The Effects of Weak Static and Pulsed Magnetic Fields on Fibroblast and Fibrosarcoma Cells Under a Controlled Magnetic Environment

by

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
Abstract

This thesis provides evidence supporting the claim that weak static and pulsed magnetic fields have measureable effects upon fibrosarcoma cells. Fibroblast and fibrosarcoma cell lines were exposed to 0, 100, and 200µT fields, with a static 45µT field as the control. Each exposure intensity, other than 0µT, was conducted at a 20 sec pulse repetition rate, as well as the normal static conditions. The cells were tested for variations in oxidative stress, membrane potential, and cell viability. The fibrosarcoma cells were also tested for cell count. The trials were conducted using an electromagnetic system that provided a controlled magnetic environment by blocking out all external magnetic fields.

It was found that the magnetic fields did indeed produce changes in cell activity, as well the creation of result variability with respect to exposure strength, but did so without an overall discernable trend. More experiments need to be conducted in order to determine which magnetic fields could be used in therapeutic devices, whether in cancer or other disease treatments. They also need to be completed to better support or deny the spin flip theory that provides a mechanism for extremely weak magnetic fields to interact with cells. The results do show though, that the exposure system detailed in this thesis is useful in conducting cellular experiments and should be used in cell incubators during other experiments to eliminate exposure to unknown magnetic fields.
Dedication

To my parents and family, who have always taken care of and loved me beyond any degree that I could ever show enough gratitude for.

And to Dr. Frank Barnes who gave me a chance to do research, entrusted me with his work, and always provided truths and stories.
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Chapter I

Introduction

In order to properly orient the reader, this introduction will first present the impetus behind the study undertaken. It will then provide a general overview of the different subjects covered and referenced in this dissertation. This induction into the motivations and background information will lead to the theory and hypothesis that in turn shaped the experiments.

Investigation Overview

This investigation is concerned with discovering how extremely weak static and pulsed magnetic fields (MF) affect fibroblast and fibrosarcoma cells. For many years, it was believed that such weak fields did not disturb cellular processes because no physical mechanism had yet been detailed that defined such an interaction. However, recently several mechanisms, including that of altering free radical electron spin states, have been connected to viable interactions and provide legitimate theories to substantiate claims that the MFs employed in this study can alter cellular function [Barnes et al., 2015]. This dissertation uses a custom made electromagnetic (EM) setup and μ-metal shield, an important element that provides a controlled magnetic environment in which to expose the subjects. In order to elucidate the effects that the fields would have on healthy vs. cancerous cells, the subjects were chosen as primary dermal fibroblasts (PDF) and fibrosarcoma
(HT-1080) cells, respectively. Oxidative stress, membrane potential, and cell viability assays were employed in order to gain a better perspective of several different processes that could be affected by extremely weak MFs.

Purpose

These set of experiments set out with five main objectives in mind:

1. Uncover cellular processes in healthy human cells that are altered by extremely weak static and pulsed MFs.
2. Uncover cellular processes in cancerous human cells that are altered by extremely weak static and pulsed MFs.
3. Determine the MF strengths and pulse repetition rates that have the greatest impact on the cells examined.
4. Compare the results in objectives 1 and 2 to determine if extremely weak MFs could be used for therapeutic purposes in cancer treatment.
5. Test the effectiveness of the shielding and exposure system described later in this thesis. If objectives 1 and 2 prove that low level fields do in fact change cellular responses, then demonstrate the value of said system.
Bioelectromagnetics Overview

The field of bioelectromagnetics is one that is not well known to the public. However, with the ever increasing ubiquity of electronic devices and pervasion of electromagnetic signals, it has become increasingly important. As its name suggests, it occupies a space between biology and electrical engineering. Therefore, this cross-disciplinary study necessitates at least perfunctory knowledge in both subjects in order to better evaluate the findings presented in this study; however some background information will be given here to supplement subjects that this thesis touches on.

i. Areas of Research

Bioelectromagnetics (BEM) pertains to the study of how electromagnetic fields (EMF) interact with and affect biological systems. Many topics fall under its sphere of influence, including: measuring and modeling tissue properties, understanding endogenous processes, imaging and diagnostic techniques, electromagnetic therapy, determining interaction mechanisms, and establishing exposure hazards. This investigation aspired to contribute mostly to the final three topics; EM therapy, interaction mechanisms, and exposure hazards. More information about the other topics is located in Appendix Section A.

ii. Area of Research: Therapy

Therapeutic devices and practices are a major end goal of BEM research. The realization that EMFs could be used for therapeutic purposes has been around for many years, but most of the possibilities yet remain untapped. However, there are a few impressive examples that evidence the
medical power of certain EMFs. Today patients suffering from non-union bone breaks can be healed using pulsed electric fields (PEF) generated by a coil wrapped around the injured limb, which mends the ill-formed bone [Gupta et al., 2009]. AC current used in electrical stimulation (ES) via implanted electrodes can help victims of spinal cord injuries regain partial control of systems such as slight motor control, perception of sensation, and bladder function (Fig. 1) [Pullar, 2003; Hamid et al., 2008]. Also, brief intense voltage pulses are used in electroporation to open cancer cell membranes to facilitate delivery of chemotherapy drugs, which provides more effective and safer oblation of tumors [Gothelf et al., 2003].

Figure 1- An implanted ES device designed to help patients regain functions lost due to spinal cord injury [He et al., 2008].

As objective (4) from the Purpose section highlighted, a desire of this study was to determine whether or not the MFs used here could kill or arrest cancer cells, while not harming healthy cells. This differential effect would provide another way that EMFs could provide a safe, localized way of treating cancer. The devices used for that act would also be fairly straight-forward and cheap if kept to the essentials.
Area of Research: Interaction Mechanisms

In order to know fully the implications of EMF effects on a biological system and be able to accurately predict reactions, the mechanisms by which the EMF directly interacts with that biological system must be known. Because of both the many characteristics of EMFs and the highly interconnected nature of biological systems, it is an arduous task to be able to predict how EMFs will modulate an organism. However, by focusing on EMFs in groups some direct mechanisms can be laid out. Strong static MFs not only have the ability to attract or repel objects, but they can reorient polar molecules as well by subjecting them to a magnetic torque. Time-varying MFs, such as radiofrequencies (RF) can generate eddy currents, which can stimulate nerves. RF fields also have the ability to generate thermal effects on tissue, based on how much energy the tissue absorbs; this is represented by the specific absorption rate (SAR)(W/kg). These are well known effects and are utilized for equipment both in and out of the medical arena, such as MRI machines and RF sealers, respectively [Ueno et al., 2007].

Historically, extremely weak MFs were taken to be insignificant in causing changes in cells because no mechanism of interaction was detailed. Later in this paper the mechanism subscribed to by our lab, the electron spin flip mechanism, will be detailed. However, other possible mechanisms could exist and should not be overlooked. The first involves an effect on the movement of electrons on large molecules, like initiating DNA-mediated charge transport. This involves the DNA acting basically as a conducting wire and with the result being the activation or deactivation of proteins bound to the DNA due to electron transfer to or from them [Schaefer et al., 2014]. A similar conformation change could occur in membrane bound proteins or possibly in molecules with
transition metals embedded in their structure. One such molecule happens to be superoxide
dismutase (SOD) a chief enzyme in the regulation of the superoxide free radical [Halliwell et al.,
2007].

iv. Area of Research: Exposure Hazards

In everyday life humans are exposed to many sources of potential harm. One of the areas of
BEMs research that is heavily funded is the evaluation of EMFs on human health, which in turn
helps inform exposure limits and device safety characteristics. Large scale epidemiological studies on
whether or not cell phones cause brain tumors seem to have become the poster child of BEMs
research. Extremely low frequency fields, such as those 60Hz generated by power lines, are also
under scrutiny. These studies garner a lot of attention; however it is extremely difficult to decipher
any sort of conclusion from the thousands of cases and all of the confounding factors introduced.
This investigation presented here was formatted so that the lessons learned on extremely weak static
MFs could be used to determine whether or not people are threatened by some of the fields that
surround them in daily life [Feychting 2007].

Cellular Systems

The fibroblast and fibrosarcoma cells used are healthy and cancerous counterparts,
respectively, of one another. This section not only provides information on free radicals and
membrane potential, but it also expounds upon how these two subjects play a role in the healthy vs.
cancerous cell discussion.
Free Radicals

Free radicals are molecules that carry with them a dual nature that is both potent and intriguing. They are vital player in many cell signaling pathways while in controlled concentrations; however, when an imbalance arises, they can disrupt these pathways and also lead to extensive damage of cellular macromolecules by directly oxidizing these molecules. Their behavior is due to a highly reactive nature, brought about by having one or more unpaired electrons. Mostly consisting of reactive oxygen species (ROS), such as (O$_2^-$, H$_2$O$_2$, OH$^-$) and reactive nitrogen species (RNS), such as (NO, ONOO$^-$), they are an unavoidable byproduct of metabolism occurring in the mitochondria. Normally they are kept in check by antioxidants or reducing enzymes, such as superoxide dismutase (SOD), which all-together play a homeostatic role in oxidative stress [Halliwell et al., 2007].

Malfunctioning ROS pathways play a pivotal role in maladies such as Alzheimer’s disease, metabolic syndrome, hypertension, aging, and many others; including tumor formation. Tumor formation can occur in the absence of proper apoptosis, programmed cell death, which can be initiated when a cell senses its DNA is too damaged to keep splitting into healthy daughter cells. Normally tumor suppressor proteins, like p53, are in the cell’s arsenal to safeguard against these events by either choosing to repair DNA, activate antioxidant responses or starting apoptosis. p53, however, is controlled by concentrations of the ROS hydrogen peroxide (H$_2$O$_2$) and when H$_2$O$_2$ levels do not represent the cell state they are supposed to, then p53 gets improper instructions with regard to apoptosis. In cancer cells metabolism is also upregulated and free radicals are present in elevated concentrations, displaying how free radicals play a vital role in the creation of cancer and in how it differs from normal cells [D’Autréaux et al., 2007].
ii. Membrane Potential

The membrane of each cell is a phospholipid bilayer that acts like a fluid mosaic with proteins, cholesterol, and other entities studding it. Between the intra and extracellular environments resides the membrane potential in the form of a voltage difference ($V_m$). $V_m$ is caused by concentrations of ions, like Na$^+$, K$^+$, Cl$^-$, and Ca$^{2+}$, existing in different concentrations across the lipid bilayer. Membrane proteins control the flow of ions in and out of the cell, which results in a modulation of membrane potential. Changing potentials beyond a threshold value is what initiates action potentials and sparks the firing of nerve and muscle cells [Yang et al., 2013]. Free radicals can modulate $V_m$ as well. Exposure to $O_2^-$ or $H_2O_2$ has been shown to initiate a transient increase, then exponential decrease in the potential of renal cells [Scott 1987].

Transmembrane potentials are also integrated into the overall physiological state of the cell. In general, the $V_m$ of cancer cells is more depolarized than of their non-cancerous counterparts; healthy non-dividing fibroblasts exist around -70mV and fibrosarcoma cells are around -40mV. This relationship though is not a simple one way street. By hyperpolarizing cancer cells researchers were able to arrest their cellular divisions, halting proliferation. They then reinitiated mitosis by artificially depolarizing the cells back to their cancerous depolarized state, showing that membrane potential itself plays a role in this healthy vs. cancerous relationship [Yang et al., 2013].
Previous Studies

The following experiments that took place previously in Dr. Barnes’s lab laid the groundwork for this study and influenced the mechanism theory detailed in the next section.

i. Weak Magnetic Fields Create Variations

One of the principle investigations was that of Cynthia Bingham. She conducted tests on mastocytoma (P815) cells by exposing them to DC and extremely low frequency (ELF) AC fields at field strengths between 0.003G and 1.0G (0.3µT and 100µT) and found that the proliferation of these cells was significantly altered differently, depending on the field strength and frequency. At the time, fields that were this weak were not expected to cause any significant changes in cell behavior, because no physical mechanism had been outlined. What this did was jump-start the investigation into weak magnetic field effects, as well as energize the search for a mechanism that these changes could be attributed to [Bingham 1996].

ii. Magnetic Field Induced Free Radical Increase Implicated in Proliferation Changes

The work of Carlos Martino further looked at extremely weak MFs and he discovered that indeed proliferation of endothelial cells could be increased by 40% by fields of 120µT. Martino went further to state that eNOS production increased and VEGF expression did not change. This means that the cells were rejuvenated, yet did not exhibit a tendency for increased tumor development, respectively. He then was able to pinpoint an increase in free radicals as the chief culprit behind the rise in cell count by introducing free radical scavengers and observing a reversal in the proliferative
changes produced by the MF. This led him to endorse an increase in free radical populations as one of the incipient reasons for the observed effects [Martino 2008].

iii. Inhomogeneous Nature of Cell Incubators

Lucas Portelli’s work focused on the inhomogeneous nature of cell incubators. During cellular experiments, the results hinge on having the environment the cells are placed in reproduced accurately from trial to trial. Portelli took temperature and MF measurements in 21 incubators at 27 locations within each incubator. He discovered that the static MFs could vary up to 450µT within the same incubator. He then went about developing a µ-metal shield system that blocks out the unaccounted for fields inside the incubator and re-induces the desired field by using coil(s) within the µ-metal enclosure. This is the origin for the exposure setup used in this investigation [Portelli 2012].

iv. Temperature Oscillations Arrest Fibrosarcoma Cells

Aditya Kausik used oscillations in temperature +/- 1.25 °C and weak magnetic fields +/- 100µT to decrease proliferation of cancerous cells in the form of fibrosarcoma up to 60%. The periods he chose of 7, 13, 20 and 25s were used to try and tap into the cell’s natural rhythm of molecular oscillations, such as Ca^{2+} and NADPH releases. Kausik also showed that these oscillations decreased levels of intracellular NADPH, an enzyme involved in cell redox processes, by 50% with the varying temperature, which strongly pointed to possibility that the oxidative stress inside the cell
was altered. He accomplished these experiments using the setup that Portelli invented in order to have complete magnetic control [Kausik 2014].

Mechanism for Change

Instead of dismissing extremely weak fields as insignificant in altering biological systems, this theory dives into the physical chemistry of electrons to show how seemingly ineffectual amounts of energy actually do have the potential to create change within cells.

i. Free Radical Pair Recombination Theory

When a radical pair is together, it consists of two molecules that are bonded by electrons in the singlet (S) state, with electron spins antiparallel to each other. In the event they split, each radical contains one unpaired electron. If the electrons maintain their antiparallel spin, the molecules will likely recombine within $10^{-6}$ to $10^{-10}$ sec. However, if one of the electrons has its spin flipped, then the electrons will now be in the triplet (T) state (Fig. 2). In this state, when the electron spins are in the same direction, the free radicals are unable to pair as per the Pauli Exclusion Principle. This extends the life of the free radicals and their drift is possible [Barnes et al. 2015].
Figure 2- The splitting of a radical pair can result in radicals with either antiparallel (S) spins or parallel (T) spins, which will determine their lifetimes as unpaired free radicals [Barnes et al., 2015].

The way that the spins could enter into the triplet state is through exposure to a weak MF, which combines with other nuclear and electronic coupling interactions corresponding to the frequencies for energy transition to a new state. The two unpaired electrons each have a different coupling to their own respective nucleus, meaning that the applied MF will affect each electron differently and the spin state of one electron compared to the other will be altered. The resulting longer free radical lifetimes can lead to increased intracellular oxidative stress and alteration of cell signaling pathways. However, it is not simply a matter of applying stronger magnetic fields in expectation of creating more free radicals by this method. As the applied magnetic field, B, increases, the energy gap that needs to be crossed to change states is widened (Fig 3) [Barnes et al., 2015]. The implications of this theory are not a way that extremely weak MFs can physically change living systems directly by itself, but rather a way it can initiate a chain of biological processes that lead to serious changes; which provides the background for the hypothesis.
Figure 3- Zeeman splitting diagram displaying the energy gaps between the triplet (T) states and the singlet (S) state. As the magnetic field (B) gets larger, the energy needed to bridge the energy gap increases and state change becomes increasingly less likely after the crossover junction circled [Barnes et al., 2015].

Hypothesis

Extremely weak static and pulsed magnetic fields have the ability to affect fibroblast and fibrosarcoma cells, with some of the effects being expressed via levels in oxidative stress, membrane potential, and cell viability.
Chapter II

Materials and Methods

The following sections closely detail the materials that were needed and the methods that were followed in order to accomplish the experiments.

Considerations

In order to guard against the possibility of contamination, the labware used was sterile and single-use. After use, disposable materials were placed in a biohazard bin, or sharps container, and autoclaved before proper disposal. All cell work and preparation of medium, reagents, or other liquid that the cells were introduced to was accomplished in a Class II Biosafety Cabinet (Labconco). Before operation of the cabinet or the EM exposure system, surfaces were cleaned with 75% 190 proof ethanol solution (Decon Labs). Objects were sprayed before entrance and upon exit of the biosafety cabinet as well. Both the fibroblast and fibrosarcoma cell lines were given the exact same treatment and environments throughout; however they did always remain separate from each other and were not worked with in the same space-time.
Cell Lines

i. Primary Dermal Fibroblasts

The fibroblast cells were primary dermal fibroblasts (PDF) obtained from healthy, adult males (ATCC, PDF). They are adherent cells that are naturally spindle shape and were selected because they are the healthy counterpart to the fibrosarcoma cells used (both in the class of connective tissue) (Fig. 4) (ATCC, PDF). Their doubling time was about 24hr; however they exhibited conscious splitting, in that they would not continue to rapidly divide over one another once they neared confluence.

Figure 4- (a) PDFs viewed using light microscopy and (b) stained with the membrane potential dye using fluorescent microscopy (Zeiss), exhibiting spindle shape morphology.
HT-1080 Fibrosarcoma Cells

The fibrosarcoma were HT-1080 cells obtained from adult males exhibiting the disease (ATCC, HT1080). This type of cells was selected because they were easy to use and had been previously worked with in experiments by Aditya Kausik, upon which this study wanted to build upon [Kausik, 2014]. The HT-1080 cells are adherent cells, but are more circular in shape than the PDFs (Fig. 5). Their doubling time was about 24hr; however they exhibited splitting that was not conscious of the cells around them, in that they would still continue to divide over one another once they neared confluence.

Figure 5- (a) HT-1080 cells viewed using light microscopy and (b) stained with the membrane potential dye using fluorescent microscopy (Zeiss).
Exposure System

The exposure system is used to block out all external EM signals, as well as provide the desired field cell exposure. These two functions are carried out by two separate sections working together, the µ-metal enclosure and the coils (Fig. 6). These are placed within a cell incubator that provides the traditional temperature, gas, and humidity settings (Forma Scientific). Two complete incubator systems were used, allowing for experiments to run in parallel.

Figure 6- Photo displaying the exposure setup, with the µ-metal enclosure nestled in the cell incubator and the two coils in their own compartments of the enclosure.
i. µ-metal Enclosure

The µ-metal enclosure is essentially a box composed of µ-metal, that is placed inside the cell incubator in order to keep the unknown fields from the laboratory environment from reaching the cells undergoing testing. The incubator itself, with its heating elements, is normally a large source of MF variation as well. The enclosure helps guard against large variations found in the magnetic environment of incubators that Portelli found to reach up to an order of magnitude higher than the Earth's average field (Fig. 7) [Portelli 2012]. The enclosure used in this investigation has a door in the front so the coils and cell trays can easily be moved or accessed. This differs from Portelli's original construction and allows for a more convenient workflow, but does sacrifice some of the improvements Portelli made. Vents are present at the top and bottom of the box in order to allow gas and temperature exchange, as well as for the passage of wires. A µ-
metal partition is placed in between the two coils to stop cross pollution of EM signals among the control and exposed coils. Under normal incubator conditions, the μ-metal shield allowed under 1μT to penetrate the interior. Measurements were taken with a fluxgate magnetometer (Walker Scientific), which was accurate up to 0.01μT.

ii. Helmholtz Coils

A pair of separate cube shaped, multi-axial coils were used simultaneously to generate the MFs that the cells were exposed to. They were both placed within the μ-metal enclosure on either side of the partition. The first coil was the control coil that induced a 45 +/-1μT static MF to mimic the Earth’s average MF [Barnes 2007]. The second was the exposure coil used to induce the experimental fields desired for the particular trial that was being run.

The coils were constructed out of four Plexiglas sides, forming cubes with two open faces, measuring 15 x15cm. Laminated 22 gauge wire was wound in two tracks in Helmholtz configuration around the control cube, giving a resistance of 2.3Ω +/- 0.01Ω per coil. Laminated 18 gauge wire was wound in two tracks in Helmholtz configuration around the exposed cube, giving a resistance of 0.5Ω +/- 0.01Ω per coil. Only the x-direction field was used in these trials, but in the future more axes can be used in a full tri-axial configuration if needed. 18 gauge wire was used in the exposure cube because it needed to be able to carry more current than the control cubes in order to provide fields up to 200 μT. The wires were all sufficiently thick to allow the needed current to flow without heating up the wires and subsequently the samples. The uncertainties of the MFs generated were about 3% and were corroborated by a fluxgate magnetometer (Cole-Parmer). During the experiment,
the doors of the µ-metal enclosure and the incubator remained close so as to not disturb the experimental conditions.

iii. Power Supplies and Switching Circuit

The coils were driven by sets of DC power supplies (Keysight Technologies, HP), with only one supply for each coil needed when the exposure field was static. When the experimental field was pulsed, two DC supplies were needed for the exposure coil in order to exhibit the positive and negative values of a square pulse (Fig 8). The two supplies ran through a switching circuit, controlled by a stimulator that acted as a clock for the circuit (Grass Technologies). The output exhibited oscillations in the field, consisting of 10 sec up and 10 sec down, creating a period totaling a 20 sec pulse. The rise time was about 5ms with oscillations going up and the fall time was about 1.3ms with a nearly linear downwards slope (See Appendix Section B). The

![Figure 8- Pulsed square waveform from supplies to coil consisting of 10s up and 10s down, as measured by oscilloscope (Tektronix).](image-url)
extended period of time, the concern about rise and fall characteristics would be the creation of a
transient electric field across the sample. Table 1 has some of the power supply settings needed for
the different exposure intensities, with the outputs double checked by a voltmeter (Wavetek).

<table>
<thead>
<tr>
<th>MF Exposure</th>
<th>Coil &amp; Resistance</th>
<th>Amperage (A)</th>
<th>Voltage (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 +/-1µT static</td>
<td>EXP_0.5 +/-0.01Ω</td>
<td>0 A</td>
<td>0 V</td>
</tr>
<tr>
<td>45 +/-2µT static</td>
<td>CON_2.3 +/-0.01Ω</td>
<td>0.17 +/-0.05A</td>
<td>0.39 +/-0.05V</td>
</tr>
<tr>
<td>100 +/-3µT static</td>
<td>EXP_0.5 +/-0.01Ω</td>
<td>0.75 +/-0.05A</td>
<td>0.29 +/-0.05V</td>
</tr>
<tr>
<td>100 +/-3µT pulsed</td>
<td>EXP_0.5 +/-0.01Ω</td>
<td>0.76 +/-0.05A</td>
<td>0.35 +/-0.05V</td>
</tr>
<tr>
<td>200 +/-5µT static</td>
<td>EXP_0.5 +/-0.01Ω</td>
<td>1.49 +/-0.05A</td>
<td>0.66 +/-0.05V</td>
</tr>
<tr>
<td>200 +/-5µT pulsed</td>
<td>EXP_0.5 +/-0.01Ω</td>
<td>1.65 +/-0.05A</td>
<td>0.75 +/-0.05V</td>
</tr>
</tbody>
</table>

Table 1 – Power supply settings for the MFs used in exposure. Coils used in the
45µT control denoted by CON. Coils used in the test fields denoted by EXP.

Cell Culturing and Splitting

Cells were cultured in a mixture of 89% Eagle's Modified Essential Medium (ATCC, EMEM),
10% Fetal Bovine Serum (Sigma-Aldrich, FBS), and 1% Antimycin (Life Technologies, AA) in 75cm²
tissue culture treated (TCT) flasks with vent caps to allow for gas exchange (CELLTREAT, Flasks).
This growth solution also had the correct buffering capability to incubate the cells at 5% CO₂
atmosphere (Airgas). Once at near confluence (~80%) the cells were removed from the source flask
and split into four more identical TCT flasks, three corresponding to trials 1-3 and the fourth corresponding for the source flask for the next set of trials. In order to remove the cells from the source flask the growth medium was first pipetted out, leaving the adherent cells attached to the surface of the flask. 5mL of Trypsin (Sigma-Aldrich, Trypsin) was then added for about 4 minutes, depending on how firmly the cells were attached to the flask. 5mL of the growth medium was then added to the solution to neutralize the Trypsin and the total 10mL was put into a conical tube and centrifuged (Beckman Coulter) at 1000 RPM for 5 min.

After centrifugation, the supernatant was removed from the conical tube and the cell pellet at the bottom was resuspended in 10mL of growth medium. 1mL was placed into a conical tube for counting to determine the density of cells in the volume. 10µL of the 1mL solution was placed into each side of the hemacytometer (Sigma-Aldrich, Hemacytometer), which was then viewed under a microscope (Nikon) and the cells were counted on its grid. This step was repeated several times, at least four, until the standard deviation was under 10%. The correct amount of cell solution was then pipetted into each TCT flask such that each flask population would be at least 4 million cells on the day of its use. Growth medium was then added to each flask until the total volume per flask was 10mL. Trial 1 would be used in 3 days, trial 2 in 4 days and trial 3 in 5 days; until then they would be put back in the normal growth incubator at 37°C +/- 1°C, 5% +/- 0.5% CO₂, and high humidity.
Cell Seeding

Cell seeding refers to the removing of cells from their trial flask and seeding them into the 48 well tray in which they are to be housed for exposure. The beginning of this process duplicates some of the steps of Cell Splitting and Culturing so those steps will be briefly told.

At the time of seeding there were three flasks available, one for each trial. The cells from trial 1 would be removed, centrifuged and counted to determine the number of cells available. Afterwards, a calculated amount of cell dense solution was mixed with a certain amount of growth medium in order to give the correct seeding density needed for 7,600 cells/well (10,000 cells/cm$^2$) to be delivered in 200µL. In two of the 6x8, 48 well trays (Denoted by rows A-F and columns 1-8), 200µL of the cell solution was pipetted into the four center wells of rows C, D, and E. 200µL was pipetted into the 4 wells above these 3 rows as well. The growth medium wells would go through the same process as the cell wells for the assays in order to determine the background fluorescence of the medium and wells. The last column, column 6, would be used for controls to double check the assay measurements read at the end. One of the 48 well trays was labeled CONT, for control, and the other was labeled EXP, for exposed (Fig 9). Afterwards, the trays were put back into the incubator for 24hrs to allow the cells to become adherent and acclimate to their new environment.
Figure 9- Layout of the wells used in 48 well plates. The first row consisted of just medium with the assays added in order to determine background fluorescent levels. Rows 2-4 had cells and assay reagents added (assay shown by the left side labels). The last well on each row consisted of a control (as shown by the right side labels).

The center most wells were used because in the exposure setup, the most homogeneous MF exists at the center of the coils. Therefore, the most uniform exposure across the wells would occur if they were as centered as possible on the tray, while the outer wells remained blank. From trial to trial the order of the rows the assays were delivered to was rotated in order to cancel out any location bias within the tray.

**Exposure Methods**

A few hours before exposure, the testing incubators were turned on to get their internal temperatures to steady state 37°C. The CO₂ was also set so that the atmosphere inside the incubator was 5% CO₂. Before the cells were to be tested, the power supplies were double checked for correct connections and were set to proper outputs. The MF field strengths within the coils were
verified using a magnetometer. After the supplies were dialed in to providing the proper current for the desired intensity, they were allowed to run for a few minutes in order to ensure their values did not waiver.

At t=0 the cells were seeded into the 48 well tray, as detailed in Cell Seeding, and left to incubate for 24hrs in order to adhere to the bottom of the wells and adjust to their new environment (Fig. 10). At t=24, the trays were placed into the exposure system within the

![Flow diagram of experiments](image)

Figure 10– Flow diagram of experiments
test incubator at 37°C and 5% CO₂. The tray designated CONT was placed in the center of the 45µT control coil and the tray designated EXP was placed in the center of the exposed coil. The 48 well trays containing the cells were placed flat in the coils such that the x-axis MF flowed across the smaller dimension side. The trays were supported by inert plastic material so that they could sit in the center of the cube, where field homogeneity was greatest (variations <1µT). The door on the µ-metal enclosure was closed, along with the doors to the incubator. These doors were not opened again until the end of the exposure period. A short time later, however, the power supplies were checked to confirm that their outputs did not alter. The exposure lasted for 24 hrs and afterwards at
t=48hrs the trays plated with cells were removed from the incubators and assayed immediately.

After the assays were read, cell counts were performed. The trays were then left to incubate for another 24hrs and at t=72hrs the 24hr cell viability test was completed, concluding the trial.

Assays

After the 24hr exposure cycle, three assays were carried out on both the PDF and HT-1080 cells using fluorimetric dyes; oxidative stress, membrane potential, and cell viability. Cell counts were also performed, but only on the HT-1080 cells, due to complications with the PDF cells (See Appendix Section D). The three dyes were each introduced to their designated wells and their protocols set into motion immediately after exposure in order to gain the best understanding of the exposure effects.

i. Oxidative Stress

This CellROX Deep Red Reagent (Life Technologies) was used to detect levels of general intracellular oxidative stress caused by free radicals, which elevate oxidative stress. Normally in its non-fluorescing reduced state, it begins to fluoresce after it is oxidized by ROS inside the cell. Therefore, the dye will display greater intensity in the presence of elevated levels of oxidative stress.

The OX dye protocol necessitated the removal of the growth medium from the OX wells and mixing the reagent with 1mL growth medium before use. 100µL of CellROX mixed with growth medium was pipetted into the just medium well, the three test wells with cells, and the control well (Fig 8). 20 µL of Menadione (Sigma-Aldrich, Menadione) in solution was also added to the control well. Menadione raises oxidative stress in the cell, which would generate a higher fluorescent
intensity during reading. If the test wells with cells had fluorescence values above the control well with menadione or below the just medium well, they would be considered invalid and discarded. The insertion of CellROX into the CONT and EXP trays was executed simultaneously and in identical fashion. After all the assays were inserted, the trays were incubated for 40min. Upon removal the liquid from the OX wells was removed and they were washed twice in 100µL PBS (Sigma-Aldrich, PBS) and ready for reading at Excitation/Emission of 644nm/655nm.

ii. Membrane Potential

DiBAC$_4$(3) (Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol) (Life Technologies) measured the cellular membrane potential. The anionic reagent’s influx into the cell increases with cellular depolarization, where it binds to intracellular proteins and membranes and fluorescent intensity increases. Conversely, hyperpolarization will cause less DiBAC$_4$(3) to enter the cell and the higher concentration of unbound extracellular probes will lead to a lower fluorescence reading. Because of the reagent’s negative charge it will not enter the mitochondria and lead to false readings due to the integration of the mitochondrial membrane. 5µL Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, CCCP) in solution was also added to the control well. CCCP acts to hyperpolarize the cells’ membrane potential, thus decreasing the dye’s fluorescent intensity during reading. If the test wells with cells had a lower intensity value than the control well with CCCP or the just medium well, then their results were deemed invalid and discarded. The control well with CCCP also needed to be above the just medium well. After all the assays were
inserted, the trays were incubated for 40min. Upon removal, the MP wells did not need washing and were ready to be read at Excitation/Emission of 490nm/516nm.

iii. Cell Viability

AlamarBlue (Life Technologies, AB) was the reagent of choice to evaluate cell viability. It should be noted, that even though it was introduced to the cells at the same time as the oxidative stress and membrane potential assays, it was read 24hrs after the exposure was completed. This was done in order to give the cells enough time to properly convert the chemical and generate a tangible understanding of viability. The reagent is non-toxic and functions by fluorescing only once it has been reduced by reducing agents in the cytosol or by NADPH and other cofactors in the mitochondria [Rampersad 2012]. Therefore, although AlamarBlue is a viability assay, it should not be viewed as a method to base cell count on for this set of experiments. This is because the reducing capabilities of the cell are altered by changes in oxidative stress generated by MF exposure. So, this assay is taken to express solely a direct correlation to metabolic activity and not cell count.

The AB dye assay protocol simply calls for the pre-mixed solution to be loaded into the AB wells at 10% volume of the existing volume. Therefore 20µL of AB was pipetted into the just medium well and the three test wells with cells; the control well was kept without dye. If the test wells with cells had a lower fluorescent intensity than control well without AB or the just medium well with AB, then their results were deemed invalid and discarded. The control well without AB also needed to be below the just medium well. After all the assays were inserted, the trays were incubated for 40min. Upon removal the AB wells did not need washing and were ready to be read; however this
reading was replaced in favor of the more accurate 24hr viability reading; which consists of another reading 24 hrs after the first set at Excitation/Emission of 570nm/595nm. During the day in between readings, the cells were placed back into the normal incubators.

iv. Cell Count

Direct count of fibrosarcoma cells was made following the reading of the assays on the day exposure ended. Counts for fibroblast cells were unable to be accomplished due to an unknown reason for unnatural affinity to the well surfaces (See Appendix Section D). Counts were accomplished by the traditional method of using Trypsin to detach the cells and generating multiple sample readings on a hemacytometer to extrapolate total cell population. At least 6 samples were taken, in order to give a standard deviation of less than 10%. The cell count was converted to cell density, which then was used to normalize assay readings.

v. SpectraMax M5 Spectrophotometer

The SpectraMax M5 Spectrophotometer (Molecular Devices) is a plate and cuvette reader that is equipped to take both fluorescent and corimetric readings. The oxidative stress, membrane potential, and cell viability assays were all measured simultaneously using this multifunction reader and its associated software, SoftMax Pro. The operation proved simple and consisted of choosing a proper setup that accommodated the 48-well plate and emission/excitation characteristics of the assays. The plate was then loaded into the spectrophotometer’s tray and allowed to run and read the fluorescent intensities. The machine read the intensities at six point locations around the well and
averaged the values; it did not read the whole well at once. The intensities obtained in the just
medium wells represented the background fluorescence of the wells. These intensities were subtracted
from the test well values in order to remove the intensity attributed to the background and leave
solely that of the cells.
Chapter III

Results and Discussion

Results Overview

The data was obtained through the steps detailed in the Materials and Methods chapter. It is displayed with all of the trials shown simultaneously. This was done because of the large variations that existed at times from trial to trial, which will be expounded upon in the discussion. Each MF is given a different color, as designated by the key.

Fibrosarcoma Experiments

The fibrosarcoma experiments include results for oxidative stress (OX), membrane potential (MP), cell viability (AB), and cell count (COUNT). The assay results (OX, MP, AB) are each normalized to the cell density obtained from the cell count and area of the well. After generating the results per density, the % Change with respect from the control cells to their exposed counterparts were calculated to give the % Change values (see Appendix Section E). The %Change was then divided by the %Change of AB, so that the value given could be with respect to the metabolic health of the cell and would help to account for the cell’s homeostatic patterns in disposing of changes. The average standard deviation of each assay with respect to the exposure intensities was looked at as
well to determine if the amount of variability in the results for OX, MP, AB, and COUNT was correlated to the field the cells were exposed to. It should be noted that only two trials were gathered of the 200µT pulsed MF, while at least three were taken with the rest of the MFs.

i. Effects on Oxidative Stress

Oxidative stress was altered significantly 100µT field (two-tailed t test, p<0.05). Figure 11 shows the whole graph and Figure 12 displays the same graph magnified to give a closer look at the majority of the values. Decent changes were also exhibited by the 200µT and the 200µT pulsed fields.

![OX - HT1080](image)

Figure 11- Changes in oxidative stress for all exposures over the trials given.
Figure 12- A closer look at the oxidative stress changes for all exposures over the trials given.

ii. Effects on Membrane Potential

Membrane potential was altered a large amount in a couple of trials, most notably the drastic decreases that occurred with 100µT and 200µT. However 0µT was the only intensity that had a semblance of a notable pattern and yet still fell short of being termed significant. Figure 13 shows the whole graph and Figure 14 displays the same graph magnified to give a closer look at the majority of the values.
Figure 13- Changes in oxidative stress for all exposures over the trials given.

Figure 14- A closer look at the membrane stress changes for all exposures over the trials given.
iii. Effects on Cell Viability

Cell viability was not altered significantly overall by any one field, however there was a drastic increase with trial 3 of the 100μT pulsed. However 0μT was the only intensity that had a semblance of a notable pattern. Figure 15 shows the whole graph and Figure 16 displays the same graph magnified to give a closer look at the majority of the values.

![Graph showing changes in cell viability for different trials and field intensities.](image)

**Figure 15-** Changes in cell viability for all exposures over the trials given.
Figure 16- A closer look at the cell viability changes for all exposures over the trials given.
iv. Effects on Cell Count

Cell count was not altered significantly for the most part. However, some trials stood out as exhibiting noted results, but no trend (Fig. 17).

![COUNT - HT1080](image)

**Figure 17-** Changes in cell count for all exposures over the trials given

v. Effects on Result Variability

The average standard deviation (%) for the results of each of the assays, with respect to exposure intensity, is plotted on Figure 18. This feature was looked at to determine if the amount of variability in the results for OX, MP, AB, and COUNT was correlated to the field the cells were exposed to. The standard deviation for all of the terms used to calculate the results were combined in order to arrive at the values displayed. The average deviation did change between exposure
settings, with 200µT static generating the largest variability between results and 200µT pulsed generating the smallest variability.

Figure 18- Average standard deviation in % for all assays over the exposures given.
Fibroblast Experiments

The fibroblast experiments include results for oxidative stress (OX), membrane potential (MP), and cell viability (AB). The fibroblast assays (OX, MP, AB) were unable to be normalized by density because cell counts could not be determined (see Appendix Section D). Therefore, the fibroblast graphs give only raw values of % difference between control and exposed cells.

i. Effects on Oxidative Stress

Oxidative stress was not altered significantly overall by any one field. There were slight changes in some fields, however the errors overwhelmed the results. (Fig. 19).

Figure 19- Changes in oxidative stress for all exposures over the trials given.
ii. Effects on Membrane Potential

Membrane potential was not altered significantly overall by any one field, however there were noticeable changes in several fields (Fig. 20).

![Figure 20- Changes in membrane potential for all exposures over the trials given.](image-url)
iii. Effects on Cell Viability

Oxidative stress was not altered significantly overall by any one field, however 0µT seemed to have the largest effect in trial 3, followed by 200µT (Fig. 21).

![AB - PDF](image)

**Figure 21-** Changes in membrane potential for all exposures over the trials given.

vi. Effects on Result Variability

The average standard deviation (%) for the results of each of the assays, with respect to exposure intensity, is plotted on Figure 22. This feature was looked at to determine if the amount of variability in the results for OX, MP, AB, and COUNT was correlated to the field the cells were exposed to. The standard deviation for all of the terms used to calculate the results were combined
in order to arrive at the values displayed. The average deviation did change between exposure settings; however these differences could not be justified as significant.

Figure 22- Average standard deviation in % for all assays over the exposures given.
Discussion

i. Fibrosarcoma Effects

HT-1080 cells' oxidative stress was significantly affected by extremely weak MFs at 100µT. Other MFs generated significant changes on a per trial basis, leading to the belief that perhaps significance should be judged in a manner other than combining all of the trials for a normal t-test. An increase in oxidative stress is consistent with Martino's observations introduced earlier [Martino 2007]. Overall however, large standard deviations worked to lessen the significance of readings. These deviations should be attributed to several things. Firstly, the oxidative stress readings give general oxidative stress, not a specific ROS. Usselman et al. found that under extremely weak RF MFs, levels of H$_2$O$_2$ rose and levels of O$_2^-$ fell (Usselman et al., 2014). This means that give and take effects like this one could be exhibited as inconsistencies if looking at oxidative stresses in general. The oxidative stress assay was the closest to the spin flip mechanism that the fields were theorized to be directly interacting with; however the other tests were further downstream from the mechanism. Free radicals do play an important role in membrane potential, as well as viability, but with the unknown concentrations of each radical and other cellular activities coming into play to maintain homeostasis the effects upon these downstream characteristics was rendered less significant. The 24hr exposure time also could have attributed to this and could have been either too long to see the peak transient effects, because cells adjusted. Or the exposure time could have been too short and stresses upon the cells were not present for enough time to become integrated into fully expressed consequences. Lastly, both positive and negative effects have been observed when EMFs activate calcium channels, showing that the cells own activities play a role in variability as well [Pall, 2013].
The variability vs. exposure showed that different MFs could lead to changes in the variability of the experiment. HT-1080 OX results saw a significant increase in variability when exposed to 200μT fields. This fact also lends weight to the argument that incident MFs could produce unintended consequences in cells.

ii. Fibroblast Effects

PDFs were not significantly altered when looking at the breadth of the study, but on a per trial basis there were some obvious changes. The variability vs. exposure relationship also did not yield significant differences, but the averages did display noticeable differences. The largest downfall of the fibroblast tests was the lack of a cell count that would allow for the % Changes to be given with respect to cell density. The lack of this not only takes away from the usefulness of the results for the fibroblasts, but also eliminates the ability to compare fibroblast to fibrosarcoma results.
Chapter IV

Conclusion

Significant effects on the fibrosarcoma cells were observed by exposure to extremely weak MFs. All of the MFs did not render significant consequences, but there were spikes in a per trial basis, leading to the belief that cells at certain states could be more susceptible to the fields and they should not be ignored. An alteration of variability in the results dependent upon the MF used also occurred. Unfortunately, not all of the goals for these experiments were met due to the fact that PDF cells were unable to be normalized properly. This removes the ability to compare the effects that extremely weak MFs had on healthy vs. cancerous cells.

Although clear cut trends for each intensity were not able to be filled out, the results do call into question the fields that cells are subjected to during experiments. If an incubator has MF variations between 0 and 450µT then the cells are contaminated by unaccounted for MFs that have the potential to modify processes and produce discernable variability [Portelli 2012]. Therefore this dissertation endorses the use of a µ-metal enclosure and coil system, or something similar, to ensure that no external fields induce inconsistency in the subjects. It also demonstrates that some of the lack of reproducibility of experiments, when they are accomplished in separate research groups, could have to do with the lack of magnetic control instituted for the samples.
Chapter V

Future Work

In order to gain a more complete understanding of this subject several avenues are available for future studies. The first involves reproducing the experiments undertaken here. The trial to trial variations were large enough be concerned about and need corroboration. The modulation of characteristics of the EM waves can be looked at afterwards. This thesis investigated fields at 0, 100, and 200µT, going forward more intensities should be added. Specifically in the range from 200µT-1mT a dose curve needs to be generated, which will fill in data points and better define trends. Also pulse repetition rates and exposure times are preliminary settings that could be altered at a later date.

The manner and identity of the assays and tests should be reevaluated as well. As seen in the Discussion section, a more precise and accurate set of methods should be enacted in further investigations in order to be able to detect subtle differences that weak magnetic fields could induce in cellular processes. A real-time analyzation of assays would allow for a better understanding of the transient effects of MFs on cells that were missed here due to the single time point measure as well. The main point however, would be to try and look at effects that are closer to the actual mechanism being affected. This would be a more direct measurement on the actual effects and could help to prove the free radical recombination theory. It could be done using different techniques, such as
nuclear magnetic resonance (NMR), like Usselman et al., in order to decipher the actual state the electrons of free radicals are in. Regardless of the techniques, though, a system to provide an environment free of unknown external EMFs should be used.
References


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15. Life Technologies. AB. "AlamarBlue, Cell Viability Reagent.”

16. Life Technologies. MP. ”DiBAC₄(3) (Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol).
http://www.lifetechnologies.com/order/catalog/product/B438

17. Life Technologies. AA. “Antibiotic-Antimycotic (100X).”
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19. Sigma-Aldrich. FBS. “Fetal Bovine Serum.”


Appendix

A. Bioelectromagnetics Areas of Study

This section gives details about the BEM topics of study not gone over in the main body of the thesis, including: measuring and modeling tissue properties, and understanding endogenous processes, imaging and diagnostics techniques,

Measuring tissues for their dielectric constants are important to determine how they would react with a given applied electric field. Dielectric, or nonmetallic materials, are measured for permittivity (\(\varepsilon\)) as well as conductivity (\(\sigma\)) at ranges of frequencies and varying environmental conditions. The general rule is that permittivity declines and conductivity rises with an increase in frequency [Gabriel 2007]. Measurements are also taken on the magnetic properties of biological material and their occurrence in species such as magnetotactic bacteria [Blakemore 1975] and birds [Deutschlander 2012]. Birds use their iron infused structures to base their geographic position on the strength of the Earth’s magnetic field and migrate in the proper direction [Deutschlander 2012]. The
dielectric and magnetic measurements are also all compiled in order to make more accurate computational models for simulating exposures on bodies [Lin and Bernardi 2007].

Endogenous processes are electromagnetic processes that take place naturally within the body. A well-known instance of this is demonstrated by cell membrane potential, which is about -50 to -100mV in most healthy cells [Pullar 2011], and occurs across cell membranes; however many other lesser known electric phenomena play a vital role in organisms. During wound healing, an electric field is set up so that current flows from the positive tissue around the cut into the negative pole at the site of damage (Fig. A1) [Nuccitelli 2007]. Electric field gradients are also created during embryogenesis [Adams 2008] and regeneration [Tanaka 2011] by H+ ions that direct the spread of growth factors and proteins.

Figure A1- A field is generated with the positive poles surrounding the cut and the negative pole located at the cut. Current flows into the cut from the tissue that has not been breached [Nuccitelli 2007].

Imaging and diagnostic equipment that rely on electromagnetic principles are widely used in medicine, including magnetic resonance imaging (MRI) machines, encephalograms, and the newer electrical impedance imaging (or tomography, EIT). MRI machines are well known and widely used for soft tissue imaging. They create strong magnetic fields (normally 1-3T) to align electron spins of water molecules and subsequently read them with RF pulses. EIT is based off contrasts in the
complex permittivity exhibited by the tissues under focus. The use of small electrodes create a relatively cheap and easy detection system compared to MRI machines [Joines et al 2007]

Coil Specifications

Figure A2- 15x15cm coil constructed of plexiglass and wrapped laminated wire for the 45µT control MF.

\[
B(Z) = 80NI \left\{ \frac{1}{\left[ 1 + \left( \frac{Z+4A}{2} \right)^2 \right] \sqrt{2 + \left( \frac{Z+4A}{2} \right)^2}} + \frac{1}{\left[ 1 + \left( \frac{Z-4A}{2} \right)^2 \right] \sqrt{2 + \left( \frac{Z-4A}{2} \right)^2}} \right\}
\]

Figure A3- Equation detailing the magnetic field produced by square Helmholtz coils. N is the number of turns, I is the current, z is the distance to the sample, 2A is the dimension of the square, \( A_0 = 1.089 \)A [Radhakrishna, 2015].
Figure A4- Oscilloscope reading of the 5ms rising slope for the 20sec pulsed MF.

Figure A5- Oscilloscope reading of the 1.3ms falling slope for the 20sec pulsed MF.
Figure A6- Diagram of the switching circuit, timed with a stimulator, that allowed two DC power supplies to operate together in order provide a pulsed waveform for the exposure coil [Radhakrishna, 2015].
Assay Protocols

DiSBAC₄(3) Kit Prep (Membrane Potential Assay)

Prepare 10 to 30mM stock solution of DiSBAC₄(3) in high quality, anhydrous DMSO. Add 4.839mL DMSO to the vial of 25mg DiSBAC to create a 10mM solution.

Aliquot DiSBAC assay and store at -20C. For every well, about 0.5uL of assay stock will be needed. Therefore put 5uL assay stock per aliquot. Buffer solution will be added before introduction to cells (see below)

Wrap stock solution and aliquots in foil to protect from light and freeze.

CellRox Deep Red Kit Prep (Oxidative Stress Assay)

Add 50uL DMSO per 50uL 2.5mM vial to get working concentration of 1.25mM.

Aliquot 5uL working solution into 20 Eppendorf tubes. EMEM will be added before introduction to cells (see below)

Wrap stock solution and aliquots in foil to protect from light and freeze.

AlamarBlue (Metabolic Rate Assay)

Aliquot solution for a couple trials and wrap aliquot stock solution in foil to protect from light and place in refrigeration.

CCCP (Membrane Potential [-] Control)

Dissolve 10.23mg CCCP into a 10mL solution of 3 parts methanol to 2 parts sterile H₂O. Dissolve the 10.23mg into 6mL of methanol and 4mL of H₂O to give a 5mM stock solution

Menadione (Oxidative Stress [+]* Control)

Add 0.172mg of menadione to 10mL of DMSO to give 100uM stock solution. Add 25uL of the stock solution/well of the 100uL oxidative stress control well.
Assay test protocol

1. Expose Cells for desired time in EM field.

2. Dissolve DiSBAC in 1.5mL (150uL/well) Hanks Buffer Solution for 20 to 40uM. This creates the working solution.

3. Add 1mL of EMEM/tube of CellROX.

4. Add 20uL of AlamarBlue/well of Count cells so that it is 10% of the well volume.

5. Remove all EMEM liquid from the membrane potential DiSBAC assay wells.

6. Remove all EMEM liquid from the oxidative stress CellROX assay wells.

7. Add 5uL/well (theoretical is 3uL) to give 100uM final concentration in 150uL of membrane potential control well.

8. Add 25uL of the stock solution/well of the 100uL oxidative stress control well.

9. Add 150uL/well of DiSBAC working solution to the membrane potential wells.

10. Add 100uL/well for CellROX/EMEM solution at a final concentration of 5uM for the CellRox assay.

11. Incubate cells for 40 minutes at 37°C.

12. Remove media from CellROX wells and wash cells three times with 100uL of PBS.

13. Take measurements with fluorescence plate reader.
   Use 493nm excitation and 516nm emission for DiBAC₄(3).
   Use 640nm excitation and 665nm emission for CellRox DeepRed.
   Use 560nm excitation and 590nm emission for AlamarBlue.


15. Take measurement 24 hours later for AlamarBlue24hr reading.
Fibroblast Count Problem

Counts for fibroblasts were unable to be obtained by the method that was employed to garner fibrosarcoma results. The fibroblasts for an unknown reason had an increased affinity to the wells of the TC trays. The normal protocols were augmented by adding more Trypsin and allowing the cells to sit in the Trypsin for up to 10 minutes, instead of the normal 4. Despite the drastic increase, the cells still did not become suspended in solution to allow for proper counting via hemacytometer.

Results Calculations

This section demonstrates how the results were arrived at from the raw intensities found from fluorescent readings. \( I_c \) and \( I_e \) are the average of the intensities found from the fluorescent readings minus the background fluorescence exhibited in the wells without cells.
i. Fibrosarcoma Calculations

\[ \frac{I_e}{d_e} - \frac{I_c}{d_c} \bigg/ \frac{I_c}{d_c} \bigg/ \Delta I_{AB} \]

- \( I_c \): control cells intensity reading
- \( I_e \): exposed cells intensity reading
- \( d_c \): control cells density
- \( d_e \): exposed cells density
- \( \Delta I_{AB} \): change in cell viability

Figure A7- Equation used to calculate HT-1080 results.

\[ \frac{\Delta I_d}{\Delta I_{AB}} \]

- \( \Delta I_d \): change in cells per density
- \( \Delta I_{AB} \): change in cell viability

Figure A8- Simplified version of equation from Figure A7 used to calculate HT-1080 results.
ii. Fibroblast Calculations

\[ \frac{I_e - I_c}{I_c} \]

\( I_e \): exposed cells intensity reading \hspace{10mm} \( I_c \): control cells intensity reading

Figure A9- Equation used to calculate PDF results.

Statistics

Two tailed T-tests were performed maintaining a p-value of 0.05 as the threshold for significance. Error propagation was performed when standard deviations needed to be combined using the formulas in Table A1. Error bars for graphs were calculated by dividing the square root of the number of samples into the standard deviation.

<table>
<thead>
<tr>
<th>Function</th>
<th>Example</th>
<th>Standard Deviation (( \sigma_x ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition or Subtraction</td>
<td>( x = a + b - c )</td>
<td>( \sigma_x^2 = \sigma_a^2 + \sigma_b^2 + \sigma_c^2 )</td>
</tr>
<tr>
<td>Multiplication or Division</td>
<td>( x = a \times b/c )</td>
<td>( \frac{\sigma_x^2}{x} = \sqrt{\left(\frac{\sigma_a}{a}\right)^2 + \left(\frac{\sigma_b}{b}\right)^2 + \left(\frac{\sigma_c}{c}\right)^2} )</td>
</tr>
</tbody>
</table>

Table A1- Formulas for combining standard deviations in arithmetic functions [Chemwiki].
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