour, stained panc-1 cells in Delta T culture dish was pulled out of incubator and again rinsed with PBS two times. Then, 1 ml phenol free DMEM was added to the dish and it was ready for the confocal imaging.

Now, this culture dish containing PEG BODIPY Dye stained panc-1 cells was to be taken to the microscope in the lab to image the panc-1 cells in order to make sure that cells are stained well and clear images can be taken. Most important part of designing this experiment is staining the cells with right amount of PEG BODIPY dye and getting clear signal to noise ratio on Microscope in order to get the analysis done on healthy cells in one plane. In the previous trials, it happened that many times cells can't be imaged well due to many factors like even distribution or absorption of dye by cells, more absorption of dye can interfere with the light and give more bright blur images. So, several experiments were performed to optimize the time and ratio of dye/PBS for staining the cells. Figure 4.9 shows some of the images of panc-1 cells stained with PEG BODIPY dye.

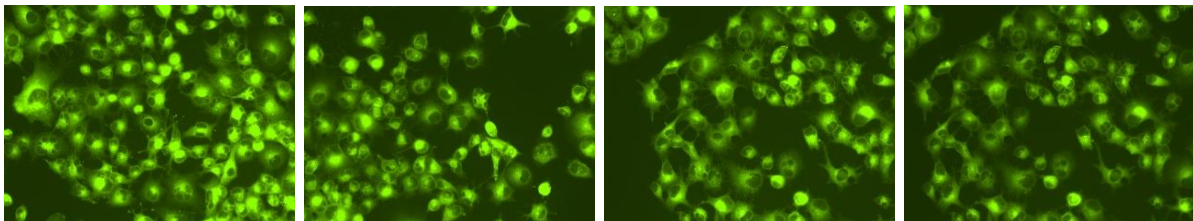


Fig. 4.9 Panc-1 cells fluorescent microscope images

When the cell images are obtained through fluorescent microscope by making sure that the pancreatic cells are well stained. Then, Nikon A1 confocal microscope with picoquant is used calculate the lifetime of the dye with change in temperature. Delta T culture dish is heated using a Bioptechs device called BioTherm DeltaT system. This system can be used to increase or maintain the temperature of the cell culture dish while taking images and doing measurements in the microscope. Software which was used to process the FLIM images was SymPhoTime software. There were many factors and settings which were fixed for all the images taken and processed during all the experiments on confocal microscope, one of which was background threshold. I fixed the background threshold to 1000 photons/pixel for every measurement taken and each decay was fit to a biexponential decay.

Synthesized BODIPY dye is a nanothermometer which gives information about the temperature change within the cells from its lifetime change as lifetime of this dye is inversely proportional to the temperature. Lifetime measurement would give the clear indication of temperature increase/decrease in cells.

Tumor Treating Fields (TTFields) are actually alternating electric fields which have low intensity (1-3 V/cm) and intermediate frequency (100-500 kHz), non-invasively applied to the patients.

I did hyperthermia study on pancreatic cells by introducing TTF in them to test whether heat play any role in killing the cancer cells or not. I hypothesized that the tumor treating field at 150 kHz frequency induce hyperthermia in pancreatic cancer cells.

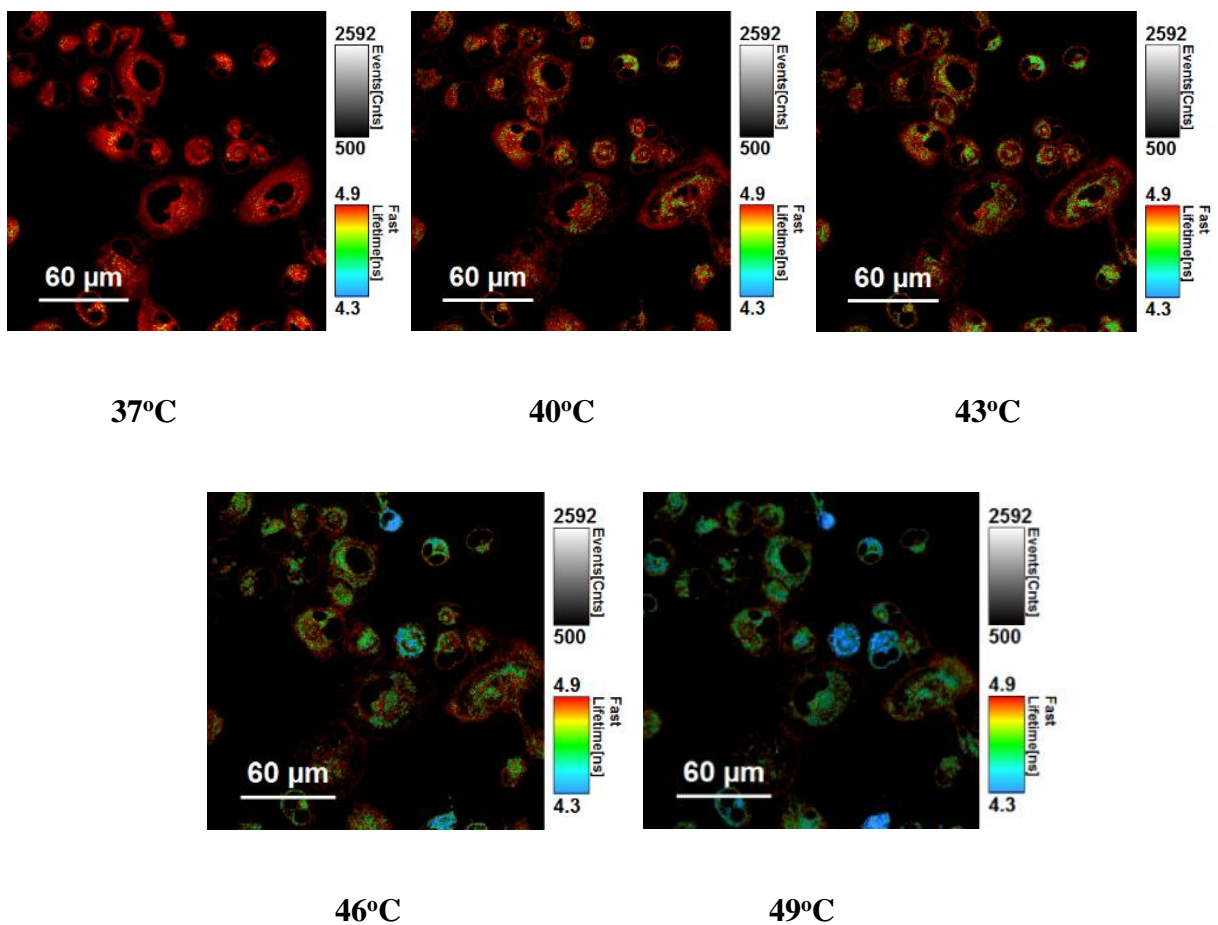


Fig. 4.10 Panc-1 cells stained with PEG BODIPY dye FLIM images at 5 different temperature

Above figure 4.10 shows hyperthermia in pancreatic cells. Pancreatic cells were imaged at five different temperature 37°C, 40°C, 43°C, 46°C, 49°C using PEG BODIPY, a nanothermometer. Temperature is increased and maintained by Delta T culture dish temperature controller which has a temperature range of ambient to 50° C. So, this set up is designed to be stable till 50° C. It was probed using picoquant in confocal microscope measurements that the lifetime of the dye decreases with the increase in temperature from 37°C to 49 °C. Change in color of pancreatic cells from red (37°C) to Green/Blue deciphers (49°C) deciphers that the lifetime of BODIPY dye is decreasing. Color which is seen in this picture is just a virtual way to depict the change in temperature. Usually, a reference color is chosen from the available color scheme in the picoquant

software which is kept as same in every measurement of that experiment in order to see the difference in color at different temperature or time. In figure 4.10, red color was chosen as the reference color as it was available in the color scheme settings at that time and at different temperature cells have changed their colors. In the last picture at 49 C, some cells are blue in color and some are green. It might be possible that the proteomics of the cells might not be same. Blue cells might have different viscosity than green color cells. Since, the dye is sensitive to the change in viscosity. Its fluorescence and hence, its lifetime is different as the viscosity of the cell changes. This is why, some cells are green and some appear blue in picture taken at 49 C. Limitation of this study is that an assumption is made that the viscosity is almost the same in all panc-1 cells. In reality, condition of all cells might not be the same and cellular changes in viscosity leads to many other changes in cells including, temperature, pH, calcium influx, cell viability etc. Also, another possibility is that the blue cells in figure 4.10 are balled up and thus, might be undergoing necrosis. Due to which, there would be change in viscosity between blue cells and green cells but not temperature change. This is the limitation of this study as its assumed that there is no viscosity change in panc-1 cells and thus change in color or lifetime represents only the change in temperature which is not true in all conditions.

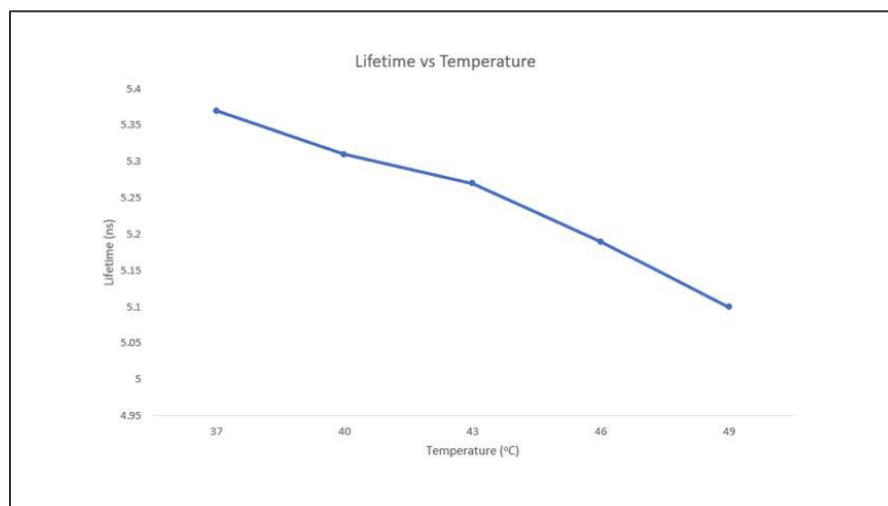
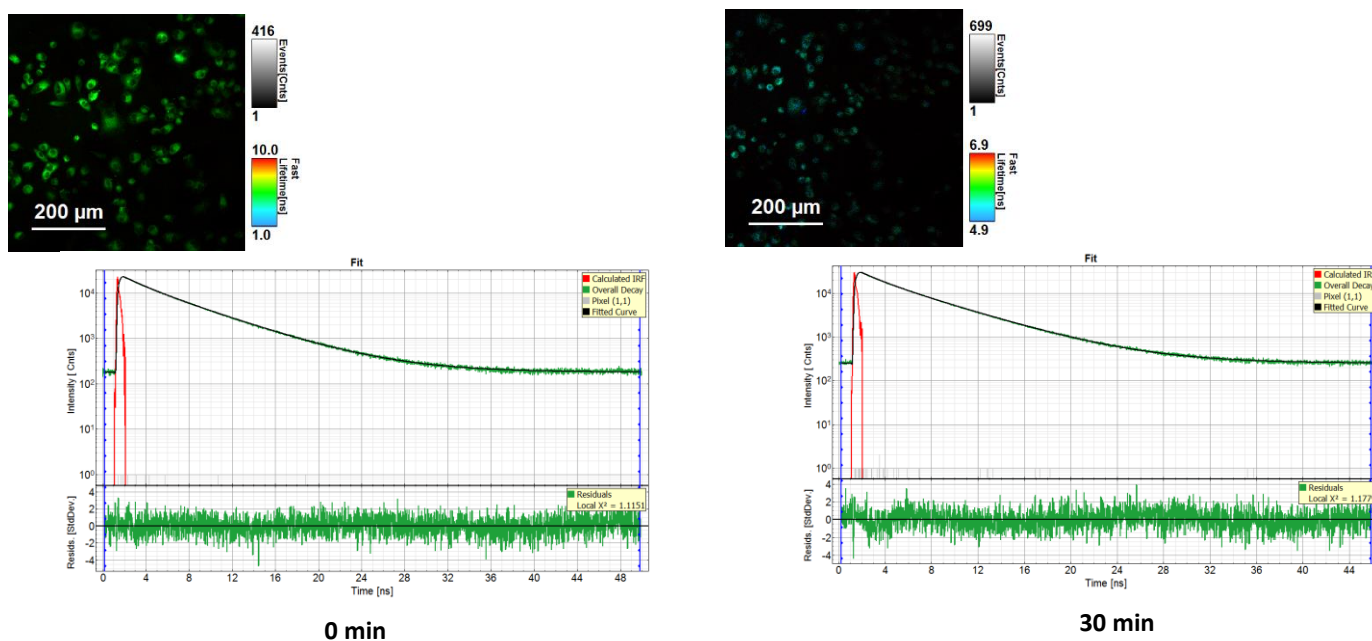


Fig. 4.11 Plot of the lifetime component versus temperature showing a quadratic dependence with temperature (blue curve)

Figure 4.11 is a graph which shows that lifetime of the dye has negative correlation with temperature. In the second set of experiment, 150 kHz TTF was induced in pancreatic cancer cells using frequency generator set up along with developed Delta T culture device (temperature controlled) developed in lab in a confocal microscopy room. Images were taken and measurements were done for four different time duration- 0 min, 30 min, 60 min, 90 min. Then, measured the lifetime of the BODIPY nanothermometer which was 5.37, 5.32, 5.29, 5.10 respectively as shown in figure 4.13.

There were many factors and settings which were fixed for all the images taken and processed during all the experiments on confocal microscope, one of which was background threshold. I fixed the background threshold to 1000 photons/pixel for every measurement taken and each decay was fit to a biexponential decay as seen in figure 4.12. It shows the overall decay of the dye with time at fixed photons/pixel.



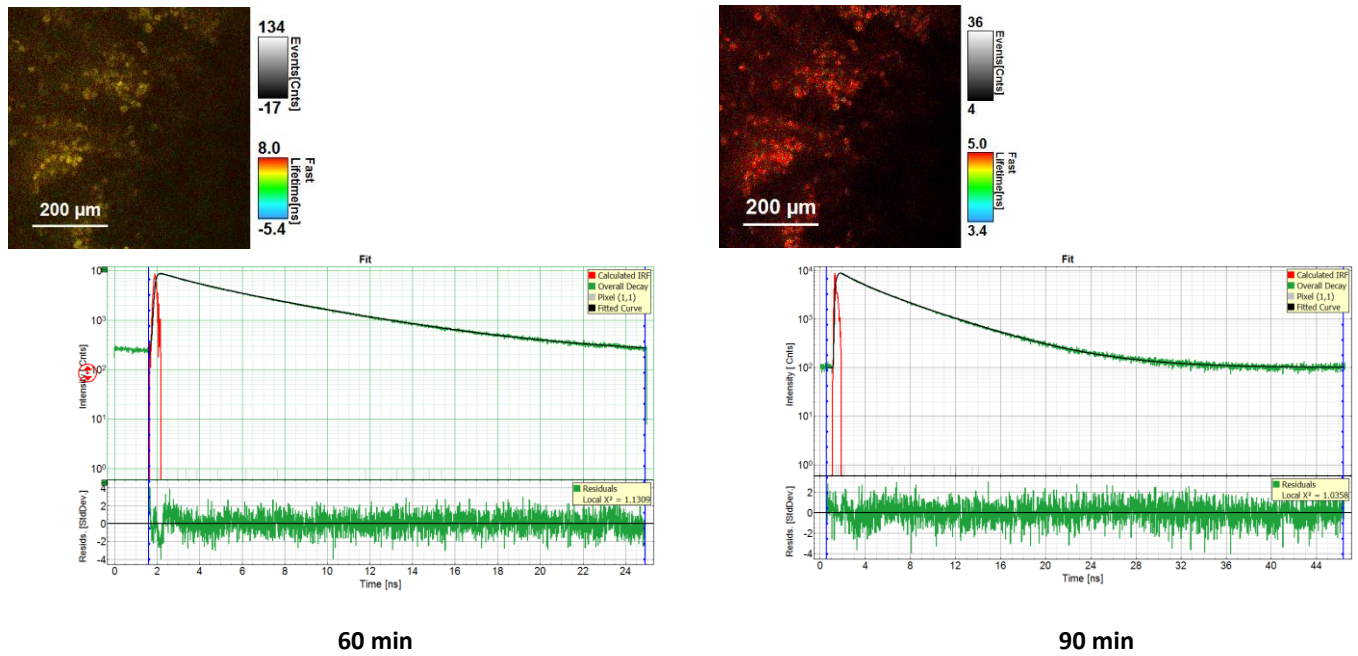


Fig. 4.12 Overall decay of the dye with time

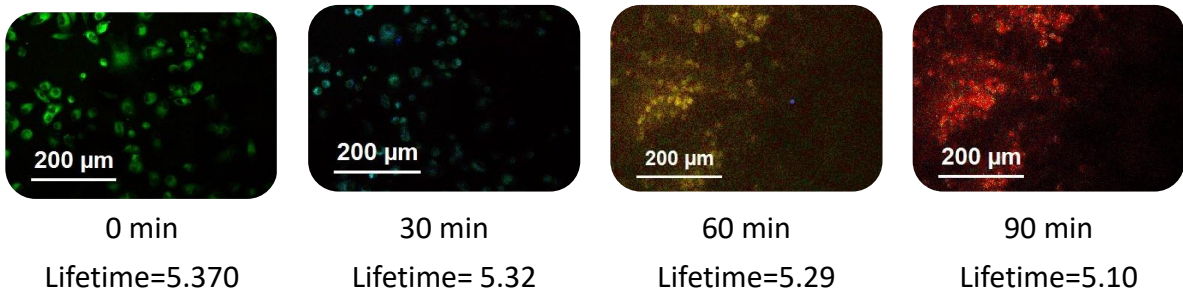


Fig. 4.13 Panc-1 cells lifetime stained with PEG BODIPY FLIM images at 4 different time duration maintained at 37 °C

So, the lifetime is decreasing here with time which deciphers that the temperature is increasing. This gave us the information that the temperature does increase with the time when 150 kHz frequency is induced in panc-1 cancer cells. In figure 4.13, green color was chosen as reference and thus as the time increased for inducing the TTF's. There was change in color from

green to red. As the reference color is changed here in comparison to figure 4.10, with increase in time, green color changed to red color which is just a pictorial or virtual representation of increase in temperature.

One of the biggest limitations of this study is that it is known from the previous study, PEG BODIPY dye is sensitive to temperature and viscosity both but it shows no sensitivity in viscous media. This dye is sensitive to viscosity only in less viscous liquids like water whose viscosity is less than 10 cp. Since, the viscosity of the cell fluid is more than 10 cp, due to which this dye doesn't show any change in its viscosity. So, its assumed that viscosity of all the cells are the same. But, during the experiments it was observed that proteomics of the cells plays a vital role. When TTF is induced in the cells or when the temperature of the cells is increased, few cells undergo necrosis which is usually characterized by nuclear condensation, organelle swelling and finally the rupture of the plasma membrane. Specific intracellular proteins are released when cell is going through necrosis which stimulate an inflammatory response. It was studied and analyzed that cells undergoing necrosis are usually balled up and have different proteomics than the live healthy cells due to which their viscosity gets changed. It might be possible that their viscosity might be less than 10 cp to which PEG BODIPY dye is sensitive. Thus, dye is no more providing the temperature change but the viscosity change. This is the possible explanation that few cells are blue in figure 4.10 whereas few are green.

Other limitations of this study is that this study is limited to only one Tumor treating field i.e 150 kHz, for only one type of cancer cell which is pancreatic cancer cell line. There are many factors which could be affecting the experiment performed during this study like change in pH, changes in electrolyte gradient, loss of membrane integrity, changes in nucleus etc. which should have studied as the cells were going through necrosis due to the introduction of TTF's.

CHAPTER FIVE

CONCLUSION

Tumor Treating Fields (TTFields) is the fourth modality which is been recently added to the conventional treatment of cancer which is surgery, chemotherapy and radiation therapy. Intermediate and low intensity frequencies are delivered non-invasively through the insulated transducer arrays incorporated in the skin patch which is placed on the skin in the region surrounding the tumor. Hydrogel which is conductive in nature is applied on the skin before placing the skin patch comprising of transducer arrays. It also helps in redness, itching and irritation of skin due to the prolonged application of TTF's around that region.

In our experiments, we have analyzed whether introduction of the Tumor treating field (150 kHz) induce hyperthermia in PANC-1 cells. Hyperthermia study of tumor treating field is not yet done. Several studies on radiation and chemotherapy in combination with TTFields have been published which provides detailed information in combination therapy effects but nothing is been published related to hyperthermia caused by TTFields. So, this is very new area of TTFields which is yet to be explored. In theory, TTFields do not stimulate nerves and muscle because of their high frequency, and do not generate heat because of their low intensity. But, there is no study to support it yet. It's still not known whether heat also play a role in inhibiting the cancer growth and killing cancer cells. Mechanism of tumor treating fields is still under research. Once, we know that TTF is causing hyperthermia in cancer cells, we have a long-term goal of doing further research on the mechanism underlying tumor treating field disrupting the cell cycle of cancer cells.

We also devised a frequency generator and electrically conductive cell culture dish device, complete engineering set up while working on this experiment. Frequency generator set up was used extensively in the confocal microscopy room for inducing 150 kHz TTF in pancreatic cancer

cells plated on the Delta T culture dish. To test whether introduction of the TTF induce hyperthermia in PANC-1 cells. Approach was to first stain the panc-1 cells with PEG BODIPY dye which is nanothermometer. It was found through experiment that lifetime of this dye is inversely proportional to the temperature of the cells. Then, induced 150 kHz frequency to the stained cells and measured the lifetime of the dye using Picoquant in FLIM microscopy. Lifetime measurement deciphered whether the temperature is increasing or decreasing within the cells. This experiment provided the information that temperature does increase with the time when 150kHz frequency is induced in panc-1 cancer cells. Complete thesis work was divided into three main parts, first was the design of engineered experimental set up and second is staining the cells with right amount of dye and getting clear signal to noise ratio on microscope in order to get the right picture and healthy cells in one plane. Third was to induce the TTF in panc-1 cells while controlling the temperature and time duration in confocal microscopy. Results of the experiment suggests that the panc-1 cells get heated with time due the TTF introduction. This finding would help in investigating further on mechanism of TTF's.

FUTURE WORK

In future, I would like to study and design experiments to get comprehensive understanding of TTF treatment and biological mechanism how it interacts with DNA which in turn contribute to cell growth inhibition. I would like to do experiments on single cell hyperthermia using FLIM Microscopy where 13.56 MHz, 1 MHz would be induced to produce hyperthermia effect which would be used to do comparison studies. Furthermore, I would develop the strategy for real time imaging. Then, comparison studies would provide detailed insight into the hyperthermia studies on different frequencies.

There is a lot to explore in the area of TTF fields which extends to include new and previously unstudied solid tumors. I would like to investigate and perform experiments to analyze the effects of TTF fields on the actively dividing noncancerous tissue [14]. In addition, there are many possible directions which could be area of investigation in future including modifications in the frequency, amplitude and the intensity of electric field applied. Optimization of the array layout is also one of the future works which could be developed using nanoengineering

It is known that due to the disruption in the mitotic activity of the cells, they become non-viable or undergo the process of necrosis with the introduction of tumor treating fields. Proteomics of the cells changes while they go through necrosis which effects many factors of the cell such as temperature which was studied during this work, intracellular pH, membrane integrity, cell viability, electrolyte gradient etc.

So, in future I would like to do detailed analysis of the change in all these factors with the introduction of TTF's in different types of cell. In order to check the viability of the cell, resazurin which is dark blue color cell permeable dye can be used. The way it works is that it gets reduced to resorufin by the active metabolism of the viable cells which can be clearly seen in the microscope as it is pink in color and fluorescent. Sodium green dye can be used to determine the

change in electrolyte i.e. sodium in the cells while going through necrosis [35]. Dye which can be used to ensure the membrane integrity of the cell is propidium iodide (PI) which gets easy access into a dead cell due to its compromised cell membrane and binds to its DNA. Once it binds to the DNA of a dead cell, its emission intensity increases and becomes fluorescent which clearly indicates the membrane integrity of a cell. There are many dyes or proteins which can be used as a pH indicator in cells. Most common one is green fluorescent proteins (GFPs) whose fluorescence intensity increases with increase in pH [36]. It is known from previous studies that pH of the cytoplasm is around 7.2, cell organelles is 5 and dying cells might exhibit lower pH due to the process of apoptosis or necrosis. Such changes which constitute the proteomics of the cells is a major consideration for the future work, which is also closely related to the work done in this thesis. It will be a good addition to the tumor treating fields research.

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VITA

Ruchi Singh was born in Kanpur, India, the daughter of Sudha Singh and Anil Kumar Singh. After completing her work in high school in India, she decided to pursue an undergraduate degree in India. She received the degree of Bachelor of Technology in Chemical Engineering from Kanpur University in July 2009. In her under graduation, she conducted research in Dr. Ashutosh Sharma lab on an industrial project of Proctor and Gamble investigating the nano polymers for self-heal paints. Thereafter, she decided to pursue Master of Science in Energy Science (Nanotechnology) from Sungkyunkwan University, South Korea. While working in the nanotechnology lab with Dr. Didier Pribat, she worked on copper polishing, its synthesis and application in NEMS devices.

After graduating from Sungkyunkwan University, Ruchi joined GSBS Master's Program in Biomedical Sciences at the University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences in Aug 2018.