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Impedance Measurements as a Means to Improve the Biological Response of Gene Electrotransfer

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

When an electric field is locally applied to tissues *in vivo* the uptake of exogenous DNA can be greatly increased. This approach to gene transfer, called electroporation (EP) or gene electro transfer (GET), has potential applications in the treatment of skin disorders, vaccinations, some types of cancer and metabolic diseases. The effect of electric fields on cells and tissues has been studied and related to the uptake of DNA. Tissue impedance changes have been measured as a result of electroporation. The aim of this study is to explore the predictive accuracy of impedance spectroscopy for the success of GET. Mice were used in this study for their histological similarities to human skin. The mice were injected with plasmid DNA coding for luciferase and given one of a series of electroporation treatments varying the number and intensity of the electric pulses delivered. The number of pulses delivered was based upon impedance measurements taken during the EP procedure. Mice were then imaged to quantitatively measure the luminescence resulting from the gene delivery procedure at intervals of 2, 4, 7, 10 and 14 days after treatment to quantitatively determine the biological response to gene electrotransfer. Increased luminescence was noticed in treatment groups compared to injection only groups, and the most effective treatments resulted from a feedback mechanism based upon percentage changes in skin impedance. The relationship between impedance change and gene expression suggested that treatment can be improved based on impedance measurements taken during the EP treatment.

CHAPTER 1

INTRODUCTION

1.1 The Cell Membrane

The cell membrane consists of a semi-permeable phospholipid bilayer into which proteins, glycoproteins and glycolipids are embedded. It only allows certain small and uncharged particles to pass through unaided [2]. The different ways that molecules can enter a cell are:

- Diffusion. This mechanism does not require any additional energy input and occurs down a concentration gradient. Its effectiveness is dependent on the concentration gradient, permeability, surface area and the distance over which a compound must travel. There are two types of diffusion: simple, through the lipid bilayer, and facilitated, which requires a protein on the cell membrane.
- Active transport. This mechanism uses energy obtained from ATP hydrolysis to activate membrane proteins that can allow the passage of compounds in and out of the cell. It can be used, for example, to drive the movement of ions against their concentration gradient in a sodium-potassium pump.

All of these mechanisms restrict the passage of large molecules such as plasmid DNA (pDNA) and some chemical compounds often used for medicinal purposes (e.g. chemotherapy drugs). The selective permeability of the cell membrane helps maintain homeostasis, but it also impedes treatments that rely on a therapeutic molecule being able to enter the cell. Hence, a number of methods have been deviced to introduce plasmid DNA and various compounds into cells.

1.1.1 Transfection

A number of disorders are caused by a lack of a specific protein. This protein may be lacking for many reasons, including incorrect folding (meaning it is produced but not in an active form) and the lack of the correct DNA code for synthesis. The insertion of nucleotides into cells is called transfection. Potential treatments for such disorders include the delivery of therapeutic

DNA coding for the specific protein. Several methods are available to this purpose, including the use of viral vectors and other physical methods. Viral vectors rely on the mechanisms used by viruses to insert DNA into the host cell. Non-viral methods, such as the gene gun, sonoporation, and magnetofection, disturb the lipid bilayer to help compounds enter the cell. A distinction exists, once the genetic material is added to the host cell, depending on whether the gene is temporarily or permanently expressed. If it is expressed temporarily and the DNA is not integrated to the cell genome, it is said that the transfection is transient. If it is transferred on to daughter cells and continues to be expressed after the initial host cell is degraded, it is said that the transfection is stable. Both types of transfection have different applications. For the purposes of the present research, transient transfection was sufficient to study the relationship between gene expression and the electrical properties of tissue. This study focused on the mechanism of electroporation.

1.2 Electroporation

Electroporation (EP) is the increased permeability of cell membranes due to an increased transmembrane voltage. This increased voltage is created through short (microsecond to millisecond long) pulsed electric fields. Pulses are typically applied *in vivo* using electrodes that contact the tissue.

1.2.1 Brief History of Electroporation

Applications of EP first appeared in the 1980s as a means of introducing DNA into bacteria [3]. The high voltages pose the risk of damage to the tissue of higher organisms, which restricted the use of electroporation for gene delivery to bacteria for some time. However, applications in drug delivery were identified and experiments proved it could improve the delivery of drugs for the treatment of cancer [10]. These studies encouraged the use of EP to transfer genes to multicellular organisms with high voltage pulses. In 1996 lacZ was successfully delivered to tumors in an animal model. This study showed that expression was limited to tissue that received an electric charge [9].

In vivo EP for DNA delivery has been investigated in preclinical studies for many types of tissues and tumors [6]. The uses include the treatment of tumors, such as melanoma, fibrosarcoma, gliomas, and hepatocellular carcinomas. There are two types of electroporation: reversible and irreversible. Reversible EP happens when the cell membrane is temporarily permeabilized

and then returns to normal. Irreversible electroporation occurs when the membane permeabilization causes cell death. This can also be used therapeutically to treat tumors, for example, because this particular instance of cell death does not alter the extracellular matrix or cause protein denaturation, both common side effects of necrotic tissue ablation. The exact mechanism is not fully understood for either type of EP, but it is widely acknowledged that cell death happens after damage to the cell membrane or the loss of homeostasis [1]. Note that the uses of reversible electroporation depend on cells remaining alive to either process a delivered drug or express the proteins encoded by delivered DNA. Irreversible electroporation can cause unnecessary damage to tissue if it happens accidentally, increasing the pain and discomfort caused by treatment as well as decreasing its effectiveness because dead cells cannot express transfected genes.

Successful treatments of gene electrotransfer have to satisfy several requirements: cause little to no tissue damage in the form of blisters, burns and ablation; result in high expression of the transfected gene; have results that can be repeated.

The variables that affect EP include tissue type, electrode, animal model and animal to animal variation. Initial studies aimed to optimize delivery through changes in the electrode [5]. Optimal electrical parameters that result in the best treatment have been determined empirically up to this point in time, and they fail to compensate for the variation found between EP treatments. In 2007 electrical impedance measurements were used to study the physical changes that occur in tissue during and after electroporation [7]. Impedance can be measured rapidly as a function of the voltage and current. It has been suggested that the changes in conductivity of electroporated tissue can be used to monitor the extent of electroporation [4], which may help to better understand the limits between reversible and irreversible EP.

1.3 Impedance

There are many differences between the electrical properties of living tissue and those of man-made electrical systems. One of the major ones is that living systems depend on an ionic gradient formed across the cell membrane to derive their electrical energy. Since charged particles are not able to freely move across the cell membrane, it acts as an electrical insulator for

sodium, potassium, chloride and calcium ions. This behavior corresponds to the concept of a physical dipole and is modeled in a circuit as a capacitance. The flow of these ions is a source of energy that triggers a number of processes in living organisms such as muscle movement and the transmission of sensory signals. Given there are ion channels distributed over it, energy sources are spread throughout the cell membrane and using this energy involves current flow across the lipid bilayer. Skin as a whole, including the layer of dead cells that corresponds to the *stratum corneum* can be modeled as a resistance in series with a resistance parallel to a capacitance. Sometimes, an inductive element will also be included in the circuit [8].

Though a physical model for cellular response to electrical stimuli has been established, the relationship between changes in tissue properties and a measure of gene expression has not been thoroughly researched to date. The aim this research is to relate the biological response observed to quantifiable physical changes in the tissue to apply the best experimental treatment based on these changes. Impedance is measured because it relates directly to the capacitance and, depending on the model, inductance of the tissue.

Previous studies, both by this group and others, have found a drop in impedance following EP treatment (see Figure 2.1). Ivorra and Rubinsky plotted the Cole parameters R_0, which is the impedance at zero frequency, and R_∞, which is the impedance at infinite frequency, as they varied over time after EP treatment. The figure shows the y axis in Ωcm as measure of resistivity rather than impedance. This is due to the fact that the Cole parameters in the graph were scaled according the probe cell constant (in cm). Both reversible and irreversible electroporation were studied and in both cases an impedance drop was observed after pulsing.

1.4 Research Aim

Electroporation is a viable method for gene delivery that has many potential clinical applications. *In vivo* EP also creates a change in tissue impedance. These two facts make impedance a parameter that could be used to ultimately guide EP applications in the clinic. Thus, the aim of this research is to explore the predictive accuracy of impedance spectroscopy for the success of gene delivery through electroporation.

CHAPTER 2

MATERIALS AND METHODS

2.1 DNA Delivery and Expression

Mice were chosen for the histological similarities shown between their skin and human skin. Both male and female mice of the BALB/C strain were used for experiments. They were between 6 and 7 weeks old at the time of treatment. The flank were used as a target tissue as it provided a large enough surface area to place the electrodes and it also provided a tissue that was easy to image.

The DNA delivery procedure was as follows:

1. The flanks of the animals were shaved
2. The mice were anesthetized in isofluorane chambers at a 2.5% concentration
3. The ears of the mice were punched for identification purposes
4. 33 gauge needles were used to inject 50μl of gWiz^{TM} plasmid DNA encoding luciferase intradermally, for a total of 100μg of DNA per injection
5. The electrode was placed over the injection site and EP was performed and/or impedance measurements were made

Luciferase expression was determined as follows:

1. The mice were anesthetized in isofluorane chambers at a 3% concentration
2. An intradermal injection of 50μl of luciferin at a concentration of 2mg/ml was delivered to their flanks
3. After 12 minutes, the mice were placed in an IVIS 4 XENOGEN Imager and the luminescence emitted was recorded as the biological response

Biological response results were collected 2, 4, 7, 10 and 14 days after injection.

2.2 Electrode

A multielectrode array (MEA) (see Figure 2.2) made out of sixteen gold-plated electrodes that were 0.5mm in diameter with flat tips arranged in a four by four grid. The electrodes were spring-loaded so they could freely retract in response to pressure. This ensured that all electrodes were in contact with skin at any given point in treatment. The array of electrodes formed a square with a side length of 6mm, containing nine subsets of 4 electrodes. Each square pattern subset was referred to as a "sector."

The power source was set to 20V, 30V and 40V to create fields of 100V/cm, 150V/cm and 200V/cm respectively. The device was calibrated before each experiment by shortcircuiting the electrodes on a stainless steel slab with sixteen indentations that corresponded to each electrode tip. Calibration of the system resulting in recorded readings of 0Ω with minimal fluctuations.

2.3 Impedance Analyzer

An impedance analyzer was custom made for this project (see Figure 2.3). The device could acquire impedance data from 0 Hz to 100 kHz. The circuit was coupled with a custom pulse generation system that consists of a chassis containing relay modules (SCXI-1600 and SCXI-1163, National Instruments, Austin, TX). The system was controlled using a laptop computer and custom program written in LabView (National Instruments). The relays, or electrically actuated switches, were used to generate electric pulses from a constant direct current (DC) power source. They were also used to electronically connect the impedance analyzer to the electrodes. This system provided precise timing of impedance measurements as well as EP pulses to electrodes. This electronic switch/pulsing system was able to measure impedance spectra as quickly as 50 milliseconds after an EP pulse.

Pulsing and impedance measurements were applied in one of two ways for this study. In both of these, 150 ms pulses were used exclusively with a 500 ms interval between successive pulses. Impedance measurements were made within the 500 ms interval between pulses as shown in Figure 2.4.

The first way was termed 4 × 4 pulsing. After DNA injection, a baseline impedance measurement was taken for reference. This involved the 4 electrodes in the first sector (upper left) of the electrode shown in Figure 2.2. An impedance measurement was made between the two left-hand electrodes and the two right-hand electrodes. Then, an impedance measurement was made between the two upper electrodes and the two lower electrodes. Then, baseline impedance measurements were taken from the second sector which was immediately below the first in the figure. The process was continued until baseline measurements were taken from all 9 individual sectors. Then, EP pulsing was carried out in the same sector by sector manner by applying 4 EP pulses from left to right followed by 4 EP pulses applied from upper to lower. Immediately after each sector had been pulsed, post electroporation impedance measurements were taken in the same manner (in both directions) as the baseline measurements were taken.

The second way of applying pulses was termed feedback pulsing. This involved first taking baseline impedance measurements sector by sector (after DNA injection) in the same in the same way they were made for the 4 × 4 pulsing. Then EP pulses were applied in a sector by sector manner. A single EP pulse was applied in each direction for the first sector. Then impedance measurements were taken in each direction in this same sector. The mean of these impedance measurements was compared to the mean of the baseline to determine if the impedance had decreased by a predetermined percentage (an experimental variable). If it had, then pulsing was discontinued. If it had not, then another pulse was applied in each direction. Impedance measurements were taken again and compared to baseline. Pulsing was continued in this first sector until post electroporation impedance was reduced by a percentage that was equal to or greater than the predetermined value. The second sector was then pulsed with impedance feedback used to reduce the impedance to the same predetermined value. The process was continued until all 9 sectors were pulsed using feedback.

2.4 Preliminary Measurements

The system described in the previous subsection was tested to ensure that impedance changes could be detected after EP. Baseline impedance measurements were made for animals after DNA injection but before pulses were administered. A representative Nyquist plot of the resulting data is shown in Figure 2.5. Baseline measurements were consistent throughout treatment groups. 4 × 4 pulses were then administered to mice, followed by another impedance measure-

ment. The resulting post EP data was different. Figure 2.6 is an example of such data. It is a Nyquist plot from an animal that received pulses at 100V/cm. Different electric field strengths resulted in different degrees of change in the impedance. Plots with more dispersed data showed smaller percentage changes in impedance relative to the baseline, while data points concentrated around the origin indicated a greater impedance drop. Thus, different treatments were seen to produce different patterns of change in impedance.

2.4.1 Experimental Settings for Data Collection

MATLAB's builtin chirp function was used in the experiments to measure impedance. This involved passing a low voltage chirp through the tissue in contact with an electrode sector. To rule out any impact of the chirp pulse on the biological response (luciferase expression). Two sets of animals had DNA delivered identically except one set had impedance measurements performed and the other did not. Both sets of animals had statistically identical biological responses, there fore the chirp was determined not to affect EP delivery.

The way that frequencies were sampled to perform the impedance measurements could be varied. Two different options were examined: logarithmic and linear. After studying the Nyquist plots obtained through both methods of frequency sampling, the differences found were not significant and hence linear sampling was used for this entire study.

Data was collected for frequencies between 50Hz and 100kHz but the data analyzed was restricted to the range between 1kHz and 3kHz. In this range, the magnitude of the impedance change was the greatest relative to the baseline. Also, the variability was the lowest. Figure 2.7 shows a plot of Impedance vs. Frequency for animals (N = 12) treated with 4×4 standard pulsing using electric fields of 50V/cm, 100V/cm, 150V/cm, 200V/cm and 250V/cm. Impedance values shown were measured immediately after the final pulse for each sector. The mean data of all sectors is the data point used for each frequency in the figure.

2.4.2 Data Analysis

Impedance data was calculated through the use of a Fast Fourier Transform (FFT) in order to change the parameter of time in the voltage and current functions for the parameter of frequency. The values obtained were phase-normalized and the magnitude of the impedance was found as a quotient of the magnitudes of the voltage and current used.

Baseline and post EP data along with the treatment parameter values was saved from each animal. A MATLAB custom program was written to retrieve this data and the following information was obtained:

- Voltage over time
- Current over time
- Impedance norm baseline values as a function of frequency
- Impedance norm values after pulsing as a function of frequency
- Number of pulses delivered to each sector

The statistical analysis was conducted with SPSS software and some of the tests used are listed below:

- Kolmogorov-Smirnov Normality Test
- Shapiro-Wilk Normality Test
- Kruskal-Wallis Test (nonparametric test equivalent to ANOVA for non-normal distributions)

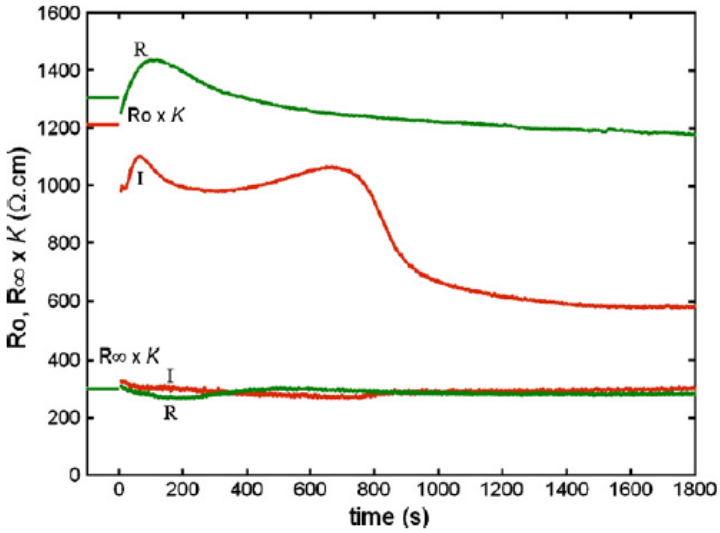

Figure 2.1: Cole Parameter Over Time after EP. [7]

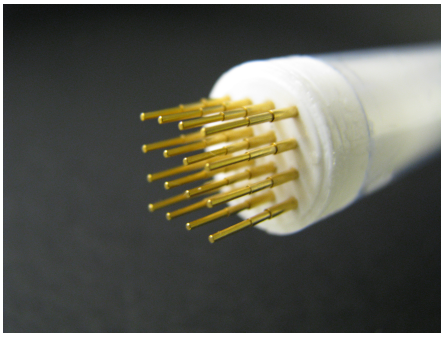

Figure 2.2: Array of Sixteen Gold-Plated Electrodes.

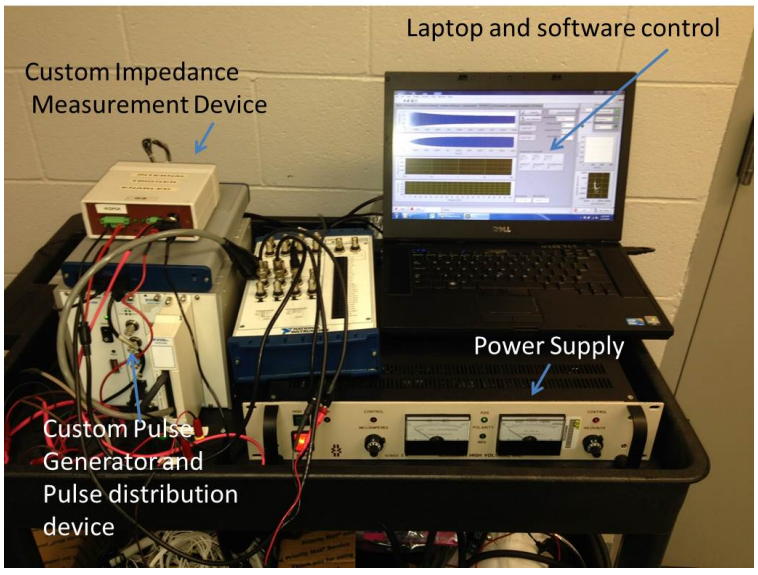

Figure 2.3: SCXI Chassis, Relay Boards, DC Power Supply, and Laptop Control

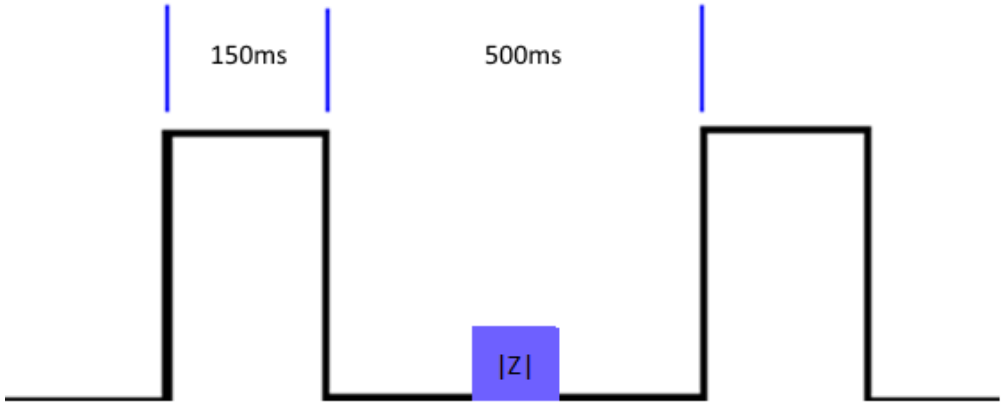

Figure 2.4: Electroporation and Impedance Parameters

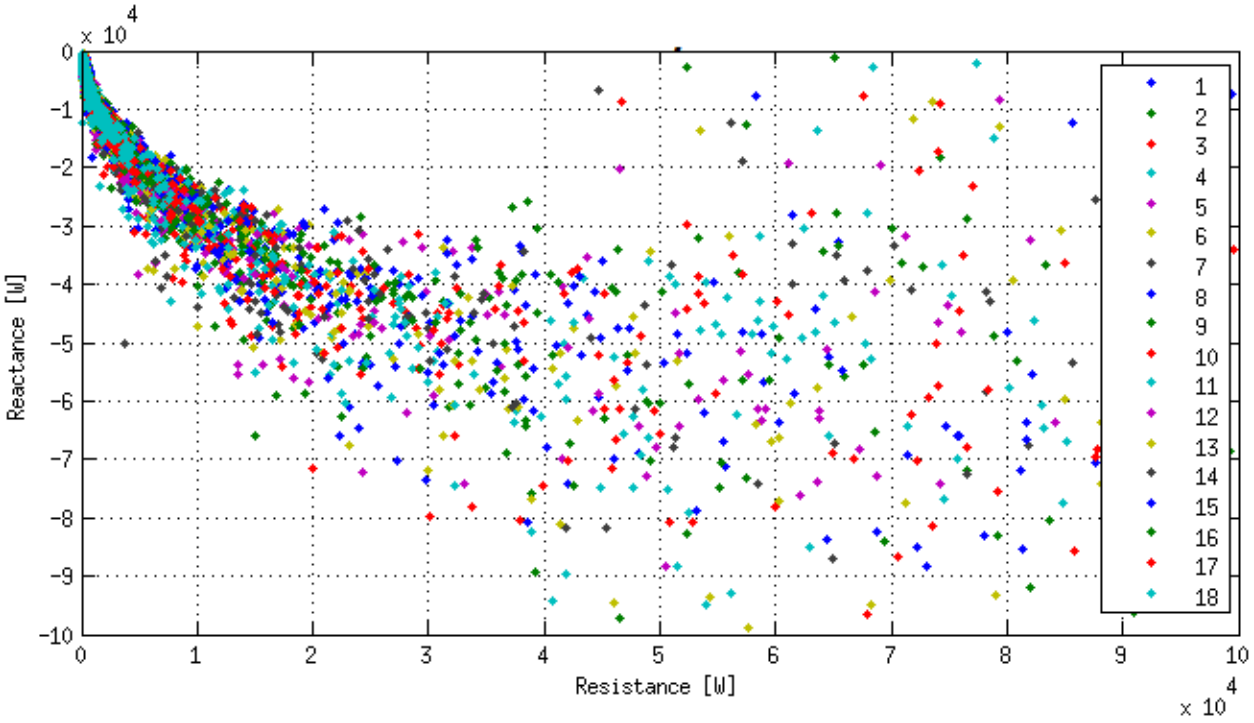

Figure 2.5: Representative Nyquist Plot for Baseline Impedance Values.

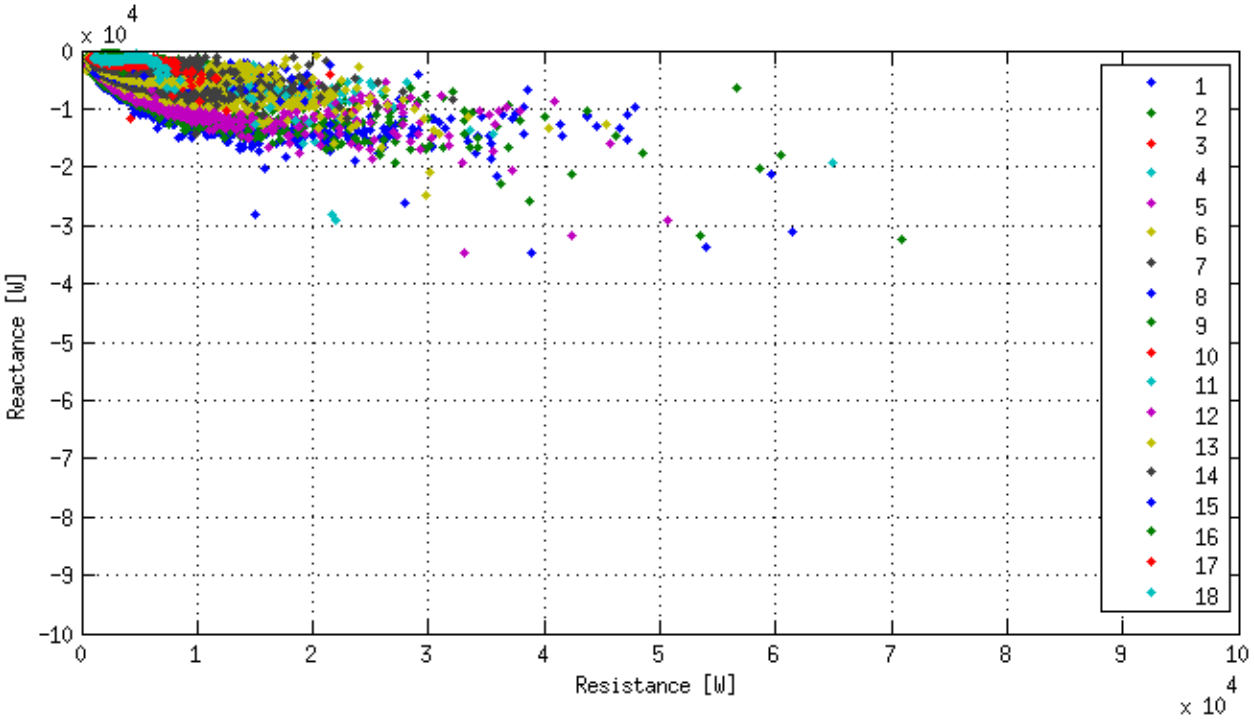

Figure 2.6: Representative Nyquist Plot for Post EP Impedance Values.

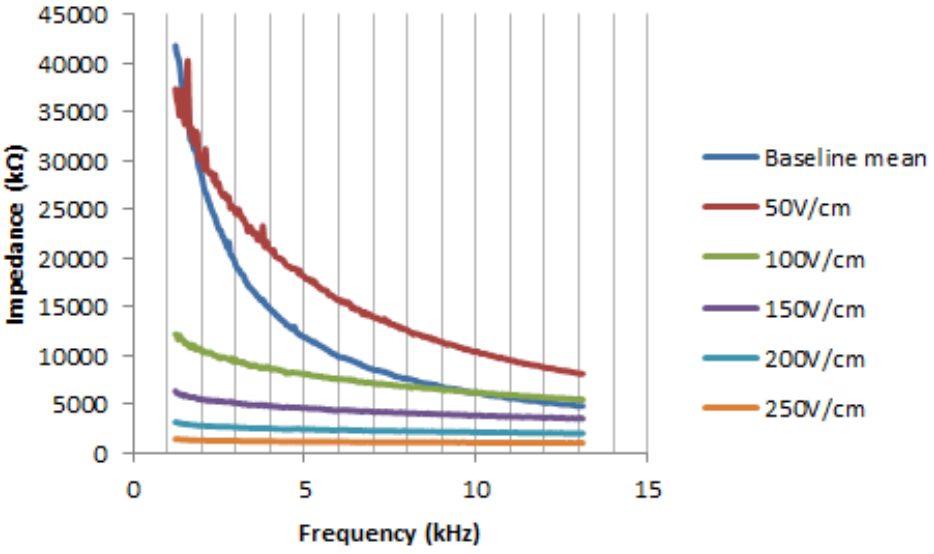

Figure 2.7: Impedance Norm After EP For Frequency Range

CHAPTER 3

RESULTS

3.1 Experimental Groups

This study was conducted by treating 11 groups. Each group contained 12 animals. The specific treatment of each group is listed below. Note that some animals received standard 4×4 pulsing (groups 3, 6 and 9). Groups 4, 5, 7, 8, 10 and 11 received feedback pulsing until a prescribed decrease in impedance relative to baseline was achieved.

1. No treatment (Control 1)
2. Injection of plasmid DNA only (Control 2)
3. 4×4 pulses in each sector at 100V/cm
4. Pulses at 100V/cm until an impedance drop of 80% or more resulted
5. Pulses at 100V/cm until an impedance drop of 95% or more resulted
6. 4×4 pulses in each sector at 150V/cm
7. Pulses at 150V/cm until an impedance drop of 80% or more resulted
8. Pulses at 150V/cm until an impedance drop of 95% or more resulted
9. 4×4 pulses in each sector at 200V/cm
10. Pulses at 200V/cm until an impedance drop of 80% or more resulted
11. Pulses at 200V/cm until an impedance drop of 95% or more resulted

The biological response, meaning the luminescence expressed as radiance, of each treatment group is shown in Figure 3.1. The group numbers at the bottom of the figure correspond to the treatment groups above. Data for days 2, 4, 7, 10 and 14 is shown. Note that the radiance for many of the treatment groups is higher than the radiance of the animals that only received a DNA injection (group 2). This indicated that the EP treatment did augment DNA delivery.

Though biological response was measured at five different points in time after treatment, only the data collected on day 2 after treatment was analyzed. The rationale for this is came from the changes in radiance over time. Figure 3.2 represents the same information from

Figure 3.1 organized by days rather than groups, shows that biological response decreased over time from day 7 onward. Day 2 and day 4 data are very similar. Therefore day 2 data was used for analysis in this study.

3.2 Data Analysis

After determining that day 2 data would be analyzed, the data analysis strategy was carefully considered. Three main factors were identified and so were three response variables.

The three factors were:

1. Voltage (combines all groups that were treated with the same electric field strength)
2. Feedback (combines all groups that were treated with the same feedback program)
3. Interaction (examines each experimental group individually)

The response variables were:

1. Biological Response
2. Impedance Drop
3. Number of Pulses

Data analysis was comprised of comparing Voltage vs. Biological Response; Feedback vs. Biological Response; the Interaction of voltage and feedback vs. Biological Response; Voltage vs. Impedance; Feedback vs. Impedance; the Interaction of voltage and feedback vs. Impedance; Voltage vs. Number of Pulses; Feedback vs. Number of Pulses; and finally the Interaction of voltage and feedback vs. Number of Pulses.

3.3 Effect of Control Factors on Biological Response

3.3.1 Voltage vs. Biological Response

Note that control groups for the treatment with voltage are Group 1 and Group 2. Table 3.1 illustrates a five statistics summary of biological response with respect to variations in voltage applied.

The data can be analyzed using the following five number summary:

- *Mean.* There is an obvious increase in the average response as voltage increases. This result is preliminary and further tests will indicate how significant it is statistically.
- *95% Confidence Interval for the Mean.* This interval can be said to contain the population mean with a 95% confidence level.
- *Median.* This value provides another central tendency of the response variable.

- *Standard Deviation.* This shows the diversity of the results obtained.
- *Minimum and Maximum.* These show the range of the results.

The first test carried out was a test for the normality of the data. The results are illustrated in Table 3.2. The Kolmogorov-Smirnov and Shapiro-Wilk normality tests were used to calculate the values in the table. The numbers in the statistics column for each show the value of the test statistics and df, which is abbreviation for degree of freedom. The numbers in the Sig. column which show the significance level (or the p-value) of the test are used for inference about the results.

For the Kolmogorov-Smirnov test, two groups had p-values greater than 0.05. It can therefore be interpreted that the scores are normally distributed with a confidence level of 95%. These two were the 100V/cm and 150V/cm field strength groups. But the biological response on two 0V/cm (no treatment) and 200V/cm groups, as indicated by the test, was not distributed normally. Therefore, non-parametric tests had to be used for further inferences on the possible difference in response through different voltage groups. Non-parametric tests use median data instead of the mean. Data for these groups is typically presented in box and whisker plots. Figure 3.3 explains what a box and whisker plot represents. Figure 3.4 shows a box and whisker plot of the response to different voltages. As indicated in the chart, higher levels of radiance were recorded on animals that were treated with higher electric field strengths.

Inferential statistical analysis was performed by comparing response through different voltage groups statistically. The results of the Kruskal-Wallis test (see Table 3.3), a non-parametric test to compare response through various non-normal samples, indicated with a 95% confidence that the response is significantly different in various voltage groups. Thus, it was concluded that voltage had an effect on the success of gene delivery.

3.3.2 Feedback vs. Biological Response

The first analysis performed was to compare descriptive statistics. It provides the computed sample mean and a 95% interval where the population mean could be, the standard deviation of the sample and sample median. Table 3.4 provides this five statistics summary of the response variable responding to different values of feedback. Table 3.5 provides the normality test

results for the distribution of response on different feedback groups. Both tests indicated that at least the group for 95% feedback pulsing was not normally distributed, so non-parametric analysis was used to make comparisons.

Once again, with a confidence level of 95%, the responses were interpreted as normally distributed in 4×4 and 80% feedback groups. But the response on two (80% and 95% feedback) groups is not distributed normally. Overall, non-parametric tests must be used for future comparisons. Figure 3.5 shows that the response on different feedback settings differ significantly. The median of responses differ between the 4×4 and feedback pulsing groups. Moreover, there is a peak in response variable at 80% feedback. That is, response increases with feedback till 80% and then decreases. Further these differences are tested for statistical significance.

With respect to inferential statistics, response is compared through different feedback groups statistically. The results of the Kruskal-Wallis test is provided in Table 3.6. The table indicates that there is a statistically significant difference between different feedback groups and the hypothesis that they are not can be rejected with a 95% level of confidence.

The results show that with 95% confidence the response is significantly different in various feedback programs since the significance value is less than 0.05. Based on Figure 3.5, it can be stated that the response does depend on feedback.

3.3.3 Interaction vs. Biological Response

The combined effects of both voltage and feedback settings on the biological response were studied. The optimal results, as will be seen, are obtained with a pairing of the optimal feedback and optimal voltage settings. Table 3.7 is the descriptive statistics summary for biological response as it was affected by different interaction settings.

Figure 3.6 shows the behavior of biological response for different interaction settings with box and whisker plots. The trend observed was that the highest response was obtained for the combination of 200V/cm and 80% feedback pulsing.

Figure 3.6 suggests there was a peak in response when feedback was set to an 80% drop in impedance. Overall, an increasing pattern is spotted through increasing field strength levels.

Table 3.8 shows that the distribution of response is normal with 95% confidence through all levels of interaction except for two groups, Group 3 and Group 9. These groups were inspected further. Normality in these two groups would allow for ANOVA, a more powerful test,

to be used for analysis. After transforming the data for these two groups with a square root function, the normality assumption was accepted with 99% confidence and the analysis is continued using parametric analysis.

Table 3.9 shows that each factors affected the response. The difference in response to different factors was statistically significant, therefore their mutual effect is also is seen to have affected the response variable. Thus, the effect of voltage settings and feedback settings combines in a straightforward fashion to yield the highest luminescence.

To better understand the impact of two variables on response, we studied Figures 3.7 and 3.8. These line graphs show the overall behavior of the biological response as it is affected by different voltages and feedback programs. One showed that 4×4 pulsing is seen to have decreasing biological response with increasing voltage. Both feedback 80% and 95% feedback pulsing produced increasing biological response results with increasing voltages. The other figure showed that 80% feedback yielded the highest level of gene expression for all feedback settings. This was consistent with observations based on the box and whisker plot.

3.4 Effect of Control Factors on Impedance

3.4.1 Voltage vs. Impedance

Table 3.10 shows the five number summary for the data collected on the effect of different voltage settings on the observed impedance drop. Next, normality tests were run to determine what kind of test to use to analyze the data further. Results are shown in Table 3.11. It was concluded that not all the data was normally distributed. Since both normal and non-normal sets were to be compared, the most appropriate test was a non-parametric one. Median values were employed.

The data was used to produce box and whisker plots, which revealed that voltage settings affected the impedance drop. Figure 3.9 revealed that higher field strengths cause a greater drop in impedance. Note that the y axis of the graph has negative values, and that a greater drop in impedance is noted by points that are lower on the y axis.

A Kruskal-Wallis test was used to determine whether the different voltage settings led to statistically significant differences between groups. The results of the test, see Table 3.12, suggest that voltage does have an effect on impedance drop.

3.4.2 Feedback vs. Impedance

The descriptive statistics for the effect of different feedback settings are shown in the table below. The feedback mechanisms are seen to have an effect on the precision of the data, compared to the data collected for biological response.

These results lead us to believe that different feedback programs result in different impedance changes, independent of what voltage was used for treatment. Higher impedance values resulted in lower variance.

Once again, to decide which test to run to compare data from different groups, normality tests were run and the fact that not all data was normal was observed. This again indicated non-parametric tests should be used.

Figure 3.11 is a box and whisker plot demonstrating that the lowest drop in impedance was actually achieved when the feedback mechanism was set to 80%. The impedance drop achieved by 4×4 pulsing was comparable to that achieved through feedback pulsing to a 95% impedance drop. The non-parametric Kruskal-Wallis test is run to find out if the impedance drop is different across different feedback settings. The result summaries shown in Figure 3.12 indicate that, with a 95% confidence it can be stated that there is a statistically significant difference between the impedance drops caused by varying feedback settings.

3.4.3 Interaction vs. Impedance

The combined effect of voltage and feedback on the drop in impedance was observed. The first set of numbers analyzed were those in the table of descriptive statistics for this data set. This five number summary shows estimates for intervals where the population values could be and how the sample values obtained compare to them.

There were high error margins and a high standard deviation. The groups were tested for normality. It was concluded with a 95% confidence that some of them are not normal, as the values obtained are less than the level of significance ($\alpha = 0.05$). The normality test (Table 3.15) indicated that the groups corresponding to 150V/cm*80%, 150V/cm*95% and 200V/cm*4×4 were not normal. Hence non-parametric tests were run in subsequent analysis. In Figure 3.13, a box and whisker plot of the effect of interaction on impedance drop shows that the behavior shown by impedance as it changed according to feedback and voltage settings independently

holds true for combinations of both. The lowest impedance drop was achieved through lower field strengths, and an 80% feedback setting caused a smaller change in impedance than 4×4 pulsing or 95% feedback pulsing. However, when compared to how interaction affects biological response, no pattern is evident where similar results in impedance drop yield similar biological responses. Based on this, it cannot be stated that a net impedance drop alone will produce high biological responses consistently.

3.5 Effect of Control Factors on Number of Pulses

Based on the analysis of the impedance drop, it was determined that impedance drop alone was not a strong predictor for the success of gene delivery through EP. Hence additional tests were performed to evaluate the effect of the control factors on the number of pulses. It can be seen that the median number of pulses does not vary much between different voltages, but higher voltages create less variance in the number of pulses needed to satisfy the feedback conditions.

3.5.1 Voltage vs. Pulses

The analysis was performed by observing trends in the box and whisker plots that compared the number of pulses needed by each voltage setting. The results are shown in Figure 3.14, a box and whisker plot that shows the number of pulses needed by each voltage treatment group.

Including the data for 4×4 pulsing skews the overall values for Figure 3.14, making the median values very similar. This skewed by the same amount in all test groups because it added the same constant number to the variance when it was calculated. Given that the most noticeable feature of the graph is the variability in the data, the analysis including these values was still valid.

3.5.2 Feedback vs. Pulses

In Figure 3.15, the effect of different feedback programs on the average number of pulses can be seen. It can be observed that both 4×4 pulsing and 95% feedback pulsing use a greater number of pulses compared to 80% feedback pulsing. The median number of pulses for 80% impedance drop is less than that for 4×4 and 95% impedance drop.

3.5.3 Interaction vs. Pulses

The results of the tests run to evaluate the effect of both voltage and feedback settings independently on the number of pulses used suggests the study of their combined effect. Figure 3.16 shows a box and whisker plot where the combined effect of the voltage and feedback settings were seen to add upon one another. The lowest impedance drop was accomplished by combining the voltage setting for the lowest impedance drop and the feedback setting for the lowest impedance drop. The overall trends observed for the independent effect of the control factors was still present. Lower field strengths required fewer pulses and 80% feedback yielded the lowest number of pulses overall.

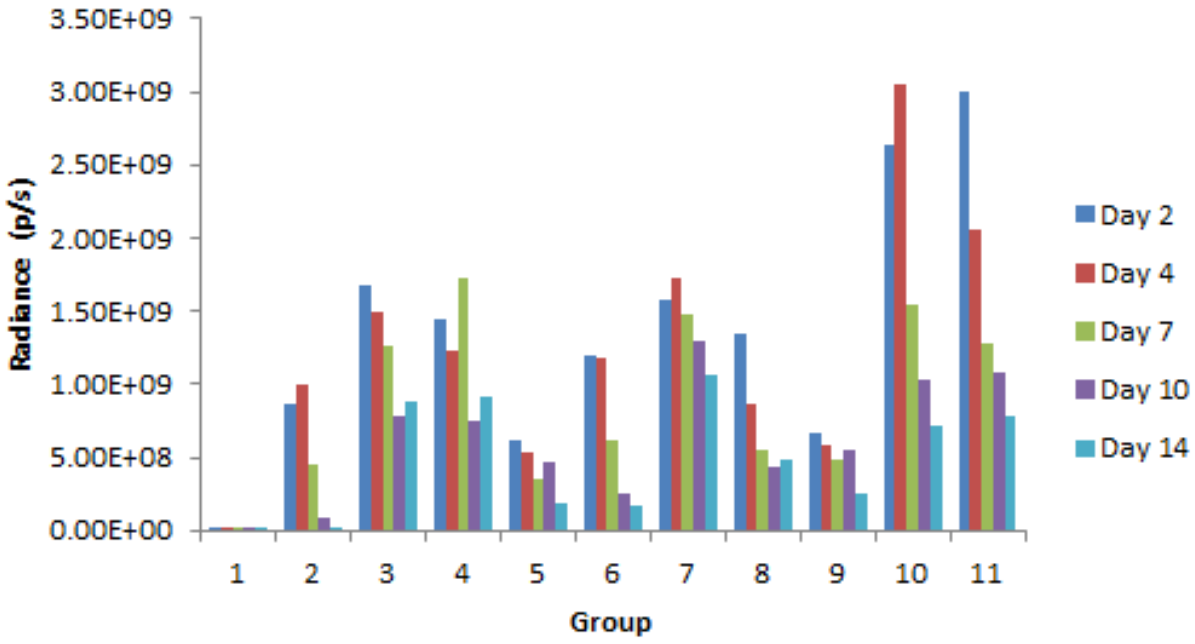

Figure 3.1: Biological Response Means

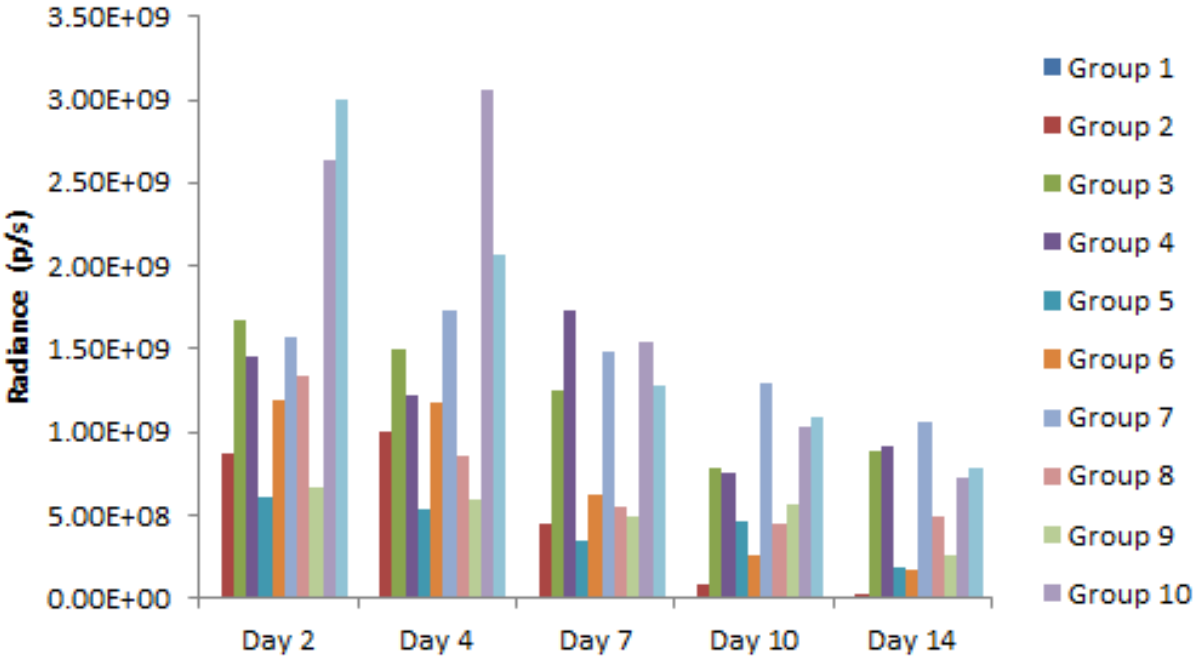

Figure 3.2: Mean Radiance By Days

Table 3.1: Descriptive Statistics for Voltage vs. Biological Response

	Voltage	Statistics						
		Mean	95% Confidence Interval for the mean		Median	Std. Dev.	Min.	Max.
			Lower Bound	Upper Bound				
Response	100V/cm	1.21651E+9	8.88179E+8	1.54484E+9	1.12600E+9	9.409943E+8	1.65106	4.009E+9
	150V/cm	1.47224E+9	1.15271E+9	1.79176E+9	1.35100E+9	9.443571E+8	1.022E+8	4.400E+9
	200V/cm	2.21568E+9	1.61115E+9	2.82021E+9	1.93450E+9	1.732586E+9	2.529E+7	6.367E+9

Table 3.2: Tests of Normality for Voltage vs. Biological Response

	Voltage	Kolmogorov-Smirnov			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Response	100V/cm	.119	34	.200*	.909	34	.008
	150V/cm	.117	36	.200*	.949	36	.095
	200V/cm	.163	34	.023	.910	34	.009
* This is a lower bound for true significance.							

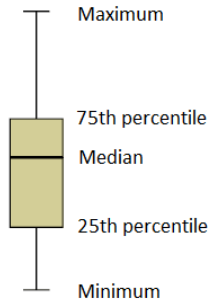

Figure 3.3: Sample Box and Whisker Plot

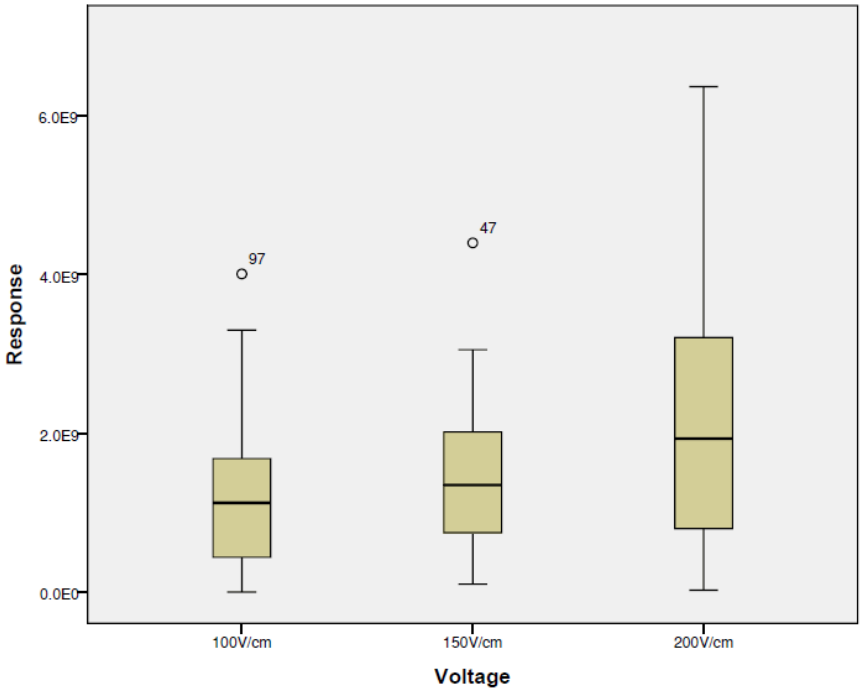

Figure 3.4: Box and Whisker Plot: Voltage vs. Biological Response

Table 3.3: Kruskal-Wallis Test for Voltage vs. Biological Response

	Response
Chi-Square	30.980
df	3
Asymptotic Significance	.000

Table 3.4: Descriptive Statistics for Feedback vs. Biological Response

	Feedback	Statistics						
		Mean	95% Confidence Interval for the mean		Median	Std. Dev.	Min.	Max.
			Lower Bound	Upper Bound				
Response	4×4	1.22467E+9	9.00098E+8	1.54924E+9	1.10050E+9	9.592666E+8	1.65106	4.009E+9
	80%	2.05921E+9	1.63573E+9	2.48268E+9	1.89000E+9	1.232770E+9	1.022E+8	5.555E+9
	95%	1.62226E+9	1.05595E+9	2.18858E+9	1.18900E+9	1.597121E+9	1.152E+8	6.367E+9

Table 3.5: Tests of Normality for Feedback vs. Biological Response.

	Feedback	Kolmogorov-Smirnov			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Response	4×4	.128	36	.145	.924	36	.016
	80%	.145	35	.061	.910	35	.007
	95%	.234	33	.000	.792	33	.000
* Marks a lower bound for true significance.							

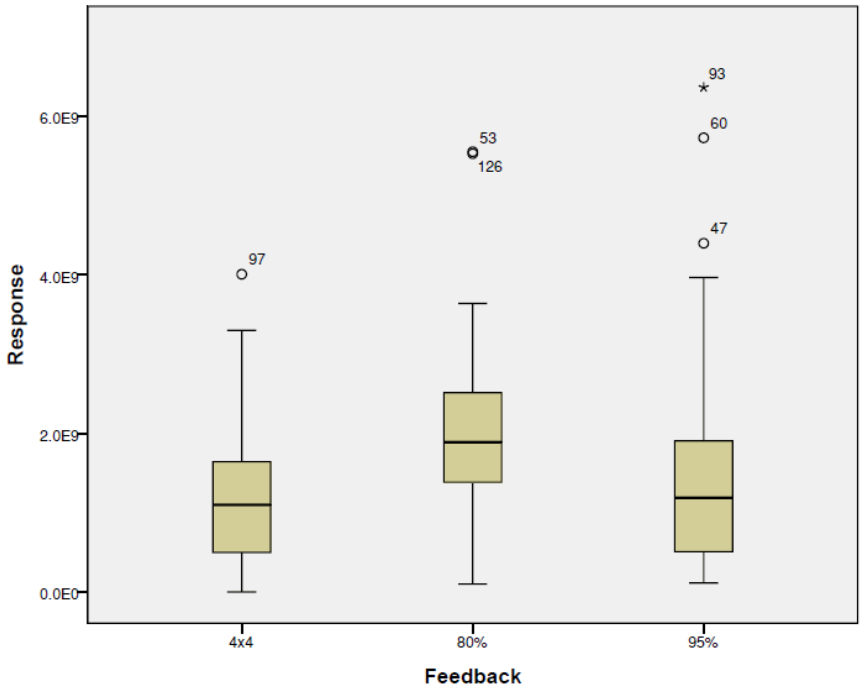

Figure 3.5: Box and Whisker Plot: Feedback vs. Biological Response

Table 3.6: Kruskal-Wallis Test for Feedback vs. Biological Response

	Response
Chi-Square	33.496
df	3
Asymptotic Significance	.000

Table 3.7: Descriptive Statistics for the Effects of Interaction on Response

	Interaction	Statistics						
		Mean	95% Confidence Interval for the mean		Median	Std. Dev.	Min.	Max.
			Lower Bound	Upper Bound				
Response	100V/cm*4×4	1.51045E+9	7.22501E+8	2.29841E+9	1.15000E+9	1.240150E+9	1.65106	4.009E+9
	100V/cm*80%	1.46754E+9	1.03808E+9	1.89700E+9	1.54700E+9	6.759192E+8	2.345E+8	2.578E+9
	100V/cm*95%	5.62530E+8	3.00519E+8	8.24541E+8	4.72200E+8	3.662662E+8	1.605E+8	1.372E+9
	150V/cm*4×4	1.21975E+9	8.15376E+8	1.62412E+9	1.22050E+9	6.364388E+8	2.040E+8	2.625E+9
	150V/cm*80%	1.78398E+9	1.14421E+9	2.42374E+9	2.00650E+9	1.006911E+9	1.022E+8	3.054E+9
	150V/cm*95%	1.41298E+9	7.05676E+8	2.12029E+9	1.25500E+9	1.113222E+9	1.152E+8	4.400E+9
	200V/cm*4×4	9.43798E+8	3.72080E+8	1.51552E+9	5.01400E+8	8.998191E+8	2.529E+7	2.470E+9
	200V/cm*80%	3.00491E+9	2.04198E+9	3.96784E+9	2.27500E+9	1.433344E+9	1.460E+9	5.555E+9
	200V/cm*95%	2.81395E+9	1.48030E+9	4.14761E+9	2.02300E+9	1.985167E+9	5.108E+8	6.367E+9

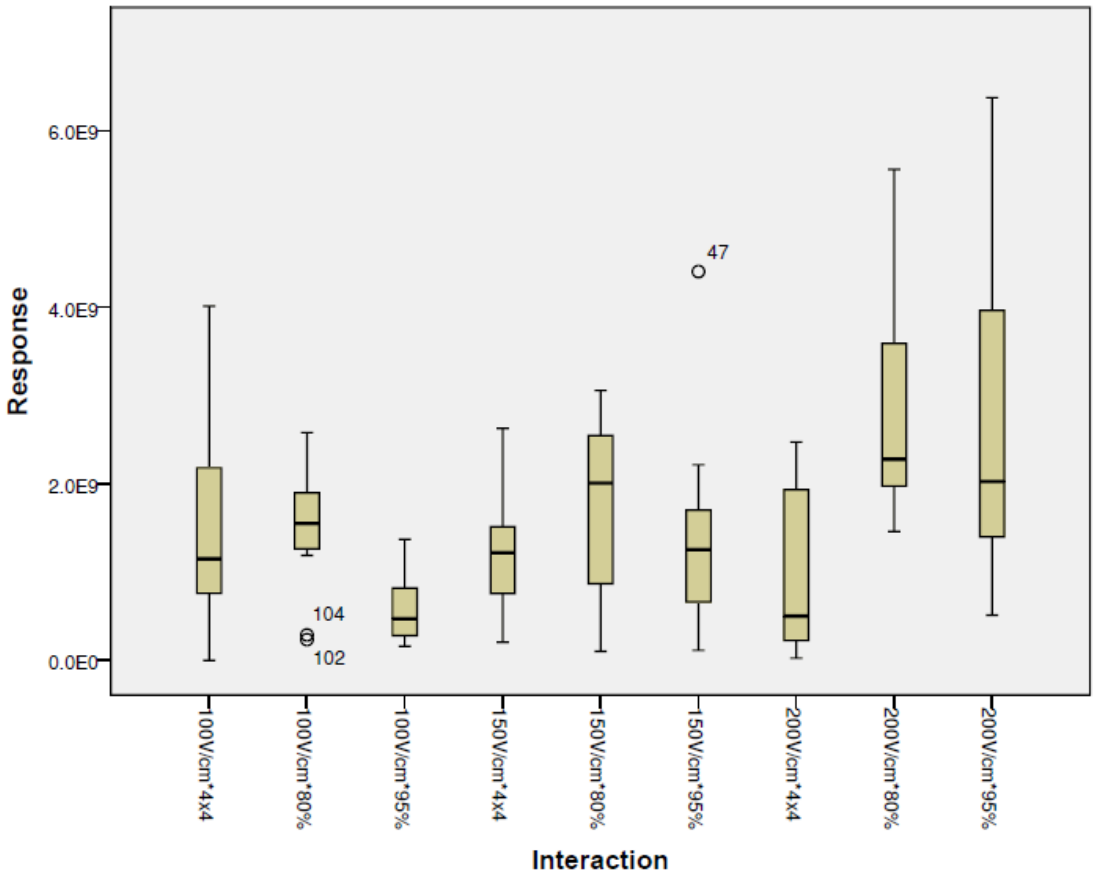

Figure 3.6: Box and Whisker Plot: Interaction vs. Biological Response

Table 3.8: Tests of Normality for Interaction vs. Biological Response

Interaction	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Control 2	.250	11	.052	.816	11	.015
100V/cm*4×4	.255	12	.031	.891	12	.123
100V/cm*80%	.173	12	.200*	.921	12	.298
100V/cm*95%	.193	10	.200*	.899	10	.212
150V/cm*4×4	.150	12	.200*	.963	12	.829
150V/cm*80%	.192	12	.200*	.923	12	.311
150V/cm*95%	.221	12	.108	.836	12	.025
200V/cm*4×4	.266	12	.019	.836	12	.025
200V/cm*80%	.240	11	.076	.848	11	.040
200V/cm*95%	.222	12	.105	.889	12	.114
*. This is a lower bound of the true significance.						
a. Response is constant when Interaction = Control 1. It has been omitted.						

Table 3.9: Test of Between-subjects Effects

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.514E+19[a]	8	6.893E+18	5.392	.000
Intercept	2.758E+20	1	2.758E+20	2.158E+2	.000
Voltage	1.984E+19	2	9.922E+18	7.762	.001
Feedback	1.331E+19	2	6.657E+18	5.207	.007
Interaction	2.430E+19	4	6.076E+18	4.752	.002
Error	1.227E+20	96	1.278E+18		
Total	4.571E+20	105			
Corrected Total	1.779E+20	104			
a. R Squared = .310 (Adjusted R Squared = .253)					

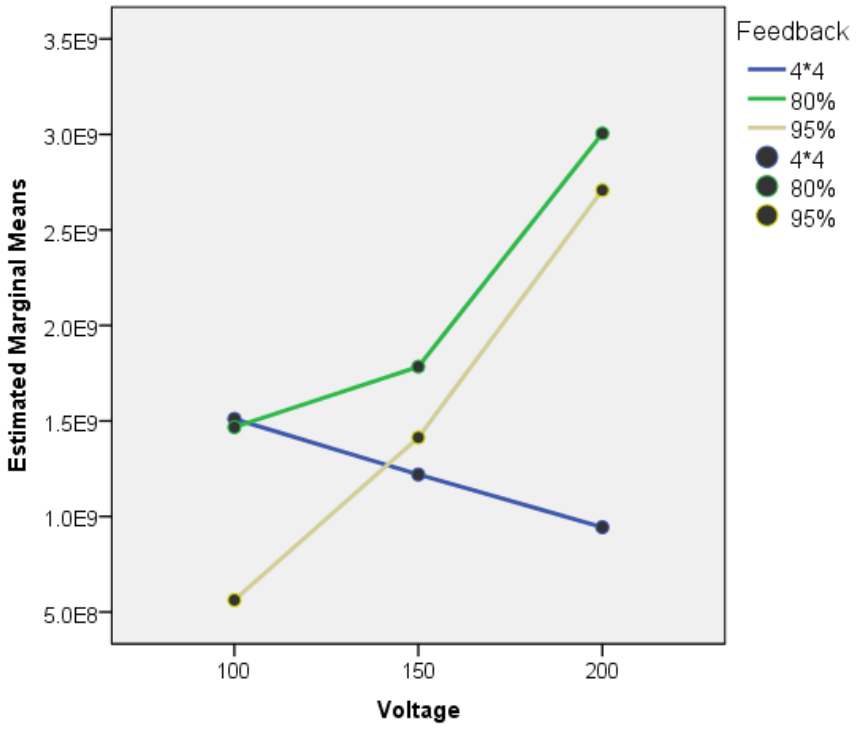

Figure 3.7: Line Graph on Response to Interaction

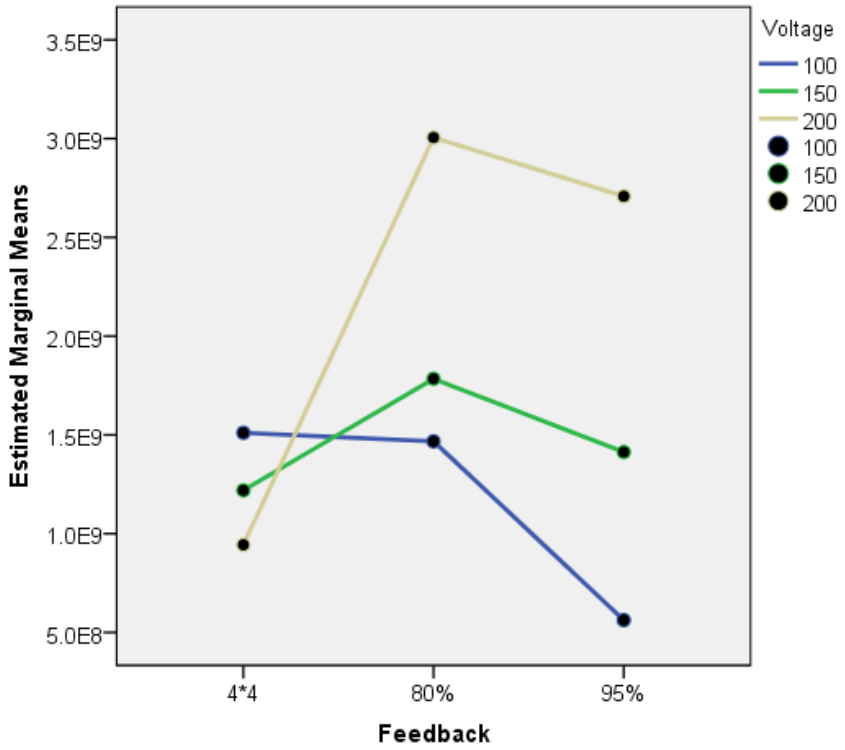

Figure 3.8: Line Graph on Response to Different Treatments

Table 3.10: Descriptive Statistics for Voltage vs. Impedance

	Voltage	Statistics						
		Mean	95% Confidence Interval for the mean		Median	Std. Dev.	Min.	Max.
			Lower Bound	Upper Bound				
Impedance	4×4	7.97113E+1	8.20114E+1	7.74112E+1	7.88825E+1	6.592026	8.976E+1	6.873E+1
	80%	8.52669E+1	8.86934E+1	8.18404E+1	8.82185E+1	1.012693E+1	9.379E+1	3.887E+1
	95%	8.96109E+1	9.71731E+1	8.20488E+1	9.37030E+1	2.167322E+1	9.817E+1	8.315E+1

Table 3.11: Tests of Normality for Voltage vs. Impedance

	Voltage	Kolmogorov-Smirnov			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Impedance	4×4	.092	34	.200*	.944	34	.081
	80%	.200	36	.001	.690	36	.000
	95%	.386	34	.000	.306	34	.117
* This is a lower bound for true significance.							

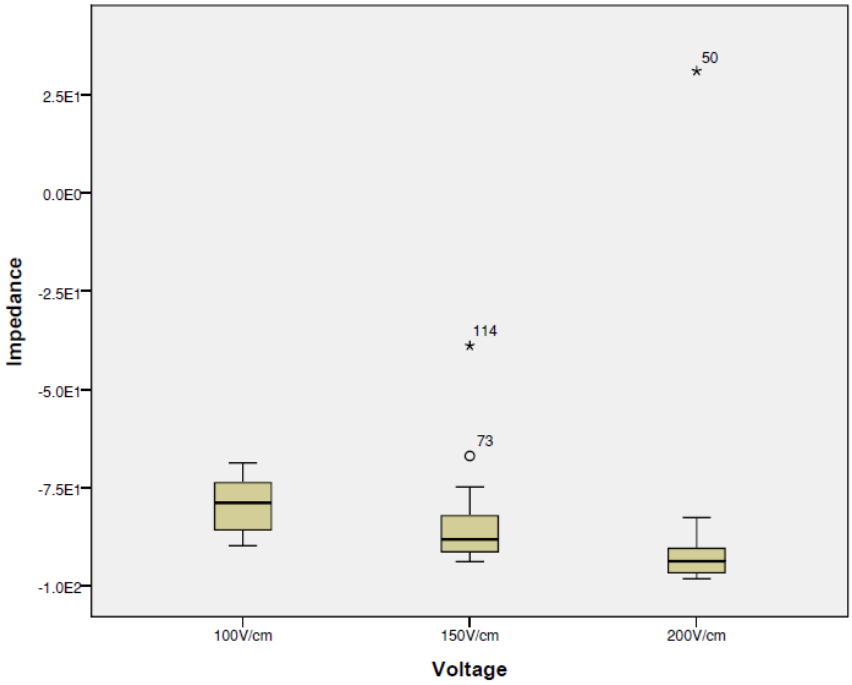

Figure 3.9: Box and Whisker Plot: Voltage vs. Impedance

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Impedance is the same across categories of Voltage.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Figure 3.10: Kruskal-Wallis Test: Voltage vs. Impedance

Table 3.12: Descriptive Statistics for Feedback vs. Impedance

	Feedback	Statistics						
		Mean	95% Confidence Interval for the mean		Median	Std. Dev.	Min.	Max.
			Lower Bound	Upper Bound				
Impedance	4×4	8.41003E+1	9.14399E+1	7.67607E+1	8.79089E+1	2.169221E+1	9.817E+1	3.110E+1
	80%	8.01118E+1	8.35890E+1	7.66346E+1	8.19992E+1	1.012254E+1	9.214E+1	3.887E+1
	95%	9.07589E+1	9.18223E+1	8.96954E+1	9.13205E+1	2.999278	9.503E+1	8.315E+1

Table 3.13: Tests of Normality for Feedback vs. Impedance.

	Feedback	Kolmogorov-Smirnov			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Impedance	4×4	.258	36	.000	.522	36	.000
	80%	.117	35	.200	.836	35	.000
	95%	.125	33	.200	.948	33	.117
* This is a lower bound for true significance.							

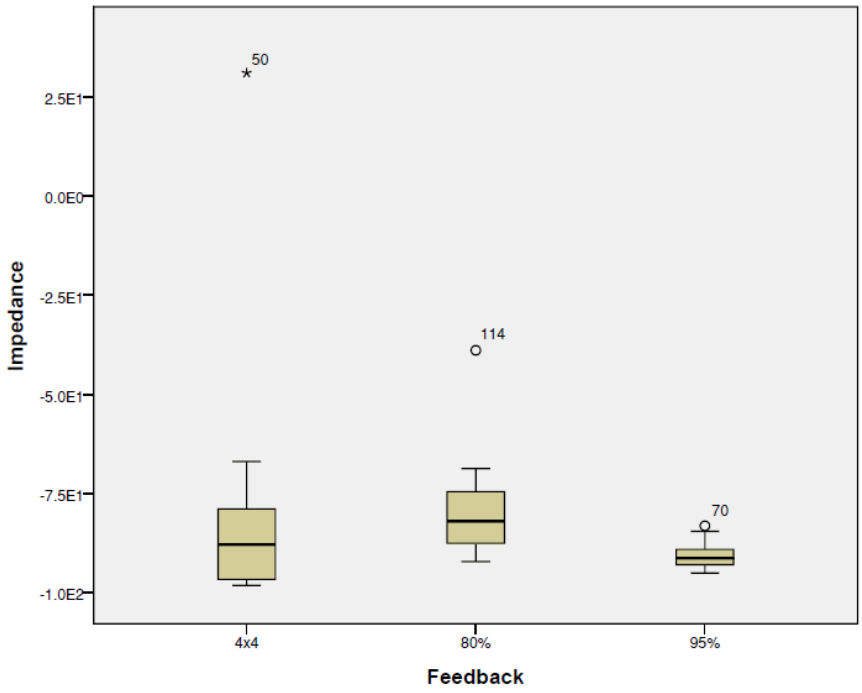

Figure 3.11: Box and Whisker Plot: Feedback vs. Impedance

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Impedance is the same across categories of Feedback.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Figure 3.12: Kruskal-Wallis Test: Feedback vs. Impedance

Table 3.14: Descriptive Statistics for the Effects of Interaction on Impedance

	Interaction	Statistics						
		Mean	95% Confidence Interval for the mean		Median	Std. Dev.	Min.	Max.
			Lower Bound	Upper Bound				
Impedance	100V/cm*4×4	-7.93584E+1	-8.20081E+1	-7.67086E+1	-7.88825E+1	4.170471	-8.673E+1	-7.152E+1
	100V/cm*80%	-7.36154E+1	-7.59616E+1	-7.12693E+1	-7.28933E+1	3.692639	-8.051E+1	-6.873E+1
	100V/cm*95%	-8.74499E+1	-8.90747E+1	-8.58251E+1	-8.79880E+1	2.271271	-8.976E+1	-8.315E+1
	150V/cm*4×4	-8.64109E+1	-9.17425E+1	-8.10792E+1	-8.90017E+1	8.391456	-9.379E+1	-6.693E+1
	150V/cm*80%	-7.86487E+1	-8.69770E+1	-7.03204E+1	-8.21503E+1	1.310775E+1	-8.861E+1	-3.887E+1
	150V/cm*95%	-9.07411E+1	-9.16791E+1	-8.98032E+1	-9.13182E+1	1.476241	-9.180E+1	-8.740E+1
	200V/cm*4×4	-8.65317E+1	-1.10071E+2	-6.29922E+1	-9.69984E+1	3.704858E+1	-9.817E+1	3.110E+1
	200V/cm*80%	-8.87948E+1	-9.07238E+1	-8.68657E+1	-8.83004E+1	2.871422	-9.214E+1	-8.258E+1
	200V/cm*95%	-9.37863E+1	-9.43542E+1	-9.32184E+1	-9.37090E+1	8.453397E-1	-9.503E+1	-9.239E+1

Table 3.15: Tests of Normality for Impedance based on Interaction

	Feedback	Kolmogorov-Smirnov			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Impedance	100V/cm*4×4	.136	12	.200	.975	12	.955
	100V/cm*80%	.158	12	.200	.951	12	.657
	100V/cm*95%	.201	10	.200	.890	10	.169
	150V/cm*4×4	.213	12	.140	.830	12	.021
	150V/cm*80%	.314	12	.002	.612	12	.000
	150V/cm*95%	.290	12	.006	.702	12	.001
	200V/cm*4×4	.521	12	.000	.342	12	.000
	200V/cm*80%	.137	11	.200	.916	11	.288
	200V/cm*95%	.124	11	.200	.961	11	.782

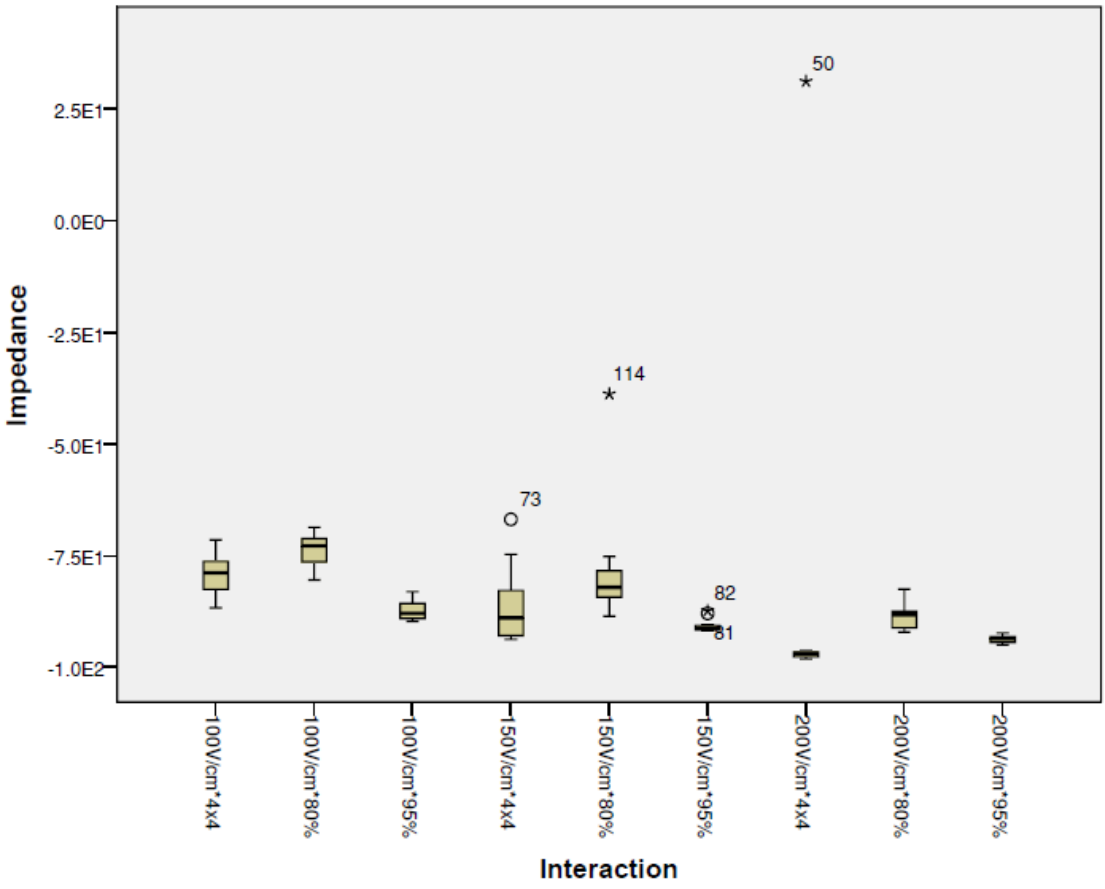

Figure 3.13: Box and Whisker Plot: Interaction vs. Impedance

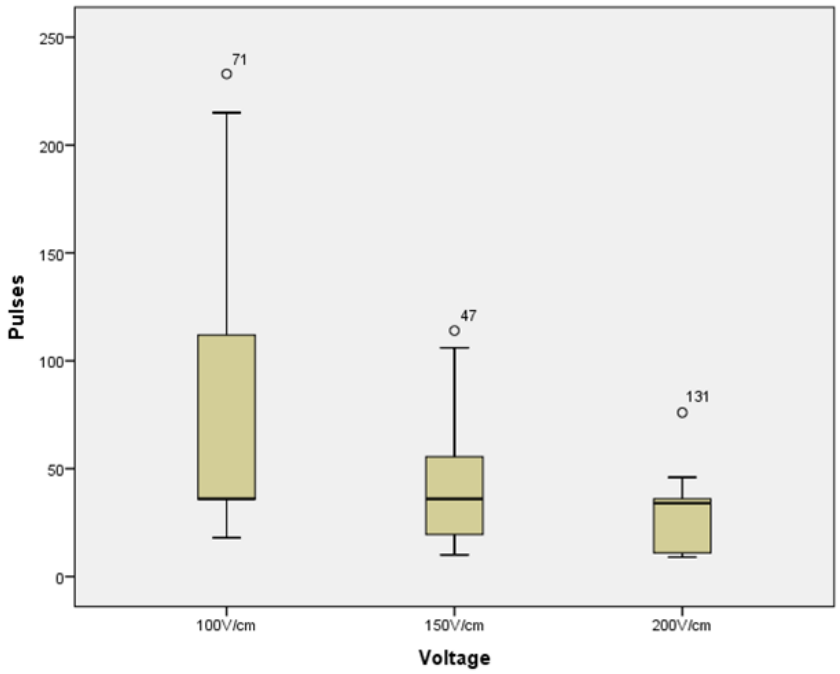

Figure 3.14: Box and Whisker Plot: Voltage vs. Number of Pulses

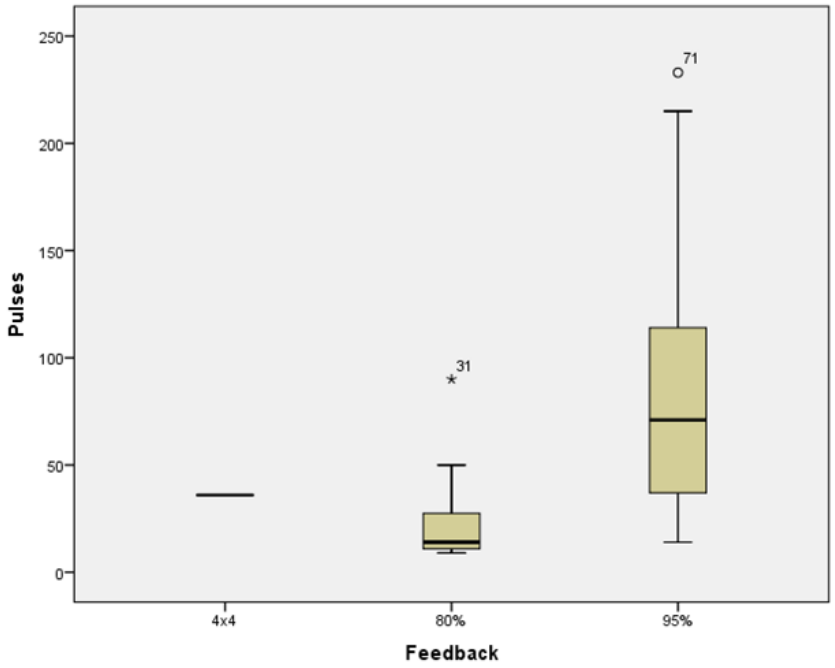

Figure 3.15: Box and Whisker Plot: Feedback vs. Number of Pulses

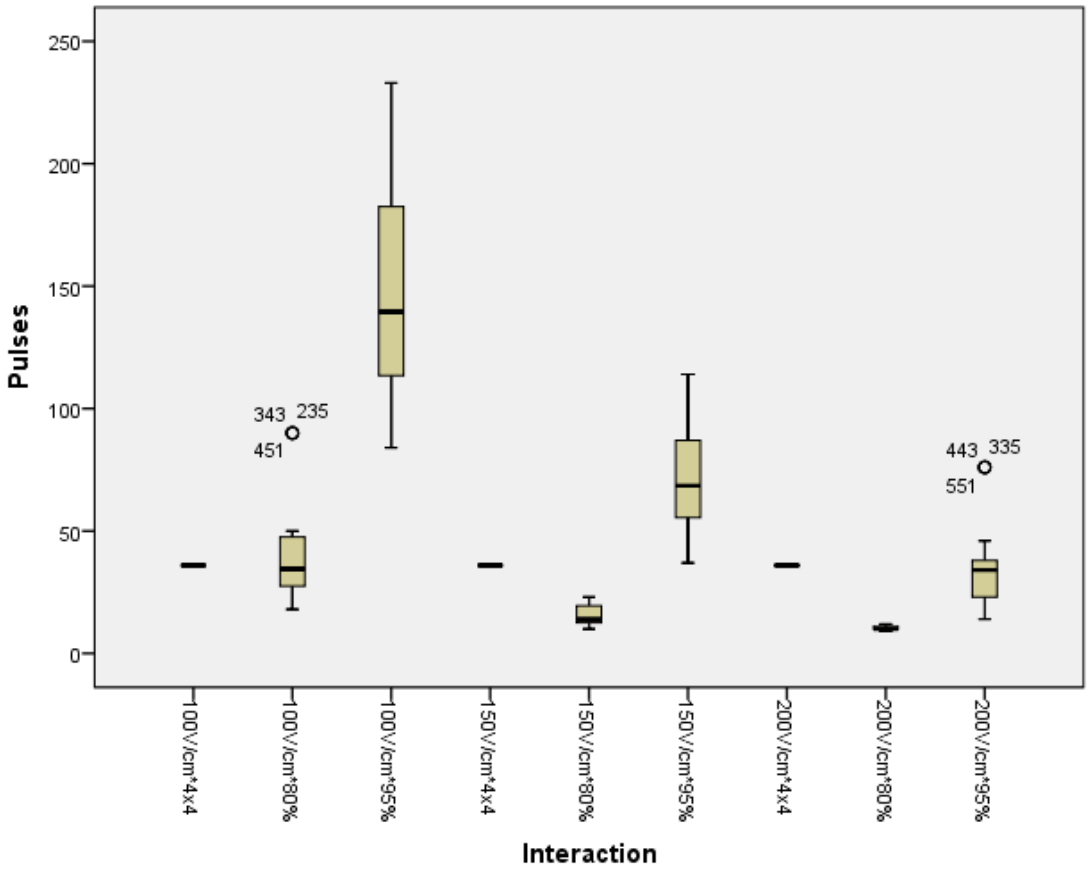

Figure 3.16: Interaction vs. Number of Pulses

CHAPTER 4

CONCLUSIONS

The results indicated that there was a statistically significant correlation between higher voltages applied during treatment and higher gene expression. The best voltage setting was 200V/cm. Similarly, the best feedback program utilized an impedance drop set to 80% because it yielded the highest luminescence. Moreover, it was observed that the combination of feedback set to an 80% impedance drop achieved through 200V/cm pulses, corresponding to Group 10 in the experiment, resulted in the best overall gene expression.

The highest-performing feedback setting corresponded to the lowest impedance drop. The highest-performing voltage setting corresponded to the highest impedance drop. This indicated that the impedance norm drop alone was not the best predictor for the success of gene delivery. It is important to note that the most successful EP treatments did not depend on the impedance norm drop alone, but rather on how this drop was obtained. Based on the analysis of how the control factors affected the number of pulses, the lowest variance and lowest average number of pulses yield the best biological response. High voltages reduce the variance and impedance feedback pulsing reduce the number of pulses needed per sector. Hence, it is not impedance alone but a combination of an impedance, leading a feedback mechanism, and field strength that yields optimum results.

4.1 Future Work

In future experiments, different feedback programs set to impedance drops lower than 80% should be explored. The data should be analyzed for both real and imaginary values of impedance separately, rather than combined as the norm of the impedance.

Changes should be made to reduce variability in the data as much as possible. Additionally, further experiments could be done testing the injection of other compounds that are metabolized differently from luciferin. This includes other genetic material as well as drugs, given that one possible use for this technology involves electrochemotherapy. A number of improvements could be made to the experimental procedure in the future.

1. Use only female mice. Male mice often fought, causing scarring on their flanks that could interfere with the precision and accuracy of the data obtained.
2. Exfoliate the skin. The initial pulses seem to be lowering the resistance of the stratum corneum. If the skin is exfoliated beforehand fewer pulses and/or a lower voltage may be required to obtain good results.
3. Modifying the user interface of the LabView program. If the group settings could be preprogrammed in the software, there would be less room for human error while changing the parameters in between treatment groups.
4. Changing the electrode tips more often. The electrode tips would periodically be damaged, possibly by trace amounts of saline solution on the skin surface causing a short circuit.
5. Changing the concentrations of luciferase injected. The amount of luciferase that reacted on early days (2 and 4, most notably) may interfere with the biological response observed in future dates.

Some variability, however, is inevitable. The factors that influenced the variability of the data include:

- Scarring due to grooming. The mice would often groom while in the cages and have patches of skin with a different texture that could have different electrical properties and skew the data.
- Skin cell shedding. Biological response rates varied as the skin cells died and moved towards the stratum corneum. The turnover rate for skin cells varied between animals.
- Animal to animal variability. Because of the social structures that were formed in each cage it was not unusual to find mice with different phenotypes. In particular, one of the mice in each group always seemed to gain more weight than the others and there would be a significant fat layer under this mouse's skin.

REFERENCES

[1] W. J. C and Chizmadzhev. *Electroporation: Biological and Medical Aspects of Electromagnetic Fields*. CRC Press, 2007.

[2] R. G. Carroll. *Elsevier's integrated physiology*. Mosby Elsevier, 2007.

[3] B. M. Chassy, A. Mercenier, and J. Flickinger. Transformation of bacteria by electroporation. *Trends in Biotechnology*, 6(12):303 – 309, 1988.

[4] R. V. Davalos, B. Rubinsky, and D. M. Otten. A feasibility study for electrical impedance tomography as a means to monitor tissue electroporation for molecular medicine. *Biomedical Engineering, IEEE Transactions on*, 49(4):400–403, 2002.

[5] L. Heller, M. Jaroszeski, D. Coppola, A. McCray, J. Hickey, and R. Heller. Optimization of cutaneous electrically mediated plasmid dna delivery using novel electrode. *Gene therapy*, 14(3):275–280, 2006.

[6] L. C. Heller and R. Heller. In vivo electroporation for gene therapy. *Human gene therapy*, 17(9):890–897, 2006.

[7] A. Ivorra and B. Rubinsky. In vivo electrical impedance measurements during and after electroporation of rat liver. *Bioelectrochemistry*, 70(2):287–295, 2007.

[8] O. G. Martinsen and S. Grimnes. *Bioimpedance and bioelectricity basics*. Academic press, 2011.

[9] T. Nishi, K. Yoshizato, S. Yamashiro, H. Takeshima, K. Sato, K. Hamada, I. Kitamura, T. Yoshimura, H. Saya, J.-i. Kuratsu, et al. High-efficiency in vivo gene transfer using intraarterial plasmid dna injection following in vivo electroporation. *Cancer research*, 56(5):1050–1055, 1996.

[10] M. Okino and H. Mohri. Effects of a high-voltage electrical impulse and an anticancer drug on in vivo growing tumors. *Japanese journal of cancer research: Gann*, 78(12):1319–1321, 1987.