Live-Cell Fluorescent Imaging of Cardiomyocytes

BGGN 266/PHYS 173
Kevin Vincent & Joyce Lee
6/24/2012

Abstract

Voltage sensitive dye responds to changes in voltage of cell membranes. Knowing that there are voltage changes during an action potential, we did an experiment in quantifying action potentials of mouse cardiomyocytes. Having built the scope and set the filters, we loaded the voltage sensitive dye, Di-8-ANEPPs, to the cultured mouse cardiomyocyte and collected data. Analyzing the data, we were unable to see a clear action potential signal through the noise and photobleaching effect. Our results also suggested that using ascorbic acid, an anti-oxidant, helps slows down photobleaching of the sample.

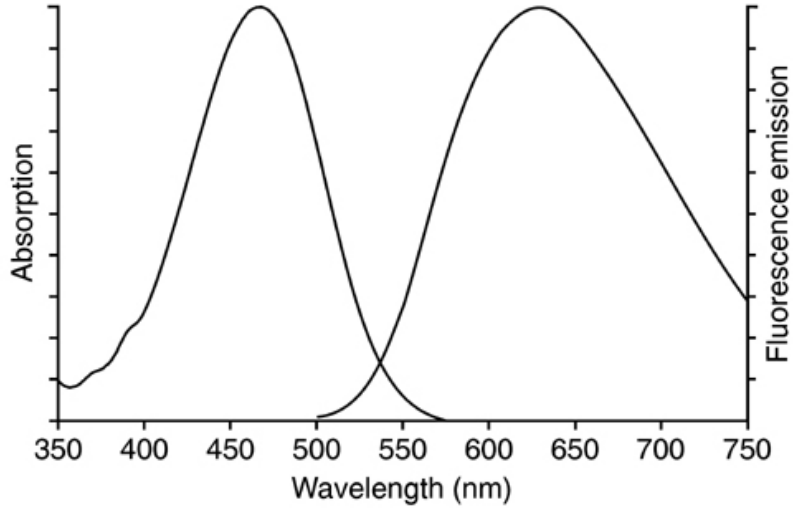
Table of Contents

Introduction

Contraction of cardiomyocytes is driven by a wave of depolarization that originates in the sino-atrial node in the right atrium. The resting membrane potential of a cardiomyocyte is around -90mV. The wave of depolarization triggers an action potential in each cell. Sodium ions rapidly flow into the cell and raise the membrane potential to around +25mV. This triggers additional voltage sensitive ion channel currents that return the cell to its resting membrane potential resulting in action potential durations of about 250ms in humans. Abnormalities in the development of an action potential on the cellular level or in the propagation of an action potential on the tissue level can result in deadly arrhythmias. Therefore, measuring transmembrane voltage can provide important experimental insight into cardiac physiology and pathophysiology.

A number of small molecule fluorescent indicators allow for quantification of transmembrane voltage. Di-8-ANEPPS, a small molecule developed by Leslie Loew and colleges at the University of Connecticut, imbeds in the cell membrane and experiences an electrochromic shift in its fluorescence excitation and emission spectra. The electrochromic mechanism allows fluorescent measurement of trans-membrane voltage. Figure 1 shows the excitation and emission spectra for the dye. Di-8-ANEPPS is often used as a ratiometric dye where excitation light is measured on either side of the peak emission wavelength. During an action potential, emitted light in the green region increases and emitted light in the red region decreases. Figure 2 contains published data measuring ratiometric action potential data for di-8-ANEPPS compared to a standard electrode recording. Ratiometric measurements have the advantage of eliminating the effects of photobleaching and uneven dye loading.[2]

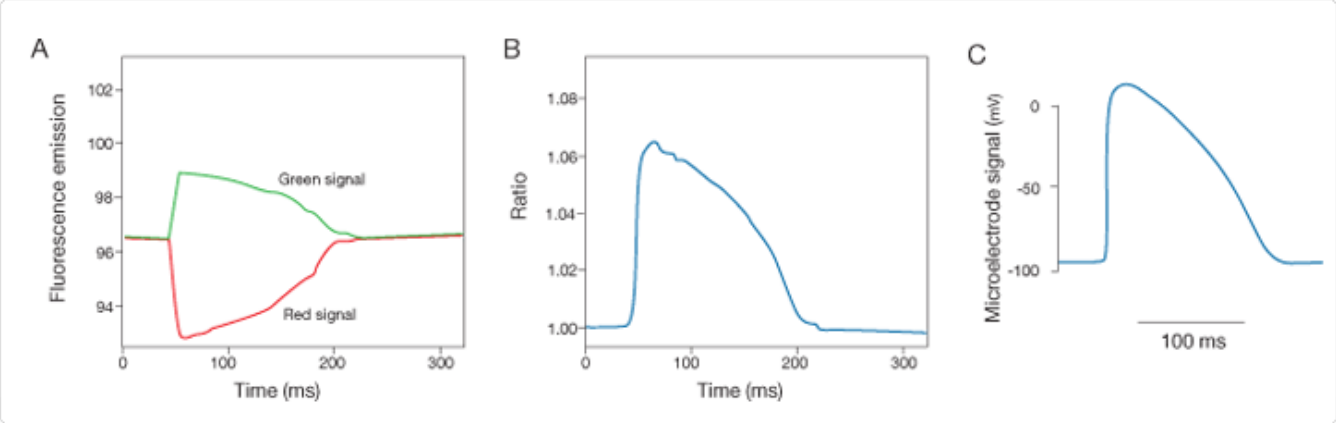
Figure 1: Excitation and Emission Spectra for di-8-ANEPPS[1]



Di-8-ANEPPS is also frequently used measuring only one emission wavelength. This is common when absolute voltage is not essential to the

measurement. For example, optical mapping studies of intact explanted animal hearts or cells layers grown in culture often use relative voltage measurements to calculate activation times and conduction velocity. Measuring fluorescence emission at one wavelength greatly simplifies the set-up and equipment needed for the experiment, but it comes at the cost of increasing the problems due to photobleaching.

Figure 2: Ratiometric Voltage Data from Knisley et. al.



Photobleaching is the permanent fading of fluorescent signal due, and it can be a major impediment to data collection with voltage sensitive dyes. Photobleaching occurs when a molecule has an electron under go the 'forbidden' intersystem crossing to the triplet state. The electron then interacts with oxygen to create the highly reactive singlet oxygen. Singlet oxygen can damage the fluorophore or other proteins in a living cell degrading signal quality.[4] There are a number of anti-photobleaching agents available commercially. Ascorbic acid (vitamin C) is one simple anti-photobleaching agent that has shown to reduce the rate of photobleaching by a factor of 3 to 5 in rhodamine and fluorescein.[5]

In this project we attempted to measure fluorescence intensity changes due to spontaneous action potentials in neonatal mouse cardiomyocytes. Additionally, we attempted to reduce the rate of photobleaching using ascorbic acid.

Methods

Microscopy

Figure 3 shows the microscopy setup. A Zeiss upright microscope was used with a 40x 0.75 NA water immersion objective, and the Andor iXon DV885 camera was used to collect data. All camera data was collected using the Kinetic Series collection mode. Excitation filters, emission filters and dichroic mirrors were measured on using a spectrometer. The final filter set was selected by comparing to the excitation and emission spectra of di-8-ANEPPS. Figure 4 show the data from the spectrometer for the filter set selected.

Figure 3: Microscopy Setup

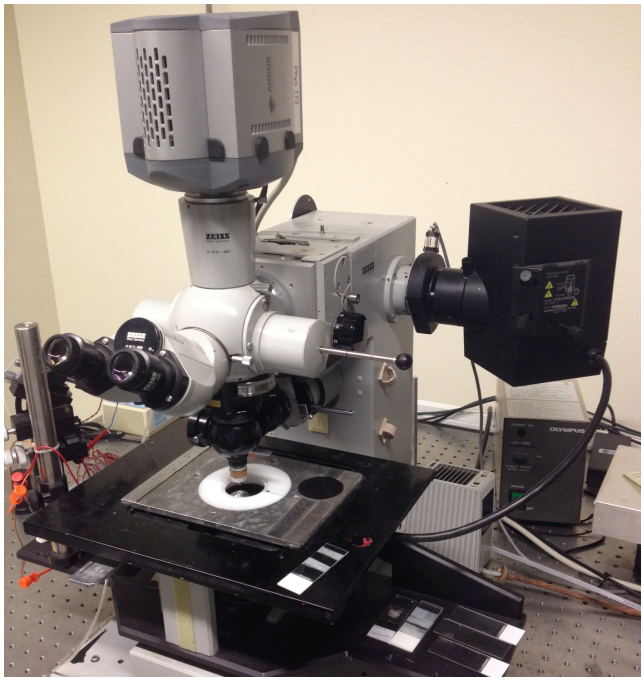
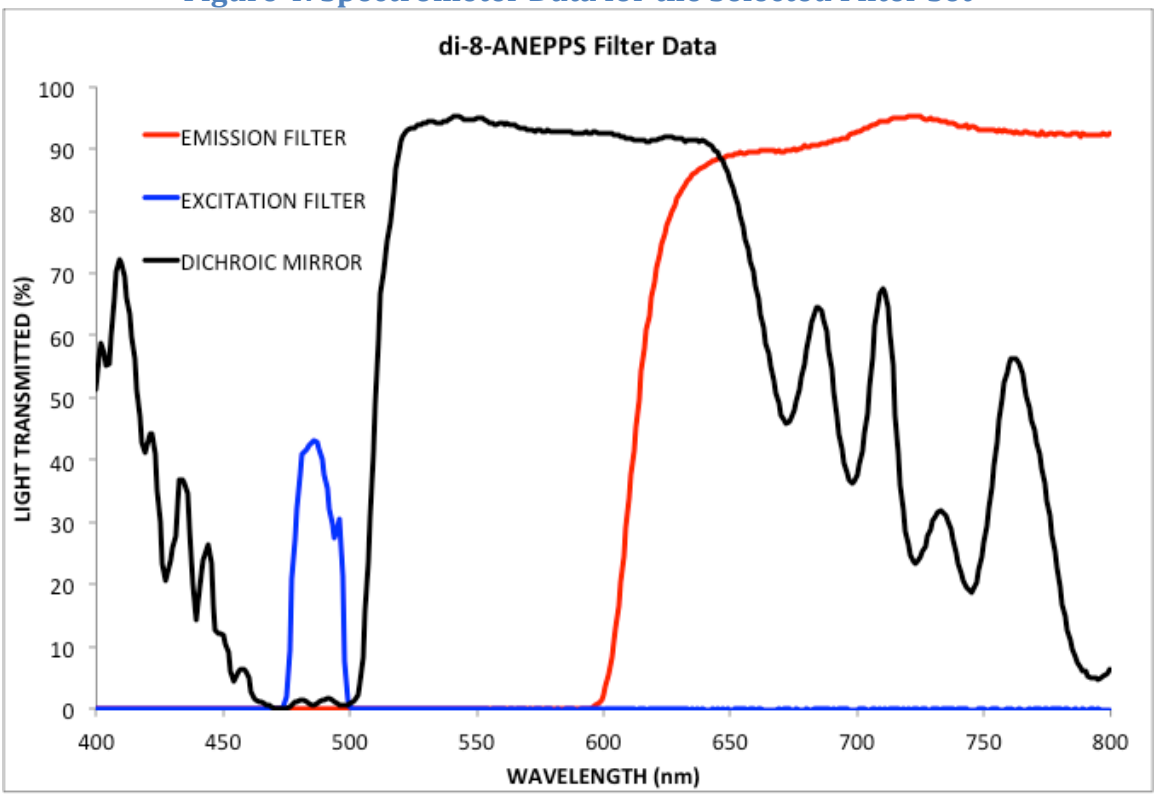


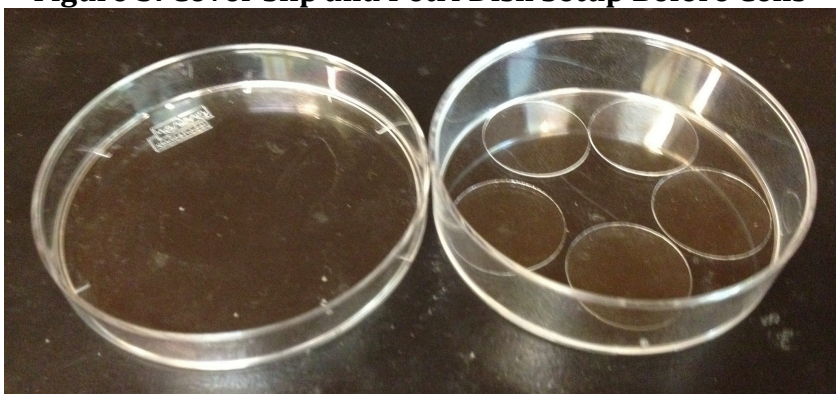
Figure 4: Spectrometer Data for the Selected Filter Set



Sample Preparation

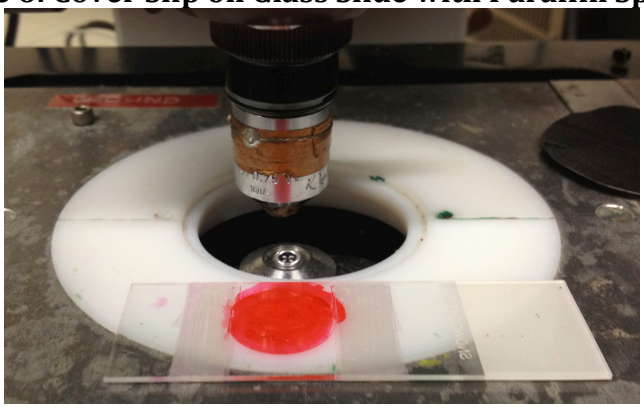
Cells from P1 (one day old) neonatal mice were provided by the McCulloch lab. To accommodate the upright microscope, cells were grown on small round coverslips inside a larger petri dish (Figure 5) as suggested by Fischer et. al.[6] The cover slips were first coated in laminin and incubated for 30 minutes to increase cell adhesion. These cells were cultured for 5-7 days in tissue culture incubators (37°C, 10% CO2 and high humidity). The tissue culture media contained 74.5% DME, 18.5% M199, 5% horse serum, 1% fetal bovine serum, and antibiotics. One day prior to the experiment, the cells were switched to an antibiotic free media. The dye solution consisted of antibiotic free media with 30 μM di-8-ANEPPS and %0.1 pluronic to help stabilize the membrane and insert the dye. The test solution was antibiotic free media.

Figure 5: Cover Slip and Petri Dish Setup Before Cells



Prior to the experiment the petri dishes were carefully transported from PFBH to the lab room and stored in an oven at 37°C and atmospheric CO_2. After the dye and test solutions were warmed to 37°C, the media was extracted from the petri dish and dye solution was added. After about 30 minutes at 37°C, the dye solution was removed and the test solution was added. The coverslips were removed with tweezers and placed upside down on a glass slide with paraffin wax strips to prevent the cells from being crushed (Figure 6 with the coverslip artificially colored red).

Figure 6: Cover Slip on Glass Slide with Paraffin Spacers



Experiments

The first experiments attempted to measure spontaneous action potentials in the neonatal mouse cardiomyocyte cells. Brightfield microscopy was used to align the slide to a location with actively contracting cells. The lamp for the brightfield microscope was filtered with a red-pass filter to decrease the amount of fluorescent excitation during this process. Once properly aligned the shutter for the fluorescent microscope was opened and data was collected. The camera was set to use frame transfer and collect at 22Hz for 2 seconds. The shutter was manually closed when not collecting data to minimize photobleaching.

New coverslips with fresh cells were used to collect photobleaching data. The scope was aligned as before to an area with contracting cells. Numerous different camera settings were attempted with 30Hz for 2 minutes being the settled on collection scheme. The photobleaching experiments were repeated with 100 μM ascorbic acid in the dye and test solutions. Background data was also collected.

Data Analysis

Data analysis was performed in Matlab. 10% of each image was removed from the left side of each image due to a large amount of noise from an undetermined source. First movies were created to easily view the data. Next, regions of interest containing only cells and only background data were selected for further analysis. The primary metric was to select a region of interest (ROI), sum the photon count in that ROI for each image in the stack and normalize the data.

Results

Membrane Voltage

Seven data sets were collected attempting to look at action potential data. No clear action potential data was visible in the data. Motion due to contracting cells was seen very clearly in the brightfield images but not in the fluorescence data. Figure 7 contains bright field and fluorescent data from a representative data set. Figure 8 quantifies the intensity of fluorescence.

Figure 7: Sample Microscopy Data

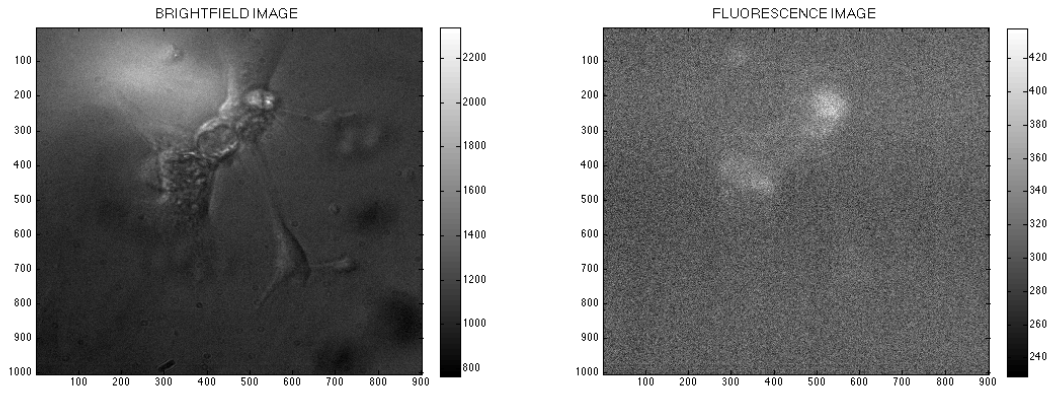
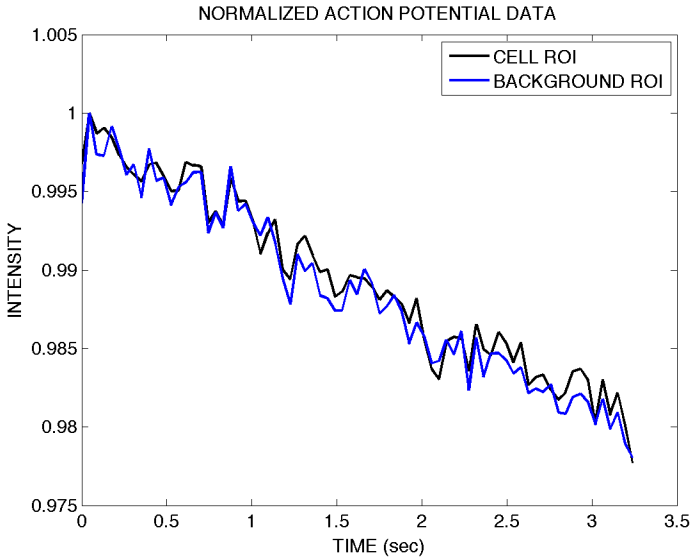


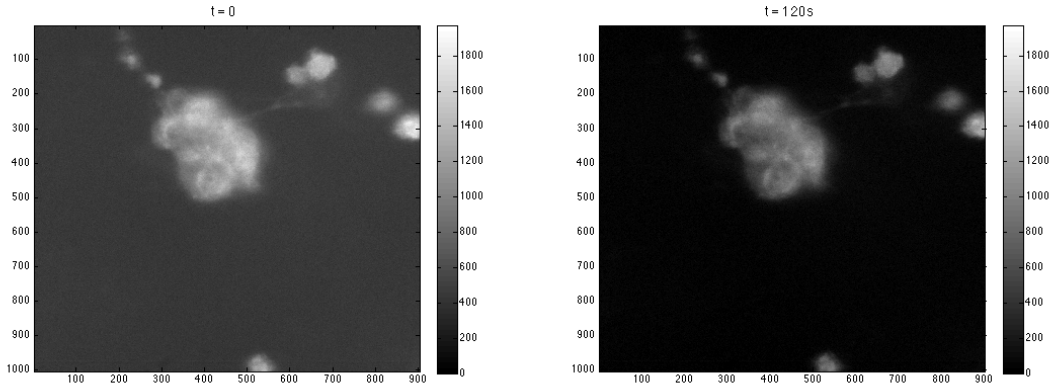
Figure 8: Action Potential Data Normalized by Maximum Value



Photobleaching

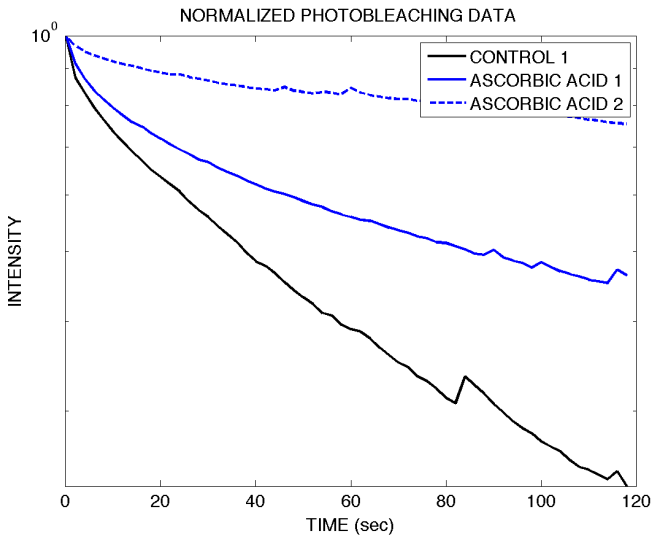
Five control and seven ascorbic acid experiments were run. Not all data sets were directly comparable as different CCD camera settings were used, but one control and two ascorbic acid runs were directly comparable. Figure 9 contains two fluorescence images with the same color scale spaced 2 minutes apart using a 0.5 second exposure time.

Figure 9: Photobleaching Image at 120s



Quantification of the photobleaching is shown in Figure 10. One control experiment is compared to two experiments with ascorbic acid in the dye and test solutions. A number of different normalization and masking techniques were tried. The ROI for this analysis was traced by hand around the cell cluster in Matlab. Intensity in each frame was summed to provide a data point, and the data was normalized by dividing by the maximum intensity value. Figure 10 shows the exponential rate of photobleaching to be greatest in the control sample and slower photobleaching in the two samples with ascorbic acid.

Figure 10: The Effect of Ascorbic Acid on Photobleaching



Discussion

Fluorescent measurements with di-8-ANEPPS were recorded, but despite numerous attempts to filter out the photobleaching effects we were not able to resolve any action potential like data. The light intensity in the measurements was extremely low. Reference data collected with the scope and the CCD information suggested that we were barely over the normal level of noise. This was very surprising since we were using a very long exposure time. The longer exposure time should collect more photons for a higher measurement, but even with long exposure times the data level was very low. The sampling frequency for the action potential data was 22Hz providing about 45ms of exposure for the CCD for each frame. While this should be a high enough sample rate for activation time measurement, it is much less than the typical sampling rates used on this dye (500-1000Hz). One day of data collection in the McCulloch lab was obtained from cells that could not be used on our scope because they were in an 8 well plate that blocked the objective. Action potentials were not observed, but in the fluorescence data the intensity of emitted light was much greater than on the Zeiss microscope.

It is possible that the filter set was did not allow enough of the emitted light to pass on the CCD. A different filter set should have been tested just to see if the intensity of the light greatly increased. Additionally, no contractions were seen in the fluorescent recordings. Using a field stimulus could ensure that the cells are contracting. Without depolarization, action potential measurements can not be made. Data measurements were almost always taken from groups of cells. Fibroblasts (non-contractile support cells) were likely present in these cell groupings. Since these cells do not beat, focusing the microscope on a fibroblast would not show an action potential. A better understanding of the dynamics of

photobleaching could have allowed for better interpretation of data and noise removal.

Figure 10 suggests that there is a measureable decrease in the rate of photobleaching when ascorbic acid is added to the dye solution and test solution for ascorbic acid. However, only three sets of data were directly comparable, and this is not enough to draw any clear conclusions. One unexplored aspect of this data results from the data being collected from groups of cells. Having multiple cells on top of each other may alter the effect of photobleaching. One-photon non-confocal microscopy is susceptible to blurring in the Z direction. If these out-of-focus cells are photobleaching at a different rate due to different light intensity or other factors, the photobleaching results may be altered.

Problems and Future Suggestions

The major problem with this project was obtaining useable cells. Cells were arranged and cultured four times, but only one set could be used to collect data. One failed incubation was killed due to a power outage and a change in CO_2 level, for another the cells did not stick to the cover slip, and for the last failed cell culture the cells died due to a lack of enough media. The media problem was due to the specific way the cells were plated to accommodate the upright microscope.

Experience with this project led to some suggestions for future groups. First, get a model system using the voltage sensitive dye that is not dependent on living cells. This would provide ample opportunity to optimize the filters, adjust CCD options, and calibrate the dye. Some of the early papers in this field used liposomes made from egg phosphatidylcholine with the dye in ethanol to test the dye. This would be an extremely valuable first step. Additionally, by reconstituting the liposomes in different concentrations of potassium or other ions, a potential difference across the membrane could be created. Second, obtain some type of tissue culture incubator for the lab so that cells can be held there at least overnight before experiments. Lastly, use an inverted microscope if possible. Many of the problems with the cell culture were due to having to plate the cells on coverslips instead of petri dishes or 8 well plates. Inverted microscopes are preferred for live-cell imaging since there is no issue getting the objective close to the cells.

Conclusions

Our microscopy set up was able to see the cells using fluorescent microscopy. However, we were not able to resolve the action potential data. Quantification of photobleaching yielded promising but inconclusive results for the effect of ascorbic acid on the rate of photobleaching. Overall, the project exposed many of the most important practical aspects of live-cell fluorescent imaging and the use of voltage sensitive dyes. Despite the mixed results, the project proved to be a valuable learning experience.

Acknowledgements

We would like to thank Drs. Phil Tsai and David Kleinfeld for their help and guidance obtaining the necessary materials and equipment for this project and for their guidance with microscopy. We also offer many thanks to Dr. Jon Driscoll for his suggestions, encouragement and guidance for the microscopy and data collection portions of this project. Lastly, we would like to thank Emily Pfeiffer, Britton Boras, and Kyle Buchholz from Dr. Andrew McCulloch's lab for providing us with cells, helping us culture the cells, and discussions about Di-8-ANEPPS.

References

1. 1. "Di-8-ANEPPS." *Life Technologies*. Web. 07 June 2012. <http://products.invitrogen.com/ivgn/product/D3167>.
2. Zhang J, Davidson RM, Wei M, Loew LM. Membrane Electric Properties by Combined Patch Clamp and Fluorescence Ration Imaging in Single Neurons. *Biophys J* 74: 48-53.
3. Knisley SB, Justice RK, Kong W, Johnson PJ. Ratiometry of Transmembrane Voltage-Sensitive Fluorescent Dye Emission in Hearts. *Am J Heart Circ Physiol* 279: H1421-H1433.
4. Lichtman JW, Conchello JA. Fluorescence Microscopy. *Nature Methods* 2: 910-919.
5. Gilon H, Sedat JW. Fluorescence Microscopy: Reduced Photobleaching of Rhodamine and Fluorescein Protein Conjugates by *n*-Propyl Gallate. *Science* 217: 1252-1255.
6. Fischer AH, Jacobson KA, Rose J, Zeller R. Mounting Live Cells Attached to Coverslips for Microscopy. *Cold Spring Harb Protoc* 3: 1-2.