

FLUORESCENCE TRACKING OF CARDIAC MYOCYTE EXCITATION

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ABSTRACT

Cultured cardiac myocytes were fluorescently tagged with fluo-4AM and stimulated with an extracellular electrode. Fluorescence levels of five cells were recorded for 2ms pulses of stimulation at 7 volts, and averaged ~1000 above the background level for a 100 second exposure.

Cardiac myocytes subsequently exposed to a 5% ethanol solution showed no detectable levels of fluorescence. However, when resuspended in a buffer solution, 20% of the cells being imaged showed a return in fluorescence, at an average level of ~1000 above the background.

Table of Contents

1. INTRODUCTION.....	3
2. EXPERIMENTAL SETUP.....	3
2.1 Instrumental Setup.....	3
2.2 Calibration of Fluorescence Measurements	5
3. EXPERIMENTAL PROCEDURE.....	7
4. RESULTS	9
5. DISCUSSION AND CONCLUSIONS	11
6. ACKNOWLEDGMENTS	12
7. REFERENCES.....	13
8. SUPPLEMENTARY INFORMATION.....	14

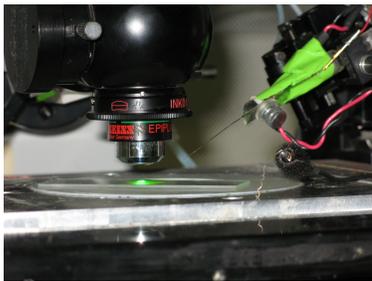
1. INTRODUCTION

Cardiac myocytes are heart muscle cells which function similarly to smooth muscle. Ca^{2+} is sequestered into intracellular vesicles, and released upon a mechanism governed by extracellular potential. This release of calcium allows muscle filaments within the cell to contract. Because cardiac myocytes are separated by an intercalated disc, which functions as a leaky membrane, the release of calcium within a cell triggers adjacent cells to depolarize as well, which produces an overall synchronized beating.

This experiment seeks to demonstrate this mechanism of cardiac myocyte contraction by introducing fluorescent tracking of calcium. First introducing a calcium-sensitive fluorescent dye to a solution of cells, and then applying an extracellular potential via an electrode, induced beating produces changes in calcium levels that can be monitored by fluorescence imaging techniques. Several experiments were carried out to observe calcium flux, each confirming prior experiments on the known function of cardiac myocytes.

2. EXPERIMENTAL SETUP

2.1 Instrument Setup



*Figure 1: diagram of experimental setup showing the positioning of the objective and electrode relative to the sample
Note : this does not show the bend in the electrode*

This experiment was carried out on a Zeiss upright microscope using both 16x 0.35NA and 4x 0.1NA objectives. The sample of cells were loaded in plastic

Petri dishes, at sufficiently disperse concentrations so as to mostly avoid spontaneous synchronized beating.

A stainless steel, monopolar electrode was positioned

on hydraulic manipulators for ultra-fine control, and pulsed current that was administered through a stimulator box controlled by a function generator. The electrode had to be bent

into a Z shape in order to fit under the objective. A mercury lamp was used in a reflected Kohler illumination setup to fluorescently illuminate the sample of cells. The emission filter, a 480/20 band pass filter, allows light between 470nm and 490nm to pass through. A 500 DRLP dichroic filter then reflects the excitation wavelength down into the sample. Fluo-4 in the sample is excited at 488nm, and reemits with a maximum emission around 530nm, which passes through objective and the dichroic and up towards the eyepiece. The emitted light is further filtered by an OG515 long pass filter, to fully eliminate any stray excitation light that may have gotten through the dichroic. The images were then captured on

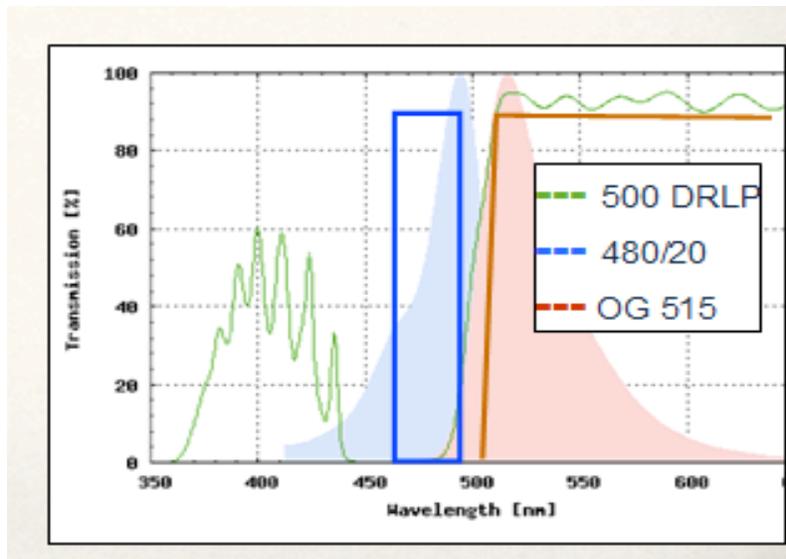


Figure 2: Graph of the rough percentage of light transmitted at particular wavelengths for the excitation filter, the dichroic, and the emission filter.

an Andor iXon camera for further analysis. The Andor camera software was utilized to control the camera shutter and to capture both single exposures, as well as stacks of exposures. ImageJ, a program available as freeware on the web, is able to analyze images

exported as Tiff files. The camera produces a high pixel-depth image, due to some noise the window and level over which the image had been produced had to be carefully chosen in order to see features of the image. For more in-depth fluorescent analysis, one can use the tools designed for measuring pixel brightness only in particular regions of interest. Measured pixel brightness above baseline corresponds to level of fluorescence.

2.2 Calibration of Fluorescence Measurements

The pixel count measured by the camera corresponds to the level of fluorescence at a particular amount of calcium bound fluo-4. To calibrate the camera's measurements, a variety of solutions were prepared and illuminated, each with varying calcium concentration. Living cells contain enzymes which catalyze the conversion of fluo-4AM to its functional form fluo-4 by cleaving two ester bonds. Because the calibration

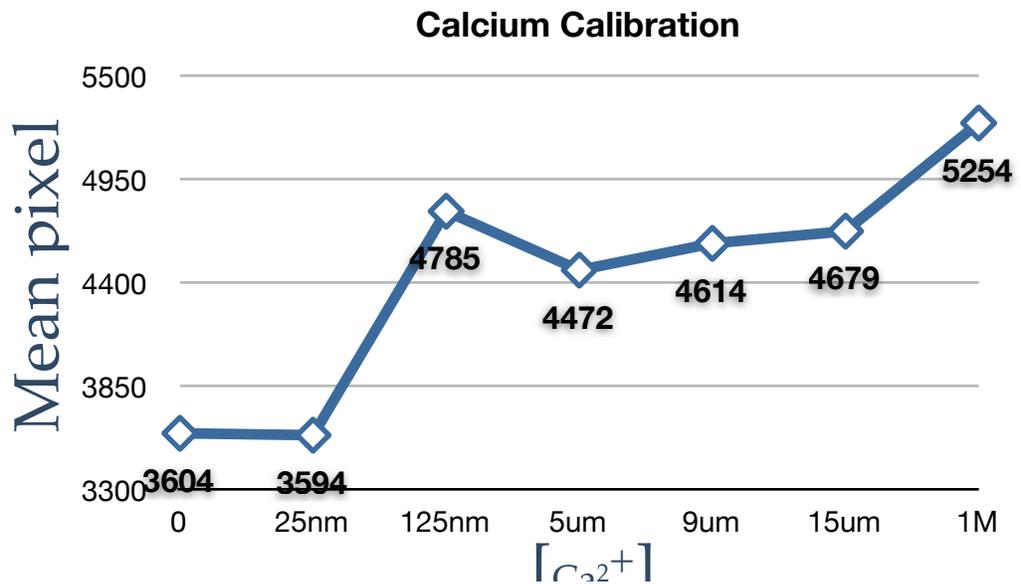


Figure 3: Pixel count vs. calcium concentration is plotted to calibrate the level of fluorescence measured during experimentation.

measurement was done in a solution lacking this enzyme, the fluo-4AM was first treated

with 1M NaOH to perform this necessary ester cleaving. To do this, we added 50uL DMSO/pluronic (a nonpolar solvent, and a detergent, respectively), 50uL Ethanol, and 25uL 1M NaOH to the dye (not suspended) and incubated for 1 hour at room temperature.

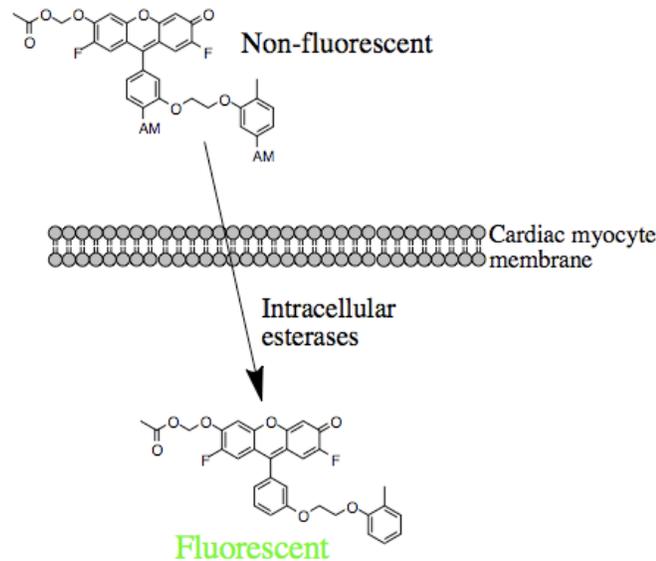


Figure 4: Method of Fluo-4 AM incorporation into the cell, as well as the intracellular cleavage of the AM by esterases.

In order to create a buffered solution with a constant concentration of calcium, we created an EGTA buffer as described by McGuigan, et al, 1991. This can not be ignored, and recipes are in the supplementary information.

The average pixel count over the entire field of view of the camera was taken as a data point for calibration. Because we were imaging in a bath, all of the recorded pixels should be about the same brightness. This curve can be compared to pixel count values obtained during cell experimentation to give a direct measurement of intracellular calcium secretion during excitation.

3. EXPERIMENTAL PROCEDURE

Cardiac myocytes were first treated with 3uM fluo-4AM added directly to their growth media. They were incubated for 30-60 minutes at 37°C to allow the cells to uptake the fluorescent tag. The cells were then washed twice (2mL each time) and 2mL fresh buffer solution was added (128mM NaCl, 1mM MgCl₂, 45mM sucrose, 10mM glucose, 0.01M HEPES, 5mM KCl) to remove extracellular fluorescent dye that had not diffused into the cell, or was stuck to the outer cellular membrane. Initially, cells were surveyed under non-stimulated fluorescence to establish a baseline of fluorescence. This should be done for the same length of exposure as the excited measurements will be. Cells were then surveyed under bright field illumination while being electrically stimulated. Contracting cells were visually identified using a 4x objective. Fluorescent imaging was carried out next, done both at 10 and 100 second exposures. During imaging, an electrode stimulated the sample at 7V for 2ms at 1 Hz.

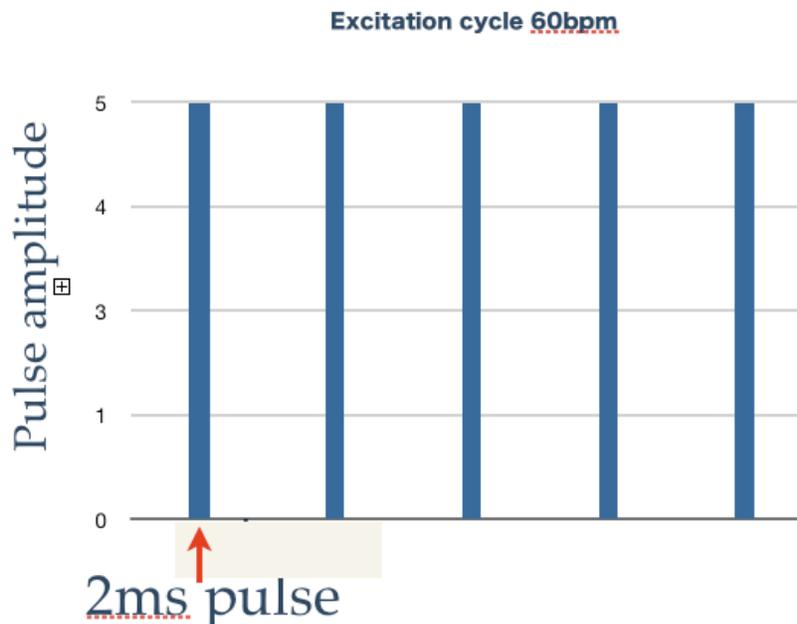


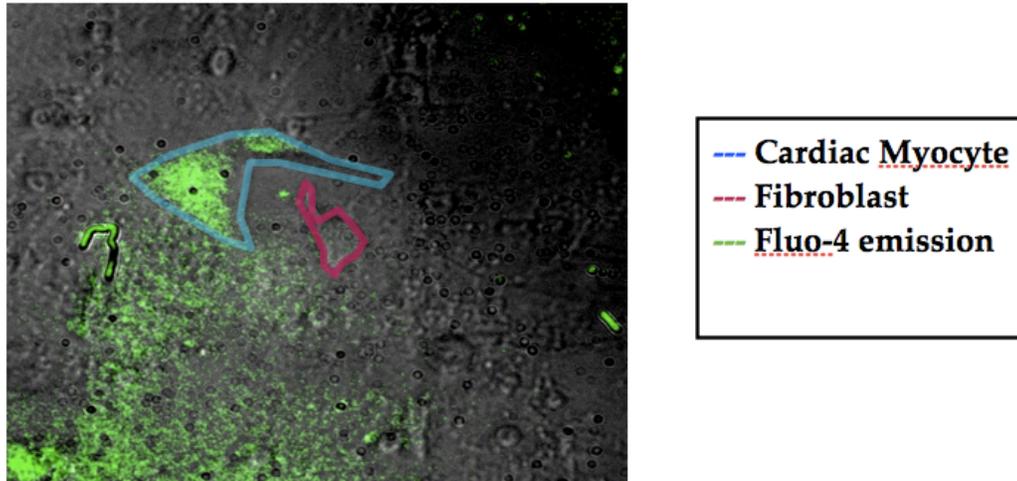
Figure 5: Excitation cycle for the cardiac myocytes

Image J was used to analyze the resulting images. The pixel count can be compared to the calibration curve previously established, to obtain a value of the quantity of the calcium emitted per cell over the 100s exposure.

As an additional experiment, we found a paper, Danziger, et al. that stated a cease in cardiac myocyte contractions due to addition of varying concentrations of ethanol. The ethanol causes a drop in calcium levels in the sarcoplasmic reticulum, as well as a decrease in release of calcium from the sarcoplasmic reticulum. Ethanol was then added to the Petri dish, bringing the concentration to 5% v/v. Cells were incubated for ~1 minute, then imaged and analyzed again in the way described above. Next, the cells were washed twice, resuspended in the original buffer solution and imaged a third time. This is expected to result in return of contractions and consequent return of fluorescence.

4. RESULTS

Experimentation done with a 16x objective allowed us to distinguish between cardiac myocytes and fibroblasts on a relatively overgrown petri dish, the former being the fluorescent cells in a 10s exposure.



16x, 0.35NA, 10s fluorescent exposure

Figure 6 : Single cardiac myocyte resolved from fibroblasts on an overgrown plate. Image overlay done on ImageJ.

Experimentation done with a 4x objective captured the fluorescence given off by 5 contracting cardiac myocytes, at an exposure of 100s. The relative fluorescence measured in pixel counts is shown below. As far as we know, this experiment has not been carried out with extracellular stimulation.

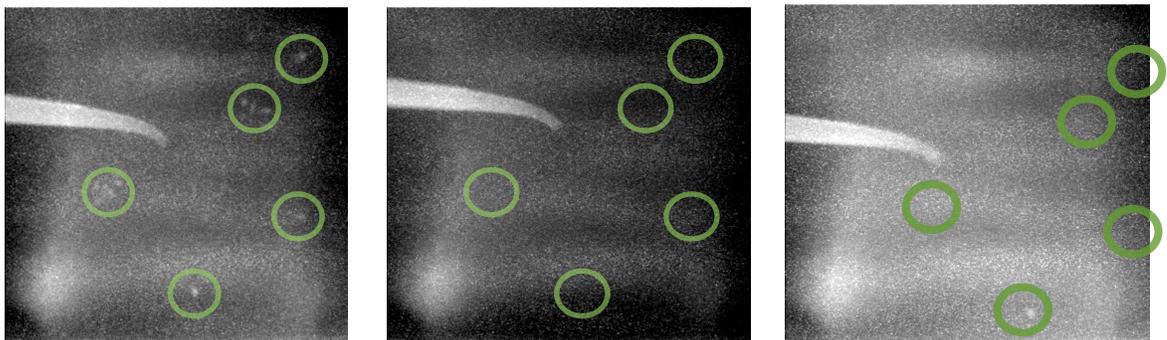


Figure 7: A) 100 second exposure of 1 Hz stimulated cardiac myocytes. B) Image of same cells after resuspension in 5% Ethanol solution and C) after being washed and suspended in original buffer solution. Cells 1-5 are numbered from the top down to the bottom of the image.

Relative Fluorescence (Mean Pixel Count)

Cell #	Before adding EtOH	5% suspension of ETOH	After Washing off ETOH
1	7499	6584	6837
2	7496	6585	6749
3	7735	6844	6860
4	7555	6684	6893
5	7766	6668	7958

5 DISCUSSION AND CONCLUSIONS

This experiment showed the successful labeling of cultured cardiac myocytes with Fluo-4AM. Furthermore, upon electrical stimulation at 7V for 2ms, at 1Hz, intracellular fluorescence is observed.

We have found, as corroborated by Danziger, et al. that the addition of ethanol to the cellular solution ceases the release of Ca^{2+} to the cytoplasm. Also, after washing away the ethanol and resuspending in the original buffer, 20% of the cells regained fluorescence.

Due to incomplete darkening of the experimental room, the long exposure of 100s introduced additional shot noise to measurements. It helps to shut off computer monitors and turn the imaging monitor away from the rig. However, our methods overall were robust, and the data obtained is in accordance with the known physiology of cardiac myocytes, therefore validating the known mechanism of intracellular calcium release.

8. WHAT WENT WRONG/FUTURE DIRECTIONS

Aligning the dichroic was a major issue for us, as in order to get the maximal amount of light through, there is about 1 degree of motion. To calibrate the angle effectively, put a drop of fluorescein on a slide and focus on it (it helps to have a cover slip on the slide so you can focus on the edge of that and then move over the fluorescein). Darken the room and make sure you have a live feed of the fluorescence on the computer. Very slowly rotate the dichroic, as you watch the mean pixel count at the bottom of the acquisition window. The goal is to find the rotation of the dichroic that gives you the highest fluorescence passed through and up to the camera, indicated by the highest mean pixel count.

Another issue we had was with the lenses we used. They were all low-NA, air objectives and we found that when there was more than about 1mL of liquid in our petri dishes, the aberration from the liquid made it almost impossible to focus on the cells. Taking out most of the liquid helps with this - though you have to keep a close watch on the plate to make sure it does not dry out during this time.

Due to differences in exposure times, it is not possible to calculate an appropriate baseline. In the future, groups should calibrate at the same exposure time that they will take images at. In our case, we did not know that we would have to take 100s exposures, and thus have incompatible calibration data.

In the future, there are some interesting directions that this project can go. It was found in a previous paper that varying pH of the buffer can impact a myocyte's ability to contract. It would be interesting to see if the change in fluorescence correlate with this change in pH, though fluo-4 has a strong pH dependance, so it may be difficult. Additionally, a condition called hypokalemia (low blood potassium levels) can cause cardiac arrest. Lowering buffer potassium may mimic this. The opposite is also the case - hyperkalemia can cause increased pulse rate, and may be replicable in vitro during this lab.

9. ACKNOWLEDGMENTS

We would like to give special recognition to Shigeki Miyamoto for providing us with cardiac myocytes at a variety of densities. Also we would like to recognize Ben Migliori for dedicating many hours during, as well as outside of the scheduled lab time to making this project work.

9. REFERENCES

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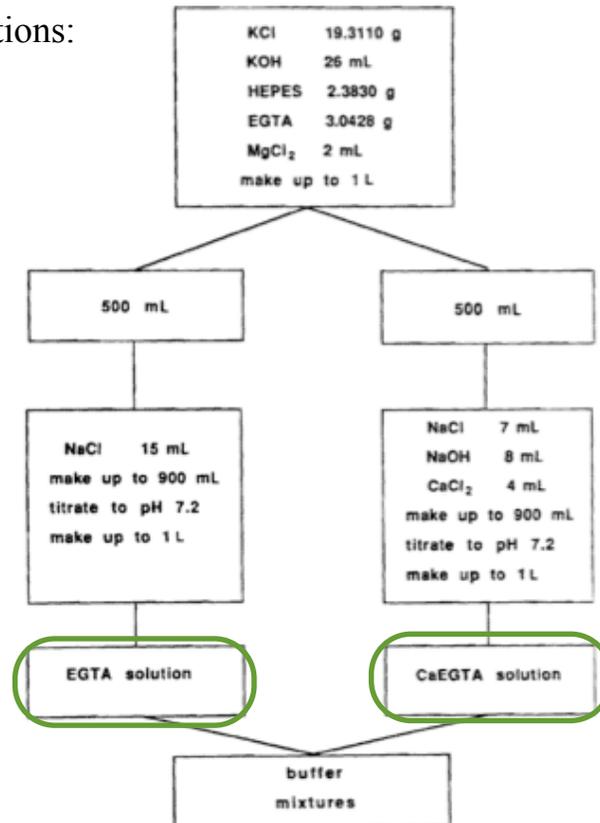
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Supplementary information :

EGTA buffer solutions:



Solution no.	EGTA/ CaEGTA	Volumes (mL)			Solution no.	pCa	Free Ca (mmol/L)	Bound (mmol/L)	Bound/free
		EGTA	CaEGTA	Total					
1	1/9	10	90	100	1	5.0455	0.009005	3.5910	398.78
2	1/8	10	80	90	2	5.1730	0.006714	3.5488	528.56
3	1/7	10	70	80	3	5.3074	0.004927	3.4951	709.32
4	1/6	20	120	140	4	5.4762	0.003340	3.4252	1025.45
5	1/5	20	100	120	5	5.6072	0.002471	3.3309	1348.08
6	1/4	25	100	125	6	5.7622	0.001729	3.1983	1849.82
7	1/3	20	60	80	7	5.9000	0.001259	2.9987	2382.21
8	1/2	50	100	150	8	6.0896	0.000814	2.6659	3276.34
9	1/1	50	50	100	9	6.3824	0.000414	1.9999	4823.63
10	2/1	100	50	150	10	6.6030	0.000249	1.3333	5343.35

These tables allow various mixtures of caEGTA and EGTA solutions to give desired concentrations of free calcium for calibration purposes. Buffers used in this experiment are boxed in green.