IMPROVED MONITORING OF SINGLE UNIT ACTIVITY IN NEURONAL CIRCUITS BY LOCALIZATION OF A HYBRID OPTICAL VOLTAGE SENSOR TO THE SOMA

by

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Improved monitoring of single unit activity in neuronal circuits by localization of a hybrid optical voltage sensor to the soma

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

by

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Bachelor of Science
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DEDICATION

This is dedicated to my loving family, my parents Richard Senior and Daniela, and my younger brother Richard Junior.

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I would like to thank my friends and family who have made this possible. My loving parents, whom supported me as I pursued my dreams and aspirations. My brave brother, who even when met with challenges in his life was there to support me. Dr. Dumas and the other members of my committee, who were an essential part of this process. Michelle Moore, who was an essential part of this thesis project. Finally, thanks to Krasnow Institute of Advanced Learning and all of the other students who aided me in achieving my goal.

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LIST OF ABBREVIATIONS

Accelerated Sensor of Action Potentials 1 ..... ASAP1
Action Potential ..... AP
Bacterial-Stab ..... bac-stab
Base Pair ..... bp
Calcium Ions ..... Ca^{2+}
Cerulean Fluorescent Protein ..... CeFP
Cyan Fluorescent Protein ..... ECFP
Dipicrylamine ..... DPA
Ethidium Bromide ..... EtBr
Fluorescence Resonance Energy Transfer ..... FRET
Fluorescent Protein ..... XFP
Fluorescent Shaker ..... FlaSH
Forward Primer ..... F'
Genetically Encoded Calcium Indicators ..... GECI
Genetically Encoded Voltage Indicators ..... GEVI
Green Fluorescent Protein ..... EGFP/GFP
Hybrid Optical Voltage Sensor ..... hVoS
Immunocytochemistry ICC
Light-Emitting Diode ..... LED
Luria-Bertani ..... LB
Miniature Excitatory Postsynaptic Currents ..... mEPSCs
Polymerase Chain Reaction ..... PCR
Reverse Primer ..... R'
Small Conductance Calcium-Activated Potassium Channel ..... SK Channel
SK Channel Gene 1 ..... SK1
Sodium Channel Protein-Based Activity Reporting Construct ..... SPARC
Teal Fluorescent Protein ..... TeFP
Transmembrane Domain ..... TMD
Voltage Sensitive Dye ..... VSD
Yellow Fluorescent Protein ..... EYFP/YFP

ABSTRACT

IMPROVED MONITORING OF SINGLE UNIT ACTIVITY IN NEURONAL CIRCUITS BY LOCALIZATION OF A HYBRID OPTICAL VOLTAGE SENSOR TO THE SOMA

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George Mason University, 2016

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Electrical activity of neurons traditionally has been measured using electrophysiological methods. In this manner, numerous important concepts regarding relationships between activity dynamics in neuronal circuits and cognitive abilities have been resolved. However, a technological ceiling has been reached in trying to apply electrophysiological recordings to monitor large numbers of neurons simultaneously as an animal performs a behavioral task, limiting further discoveries. Voltage- and calcium-sensitive dyes (VSDs) proved successful in increasing the recording throughput, providing the impetus to develop genetically encoded probes. Genetically encoded voltage and calcium sensors allow for cellular specificity with a few constructs displaying the temporal resolution required to track action potentials (APs) in neural circuits, including the hybrid optical voltage sensor (hVoS) system. The poor signal-to-noise ratio, due to cell-wide expression, limits the specificity of neuronal recordings, leading us to develop a novel

hVoS sensor (termed Firefluo) where the membrane-anchored fluorescent protein is fused to the carboxy terminus of the SK1 potassium channel, directing surface expression to the neuronal soma only. It is believed that Firefluo will greatly increase the signal-to-noise ratio for AP dynamics in large neuronal populations by removing background noise from neuronal processes and more tightly coupling the fluorophore to APs due to it's soma localization.

CHAPTER 1: INTRODUCTION

1.1 Neuronal Recordings

In order to achieve a better understanding of the function of neuronal circuits, it is imperative to be able to record from large number of neurons simultaneously. Numerous electrophysiological and optical approaches to neural recording have been attempted (Wang, McMahon, et al. 2012; Wang, Zhang, et al. 2010). Advancement in the functional recording from near complete neural circuits will lead to a more complete understanding of how the brain enables learning and memory.

In the 1970s, neural recordings facilitated methods of measuring the electrical response of a single neuron by manipulation and fixation of tetrodes into the brain (Bai and Wise 2001; O'Keefe and Dostrovsky 1971). This technique yielded the ability to record dozens of isolated cells in behaving animals leading to the identification of "place cells", neurons that code for locations in space and elements of a cognitive mapping system. Since then, investigators have implanted up to 40 independently adjustable tetrodes which allowed for simultaneous recording from roughly 250 hippocampal neurons enabling identification of neural dynamics related to route planning (Pfeiffer and Foster 2013). In essence, the monitoring of increased number of neurons allows for more sophisticated discoveries of neural circuit phenomena. However, a ceiling to the number

of neurons recorded simultaneously has been reached using electrophysiological approaches and new techniques are required.

In order to record the electrical output from larger networks, new methods were developed including optical imaging (Larsch, et al. 2013). Optical imaging of neurons measures voltage changes as changes in fluorescence. This methodology includes the early voltage-sensitive dyes (VSDs), genetically encoded calcium indicators (GECIs), genetically encoded voltage indicators (GEVIs), and most recently hybrid optical voltage sensors (hVoS) (Kaestner, et al. 2015). Many of these methodologies take advantage of fluorescence resonance energy transfer (FRET) interactions, which links the intensity of fluorescence to changes in membrane potential. For the hVoS system, FRET interactions occur between a synthetic fluorescent label near the membrane surface and a negatively charged, lipophilic fluorophore housed within the hydrophobic core of the lipid bilayer (González and Tsien 1995) (Figure 1).

1.2 Voltage Sensitive Dyes

The earliest method of optical imaging is staining the cell membrane with VSDs (Wang, Zhang, et al. 2010). Older VSDs produce linear responses to voltage changes with microsecond response times (Jin, Zhang and Wu 2002). Newer VSDs are based on FRET interactions, which have the potential to yield much larger fractional changes in fluorescence than older dyes, improving the signal to noise ratio (Jin, Zhang and Wu 2002). However, VSDs stain cells indiscriminately and excess dye remains in the solution bathing the tissue creating a large amount of background noise with no cell type

specificity, hindering accurate interpretation of images recorded in complex neural tissues (Chanda, et al. 2005; Wang, Zhang, et al. 2010).

1.3 Genetically Encoded Calcium Indicators

GECIs provide faster response times measuring changes to intracellular calcium ions (Ca^{2+}) in neurons (Akerboom, et al. 2012). GECIs allow simultaneous detection of activity in large or disperse populations of neurons, reduce mechanical disturbance of brain tissue, and have proven invaluable for investigating AP firing, synaptic input, and activities of neuronal somata (Akerboom, et al. 2012; Chen, et al. 2012). *In vivo* studies involving GECIs, such as GCaMPs, were accomplished using a high-speed, miniaturized epi-fluorescent microscope comprised of light-emitting diodes (LEDs) and nanofabricated semiconductor sensors (Alivisatos, et al. 2013). The microscope, weighing less than 2.0 grams, was easily mounted on the head of a behaving adult mouse (weighing about 20-30 grams) for monitoring of calcium dynamics in approximately 1000 neurons per mouse (Alivisatos, et al. 2013). GCaMPs expression in the hippocampus coupled with this miniaturized microscope allowed for recording of the same neurons across many days and showed that a vast majority of place cells are very stable across time and only a subpopulation displays task-related adjustments (Chen, et al. 2012). GECIs offer many advantages in advancing monitoring of neural circuits, but are still too slow to keep pace with individual AP (bursts of APs are necessary to raise intracellular calcium to a level that can be detected with this system). Thus, the neural correlates of route planning would not be observable with this method because it involves sparse encoding (Chen, et al. 2012).

1.4 Genetically Encoded Voltage Indicators

GEVIs have the potential to track individual APs in specific neuronal cell populations (Wang, Zhang, et al. 2010). GEVIs evolved from early models including FlaSH voltage sensor (Siegel and Isacoff 1997), and SPARC voltage sensor (Ataka and Pieribone 2002), to more sophisticated models such as ArcLight (Jin, et al. 2012) and Accelerated Sensor of Action Potentials 1 (ASAP1) (St-Pierre, et al. 2014). The fluorescent intensity of ArcLight in response to 100mV depolarization, increased by 35%, which is more than five times larger than signals from previously reported fluorescent protein voltage sensors (Jin, et al. 2012). ASAP1 voltage sensor was able to reliably detect single APs, and subthreshold potential changes. This sensor is also able to track AP waveforms up to nearly 200 Hz in a single trial, but the fluorescent signal is degraded at higher frequencies to due to a suboptimally slow recovery time (St-Pierre, et al. 2014). These probes need to be further refined in order to produce larger fluorescent changes and, faster response times, perhaps via localization of the fluorophore to selected neuron compartments (Wang, Zhang, et al. 2010).

1.5 Hybrid Optical Voltage Sensors

Three common approaches to achieving the FRET interaction are as follows: fusing the two fluorescent proteins to the end of the voltage-sensing domain of a voltage gated ion channel (Sakai, et al. 2001); fusing the two fluorescent proteins to the end of a voltage-sensing domain of a protein phosphatase (Tsutsui, et al. 2008); or a diffusible acceptor molecule that becomes embedded in the cytosolic membrane. The latter, hybrid optical voltage sensors (hVoS), are largely based on the FRET method developed by González and Tsien, and are currently the most promising system for neural recordings in

large networks (Wang, Zhang, et al. 2010; González and Tsien 1995). hVoS preserves the fundamental FRET interaction utilized by VSDs by replacing the surface label with a membrane-anchored enhanced GFP, and the lipophilic fluorophore with dipicrylamine (DPA), a synthetic FRET acceptor and voltage-sensing molecule that partitions into the plasma membrane (Wang, Zhang, et al. 2010; Chanda, et al. 2005). DPA shuttles quickly across the cell membrane, which is translated via FRET into a large changes in the fluorescence signal (up to 34% change per 100 mV), with a fast response and recovery time (0.5ms) (Chanda, et al. 2005).

Advantages of the hVoS method are due to the large signal to noise ratio and the fast response time of the fluorescent signal. Improvements on previously created hVoS probes were pursued by the lab of Meyer B. Jackson, who generated a number of XFP variants with different optical properties and membrane attachment motifs with the most promising construct being the GAP43-CeFP-t-h-ras construct, designated hVoS 2.0 (Wang, Zhang, et al. 2010). The cerulean fluorescent protein (CeFP) used in hVoS 2.0 is a truncated cyan fluorescent protein (ECFP) variant. Further refinement to improve the specific monitoring of APs *in vivo* can be accomplished by localizing the membrane-anchored XFP to the site of AP initiation.

1.6 Localization of Hybrid Optical Voltage Sensors

This study seeks to improve upon Jackson's hVoS 2.0 construct for recording APs *in vivo* by targeting the fluorophore to the neuronal soma. This compartmentalization serves to increase the efficiency of voltage-fluorescence coupling by placing the XFP at the site of AP origination and improves the signal to noise ratio by eliminating dendritic

and axonal fluorescence. Our novel hVoS sensor (termed FireFluo) contains a membrane-anchored CeFP fused on either end to the last transmembrane domain and carboxy terminus of the $K_{Ca}2.1$ (SK1), a calcium-activated potassium channel. Fusion of this SK1 fragment results in localization of CeFP to the neuronal soma excluding it from processes (Corrêa, et al. 2009; Fletcher, Bowden and Marrion 2003).

The FRET interaction of FireFluo corresponds to the extracellular expression of the hVoS probes investigated by Chanda et al., in 2005. The FRET interaction is produced by the spectral overlap of CeFP emission, and DPA absorbance (Wang, Zhang, et al. 2010). The voltage change that occurs during the firing of AP drives DPA across the membrane, modifying the distance of the donor and acceptor (Figure 1).

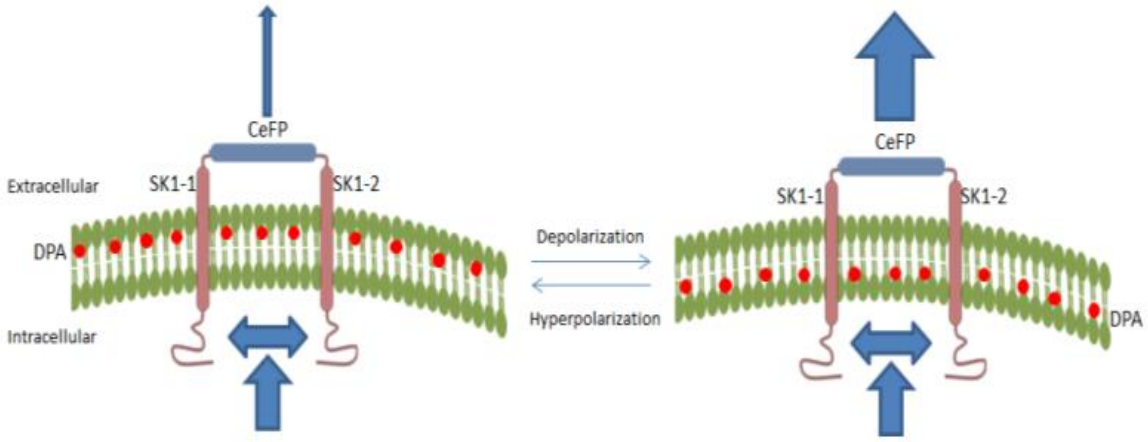

FIGURE 1 Schematic illustration of the FireFluo method. Membrane hyperpolarization (left) leads to an accumulation of DPA on the outer leaflet of the cell, quenching the fluorescence produced by CeFP. Subsequent depolarization (right) translocates DPA to the inner leaflet of the membrane, which reduces the FRET interaction and leads to an increase in fluorescence intensity of CeFP.

CHAPTER 2: MATERIALS AND METHODS

2.1 Construct Design

The SK1 sequence was isolated from the N-terminal pFLAG-tagged rat $K_{Ca}2.1$ (pFLAG-$K_{Ca}2.1$) plasmid kindly provided by Neil V. Marrion (School of Physiology and Pharmacology, University of Bristol; Bristol, UK), as previously described (Fletcher, Bowden and Marrion 2003). The hVoS_2.0_Thy-1 plasmid was purchased from Addgene and used to isolate the CeFP sequence for FireFluo. Meyer B. Jackson (Department of Neuroscience, University of Wisconsin; Madison WI) kindly provided the pEGFP-N1 plasmid.

The plasmid design includes two SK1 sequences in opposing orientations, flanking a CeFP sequence and functioning as soma-specific transmembrane anchors. The plasmid insert starts with a truncated SK1 sequence in the C-terminus to N-terminus orientation (SK1-1), which serves as one side of the transmembrane anchor. Downstream of SK1-1 is the CeFP sequence, which is followed by the second truncated SK1 sequence in the N-terminus to C-terminus orientation (SK1-2); this anchors the other side of the CeFP sequence to the membrane. The entire cassette was constructed in the vector plasmid pEGFP-N1 provided by Meyer B. Jackson (Department of Neuroscience, University of Wisconsin; Madison WI) (Figure 2).

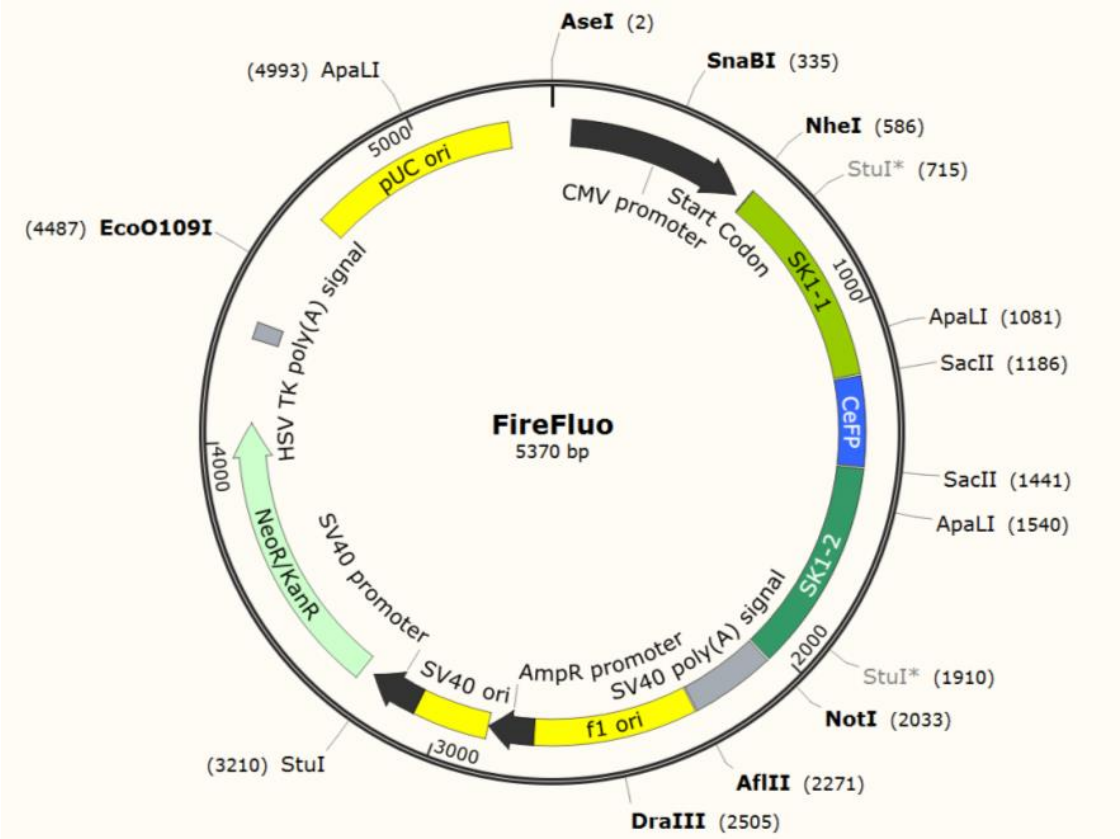

FIGURE 2 FireFluo construct map. SK1-1, SK1-2, and CeFP were cloned into the vector plasmid pEGFP-N1 producing FireFluo. The numbers indicate the nucleotide position. Note that the SK1-1 fragment is inserted and expressed in a 3' to 5' orientation and includes an ATG start site at the 3' end.

The FireFluo plasmid begins at the AseI restriction site, followed by an immediate early promoter of CMV (P_{CMV}) from pEGFP-N1. The SK1-1_CeFP_SK1-2 sequence was inserted using the NheI and NotI restriction sites, effectively excising the multiple cloning site (MCS), ATG start codon, and EGFP from pEGFP-N1. An ATG start codon was added to the sequence after the NheI restriction site prior to the SK1-1 sequence. Downstream of the NotI restriction site are the SV40 polyadenylation signals, which direct proper processing of the 3' end of the mRNA. The entire construct (minus

the CMV promoter) can be excised using the NheI and AflII restriction sites for ligation into the pTRE-HA vector to create transgenic animals.

2.2 Primer Design

The mutagenic PCR primers consisted of a four to six bp platform sequence, a restriction site, and an eighteen bp hybridization sequence. SK1-1 was inserted into the pEGFP-N1 vector by NheI and SacII restriction sites. SK1-2 was inserted using SacII and NotI restriction sites. The SacII restriction site was used to trap the CeFP between SK1-1 and SK1-2 in the FireFluo plasmid. Table 1 shows primer designs for SK1-1, SK1-2, and CeFP.

TABLE 1 hVoS probes for evaluating performance. Oligonucleotide primers were used for assessing hVoS performance. The sequences that encode the restriction sites are shaded in light gray and the ATG start codon is highlighted in dark gray. The nucleotides to the left of the RS are the leader sequence and are removed in the final construct. The left column indicates how these primers are referred to in the text.

Designation	Restriction Site	Probe Design
SK1-1 F'	SacII	TATGGGCCGCGGGTGTGTCTGCTCACTG
SK1-1 R'	NheI	AATGGCGCTAGCATGTCACCCACAGTCTGATC
SK1-2 F'	SacII	TATGGGCCGCGGGTGTGTCTGCTCACTG
SK1-2 R'	NotI	AATGGCGGCCGCTCACCCACAGTCTGATC
CeFP F'	SacII	GTGAGCCCGCGGGAGGAGCTGTTCACCGGG
CeFP R'	SacII	GCCTTCCCGCGGGGCGGACTTGAAGAAGTC

2.3 Bacterial Cell Transformation

pFLAG-K_{Ca}2.1 (SK1), hVoS_2.0_Thy-1 (CeFP), and pEGFP-N1 (GFP-N1) were transformed into NEB 5-alpha competent *E. coli* (high efficiency). pUC19 was transformed into NEB 5-alpha competent cells as a positive control, and non-transformed cells were used at the negative control. The cells were plated at 1x, 100x, and 1000x

dilutions, and allowed to grow overnight at 37^{o}C on LB agar plates with ampicillin or kanamycin.

2.4 Mini-Culture

Mini-cultures were grown in LB broth with ampicillin or kanamycin in a rotating incubator at 37^{o}C at 200 rpm. SK1 is a low copy plasmid and was allowed to grow for 20 hours, CeFP and pEGFP-N1 were high copy plasmids and were grown for 16 hours. Single colonies, from the hVoS_2.0_Thy1, pFLAG-K_{Ca}2.1, and pEGFP-N1 transformation plates, were used to spike mini-cultures.

2.5 Alkaline Lysis Miniprep

Isolation of the plasmid DNA, was performed by alkaline lysis mini preps of the mini-cultures. A spectrophotometer was used to determine the concentration and purity of the mini prep samples using the 260 nm and 280nm wavelengths as well as the 260:280 ratio.

2.6 Gel Electrophoresis

Visualization of DNA was done on a 0.8-1% agarose gel, containing ethidium bromide (EtBr) or SYBR safe, in 1x TAE buffer (20mM Tris Acetate, 10mM sodium acetate, 0.5mM EDTA). Electrophoresis was performed at beginning voltage of 90 V for 10 minutes and sped up using a final voltage of 120 V.

2.7 Polymerase Chain Reaction (PCR)

Isolation and amplification of SK1-1, SK1-2, and CeFP was achieved by PCR using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific; Waltham, MA). The PCR reaction was initially denatured at 98^{o}C for 30 seconds and ran for 33 cycles

with a denaturing temperature of 98^{o}C for 10 seconds, annealing of 64^{o}C 20 seconds, and extension of 72^{o}C of 20 seconds. PCR products were separated on a 1% agarose gel visualized with SYBR Safe (ThermoFisher Scientific; Waltham MA) and excised using a non-UV protocol. PCR products were purified using a gel extraction kit (Introvigen; Grand Island, NY).

2.8 Restriction Digest

Restriction digest of pEGFP-N1 was performed using NheI/NotI. SK1-1 was digested with NheI/SacII, SK1-2 was digested with SacII/NotI, and CeFP was digested with SacII. The double digests were visualized in a 1% agarose gel with uncut and single digest controls. The expected bands were excised from the gel using a non-UV protocol and purified with a PCR clean-up kit (Introvigen; Grand Island NY). The samples were then frozen for use in a ligation reaction.

2.9 Vector Ligation

Ligations are performed using T4 DNA ligase incubated at 16^{o} C for 16 hours, with heat inactivation at 65^{o}C for 10 minutes. Ligation products were visualized using gel electrophoresis. Two ligation strategies were attempted; a two-step ligation and a single step ligation reaction.

The two-step ligation reaction ligated SK1-1 and SK1-2 into pEGFP-N1 during a single reaction. SK1-1, SK1-2, and pEGFP-N1 restriction digest products were visualized with EtBr and extracted from a 1% agarose gel for use in the ligation reaction. Ligation was attempted using a 1:3 and 1:10 vector to insert ratio. The ligation products were transformed into NEB 5-alpha competent *E. coli* (High Efficiency). Plasmid

identity was tested using restriction enzyme digestion, with the original plasmids as controls. CeFP was extracted from an EtBr agarose gel in preparation for the second ligation reaction.

The single step ligation reaction ligated SK1-1, SK1-2, and CeFP into pEGFP-N1. SK1-1, SK1-2, CeFP, and pEGFP-N1 restriction digest products were visualized using SYBR Safe (ThermoFisher Scientific; Waltham MA) and extracted from a 1% agarose gel using a non-UV protocol for use in the ligation reaction. Ligation was attempted using a 1:1:1:1 and 1:1:1:2 vector to inserts ratio. The 1:1:1:1 ligation ratio was attempted at 0.02pmol and 0.04pmol concentrations. The ligation products were transformed into NEB 5-alpha competent E. coli (High Efficiency). Plasmid identity was tested using restriction enzyme digestion, with the original plasmids as controls.

2.10 Commercial Company Ligation

GenScript is the biotech research service company used to facilitate the synthesis of SK1-1, CeFP, and SK1-2 as a single unit for subcloning in pEGFP-N1 with kanamycin resistance gene. pEGFP-N1 was prepared in three 10µL aliquots at 100ng/µL concentration spotted on filter paper for shipping to GenScript. The finalized FireFluo sequence was also provided to the company for plasmid verification through sequencing (Figure 3). The finalized product was ordered as plasmid DNA and as a bacterial-stab (bac-stab).

The standard delivery was comprised of one vial containing approximately 4 µg of lyophilized plasmid DNA which was stored at -20^{o}C for long-term storage. The plasmid DNA was prepared for transformation following the protocol provided by

GenScript. The vial was centrifuged at 6,000 x g at 4^{o}C for 1 minute, 20 µL of sterile water was added, placed on the vortex for 1 minute, and heated at 50^{o}C for 15 minutes. The plasmid DNA was then transformed into *E. coli* TOP10 and plated on kanamycin selection plates. The bac-stab comprised of the gene product transformed into *E. coli* TOP10 cells which were placed in short-term storage at 4^{o}C. LB was added to the bac-stab and plated on kanamycin selection plates. Plasmid identity of the plasmid DNA and bac-stab was tested by restriction enzyme digestion with AseI and NotI. The restriction digest was performed using uncut and single digest controls.

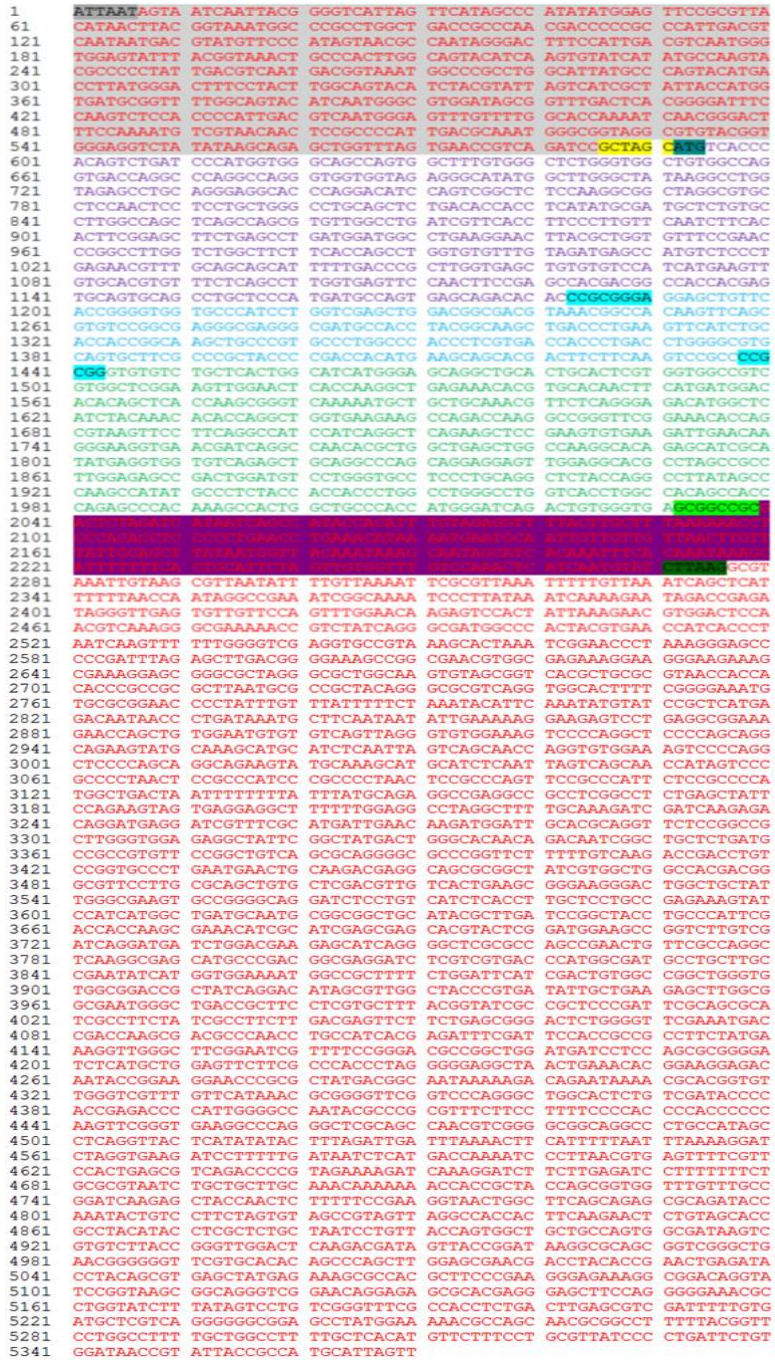

FIGURE 3 The FireFluo plasmid sequence. The FireFluo final construct sequence is presented with color coding to highlight key components. Red text is the pEGFP-N1 vector sequence. The pCMV promoter region of pEGFP-N1 is highlighted in light gray, the start codon is highlighted in teal, and the PolyA SV40 is highlighted in purple. SK1-1, CeFP, and SK1-2 sequences are in purple, blue, and green respectively. Restriction sites are highlighted in black text. AseI, AflII, NheI, SacII, and NotI are highlighted in dark gray, dark green, yellow, blue, and bright green respectively.

2.11 Preliminary Evaluation

The FireFluo plasmid was transformed into DH5alpha cells (New England Biolabs) on kanamycin resistance plates with coverslips embedded in the agar for preliminary evaluation of fluorescence. Twenty-four hours after plating the coverslips were extracted from the agar and placed on microscope slides. The transformed colonies were visualized by bright field and epifluorescent microscopy (CFP-experimental and GFP-control filters) using a QI Imaging Retiga EXi Fast 1394 camera (2,000 frames per second) provided by J.R. Cressman's Lab (George Mason University, Department of Neuroscience, Fairfax, VA) in the absence of DPA. Additional evaluation of fluorescence was performed by M.B. Jackson's laboratory. FireFluo was expressed in PC12 cells by electroporation. A CCD-SMQ camera (Redshirt Imaging, Decatur, GA) mounted on an Olympus BX51 fluorescence microscope equipped with ECFP/CeFP, EGFP, and EYFP filter cubes was used to image fluorescence (Wang, Zhang, et al. 2010).

CHAPTER 3: RESULTS

3.1 Uncut Plasmids

The pEGFP-N1, pFLAG-K_{Ca}2.1, and hVoS_2.0_Thy-1 transformations were grown on ampicillin selection plates. Individual colonies from the 1000x diluted transformation plates were selected for mini-culture preparation. Uncut plasmids were run on a 1% agarose gel to serve as a baseline for PCR and digest reactions (Figure 4). Uncut plasmids typically display between two to four bands. Bands produced by uncut plasmids typically consist of supercoiled and relaxed/nicked plasmid, however, bands for linear and single stranded circular plasmids are also apparent.

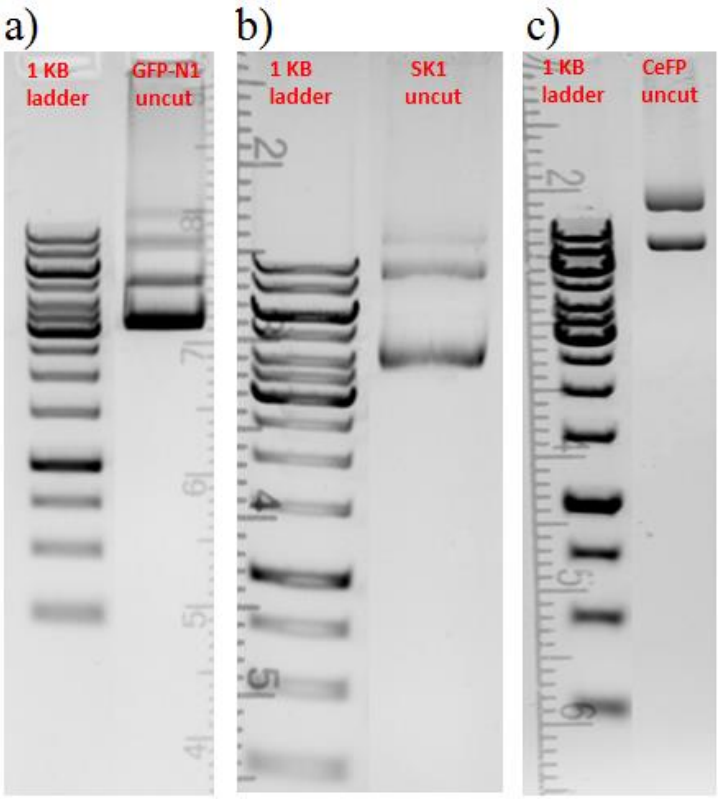

FIGURE 4 Uncut donor plasmids. Uncut donor plasmids; pFLAG-K_{Ca}2.1, hVoS_2.0_Thy-1, and pEGFP-N1 were run through three different 1% agarose gels. A 1 kb ladder was included for reference. **a)** pEGFP-N1 uncut plasmid exhibiting 4 bands **b)** SK1 uncut plasmid exhibiting 3 bands **c)** CeFP uncut plasmid exhibiting 2 bands.

3.2 pEGFP-N1 Restriction Digest

pEGFP-N1 was prepared for ligation by restriction digest with NheI and NotI including uncut and single digest controls (Figure 5a). The restriction digest of pEGFP-N1 with NheI and NotI produced two bands: the pEGFP-N1 backbone consisting of 4,000 bp and the EGFP fragment of 700 bp. The 4,000 bp band was extracted from the agarose gel, and purified using a gel extraction kit. The purity of the sample was determined by gel electrophoresis, which produced a single band at 4,000 bp (Figure 5b).

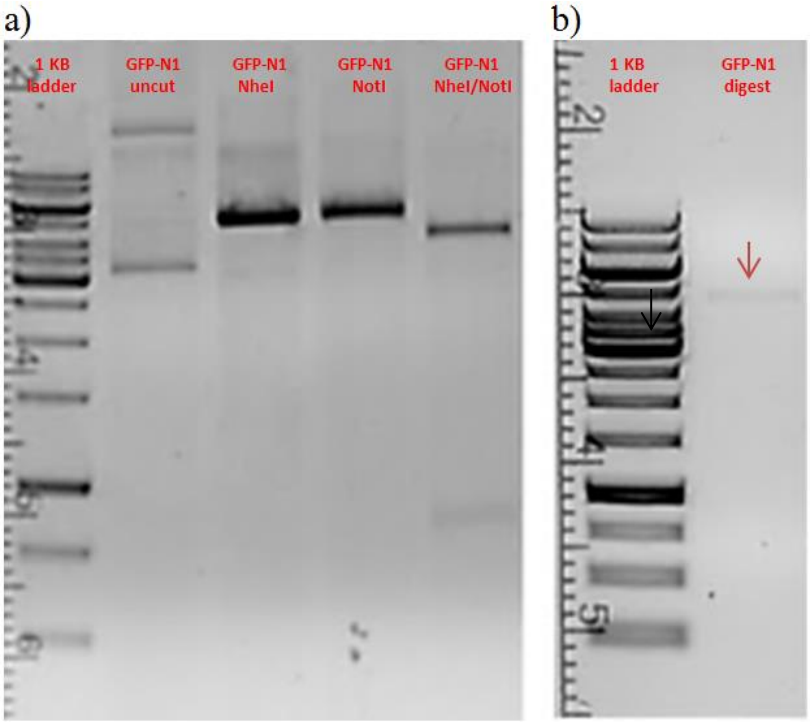

FIGURE 5 Characterization of pEGFP-N1. Restriction enzyme analysis of pEGFP-N1 and subsequent gel band extraction were examined by 1% agarose gels **a)** pEGFP-N1 was digested with NheI and NotI in single digest controls ensuring proficient cutting of the enzyme, and a double digest for extraction of the 4 kb band, **b)** the extracted 4 kb band (indicated by the arrow) was run through another gel to ensure purity of the sample. A 1 kb ladder was included in both gels for reference.

3.3 SK1-1, SK1-2, and CeFP PCR

Isolation and amplification of SK1-1, SK1-2, and CeFP fragments were accomplished using a PCR protocol. The isolation of SK1-1 and SK1-2 from pFLAG-K_{Ca}2.1 was accomplished using the SK1-1 F'/R' primers and SK1-2 F'/R' primers respectively. CeFP was isolated from hVoS_2.0_Thy-1 using the CeFP F' and CeFP R' primers. A comprehensive list of primers is included in Table 1. The PCR products were run through a 1% agarose gel to verify fragment identity and length. The expected band lengths of 600 bp for SK1-1 and SK1-2 and 261 bp for CeFP were verified (Figure 6a).

A restriction digest was performed on the PCR products in preparation for ligation: SK1-1 was digested with NheI and SacII; SK1-2 was digested with SacII and NotI; and CeFP was digested with SacII. All three digests were run through an agarose gel, and the appropriate band sizes were extracted. Purity of the extraction was verified by gel electrophoresis (Figure 6b).

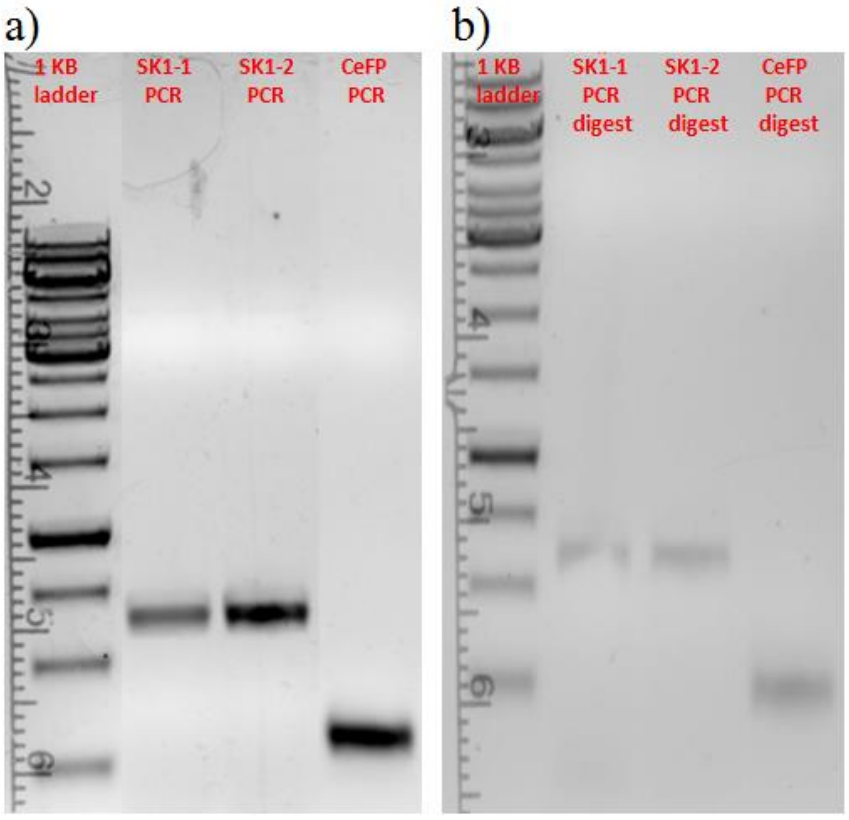

FIGURE 6 Analysis of PCR amplification products. SK1-1, SK1-2, and CeFP PCR products and restriction enzyme digests were run through a 1% agarose gel; **a)** SK1-1 uncut, SK1-2 uncut, and CeFP uncut PCR products (lane 2, 3, and 4 respectively), **b)** Gel extraction of: SK1-1 digested with NheI and SacII, SK1-2 digested with SacII and NotI, and CeFP digested with SacII (lane 2, 3, and 4 respectively).

3.4 Two-Step Ligation

The two-step ligation reaction was accomplished using pEGFP-N1, SK1-1, and SK1-2 extracted from a 1% agarose gel in the presence of EtBr. The transformations were attempted using a 1:3 (0.02 pmol:0.04 pmol) and 1:10 (0.02 pmol:2.0 pmol) vector to insert ratio. The completed ligation reaction was transformed into NEB 5-alpha cells with appropriate controls. Individual colonies from the transformation plates were selected for mini-culture preparation.

Identity of the transformed plasmid was tested using restriction digest with NheI and NotI (Figure 7). The uncut plasmid displayed 3 bands. The single digest controls were expected to produce a single band, however, the NheI digest produced two bands and the NotI produced four bands. The NotI single digest showed the same band pattern as the uncut control. Double digests with NheI and NotI were expected to produce two bands at 4,000 bp and 1,200 bp. All of the double digests produced two bands with the same band pattern as the NheI single digest. The two bands produced were at 8,000 bp and 3,000 bp, which are significantly larger than expected.

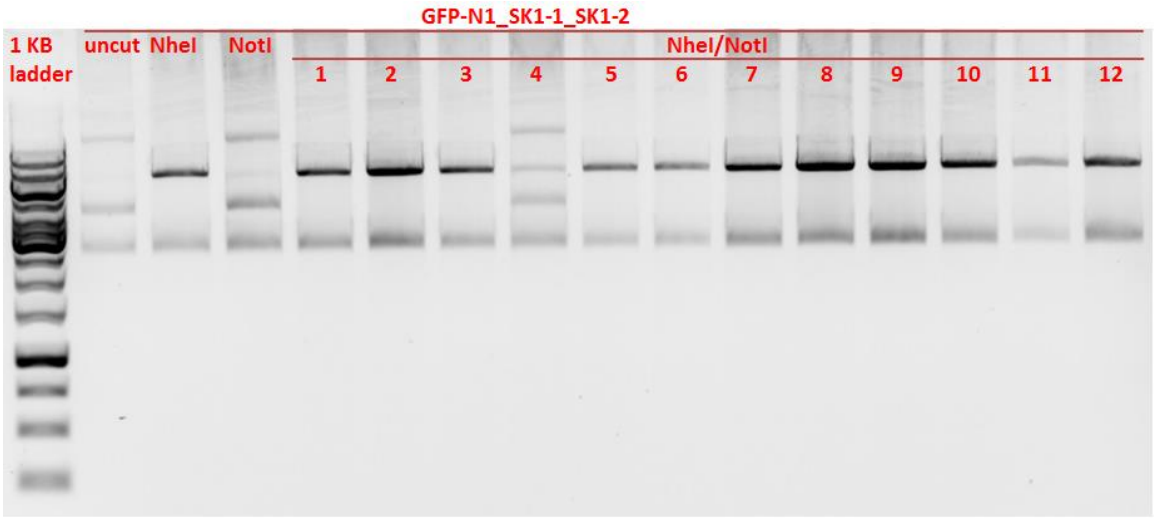

FIGURE 7 Two-step ligation. pEGFP-N1, SK1-1, and SK1-2 were extracted from a 1% agarose gel in the presence of EtBr. The ligations were completed at 1:3 (lanes 1-6) and 1:10 (lanes 7-12) ratios and digested with NheI and NotI.

3.5 One-Step Ligation

The one-step ligation reaction for FireFluo synthesis was accomplished using pEGFP-N1, SK1-1, and SK1-2 extracted from a 1% agarose gel in the presence of SYBR safe. The ligation reactions were performed using a 1:1:1:1 (at 0.02 pmol and 0.04 pmol concentrations) and 1:1:1:2 vector to insert 1 (SK1-1), to insert 2 (SK1-2), to insert 3 (CeFP) ratio. The completed ligation reaction was transformed into NEB 5-alpha cells with appropriate controls. Individual colonies from the transformation plates were selected for mini-culture preparation.

Identity of the transformed plasmid was tested using restriction digest with NheI and NotI (Figure 8). The double digests were expected to produce two bands at 4,000 bp and 1,400 bp. Instead, the double digest controls produced two bands at 3,700 bp and 1,600 bp, which are not the expected sizes.

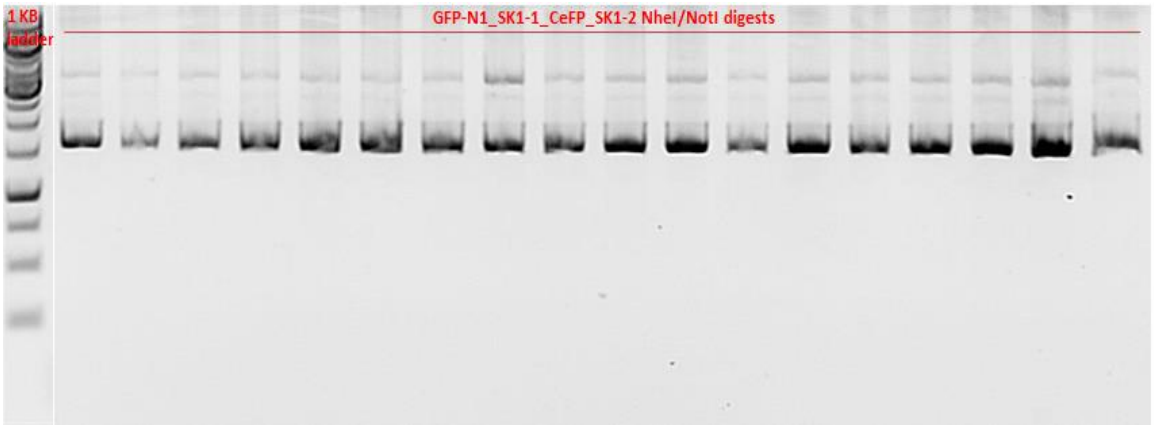

FIGURE 8 One-step ligation. pEGFP-N1, SK1-1, CeFP and SK1-2 were extracted from a 1% agarose gel in the presence of SYBR Safe. pEGFP-N1, SK1-1, CeFP, and SK1-2 ligation reactions were completed at 1:1:1:1 ratio at 0.02 pmol (lanes 1-6), and 0.04 pmol (lanes 7-12), and a 1:1:1:2 ratios (lanes 13-18) and digested with NheI and NotI.

3.6 Commercial Company Ligation

The FireFluo bac-stab and FireFluo plasmid DNA transformation were grown on kanamycin selection plates. Individual colonies from the transformation plates were selected for mini-culture preparation. Uncut plasmids were run on a 1% agarose gel to serve as a baseline for digest reactions (Figure 9a). The band pattern was consistent across both the plasmid DNA and bac-stab with the exception of the plasmid DNA mini prep number one.

The identity of the FireFluo plasmid DNA and FireFluo bac-stab were tested using restriction digest with AseI and NotI (Figure 9b). The single digests were expected to produce a single band at about 5,300 bp. The double digests were expected to produce two bands at 2,000 bp and 3,300 bp. Plasmid DNA mini prep number three and bac-stab mini prep number one were used for restriction enzyme ligation. The single digests

produced the expected band at 5,300 bp, however, the plasmid DNA produced bands in addition to the expected band. The double digests produced the expected bands at 2,000 bp and 3,300 bp; however, the plasmid DNA also produced additional bands.

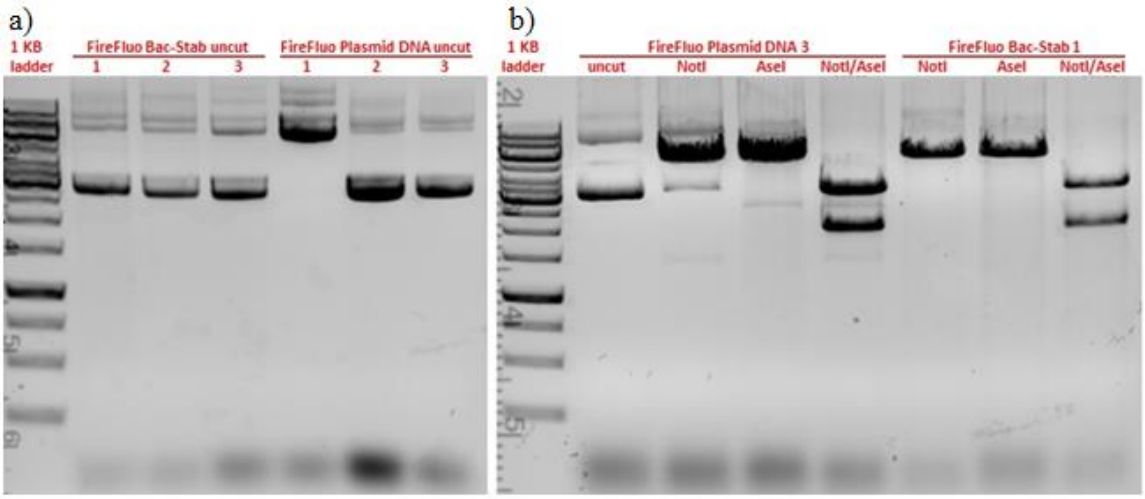

FIGURE 9 GenScipt FireFluo digest. FireFluo bac-stab and FireFluo plasmid DNA transformation were run through a 1% agarose gel; **a)** FireFluo bac-stab uncut (lane 2, 3, and 4) and FireFluo plasmid DNA transformation uncut (lane 5, 6, and 7), **b)** FireFluo bac-stab sample 1, and FireFluo plasmid DNA sample 3 were digested with AseI and NotI with single digest and uncut controls.

3.7 Preliminary Evaluation

FireFluo was transformed into DH5alpha cells (New England Biolabs) that were then grown on kanamycin selection plates with coverslips embedded in the agar. Twenty-four hours after plating, coverslips were extracted from the agar, placed on microscope slides, and colonies were visualized by bright field and epifluorescent microscopy (CFP-experimental and GFP-control filters) in the absence of DPA. The basal level of fluorescence was not determined for FireFluo due to the absence of fluorescence in the colonies (see appendix Figure 10). Additional testing in PC12 cells

performed by Meyer B. Jackson (University of Wisconsin) yielded only a few cells with slightly higher fluorescence then background, however, the fluorescence did not appear to be localized to the plasma membrane.

CHAPTER 4: DISCUSSION AND FUTURE WORK

4.1 FireFluo Assembly

Assembly of SK1-1, CeFP, and SK1-2 into the pEGFP-N1 vector can be accomplished through in-house restriction enzyme ligation or by synthesis and subcloning by a commercial company such as GenScript. We received the final construct from GenScript prior to completion via in-house efforts. Restriction enzyme ligation and synthesis of FireFluo by a commercial company facilitates the versatility of restriction sites allowing for future subcloning or modification of FireFluo components.

The final sequence for the FireFluo construct includes restriction sites flanking each of the individual components (Figure 2). The pCMV promoter region of pEGFP-N1 is led at the 5' end by the AseI RS. The NheI restriction site is located at the 3' end of the promoter region, and is immediately followed by the ATG start codon. Following the start codon is the FireFluo sequence; SK1-1_SacII_CeFP_SacII_SK1-2_NotI. After the NotI restriction site is the PolyA SV40 region of pEGFP-N1, which includes the AflII restriction site at the 3' end.

4.1.1 Restriction Enzyme Ligation

Restriction enzyme ligation is a classic molecular cloning technique using restriction enzymes to cut double-stranded DNA for the fusion of complimentary strands in a ligation reaction. The assembly of FireFluo was attempted using this method. Restriction digests were performed on the SK1-1, CeFP, and SK1-2 PCR fragments, as

well as, pEGFP-N1 to produce 5' and 3' single-strand overhangs. Fusion of complimentary overhangs was attempted in several T4 ligation reactions. The restriction enzyme ligation produced the wrong construct which can occur because of poor template quality, potential contaminants, long storage time, prior handling, or chemical modification.

4.1.2 Commercial Company Ligation

The assembly of the FireFluo plasmid was prepared for commercial company ligation at GenScript. GenScript is a biotechnology company that was selected for the gene synthesis of the FireFluo gene and subcloning into the custom vector, pEGFP-N1. pEGFP-N1 was prepared and sent to GenScript on filter paper along with the vector sequence.

The gene synthesis was completed by a Ph.D.-level project manager based on the provided gene sequence. GenScript provided codon optimization, in order to increase the protein expression in any host. Commercial company ligation produced a ready-to-use FireFluo plasmid, which eliminates the need for digestion, ligation, PCR-amplification, gel purification, transformation, screening, prepping, and sequencing of the restriction enzyme ligations. GenScript provided sequence verification with delivery of the finalized product, eliminating the risk of getting the wrong construct.

4.2 Transformation and Preliminary Evaluation

After receiving the completed FireFluo plasmid from GenScript, the FireFluo plasmid was transformed PC12 cells for preliminary evaluation. The FireFluo transformed cells displayed the same level of fluorescence as the PC12 controls.

Additional evaluation performed by Meyer B. Jackson, showed a non-significant elevation of fluorescence in PC12 cells expressing FireFluo and noted no soma localization.

4.3 Future Evaluation

FireFluo protein expression in PC12 cells will be determined by cell culture and immunocytochemistry (ICC). The antibody chosen for ICC will be able to recognize native and denatured forms of ECFP and its variants. An antibody proven in ICC will be selected based on the portion of the XFP that serves as the antigen for the antibody (Cat No. 632375; Clontech Laboratories).

Upon verification of protein expression, FireFluo will be transformed into appropriate cells for reevaluation of fluorescence. Prior to addition of dipicrylamine, transformation rate and soma localization will be assessed. Upon verification that the fluorophore resides only at neuronal somas, dipicrylamine will be included and neuronal activity will be manipulated via addition of tetrodotoxin (to reduce AP discharge rate) or bicuculline (to increase AP discharge rate) to the bathing media. Fluorescence of transfected cells will be quenched by the addition of 4% DPA solution. Fluorescence levels of pEGFP-N1 and FireFluo will be compared to determine if the same level of quenching is achieved, with the addition of DPA.

Further evaluation of probe performance will be conducted by voltage imaging and patch-clamp recordings at Meyer B. Jackson's lab. The voltage imaging will be conducted using a CCD-SMQ camera (Redshirt Imaging, Decatur, GA) mounted on an Olympus BX51 fluorescence microscope equipped with ECFP/CeFP, EGFP, and EYFP

filter cubes (Wang, Zhang, et al. 2010). The whole-cell patch-clamp recordings will performed with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) (Wang, Zhang, et al. 2010). The performance of the probe will be compared to hVoS 2.0 to evaluate if membrane targeting has improved probe performance.

4.4 Future Applications

FireFluo has the potential to contribute to a variety of future studies including applications in photoacoustics, neural network mapping, and transgenic animals.

4.4.1 Photoacoustics and Neural Network Mapping

A promising new mode of information collection combines optics and photoacoustic imaging. Photoacoustic imaging, also known as optoacoustic or thermoacoustic imaging, has potential application in imaging animal or human organs, with high contrast and high spatial resolution (Xu and Wang 2006). Biomedical applications of this technique includes, laser-induced photoacoustic imaging of small animals. This technique measures unique optical absorption contrasts, which provide better resolution in deep tissues than current optical imaging techniques (Xu and Wang 2006). The 3D perspective provided by photoacoustic imaging will facilitate a more complete understanding of the entire neural network as a system opposed to small clusters of neurons.

Photoacoustic imaging paired with hVoS probes has the potential to offer non-invasive imaging of neural networks. The majority of modern techniques, including patch-clamp and electrode imaging is limited to imaging a few hundred neurons at the same time, photoacoustic imaging, has the potential to image thousands. In order to

ascertain the optimal wavelength for photoacoustic imaging, several fluorescent proteins will need to be tested for the highest quality resolution. FireFluo currently includes CeFP, additional constructs will be created replacing CeFP with yellow, green, red and teal fluorescent protein (EYFP, EGFP, ERFP, and TeFP respectively). These five constructs will be sent to Parag Chitnis' Lab (George Mason University, School of Bioengineering, Fairfax, VA) to determine the optimal construct design for photoacoustic imaging.

4.4.2 Transgenic Animals

A FireFluo line of transgenic mice will assist in behavioral and physiological studies of neural circuitry and their implication in learning and memory through imaging of electrical activity in brain slices. Recording signals from populations of neurons is dependent on the interaction of DPA eliciting the FRET interaction with the membrane anchored CeFP (Wang, McMahon, et al. 2012). Successful imaging of the hVoS probes in transgenic mice demonstrates the distinct possibility for studying the electrical activity of intact neural circuits using genetically targeted cells (Wang, McMahon, et al. 2012). FireFluo provides an alternative strategy for more precise localization of hVoS probes to population of neurons in the hippocampus (Huang, et al. 2010; Miyoshi and Fishell 2006). FireFluo transgenic animals provide a gateway to imaging experiments to study neural circuit activity, and the relationships between the electrical activity of distinct neuronal populations (Wang, McMahon, et al. 2012).

4.5 Conclusion

Neuronal recordings have benefited from the advancement of imaging techniques from the earliest voltage sensitive dyes to the modern hybrid optical voltage sensors. FireFluo hopes to contribute to the advancement of the field by localization of a hVoS to the cell bodies of neurons in the hippocampus increasing the specificity of the previously created hVoS 2.0 (Wang, Zhang, et al. 2010). Future collaboration with Meyer B. Jackson and Parag Chitnis, will evaluate the performance of FireFluo, and test applications in photoacoustic imaging respectively. Imaging of neurons is currently limited to a few hundred at a time, FireFluo and other hVoS sensor hope to aid in advancing entire neural network mapping.

APPENDIX

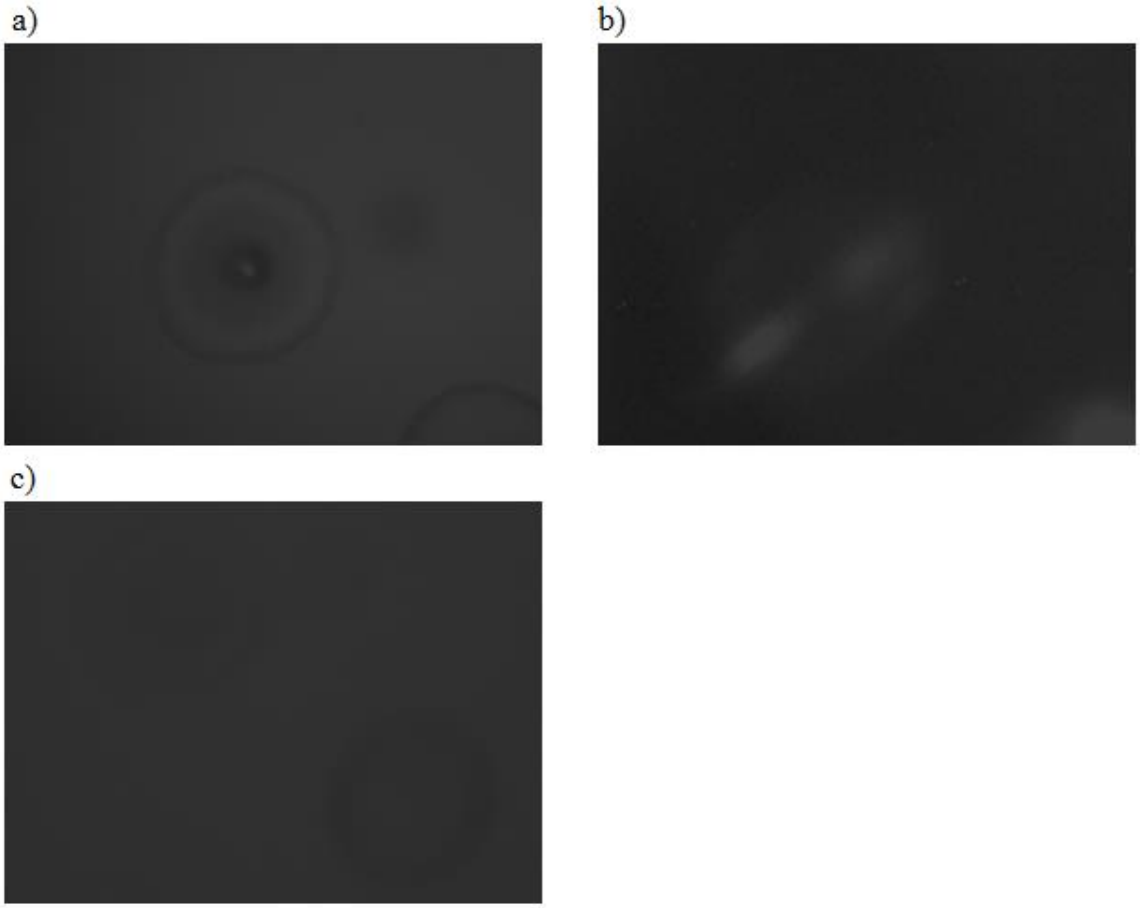

FIGURE 10 FireFluo fluorescence. FireFluo DH5alpha bac-stab cells were plated on kanamycin selection plates embedded with coverslips. The transformed colonies were imaged using a QI Imaging Retiga EXi Fast 1394 camera in the absence of DPA; a) a bright-field image of a single transformed colony, b) the same colony imaged using a CFP fluorescence filter, c) the same colony imaged using a GFP fluorescence filter.

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BIOGRAPHY

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