OPTICAL METHOD OF RECORDING ELECTRICAL ACTIVITY IN ISOLATED RABBIT HEARTS

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(ABSTRACT)

A recently developed optical method utilizes a single, implantable, optical fiber to record electrical activity from isolated hearts stained with voltage-sensitive dyes. This optical technique generates recordings of transmembrane potential from excitable myocardial tissue, and remain free from stimulus artifacts that accompany electrostimulation and hinder all standard electrode recording methods during the application of high-voltage electrical shocks. The fiber optic system uses a 100μm diameter core fiber which can record from epicardial surface, internal mid-myocardium, or endocardial surfaces. The stained tissue is excited through the fiber and the resulting fluorescence is transmitted through the same fiber to a photomultiplier tube. Changes in fluorescence accompanying normal cardiac action potentials usually range from 1-5%. Substantive motion related signals accompany normal beating hearts, drowning out the actual signal that corresponds to change in membrane potential. When added to the nutrient solution of the heart, an excitation / contraction decoupler restricts motion and reduces the motion related signal making it easier to isolate the true membrane potential signal.
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1. Introduction

The ability to assess the electrical activity of cells ranks high among the many requisites for research in cardiology, electrophysiology, neurology, and various other medical sciences. Whether they are myocardium cells or nerve axons, researchers must have the capability of measuring the intracellular electrical potential from one cell up to large groups of cells. Some of the classical methods for recording bioelectric activity include extracellular electrodes that measure potential differences between separate areas of the sample (electrocardiograms), and intracellular microelectrodes that penetrate the cells and measure the potential across the cell membrane [1,2]. A more modern technique gaining popularity, the Monophasic Action Potential (MAP) probe, measures electrical activity from cells within a radius of approximately $1/32$ of an inch (0.7mm) by placing it in direct contact with the tissue [11]. Recent developments in optical recording techniques have proven to offer more direct information than extracellular recordings and do not require penetration and destruction of the cell as with microelectrodes [2].

This optical method bases itself on dyes that produce linear changes in absorption of transmitted light or fluorescence from excitation light in response to changes in membrane voltage or ionic changes in the cell [3]. Over 1800 dyes have been tested for appropriate optical response and for toxic effects on various specimens and of the 1800, 200 exhibit significant voltage-sensitive optical changes without causing excessive pharmacological side effects or photodynamic damage [1,2]. Many of the dyes fall under the classification of fast response dyes which act on a microsecond time scale to membrane potential changes [2,12]. For the use of voltage sensitive dyes in animals, most applications require extensive dissection because of the necessity of a direct line-of-sight path between the living tissue and the excitation/recording system [3]. The incorporation of optical fibers has alleviated much of this constraint, as the fiber provides a transmission medium excitation light and returning fluorescence over long distances without a path free from obstacles.

Optical techniques are gaining great favor in cardiology research labs across the country due to its advantage of immunity from stimulus shock artifact. Some of the advantages of the optical method over conventional electrical recording techniques include (i) the recordings are not subject to stimulus artifacts from pacing or defibrillation
shock pulses [4,5], (ii) optical fibers have the potential for implantation [5], (iii) the ability to make intracellular recordings simultaneously from many sites that could not be penetrated by microelectrodes [3], (iv) the signals have long-term stability so recordings of electrical activity from the same location last upwards of an hour, (v) the capability to look at the membrane potential of one single cell (100μm diameter), (vi) measurement of intracellular events without penetration of the cell.

Disadvantages include (i) motion artifacts that compromise the quality of the optical signal, (ii) the inability to calibrate the optical signals directly to voltage; only the relative magnitude of the electrical pulse and its duration can be measured [5], (iii) loss of signal intensity due to bleaching of the dye by the excitation light source, (iv) photodynamic damage to cells and dye toxicity, and (v) high sensitivity to position, sometimes requiring 10 to 15 attempts before locating a good signal.

Cardiac fibrillation, irregular contractions of myocardium (heart muscle) that make it impossible for the heart to pump blood, is a very serious condition and can be fatal. In fact, sudden death due to ventricular fibrillation kills 400,000 people a year in the United States [15]. The application of a strong electrical shock across the heart has long been known to terminate ventricular fibrillation [4]. A major goal of researchers of defibrillation mechanisms is the evaluation of safety and effectiveness of defibrillator waveforms, waveform intervals and electric field geometry. In addition to medical researchers, the Food and Drug Administration has acute interest optical methods used in defibrillation studies as they regulate and approve all implantable defibrillators for use in human patients.

The fact that the optical method directly and exclusively senses cardiac membrane voltage warrants considerable attention these researchers of pacing and defibrillation wave forms. By design, conventional electrical recording systems sense electrical impulses on the order of millivolts. Pacing and defibrillation shock pulses are on the order of tens of volts to hundreds of volts. Electrical artifacts attending these shocks hamper investigation into their electrophysiological effects [4]. The voltage gradients produced by the shocking current are large enough to overload and saturate the conventional recording instruments during the application of the shock. In addition to not having the ability to record the membrane potential at the instance of the shock, restoration of normal recording is delayed by lingering electrode artifacts and electronic recovery of the amplification system [4]. Improvements continue to develop in designing
special instruments that permit rapid recovery of normal recording, but it remains physically impossible to record the true cellular potential at the point of the shock with conventional electronic recording systems.

Researchers currently use this optical technique to study how defibrillation thresholds change with shock intensities, and shock durations, and how myocardium reacts to shocks delivered during various phases of the cardiac cycle. The results of their research are directly applicable to the clinical realm of medicine. In addition to the use of voltage sensitive dyes, calcium sensitive dyes are also implemented to investigate the hypothesis that cellular damage to the myocardium occurs during the presence of the large electric fields created during defibrillation. The ultimate goal of the optical method is to have the ability to implant and optical fiber through a vein without resorting to major surgery, and use it as a diagnostic tool in evaluating the electro-physiology, especially the defibrillation characteristics, of individual patients.

This thesis deals with the design and implementation of an optical recording system that utilizes a single fiber for both excitation and detection of electrical impulse-related fluorescence changes in cardiac tissue in a live rabbit heart. A helium-neon laser excites the dye, which has been perfused into the rabbit heart. With this system, we have succeeded in providing a system that reliably records cardiac electrical activity with long term stability on the order of hours. A MAP signal provides a comparison for the optical signal during various stimulation paradigms, including pacing and defibrillation shocks varying in intensities of 50V to 150V. In every case the optical signal maintained its measurement of strictly the membrane potential while the MAP signal many times saturated off scale and returned to normal operation after several seconds. To overcome the problems with motion we employed a pharmaceutical agent that decouples the contraction of the heart muscle from its excitation. The agent used in this project is Diacetyl Monoxime (DAM).

Normal electrical impulses of the heart have generated a 1% to 5% change in the returning fluorescence from the muscle. In a recent abstract to be submitted to the American College of Cardiology we documented a significant effect of DAM on the electrophysiology of the cardiac defibrillation. This is an unfortunate discovery because it might invalidate data that researchers find using the optical technique in conjunction with this agent.

In addition this thesis will discuss some experiments using a calcium ion sensitive
dye with the same optical equipment. The experiment's purpose investigated a hypothesis that high voltage shocks damage the integrity of the cell membrane and would allow large amounts of calcium to enter the cell and therefore change its internal calcium concentration. Past experiments by many investigators have shown the changes in internal concentration of calcium are normal to cardiac muscle contraction. The experiments with a rabbit heart did not show the internal fluctuation in calcium from normal cardiac activity or after defibrillation strength shocks.
2. Background

2.1 Physiological Concepts

To fully understand the principle and motivation of the optical method, there are some basic concepts in physiology that are helpful to know. The following provides a basic introduction to cardiac physiology and electrophysiology, and is not absolutely necessary to read, but will provide definitions and explanations of terms and concepts used throughout the paper.

2.1.1 The Myocardium

The muscles of the heart that allow it to contract and pump blood throughout the body are termed myocardium. The myocardium is composed of specialized striated muscle cells and interconnective tissue. Specialized paired-membrane junctions, intercalated discs, join the cells, end to end, into long fibers. The intracellular fluid of the cells is isolated from the extracellular fluid, i.e. blood, by a plasma membrane, sarcolemma, and a mucopolysaccharide-rich basement membrane [6]. The membranes together are about 75Å-100Å thick and a typical cell has a diameter of around 100μm [8].

The fibers are separated from one another by intercellular spaces, of varying widths, containing collagen, fibroblast and numerous capillaries. Sheaths of connective tissue separate groups of muscle fibers in long bundles which also interconnect. The muscle fibers within each bundle are parallel, however, adjacent bundles may be parallel, oblique, or even transverse to one another [6].

There exists a specialized conduction system in the heart made up of unique pacemaker nodes that generate the initial stimulus for a beat of the heart, and distinct conducting fibers that act as the highway for delivering the stimulus to the myocardium in the proper sequence for the heart to execute one correct beat. This thesis will not go into the specifics of the conduction system or the contractile elements of the myocardium that allow the fibers the contract. For more information consult references [6] and [7].
2.1.2 The Action Potential and its Ionic Basis

The initial stimulus created in the pacemaker nodes is and electrical signal called an action potential (AP), resembling a square wave (see Figure 2.1). Arrival of the action potential at the contractile fibers of the heart initiates contraction (see Figure 2.2). This electrical signal is best understood by recording the transmembrane potential by use of intracellular microelectrodes [6].

The basis for transmembrane potentials lies in the fact that the intracellular ionic composition differs from that found in extracellular fluid [6,7]. For the scope of this thesis the most important ions are potassium (K⁺), sodium (Na⁺), and calcium (Ca²⁺). The intracellular v.s. extracellular concentrations of the ions in frog muscle are as follows (note: and ion in square brackets denotes concentration of that ion, ie [K⁺] = concentration of potassium ion) [8]:

<table>
<thead>
<tr>
<th>Ion</th>
<th>Intracellular [ ]</th>
<th>Extracellular [ ]</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺</td>
<td>124 mM</td>
<td>2.25 mM</td>
</tr>
<tr>
<td>Na⁺</td>
<td>10.4 mM</td>
<td>109 mM</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>4.9 mM</td>
<td>2.1 mM</td>
</tr>
</tbody>
</table>

The intracellular potassium concentration is much greater that the extracellular concentration, while intracellular sodium is much less than extracellular sodium [6,7,8,9] (see Figure 2.3: Note that the relative sizes of the symbols illustrate the relative concentrations). During resting conditions the membrane permeability to K⁺ is much greater than the permeability to Na⁺ [6,7,8,9]. A concentration gradient exists that necessitates the outward flow of K⁺ and the inward flow of Na⁺, but due to the fact of a greater membrane permeability towards K⁺ at rest, the resting membrane potential is determined much more on the basis of potassium movement than sodium. The outflow of K⁺ down its concentration gradient polarizes the resting membrane to approximately -90mv. In other words the intracellular fluid is 90mv less than the extracellular fluid [6,7]. An active transport pump sets up the concentration gradient and removes excess ions after an action potential. The pump is often called the sodium/potassium ATP pump, because it requires ATP to pump against the concentration gradients. To still maintain the polarized membrane potential more Na⁺ is pumped out than K⁺ is pumped in [7].

In addition to the chemical forces (i.e., the concentration gradients) on the ions,
**PHASE**

0 - rapid upstroke of AP
1 - brief repolarization
2 - plateau
3 - gradual repolarization
4 - return to normal resting pot.

C - D absolute refractory period
D - E relative refractory period

Figure 2.1 Phases of the Action Potential [7]

---

Action Potential

---

Muscle Twitch

---

Time (ms)

Figure 2.2 Time correlation between excitation and contraction [7]
Potassium

\[ [K^+]_i >> [K^+]_o \]

High membrane permeability to potassium

2 OPPOSING Forces

1) chemical - K+ flows down the concentration gradient OUT of the cell

2) electrostatic - Negative anions can't leave the cell, so the net diffusion of K+ causes the interior to be electronegative. The negative pot. attracts K+ INTO the cell

@ Equilibrium \( E_k = -61.5 \log \left( \frac{[K^+]_i}{[K^+]_o} \right) \)

Theoretically \( E_{rest} = E_k \) but it is higher due to a small inward current of Na+

Sodium

\[ [Na^+] << [Na^+] \]

Low membrane permeability to Na+

2 COMPLIMENTARY Forces

1) chemical - Na+ wants to flow INTO cell

2) electrostatic - Na+ is pulled INTO cell by negative resting potential

ATP pump

Pumps Na+ out and K+ in against the concentration gradients

Uses ATPase as its power source

Figure 2.3 Ionic basis for resting potential [7]
there are also electrostatic forces that govern the motion of the ions. Negative ions cannot leave the cell because of their large size, and due to the net diffusion of K⁺ out of the cell, the interior of the cell becomes electronegative. This electrostatic force attracts K⁺ and Na⁺ back, though Na⁺ can't really pass through the membrane during rest. Equilibrium between the ATP pump, the electrostatic force and the concentration gradient maintain the resting intracellular and extracellular concentrations of K⁺.

A formula called the Nernst Equation relates membrane potential to the intracellular and extracellular concentrations of and ion. For example, the potassium equilibrium potential is [7,8,9]:

\[ E_K = -61.5 \log \left( \frac{[K^+]_i}{[K^+]_o} \right) \]

where \( E_K \) = potential created by the potassium ion
\([K^+]_i = \) intracellular concentration of potassium
\([K^+]_o = \) extracellular concentration of potassium

Theoretically \( E_{rest} = E_K \) but due to a small inward current of Na⁺ the resting potential is slightly less negative than \( E_K \).

It is interesting to note that the actual amount of K⁺ needed to leave the cell to polarize it to -90mV is actually quite minuscule. The following is a demonstration to illustrate this point.

**Given:**  
**Area Specific Capacitance:** \( C=1\mu F/cm^2 \)

**Example cell diameter = 20\mu m**

\([K^+]_i = 100mM\)

\([K^+]_o = 2.5mM\)

**Problem:** Determine the amount of K⁺ needed to leave the cell in order to create a resting potential of \( \approx -90mV \).

**Solution:**

\[
\text{Area} = 4\pi r^2 = 1.257E-9 \text{ m}^2 \\
\text{Vol} = \frac{4}{3}\pi r^3 = 4.188E-15 \text{ m}^3 \\
E_K = E_K = -61.5 \log \left( \frac{[K^+]_i}{[K^+]_o} \right) \\
= -98.52 \text{ mV}
\]
Total Cap. = (1μF/cm²)·(100cm/1m)²·1.257E-9m²
= 1.257E-11 F

Charge (Q) = C·V
= 1.238E-12 coulomb

Faraday's# = 96.487 coulombs/mole
Q / F# = 1.283E-17 moles K⁺ needed to leave.
Tot. Moles In = (.1M/L)·(1L/1000cm³)·(100cm/1m)³
= 100 moles inside

% K⁺ to leave = 1.283E-17 %

With the onset of excitation, the permeability characteristics of the membrane changes dramatically [6,7,9]. During phase 0 permeability of the membrane to Na⁺ increases dramatically. Both the electrostatic and chemical forces favor the influx of sodium into the cell. Berne and Levy explain the regulation of sodium by means of channels with 'm' and 'h' gates in the cell membrane (see Figure 2.4). At phase 0, m gates begin to open, increasing the permeability to Na⁺, allowing sodium to enter the cell, and in turn depolarizing it. As the membrane becomes more depolarized, more m gates open, allowing more sodium to enter. This regenerative process allows the membrane potential to depolarize to ≈20mv. The positive membrane potential wants to oppose more Na⁺ entering the cell, but as long as the sodium concentration gradient is greater than the electrostatic force, sodium will continue to enter the cell [7].

The h gates are governed by the membrane potential and at ≈20mv they begin to close, though at a rate that is about ten times slower than the m gates open. Phase 0 is totally terminated when all the h gates have closed. These h gates will remain closed, blocking any influx of sodium, until the cell has partially repolarized. Until the h gates reset, the cell is in its absolute refractory period, and it is physically impossible to induce another action potential. Once some the h gates have reset, the cell is in its relative refractory period, where another AP can be induced but requires a greater excitation voltage than was needed when the cell was at rest.

During the plateau (phase 2) of the AP, a flow of Ca²⁺ and a weaker flow of Na⁺ enters the cell. In addition, a concentration gradient of K⁺ that greatly exceeds the electrostatic force still exists, causing K⁺ to leave the cell as well. The combination of the influx of Ca²⁺ and Na⁺ and efflux of K⁺ balance each other out, creating the plateau.
At Rest

Na⁺

At rest, the total electro-chemical force favors and influx of Na⁺.
The [m] activation gates are closed while the [h] inactivation gates are open. Assume that the inward Na⁺ current is negligible.

* Any process that tends to make Vm less negative tends to open more [m] gates.

At Threshold (phase 0)

Na⁺

At threshold, the Vm has depolarized enough to partially open the [m] gates. Na⁺ enters and neutralizes some of the negative anions, which depolarizes Vm more and in turn opens more [m] gates, creating a REGENERATIVE PROCESS.

Although the Na⁺ entering the cell will change the Vm by more than 100mv, the actual amount is too small to change the intracellular [Na⁺] measurably, therefore the chemical force remains the same.

At phase 1

As Na⁺ enters the cell, the negative anions are neutralized, but the concentration gradient is still large enough to force more Na⁺ into the cell, making it positively charged. The positive charge will tend to oppose the influx of Na⁺, but as long as the gradient is > the electrostatic force, Na⁺ will continue to enter the cell.

As the cell becomes more positively charged, the [h] inactivation gates begin to close, curtailing the influx of sodium. The [h] gates are governed by Vm, but they close 10X slower than the [m] gates open.

The [h] gates remain closed until the cell has partially repolarized at phase 3. After a certain amount of repolarization has occurred, the [h] gates open again. Until the [h] gates begin to open, the cell is in its ABSOLUTE REFRACTORY PERIOD.

**DURING PLATEAU** A weak influx of Na⁺ and Ca⁺⁺ balance out an efflux pf K⁺ that flows down its concentration gradient.

**REPOLARIZATION** There is a slowdown in the influx of Ca⁺⁺ and Na⁺⁺, but there is a regenerative increase in the membrane permeability to K⁺. The net effect is a fast repolarization back to resting potential. During phase 3 the [m] gates and the [h] gates return to fully open, setting the cell up to be stimulated again.

**Figure 2.4 m and h gates during action potentials [7]**
During phase 3 the inward current of calcium and sodium slows down and another regenerative process occurs, this time with K\(^+\). The permeability of the membrane to K\(^+\) is dependent on the membrane potential, and it increases as the membrane potential decreases. This creates a regenerative repolarization of the membrane down to the resting potential where all the forces are in equilibrium at -90mv. During resting the ion pump is also eliminating any excess Na\(^+\) in the cell that entered during phases 0, 1 and 2.

2.1.3 Conduction in the Cardiac Fiber

Propagation of the impulse occurs because the large change in transmembrane potential at one location, in conjunction with its action potential's upstroke, causes a local longitudinal potential difference. This produces a local flow of current across the membrane in advance of the action-potential upstroke, resulting in excitation of the next cell of the fiber [6] (see Figure 2.5).

2.1.4 Fibrillation / Defibrillation

Fibrillation is defined as irregular contractions of the heart. Ventricular fibrillation is fatal in a matter of minutes, as it keeps the ventricles from pumping blood to the body, and hence the brain is left without oxygen. One theory as to the cause of fibrillation is that of reentry. A stimulus pulse travelling down the myocardium reaches an area where conduction is blocked, such as group of dead cells. A normal reaction to a blocked area would be for the stimulus to split off in two directions, go around the block, and meet again on the other side. Sometimes the block is a unidirectional block so that a pulse will go around it on one side of the block and then circle back up to the point before the pulse hit the block (see figure 2.6) [7]. In this situation a muscle cell will be stimulated by this returning wavefront, and if the cell is in its relative refractory period and enough h gates have reset, a new AP will be induced. The end result is one pulse after another which causes the muscle fiber to look like it is twitching. A heart in fibrillation has many of these reentry wavefronts throughout the myocardium causing the entire ventricle to twitch and flutter. When in this state, the heart cannot pump blood normally.

Strong electrical shocks on the order of hundreds of volts have been shown to
Depolarization of cell creates local currents at the border between the depolarized cell and an adjacent cell. The local currents tend to depolarize the region adjacent to the border, and once this adjacent region has been depolarized to threshold, it will undergo an AP.

**Figure 2.5  Conduction in cardiac fibers [7]**

The unidirectional block causes the action potential train to double back on itself and proceed to excite tissue that is in its relative refractory period, which can lead to fibrillation of the muscle.

**Figure 2.6  Re-entry in muscle tissue leading to fibrillation [7]**
extend the absolute refractory period so that the returning wavefront should hit the cell at a time when not enough h gates have reset to allow another AP to be induced [4].

2.2 Classical Methods of Recording Cardiac Electrical Activity

2.2.1 The Intracellular Microelectrode

The use of microelectrodes for recording transmembrane potentials dates back to Ling and Gerard in 1949 [10]. The principles of the intracellular recording have remained unchanged, though modern developments have made the method much easier and convenient.

The microelectrode is made from a glass tube whose end has been drawn into a fine-tipped capillary tube called a micropipette and is filled with an electrolyte solution (see Figure 2.7). The actual microelectrode is the junction between metal, usually silver, and electrolyte, usually KCl in the stem of the pipette or just outside [10]. The microelectrode is a salt-bridge between the intracellular fluid and electrical recording equipment.

The most important feature is its external diameter near the tip. It needs to be 0.5% or less of the diameter of the cell which it is to impale [10]. Thus a cell 100μm may be impaled, without gross damage to the cell, by a pipette of 0.5μm tip diameter, just large enough to resolve in a light microscope. Most cells are smaller and the pipettes used have tips that cannot be seen in a light microscope, though electron microscopes show the tip region sufficiently well.

Because a potential difference is being measured an indifferent electrode is necessary to provide a reference. For isolated cells the indifferent electrode is connected to Earth as in Figure 2.7, but for whole animal studies the indifferent electrode is usually a strip of silver buried in the musculature [10].

The microelectrode is kept in place by a micromanipulator. A high impedance preamplifier passes the voltage signal from the microelectrode to the oscilloscope. The preamplifier also serves to prevent any significant current from flowing through the microelectrode, and it must not draw any current from the microelectrode. If a current draw did take place a voltage drop would occur across the resistance of the microelectrode and the potential recorded would not be a faithful replica of the cell. 
Figure 2.7 (a) Parts of a micropipette. (b) Schematic of recording system [10]

Figure 2.8 Schematic of MAP electrode [11]
Other output devices include pen, tape recorders, DMM's and audio monitors that signal whether the cell has been impaled or not.

One of the problems with microelectrodes are is inherent fragility. A 0.5μm glass tip can break very easily just from inserting it into tissue too forcefully and the manufacturing of microelectrodes, though not difficult, can be time consuming and delicate work. The cell penetrated with a microelectrode is very susceptible to vibrations and precautions such as suspension tables for the apparatus must be implemented. The fragile nature of the pipette tip also restricts microelectrodes from being used in cardiac catheter techniques.

The stability of microelectrodes signal is not very good over time in comparison to MAP, EKG or optical recordings, and the signal is sensitive to nearby alternating magnetic fields. In addition, the preamplifier saturates during pacing and defibrillation shocks and true membrane potential cannot be recorded during the shocks [4]. A significant advantage over MAP and EKG recordings is the microelectrodes ability to record from a single myocardial cell and measures the true transmembrane potential.

### 2.2.2 The MAP Electrode

In 1966 a suction electrode catheter was introduced that allowed repolarization time course of the human myocardium to be studied [11]. Currently a non-suction contact electrode method yields long term MAP recordings from a single endocardial site for periods of up to 1 hour and more, and is used extensively in research of defibrillation waveforms.

The catheter has an outer diameter of 1.5 mm. It's distal end contains two sintered silver-silver chloride electrodes, one of 1 mm diameter forming its tip and one located in the catheter wall 3 mm proximal to the tip (See Figure 2.8). The catheter is usually held by a micromanipulator which presses the catheter gently upon the surface of the myocardium until the tip electrode and the surrounding catheter wall are in intimate contact with the tissue. [11]. As with a microelectrode, the MAP electrode requires a preamplifier to boost the signal before it sent to any variety of output devices and an indifferent electrode placed somewhere in contact with the tissue.

The advantages of the MAP electrode are its longterm stability over time and its ability to be used in catheterization techniques in the clinical setting. In addition, the
electrode itself is robust and not as fragile as microelectrodes and optical fiber are.

The MAP electrodes, like microelectrodes and electrocardiograms, are susceptible to outside electrical influence. Pacing and defibrillation shocks create electrical artifacts that can saturate the MAP amplifiers and distort the electrical signal. In addition, due to the large diameter of the electrode tip, the MAP electrode records electrical activity from many cells [11]. The signal is also very sensitive to the pressure placed on the electrode tip. During recording the pressure must be adjusted several times as it changes from the beating of the heart.

2.2.3 The Electrocardiogram

While microelectrodes, MAP electrodes and optical techniques are used to study the electrical activity of myocardium at specific sights, electrocardiograms are used to study the activity of the entire heart as a whole.

Depolarization and repolarization of the cardiac myocardium produce electric fields that reach the surface of the body where electrodes are located, usually in areas such as at the right arm, left arm, right leg, and left leg [6]. The ECG (or EKG) signal is divided into the P, Q, R, S, and T waves (see Figure 2.9). These complexes tell when atrial depolarization, ventricular depolarization and myocardium repolarization.

As with all the electrical recording techniques, ECGs are also susceptible to electronic shock artifact from pacing and defibrillation shocks. The ECG is important to cardiac researchers using optical methods because it is the only way of verifying whether a heart is in true fibrillation, versus ventricular tachycardia (very fast beating of the ventricles). MAP, microelectrodes, and optical methods only show action potentials from one sight or one cell, and ventricular fibrillation is almost impossible to distinguish from ventricular tachycardia by looking at one sight. The sight might be in an episode of reentry or tachycardia. Because the ECG looks at the heart as a whole, a heart in fibrillation will produce a very chaotic ECG because there are many episodes of reentry in a fibrillating heart.

2.3 Animal Preparation

Rabbits are anesthetized with injections of pentobarbital dosed to their particular
Figure 2.9  EKG Waveform with P, Q, R and S complexes  [9]
weight (30-50mg pentobarbital/kg) [21]. In addition, an anticoagulant is injected to keep blood from coagulating in heart passages. Reflex tests on the pupils and the feet are used to ensure that the rabbit is fully under the anesthesia before extraction begins.

Once extracted from the rabbit, the heart is cannulated to a Langendorf Perfusion setup through the aorta. Once cannulated a Modified Krebs's Solution is perfused through the heart to provide it with the necessary nutrients. The solution is made from the following items and concentrations: NaCl (110mM), KCL (4mM), NaH2PO4 (1.2mM), Dextrose (5.5mM), MgSO4.7H2O (1.2mM), Pyruvate (2.0mM) and Insulin (10 μ/L). The temperature of the heart must be maintained in a range from 36 to 38 degrees celsius.
3. Optical Setup and Experimental Procedure

3.1 General Principle

Nearly all optical recording techniques have the following in common: some molecular probe that is dependent on a variable and expresses its dependence optically, an excitation system for this probe, and a detection / signal processing system for the returning signal that displays the changes in the variable.

In this case the probe is a dye that is perfused into the heart along with the Krebs solution that feeds the myocardium its required nutrients. This dye binds itself to the membrane and is sensitive to changes in membrane voltage. We also tried a calcium sensitive dye that registers changes in internal calcium concentration and therefore must penetrate the membrane itself. Both of these dyes indicate their respective changes in the dependent variable by changing their emitted fluorescence when excited by some light source.

Laser light directed through an optical fiber inserted directly into the myocardium serves to excite the dye. The fluorescence returns through the same optical fiber and with the use of optical filters and beam splitters is directed into a detection circuit. The dye's reflected fluorescence will change in response to fluctuations in membrane voltage (or whatever the dye is sensitive to). The intensity of the fluorescence is transduced into an electrical signal by some light detection device. Finally this electrical signal undergoes processing before it advances to any number of display devices. The convention for assessing the amplitude of the optical signal is to look at the ratio of the change in fluorescence to a baseline fluorescence (delta-F/F %) See Figure 3.1 for a schematic of the optical system.

3.2 Excitation

The excitation light source consisted of a green (543nm wavelength) helium-neon laser (Siemens, Iselin, NJ; Model LGK-7770). The laser supply was powered by a 12V DC power source, the laser had the following specifications:
Figure 3.1 Schematic of Optical Setup
output power, min 0.5 mW  
beam diameter 0.71 mm  
beam divergence ≤ 1.2 mrad  
polarization random  
firing voltage ≤ 8kV  
operating current 6.5 mA

The laser light intensity was attenuated by a factor of 10 by a #1 neutral density (ND) filter (Schott, Duryea, PA; Model NG9100). The neutral density filter is used to limit dye bleaching, which will be discussed in more detail later, and because the photomultiplier tube detection system has such a high sensitivity that without the ND filter the signal would saturate.

The light then passed through a round short pass filter of 22mm diameter (Omega, Battleboro, VT; Model #555DRSP-9324) that is held in a rotating stage at ≈45 degrees. When placed at such an angle the filter acts as a dichroic beam splitter, allowing the excitation light (543 nm) to pass through the filter, while reflecting the longer wavelength returning fluorescence into the detection system. The alignment of the dichroic beamsplitter with the detection system demands the utmost attention. If the alignment is off some of the returning fluorescence would not enter the detection system and would be lost. The resulting optical signal would be much weaker than if all the fluorescence were directed into the detection unit.

The Omega filter has a cutoff wavelength of 555nm (See Figure 3.2 for a transmission spectra provided by Omega). The returning fluorescence has a wavelength of ≈620nm for Di-4-ANEPPS and ≈570nm +/- 10nm for Calcium Orange dye. According to Figure 3.2, 620nm corresponds to ≈99% transmission while 570nm corresponds to ≈87% transmission, therefore 99% of the returning fluorescence from Di-4-ANEPPS and 87% from Calcium Orange is directed into the detection system.

In early experiments a shortpass filter with a cutoff wavelength of 575nm was used (Oriel, Stratford, CT; Model SP575). This filter only allowed ≈80% of the returning fluorescence to be directed into the detection system. Changing this filter improved the results by increasing the amplitude of the returning signal.

After passing through the beam splitter the light was focussed to the endface of an optical fiber by a X20, 0.40 - numerical aperture microscope objective held in a fiber coupler (Newport, Fountain Valley, CA; Model F-1015).

The fiber was a multi-mode, 100μ core diameter (Newport; Model F-MLD) of
Figure 3.2 Spectrum of Short Pass Filter
approximately 6 feet in length. The fiber has a numerical aperture of 0.3, a cladding diameter of 140µm and a jacket diameter of 500µm (see Figure 3.3).

To prepare the fiber for cleaving, the protective jacket needs to be stripped off the tip. The fiber end was dipped in a small bottle of simple household furniture stripper for a few minutes. The part of the jacket that had been submerged in the stripper slid right off the fiber with gentle pressure from the fingertips. The fiber was cleaved by placing the exposed fiber under tension, scribing it with a diamond blade perpendicular to the axis and then pulling the fiber apart to produce a clean break. Newport manufactures a high quality fiber cleaver. A fiber cleaved properly will emit a circular far field radiation pattern with a gaussian intensity distribution. Any impurities in the cleave will produce a non-uniform distribution of light. Finally the tip of the fiber should be dipped in acetone and wiped with a soft tissue to ensure its cleanliness.

3.3 Detection

Before the light hits a detection system it is filtered to eliminate the green excitation light. A long pass 3mm thick orange glass filter with a cut-on wavelength of 570nm (Schott; Model OG570. See Figure 3.5 for a transmittance spectrum) was used with Di-4-ANEPPS which had a reflected fluorescence of 620nm. For experiments using Calcium Orange a 3mm thick bandpass filter with transmittance range of 569.7 +/- 8.8nm (see Figure 3.6 for a spectrum) was used (Oriel; Model #54385). Detection of the light intensity is accomplished by either a photomultiplier tube or a photodiode circuit.

3.3.1 The Photomultiplier Tube

The light detection system currently in use is a -1500 V photomultiplier tube (Hamamatsu, Bridgewater, NJ; Model R1333). This type of PMT is different from the photomultiplier tubes used in night scopes. The output of this PMT is an electrical current as opposed to an optical image. The input to the PMT is a hole with a 1 inch radius. Only light entering the hole will be transduced into an electrical signal in the form of current. All other light missing the hole is lost, therefore alignment is very important.

The radiant sensitivity as provided by Hamamatsu is shown in Figure 3.7. This
Figure 3.3 Schematic of Optical Fiber [Newport]

Figure 3.4 Molecular Structure of Di-4-ANEPPS [13]
Figure 3.5  Transmission Spectrum for OG570 Long Pass Filter  [SCHOTT]
Figure 3.6 Transmission Spectrum for 570 nm Band Pass Filter
type of detection system is extremely sensitive to light and has the ability to detect as little as one single photon. In order to limit the room light reaching the PMT and also reduce air currents which might affect the coupling of the laser beam into the fiber, most of the optical setup is contained in a light tight box [1]. In fact, if the PMT were switched on with the lid of the box up it would destroy itself in a blue flash.

The photomultiplier tube converts photons to current and basically acts as a current source. A 10k precision load resistor is used to convert the current signal into a voltage signal. The PMT is currently calibrated at 0.22 amps/microwatts.

The advantages of a photomultiplier tube are its high sensitivity and its large input size compared to a photodiode. High sensitivity is also a disadvantage of the PMT because of the need to maintain a light free environment for it. The cost of the PMT is very prohibitive compared to a photodiode.

3.3.2 Photodiode

A simple photodiode circuit can also be utilized to record the intensity of the returning fluorescence [16]. In addition a photodiode is also used to calibrate the system for an experiment. The light detection circuit uses a photodiode (Hamamatsu; Model S2386-45K) hooked into an LF356N operational amplifier set up as a current-voltage converter (see Figure 3.8). A five position switch allowed for a variable feedback resistances to change the amount of gain. The photodiode had a radiant sensitivity of 0.3 (A/W), an inherent noise (dark noise) of 9.063mV when the feedback resistor was 0.1 M ohms, and a photosensitive surface of 17.9 mm². In addition, the diode was calibrated at 100 milliwatts/volt, and the circuit itself had a saturation voltage of 13.38 volts.

The advantages of the photodiode are its simplicity and cost effectiveness. Because the circuit is powered by an operational amplifier it can only reach a certain saturation voltage if the light intensity is too high and cannot destroy itself from like the PMT. A disadvantage is the small size of the photosensitive surface. An optical microscope objective must be used to focus the returning fluorescence onto the detecting surface of the photodiode. This is easily accomplished if the light is in a nice, tight configuration such as that emitted from a laser. Unfortunately the returning fluorescence reflected off the dichroic beam splitter is not as concentrated as laser light. It is distributed over an area the size of a circle with a radius of approximately 1/2". This can
Figure 3.7 Radiant Sensitivity of Photomultiplier Tube [Hamamatsu]
Figure 3.8 (a) Photo Diode Detection Circuit [16]  (b) Schematic of PD [Hamamatsu]
be overcome with an additional focussing lens. Noise can also be a large disadvantage with photodiodes.

3.4 Dyes

The voltage sensitive styryl dye Di-4-ANEPPS (Molecular Probes, Eugene, OR) is used to study the electrical activity of the myocardium, while Calcium Orange (Molecular Probes) is used to study the internal calcium concentration.

3.4.1 Di-4-ANEPPS

The ability of the dyes to change with electricity is called electrochromism. According the Loew, since the coupling of an electric field to the electronic states of a chromophore (a molecular building block of the dyes) is instantaneous on the timescale of ion currents or even intramolecular charge separations, electrochromism should faithfully follow the kinetics of a potential change associated with any physiological event [12]. The fast response of styryl dyes is caused by a direct potential-sensitive change in the electronic distribution within the dye, rather than by a redistribution of the probe [13]. The styryl dyes are designed to localize in the outer leaflet and are essentially nonfluorescent unless bound to tissue [13]. Dr. Krauthamer showed that the dye is 10 times as fluorescent when bound to tissue versus unbound [3]. In addition, the photoexcitation-induced charge displacement must be perpendicular to the membrane surface for a maximum electrochromic effect [12]. Previous experiments showed that the fiber tip needs to be very close to the excitable tissue to detect a signal (within 100 microns).

If a typical myocardial cell membrane is 100Å thick and the magnitude of its action potential is 100mv then an electrical field of 10,000 volts/cm exists across the membrane during the action potential. When the molecules (See Figure 3.4 for molecular structure [13]) of Di-4-ANEPPS insert themselves in the myocardial cell membrane and sit inside such a large field they undergo the aforementioned conformational change, the details of which are beyond the scope of the this discussion. This transformation in structure causes the reflected fluorescence of the dye to change. The intensity of the returning fluorescence of Di-4-ANEPPS decreases with increased membrane voltage. According to Haugland, Di-4-ANEPPS has a fairly uniform 10% change in fluorescence
intensity per 100mV, however our results were from 2 to 5 times less.

Dr. Krauthamer performed some spectrum analysis on Di-4-ANEPPS at the Food and Drug Administration's Center for Devices and Radiological Health in Rockville, MD. Figure 3.9 shows the excitation spectrum with the X axis being excitation wavelength and the Y axis representing the intensity of the 620nm fluorescence. The current excitation of 543nm produces and intensity of more than three and a half times less that of the maximum intensity which occurs when excited with a wavelength of about 480nm. The actual intensity of the returning fluorescence isn't as important as the change in intensity during an action potential, so having 3.5 fold increase in intensity for fluorescence is not of paramount importance except for increasing the signal to noise ratio. Figures 3.10, 3.11, and 3.12 show an emission spectrum for Di-4-ANEPPS when excited at 488nm, 514nm and 543nm respectively.

3.4.2 Calcium Orange

The probe used to attempt measurements of internal calcium ion concentrations is Calcium Orange (Molecular Probes). The emission spectrum of Calcium Orange is $\approx 570\text{nm } +/-\text{ }10\text{nm}$ (See Figure 3.13 [13]).

Unlike Di-4-ANEPPS, which only has to bind to the membrane, Calcium Orange must bind to calcium itself. Therefore, in order for the dye to register changes in internal calcium ion concentrations it must somehow pass through the cell membrane [13]. This characteristic might explain why our experiments with the rabbit heart using Calcium Orange failed to show the changes in internal calcium concentration during normal heart operation. In dealing with cells in a Petri dish it is much easier to soak the dye with cells for a period of time (20 minutes), in such a way that it can pass through the interior of the cell membrane, whereas perfusing the dye into the heart as with Krebs solution makes it much harder for the dye to work its way through the membrane to the interior of the cell.

3.4.3 Dye Bleaching

Dye bleaching occurs when dye is subjected to intense illumination and results in a loss of fluorescence and hence a loss in change in fluorescence resulting from an action potential. In experiments it was found that after twenty to thirty minutes of recording
Figure 3.9 Excitation Spectrum of Di-4-AEPPS
Figure 3.10 Emission Spectrum of Di-4-ANEPPS when excited by 488 nm
Figure 3.12 Emission Spectrum of Di-4-ANEPPS when excited by 543nm
Figure 3.13 Emission Spectrum of Calcium Orange [13]
from one site the amplitude of the signal would begin to steadily decrease. Dye bleaching needs to be distinguished from dye elusion: bleaching occurs at one spot, while elusion occurs over the entire heart. After adding more dye to the heart the signal returned to its original amplitude within 30-45 seconds. To minimize the bleaching of the dye the #1 neutral density filter was added into the optical setup. It was found that decreasing the intensity of excitation light did not decrease the change in fluorescence generated in the dye by an action potential at the cell. In addition, the laser has a shutter that can be controlled manually or electronically which allows the laser light to be blocked from the dye without removing the fiber from the tissue. This allows the user to maintain a "good spot" without unnecessarily bleaching the dye.

3.5 Signal Processing and Data Storage

A variety of passive filters are utilized to reduce noise and smooth the final signal. After termination to ground with the 10k resistor, the signal is first sent into a unity gain operational amplifier buffer to limit the output voltage to +/- 15 volts in order to protect other equipment from being damaged by a high voltage spike if the photomultiplier tube is accidently exposed to intense light.

The output of the buffer is then sent to the first of two variable gain amplifiers (A1) to allow signal boosting on various levels (see Figure 3.14 for a schematic). This boosted signal then progresses to a digital multimeter where the baseline fluorescence is read. A sufficient amplitude indicates if the end of the fiber is placed in a region containing stained tissue. From the DMM the signal passes through a passive RC high pass filter (t=10s) (see Figure 3.15(a)) to remove the DC component (the baseline fluorescence) to allow the emphasis of analysis to be placed on the change in fluorescence and permit high gain amplification.

The signal then proceeds into a second variable gain stage (A2) and then into a variable RC low pass filter (f=53 Hz typ.) to remove high frequency components of noise (see Figure 3.15(b) for a schematic). Finally, the signal advances through one last active buffer stage before entering the recording equipment. This final buffer stage acts to sink currents from the input leads of the A/D board.

An oscilloscope equipped with an image freezing phosphorous screen and polaroid camera, and a strip chart recorder are all utilized to record the signal. In addition
Figure 3.14  Variable Gain Amplifier
Figure 3.15 (a) High Pass Filter (t=10s)
(b) Variable Low Pass Filter (fx=53hz typ.)
to the more conventional recording an A/D board connected to an Apple computer is used in conjunction with a Virtual Instrumentation software package called Lab View. Kevin Milne, of the Cardiac Research Lab programmed a number of recording and viewing programs to allow the analysis of two signals at a time. These programs make it very easy to compare the optical signal to the MAP signal or an EKG signal. All episodes are saved on an high density CD disk for permanent storage.

3.5.1 Convention for Optical Waveforms

As mentioned previously the amplitude of the optical signal is not as important as the ratio of the change in fluorescence to the baseline fluorescence (delta-F/F). Because the photomultiplier tube is powered by negative 1500 volts the intensities of the fluorescence are measured in negative voltage. Di-4-ANEPPS decreases its reflected fluorescence with increased membrane voltage, therefore an action potential produces a positive (less negative) change in the intensity and in turn creates a signal that resembles an actual action potential. If a photodiode were used, the signal would appear as an inverted action potential because the decrease in fluorescence would translate in a decrease in intensity voltage and hence an inverted signal.

One must remember that the signal had been amplified by A1 and A2. Because both the baseline F and the delta-F were affected by A1 it reduces out of the calculation of percentage change in delta F (%delta-F), but A2 amplifies just delta-F. Therefore, the correct formula for %delta-F is (delta-F/A2) / F, where F is read off the DMM (see Figure 3.16).

3.5.2 Electrical Noise in the Optical Signal

The incorporation of the A/D board and the virtual instrumentation software adds about 20mv of noise to the optical signal. Intensive investigation revealed that the A/D board generates 20mv of noise in its circuitry which is added to an incoming signal. Due to the relatively small amplitude of an unmodified optical signal, around 30mv, the noise is very disturbing to the integrity of the signal. This noise is also included in the MAP signal that is sent into the A/D board but is not noticeable due to the larger amplitude of the MAP signal compared to the optical signal.
To determine the percentage change in delta-F, divide the amplitude of the optical signal by the gain of the second gain stage (A2) and divide by the absolute value of the reading on the DMM (Baseline F).

Figure 3.16 Convention for reading optical signals.
Because the noise is generated at the A/D board, any modification to the signal before reaching the board will not change the amount of noise to the signal. By placing the second gain stage after the DC component of the fluorescence is filtered out by the high pass filter, we are able to boost the amplitude of the optical signal, to use the full range of the A/D without increasing the noise, therefore increasing the signal to noise ratio.

The noise arose from the input of the A/D board or across the resistor in the high-pass filter; adding a low output impedance amplifier after the high-pass filter served the dual purpose of sinking the current from the A/D board and increasing the signal.

In experimentation with large amplitude defibrillation shocks, a problem of crosstalk cropped up between the MAP trace and the optical trace. During incursion of the shock, the MAP signal would show a voltage spike equal to the saturation voltage of its preamplifier (about 12-14 volts), but this spike would also show up on the optical trace as well. This spike is counter to the whole motivation of the optical method in its ability to withstand electrical shock artifact and show only the membrane potential. In fact, it is physically impossible for the optical method to record such a large voltage because there is no way that enough ions could flow in and out of a cell to generate such a large amplitude signal. Therefore there had to be some form of cross communication between the MAP and the optical signal at the A/D board, because that was the only place where the paths of the two signals met. After considerable effort, the problem turned out to be an impedance mismatching between the output of the passive low-pass filter and the A/D board. By adding an active operational amplifier buffer amp, a suitable output resistance of the signal processing circuitry was matched to the input resistance of the A/D board.

3.5.3 Optical Noise

Because the system works with light, any additions to the excitation light other than the laser cause a distorted signal to be recorded. The overhead lights in the lab were 60 Hz fluorescent bulbs. During experimentation, the room lights would add a 120 Hz component of noise to the signal (see Results section, Figure 4.2). In order to get the best quality signal, the room lights had to be turned off during experimentation.

The distortion in the optical signal caused by motion of the fiber tip due to muscle contraction (motion artifact) will be discussed in the results section.
3.6 Experimental Procedure

3.6.1 Calibration / Testing of the System

Before an experiment can begin the optical system must be calibrated and tested to ensure proper operation. The calibration procedure determines what the intensity of the excitation light is, and lets the experimenter verify that the fiber is allowing a suitable amount of light to pass through it. In addition, the detection capabilities of the system are tested before experiments with a heart begin.

The fiber tip must have clean cleaves on both ends, and freedom from impurities. The tip should be cleaned in acetone, and if the laser light is not emitted in a Gaussian pattern the fiber tip should be recleaved.

A photodiode circuit calibrated to 100 milliwatts of light/volt is used to measure the excitation light intensity. The fiber is placed in a fiber chuck and focused using a microscope objective on the photosensitive area of the photodiode. A reading of between 3 and 5 volts with the neutral density filter removed is sufficient.

To ensure proper alignment of the dichroic beamsplitter with the PMT, a red laser pointer is used to simulate a very high intensity returning fluorescence. The pointer is directed into the fiber tip, and a white piece of paper is placed between the long pass filter and the entrance of the photomultiplier tube to show where the red light is directed by the beam splitter. As much of the returning light as possible should be directed into the photomultiplier tube.

Fluorescent dots made from melted fluorescent crayons simulate stained tissue in order to test the detection capabilities of the system before an experiment. The fiber chuck is placed in a micromanipulator such that the fiber tip is almost touching a dot. Moving the manipulator up should decrease the magnitude of the baseline fluorescence reading on the digital multimeter. If the manipulator is moved up and down fast enough a waveform can be seen on the oscilloscope. Another interesting method of verifying the detection ability is to direct the fiber tip onto the screen of the oscilloscope. As the optical signal passes underneath the fiber tip, the returning light generated by the signal on the screen will register in the photomultiplier tube and in turn, the trace on the scope will show a small dip representing the sudden increase in fluorescence seen by the fiber tip.
The actual detection capability of the system is very robust and simple; malfunctions are very rare. Almost all failures to find a signal are due to ineffective binding of the dye to excitable membrane rather than malfunctions in the detection system.

3.6.2 Experiment Protocol

Once the optical system is calibrated and the heart hung on the Langendorf perfusion system, an experiment can proceed. After the heart's vital signs stabilize and the temperature and oxygen content of the nutrient solution are normal, emergency defibrillation patches need to be attached to the heart. A heart can spontaneously go into fibrillation at any time and the patches along with the necessary software on the computer must be ready at all times to issue a defibrillation rescue shock. A MAP electrode can also serve as a defibrillation patch when the need arises. Before optical recording proceeds a stable MAP signal was necessary for comparing to the optical signal.

Almost all optical experiments use DAM in order to get an action potential signal free from motion artifacts. DAM is mixed in concentrations of 10mM, 15mM and 20mM depending on what the experiment is, but usually 20mM was most effective in restricting motion. The DAM mixes in Krebs solution at a ratio of 4g/2L for a 20mM concentration. Once dissolved the jug of DAM/Krebs solution was put in place of the normal Krebs solution. The DAM takes effect rapidly and wash in times were on the order of 8-12 minutes. It is really interesting to see the heart almost rock solid then look at the oscilloscope and still see action potential trains. Usually the atria still beat slightly but the ventricles show no signs of motion. When DAM is used the heart tends to fill with solution and becomes adenomas. To keep the heart from getting too bulbous a small tube was placed in the mitral valve to vent some of the solution and a large gauge syringe connected to a long tube was inserted through the apex of the heart into the left ventricle chamber (see Figure 3.17 for a figure of the heart). A siphon is started through this tube to help remove fluid from the chamber.

Di-4-ANEPPS is mixed to a concentration of 52µM by combining 200µL of dye dissolved in DMSO with 20ml of Krebs solution. The dye is placed in a syringe without a needle tip added to the perfusion at an input port just before the solution enters the aorta. It is crucial to make sure that no air bubbles enter with the dye, for it could make
the heart ischemic and eventually kill it. Within seconds the heart begins turning slightly orange in color. Dye can be added in higher concentration to any part of the heart through a needle and syringe if desired.

First a region of high fluorescence needs to be found. Simply inserting the fiber tip into the myocardium is all that is necessary. Care must be maintained when inserting the fiber, as the exposed tip can break off if it catches surface tissue upon insertion. During normal beating the fiber tip often shakes loose from the myocardium, but under influence of the DAM the tip remains quite secure in the tissue. The digital multimeter displayed the baseline fluorescence. With A1 set to 30 a baseline fluorescence magnitude of 3 volts or greater indicated a respectable sight. In cases where F was appreciably less than 3 volts signals, if any, generated very small changes in fluorescence. With so little dye in the area there is even less that has bound to the membrane to be able to generate a discernable signal.

The presence of a sizeable baseline F is not enough to ensure the establishment of an action potential signal. As mentioned earlier, the dye can produce a high fluorescence when bound to tissue, but the tissue doesn't necessarily have to be excitable. In order to generate a signal corresponding to an action potential, the dye at the excitation sight must be bound to the membrane of excitable tissue such that its molecular structure will transform with changing membrane voltage. This requires upwards of 15 attempts to find a suitable recording sight.

One hypothesis as to the difficulty of finding a signal is that the fiber is exciting non-excitable tissue, such as connective tissue, small blood vessels, endothelial tissue, or damaged muscle tissue, most of the time. In addition, the tip of the fiber needs to be very close to the excitable cardiac muscle, within 100 microns, to detect a signal. Once found, a site can continue to provide stable action potentials for hours regardless of what type of electrical stress (i.e. pacing pulses, fibrillation induction, or defibrillation shocks) is placed on the heart. Before storing the waveform the room lights are turned off to remove the 120hz noise element caused by the overhead fluorescent bulbs. It should be noted that once the lights are switched off one had to wait at least ten seconds for the signal to stabilize due to the long time constant of the high pass filter. The amplitude of the signal will decrease with increasing dye bleaching and is easily returned to normal amplitude by perfusing more dye into the heart.

Throughout the entire experiment the condition of the heart requires careful
Figure 3.17  Diagram of the Heart
monitoring. Slight fluctuations in temperature or oxygen content can kill a heart in minutes. One person has to watch the heart at all times to issue a defibrillation shock in the event that the heart spontaneously enters fibrillation. Many other complications can arise at any moment without warning. The delicacy of the rabbit heart sometimes makes research with the optical method very difficult.

3.6.3 Experiments to Detect Effect of DAM

In the proposed abstract to the American College of Cardiology in the effect of DAM on the electrophysiology of the heart, a protocol was designed to study the effect of DAM on fibrillation cycle lengths, and DAM’s effect on the almost linear relationship between pacing interval and action potential duration.

A heart is extracted and hung on the standard Langendorf perfusion setup, using normal Krebs solution. The normal sinus rhythm (NSR) of the heart is recorded using a MAP contact electrode and stored and measured with LABVIEW software. The heart is then paced at 50ms less than NSR for at least 10 pacing pulses and again stored in the computer. This pacing interval is decremented by 50ms each time until the heart reaches its minimum pacing interval. The procedure is repeated once more starting from NSR again. Once the second round of pacing is completed, three instances of fibrillation are induced and recorded. The episodes of fibrillation are verified through an EKG strip chart recording to distinguish them from ventricular tachycardia.

The Krebs solution is replaced with 20mM solution of DAM and once the beating of the heart stops, the entire pacing and fibrillation procedures are repeated. The perfusate is then changed back to Krebs and the DAM is washed out of the heart. Once normal beating resumes, the entire protocol is repeated again.

Action potential durations corresponding to their pacing intervals and fibrillation cycle lengths for the cases of NO DAM (normal Krebs solution as perfusate), DAM (Krebs solution mixed with 20mM DAM), and WASHOUT (return to normal Krebs perfusate) are measured with the computer. The differences in the relationship of APD to pacing interval and the changes in fibrillation cycle length among the different cases indicate how DAM affects the electrophysiology of the heart.
4 Results and Discussion

4.1 Optical Signals Without DAM

Figures 4.1 and 4.2 illustrate signals generated in a rabbit heart perfused with only normal Krebs solution. The top trace is from a MAP electrode located in the epicardium of the middle, left ventricle, while the bottom trace is from the optical fiber located in a site near the MAP electrode. Figure 4.1 had a F with a magnitude of 2.7 volts and an delta F of 400mv with the rooms lights off. It was generated before the second gain stage was added, therefore the % change in F was .4/2.7, or 14.8%. Figure 4.2 represents the same location of the heart as 4.1, but the room lights were on and the gain of the oscilloscope was turned up. Notice the 60hz component of noise in the optical signal.

The excitation voltage was calibrated at 4.5 volts without the #1 neutral density filter. At 100 milliwatts/volt, the excitation power with the #1 filter in place was 450 milliwatts. The power of the returning fluorescence =

\[(\text{delta-F} / \text{A1} / 10\text{kohms}) \times \text{(sensitivity of PMT)}\].

The reflected fluorescence generated a delta-F of 0.4 volts with A1=30, which corresponds to a power of 6.06 E-12 watts.

4.1.1 Motion Related Signals

Signals obtained from a beating heart contained motion related artifacts rather than signals created by the change in membrane potential accompanying a cardiac action potential. The motion artifact must be related to the dye because none appear in unstained tissue [1,2]. The initial upstroke of the of the optical signal in Figures 4.1 and 4.2 could be caused by the depolarization of the membrane and the fact that it occurs a few milliseconds after the upstroke of the MAP signal could be because the fiber tip was located in tissue that is excited after where the MAP is located. But a %delta-F of 14.8% is almost 5 times the largest ever recorded signal generated with a DAM perfused heart where the motion was stopped, which generates some doubts as to its actual cause. In addition, the slope of the optical upstroke is not as high as in the MAP signal. The depolarization is a very fast, regenerative process and shouldn't have a slope of anything less than vertical.
In Figure 4.3, the succession of cardiac conduction of the different areas of the heart is shown [6]. If one considers the optical and MAP signals in Figures 4.1, 4.2 to correspond to the ventricular muscle in 4.3, the first upstroke of the optical signal occurs almost exactly at the point in time when the atrial muscle has reached the end of its action potential. This time relation matches the time relation of muscle contraction to excitation, as in Figure 2.2. This supports the hypothesis that the first upstroke in the optical signal is caused by motion generated during contraction of the atria.

The second upstroke of the optical signal occurs after the MAP signal has completely repolarized and hence corresponds to a contraction of the ventricle. As explained earlier an upstroke in the optical signal coincides with a decrease in fluorescence at the fiber tip. This decrease in F is possibly created when the contraction causes the fiber tip to move and illuminate an area with less dye or to an area without excitable tissue.

A second and more probable cause to the decrease in F is the bending of the fiber that occurs with large contractions of the rabbit heart. By bending the fiber by hand as it would bend in a beating heart a 10-15% decrease in fluorescence usually results. Conventional ray optics theory and mode theory explain the attenuation losses of fiber bending [17]. In previous experiments with frog hearts motion related signals generated smaller percentage change in F because the beating was not vigorous enough to bend the fiber [1,2].

Almost all motion related signals were in the direction of decreased fluorescence, most likely caused by fiber bending. In previous work most of the motion artifacts were in the direction of increased fluorescence [1,2]. Two major reason cited were 1) the muscle contraction moves the surface closer to the fiber causing a large increase in the amount of fluorescence collected, and 2) lateral displacement of the muscle brings 'fresh' (unbleached) dye into the narrow field-of view of the fiber causing a temporary increase in the amount of fluorescence [1,2]. It is unknown why these two phenomena do not occur more regularly in the rabbit heart. More experiments are needed to determine exactly what causes these movement-related signals.

The signal artifacts caused by motion are the single most debilitating problem with the optical method. The most common circumvention of the contraction related signals is using a pharmaceutical agent to decouple contraction of a muscle from its excitation and in effect stop the beating of the heart without halting the train of action.
Figure 4.1 Optical Signal with Motion Artifact (No DAM)
Figure 4.2 Optical Signal with Motion Artifact (No DAM)
potentials. Several other methods of overcoming the motion predicament are being investigated as well and will be described in more detail in the conclusion.

4.2 With DAM

Diacetyl Monoxime is described as a negative inotropic agent that works at the myofibril level of the muscle to decouple the excitation from the contraction of muscle tissue [14, 20]. A similar agent also used in optical methods is D-600 (verapamil) [4].

The DAM acts to stop the beating of the heart within a few minutes of its introduction to the heart. An useful property of DAM is that it can be washed out of the heart by replacing the DAM perfusate with normal Krebs solution. With in 10 minutes the heart resumes normal beating.

Figure 4.4 shows a heart with 10mM of DAM solution as perfusate. At 10mM the ventricles continue to beat slightly while the atria beat a little less then normal. The optical signal still does not match the MAP action potential very well. It lacks the characteristic sharp upstroke and looks rather like a motion artifact than an action potential. The delta-F/F is around 3.5% which is much less than other motion signals seen without any DAM.

Figure 4.5 depicts a DAM concentration of 15mM. At 15mM almost all beating has ceased. Only the atria continue to move minutely. This optical signal has a F of 5.1 volts and a delta-F/F of 0.8% Most signals have a delta-F/F of 1-3% but there where some isolated incidences of a 5% change for a normal action potential. It has all the characteristics of a normal action potential with a sharp upstroke and a slight plateau region and a more gradual repolarization. The setup at that time lacked the second gain stage and therefore had a higher ratio of signal to noise.

DAM concentrations of 15mM or greater proved strong enough to deter any motion artifacts and all future experiments used 20mM DAM to restrict motion related signals. Knisely et. al also determined that 20mM was sufficient to limit the motion enough to remove all motion artifact [20].

4.2.1 Optical Signals During High Voltage Shocks

As mentioned earlier, one of the greatest advantages of the optical method over
conventional recording techniques is its ability to continue recording action potentials without interruption during high voltage shocks. Figure 4.6 was taken from a strip chart recording from a MAP electrode (top) and an optical probe (bottom). This heart was being paced at a certain time interval by pacing shocks of around 5 volts. The electrical artifact from the pacing shocks can be seen as spikes on the MAP waveform. After 8 shocks the pacing machine was instructed to deliver a 20msec monophasic, 25 Voh shock in the middle of the repolarization of the action potential. This shock, known as an S2 shock is clearly seen on the MAP recording. It is absolutely impossible to tell from the MAP waveform what happens to the action potential, because the electrical shock artifact dominates the signal.

The optical trace shows no spike during pacing and at the occurrence of the S2 shock there is no electrical artifact. If one looks closely at the AP during which the S2 occurs, it can be seen that the action potential duration was extended a few milliseconds by the S2 shock. There is positively no way that the MAP recording could have determined this.

The phenomena of lengthening action potential durations from large shocks has been a hypothesis for why large amplitude shocks stop a heart from fibrillating, and is an important topic being researched by cardiac research labs across the country using optical methods, such as Steve Dillon’s paper on prolonging the duration of depolarization and the refractory period through defibrillation strength shocks [4]. The basic hypothesis is that large shocks extend the absolute refractory period of the action potential, making it impossible for returning reentry wave fronts to induce another action potential. The ability to see exactly what occurs during large shocks would be impossible without the optical technique.

The optical signal in Figure 4.6 was generated after the second gain stage was added. The signal is on the order of volts versus earlier traces that were on the order of millivolts. Much of the low amplitude noise has been reduced by increasing the signal amplitude without increasing the amplitude of the noise.

Figures 4.7 and 4.8 show MAP and optical traces where a large shock was introduced. In Figure 4.7 the electrical shock saturates the MAP amplifier and affects the offset of the signal. It took a few seconds for the MAP signal to reset itself to normal. The optical trace showed that the shock had no actual affect on the action potential.

Figure 4.8 was taken from the front panel of a LABVIEW program written by
Figure 4.4 Optical Signal using 10mM DAM
Figure 4.5  Optical Signal using 15mM DAM
Figure 4.7  Optical Signal with High Voltage Shocks
Kevin Milne of the V.A. Hospital Cardiac Research Department. The shock was a 25 volt 20millisecond bi-phasic shock that completely saturated the MAP signal and caused it to cease functioning for 500msec and changed the offset of the signal. The optical signal shows that the shock induced another action potential before the first had fully repolarized and put the heart into fibrillation.

In studying fibrillation and defibrillation and how electrical shocks of varying amplitude and geometries affect them, the optical method has proven invaluable to researchers. In conjunction with MAP and EKG data, the intricate workings of the cardiac tissue in large electric fields can now be analyzed in much more depth.

4.2.2 Endocardial Versus Epicardial Recordings

All of the optical recordings previously shown are from the epicardium (the outside of the heart) of intact hearts. Other optical systems are too large to penetrate the heart tissue. Using the single fiber optical method to record action potentials from the endocardium (the muscle on the inside of the heart chambers) had never before been published. Our endocardial recordings are the first time, known to us, anyone has used an optical fiber to access the electrical activity of the endocardium of an intact heart.

Figures 4.9 - 4.11 depict a MAP signal taken from the epicardium of the middle left ventricle and an optical signal generated from the endocardium of the left ventricle. The optical fiber was inserted through the pulmonary artery into the endocardial tissue of the right ventricle. Inserting the fiber through the artery proved rather difficult because the tip of the fiber kept snagging itself on tissue around the artery.

The heart is in normal sinus rhythm in Figure 4.9, while 4.10 shows the heart after inducing fibrillation with a mild electric shock, and finally 4.11 shows it after a defibrillation shock has restored normal sinus rhythm. In 4.9 and 4.11 notice how the optical signal and the MAP signal are synchronized, but during fibrillation they are not. This adds support to a hypothesis that fibrillation is made up of many separate wavefronts involved in the phenomenon of reentry. Because the optical and MAP traces are at different locations they are recording separate reentry wave fronts, but during normal rhythm the action potentials at these locations are synchronized.

Being able to record from two different sights at the same time is invaluable in studying the conduction patterns of the heart, and now the ability to record optically from
Figure 4.9 Optical Signal from Endocardium in Normal Rhythm
Figure 4.10 Optical Signal from Endocardium in Fibrillation
Figure 4.11 Optical Signal from Endocardium in Normal Rhythm
inside the heart has added to the advantages of multiple sight recordings.

4.3 Experiments to Detect Effect of DAM

The preliminary results of the proposed abstract for the American College of Cardiology showed, through the use of a t-test of regression lines and a t-test of significance, that the DAM does not significantly affect the relationship between action potential duration and pacing interval, but does produce a significant change in fibrillation cycle length between without DAM, with 20mM DAM and after washout. The fibrillation cycle lengths increased by an average of ≈20% between control and DAM, while the washout fibrillation cycle lengths returned to approximately control lengths.

The slowing of the fibrillation cycle lengths has not been proven to be a direct effect of the DAM as of yet. When under influence of the DAM the heart becomes quite bulbous and swollen, because the lack of beating allow perfusate to collect in the chambers of the heart. Venting through the mitral valve and through the apex of the heart have not proven effective in completely decreasing the swelling to acceptable levels. The slowing of the cycle length could be as a result of the swelling, which is indirectly caused by the use of DAM.

A change in the electrophysiology of the heart due to DAM might affect data collected using DAM in conjunction with the optical method. If the change in fibrillation cycle lengths proves to be unacceptable for cardiac physiology, DAM and other agents like it would not be considered acceptable in research.

The possible conflicts that DAM creates puts much more emphasis on finding a solution to the problem of motion artifact. True, the longterm goal of the optical method is to make it feasible to implant the fiber in a human, and stopping the motion of the heart to allow the optical method to work would be ludicrous, but for the time being a decoupling agent seemed a perfect solution to allow the research to continue. Some ideas for solving the movement related signals will be presented in the conclusion.

4.4 Results of Calcium Orange

An experiment using the dye Calcium Orange to record the intracellular change of
calcium during an action potential failed to produce any optical signal. 5 ml of a concentration of 5μM were perfused in the heart without producing even a baseline fluorescence. After local injection with a syringe a baseline fluorescence of 3.9 volts was detected, but again without any delta-F generated.

The Calcium Orange dye must enter the membrane and bind to calcium ions inside the muscle cell, as opposed to Di-4-ANEPPS which need only bind to the outside of the membrane. The difficulty of the dye to pass through the membrane would explain why no signal could be produced.

Dr. Krauthamer, of the F.D.A., has had good results using other similar calcium dyes with cultured chick-embryo heart cells in petri dishes in conjunction with a charge-coupled device (CCD) camera to detect the changes in dye fluorescence. The dye seems to have a more difficult time in passing through the membrane of the cardiac tissue of the rabbit heart than in the cultured cells. Dr. Krauthamer lets the dye incubate with the cultured cells for 20 minutes, which allows direct access of the dye molecules to the cultured cells. If the dye were allowed to perfuse for 20 minutes the dye might have been able to pass into the membrane. Another problem may have been the DAM, which works on the basis of blocking some calcium channels in the muscle tissue. Even if the cells were loaded with Calcium-Orange the signal might have been very weak, because many of the Ca channels were blocked. A protocol designed to maximize the passage of the dye through the membrane is being worked on.
5. Conclusion

5.1 Summary

Utilizing voltage sensitive molecular probes in conjunction with fiber optic technology, has succeeded in recording action potentials from a live rabbit heart under the influence of diacetyl-monoxime. The optical signals are impervious to electric shock artifacts that accompany large electric fields created by fibrillation induction shocks, defibrillation shocks and pacing shocks. Unlike conventional electrical methods, such as EKG and MAP electrodes, which are unable to record during the shock and for a little time afterwards, the optical method records the membrane potential of the cardiac muscles through the entire shock episode.

Normal action potentials in the rabbit myocardium create a 1-5% change in reflected fluorescence with excitation power on the order of 450µW and returning fluorescence on the order 6E-13 watts.

The method is providing physiologists a unique insight into the reaction of the myocardium to defibrillation strength shocks of varying amplitudes and geometries that conventional recording methods could never give. The major disadvantage of the optical method lies in the strong motion artifacts that accompany a vigorously beating heart. The DAM agent used to decouple the excitation from the contraction of the muscle has been shown to slow the fibrillation cycle length of the heart and could adversely alter results of experiments where the agent is in use.

5.2 Future Work

This optical method is still in its infancy and far from the overall goal of having a diagnostic technique that can be used in humans. There is still much to be done to increase the clarity of the signal and make it easier to find a signal. Before the optical method could ever be used in human hearts a method of overcoming the motion artifacts without resorting to an excitation-contraction decoupler must be developed.

To increase the clarity and strength of the signal requires increasing the delta-F that is generated by an action potential. Exciting the dye at the wavelength which produces the maximum fluorescence intensity might increase the change in fluorescence...
generated by an action potential. A tuneable laser with the capabilities of generating a light beam between 460 nm and 540 nm would be an ideal excitation for Di-4-ANEPPS (see Figure 3.9). Once the ideal excitation wavelength were found, then the dichroic beam splitter could be customized to the excitation beam, and the long pass filter before the photomultiplier tube could be changed to isolate the returning fluorescence to maximize the delta-F/F signal.

A possible improvement to the detection of the returning fluorescence might be replacing the photomultiplier tube with a photodiode detection circuit. It would certainly decrease the fear of destroying a PMT by exposing it to room light. Some researchers have opted to use multiple fibers in conjunction with a photodiode array for detection of the fluorescence [4].

Having to turn the lights off before each recording can be quite inconvenient. The 120 Hz noise from the overhead lights could be filtered on the computer by processing the signal in the frequency domain.

Fiber bending seems to be the major factor in motion artifacts. Increasing the fiber core diameter might increase the stiffness of the fiber and decrease the bending associated with muscle contraction. A larger core diameter would also increase the amount of excitation light reaching the stained tissue but it would decrease the spatial resolution of the optical signal because the current fiber diameter (100μm) is about the same size as a single muscle cell. A larger diameter would possibly detect action potentials from more than one cell at time.

Once the system progresses to experiments in working hearts, pumping real blood as opposed to Krebs nutrient solution, problems might arise with the interaction between blood and the glass fiber tip. Blood could coagulate on the fiber tip and block the excitation light and returning fluorescence. Recent developments in sapphire fibers might solve this potential complication because blood does not coagulate on sapphire tips. For the time being the cost of this technology keeps it out of reach.

One possible solution to the motion artifact without resorting to pharmaceuticals is a ratio method between a voltage sensitive dye and a non-voltage sensitive dye. In theory the inclusion of a non-voltage sensitive dye would allow differentiation between motion related signals and voltage related signals. There are a large number of technical problems, however:

1) Finding an excitation wavelength in which to measure only motion and
another wavelength that will contain the voltage signal would be difficult.

2) If two dyes are used, one voltage sensitive and the other non-voltage sensitive, the two dyes must distribute themselves the same way and have a similar time course of bleaching.

3) Noise is always a problem. As mentioned previously, the signal is very sensitive to fiber position, and if a heart cell moves away from the fiber then the voltage sensitive part of the signal would disappear leaving only the motion signal. In theory the dual dye method seems feasible, but it would require a lot of effort.

Mechanical methods are also being considered in preventing the motion artifact. One group of researchers developed a suction method where the fiber passed through a syringe and the suction of the syringe helped maintain the fiber position [18]. Another group used a mechanical feedback system where a piston moved in synchrony with the heart to make the relative differences in position between the fiber and the heart as small as possible [19].

Other ideas include attaching a barb, at the end of the fiber tip to help keep it in place, or using wet adhesives such as the barnacle derivative Cell-Tak to attach the fiber tip to the heart (note: once Cell-Tak touches tissue, it can not be re-used). Applying signal processing techniques used to stabilize moving images might provide a non-mechanical method of filtering out motion artifact.

A few months ago this method had never worked in a live rabbit heart and now cardiologists at the V.A. Medical Center are using it in studies that will affect heart patients. This method is still 10-20 years from being used in humans and as with most cutting edge research improvements are made very slowly, one step at a time.
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Vitae

Ashwin Amanna was born to Ralph and Urmilla Amanna on June 4, 1968 in Johnson City, New York. After relocating to Northern California in 1973 he eventually earned a B.S.E.E. at the University of California, Davis.

Upon graduating he worked as a post graduate research engineer on a High Mileage Vehicle and drove the vehicle to the United States Record in gasoline. After acceptance to Virginia Polytechnic Institute he, along with 3 others embarked on a two tandem bicycle crossing of the U.S. from the Washington coast to the New Jersey Coast, raising money for a women's shelter in Sacramento.

With his graduation from VPI with a M.S.E.E, Ashwin hopes that his engineering background will provide a unique perspective and motivation as he continues to pursue his overall goals of becoming a medical doctor and racing mountain bikes.