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Non-Invasive Manipulation of Membrane Potential in Intact Living Cells

Robin Dando
University of South Florida

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Non-Invasive Manipulation of Membrane Potential in Intact Living Cells

by

Robin Dando

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Physics
College of Arts and Sciences
University of South Florida

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Non-Invasive Manipulation of Membrane Potential in Intact Living Cells

Robin Dando

ABSTRACT

All living cells contain the electrogenic enzyme Na/K ATPase, whose function is to pump ions against the electrochemical gradient, in order to provide potential energy which is later used for cellular processes such as action potentials, muscle contraction and facilitated transport. Using a technique developed in our lab, exploiting the molecule’s voltage dependence, it is possible to increase this pump function by many folds. Optical measurement of the membrane potential of living cells was made using a potentiometric dye, with successful manipulation of the ionic concentration and membrane potential reported. Additional supporting results are presented, along with extension of this field to the study of cardiac Myocytes, representing a progression to Mammalian cells, with advantages to future clinical research evident. Successful manipulation of membrane potential was also achieved using cells in a two dimensional tissue matrix, which more closely approximates the living system, and hence is closer to an eventual clinical application. Also, expedited recovery from electrical injury was recorded, demonstrating a possible therapeutic application of the technique.
Chapter 1 – Introduction and Background

In this chapter, the basic concepts of biological membranes, membrane transport and electrochemical gradient are introduced. The function of the molecule Na/K ATPase is presented, along with the mechanism and purpose of our synchronization technique, supported by previously obtained results. Methodologies for both the application of our technique, and the measurements of evoked effects are described in detail.

1.1 - Introduction

Figure 1.1 is a simple representation of a cell. We can see that there is a difference in constituents, as we move from the inside to the outside of the cell. This difference, or chemical gradient, is essential for normal function of the cell. By this, we mean processes such as the maintenance of cellular volume, cellular pH control, and signaling processes, for example the production of action potentials. The differing constituents are separated by a barrier, the plasma membrane, ensuring this concentration gradient remains.
Just as a balloon separates a higher concentration (or pressure) of constituents from a lower, when a channel is opened up across this barrier, the constituents will escape down the concentration gradient, and out of the cell. In order for material to be forced into the cell, and hence up this concentration gradient, we must use a form of pump, which will expend another form of energy in order to accomplish this feat. If we now consider that these cellular constituents are in fact ions, and therefore hold a charge, then we can consider that this separation across a resistive barrier would represent a voltage, indicated in Figure 1.1 as $V$, and any movement of these ions through either the channel or pumping molecules across this barrier, would represent a current, $I$. 

*Figure 1.1 – Representation of a simple cell*
1.2 - Biological Membranes

Every type of biological cell in existence is held together by a cell membrane, in the vast amount of cases a phospholipids bilayer. This intensely studied system is often simplified to little more than a solid wall preventing cellular components from escaping the cell, whereas in actual fact it is a constantly changing two dimensional form of entropic self assembly, held in place by the hydrophobic effect, allowing cellular components to enter or leave the cell as and when needed, as in Figure 1.2.

Figure 1.2 – A “fluid mosaic”, demonstrating peripheral and integral membrane proteins. (Universita della Tuscia)
This is accomplished by an array of membrane proteins acting as selective gateways for ionic species to navigate this barrier, and biological pumps facilitating transport in directions normally opposing the natural flow of said species. The cellular membrane was first termed a fluid mosaic, by S.J. Singer (Singer and Nicolson, 1972), expressing the relatively free diffusion of some membrane proteins within the plane of the membrane. Proteins can be physically attached to this membrane, or can merely span the membrane, according to the principles of the hydrophobic effect, with no physical means of attachment save for thermodynamics.
1.3 - Membrane Transport

The degree of diffusion allowed by the membrane is determined by the relative permittivity of the membrane to a species, a non-linear relationship linked to the electrical properties of the specific cell. The electrical properties of various cells rely on the establishment of a potential difference from the inside to the outside of a cell, across this plasma membrane. This is termed the transmembrane potential, membrane potential, or simply Vm. In addition to the transport of chemical species through the cellular membrane, membrane proteins play a fundamental role in determining the electrical characteristics of a cell. As the cellular fluid is populated by ionic species, a differing chemical concentration of species will relate to a potential difference across the cell, a voltage within the cell. Any passage of a charged particle across the resistive barrier of the plasma membrane will comparably be analogous to a current. The most readily studied membrane proteins belong to the class of ion channels. The existence of ion channels was first hypothesized by Hodgkin and Huxley (Hodgkin and Huxley, 1952), in work which would later be rewarded with a Nobel Prize. This hypothesis was confirmed in the 1970s by Sakmann and Neher (Sakmann and Neher, 1976), with their work in patch clamping, which would also later be rewarded by the Nobel foundation. Ion channels are an arrangement of several proteins, forming a circular structure arranged around an aqueous central pore, as in Figure 1.3.
Figure 1.3 – An ion channel allowing sodium ions to pass through the hydrophobic lipid bilayer, through an aqueous pore. (Michael D. Hilborn)

Due to the hydrophobic nature of the lipid bilayer, a structure with an aqueous pore capable of spanning the membrane is of imperative significance, allowing a level of permeability of the membrane to aqueous species incapable of navigating the plasma membrane. This level of permeability is dictated by the fact that the channel is both selective to a particular ionic species, and that the channel is often gated, either mechanically, chemically, or in this case, electronically.
1.4 - Electrochemical Potential and the Na,K ATPase

The constituents of the intra- and extra-cellular fluids often differ vastly, resulting in a chemical gradient over the cellular membrane, imparting a driving force for chemical diffusion across the membrane, dictated by the Nernst equation (Waggoner, 1979). In the following equation R represents the universal gas constant, T is the temperature of the system, the a superscripts are the relative chemical activities, F is Faraday’s constant, n is the number of electrons, $E^0$ is the electrode potential, and P is the permeability of the ion M, whereby,

$$E = E^0 - \frac{RT}{nF} \ln \frac{a_{\text{red}}}{a_{\text{OX}}}.$$  \hspace{1cm} (1.1)

The Nernst equation differs with respect to which ionic species is under consideration, therefore the Goldman-Hodgkin-Katz equation, or GHK equation is applied for multiple ions,

$$E_m = \frac{RT}{F} \ln \left( \frac{\sum_i^N P_{M_i^+}[M_i^+]_{\text{out}} + \sum_j^M P_{A_j^-}[A_j^-]_{\text{in}}}{\sum_i^N P_{M_i^+}[M_i^+]_{\text{in}} + \sum_j^M P_{A_j^-}[A_j^-]_{\text{out}}} \right).$$  \hspace{1cm} (1.2)
Within this *electrochemical potential*, we have two driving forces attempting to return the system to equilibrium, across a membrane which we already know to be permeable to these species, and yet the potential is sustained across the membrane. Clearly, there is another factor not yet accounted for. This is the contribution from the electrogenic ion exchanger proteins, moving ions across the membrane against the electrochemical potential, in an active transport process. The energy for this transition is provided via chemical potential energy, through for example the consumption of ATP by these proteins. In the most common of these proteins, the sodium:potassium ATPase molecule, discovered in 1957 by Danish Nobel laureate Jens Skou (Skou, 1957), three sodium ions are transported across the membrane and out of the cell, into a region of higher sodium concentration, with two potassium ions brought into the cell, where the potassium concentration is again higher, at the expense of one ATP molecule, whereby chemical potential energy is taken from the system via the dephosphorylation of the ATP molecule to ADP, and ions are moved against both the chemical and potential gradient. The molecular domains of this molecule are shown in Figure 1.4.
To give an idea of the importance of study of this process, it is estimated that around 40% of a human body’s energy when at rest goes to maintaining this gradient. The net electrical difference inherent in this process is vital to the notion of membrane potential, in that there is one more monovalent ion pumped out of the cell than those taken in, i.e. there is a net current out of the cell (Post and Jolly, 1957; Garragan and Glynn, 1967). For this reason, the process is termed electrogenic.

Much has been investigated in terms of the interaction of the protein with electric fields (Teissie and Tsong, 1980; Serpersu and Tsong, 1983; Blank and Soo, 1989; Weaver and Astumian, 1990). The process is a voltage dependant one, whereby the rate limiting step, extrusion of the three sodium ions from the cell, can be facilitated by a depolarization of the membrane potential. However, this applied membrane potential will similarly inhibit
the subsequent pumping in of the two potassium ions, due to the opposite direction of charge movement. These ions would be facilitated in crossing the membrane with a hyperpolarization, however, the rate limiting step is determined as the extrusion of sodium ions, which is deemed to have a larger effect, meaning that the net effect of a depolarization of the membrane is accelerated action of the Na/K ATPase molecules. The relative contributions of these two ions are not discernable from each other, as one cannot occur without the complementary effect occurring sequentially. Moreover, observation of this effect in physiological situations is made difficult by the very nature of the system, if a potential is applied to one side of a living cell, unless an electrode is placed within the cell itself; the opposite side of this cell will be at the opposite potential, and the net acceleration of pump molecule action will be cancelled by a deceleration on the opposing face or hemisphere. The Na/K ATPase molecule physically transports individual ions across the membrane, unlike the ion channel’s opening effect, where many thousands of ions rush through the channel with each action. The ATPase binds three intracellular sodium ions, the ATP is hydrolyzed, undergoes a conformational change whereby the sodium ions are exposed to the extracellular region, binds two potassium ions, and again reorients, to where it can release the potassium ions into the intracellular fluid. This process represents the pump “turning over” once, with ionic transport of 3 sodium ions, 2 potassium ions, and consumption of one ATP molecule, the fuel for this process. However, there are millions upon millions of these molecules in a cell. Each individual Na/K ATPase molecule operates independently from its neighbors, working at a seemingly random and varying pumping rate, with each molecule showing no phase relationship with those close by, working constantly, not reliant on a signaling
process, as in ion channels. The collective rates of all of these molecules are arranged in a statistical distribution determined by the thermodynamics of the system, in a Gaussian distribution. Under physiological conditions, it is estimated that the peak of the distribution is estimated to be located at around 50Hz, meaning that a thermodynamically average ATPase molecule would be turning over 50 times in a second, when acting at rest. It is possible, however, for these molecules to perform at a highly accelerated pace, for example when recovering from the opening of sodium or potassium channels, occurring in the propagation of an action potential. The action potential is a propagating electrical discharge, used in biological signaling. The high density of sodium channels, potassium channels, and Na/K ATPase molecules in nerve fibers is due to a necessity in the nervous system for a high degree of electrical activity, for example for the generation of action potentials. The action potential process allows for an ionic current in the direction of the chemical gradient, meaning a localized lowered ionic concentration. In order for this concentration gradient to be reestablished, the ATPase pump molecules must work at many times their resting turnover rate required to maintain an equilibrium. Here, the situation is different from that of the typical physiological situation, and the distribution is shifted, with the average turnover rate now much higher.
1.5 - The Synchronization Modulation Technique

The theoretical basis of this work builds upon research from many labs, reported in many cited journal articles, in many pioneering articles (Robertson and Astumian, 1991; Astumian et al, 1987; Astumian, 1997; Tsong and Chang, 2003; Astumian, 2003). In a generalized membrane transporter, exchanging m ions A from one side of the membrane with n of another ion B from the opposing side, a six state model is used to demonstrate the respective binding, transport and unbinding processes (Albers, 1967; Post et al, 1972; Weiss, 1996). In the below simplification of the Post Albers loop, Figure 1.5, the left side of the diagram would represent the intracellular region, and the right the extracellular. The ions A are the 3 sodium ions, and B represents the 2 potassium ions. This loop is termed an asymmetric model, due to differing binding affinities at each side of the membrane.
Figure 1.5 – Asymmetric six state model.

The other four non-translational steps in the loop, which do not involve movement of charge, are not dependant upon the membrane potential. It is useful in a theoretical sense to reduce these steps to representative algebraic equations, in order to predict eventual behavior. The two ions A and B, have respective fluxes as shown below, for $\varphi_1 \varphi_2$. The total flux within the protein must be equal to zero, and the transporter conservation equation must hold, therefore we have (Chen, 2007),
\[
\phi_1 = c_{E_1m_A} \alpha_1 - c_{E_2m_B} \beta_1 \\
\phi_2 = c_{E_1m_B} \beta_2 - c_{E_2m_A} \alpha_2 \\
\phi_1 + \phi_2 = 0 \\
\sum_{i=6}^{c_i} = c_{ET} \] (1.3) (1.4) (1.5) (1.6)

The aforementioned non-translational interfacial events occur at such a rapid rate in comparison with the other steps that they can be accurately represented by their dissociation constants \(K^{i_mA}, K^{i_nB}, K^{o_mA}, K^{o_nB}\) (Smith and Crampin, 2004).

The pumping flux, derived in greater detail in other work, is,

\[
\phi_1 = C_{ET} \frac{c_5 e^{(A_1-A_2)V} - c_6 e^{-(B_1-B_2)V}}{c_1 e^{A_1V} + c_2 e^{-B_1V} + c_3 e^{-A_2V} + c_4 e^{B_2V}}.
\] (1.7)

Where parameters \(C_n\) are sensitive only to the relative ionic concentrations, and not the membrane potential. This final equation gives us a theoretical prediction of the behavior of the pumping flux as a function of the membrane potential. Unfortunately, due to the fact that in most common ion transporters the two forwards and the two backwards reaction rates are of similar magnitude, the result of this model is that a DC change in membrane potential can never bring about a very large change in pumping flux. What is needed is a situation whereby the first reaction rate is significantly larger than the second, allowing a larger potential sensitivity. In earlier work reported from our lab (Chen and Zhang, 2006; Chen et al., 2006) a technique was derived from these basic physical principles, which stated that an alternating current can be applied to the membrane of a
living cell, at a frequency approximating the physiological rate, whereby the depolarizing half cycle of the AC field can be used to facilitate transport of the sodium ions out of the cell, and sequentially, the hyperpolarizing half cycle can be used to facilitate the subsequent potassium transport into the cell. In addition to the consideration of the behavior of a single isolated molecule, an entire section of tissue could be treated in this way with a pair of electrodes, and the subsequent acceleration of pumping rate would be matched on the opposite side of a cell, 180 degrees out of phase. This would lead to a net increase in pumping speed across the entire cell. A representation of this process is given in Figure 1.6 below, where initially the rate and phase of a pump molecule is randomly arranged, until the synchronizing field is applied, making each pump molecule work in time and in phase with one another across the same area.

*Figure 1.6 – Randomized pumping phases of the Na/K ATPase molecules are synchronized through an oscillating dynamic entrainment field.*
Work carried out in our lab employing a voltage clamp system, showed that it was possible to observe this phenomenon in skeletal muscle fibre, with the outward and inward currents from sodium and potassium transport respectively possible to resolve, where there would in a usual situation only be an outwards current, from the net single ion transport. This resolution is due to the pumps all working in phase with one another, responding to the AC field applied to the cell from within. Another way of visualizing this situation is as below:

*Figure 1.7 – On application of a voltage pulse, in the upper trace, the inward and outwards pumping events, with related measured ionic currents, are shown for a non-synchronized and synchronized systems.*
In the first frame, a depolarizing pulse is applied to the membrane, as seen in the upper line. Each individual pump’s contribution to the overall measured membrane potential can be seen in the middle and lower lines respectively, showing that the smaller inward currents from the potassium currents are not resolvable, as they are masked by the accompanying larger sodium currents, leaving a simple outward current measurable by the voltage clamp. In the second frame, the pumps are synchronized, and working in phase. Now, an inward current is visible in the readings, the magnitude of which reflects the stoichiometry of the system, i.e. 3:2. It is worth noting that this situation cannot be reached immediately, but a period of rhythmic entrainment is needed for the pumps to adopt the synchronization, therefore a few seconds of pre pulses of the same frequency would be applied to the system before the system would be reflected by the second frame.

In addition to this mere synchronization of the pumping phase, previous results suggest that the turnover rate itself can be altered to match the applied field, for those molecules whose pumping rate are within a limit of between one half, and double, the applied field frequency. This would lead to an interesting question; if it is possible to entrain those molecules operating at a lower turnover rate to adopt that of the specified frequency, would it be possible, once the molecules are fully synchronized, which has been shown to occur after around 100 cycles, to then entrain the molecules, stepwise, to a progressively higher turnover rate, sequentially increasing the action of the Na/K ATPase molecules with this applied frequency. A representation is shown in Figure 1.8:
Figure 1.8 – On modulation of the entrainment field to a higher frequency, pumping events are more closely occurring, resulting in a higher measured ionic current, in a shorter time.

Our measurements, using the improved double vaseline gap voltage clamp technique previously described, say yes. A significantly expedited action was demonstrated, via a measured pump current after this modulation pulse equivalent to six times the measured pump current of the same fiber before application of the synchronization and modulation pulses. In addition to this, the technique takes advantage of the fact that it is impossible to expedite the pumping action of both hemispheres of a cell, when unable to use an internal electrode, as shown in Figure 1.9 below.
As one hemisphere of the cell experiences a hyperpolarization, the opposing hemisphere is depolarized, thus canceling any effect on the pump molecules. However, when applying an oscillating field, both hemispheres will experience both a hyperpolarization and a depolarization within one cycle. As the cycle of the pump itself is only sensitive to the membrane potential in one particular step, and not for others, the pump molecule may receive the entire of any accelerating effect from the field within this half step (or in actuality much less than one half step), and therefore both hemispheres will experience accelerated action. It is for this reason that a symmetrical field was chosen for the
stimulation. A theoretical treatment was prepared whereby a Na/K ATPase molecule was modeled, with appropriate variables, from the equations derived earlier.

![Graph](image.png)

*Figure 1.10 – Predicted Na/K pumping flux, with varying Membrane Potential.*

As suggested earlier, according to this result, upon application of an increasing membrane potential upon the cell, the sigmoid shaped curve will quickly saturate, allowing little increment to the flux with greater potential. There is even possibly a negative slope above a certain threshold value, suggesting a definite upper limit upon the flux theoretically allowed. This would tie in with previously obtained theoretical and experimental results (Rakowski et al, 1997; Lauger and Apell, 1986; Glynn, 1984; Pedemonte, 1988; De Weer, 1988; Gadsby and Nakao, 1989; Nakao and Gadsby, 1989; Rakowski et al, 1991; Chen and Wu, 2002; Apell, 2003). If we now replace the reaction
rates in the earlier equation with those hypothesized for the synchronized molecule, we are left with the result,

\[
\phi_1 = C_{ET} \frac{C_5 e^{(A_1+A_2)V} - C_6 e^{-(B_1+B_2)V}}{C_1 e^{A_1V} + C_2 e^{-B_1V} + C_3 e^{A_2V} + C_4 e^{-B_2V}}
\]

This critical alteration to the equation will now allow us to drastically increase the numerator, whilst the denominator will be largely unchanged, giving the molecule a greater potential sensitivity upon the protein. The theoretical results are now quite different, as seen below.

*Figure 1.11 – Predicted Na/K pumping flux of synchronized molecules, with varying Membrane Potential.*
This now exponential dependence upon the membrane potential should allow us to greatly increase the pumping flux by synchronizing the membrane proteins to pump as one, at a far greater sensitivity. Comparatively, when depolarizing the cell from its resting potential of -90mV to a value of -50mV, in both schemes, the flux seen under synchronization would be over 10 times that of unsynchronized proteins, with possibly even larger differences for higher values. A subsequent question pertinent to our research would be can this expedition of pump turnover rate and related increase in pump current be used to “charge up” the membrane potential of the cell, thus hyperpolarizing the living cell, or indeed leading to an expedited recovery from the cells natural physiological recovery from a period of extended depolarization? The body of the work in this thesis concerns this question. The work of our lab concerns the initial theoretical derivation of this relationship, the fundamental readings of current taken using the voltage clamp, and the application of this technique to hyperpolarize intact cells. The work described in this thesis is concerned only with the final aspect of this research, with other aspect being investigated by other members of the group.
1.6 - Anticipated Public Benefits

There are many diseases and disorders common in patients in which either the membrane potential, or the action of the Na/K ATPase molecules, or both are significant (Clausen, 1998; Rose and Valdes 1994). A concise but by no means comprehensive list would include heart failure, Alzheimer’s disease, diabetes, cystic fibrosis, heart murmur, Hyperthyroidism, Huntington’s disease, McArdle’s disease, myotonic dystrophy and central nervous system disorder. It is plain that any advancement in the laboratory which could offer a route to a clinical treatment could be imperative to the treatment of these conditions.

To this end, our previous studies, the work presented in this thesis, and the suggested continuing work, demonstrate a capability within our technique to hyperpolarize the biological membrane of a number of differing living cells, at the level of a single cell, a group of cells, and the potential to repeat for a whole muscle, or even on the intact body. Our early studies, where the fundamentals and capabilities of this technique were revealed, concern skeletal muscle fibers. These skeletal muscle fibers were hand dissected under a microscope from the Semitendinosus muscle of the leopard frog, *Rana Pippiens*. Once isolated, these muscles were fluorescently stained with a potentiometric dye, in order to ensure a level of invasiveness superior to that of the previous voltage
clamp techniques, and the membrane potential monitored confocally, as the stimulation and subsequent modulation pulses were applied. Initial results were taken using selective blocking agents for the ionic channels within the cell, however, as results progressed, measures were taken to closer approximate the physiological conditions of a system comparable to ours, with success. Channel blockers were finally eliminated from the experiment completely, to leave the situation as close to a physiological condition as possible within the isolated cellular level. In Figure 1.12 below, some of the conditions relevant to this technique are described, with data from the CDC.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Deaths</th>
<th>Affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Failure</td>
<td>46484</td>
<td>4000000</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>58866</td>
<td>240000</td>
</tr>
<tr>
<td>Diabetes</td>
<td>73249</td>
<td>385000</td>
</tr>
<tr>
<td>Cystic Fibrosis</td>
<td>485</td>
<td>30000</td>
</tr>
<tr>
<td>Hyperthyroidism</td>
<td></td>
<td>2500000</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td></td>
<td>30000</td>
</tr>
<tr>
<td>Myotonic Dystrophy</td>
<td></td>
<td>35000</td>
</tr>
</tbody>
</table>

*Figure 1.12 - Various diseases and disorders related to the membrane potential and action of the Na/K ATPase molecules, taken from various sources. Deaths and affected are from USA, in most recent year available.*
1.7 - Fluorescence Methodology

Fluorescence is a form of luminescence seen throughout the world, from natural phenomena, to commercial man made applications, and through to multi million dollar pieces of scientific equipment, such as the laser scanning microscope. A molecule susceptible to fluorescence can be electronically excited to a higher energy state, whereby a finite amount of energy is subsequently lost, through heat and molecular vibrations, before the remaining energy is released in the form of a photon, leaving the molecule reverted to its original state, signified by,

\[ S_{\text{EXC}} \rightarrow S + \text{hv}. \]  

(1.9)

Where \( \text{hv} \) represents the emitted photon. The excitation may occur via a chemical, mechanical, or in our case a physical event, the absorption of a photon of light of a higher energy. As this light is of a higher energy, a fraction of this energy will be lost due to the particular emission pathway taken by the electron, and the subsequent emitted photon will be of a lower energy, or a longer wavelength than the initial excitation beam. The difference between these two wavelengths varies dependent upon the molecule in question. If a fluorescent molecule could be functionalized, it could be used as a fluorescent “tag”, which would specifically attach itself to a target molecule or cellular
component, and information could be gleaned on a particular characteristic of this molecule or component. Such a molecule is termed a fluorescent dye. The principle justification for us using a fluorescent dye in order to measure ionic or membrane potential change is that this method offers us an entirely non-intrusive method to obtain these results. All alternative methods will necessitate a level of destruction of the cells, or at least an alteration from their naturally functioning form, which would represent a departure from the ideal intact living cell situation. Even the use of a patch clamp would require a degree of invasiveness, with a finite period between rupture of the membrane and full dialyzing of the intracellular material with the pipette solution. With an optical reading, the cell is allowed to function in its natural state, with only a small amount of phototoxicity from the interaction between the laser and the dye.
1.8 - Selection of Fluorescent Dye

The use of a fluorescent indicator to determine the membrane potential of a cell discloses several advantages to traditional patch clamp or micropipette impalement techniques, such as the absence of complicated manipulation problems, and the possibility of application to very small cells (Gross & Loew, 1989). The dye selected for study of the global variation in membrane potential was Tetra Methyl Rhodamine Ethyl Ester, TMRE. This belongs to a class known as Nernstian dyes, initially developed by Waggoner (Sims et al, 1974; Waggoner, 1976), which distribute themselves across the membrane according to the modified Nernst-Planck equation,

\[ V_m = \frac{RT}{ZF} \ln \left( \frac{C_o}{C_i} \right) \quad (1.10) \]

Where \( R \) is the gas constant, \( T \) is the absolute temperature, \( Z \) is the valence of species, \( F \) is the Faraday constant, and \( C \) represents the concentration of specified ion. In contrast to many fluorescent dyes which exhibit fluorescence upon binding with specific molecules, TMRE does not fluoresce as a result of structural rearrangement upon bonding with another species. Instead, TMRE will always fluoresce. Due to its cationic state, TMRE molecules will be drawn into the parts of a cell with more negative potential. This redistribution process of the dye molecules is dependant on
the changes in the membrane potential across the cell membrane. The ratio of the concentrations of the dye molecules and therefore their fluorescence intensity inside the cell to those outside the cell can be directly related to the membrane potential via the following equation, a verification of the Nernst equation at room temperature,

$$Vm = -60 \cdot \log \left( \frac{F_{\text{in}}}{F_{\text{out}}} \right) \quad \text{mV} \quad (1.11)$$

where $F_{\text{in}}$ and $F_{\text{out}}$ represent the fluorescences measured in the intracellular and extracellular regions respectively. This dye is also known as a slow dye. When the muscle fibers are exposed to an applied oscillating electric field, the membrane potential will be a combination of the membrane resting potential and the applied oscillating electric field. The latter is not our interest except when calibrating the magnitude of the applied membrane potential. We are interested in the changes in the membrane resting potential, which will depend on the functions of the Na/K pump molecules. Activation of the pump molecules slowly increases the ionic concentration gradient across the cell membrane, therefore hyperpolarizing the membrane potential. The goal of this experiment is to verify how the activation of the pump molecules by the synchronization modulation technique affects the ionic concentration gradient and the membrane potential. It takes time for the pump molecules to rebuild the ionic concentration gradient, and to redistribute the dye molecules dependent on the dimensions and type of the system. In other words, we are interested in the slow change, or the DC component in the membrane potential. Therefore the slow dye TMRE fits our requirement very well. It is
not sensitive to the transient changes in the membrane potential induced by the applied electric field. Instead, it is very sensitive to the slow change in the membrane potential.

The lipophilicity of this molecule, combined with the delocalization of the molecule’s positive charge allows TMRE to pass through the membrane with ease, leading to the exhibition of good membrane permeability from this dye (Loew, 1993). This allows a reversible Nernstian equilibrium to be set up across the membrane. However, the dye will aggregate in a charged environment, for example the mitochondria, if left for too long. Typical reported applications of this dye are mitochondrial staining, but it is also utilized in studies similar to ours (Ehrenberg et al 1988), in addition to applications such as drug delivery (Chen & Simon 2004; Rajagopal & Simon 2003), studies on calcium transport, (Raraty et al 2000; Leski et al 2002), and cellular apoptosis (McClintock et al, 2002; Rosenstock et al, 2004). Conversely, fast dyes do not operate with a global redistribution through the membrane upon potential change, but actually embed in the phospholipid bilayer via a hydrophobic tail, and use charge shift phenomena such as electochromism in order to sense local potential variation. This makes the fast class of dye sensitive to more localized variations in membrane potential, useful in many applications, but not for ours. Also, typical charge shift probes such as di-4-ANNEPS, or di-8-ANEPPS show only approximately a 10% change in reaction to a membrane potential variation of 100mV. Slow dyes tend to exhibit superior sensitivity to their faster counterparts, showing orders of magnitude higher fluorescence under a similar potential change. The chemical structure of TMRE is shown in Figure 1.13 below
Figure 1.13 – The TMRE molecular structure. Conjugation of the central aromatic rings results in fluorescence.

The double bond on the upper nitrogen can be thought of as delocalized over the 3 ring structure, resonating between the nitrogen bonds, which are covered by hydrophobic methyl groups. This, combined with the molecule’s ester group combine to cover the partial positive charge, rendering the dye membrane permeable.

This category of dyes is primarily used to measure changes in potential rather than an absolute potential measurement, simplifying calibration of the dye. As the intracellular membrane potential increases, hence the concentration of the dye inside the fibre, and the subsequent dye fluorescence, will be seen to increase, whilst the net effect from reduction of dye concentration outside the fibre will be negligible, due to the significantly higher volume of the dye reservoir. The ester component of this dye allows membrane permeation, and the dye will not aggregate, and interact with the membrane only minimally. The other factors which make this dye an ideal choice for this application are
that its spectral properties are independent of environment, that it gives strong fluorescence, and that it carries a low rate of phototoxicity. Analysis using TMRE is not carried out ratiometrically, as the spectral properties of TMRE do not change significantly as a result of factors such as pH, or membrane potential.
1.9 - Optical Setup

An Olympus IX81 inverting confocal microscope utilizing the Fluoview 500 Tiempo V4.3 analysis package was employed for data collection, with a 10x dry objective used, and a confocal aperture of 80um, giving a resolution in the X and Y directions of 0.621µm, and a Z resolution of 3.09µm. Standard Rhodamine optics of excitation under a green HeNe at 543 nm and detection with a photomultiplier and barrier filter at 560 nm were employed to graph the observed fluorescence as a two dimensional map, varying with time. The maximal pixel density was 2048 x 2048, with significantly lower used at high acquisition speeds. Various temporal protocols were observed during scanning, with relevant details given by section in the following text. The experimental setup for confocal excitation and detection is shown in Figure 1.14 below:
Figure 1.14 – 6 laser lines are available to us through the confocal system, ranging from 442nm to 633nm, selectable though a system of filters and shutters, leading to a high sensitivity PMT.

Excitation is provided by a choice or combination of 4 lasers, respectively HeCd, Red HeNe, Green HeNe, and a multi-line Argon Ion laser, through a combination of mirrors and dichroics to the scope, and sample. The returning fluorescence is filtered with a dichroic mirror, passes through the confocal aperture and a barrier filter, in this case
operating at 560nm, to the detector, a PMT. A PMT or Photo Multiplier Tube is an extremely high sensitivity photon detection system, with a range selectable with window material. Inherent to the function of the PMT is a high degree of internal amplification of incident photons, as much as $10^8$, dependant upon window material, allowing for single photon detection in some cases. This combination of high gain and wide response, coupled with low noise and a wide range of operational temperatures, offers an ideal lab bench detection solution.
1.10 - Application of Oscillating Electric Field

Previous work in this field undertaken by our lab utilized single cut skeletal muscle fibers, mounted on a double Vaseline gap voltage clamp. This setup must now be altered, in this work, to allow results to be obtained from intact, living cells, in order to more completely mimic the living system. This presents us with many methodological problems, in that the cells can no longer be simply perforated, either chemically or mechanically with a microelectrode, the field must in fact be applied to the undamaged cell as a whole, in a way similar to if the field was to be applied to a patient. This is accomplished by surrounding the cell with a pair of silver:silver chloride electrodes, with a uniform field of a calculated magnitude applied across the whole intact fiber, with the membranes themselves supplying a resistance to the field calculated so as to ensure the relevant field magnitude across each membrane, with precise calculations indicated for each arrangement, further explained in relevant sections.
Chapter 2 – Amphibian Skeletal Muscle Fibers

Primary results are taken from amphibian skeletal muscle fibers, a well characterized system used previously in our lab for single cell studies. Results are presented both temporally and spatially, showing a hyperpolarization of the membrane occurring specifically at the point of application of the field. Various techniques are applied in order to isolate and remove the action of the Na/K ATPase molecule, therefore acting as controls, and therefore through comparison, we are able to attribute the measured hyperpolarization of the cell membrane to the action of the Na, K ATPase molecule. This we hypothesize is experiencing a period of accelerated action due to the applied field, through the synchronization modulation technique.

2.1 - Study of Single Intact Amphibian Skeletal Muscle Fibers

A well characterized cell in the field of electrophysiology is the skeletal muscle fibers of various frogs such as the leopard frog, and grass frog. These cells are primarily used as they represent a single cell, yet are macroscopic to a degree that they may be hand dissected with suitable microdissection tools. The cell’s high degree of excitability is due to them containing a particularly high concentration of membrane proteins, easily
examinable using voltage clamp techniques. This is a mature field of study, with innumerable papers and books published within the topic, offering an ideal starting point from which to model the action of living excitable cells.
2.2 - Dissection Procedure

The twitch skeletal muscle Semitendinosus was hand dissected from the leopard frog *Rana Pippiens* in relaxing solution, as in prior work (Chen and Lee, 1994). The fiber was then transferred to an experimental chamber, and held, stretched by two clips at a distance between the two clips of about 3 mm. A coverslip was then placed on top of the clips in order to reduce the depth of the bathing solution around the fiber. The purpose of this was to increase the resistance of the bathing solution in order to reduce Joule heating effects. The depth of the bathing solution was estimated at under 1 mm, with further information on this later. Finally, the fibers were stained with 1 µM TMRE in Normal Ringer solution. All euthanization and dissection procedures strictly followed the university IACUC regulations, with all suitable protocols for the use of live animals received and approved.

The compositions of solutions are as follows:

Relaxing solution – 120mM K-Glutamate, 5mM K$_2$PPIPES, 1mM MgSO$_4$, 0.1mM K$_2$EGTA.

Ringer solution (Normal Ringer solution) – 120mM NaCl, 2.5mM KCl, 2.15mM Na$_2$HPO$_4$, 0.85 mM NaH$_2$PO$_4$$\cdot$H$_2$O, 1.8mM CaCl$_2$. 
Dye solution, same as ringer with 1μm TMRE. All solutions were titrated to a working pH of 7.0.
2.3 - Electrical Stimulation

An electric field was initially applied to the fiber using a TENMA UTC 72-5085 function generator connected via two Ag/AgCl wires parallel to the fiber. The small cross-section of the bathing solution surrounding the fiber comparing to the distance between the two wires of about 1 cm assured that the applied electric field was relatively uniform.

After initial experimentation, employing hand alteration to vary the stimulation frequency, it was decided that a more advanced technique would be needed, and a LabVIEW program was designed to meet our needs. Several versions of this program have been written, with differing applications in mind, with each having more features than the last. Essentially, a user may input a starting and final frequency for stimulation, in addition to the time the stimulation is required to run at these initial and final frequencies. Then, when entering an electrode separation, fibre diameter, the amplitude of membrane potential required, and finally the percentage change per variable number of cycles, the program will calculate from the geometry of the system the necessary applied voltage, and output a waveform, either directly or through an amplifier, onto the fiber. Further details of this program are provided in the appendices.
2.4 - Stimulation Geometry

Below, in Figure 2.1, is a representation of the stimulation of a single skeletal muscle fiber.

![Diagram of stimulation geometry](image)

*Figure 2.1 – The purple box represents the dyed fiber, of diameter d, surrounded by electrodes with a separation D.*

If we assume, due to the geometry of the system, that the field provided by 2 electrodes separated by a distance D is uniform, it can be represented by,
\[ V = ED \quad . \quad (2.1) \]

There should be 2 equal voltage drops across the cell, representing the 2 membranes, with each carrying a membrane potential of

\[ V_m = \frac{V_d}{2D}. \]

Therefore for a membrane potential of 40mV;

\[ V = 2 \frac{V_m D}{d} \quad , \]

\[ = 13.3 \text{ V peak to peak}. \]

Therefore, applying this to, for example, a high magnitude electroporation pulse of 300mV,

\[ V = 73.3 \text{ V peak to peak} \]

This will present a problem, therefore some change will be needed to the geometrical arrangement of the experiment in order to electroporate the cell.

The other problem concerning the geometry of the experiment concerns the Joule heating of the cell under synchronization. As our field is oscillating, there will be no polarization effect observed within the electrodes or solution, therefore the lower electrochemical
effect will allow the fiber to remain healthier than with a DC field, however, Joules Law states that an amount of heat $Q$ will be imparted upon the cell, whereby,

$$Q = P \cdot t = I^2 R \cdot t = I \cdot V \cdot t = \frac{V^2 t}{R}$$  \hspace{1cm} (2.2)$$

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

therefore,

$$Q = \frac{V^2 t \cdot \sigma A}{L}$$

In our case, the applied voltage would be represented by an RMS value. We see now, that in order to minimize the Joule heating on the system, assuming we cannot change the applied voltage or time of application, we can either increase the separation of the electrodes, working against the earlier result, or decrease the cross sectional area of the conductance pathway through the chamber. Through many iterative improvements, a configuration was found that would limit the cross sectional area sufficiently whilst still allowing the electrodes to be sufficiently close to each other to give us the high membrane potential needed for electroporation. No bubbles were observed around the electrodes, indicating a relatively low Joule heating level, and testing of the extracellular ph change indicated little electrochemical effect.
2.5 - Results

The results I will present in this section are in one of two forms. Firstly, there are scans of intensity variation with respect to x, the direction directly across the skeletal muscle fiber, showing the variation in membrane potential with distance across the fiber at a specific time. Secondly, there are time dependant scans whereby a sequence of scans is taken with a specified time delay, with a two dimensional area of the fiber averaged, to show the overall membrane potential of the fiber’s variation with time. A typical skeletal muscle fiber would resemble Figure 2.2:
Figure 2.2 – Fluorescent response of the TMRE stained skeletal muscle fiber.

Intracellular organelles (myofibrils) are visible in the scan. The yellow line represents the data acquisition line for later results.

The yellow line represents the data acquisition line in the x dependant scans, with a box being placed over the entire width of the fiber for the time dependant scans. Controls were taken with this arrangement, and it was found that the dimensions of the cell meant that around 20 minutes was needed after the initial staining of the cell in order for equilibrium to be reached. A smoothing function was applied to the data, a simple averaging of adjacent points, in order to remove a certain degree of localized variation that can be visualized from the above image, due to the presence of Myofibrils within the fiber. This function is demonstrated below:
Figure 2.3 – The measured and smoothed fluorescence from the skeletal muscle fiber, in order to remove artifactually higher regions caused by vertically traveling organelles.

Subsequently, scans were taken with a specific Na/K ATPase inhibitor, ouabain, as a control. Initial scans also contained channel blockers. The results shown each represent a number of concurrent results. When the stimulation/modulation field was applied to the fiber, little or no variation was noted, as seen below.
Figure 2.4 – Attempted modulation of pump turnover rate under presence of selective Na/K ATPase blocker ouabain.

Scans are shown for the initial, and then at 5 minute interval, with field applied from zero to five minutes. The same data is shown in Figure 2.5, with respect to time:
Time dependant fluorescence with Ouabain

Figure 2.5 – Time dependant fluorescence variation of fiber with ouabain under modulation pulse train.

Next, further scans were made of fibers, without ouabain, but with no extracellular potassium, therefore making it theoretically impossible for the Na/K ATPase pump to turnover, and hyperpolarizing the membrane. In real terms, the presence of intracellular potassium would ensure that there was not a total lack of potassium in the extracellular fluid, but activation of the pump molecules should nevertheless be impossible. The normalized time and space dependant results are shown in Figure 2.6 and 2.7 respectively.
Figure 2.6 – Spatially resolved fluorescence variation, absence of Potassium in extracellular fluid should prevent pump turnover.
Again, it is demonstrated that the application of a field which has been proven in earlier voltage clamp experiments to accelerate the function of the ATPase molecules, in application to a whole cell, will have little to no effect upon the remainder of the system. Therefore, if the cell is now returned to a more physiological condition, containing only channel blockers, any effect measured should represent that of the pump molecules responding to the applied field. In Figure 2.8 below, the synchronization/modulation field is applied to the fiber after the initial scan, and removed at the 5 minute scan. There is a large elevation in membrane potential, initially localized at the membrane, which disperses within the cell as the field is removed, concurrent with our theory of the membrane proteins, which are of course located within the border of the cell, working at an elevated rate for the first five minutes, and then resuming normal operation. Due to

Figure 2.7 – Time resolved variation with no extracellular potassium, stimulation as marked
the density of intracellular constituents, diffusion of the ions within the cell would be relatively slow, in agreement with that observed. It should be noted that each fiber is different, both in physical dimensions and in exact membrane constituents, therefore the actual value increased each time showed some variation.

Figure 2.8 – Fluorescence variation under stimulation/modulation entrainment field. Original fluorescence intensity at $t=0$ is seen to undergo large, localized concentration increase at membrane, after 5 minutes. Stimulation removed at $t=5$, localized concentration increase is seen to diffuse inwards to the remainder of the fiber by $t=15$. 

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Whilst this can be seen as a success, in that we were able to increase the membrane potential by a discernable amount, this situation is not that of physiological situations, due to the presence of channel blockers. The respective sodium and potassium channel blockers, tetrodotoxin and 3,4 diaminopyridine, are both highly potent neurotoxins, not something which can be used in a clinical environment. The important question now, would be “if we remove the channel blockers, will we still see a membrane potential change?” The channels are both voltage gated, and hence we should see some contribution from the channels opening, most likely highly localized, in the scans. The potassium channels may have only a moderate effect upon the system, but the sodium current will most likely be very large. Can this be overcome by activation of the membrane proteins? In actuality, channels will undergo inactivation after a specified interval, limiting the magnitude of the membrane depolarization through channel current. We will demonstrate with the following data that it is possible to utilize this inactivation as a naturally occurring form of channel blocker. The data acquisition protocol was modified to provide improved temporal resolution, with the image now not of the entire fiber, but of a small region close to the membrane. In Figure 2.9, it is possible to resolve a reduction in the membrane potential when enduring a 50Hz stimulation with no modulation, but a discernable increase when this applied field was quickly modulated up to 200Hz, see below
Figure 2.9 – Localized membrane fluorescence variation under 50Hz stimulation field, and under stimulation with fast modulation up to 200Hz, showing contribution of ionic channels.
This is however only indicative of the situation right at the membrane surface, therefore scans were taken during modulation for the whole cell, with a spatially dependent scan of this setup yielding the results shown in Figure 2.10, with scans for 0, 5, 10 and 15 minutes shown:

![Fluorescence Intensity during stimulation and modulation of Pump Molecules](image)

**Figure 2.10 – Fluorescence with no channel blockers, showing smaller increase under synchronization/modulation entrainment field, lasting 5 minutes.**
The degree of change in membrane potential observed is not as high as that in scans obtained with the presence of channel blockers, as would be expected. However, as there is undeniably an increase, we can conclude that the channels are inactivated sufficiently to allow the accelerated action of the ATPase molecules to outweigh the effects from any ion channel opening. The variation with time of the whole cell can be seen below:

![Time dependant scan of Intact fibre under stimulation and Modulation Field](image)

**Figure 2.11 – Time resolved fluorescence variation with no channel blockers, under 5 minute stimulation, as marked.**
2.6 - Conclusions

If the values shown are calibrated to the estimated membrane potential based upon the Nernst equation, with appropriate substitutions for background and extracellular fluorescence, the final averaged fluorescence intensity and membrane potential changes measured through our technique, in semi-intact skeletal muscle fibers with no channel blockers, are shown below:

![Fluorescence Intensity Before and after Activation](image1)

![Initial and activated membrane potential](image2)

*Figure 2.12 – Distribution of average initial and activated intensity and membrane potential, calculated through the Nernst Equation, with standard deviation indicated.*
This value, of around 5mV, however small it may seem, may still be significant within the body. As an example, the action potential, the body’s messaging system, operates on a threshold principle. This threshold is typically around 15mV above the cell’s resting potential, therefore the ability to manipulate a cell’s resting potential by just this small amount may certainly become very interesting, to say the least. It is, however, hoped that in further work we can push this number higher, it is not uncommon for this number to be as high as 15-20 % of the cell’s resting potential, the number given is an average, and is conservative.
Chapter 3 – Recovery from Electrical Injury

In this short chapter, the technique was applied as an application, with thoughts of employing as a form of treatment, in order to show a recuperative effect upon a damaged cell. Successful results were obtained demonstrating not only an accelerated recovery, but recovery to a higher degree than initial, with relevant controls showing this recovery is absent without action of the Na/K ATPase molecules.

3.1 - Study of Recovery effect from Electrical Injury

The following results are presented specifically with application of this technique in mind. If we eventually aim to use our technique to externally raise the membrane potential of a cell or group of cells which has been adversely effected by some form of disorder, leading to an artificial depolarization of the membrane, we need to artificially model this process. In order to manufacture this situation, we use the well studied field of electroporation. Much work has been undertaken by other labs into the characterization of cells when electroporated by a hyperpolarizing field, causing resealable pores to form in the membrane, through which cellular material will rapidly diffuse, causing a reduction in membrane potential. It is worth noting that any reduction in dye intensity within the cell will follow a time course that will not characterize the electroporation, but instead
will be specific to the dye, as this is a slow dye, and will need time to catch up with the almost instantaneous depolarization of the membrane. The electroporation protocol was a group of 4 pulses, with a duration and separation of 4 ms, to match that of electrical line frequency. The magnitude of stimulation which was empirically observed to show maximal electroporation effect, whilst not permanently damaging the cell, was determined to be 280 mV. A single set of pulses was delivered in selected experiments, whereas others are continuously electroporated for 20 seconds, in order to show sufficient decrement of fluorescence. The slower recovery process, however, should reflect the actual situation. When comparing stimulation of the cell at 50 Hz, with and without ouabain, in the near-membrane region, the Figure 3.1 is obtained.

Figure 3.1 – Localized response of the fiber to stimulation, with and without ouabain, in comparison to response to electroporation. Time constants seem comparable, however electroporation seems more traumatic, as would be expected.
The time course and magnitude of the electroporation response appears faster and more pronounced, as would be expected. The membrane potential is reestablished in the cases of both stimulation and electroporation, however, when ouabain is present, this recovery is interrupted, confirming that this process is reliant on the Na/K ATPase molecules.

Looking more closely now at the electroporation phenomenon in Figure 3.2, accelerated recovery was now observed when using our stimulation/modulation technique, which was not only absent, but actually led to further deterioration when under the presence of ouabain, see below. In addition to this, the final membrane potential reached was higher than the initial resting potential, suggesting a further hyperpolarization.

![Fluorescence Response to Electroporation](image)

**Figure 3.2 – Improved recovery time from electroporation is observed with application of entrainment field. Response with ouabain also indicated.**
In the figures obtained from further scans taken as described, an average staining time for fluorescence intensity to reach a maximum would be around 20 minutes from addition of the dye molecules. This would represent an equilibrium state, whereby the driving force to pull the dye molecules into the cell has reached an equilibrium, and the concentration ratio in:out is representative of the concentration gradient of the potassium ions. Figure 3.3 shows a typical fiber recovering after a brief electroporation. The figure is taken from an acquisition box close to the membrane of the fiber, so as to exhibit the maximal effect of electroporation. A box taken closer to the center of the fiber would exhibit a lower degree of decrement. The lighter lines on the figure represent the standard deviation from this average of 5 figures, with the darker line representing an average of 5 skeletal muscle fibers, isolated from different frogs. Electroporation takes place after a control period of 10 seconds, with a time resolution of 0.05 seconds.
The average intensity of fluorescence originating from inside the fiber, and hence the potassium concentration, and further, the transmembrane potential of the fiber, is clearly seen to decrease sharply in all of the traces, almost immediately following electroporation. A roughly 4% averaged decrement is observed, which is compensated for in a slower time course than the initial decrement. Compensation occurs to a slightly lower level than originally, suggesting the fiber would be unable to completely recover if another stimulation was given before this refractory period was complete. If a further comparison were to be made of this data with that of an unstimulated fiber, we can see from Figure 3.4 that the initial period of staining of the fiber lasts several minutes from
when the scanning had begun, and once the electroporation is delivered, indicated by the dashed line, a significant decrease, plainly discernable to be many times that of the natural variation due to noise, etc, is seen to occur.

*Figure 3.4 – Fluorescence variation in response to a single set of electroporation pulses, in comparison to fiber’s natural noise variation, with staining kinetics indicated.*

It is possible to spatially resolve data from a single electroporation event with respect to time in Figure 3.5, where we plot a three dimensional graph, showing x and y with time. If we focus on a single membrane boundary with time, we can clearly observe, in the
following figure, the membrane potential at the boundary area dropping dramatically at specifically the time when the electroporation field was delivered.

Figure 3.5 – Two dimensional variation in fluorescence intensity at membrane boundary, with time, after delivery of single burst of electroporation pulses.

The fiber, when electroporated, is observed to drop very quickly in fluorescence intensity, from which we infer a drop in membrane potential. The subsequent repolarization is a slower process, and seems to reach a slightly lower level than that of the initial. Therefore, if we were to apply another electroporation to the fiber after this
recovery, an even lower resultant level of fluorescence would be measured. We can also infer that if this further electroporation occurred even closer to the first, a more dramatic decrease in fluorescence could be measured. It would be possible, then, to over time apply a number of electroporations, one after the other, that would bring the membrane potential of the cell as a whole to a significantly depolarized level, from which the cell would not be able to naturally recover. Therefore, we imposed a lower time resolution upon the scan, set to 1 second, in order to examine effects in the long term. These scans were taken with the entire fiber in the acquisition box, therefore enforcing a lower time resolution, with the data obtained representing an averaging over the entire fiber, not just the area proximal to the membrane. These two factors combine so as to not show the individual electroporation effect, which has already been documented, but to show that of a repeated electroporation over a prolonged period. After an initial 10 second control period, indicated under the abscissa as 1, the fiber is repeatedly stimulated, from 10 seconds to 30 seconds, as indicated by the pair of arrows under the horizontal axis and as 2. At 30 seconds, the electroporation field is removed, and the synchronization modulation field is applied to the fiber, in period 3. This, as described in the methodology section, was a 10 second synchronization followed by gradual modulation of the pumping rate, up to a value of 400Hz. At this point, the frequency of stimulation, and hence that of the pumps is maintained at an elevated rate of 400Hz in period 4 for a duration of just over 200 seconds, to a total time of 300 seconds for the entire stimulation protocol. All stimulation is then removed, and the fiber is scanned for a further minute, as shown in Figure 3.6 below. The average of seven fibers is shown, along with the standard deviation. Some variation would be expected in individual response, due to
differing dimensions, and to a natural variation in density of membrane proteins observed from fiber to fiber, and from different frogs.

Figure 3.6 – Improved fluorescence response due to synchronization modulation field, showing hyperpolarization of membrane potential following prolonged electroporation period.

In the control period, no significant change is seen in the fluorescence observed from the fiber. After electroporation, the intracellular fluorescence drops significantly, until shortly after the synchronization modulation field is applied, at which point an increase in
fluorescence is observed until the point where the stimulation is removed. This would seem to indicate a recovery by the fibers which occurs concurrent to the application of the field, which goes as far as to actually hyperpolarize the membrane potential. In the appendices, a figure is shown to display each individual trace before averaging. In the final figure of this section, ouabain, a selective blocker of the Na, K ATPase molecules was added to the bathing solution, as described in the methodology section, hereby removing any contribution to the membrane potential given by the ATPase molecules. The fiber was repeatedly stimulated in the presence of ouabain, allowing the membrane potential to fall a significant amount, with subsequent application of the synchronization modulation field in the same manner as earlier, but this time without the action of the Na, K ATPase molecules, as seen in Figure 3.7 below, with the electroporation, indicated by a vertical dashed line, taking place at 50 seconds.
In Figure 3.7, a control experiment is also shown, in order to establish a control for comparison with the depolarized, electroporated fibers. There is a plain and significant difference from the 4 electroporated fibers, which show a 10-20% decrease in fluorescence from this repeated stimulation, to the control’s fraction of a percent at most. Variation such as this in the depolarization measured from fiber to fiber would be expected due to an inherent variation in cellular diameter, which can easily vary as much as 40% in skeletal muscle fibers taken from the same muscle of the same frog. More significant than the decrease in fluorescence, from which we infer a depolarization, is the fact that there is no subsequent repolarization, which was observed in fibers not treated
with ouabain. This would suggest that the recovery and subsequent hyperpolarization indicated earlier would be dependant upon the action of the Na,K ATPase molecules, as this is the only difference in the two sets of data. It can be noted that the membrane potential continues to fall even after the electroporation has been removed, and the synchronization modulation field has taken its place. This is possibly due to the opening of potassium channels through the depolarization inherent in the synchronization modulation field, which seems to slowly reach a saturation.
3.2 - Conclusions

It is demonstrated that cells electroporated with a simulated electrical injury can recover not merely at an accelerated rate, but in fact to a higher baseline than the original value of the healthy cells, prior to this injury. Control experiments confirm that this effect is indeed due to the action of the Na/K ATPase molecules, due to the application of the synchronization modulation field. This proves the theoretical practicality of this technique in a clinical setting, with a degree of recovery attained which was impossible with the cell’s natural processes. Most important now, however, is the distance this experiment represents from a clinical system, which is now assessed in subsequent experiments.
Chapter 4 – Mammalian Cardiac Cells

The experimental protocol was altered to consider the application of the synchronization modulation technique to mammalian cells. These cells would require much altered stimulation, dissection and handling, which are all thoroughly described. A greater degree of hyperpolarization was measured, again with relevant controls, with appropriate reasoning for this increase presented.

4.1 - Study of Intact Mammalian Cardiac Cells

Our work was next extended to the analysis of cardiac cells, offering the maximum benefit in terms of human disease physiology. Bovine cardiomyocytes have been identified as offering acquiescence between ease of acquisition and similarity with human anatomy and physiology. The heart is a center of electrical activity for the whole body, undergoing a periodic excitation on a scale so large it is routinely measured from outside the body, in the familiar ECG. There is also a greater density of pump molecules within cardiac tissue than that found in skeletal muscle. It follows logically, therefore, that an elevated concentration of disorders in membrane proteins which govern electrical activity may occur within the heart. In addition, an existing probability of a disorder may be felt more acutely within such an important and sensitive organ. If a similar, or even an
improved effect can be measured within heart cells to that found in skeletal muscle fibers, this would highlight the scope of our technique. Bovine cardiac cells can be relatively easily cultured from newly slaughtered cattle, which were obtained from a local source, according to university tissue protocol IACUC regulations.

A further motive for shift to cardiac cells is their size. As cardiac cells are several orders of magnitude smaller than skeletal muscle fibers, just 20 to 50 um on average, the surface to volume ratio will be far superior. As the ATPase molecules are distributed throughout the surface of the cell, and the membrane potential determined by the contents of the volume, it would follow that a smaller cell should yield a more significant result, or at least a faster one.
4.2 - Dissection Procedure

Slaughterhouse derived bovine cardiac tissue was obtained on ice immediately after euthanizing, from a local source, with all subsequent isolation procedures taking place at 4°C unless otherwise stated, following the work of other labs (Kaminsky and Wolin, 1994; Harrison et al, 1986; Monticello et al, 1995; Spina et al, 1975). All fat, epicardial and endocardial tissue was removed from the ventrical tissue, which was then finely cut with a scalpel, and enzymatically dissected using a type 2 collagenase solution obtained from Worthington Biochemicals. The cells were incubated at 37°C, with 5% CO₂, for a periods of 30, 60 and 45 minutes respectively, with the Collagenase solution centrifuged off at 1500RPM, and replaced with fresh solution. After the final incubation, the centrifuged pellet was washed several times with Krebs HEPES solution, then passed through a 95µm nylon sieve, recentrifuged and incubated in cell culture medium, in several laminin coated optical culture dishes. Subsequently, after what was deemed a sufficient time for coating of the cells, culture medium was removed from the dishes, replaced with experimental solution containing the Nernstian dye TMRE. Culture dishes were examined with transmitted light for viable cells, with suitable cells placed under a cover slip offering a solution depth of less than 100µm, in order to reduce subsequent joule heating of solution. Also reservoirs of solution were formed outside of cover slip, to further combat this problem. The solutions used are listed below, in mM:
Krebs HEPES solution: 118 NaCl, 10 HEPES, 4.7 KCl, 1.5 CaCl$_2$, 1.1 MgSO$_4$, 1.2 KH$_2$PO$_4$, 5.6 Glucose,

Collagenase solution: as KH solution with 5% type 1 Collagenase;

Experimental solution: as KH with 1µM TMRE, 1µM TTX;

Culture medium (DMEM with 15% FBS and 1% pen/strep)

All solutions titrated to pH 7.4 at 25°C.
4.3 - Electrical Stimulation

Ag:AgCl electrodes were placed at each end of the cover slip, 10mm apart, to provide stimulation, via a purpose built amplifier, and a PC running National Instruments LabVIEW 7.2. Fluorescence images were taken using standard rhodamine optics, using a green HeNe laser, and an Olympus IX81 confocal microscope system, with the Fluoview Tiempo analysis package. Three Dimensional scans were taken every 30 seconds, with the intensity maximum, assumed to eliminate any movement of the cell upon stimulation, extrapolated, and subsequently plotted with respect to time. After a control period of 60 seconds, 50Hz synchronization pulses were applied for 10 seconds, at a magnitude calculated to be 80mV peak to peak, followed by modulation of the frequency of applied field from 50 to 400Hz, increasing by 1% every 0.1 seconds, resulting in a total modulation time of 72 seconds. There were several stimulation protocols used for the varying experiments undertaken, with the details of each presented in the corresponding sections.
4.4 - Stimulation Geometry

An area in this new experiment addressed to ensure artifact free results was the possibility of fiber movement upon application of the field. As all cells under consideration in this body of work are excitable cells, the mechanism for excitation being a voltage trigger, it is only natural that, through EC coupling, the fiber may experience contraction upon application of the synchronization field. Whilst the measured effect could never be confused with our results, as this would be a transient event, occurring within the time resolution of a single scan, this effect is still deemed undesirable, and its removal is essential if we are to closely track the time dependant development of fast events such as channel opening and inactivation behavior. Whilst a movement in the x or y direction is only evident upon examination of the images, it is also easy to eliminate, via averaging an area slightly larger than the cellular area. A movement in the z direction may be more difficult to observe, or compensate for. A movement in the z direction will, however, result in a significant change in measured fluorescence, as seen in Figure 4.1 below.
Figure 4.1 – Variation in image possible from three-dimensional artifacts

An image taken with confocal microscopy is a two dimensional image, with a small depth resolution. If a typical cell could be represented by a sphere, with the focal plane adjusted to be directly at the center of the sphere, if the cell moves in either the x or y direction, there will be no effect on the averaged intensity of the image, however, if there is any movement in the z direction, the circular image obtained by the confocal microscope will be of a smaller radius after the movement than before, resulting in a lowered average intensity. It is apparent that a slight movement in the z direction from the point deemed the center of the cell would result in a significantly lowered measured fluorescence intensity value.

The solution to this is to take a stack of images in the xy plane, varying the depth. This would give us a distribution of averaged intensities, with the peak of the distribution
representing the highest average intensity, and therefore the “center” of the cell. If then, a stack of images were taken every minute, and the peaks extrapolated, this would trace how the center of the cell varies with time, independent of any movement. There is a degree of trade off of temporal resolution made in order to analyze our data in this way, however, as none of our measurements require the tracking of transient events, this was a worthwhile sacrifice. Figure 4.2 is a typical scan with the peaks extrapolated from the raw data, showing the membrane potential variation upon stimulation.

![Raw data and Peak Extraction for long Stimulation](image)

*Figure 4.2 – Peak extrapolation to remove three dimensional artifacts.*
4.5 - Results

Initially, a number of control scans were taken on the cardiomyocytes, in a number of configurations. The initial control is the cell itself, acting as its own control. A cell is stained with the fluorescent dye, then mounted onto the microscope, and fluorescence readings taken. As the dye leaks into the cell from the external solution, a process taking from 10 to 20 minutes, the measured intensity of the cell increases to a plateau, which should subsequently show little to no variation in intensity. In this way, we can insure that each cell is viable up to the point of application of stimulation. Any cells showing instability after staining are discarded. Next, a set of controls was taken from the cardiomyocytes under the application of a field, with the presence of the selective Na/K ATPase inhibitor molecule ouabain, as in the skeletal muscle fiber experiments. This is to track the behavior of the cells when under the exact conditions of synchronization/modulation, with the exception that the Na/K ATPase molecules are not functioning as they would usually be. The individual results are shown in Figure 4.3.
Figure 4.3 – Cardiac cell fluorescence response with ouabain to synchronization modulation electric field. Notice no increase is seen, in fact, a small decrease is evident.

The statistical analysis of these results is given in Figure 4.4.
Under the application of a synchronization/modulation field, with the Na/K ATPase pump molecules selectively blocked, there is no observed increase in Fluorescence intensity within the cell. Moreover, there was actually an observable decrease in the dye intensity, signifying a reduction in membrane potential. This would be in agreement with our theory, and with earlier results, as we are not using a blocker for the voltage gated...
Potassium channels present within the plasma membrane, and hence we would expect to see a slight reduction in membrane potential upon application of a sustained potential of this type. In addition to this, there may also be a slight rundown of the membrane potential due to the fact that the Na/K ATPase molecules are no longer functioning to maintain the membrane potential. Therefore, any increase in membrane potential which is observed with these molecules active, would indicate an accelerated action to the point of overcoming this observed decrease in Vm due to the unwanted potassium channel current, in addition to any increase seen.

A further step in the establishment of a reliable control was to apply a stimulation/modulation field to the cells, in the opposite configuration to the experimental conditions. This would mean a synchronizing field at our originally final accelerated frequency, in this case 400Hz, followed by modulation down to what used to be the initial value of 50Hz, at the same modulation rate as the forwards modulation, with this final frequency of stimulation maintained for the same time period as the final frequency in the forwards modulation. The purpose of this backwards modulation would be to observe if the mere application of a field to the cells will cause an increase in membrane potential, irrespective of any synchronization phenomena. We are not expecting to be able to observe a significant slowing down of the pump functions, as our initial synchronization pulse is at a frequency where we would not expect there to be a large number of pump molecules operating. The results are shown in Figure 4.5 below:
Figure 4.5 – Cardiac cell fluorescence response to backwards modulation of synchronization electric field. Again, no increment in the membrane potential is observed.

The statistical analysis of these figures is shown in the panel below:
The recorded decrease observed in Figure 4.6 confirms that indeed any increase seen upon application of the synchronization/modulation field is dependant upon the direction of the modulation, and not merely due to the fact that we are applying the field in itself to the cell. Combined with the previous figure, we can therefore state that any increase such as that observed with the skeletal muscle fiber, is (i) due to the action of the pump
molecules, and (ii) dependant upon the properties of the field applied, hence due to the modulation effect of the field. If we compare the figures for the two controls, backwards modulation and ouabain, as seen in Figure 4.7, the two decreases are noted to be almost identical:

Figure 4.7 – Comparison between fluorescence responses of backwards modulation and ouabain traces.

This would signify that the membrane potential reduction experienced by the cell under backwards modulation is primarily due to the potassium current experienced by both
cells, not by any significant slowing down of the pump turnover rate. A degree of rundown in the ouabain treated fibers arising from the lack of action of the Na,K ATPase molecules may show an effect in addition to any backwards modulation effects in the other trace. In actuality, a slightly larger effect is observed with ouabain than with backwards modulation, as the effects of the pump molecules are completely removed, instead of minimized. Now, we applied the synchronization modulation field, in order to compare the effects with those of the prior controls, as seen in Figure 4.8.

\[ \begin{align*}
\text{Fiber Response to Long Stimulation} \\
\text{Time (sec)} & \quad 0 & 500 & 1000 & 1500 & 2000 \\
\text{Intensity (Arb)} & \quad \text{70} & \text{80} & \text{90} & \text{100} & \text{110} & \text{120} & \text{130} & \text{140} & \text{150} & \text{160} \\
\end{align*} \]

Figure 4.8 – Synchronization modulation of cardiac cells, exhibiting a pronounced increase in membrane potential upon application of field, hyperpolarizing the cells to a significantly higher level than the control.
Seven such experiments are shown, with each cell increasing to a significantly higher level of dye concentration. This does not represent a simple reestablishment of membrane potential, but a hyperpolarization to a noticeably higher level. The statistics of this experiment are shown in Figure 4.9.

![Fiber Statistical Response to Long Stimulation](image)

*Figure 4.9 – Statistics of synchronization modulation of cardiac cells.*

The bars in Figure 4.9 above represent the standard deviation. The large deviation is due to variation in cellular size on which different membrane potentials were induced by the
electric field, in addition to an inherent variation in membrane protein density from fiber to fiber. The average increase in fluorescent intensity after 30 minutes of field application is about 20%, a far superior figure to that of the skeletal muscle fibers from earlier experiments.
4.6 - Conclusions

This significantly improved measured increase would fit in with earlier predictions, attributed to a significantly higher surface to volume ratio, and a hypothesized superior electrogenic protein density. In addition to this, the cells remained healthy for extended periods, allowing the duration of stimulation to be higher than with earlier cells. Our controls would again indicate that the improvement seen is entirely due to the action of the Na/K ATPase molecules, due to the application of the synchronization modulation oscillating field. This result is positive, as it shows a significant change can be made to mammalian intact living cells, and so is encouraging to our goal of an eventual clinical application. Subsequently, now that we have succeeded in raising the membrane potential of different categories of intact living cells, a view must be taken towards application, and a model must be found to more fit in with a whole body, or clinical system.
Chapter 5 – Mammalian Tissue Slices

A step up in complexity, along with a step towards a physiological situation was made, with results taken from 2 dimensional tissue samples. These samples were obtained from rat ventricle muscle, using an oscillating tissue slicer. Once again, relevant controls were carried out to ensure effect recorded could be attributed to our technique, proving the technique can accelerate the action of membrane proteins whilst in the tissue environment. A degree of interaction was evident, with the samples exhibiting a reduced effect, however the increment remained significant.

5.1 - Study of Mammalian Tissue Slices

The decision was made, in the search for an iteration towards a clinical application, that we would move from a cellular level, to the tissue level. This would represent one step below the organ level, whereby we would attempt to control the membrane potential of an entire organ extracellularly, which would introduce a number of self sustaining systems, which would in turn attempt to correct this change, but would hence correspond to a significantly more complex system than that which we have been previously dealing. Utilizing an oscillating tissue slicer, we were able to take microscopic sections of a heart, dissected from a laboratory rat, according to all relevant university IACUC guidelines.
This is a significant step up in complexity, as each cell will have a degree of interaction with its neighbors, as well as with the extracellular fluid surrounding it. It is hoped that, given adequate results in such a system, work could be undertaken subsequently on a whole organ basis. Taking fluorescence from a tissue slice has been done many times before, with similar or identical probes as ours (Horiguchi et al, 2003; Kowaltowski et al, 2000; Combs and Balaban, 2001; Bindokas et al, 1998). Figure 5.1 below is an image of a typical slice, with individual myocytes observable. Striations are visible within the individual cells, and some outer cells are observed during testing to beat. This would indicate that the cells are still alive, and viable for experimentation.
Figure 5.1 – Transmission image of tissue slice, many individual cells are evident, woven into a two dimensional lattice.

An alternative dye, di-4-ANEPPS, was used as a marker in order to image the individual cells in a slice. This styryl dye is designed to bind to the surface of the plasma membrane of a cell, then fluoresce strongly. We were able to image the membranes of dozens of cells in the two dimensional matrix by this method, with results shown in Figure 5.2.
Figure 5.2 – Surface stained cardiac cells within slice.

Many dozens of cells are evident within the slice via this staining method not visible to us through transmission images, appearing as “bricks in a wall”, within the tissue slice. A more typical fluorescence image using the slow dye TMRE, would be as shown in Figure 5.3, in parallel with a transmission image.
Figure 5.3 – Fluorescence and transmission images, respectively, of tissue slice, in parallel

Again, within the transmission image, many cells and structures are evident, however, in the fluorescence image much of this detail is lost. Larger gaps than the typical interstitial gaps are evident within the fluorescence image, however the smaller gaps cannot be resolved, meaning that it is not possible to merely take measurements of only the intercellular areas of fluorescence, as the resolving this area is not always possible. In order to resolve this quandary, we took a high definition image of the tissue slice, as shown in Figure 5.4 below.
In this image, we can clearly resolve the interstitial gaps within a whole band of cells. Therefore, we averaged the fluorescence over an area of around 20 of these cells, including the interstitial fluid in addition to the intracellular region, and compared it with the combined fluorescence of the individual discrete cells, with no interstitial region included. The difference between these two figures was below 3%, and was therefore judged to be negligible. From this point on, when imaging a slice, we were satisfied that the fluorescence given off by a region of cells was representative of the dye molecule density within this particular set of cells.
5.2 - Dissection Procedure

An Electron Microscopy Sciences 4500 oscillating tissue slicer was utilized in the harvesting of samples, taken from adult male Sprague Dawley rats, weighing between 200 and 300 grams. The rats were previously anesthetized using Inactin, obtained from Sigma, and the hearts quickly dissected from the pericardium, and placed in cooled Phosphate Buffered Saline solution (PBS), oxygenated with 95% oxygen, 5% CO$_2$. The fat, arteries and aorta are removed in solution, with the superior region of the heart dissected completely, releasing any blood from the now open chambers. This dissected tissue is briefly dried, and brought into contact with the stage, which is coated with industrial adhesive. 15 seconds is sufficient to assure adequate adhesion, and the tissue is subsequently resubmerged in PBS, this time in the slicer bath. The OTS 4500 system allows for slices to be taken on the order of microns, dependent upon tissue type. For our application, slices of 150 micrometers were deemed to be adequate, allowing a thin enough slice for the application, but the slice will still be thick enough to approximate a three dimensional region of tissue, nearer to the clinical situation. Similar solutions to the earlier cardiac work are utilized, listed below, with the channel blocker TTX used to remove contribution from the sodium channels.
Krebs HEPES solution: 118 NaCl, 10 HEPES, 4.7 KCl, 1.5 CaCl₂, 1.1 MgSO₄, 1.2 KH₂PO₄, 5.6 Glucose,

Collagenase solution: as KH solution with 5% type 1 Collagenase;

Experimental solution: as KH with 1µM TMRE, 1µM TTX;

Culture medium (DMEM with 15% FBS and 1% pen/strep) 

All solutions titrated to pH 7.4 at 25°C.
5.3 - Electrical Stimulation

Stimulation of the tissue slice occurs using a modified version of the earlier program, with the calibrated result of the modulation program outputted to a DAGAN TEV 200A voltage clamp. This allows for more precise control of the voltage across the cells themselves than earlier, if we assume a seal is formed by the slice (see below). The voltage clamp acts upon the slice injecting current into the cells balanced by a measurement of the resistance across the slice, to ensure an accurate voltage level at all times. An obvious limitation, however, would be the fact that our slice is taken to be several cell layers thick, and therefore the voltage across the cells may be divided between several layers. If a slice thickness of 150 microns is taken, we can see from the image below of a single myocyte, indicating the approximate typical size, that as many as 3 or 4 cells may be in this layer, as illustrated by the dimensions of a single cell as in Figure 5.5.
It is unlikely, however, that the cells on the outer most layers, at the top and bottom, of the tissue slice would fully retain their integrity, due to the slicing procedure, which is highly damaging to the outermost cells. It would be prudent, however, to assume that there is more than one cell within the thickness of the tissue slice. It is assumed that around 3 or 4 cells remain intact within the thickness of the slice, necessitating an increased applied membrane potential to the slice of 3 to 4 times. In addition to this, there would be an unknown degree of resistance measured within the interstitial fluid in
between individual cells of the slice, which would again result in a voltage drop across the slice. A suitable clamp value for potential across the entire slice was deemed to be 200 mV, which should give us sufficient voltage across the individual cells, where around 30 mV is seen as acceptable.
5.4 - Stimulation Geometry

The challenge of providing an adequate stimulation field to both sides of the dissected tissue slice was a significant consideration in the design of this experiment. The chamber designed must allow fluorescence to pass through its base, whilst allowing a minimal distance from the base to the slice, allowing the objective to function within its normal range of working distance. There must be an electrical contact at the base of the chamber, whilst still allowing fluorescence to pass through the glass to the optical detector. The slice must be sealed in an airtight manner, in order to electrically isolate the top from the bottom side of the slice, to allow the voltage clamp to operate. Finally, there must be an adequately large reservoir of dye molecules on both the top and bottom of the slice, allowing any change in membrane potential which the cardiac cells experience to be followed by a change in dye molecule concentration, and thusly a change in fluorescence. This reservoir must not be over 1.3mm in height, so as to avoid problems with the microscope working distance. The design chosen, a modified Ussing chamber, is shown below, in Figure 5.6.
This design was found through experimentation to provide a more than satisfactory sealing resistance, with no problems in imaging provided by the dye reservoir. Electrical contacts were made with agar bridges, in order to minimize junction potential.
5.5 - Results

Many images of the cardiac slices were taken prior to experimental fluorescence being measured, ensuring that the cells taken were healthy, and of a representative quality. When mounted within the Ussing chamber, the key factor in determining experimental viability was the seal test available to us through the voltage clamp system. Through a small applied square wave, the system internally calculates a running average of resistance for the system, which will in turn represent the quality of the seal from the top to the bottom of the slice, which in turn represents the ability of the voltage clamp to adequately apply a voltage to the cells. The empirically determined value for a suitable slice was taken as 1MOhm, although value of much larger than this were not uncommon. This figure is in agreement with normal operation in other applications of the voltage clamp occurring within our lab. As before, controls were taken using ouabain in the bathing solution, in order to represent the response of the cells to the applied field when the pump molecule’s operation was removed. This data is shown in Figure 5.7 below.
In this average of seven results, from seven different tissue slices, with standard deviation shown, no increase was measured in any fiber on application of the field, indicated by a dashed line. In actuality, a decrease in dye concentration was measured in each experiment, indicating that with the pumps not operating, the cells within the tissue slice will lower their membrane potential upon application of the synchronization modulation field. We would expect to see a decrease such as this from potassium channel opening events, whereby the membrane potential would be seen to drop. Next, we applied the synchronization modulation field with modulation working backwards, taking the
oscillation frequency from 400Hz down to 30Hz, with the same modulation frequency as before. The results are shown in Figure 5.8 below.

![Slice Response with Backwards Modulation](image)

*Figure 5.8 – Tissue slice, backwards modulation.*

Again, in an average of seven results, with standard deviation shown, no slice was observed to increase in fluorescence on application of the field, indicated again with a dashed line. Indeed, the cells were again observed to decrease in fluorescence, indicating a lowering of membrane potential, which would be attributed again to the potassium channels, or alternatively to a contribution of this and an effect resulting from the synchronization modulation field actively lowering the turnover rate of the Na/K ATPase
molecules from their resting rate, leaving them unable to maintain the resting membrane potential. The two controls are now compared in Figure 5.9.

![Slice Response with Ouabain/ Backwards Modulation](image)

**Figure 5.9 – Tissue slice, backwards modulation and ouabain**

If the two controls are plotted on the same figure, as above, it is interesting to note that, as with the cardiac controls, the backwards modulation figure shows a lesser degree of depolarization on application of the field than the ouabain trace. This would seem to make sense, as in the case of the ouabain trace we hypothesize that the pumps are entirely prohibited from functioning, and when, inevitably, the cell becomes depolarized due to channel opening phenomena, the cell is entirely unable to recover. This would not be the
case, necessarily, with the backwards modulation trace. In this case, the cells would still partially recover, as the pumps are still able to work, just at a lower rate than they normally would. We do not, in any case, suppose that the pump molecules are actually synchronized to this lower frequency, as the initial synchronization frequency was extremely high, 400Hz, where few of the pump molecules would be naturally functioning. This would mean that as the frequency is modulated down to 30Hz, not all of the pump molecules would be captured, and follow the modulation frequency, and hence some would be functioning normally, allowing for a degree of recovery. Below, data is shown for the slice response to forwards modulation, with no ouabain.

![Figure 5.10 – Tissue slice response to modulation with no ouabain](image-url)
An increase of around 3% is evident in Figure 5.10. This would represent a slightly lesser degree of depolarization than that measured in single cells. We feel this can be adequately explained due to the fact that the stimulation geometry enforces a lower membrane potential to be impinged upon the cells. This is due to the maximum voltage deliverable by the voltage clamp being 200mV. The figure below more thoroughly illustrates this explanation.

Figure 5.11 – Tissue slice schematic representation
A maximum of 200mV is available to us through the voltage clamp system, which in most cases is more than enough, the potential across a typical plasma membrane being less than half of this. However, in our particular situation, we take a slice of around 150um. This would suggest that with dimensions of typically 30 to 50 microns per cell, there is in fact 3 to 5 cells thickness in each slice. This would consequentially mean that there are between 6 and 10 membranes across which this 200mV potential is shared. Subsequently, the maximum potential we could expect to be applied across a single membrane would be around 30mV, although this would be the maximum available. This figure could conceivably in fact be only 20mV, if more cells were present, dependant upon the orientation of cells within the slice. In fact, efforts were made when preparing the samples to slice the cardiac tissue longitudinally, which would in fact mean that this lower figure would be more likely. In addition to this fact, the membrane potential across an individual cell would be further lowered by the fact that there is a conduction pathway across the slice which passes through the interstitial fluid of the slice. This effect was assumed to be small however, due to the high sealing resistances necessary for each healthy sample used in these results. Another factor which must be considered when investigating a two dimensional tissue matrix such as this is that we are unaware of the extent of interactions between neighboring cells within the slice. In particular, would cells from further within the slice draw dye molecules from those closer to the dye reservoirs, thus effecting the fluorescence intensity measured, or would the dye satisfactorily be transported though diffusion along the interstitial fluid gaps between each cell? Efforts were made to measure cells from at least one layer into the slice, so that we could observe a situation closer to the physiological one, and therefore measured
results could be lower than those if we had measured the outer most layer of cells. Yet another factor which may contribute towards this apparent decrease in hyperpolarization observed would be the fact that each individual cell within the matrix, particularly in cardiac tissue, is interconnected, both electrically and chemically, by a series of gap junctions or nexi, known as a pair of connexons, or hemichannels. A nexus such as this would provide a direct chemical and electrical signaling pathway between neighboring cells, and hence the majority of cells within the slice, which is particularly important for the propagation of electrical signals within the heart. This would mean that when we apply the voltage to the slice, which would only occur within the center section of the tissue slice, the voltage encountered by the cells would actually be much lower than the 200mV which we apply, as this would be spread laterally within the slice, dissipating as illustrated in Figure 5.12.
In cell number 1, the membrane would experience the full magnitude of the applied field. This would not be the case, however, in cell 2 or 3. As the chamber window is small in comparison with the total size of the slice, some cells will be partly, or totally occluded, and will not be in electrical contact with the solution, therefore not experiencing the field. In addition to this, when an increase in chemical concentration is experienced by a cell, due to the applied field, this increased concentration would also dissipate into the cells outside of the field, or at least to those which experience a much reduced field strength, again lowering the observed hyperpolarization. The final, and most obvious reason for the reduced magnitude of hyperpolarization in the tissue slice experiment is that the field in earlier experiments was simply applied for a longer duration. This would naturally
lead to a greater effect being observed, as the hyperpolarization takes time to occur. In the single cardiac cells measured earlier, the field was delivered for a total of 30 minutes in each experiment. Here only 5 minutes of stimulation is delivered. This is due to the fact that the tissue slices simply will not stand a maintained stimulation like single cultured cells will without substantial deterioration. If we plot the results taken from the tissue slice, along with the results from the controls and long stimulation in single cells, as seen below, it would appear that the time courses for each cell are not drastically different, see Figure 5.13.
The tissue slice exhibits more than 50% of the hyperpolarization that the single cells do within the opening 5 minutes of stimulation. When considering all of the aforementioned factors which would contribute to the hyperpolarization observed being measurably lower, these results seem reasonable. It was, however, deemed prudent that more experiments should be carried out with a higher field strength, delivered to the slice through LabVIEW, and a custom designed program, in addition to a longer duration of stimulation. This would unfortunately mean that we can no longer track the sealing resistance of the chamber, in order to ensure that no changes have occurred from the

Figure 5.13 – Tissue slice vs single cardiac cells.
beginning to the end of each individual experiment. There would, however, now be a much higher range of membrane potential available to us, unfortunately at the sacrifice of some of the accuracy of determination of the field we are applying, as the slice is no longer voltage clamped. The stimulation was roughly empirically calibrated to our earlier levels, then membrane potentials of around double what was previously applied, which we would estimate to be about 50mV, were applied. In order to enable a longer stimulation duration, the slices would need to remain viable for longer. In order to ensure healthier cells, the heart was sliced immediately after euthanization of the rat, in oxygenated PBS solution, with the slices subsequently placed in cellular medium, as used earlier in the culture of single cardiac cells. This medium was pre heated to 37°C, with the slices incubated with 5% CO₂, in order to maintain cellular health. Even with these precautions, slices showed a noticeable deterioration with time from initial isolation, with the majority of useable slices obtained within an hour from isolation. However, inside this period the cells showed markedly improved health, with measurably higher levels of hyperpolarization, as shown in Figure 14.
Fluorescence Variation of Tissue Slice with Synchronization Modulation Field

There is a clearly improved increment to the membrane potential on earlier results, this time to around 9%. This is in agreement with our earlier results for single cardiomyocytes, as we would expect it to be more difficult to hyperpolarize the living cells when they were not isolated from the tissue, due to intracellular interactions, and a more difficult stimulation.

*Figure 5.14 – Cultured tissue slice with field.*
5.6 - Conclusions

Significant results were obtained from a two dimensional tissue slice, which were determined, through relevant controls, to be due to application of synchronization modulation electric field. Around a 9% increment in fluorescence intensity from within the tissue slice was measured, which would be approximately in line with earlier results, as the scans of single cardiac cells taken in the previous section lasted for a period of 30 minutes stimulation, and showed around a 20% increase, in comparison with the 15 minute stimulation used in the tissue slice experiments. The necessity of a shorter stimulation duration for tissue slices however, highlights one of the key problems faced. This change to the experimental protocol was necessary in order to keep the cells healthy whilst being stimulated. This would be a much more significant problem were the cells to be within a living animal, or patient. Here, deterioration of the cells would mean danger to the patient. It is, however, the preparation itself which causes this tissue damage, not the synchronization modulation technique, therefore this would represent a procedural problem, rather than a fundamental one.
Chapter 6 - Conclusions

A significant and previously unrecorded effect is presented, with results showing in ideal situations over a 20% increase in ionic concentration measured, with around a 10% change in situations closer to physiological conditions. The technique also shows great promise in application as a form of therapeutic treatment, with measurable recovery illustrated in damaged cells.

6.1 - Summary

In delicately controlled self regulating systems such as those found in the body, any electrophysiological changes, however small, can have myriad effects upon the system as a whole. We have successfully demonstrated through the course of this work, that the synchronization modulation technique can be used to manipulate both the ionic concentration and the membrane potential of intact living cells. Figure 6.1 shows our results for the measured membrane potential changes recorded across various cell types, along with their related ionic concentration changes.
Figure 6.1 – Table indicating ionic concentration and membrane potential change for various cell types.

As can be seen in Figure 6.1 above, a significant increase in recorded effect was evident in the cardiac cells, most likely due to a combination of the cell’s superior surface to volume ratio, higher pump densities and a longer period of stimulation. When results were taken from these cells within the cellular matrix, a net lower change was observed. This would most likely be due to interactions between the cells, difficulty of stimulation within this configuration, and cellular interactions, for example through gap junctions.
The fact that there was a marked difference in results from those obtained from single cardiomyocytes and those from tissue slices isolated from cardiac tissue from an anatomically similar animal may however be significant. We would expect that when stepping up from a single cell to a two dimensional tissue matrix there would be a decrement in the performance of the field, if only due to the uncertainty in the exact arrangement of the field in comparison with single cells. There may in all probability be a more complicated relationship, as there are undoubtedly interactions between adjacent cells which need to be taken into consideration, which would be extremely difficult to quantify and correct for. Therefore, it would follow that the further degree of complexity added when applying the field to a full three dimensional tissue matrix, would come with an accompanying decrement in performance. As the initial step to a two dimensional tissue halved the recorded increase in ionic change, therefore, this further step would undeniably come with a more significant decrement. It is possible, due to the nature of the gap junctions, however, that we may be seeing an exaggerated effect, due to dispersal of the ions added due to the synchronization modulation field across the cells, in the direction of the slice. As the gap junctions offer a conductive pathway for ions, allowing fast propagation of the electrical signal in the heart, it is possible that they would allow any increment in ionic conduction built up by the field to also propagate, which means it would travel to outside the influence of the field, “diluting” the effect observed. In a three dimensional tissue sample, all of the sample would have to be within the effect of the field, therefore it is possible that we would see an increment of actually larger proportions. This would, however, need to be further investigated.
If the potential public benefits of this technique can ever be fully realized, then a major new field of therapy could be pioneered, where a non-invasive treatment could be used to combat the myriad of disorders which contribute to a deterioration in the quality of life, possibly leading to fatality, in over 7 million patients in this country alone. However, before any therapeutic benefits can come to fruition, this technique must be fully tested, and extended from the cellular, laboratory level, to a level closer to a clinical situation. Our target was to obtain results from a tissue level, an already ambitious goal. We have observed promising results from our analysis of intact skeletal muscle fibers, and have expanded to an even more impressive level with cardiac cells. This was applied to an application mimicking electrical injury, with impressive results. Our technique was able to recover from this shock, and even go so far as to hyperpolarize the membrane to a level higher than the initial. Experiments were successfully taken to a two dimensional tissue matrix, with increase in membrane potential again measured, promising indications for the transition of this technique into a fully three dimensional tissue matrix, and then, theoretically on to a clinical level. This would represent the first time that research of this kind has been performed, with successful results for this entirely new technique demonstrated across several classes of cell, and cellular situation.

Another issue paramount in this research is the effect observed in the electroporated cells from the first section. In this section, cells were damaged using a high field stimulation, designed to model that of a power line frequency electrical injury. In the United States of America, there are roughly 2.1 deaths per million inhabitants due to electrical injury, with countless more non-fatal injuries. Recovery from these non-fatal injuries is a process not
fully understood, however it is clear that additional work which could be extracted from the Na/K ATPase molecule to reestablish ionic concentration and membrane potential would be of interest. We were able to demonstrate that an expedited recovery was possible through the application of our technique, to the point where the cells were hyperpolarized from their original position. This could serve not only as a model for the recovery from electrical injury, but also to any disorder whereby the membrane potential was not maintained at a sufficient level, as discussed before.

The key iteration taken by this research from that of previous work, is that all of our results were obtained from fully intact living cells, not from perforated or damaged cells. This would represent a transition from a purely laboratorial setting to a more clinical environment, which must be seen as the final goal for this field.
6.2 - Suggested Future work

The next logical step to the eventual goal of a clinical treatment, would be to extend our study away from the single cell, laboratory type of study, and more to an approximation of a clinical environment. This would mean our studies would have to move closer to a full body approach. This would represent a step up in complexity comparable to the step of moving to three dimensional study. The level of complexity we will encounter with this step up cannot be predicted, however, initial problems identified would be the increased Joule heating effects which may be seen with larger length scales, and the fact that there will simply be more membranes now for the applied field to act upon, bringing the applied voltage on a single membrane down significantly, necessitating a higher field strength to be applied to the tissue in question. There are in fact many labs that utilize Langendorf perfusion techniques in order to spatially map the heart as it beats, subsequent to removal from the body. Typically, however, they would measure a much faster event, using a fast dye such as di-4-ANEPPS. This charge shift dye has considerable use in many labs in order to track the two dimensional conduction of action potentials across the surface of the heart. A problem that may be encountered here, however, would be that our measurement would be typically much smaller than an action potential event, and would represent a DC change in membrane potential, which would be superimposed on top of these AC action potentials. Another source of trouble would
obviously be that in this arrangement, a degree of movement would be inevitable as the heart beats. This would translate into artifacts in measurement within our system, as the extremely small depth of focus of the confocal microscope would mean that there would be a significantly different image in focus with each movement.
Publications

The following papers have been completed within this work.


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Appendices
Appendix A – Chemical Data

Figure A1 – Chemical structure of Na/K ATPase blocker ouabain

The glycosylated steroid ouabain shows selective binding to the alpha subunit of the Na/K ATPase molecule, inhibiting the pump transport activity. It is thus widely used in research to isolate the function of these molecules via comparison of response with and without ouabain. It is most commonly found in the seeds of several rare African plants, such as the Strophanthus Gratus tree. Although digoxin is the preferred prescribed drug for cardiac problems such as atrial fibrillation and CHF, its use still continues in several
countries. Although pico mole concentration of ouabain have been observed naturally produced by the adrenal gland, its exact function within the human body is as yet unknown.
Appendix B – LabVIEW Program

The LabVIEW programs designed in the course of this work followed a number of designs with a number of pre-run calculations made by the program based on the requirements of the user, and the geometry of the system in question. This is vital, as around 12 different geometric arrangements of sample and electrodes have been used up to this point, with different equipment needed to be calibrated for in addition to the sample and arrangement. Field calculations are made based on this geometry, and the desired membrane potential, with parameters such as offset and amplifier gain also inputted, with the program outputting the desired voltage magnitude, pre and post amplification. The modes available across the set of programs include electroporation, invariant stimulation, modulation (both forwards and backwards), and tissue slice mode, with a combination of these modes also available. The first step in the program is the electroporation step. The number of electroporation pulses can be varied, in addition to the delay between successive pulses. Next, a wait time between this step and the stimulation step occurs, whereby the user can observe the effect of the electroporation. Subsequently, a synchronizing pulse, in our case of 50Hz, is applied to the sample, for a pre specified duration. This pulse can now be modulated from this initial frequency to a second frequency, with steps specified in percentage of the present frequency, varying up to the final frequency, which will be held until the end of the program. The two main
versions of the set of programs used in this work are shown in the diagrams below, indicating both front panel controls and block diagram functionality, with several steps in sequence structures visible where this is applicable.

**Figure A2 – Front panel 1**
Figure A3 – Block diagram 1
Figure A5 – Block diagram 2a
Figure A6 – Block diagram 2b
Appendix C - Cyanide Pathology

Attempts were made to examine the reaction of living cells to the toxin sodium cyanide. Upon addition of cyanide to skeletal muscle fibers, within 10 to 20 minutes, visible surface membrane disruption was visible, as the mitochondria of the muscle cells are attacked, and shut down. Our hypothesis initially, was that our technique could drive the Na/K ATPase molecules within the cellular surface, prolonging the life of the cell as less ATP became available from these mitochondria. However, with no observable discrete signal for cellular breakdown beyond a qualitative assessment of cellular health based on membrane condition, no solid control could be established, and this experiment was abandoned.
Appendix D – PC12 Cells

A further experiment carried out was into the response of the neuroendocrine cells PC12, a cell line taken from rat Pheochomocytoma, a cancerous tumor in the medulla adrenal gland. These cells, as the term neuroendocrine implies, represent a mid point between the nervous system and the endocrine system, with properties which can be directed towards one system or the other. This cell line will continuously divide due to its cancerous characteristics, and it is said that many of the researchers working with these cells are now younger than the cell line itself, due to its isolation and successful maintenance from over 30 years ago. The dividing of the cell line can be stopped manually by the researcher, upon addition of the factor NGF, or nerve growth factor, a polypeptide naturally secreted by the body which causes the halting of this dividing, and the differentiation of the PC12 cells into distinct neuronal cells. Our original hypothesis was that with this differentiation comes a transformation of the cells from non-excitatable to excitatable cells. This conversion must come with the ribosomal production of many more membrane proteins, in order to maintain this excitability, and therefore there must be many Na/K ATPase molecules produced and added to the cell membrane. Typically, this transformation is complete in a matter of a few days. If results could be taken from identical samples of cells before and after the addition of NGF and the resultant differentiation of the PC12 cells, interesting information could be extracted on the nature
of this differentiation. Unfortunately, however, the results were not promising, in all probability due to the fact that the degree of excitability of the cells, whether differentiated or not, was not known. The results obtained did show some differences, however it was not deemed significant.
Appendix E – Electroporation Figures

In the figure below, individual electroporation traces are shown before signal averaging, for seven traces.

![Individual Traces showing EP and Recovery with Synchronization and Modulation](image)

*Figure A7 – Individual traces showing electroporation recovery with field*
Appendix F – Equipment

In the following section, detailed schematics and photographs are given of each piece of equipment used through the course of this research. The figures are respectively, the confocal microscope, the tissue slice, and the chamber.

Fig A8 – Confocal microscope and stimulation equipment
Figure A9 – The oscillating tissue slicer OTS 4500
Figure A10 – Slice chamber showing stimulation equipment
About the Author

Robin Dando graduated from Sheffield Hallam University in the UK with an undergraduate degree in Physics, and from the Universities of Sheffield and Leeds with an MSc in Nanotechnology. He traveled to the United States to complete his PhD at the University of South Florida, where he has authored several scientific papers in the field of Biophysics.