ENHANCED RECORDING OF DISCHARGE EVENTS BY LOCALIZING GENETICALLY ENCODED VOLTAGE INDICATORS TO THE NEURONAL SOMA

by

Ryan J. Purwin

A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

Committee:

_____	Dr. Theodore Dumas, Thesis Director
_____	Dr. Nadine Kabbani, Committee Member
_____	Dr. Patrick Gillevet, Committee Member
_____	Dr. Iosef Vaismann, Acting Director, School of Systems Biology
_____	Dr. Donna M. Fox, Associate Dean, Office of Student Affairs & Special Programs, College of Science
_____	Dr. Peggy Agouris, Dean, College of Science
Date: _____	Spring Semester 2017 George Mason University Fairfax, VA

Enhanced Recording of Discharge Events by Localizing Genetically Encoded Voltage Indicators to the Neuronal Soma

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

by

Ryan J. Purwin
Bachelor of Arts
Western Connecticut State University, 2014

Director: Theodore Dumas, Professor
Department of Psychology

Spring Semester 2017
George Mason University
Fairfax, VA

DEDICATION

This is dedicated to wonderful family, my parents David and Leslie, and my sister Ashley.

ACKNOWLEDGEMENTS

I would like to thank all my friends and family who have helped me get to where I am today. My parents, who are always pushing me to be the best that I can be. My sister, who has always looked out for her nerdy little brother growing up. Dr. Dumas, for giving me this opportunity to expand my knowledge with this invaluable experience. Dr. Kabbani and Dr. Gillevet, who have been crucial in helping me form this thesis. Justin King, for helping us out with the transfections. All my classmates, clubmates, and labmates from the PBNJ lab, for making me feel welcome and a part of the team. Finally, thank you to my lab partners Ahmad Elsayed and Kevin Hynes; I couldn't have done any of this without you guys.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

Adenosine Triphosphate ..... ATP
Base Pairs ..... bp
Basic Local Alignment Search Tool ..... BLAST
Centimeter ..... cm
Cerulean Fluorescent Protein ..... CeFP
Cyan Fluorescent Protein ..... CFP
Degrees Celsius ..... ℃
Endonuclease A Positive ..... EndA+
Ethylene glycol-bis(β-aminoethyl ether)- N,N,N',N'-tetraacetic acid ..... EGTA
Ethylenediaminetetraacetic acid ..... EDTA
Förster Resonance Energy Transfer ..... FRET
Gamma-Aminobutyric Acid ..... GABA
Genetically Encoded Voltage Indicator ..... GEVI
Glucose, EDTA, Tris ..... GET
Green Fluorescent Protein ..... eGFP/GFP
Hybrid Optical Voltage Sensor ..... hVoS
Luria Bertani ..... LB
Microliter ..... µl
Milliliter ..... ml
Nerve Growth Factor ..... NGF
Pheochromocytoma 12 Cell Line PC12
Piperazine-N,N'-bis(2-ethanesulfonic acid) ..... PIPES
PIPES, EGTA, MgSO4 ..... PEM
Polymerase Chain Reaction ..... PCR
Region of Interest ..... ROI
Revolutions Per Minute ..... RPM
Roswell Park Memorial Institute ..... RPMI
Small Conductance Calcium-Activated Potassium Channel Gene 1 ..... SK1

ABSTRACT

ENHANCED RECORDING OF DISCHARGE EVENTS BY LOCALIZING GENETICALLY ENCODED VOLTAGE INDICATORS TO THE NEURONAL SOMA

Ryan J. Purwin, M.S.

George Mason University, 2017

Thesis Director: Dr. Theodore Dumas

Genetically Encoded Voltage Indicators (GEVIs) convert changes in the electrical potentials of cell membranes to changes in the intensity of fluorescence emission. Numerous types of GEVIs have been created and shown to track neuronal action potentials with high fidelity and a sufficient signal to noise ratio in cultured neurons and simple organisms. Problems have been encountered when even the best GEVIs have been expressed in complex mammalian circuits, the most concerning being a low signal-to-noise ratio resulting from cell-wide expression of the GEVI and high density and overlapping neural architectures from different cells. A way to rectify this issue is to restrict the surface expression of the GEVI to a specific neuronal compartment; for action potentials, the optimal compartment is the neuronal soma. Restriction of any GEVI to the neuronal soma can be achieved by fusing it to a soma-localizing motif. Differentiated PC12 cells transfected with our construct, FireFluo, have been shown to emit

fluorescence that is largely contained in the soma. Future directions include soma localization of additional GEVI types, testing of soma-restricted GEVIs in complex circuits, and eventually tracking of thousands of neurons in behaving animals, leading to a better understanding of relationships between activity patterns in neural circuits and specific cognitive abilities.

CHAPTER ONE: INTRODUCTION

1.1 Action Potential Discharge Events

A core principle in neuroscience is the initiation and propagation of action potential discharge events. An action potential is a wave of electrical activity that travels down the membrane of a neuronal axon. Action potentials carry information between cells and tissues in living animals. Action potentials are initiated in a specialized compartment of the soma directly adjacent to the axon known as the axon hillock. At this point, the collective input of excitatory and inhibitory signals will determine if an action potential will fire in a process called summation. This process of collecting to transmitting information is the basic component of all neural connections. Therefore, the tracking and recording of these discharge events is critical in developing a complete understanding of complex neural circuits.

1.2 Discharge Event Recordings

Discharge events in animals have historically been recorded using electrophysiology. However, a ceiling has been reached as to how many neurons can be simultaneously recorded electrophysiologically, leading to difficulties in tracing complex circuits. Optical technologies are a promising route to surmount this issue. One successful approach is the use of calcium sensitive fluorophores. These fluorophores report the calcium transients associated with action potential discharge. This technique has been used to visualize action potential firing patterns when a mouse explores a circular arena

Although promising, calcium sensors produce relatively weak signals for single action potentials and are not fast enough to track individual action potentials in a rapid burst.

1.3 Genetically Encoded Voltage Indicators (GEVIs)

GEVIs directly report changes in membrane potential as changes in the intensity of fluorescence emission. Typically, a GEVI involves the implementation of a membrane-bound fluorescent protein or proteins. The fluorescence emission can be coupled to membrane potential by several possible mechanisms. In the Hybrid Optical Voltage Sensor (hVoS) system, the fluorescence of the membrane-bound protein is coupled with the voltage-dependent movement of dipicrylamine, a membrane-embedded hydrophobic anion. Förster Resonance Energy Transfer (FRET) between the dipicrylamine anions and the membrane-bound fluorophore links changes in fluorescence intensity to changes in membrane voltage.

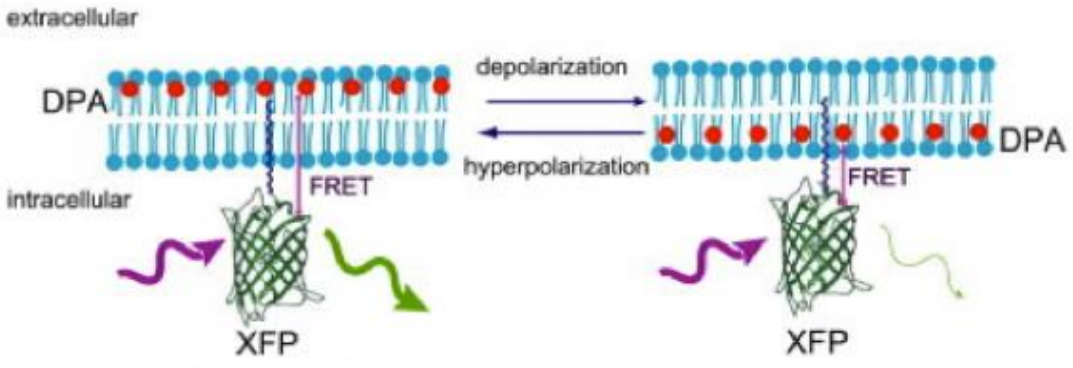

Figure 1: Illustration of the original hVoS system.
A fluorescent protein (XFP) is anchored to the intracellular surface of the cell membrane. Hyperpolarization (left) shifts dipacrylamine towards the extracellular surface of the membrane, while depolarization (right) drives the dipacrylamine closer to the XFP. This proximity increases FRET efficiency, decreasing fluorescence.
Image Credit: Meyer Jackson Laboratory, University of Wisconsin

Constructs that do not require a secondary FRET-acceptor have been created. These sensors involve a membrane bound rhodopsin protein that undergoes FRET interaction with a second tethered fluorescent protein. Perhaps the best of these GEVIs, the ACE-mNeon constructs created in Dr. Mark Schnitzer's laboratory at Stanford University, have been shown to produce bright and quick responses to discharge events in awake mice and flies. However, all current GEVIs suffer a low signal-to-noise ratio when tested in more complex neural circuits because the GEVIs are expressed throughout the transformed cells and transformed cells overlap spatially in the field of view. Therefore, new GEVIs must be developed that can bypass this problem and increase the signal-to-noise ratio.

1.4 Soma Localization

A large problem that arises from the use of GEVIs in complex networks in whole animal preparations is the low signal-to-noise ratio. This results in part from fluorescence emission in all areas of the neuron leading to ambiguity in signal source with a given neuron and contamination of signals across neurons. A way to overcome this problem when recording action potentials is to restrict the GEVI to the soma. SK1 is a calcium-activated potassium channel $K_{Ca}2.1$ that contains an internalization inhibition motif within the intracellular carboxy terminus resulting in surface expression of the channel at the soma only. SK1 channels are rapidly internalized in neuronal processes in a clathrin-dependent manner. Fusion of the internalization inhibition sequence of SK1 to a GEVI should result in a soma localized membrane-bound fluorescent protein.

The carboxy sequence and most proximal transmembrane domain of SK1 can be fused to a fluorophore to create a soma-restricted hVoS system. Constructs created in the Principle Investigator's laboratory, FireFluo 2.0C (CeFP), FireFluo 1.0C (CeFP), and FireFluo 1.1G (eGFP), make use of this approach. Also, the internalization sequence of SK1 can be attached to the intracellular tail of an ACE construct (FireFluo 1.2A). The SK1 internalization sequence should result in localization of these ACE constructs to the neuronal soma. The resulting increase in signal-to-noise ratio will allow for greater ease of recording discharge events in complex neuronal circuits in behaving animals.

CHAPTER TWO: METHODS

2.1 Subcloning of Plasmid Constructs

2.1-1 Bacterial Transformation

Initial transformation of the pFLAG-KCa2.1 and hVoS_2.0C_Thy-1 plasmids occurred in DH5α ultra-competent *Escherichia coli* cells (Thermo Fischer Science). 3 µl of each plasmid was added to 30 µl of competent cells and chilled on ice for 30 minutes. The cells were heat shocked at 42° C for 30 seconds in a Precision Microprocessor Controlled 280 Series Water Bath (Thermo Fischer Science), followed by a 5-minute chill on ice. 950 µl of SOC outgrowth medium was added to the cells, which were then shook at 250 rpm/37°C for 1 hour in a New Brunswick Innova 4300 Incubator Shaker (Marshall Scientific). Serial dilutions of 1x, 100x, and 1000x were created in SOC outgrowth medium. 100 µl of diluted sample was placed on an ampicillin-infused agar plate and spread with a flame-sterilized glass spreader. Plates were then stored overnight at in the New Brunswick incubator at 37°C with no rotation.

Sub-sequential transformations involving constructed hVoS plasmids were transformed into DH10β ultra-competent *Escherichia coli* cells (Thermo Fischer Science) and spread on a Kanamycin-infused agar plate. Ace-2n-mNeon transformations occurred using One Shot Stbl3 ultra-competent *Eschericia coli* cells (Thermo Fischer Science), followed by growth on ampicillin-infused agar plates.

2.1-2 Bacterial Miniculture Preparation

Single, isolated colonies from plates were scraped using a sterile toothpick. The entire toothpick was placed in glass test tubes containing 3 ml Luria-Bertani (LB) Broth and 3 µl antibiotic (ampicillin or kanamycin). Tubes were shook at 200RPM/37°C overnight in the New Brunswick incubator.

2.1-3 Isolation of Plasmid by Alkaline Lysis Miniprep

1.5 ml from opalescent minicultures were added to 1.5 cm micro-centrifuge tubes. Cells were pelleted through an 8000RPM/RT/5-minute spin in an Eppendorf 5417R centrifuge (Eppendorf Biotools), followed by supernatant aspiration. The cells were resuspended in a master mix of 100 µl GET buffer and 10 µl RNAse and chilled on ice for 5 minutes. 200 µl of Lysis buffer, 150 µl of Neutralization buffer, and 450 µl of 5M Lithium Chloride were sequentially added with 5-minute ice chills after each addition. Samples were spun at 14000RPM/4°C/5 minute and the supernatants were transferred to new 1.5 cm centrifuge tubes. 650 µl 100% isopropanol was added and the samples were chilled at -80°C for 15 minutes. Samples were spun at 14000RPM/4°C/5 minutes and supernatants were aspirated off. 70% Ethanol was added and the samples were spun again at 14000RPM/4°C/5 minutes. Supernatants were aspirated a final time and the tubes were left to air dry for 25 minutes. DNA was eluted in 32 µl sterile water.

2.1-4 Gel Electrophoresis

Quality of isolated plasmid DNA was observed using agarose gel electrophoresis. Gels were prepared with either a 1.2%, 1.0%, or 0.8% agarose concentration in a tape-enclosed gel tub. DNA bands were visualized using either Ethidium Bromide of SYBR

Safe DNA gel stain (Thermo Fischer Science). Separation occurred in a Mini-Sub GT Cell Electrophoresis Chamber (Bio-Rad) filled with 1x TAE. Electrical current was supplied by PowerPac Basic Power Supply (Bio-Rad). Gels were initially run at 70 mV and increased to 120 mV after dye bands showed clear separation. Gels were observed and imaged in a G:Box fluorescence and chemiluminescence (Syngene) and analyzed using GeneSys software.

2.1-5 Polymerase Chain Reaction

Polymerase Chain reaction using Phusion high-fidelity DNA polymerase (New England Biolabs) was used to amplify the fluorophores and SK1 internalization sequence. The reactions begun with an initial melting temperature of 98°C for 30 seconds, followed by 33 cycles of 98°C denaturing for 10 seconds, 64.5°C annealing for 20 seconds, and 72°C extension for 20 seconds. Products were confirmed and visualized using 1.2% agarose gel electrophoresis with ethidium bromide. Band sizes of 728 bp, 261 bp, and 596 bp appeared for eGFP, CeFP, and the SK1 internalization sequence respectively

2.1-6 Restriction Digest

Restriction digests were performed using the required enzyme to cut each plasmid at specific sites. 10,000 ng DNA, 2 µl selected buffer (2.1 Buffer, 3.1 Buffer, Cutsmart Buffer, New England Biolabs), and 1 µl of each restriction enzyme (Nhe1, SacII, AceI, EcoRI, NotI, New England Biolabs) were added to micro-centrifuge tubes on ice. The tubes were brought up to 20 µl with water. Tubes were incubated in a water bath at 37°C for 1 hour. Samples were analyzed by running them through an agarose gel.

2.1-7 Backbone Linearization

Linearization of the backbone of each construct was performed by restriction digest. Segments were separated by using 0.8% agarose gel electrophoresis visualized by SYBRSAFE (Thermo Fischer Science) gel stain. The backbone was extracted from the gel using a razor blade and was purified using a gel extraction kit (New England Biolabs).

2.1-8 Ligation

Ligation reactions with vector:insert ratios of 1:3 and 1:10 were run. Reactions were run concurrently with control reactions containing vector only or insert only. Reactions were placed in a Microcooler II (Boekel Industries Inc.) at 16°C for 16 hours using T4 Ligase (New England Biolabs) supplemented with ATP. Ligation products were transformed into DH10b cells and resulting colonies were mini-cultured in LB broth as described above. DNA products were analyzed by running a restriction digest at certain sites and comparing gel band sizes with predicted band sizes.

2.1-9 Commercial Sequencing

Sequencing was performed by the commercial company Genscript. Samples of FireFluo 1.0C and 1.1G were sequenced using the sanger method in an ABI 3730xl DNA analyzer (Thermo Fischer Science).

2.2 Transfection into PC12 Cells

Pheochromocytoma line 12 (PC12) cells were plated on a 24-well glass-bottom culture plate (Life Technologies) coated with collagen (50ug/ml) or poly-d-lysine (100ug/ml). 500ng of plasmid DNA per well was preincubated with Lipofectamine-2000

(Life Technologies) in 1/8 the final volume of RPMI media for 30 minutes at room temperature. Plasmid samples were brought up to full volume with warmed media and placed over cells for 6 hours at 37°C. PC12 cells were differentiated by addition of 50ng/ml 2.5s mouse nerve growth factor (Merck Millipore). Cells sat for 2-4 days to allow ample differentiation and expression. Cells were fixed in 1x PEM containing 0,3% glutaraldehyde and observed using a Nikon eclipse 80i confocal microscope.

2.3 Immunocytochemistry

Immunocytochemistry was performed to confirm the surface expression of CeFP and GFP in our constructs. Cells were fixed in 1x PEM containing 0.3% glutaraldehyde. Half of the cell samples were permeabilized using 10% Triton X-100. Glutaraldehyde was quenched using 2 mg/ml sodium borohydride before the addition of 500 µl blocking media (5% donkey serum, 5% goat serum). Anti-GFP was used to detect GFP in FireFluo 2.0C. Antibodies were visualized by conjugation with a red Alexa-Fluor dye and imaged using a Nikon Eclipse 80i confocal microscope.

2.4 Confocal Imaging of Fixed Cells

Images of differentiated PC12 cells were captured using confocal microscopy. FireFluo and control fluorophores emit blue or green light while secondary antibodies used to test surface expression and determine compartment boundaries emit red light. All image analysis was performed with ImageJ (National Institutes of Health). Each image was split into blue, green, and red channels. Regions of Interest (ROIs) were drawn along the soma and processes using the color coding for the control non-localized fluorophore. These regions were then transferred to the images for the other (blue) channel. (Figure 2)

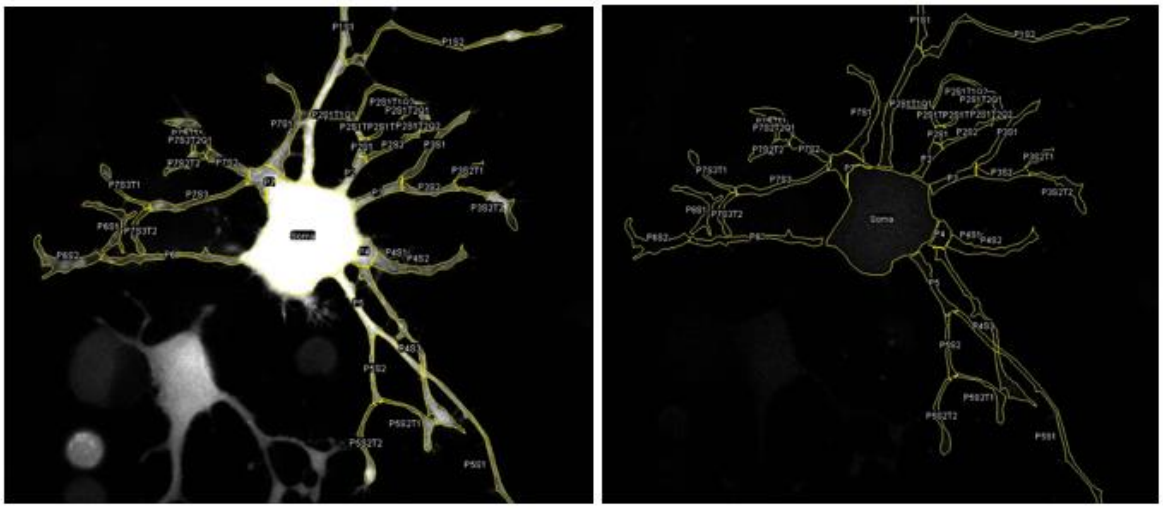

Figure 2: Regions of interest around differentiated PC12 cells.
The images were split into green (left) and blue (right) channels. The green channel shows GCaMP5 expression, while the blue channel shows FireFluo 2.0C expression.

Area, average light intensity, minimum light intensity, and maximum light intensity were recorded from each ROI for all green channels. The backgrounds of each image were measured as well. Each ROI value had the background intensity of its respective image subtracted from it to avoid discrepancies in image brightness differences. The average light intensities of each process ROI were divided by the intensity value for the corresponding soma ROI and averaged according to branch order (i.e. primary, secondary, tertiary, etc.). The average normalized intensity values for the soma and all branch orders were graphed to analyze relative brightness in each process.

CHAPTER THREE: RESULTS

3.1 FireFluo 2.0C

3.1-1 FireFluo 2.0C Construction

Previous work involved the construction of the plasmid FireFluo2.0C in a GFP-N1 backbone (Figure 3).

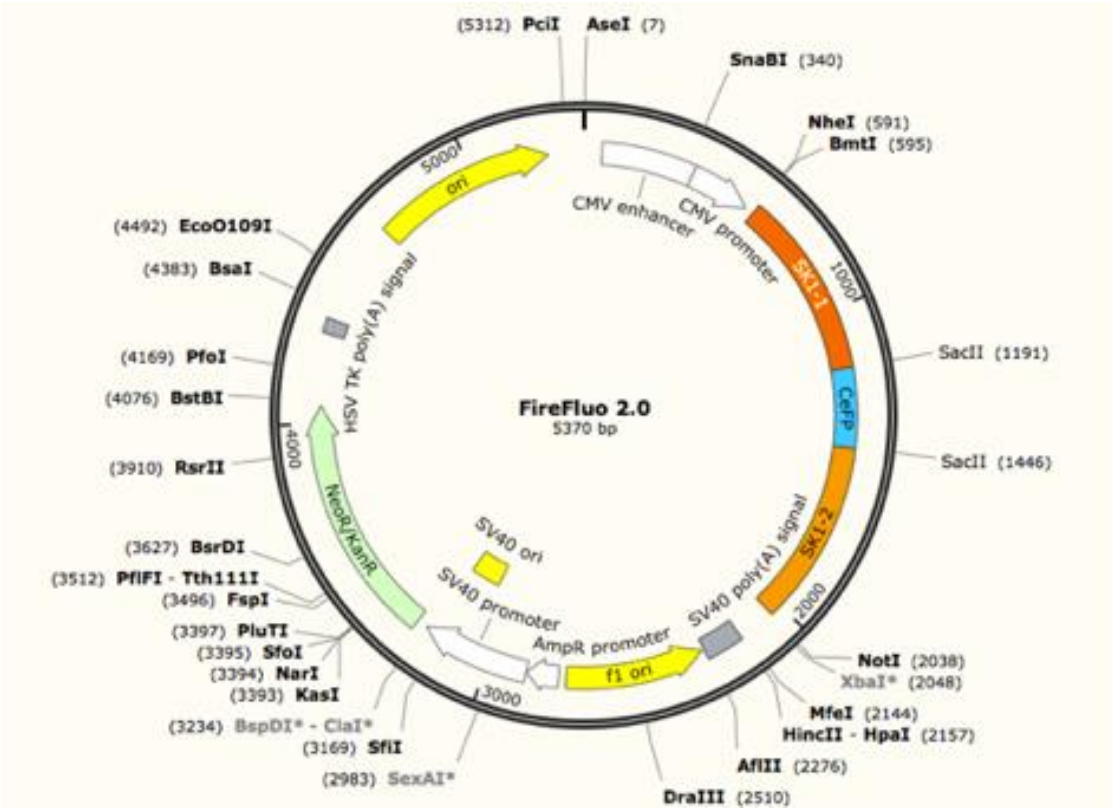

Figure 3: Restriction map of FireFluo 2.0CC.
CeFP is flanked by two adjacent SK1 sequences.

The contents of FireFluo include a CMV promoter, reversed and forward SK1 segments flanking the dNDA for CeFP, and an SV40 polyadenylation sequence. Additionally, the backbone contains an SV40 origin of replication sequence and a kanamycin resistant gene with a HST TK poly(A) sequence. The plasmids pFLAG-KCa2.1 and hVoS_2.0C_Thy-1contained the SK1 sequence, the CeFP sequence, respectively, and were used for PCR. This original Firefluo plasmid was named "FireFluo 2.0C" due to the two SK1 sequences flanking CeFP. This plasmid was initially constructed by a commercial company

The upstream SK1 sequence (SK1-1) is non-functional in this plasmid due to an error in its commercial construction. The complete SK1 sequence was reversed and ligated into the plasmid instead of the corresponding codons. This resulted in the upstream SK1 sequence not being tethered to the cellular membrane. However, the downstream SK1 sequence still anchors CeFP to the membrane along with localizing the fluorophore to the soma.

3.1-2 FireFluo Transfection into PC12 Cells

FireFluo 2.0C was transfected into NGF differentiated PC12 cells co-expressing the calcium sensitive fluorophore GCaMP5 to observe soma localization **(Figure 3)**. Note that GCaMP5 diffuses throughout the entirety of the cell, while FireFluo 2.0C is largely restricted to the soma.

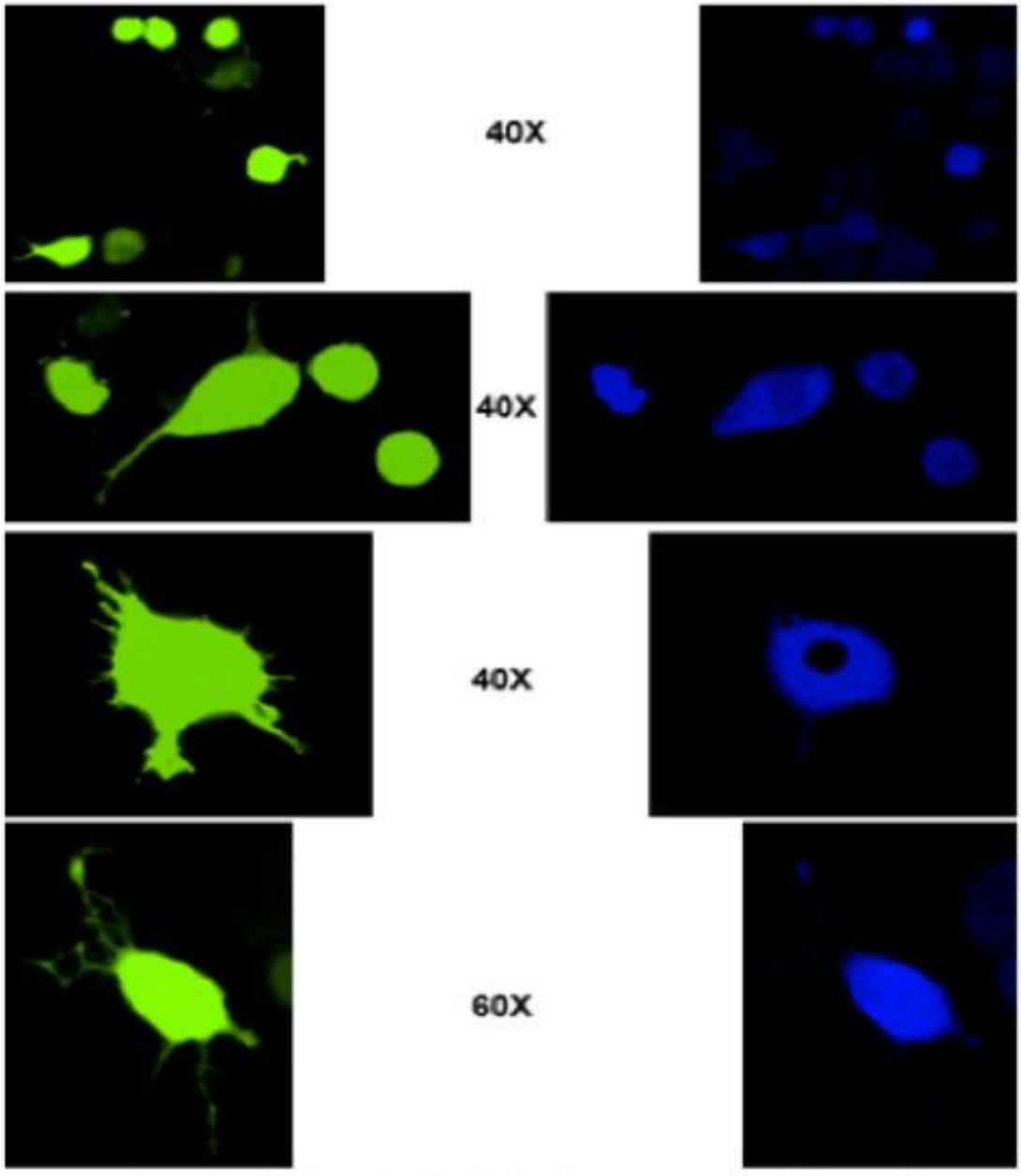

Figure 4: Differentiated PC12 cells co-expressing GCaMP5 and FireFluo 2.0C.
GCaMP5 (green) diffuses throughout the entire cell, while FireFluo 2.0C (blue) restricts to the soma.

Soma Localization was quantified in transfected cells by measuring the mean intensity values of ROIs in blue and green channels. The ROIs of each process were

divided by their respective somas and organized based on branch order. These values were then graphed to show respective process brightness (Figure 5). Two-way repeated measure ANOVA confirmed a significant difference between GCaMP5 and FireFluo 2.0C in secondary, tertiary, and quaternary branches.

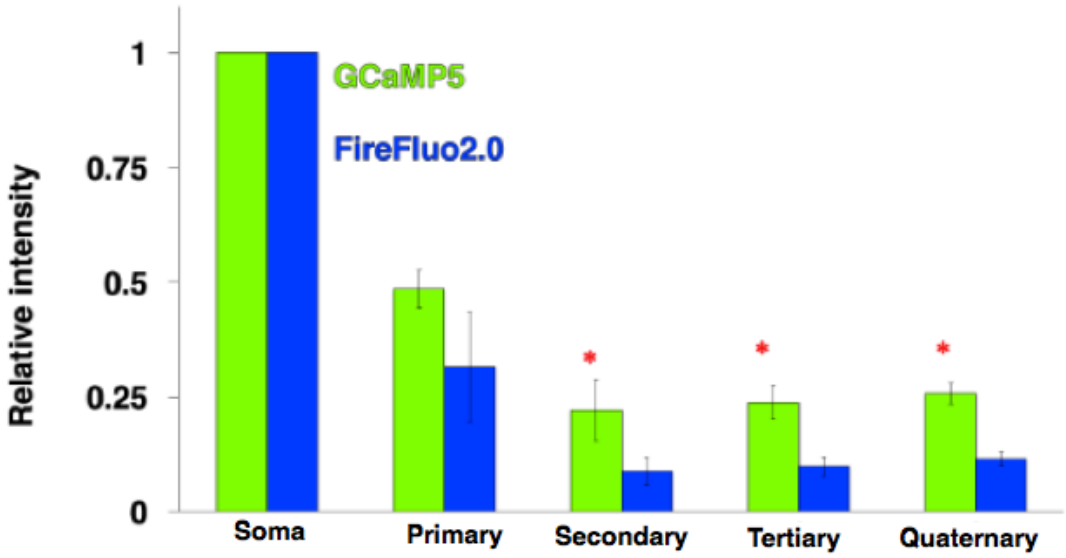

Figure 5: Intensity values of processes normalized to their soma.
Mean intensity values of each process level was normalized to their respective soma intensity. Data was collected from ROIs of cells co-expressing GCaMP5 and FireFluo 2.0C. Secondary, tertiary, and quaternary branches showed significant differences between GCaMP5 and FireFluo 2.0C.

3.2 FireFluo 1.0C and FireFluo 1.1G

3.2-1 FireFluo 2.0C Backbone Extraction

To prepare the backbone for FireFluo 1.0C and 1.1G, the upstream SK1 site and CeFP site were excised from FireFluo2.0C. This involved a restriction digest using the

restriction enzymes NheI and SacII. The extracted gel band was then purified and the isolated DNA was eluted with water. (Gel Extraction kit, New England Biolabs).

3.2-2 PCR and Ligation of Inserts

Polymerase Chain Reaction was performed to amplify the CeFP and eGFP inserts. Unique mutagenic primers were designed having a six-nucleotide platform and an added restriction site. The CeFP and eGFP reaction products were digested with their respective enzymes and ligated into the digested FireFluo 2.0C backbone at the SacII and NheI sites. The ligation reactions were transformed into DH10β ultra-competent *Escherichia coli* cells (Thermo Fischer Science).

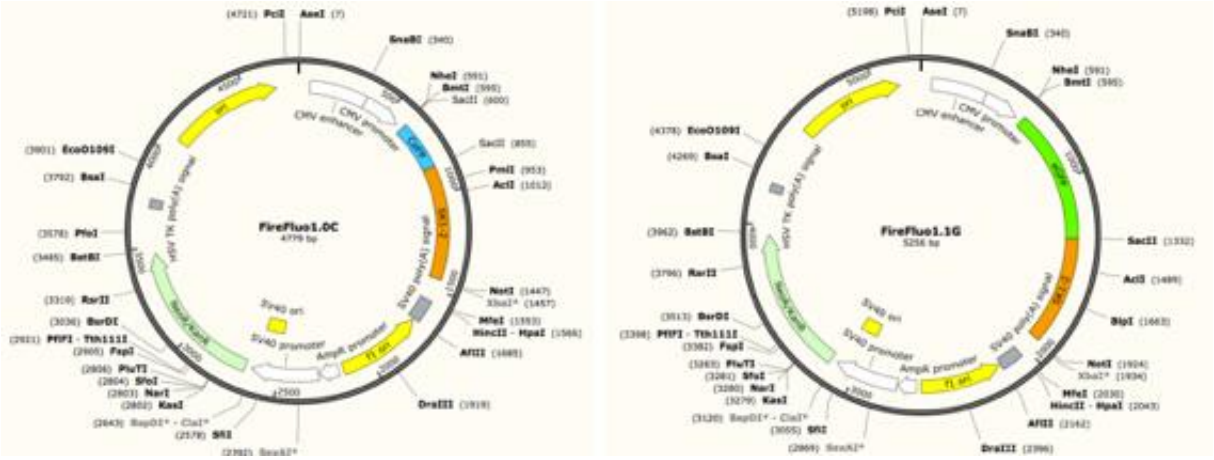

Figure 6: Restriction maps of FireFluo 1.0C and FireFluo 1.1G.
The SK1-1 and CeFP sequences of FireFluo 2.0C were excised and replaced with a new CeFP sequence and an eGFP sequence to make 1.0C (left) and 1.1G (right) respectively.

3.2-3 Preparation of Ligated Product

Minicultures were created by selecting independent colonies on the 1:3 and 1:10 plate with a sterile toothpick. Alkaline Lysis Mini-prep was performed on the minicultures to isolate the plasmid DNA.

3.2-4 Diagnostic Restriction Digests

Restriction digests were performed to confirm positive clones following ligation. All digested samples were run adjacent to uncut samples as controls. The uncut samples display multiple confirmations descriptive of a circular plasmid. FireFluo 1.0C and 2.0C were digested concurrently with Acl1. The Acl1 restriction site lies within the SK1 sequence. Being that FireFluo2.0C has two SK1 sequences, the digest cut the plasmid into two segments of 573bp and 5157bp. When cut, FireFluo 1.0C was linearized into one band at 5130bp due to only one Acl1 site in its single SK1 sequence. FireFluo 1.1G was digested with SacII and Nhe1 simultaneously, separating the eGFP insert from the backbone. This produced two fragments of 743bp and 4513bp.

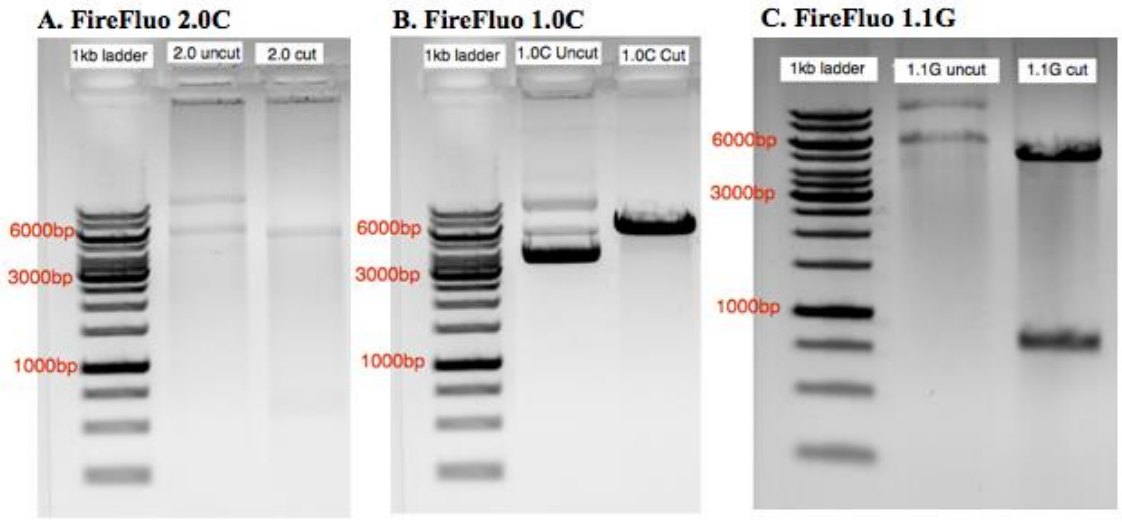

Figure 7: Diagnostic restriction digests of FireFluo 2.0C, FireFluo 1.0C and FireFluo 1.1G.
(A) FireFluo 2.0C was cut with Acl1. Uncut 2.0C shows multiple confirmations of a circular plasmid. Cut 2.0C displays two faint bands at 573bp and 5157bp, as expected.
(B) FireFluo 1.0C was also cut with Acl1. The plasmid linearized at 5130bp
(C) FireFluo 1.1G was cut with SacII and Nhe1. The uncut plasmid shows a circular and supercoiled formation. The cut plasmid shows the expected band sizes of 743 bp and 4513 bp.

3.2-5 Commercial Sequencing

Sequences of FireFluo 1.0C and 1.1G were shipped to the biotechnology company Genscript, where they underwent the sanger sequencing method. A pEGFP-N-5 primer began the sequence around 10 base pairs upstream of the CeFP/GFP ligation site. Constructed plasmids were compared to the expected sequence using Basic Local Alignment Search Tool (BLAST) provided online from the National Center of Biotechnology Information (National Institute of Health). FireFluo 1.0C and 1.1G both expressed a 99% base pair overlap between constructed and expected sequences. (Figures 8 & 9).

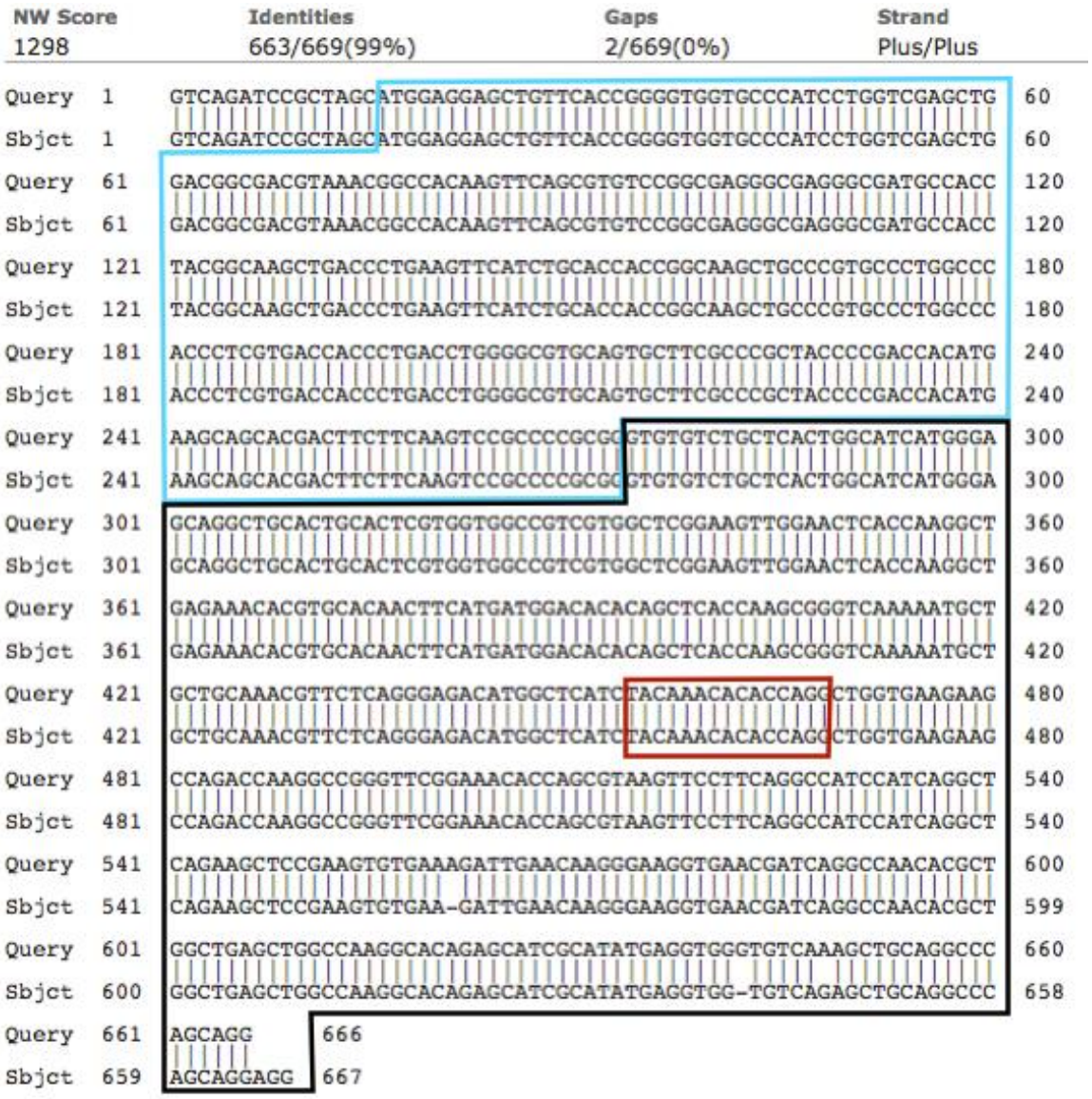

Figure 8: Comparative BLAST of FireFluo 1.0C.
BLAST compared developed construct (Query) and expected sequence (Sbjct) for FireFluo 1.0C. Both sequences share 99% identity matches. Sequence contains inserted CeFP (blue), beginning of SK1 sequence (black), and internalization inhibition motif (red).

Figure 9: Comparative BLAST of FireFluo 1.1G.

BLAST compared developed construct (Query) and expected sequence (Sbjct) for FireFluo 1.1G. Both sequences share 99% identity matches. Sequence contains inserted eGFP (green), beginning of the SK1 sequence (black), and internalization inhibition motif (red).

3.3 Immunocytochemistry to Verify Surface Expression

Immunocytochemistry was performed to determine surface expression of our constructs. Half the cells were treated with Triton-X-100 to permeabilize the membrane. PC12 cells were primarily treated with Anti-GFP, which were both conjugated with a red Alexa-Fluor dye. Triton-X-100 treated cells show uniform expression of GFP. Cells not treated with Triton-X-100 appear to compartmentalize GFP. (Figure 10) These cells possibly indicate surface expression of FireFluo 1.1G.

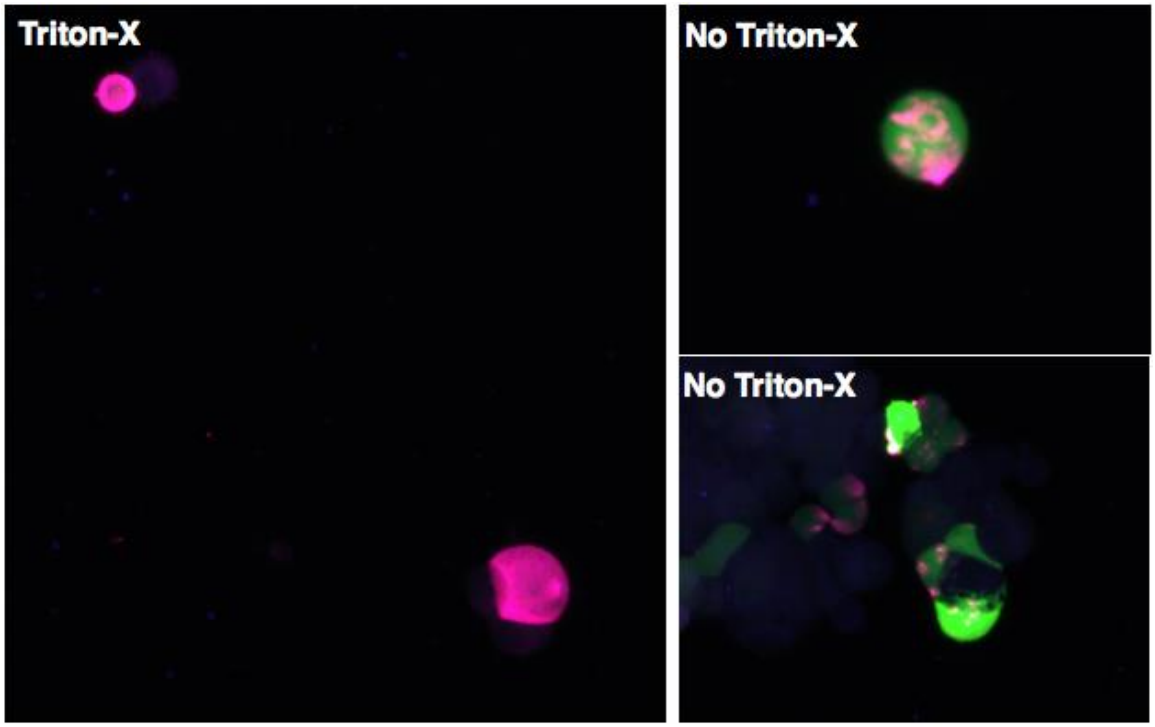

Figure 10: Immunocytochemical labelling of GFP in paraformaldehyde fixed PC12 cells transfected with FireFluo 1.1G

Half of the fixed PC12 cells were treated with 10 µl/ml Triton-X-100 in PBS, while half were treated with PBS alone. Cells were then immuno-stained with an anti-GFP antibody, which was conjugated with red Alexa-Fluor dye. Red labelling represents anti-GFP binding to the construct, while green is the fluorescence of our unbound construct. Triton-X-100 treated cells show uniform expression of the anti-GFP labelled construct. Without Triton-X-100, the anti-GFP labelling is patchy and co-localized with green. This represents surface expression of FireFluo 1.1G.

CHAPTER FOUR: DISCUSSION

4.1 Soma Localization of FireFluo 2.0C

FireFluo 2.0C has been shown to localize to the soma in PC12 cells following transfection. The extent of the localization can be quantified and observed by examining the mean light intensity of each process and normalizing it to its respective soma. The data shown in Figure 4 confirm that normalized mean light intensity is decreased in FireFluo compared to GCaMP5. This indicates the SK1 sequence is in fact localizing the fluorophore to the soma. Additional studies and tweaks must be performed to increase the overall brightness of the fluorophore and further minimize expression in each process. When the upstream SK1 sequence is corrected, it will bind CeFP more tightly to the membrane. This will allow for a higher FRET efficiency with dipacrylamine when due to its closer proximity to the extracellular surface.

4.2 Completion of FireFluo 1.0C and 1.1G

FireFluo 1.0C and 1.1G were successfully constructed using the subcloning techniques described above. The success was confirmed using diagnostic restriction digests and commercial sequencing. The BLAST shows a few innacuracies in sequencing. However, due to their location downstream, this could have been caused by sequencing errors. Multiple alignments may confirm that these are sequencing errors instead of errors in our subcloning techniques. The internalization inhibition motif is unaffected by gaps, deletions, or point mutations. Immunocytochemistry has been

successful in detecting GFP in FireFluo 1.1G. As shown in Figure 8, the patches of GFP may indicate the surface expression of this construct.

The extent of FireFluo 1.0C and 1.1Gs' soma localization has been difficult to confirm. FireFluo 1.1G has expressed fluorescence in transfected PC12 cells, yet a lack of differentiation beyond primary processes has made analyzing the soma restriction difficult. Transfection of FireFluo 1.0C into PC12 cells have been unsuccessful so far, but more transfections and diagnostics are being performed to rectify this.

4.3 Future Work

4.3-1 Completion of FireFluo 1.2A

FireFluo1.2A is currently being developed by fusing the SK1 internalization sequence to the cytosolic tail of Ace-2N-4AA-mNeon. This plasmid has been provided by Dr. Mark Schnitzer's laboratory at Stanford University. This plasmid can only be transfected into Stbl3 *Escherichia coli* cells (Thermo Fischer Science). Stbl3 cells are EndA+, meaning that a nonspecific endonuclease co-purifies with prepared plasmids. This has led to difficulty in successfully preparing the construct prior to digestion and ligation. Methods of removing the endonuclease before it degrades the plasmid are being investigated. Following the successful preparation, the Ace-2N-4AA-mNeon in the absence of the endonuclease, Firefluo 1.2A will be constructed using similar subcloning methods.

The SK1-Carboxy sequence will be amplified by Polymerase Chain Reaction. Restriction digests using EcoRI will create sticky ends on the SK1-Carboxy segment and linearize the Ace-2N-4AA-mNeon plasmid. The SK1-carboxy sequence will then be

ligated into the linearized Ace-2N-4AA-mNeon. The ligation product will be transformed using Stbl3 *Escherichia coli* cells (Thermo Fischer Science), followed by miniculutre creation and alkaline lysis mini-prep to isolate plasmid DNA. These mini-preps will be handled with extreme care to avoid endonuclease activity. This construct will also be transfected into PC12 cells and cultured neurons to analyze its soma localization.

4.3-2 Voltage Sensitivity Diagnostics

Voltage sensitivity of our constructs will be diagnosed following transfection into cultured neurons. Dipacrylamine will be added to cultures containing FireFluo 2.0C, FireFluo 1.0C, and 1.1G to allow fluorescent expression of discharge events. Tetrodotoxin, a sodium channel blocker, and bicuculline, a GABA antagonist, will be used to suppress and stimulate action potentials respectively. The effects will be observed using ultra-high-speed imaging in collaboration with the Cressman lab at George Mason University.

4.4 Possible Applications

4.4-1 Trajectory Events

A possible use of these constructs is the recording and tracing of hippocampal trajectory events. The hippocampus is known to be associated with spatial learning, memory, and decision making. In mice, hippocampal neurons were discovered to act as place cells. Place cells fire only when the mouse is in a specific location in space known as a place field. The coding of each specific neuron in correlation to the animal's place field creates a cognitive map of the animal's environment. When these transient place cell firing patterns are lined up with the animal's navigational movement, two phenomena are

observed: The place cells signal the trajectory the animal just took, and the place cells signal the trajectory the animal is planning to take next. Our constructs could allow improved monitoring of action potential discharges regarding these trajectory events.

4.4-2 Photoacoustic Imaging

A new promising method of data capture is the use of photoacoustic imaging, which can non-invasively provide images of internal tissues or organs. This process utilizes the photoacoustic effect, which is the generation of acoustic waves by absorption of electromagnetic energy. Ionizing laser pulses are fired into tissues which will absorb the oncoming energy. Some of the energy is converted into heat, which leads to ultrasonic wave generation caused by thermal expansion. The waves are detected using ultrasonic transducers, and an image is generated from the information. The combination of GEVIs and photoacoustic imaging can allow the activity of complete neuronal circuits to be observed non-invasively.

4.4-3 Other Compartmentalized Fluorophores

FireFluo's soma localization provides many opportunities for behavioral studies in live animals. The process of ligating a localization domain to a fluorescent protein can be further explored using other cellular compartments. For example, a fluorescent protein can be attached to a PDZ domain that can localize a fluorescent protein to a dendritic synapse. Using compartmentalized fluorophores will allow greater ease in observing specific sections and cellular activities.

4.5 Conclusion

Genetically Encoded Voltage Indicators will lead to novel discoveries in neuronal systems. Localization to the soma will greatly decrease the signal-to-noise ratio, making

observations using GEVIs much easier to interpret. The SK1 sequence of FireFluo has been shown to restrict the location of the surface protein to the soma of differentiated PC12 cells, decreasing the fluorescence of the processes. Different models of FireFluo can be created using different color fluorescent proteins, but more studies must be done to determine their extent of soma localization. Eventually, FireFluo may be used in future in-vivo studies to assist in the understanding of complex neuronal circuits.

REFERENCES

Ziv, Y., Burns, L. D., Cocker, E. D., Hamel, E. O., Ghosh, K. K., Kitch, L. J., ... & Schnitzer, M. J. (2013). Long-term dynamics of CA1 hippocampal place codes. Nature neuroscience, 16(3), 264-266.

Kim TH, Zhang Y, Lecoq J, Jung JC, Li J, Zeng H, Niell CM, Schnitzer MJ. (2016) Long-Term Optical Access to an Estimated One Million Neurons in the Live Mouse Cortex. Cell Rep. 17(12):3385-3394.

Kim TH, Zhang Y, Lecoq J, Jung JC, Li J, Zeng H, Niell CM, Schnitzer MJ. (2016) Long-Term Optical Access to an Estimated One Million Neurons in the Live Mouse Cortex. Cell Rep. 17(12):3385-3394.

Inagaki, S., & Nagai, T. (2016). Current progress in genetically encoded voltage indicators for neural activity recording. Current Opinion in Chemical Biology, 33, 95-100.

Gonzalez, J. E., & Tsien, R. Y. (1995). Voltage sensing by fluorescence resonance energy transfer in single cells. Biophysical journal, 69(4), 1272.

Wang, D., Zhang, Z., Chanda, B., & Jackson, M. B. (2010). Improved probes for hybrid voltage sensor imaging. Biophysical journal, 99(7), 2355-2365.

Gong, Y., Wagner, M. J., Li, J. Z., & Schnitzer, M. J. (2014). Imaging neural spiking in brain tissue using FRET-opsin protein voltage sensors. Nature communications, 5.

Gong, Y., Huang, C., Li, J. Z., Grewe, B. F., Zhang, Y., Eismann, S., & Schnitzer, M. J. (2015). High-speed recording of neural spikes in awake mice and flies with a fluorescent voltage sensor. Science, 350(6266), 1361-1366.

Corrêa, S. A., Müller, J., Collingridge, G. L., & Marrion, N. V. (2009). Rapid endocytosis provides restricted somatic expression of a K+ channel in central neurons. Journal of cell science, 122(22), 4186-4194.

O'Keefe, J., & Dostrovsky, J. (1971). The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. Brain research, 34(1), 171-175.

Pfeiffer, B. E., & Foster, D. J. (2013). Hippocampal place-cell sequences depict future paths to remembered goals. Nature, 497(7447), 74-79.

Xu, M., & Wang, L. V. (2006). Photoacoustic imaging in biomedicine. Review of scientific instruments, 77(4), 041101.

King, J. R., Nordman, J. C., Bridges, S. P., Lin, M. K., & Kabbani, N. (2015). Identification and characterization of a G protein-binding cluster in α7 nicotinic acetylcholine receptors. *Journal of Biological Chemistry*, *290*(33), 20060-20070.

Greene, L. A., & Tischler, A. S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proceedings of the National Academy of Sciences*, *73*(7), 2424-2428.

Pastrana, E. (2013). Sensors and probes: Calcium sensors reach new heights. *Nature methods*, *10*(9), 824-824.

BIOGRAPHY

Ryan J. Purwin graduated from Pomperaug High School in Middlebury, CT in 2010. He graduated from Western Connecticut State University in 2014 with a Bachelor of Arts in Biology and a minor in Chemistry.