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EVOLUTION OF THE AXON INITIAL SEGMENT: FINDING THE MEMBRANE DIFFUSION BARRIER IN CNIDARIANS

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ABSTRACT

Polar neurons with functionally distinct axons (to send info) and dendrites (to receive info) were a key innovation for directional signaling in complex nervous systems. There are two key features of polarity in neurons – distinct microtubule polarity that allows differential trafficking and the axon initial segment (AIS) and its membrane diffusion barrier. Axon initial segments are specialized for action potential initiation. This is accomplished by the requirement of ion channels for action potentials and a membrane diffusion barrier in order to keep other somatodendritic ion channels into the axon. The evolutionary origins of neuronal polarity are largely unknown. It is not clear whether polarity evolved with centralization, or whether it preexisted in simpler nervous systems such as those of Cnidarians that have traditionally been viewed as being comprised of unpolarized neurons.

The anthozoan (Cnidarian) *Nematostella vectensis* is a sea anemone whose primitive net-like nervous system predates complex central nervous systems present in bilateral metazoans. Cnidarian neurons have traditionally been viewed as unpolarized, but differentiated axons in *Nematostella* were recently identified. If the axons of *Nematostella* in polarized cells are functionally analogous to the axons in vertebrates, then the AIS should be present. This would be the first evidence to suggest that axon specialization occurred before nervous system centralization. *Nematostella* have the gene that encodes an ortholog of ankyrin-G, a protein that allows for the organization and functionality of the barrier in humans, leading to the assumption that they likely have axon initial segments and therefore the membrane diffusion barriers characteristic of axons. We hypothesize that the diffusion barrier will be present in some classes of *Nematostella* neurons because *Nematostella* have many of the signaling genes used

differentially in axons and dendrites. In this project we addressed the hypothesis by looking for a diffusion barrier characteristic of the AIS.

There exist multiple classifications of neurons including branched and unbranched neurons. Through experimentation we determined that the unbranched neurons do not contain membrane diffusion barriers. We hypothesize that further analysis of the branched neurons with multiple neurites will exhibit the same mechanism of establishing functional polarity as more complex bilaterians by means of the diffusion barrier and presence of the axon initial segment.

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AIS – Axon Initial Segment

dNTP - Deoxynucleoside Triphosphates

FRAP – Fluorescence Recovery After Photobleaching

GFP – Green Fluorescent Protein

PCR – Polymerase Chain Reaction

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Chapter 1 : Introduction

Neuronal Polarity

The directional polarity of information flow in complex neural networks depends on the functional polarization of neurons via the formation of axons (to send signals) and dendrites (to receive signals). At the center of a neuron exists the central cell body containing the nucleus as well as the mechanisms for synthesizing proteins.[1] Axons and dendrites extend from the cell body and facilitate signaling in the cell; without these three primary subcellular compartments, proper neuronal function would not be possible.[2] Axons require a distinct set of signaling proteins specialized to send signals over distances and to release neurotransmitters. An asymmetric distribution of proteins to distinct domains in the plasma membrane is critical for the function of many polarized cells.[3] Polarized neurons work to maintain axonal and somatodendritic plasma membrane domains.[3] Near the cell body, axons contain axon initial segments where action potentials are initiated.[4] Experimentation shows that membrane proteins experience reduced mobility as they move through axon initial segments.[3] This reduced mobility is indicative of a membrane diffusion barrier in the cell membrane of the axon initial segment. In the discovery of the diffusion barrier it was found that it is developed in neurons through the accumulation of transmembrane proteins anchored to the membrane skeleton.[5] In vertebrates, this membrane diffusion barrier plays a key role in axonal differentiation by blocking the diffusion of somatic membrane proteins in the axon.[6]

It was previously postulated that, given the significant variation in the organization of the invertebrate nervous system compared to that of vertebrates, the mechanisms for polarized sorting of the nervous systems must also vary.[7] We predict that invertebrates may have similar mechanisms for establishing neuronal polarity as vertebrates. An enhanced understanding of the presence or absence of the diffusion barrier in Cnidarians will allow for a better explanation of when and how this diffusion barrier evolved.

Nematostella vectensis

The model we used was an anthozoan (Cnidaria) *Nematostella vectensis*: a sea anemone. Its nervous system evolutionarily predates complex central nervous systems present in bilateral metazoans (Figure 1), having instead a primitive net-like nervous system distributed around the body.[8] It has been found that *Nematostella* and other Cnidarian nervous systems involved two nerve nets with both bipolar and multipolar neurons.[9] These neurons allow for signals to be conducted in either direction from one part of the nerve net to another. While it is generally accepted that these simple nerve-nets preceded centralized nervous systems, it is unclear when the evolution of the central nervous system occurred.[9] Until recently it was suggested that the axon initial segment, a cytoskeletal feature that allows for sorting through polarization, existed exclusively in vertebrate neurons;[10] however, the Jegla and Rolls labs have recently identified invertebrate neurons with differentiated axons.[1] It was recently found that specializations similar to the axon initial segment are present in *Drosophila*.[2] Following this conclusion, the lab hypothesizes that axons and dendrites in all bilaterians may be generated following the same mechanisms. We anticipate that the nerve net of *Nematostella* may contain polarized neurons.

Alternatively, we do acknowledge the potential for the evolution of polarity to have occurred more recently with the development of the central nervous system.[1]

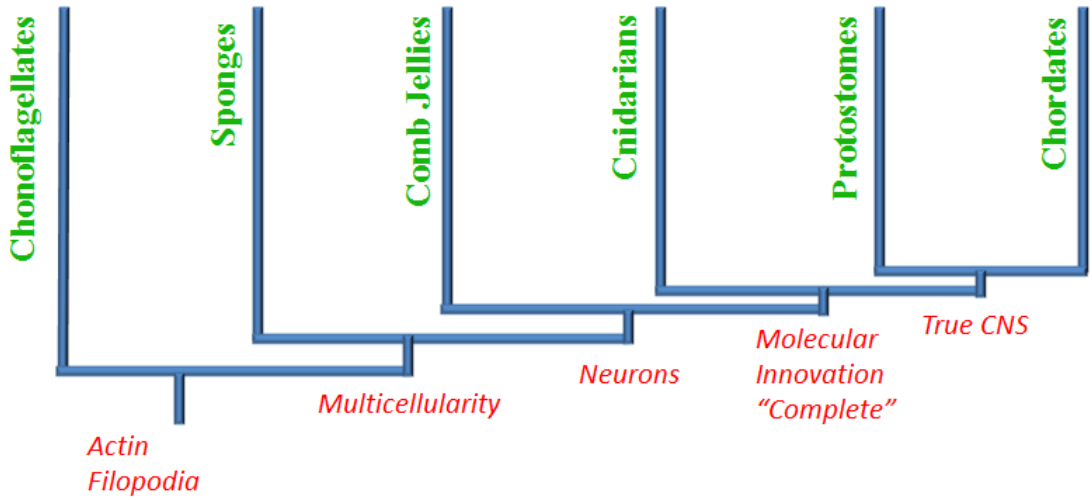

Figure 1: Nervous system development phylogeny tree

We are trying to better understand the evolution of the establishment of neuronal polarity by determining whether or not neurons of *Nematostella* have the same mechanisms for establishing functional polarity as neurons of higher animals. First, the lab identified *Nematostella* neurons in order to determine microtubule cytoskeletal polarities, which in higher order animals are known to have distinctive orientations in axons and dendrites. In determining whether or not *Nematostella* have neurites that function like axons and dendrites in higher animals, the lab found that *Nematostella* neurons have predominantly axon-like processes. One key functional feature of an axon in higher animals is that it is a molecularly separate entity with a membrane diffusion barrier to separate axonal from somatic membrane proteins.[3] This barrier

helps it become electrically specialized to perform its function by segregating ion channels of various types. The Rolls lab identified a specialized region in the neurons of *Drosophila melanogaster* that allows for the transport and segregation of neuropeptide vesicles from the cell body,[6] and for similar reasons we believe that membrane diffusion barriers or similar mechanisms that perform related functions may be present in *Nematostella vectensis.*

Indications of Membrane Diffusion Barriers

Membrane proteins such as spectrin, actin, and ankyrin comprise the skeleton of the axon initial segment, which is important for anchoring membrane proteins including neurofascin and sodium channels, but the skeleton also creates the membrane barrier for other membrane proteins.[11] Barrier proteins help to anchor the axonal channels to the axon initial segment to specialize it for action potential initiation.[11] The lab has identified the key proteins that comprise the diffusion barrier in flies and vertebrates including an ortholog of ankyrin G in *Nematostella*. The loss of ankyrin G is known to cause axons to exhibit dendrite-like characteristics.[9] We predict that the finding of an ortholog of ankyrin G (a vertebrate-specific member of the ankyrin gene family) in *Nematostella* neurons indicates the presence of an axon initial segment. Following indications of the presence of ankyrin G, preliminary experiments in the Jegla lab have identified neurons with differentiated axons. From these initial findings, we expect that specific neuronal processes in *Nematostella* may function like axons, and, as such, contain a membrane diffusion barrier. We believe the presence of the diffusion barrier could be a fundamental feature of an axon identity rather than a specialization for segregated signaling in central nervous systems.

Searching For Membrane Diffusion Barriers

In order to evaluate the presence of the membrane diffusion barrier, constructs had to be made to specifically label neurons for bleaching when injected *in vivo*. Three different constructs containing mouse CD8 antigen (mCD8), a transmembrane glycoprotein, were created so as to label neurons so that a bleaching experiment could be performed in order to determine if membrane diffusion barriers are present in *Nematostella* axons.[12] mCD8 was integral in this function of the reporters as it acts to homogeneously label the membrane of entire neurons.[13]

The constructs vary in their fluorescent proteins as different fluorophores may be more or less effective depending on the filters of the microscopes used. mCD8-GFP is a common construct used for labeling mosaic clones and has been used to better understand regulation of dendritic and axonal development.[14] mCitrine was used initially as it is one of the brightest fluorescent proteins (49 mM^{-1} cm^{-1}) and has a relatively high photostability (49).[15] The second reporter contained mCherry as it is used for its superior photostability over other fluorescent proteins (96) although its brightness is significantly reduced from mCitrine (19 mM^{-1} cm^{-1}).[15]

The third construct contained the photostable mCherry fluorescent protein separated from bright mCD8-mCitrine reporter by a 2A peptide sequence. The 2A "self-cleaving" peptide sequence was first found in the picornavirus in order to mediate protein cleavage.[16] This sequence is uncommon and results in cleavage between the 2A glycine and the 2B proline.[17] Historically, bicistronic expression vectors were used when the expression of two genes was required; however, due to their small size and high cleavage efficiency, 2A peptide sequences are favorable.[18] While normal bonds cannot be formed between these two amino acids, translation of 2B is uninhibited.[19] The design of the construct allows for the 2A-linked mCherry to be used for sorting while mCitrine may be used for photobleaching.

These constructs were then placed in the NvT vector, a transgenic vector designed for insertion. In order to drive the expression of the reporter, the Elav1 promoter was placed upstream of the mCD8 reporter. Elav1 is a member of a highly conserved family of genes that encode RNA-binding proteins.[20] It allows for cell growth and proliferation through regulation of mRNA stability.[20] Elav1 was used as it is a general promoter that is related to and has similar genomic organization to the neural-restricted members of the Elav family, and it allows the reporter to non-specifically label all types of neurons.[20]

Fluorescence Recovery After Photobleaching

After the constructs were created using molecular cloning techniques, they were injected into live *Nematostella* oocytes. The vector is designed for insertion using the I-SceI meganuclease system. The I-SceI meganuclease system is one that has historically been successful with mammalian cells, fish, and *drosophila*.[21] The I-SceI meganuclease system has been used to allow for the integration of the reporter into the *Nematostella* genome.[22] I-SceI recognizes an eighteen basepair nonpalindromic sequence of nucleotides.[23] The I-SceI then provides a sequence-specific cut, followed by integration of the inserted sequence into the genome.[23] This mechanism allows for fast, somewhat inefficient insertion.[24] In our vector, the I-SceI sites surround the expression cassette. The nuclease is used to cut the expression cassette out of the plasmid and prevents it from forming large rafts. Genome insertion is significantly more efficient for linear non-concatenated products. Following injections, the animals were screened for mosaic animals that successfully incorporated the construct. The mosaic animals

were then used to determine the presence or absence of membrane diffusion barriers in the neurons of *Nematostella* through photobleaching experiments.

Classifications of Neurons

Neurons are "polarized secretory cells specializing in directional propagation of electrical signals leading to the release of extracellular messengers."[25] A common vertebrate neuron is the multipolar neuron with a cell body giving rise to a single axon and multiple branched dendrites.[1] In most polar neurons, the microtubules of dendrites have mixed polarity, and the microtubules of axons have all plus-end-out polarity.[26] *Caenorhabditis elegans* has predominately unbranched neurons, and we expect to see a similar proportion in *Nematostella*. *C. elegans* have branched dendrites where minus-end-out microtubules are present, which we also expect to find in *Nematostella*.[1] Initial experimentation using an EB1-GFP reporter has allowed the lab to identify and classify many different types of *Nematostella* neurons. It is evident that both branched and unbranched neurons are present in *Nematostella.* This distinction is important as we hypothesize that unbranched neurons will not contain the membrane diffusion barrier and that the branched neurons will contain the barrier.

Chapter 2 : Materials and Methods

Molecular Cloning

NvElav1-mCD8-mCitrine Reporter

In order to create a construct designed for membrane diffusion and integration *in vivo*, we first created an mCD8-mCitrine reporter and a transgenic vector to express the reporter in neurons. In the creation of the reporter, the green fluorescent protein mCitrine was attached to the mouse mCD8 gene through polymerase chain reactions (PCR). PCR allows for the in vitro replication of a particular segment of DNA through multiple repetitions (25-35 cycles) of the denaturation, annealing, and extension cycle. Denaturation causes the hydrogen bonds involved in the formation of the double helix from the two strands of DNA to break at high temperatures (95°C), allowing for the creation of two single-stranded DNA templates.[27] Annealing occurs at a lower temperature (50-65°C) and allows for the binding of complementary primers to the single-stranded DNA.[27] Extension depends on the activity of DNA polymerase and its ability to extend primers using nucleotides.[27] This step occurs at the ideal temperature for DNA polymerase activity (72°C).[27] In the creation of the mCD8-mCitrine reporter, the template DNA for mCD8 and mCitrine was mCD8 and NvT, respectively. The oligonucleotides designed for the mCD8 reaction were mCD8 met and mCD8 stop. The oligonucleotides designed for the NvT reaction were mCD8 mCitrine sense and FP stop. The enzyme used to promote the replication was Herculase. 5X Herculase reaction buffer created ideal salt concentrations in order to stabilize DNA. High quality deoxynucleoside triphosphate (dNTP) mix was added to provide the base

pairs for the elongation step of PCR. This reaction allowed for the creation of hundreds of millions of copies of the mCD8-ctirine reporter.

The PCR product mCD8-mCitrine reporter was then analyzed using gel electrophoresis on a 1% agarose gel to ensure that the reaction was successful. The expected band size was approximately 800 base pairs for the cDNA PCR and approximately 800 base pairs for the NvT PCR. The two bands were then excised from the agarose gel and purified using a Qiagen QIAquick Gel Extraction Kit, a kit used to clean-up 10 micrograms of DNA ranging in size from 70 base pairs to 10 kilobase pairs (https://www.qiagen.com/us/products/catalog/sample-technologies/dna-sample-technologies/dna-cleanup/qiaquick-gel-extraction-kit)**.**

The purified reporter was then placed in a transgenic NvT vector with the Elav1 promoter upstream in order to drive the expression of the reporter in *Nematostella* neurons. The reporter was cloned into the vector by use of a restriction digest. The restriction digest allowed for both plasmids to be cleaved by the same two restriction endonucleases (Asc1 and Xba1) at restriction sites by means of recognition sequences. The recognition sequence for Asc1 is 5'…GGCGCGCC…3' (https://www.neb.com/products/r0558-asci), and the recognition sequence for Xba1 is 5'…TCTAGA…3' (https://www.neb.com/products/r0145-xbai). Asc1 and Xba1 were added to the template DNA in the presence of the 10X CutSmart buffer. The CutSmart buffer includes potassium acetate, tris-acetate, magnesium acetate, and bovine serum albumin (BSA) at pH 7.9 (https://www.neb.com/products/b7204-cutsmart-buffer). The CutSmart buffer provides optimal conditions for cleavage of the template DNA. The mixture was then incubated at 37°C. The two digests were then run on a 1% agarose gel to see that the products of the restriction digests were the expected sizes of 1.5 kilobase pairs (cDNA) and 3 kilobase pairs

(NvT template). The bands were then excised from the gel and purified using the Qiagen QIAquick Gel Extraction Kit.

Following the purification of the restriction digests, a ligation reaction was performed in order to create the NvElav1-mCD8-mCitrine construct. The ligation mixture included the two purified digests, T4 ligase, and the T4 reaction buffer. T4 ligase is used to catalyze the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl of the DNA (https://www.neb.com/products/m0202-t4-dna-ligase). The enzyme acts to bind blunt and cohesive ends from the restriction digest. The reaction mixture was then incubated at 25°C.

The in vitro plasmid was then transfected into premade *Mix & Go* chemically competent *E. coli* cells. The chemically competent cells were thawed on ice, and the ligation was added following the Zymo Research protocol (https://www.zymoresearch.com/e-coli/chemically-competent-cells/strain-zymo5alpha). The mixture was then plated on a lysogeny broth (LB) agar plate containing the antibiotic ampicillin and incubated at 37°C overnight. The antibiotic allows cells containing only plasmids with the antibiotic resistance to grow. Organisms that are not resistant to ampicillin are unable to grow on the LB plate allowing for the selection the desired construct.

After the colonies were left to grow, individual colonies were swiped and grown in Terrific Broth (TB) containing ampicillin, a nutritionally rich media designed for bacterial growth while the ampicillin allowed for only the growth of antibiotic resistant cells. The cells were grown on a shaker overnight at 37°C. MiniPreps were then performed using Qiagen QIAprep Spin MiniPrep Kit in order to isolate the construct from the bacterial cells. The MiniPrep kit required the concentration of the bacterial cells into pellets by centrifugation. Subsequently, the kit allowed for the binding of up to 20 micrograms of DNA onto a silica

membrane when the chaotrophic salt concentrations are high (https://www.qiagen.com/us/products/catalog/sample-technologies/dna-sample-technologies/plasmid-dna/qiaprep-spin-miniprep-kit/). The DNA eluted from the column is concentrated and relatively pure.

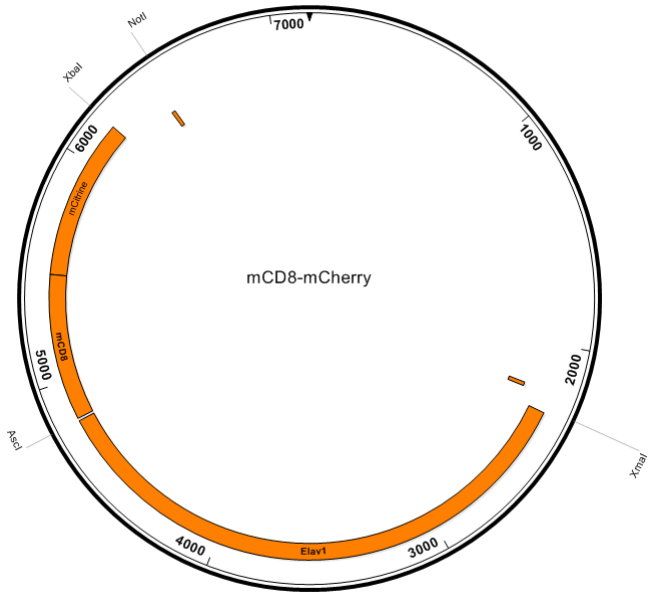

Figure 2: NvElav1-mCD8-mCitrine Plasmid Map

A check digest was performed on the NvElav1-mCD8-mCitrine construct to ensure that the construct was the desired product based on the banding pattern of the digest. The construct was digested with Asc1 and Xba1, and the expected sized bands of 1.5 and 3 kilobase pairs were observed.

In order to verify that the construct had the correct sequence, the construct was sent to sequencing with oligonucleotides to ensure that no mutations had arisen during the cloning process in the DNA. The sequence-verified construct was then grown as a maxi using Qiagen Plasmid Maxi Kit to ensure a larger quantity (up to 500 micrograms) of the construct as well as

an increased purity (https://www.qiagen.com/us/products/catalog/sample-technologies/dna-sample-technologies/plasmid-dna/qiagen-plasmid-kits/).

NvElav1-mCD8-mCherry Reporter

NvElav1-mCD8-mCherry was then created using the same molecular cloning strategies except fluorescent protein mCherry replaced mCitrine. mCD8 and mCherry were fused using PCR and then digested with Asc1 and Not1 restriction enzymes. The NvElav1-mCD8-mCitrine construct was also digest with Asc1 and Not1. The mCD8-mCherry was then ligated with the NvElav1 plasmid to form NvElav1-mCD8-mCherry. This construct was created in order to determine which fluorescent protein provides the best visualization in the screening and bleaching of the *Nematostella* neurons.

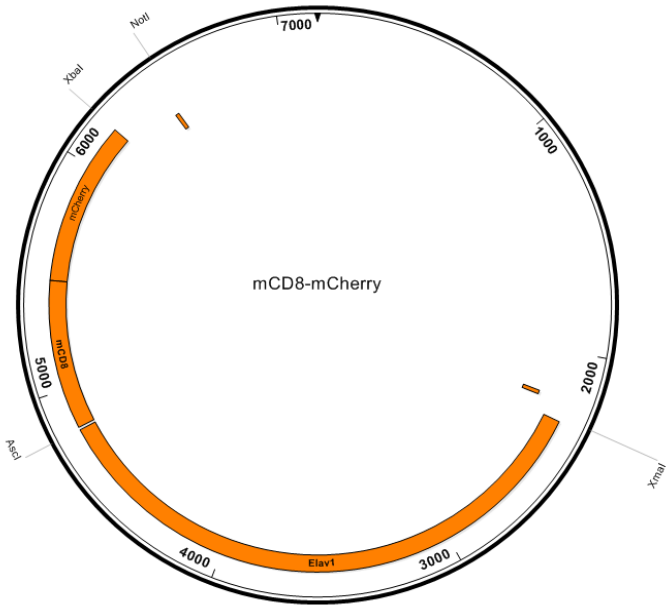

Figure 3: NvElav1-mCD8-mCherry Plasmid Map

NvElav1-mCD8-mCitrine-2A-mCherry Reporter

NvElav1-mCD8-mCitrine-2A-mCherry reporter was then created using the NvElav1-mCD8-mCitrine and the NvElav1-EB1-GFP-2A-mCherry constructs. The cloning mechanism was similar to that of NvElav1-mCD8-mCitrine and NvElav1-mCD8-mCherry. Using the eighteen basepair 2A peptide sequence, this reporter allowed for the fluorescent proteins to be used in two different aspects for the analysis of the transgenic animals: screening and photobleaching.

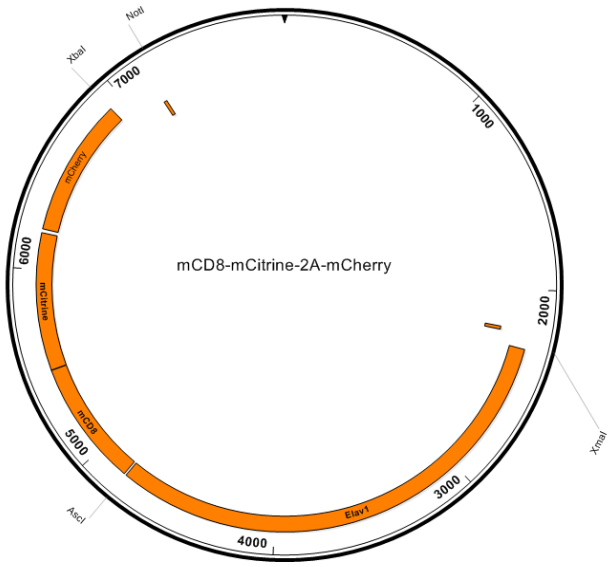

Figure 4: NvElav1-mCD8-mCitrine-2A-mCherry Plasmid Map

Creation of Mosaic Animals

In order to construct transgenic animals, we used an established meganuclease-based technique to insert the mCD8-mCitrine reporter into *Nematostella* polyps.[22] The *Nematostella* are placed on light boxes overnight to stimulate spawning. Once the animals have spawned, the

oocytes are separated into individual polyps using a cysteine solution at a pH of 7.5 so as to not denature the polyps. Individual polyps are then injected with the sequence verified reporter that has been digested with Sce1 enzyme. Using this technique, we drove the expression of the reporter using the promoter Elav1.[8]

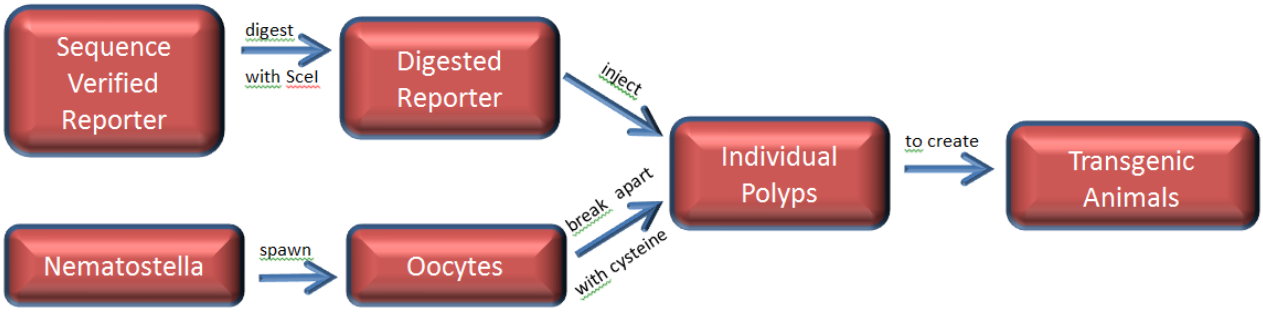

Figure 5: Pathway to create a mosaic animal

Injection of a digested sequence verified construct is a complicated process that depends on the preparation of the oocytes. Between 200 and 400 animals may be injected following a spawning depending on the preparation of the oocytes. Approximately three hours after fertilization of the spawned oocytes, the cell begins to divide limiting the amount of time allowed for injections.

Screening of Mosaic Animals

Screening of mosaic animals may occur two to three weeks following the injection of the reporter into the polyps. Screening relies on the intensity of the fluorescence of the mCitrine or mCherry fluorescent marker in order to sort the mosaic animals from the animals that may not

have been injected or did not integrate the construct. The screening is conducted using a fluorescent macroscope. The macroscope allows for visualization of the fluorophores via fluorescent filters. The mosaic animals are not entirely transgenic animals as any number of the neurons in the animal may be tagged with the fluorescent marker.

Fluorescence Recovery After Photobleaching

Fluorescence Recovery After Photobleaching (FRAP) is a technique utilized in the Rolls' lab to identify axon initial segments in *Drosophila*. Before FRAP can be performed, the mosaic animals are placed in a 2% urethane in a water solution that is one third sea water to anaesthetize the *Nematostella* and avoid muscle contraction, which can easily be visualized when watching the nematocytes relax and stretch out away from the body.[28] The mechanisms of anesthetics is currently unknown; however it is speculated that they gain their effect either from enhancement of inhibitory synaptic neurotransmission or from inhibition of excitatory neurotransmission.[29] Urethane is used as it has nominal effects on cardiovascular and respiratory systems and maintenance of spinal reflexes.[29] If the animals are not sufficiently relaxed, the bleaching experiment may be impossible to perform as the targeted bleaching region moves sporadically. These animals were then visualized under the epifluorescent bleaching microscope.

FRAP allows for the bleaching of fluorescent proteins covalently bonded to a molecule targeting a specific site in the cell. Each fluorescent protein emits light of a specific wavelength upon absorption of another wavelength. For mCitrine a wavelength of 529 nanometers is emitted following the absorption of a wavelength of 516 nanometers.[30] For mCherry, a wavelength of 610 nanometers is emitted following the absorption of a wavelength of 587 nanometers.[31] The

high intensity of the light emitted from the laser causes the protein to become photobleached and unable to fluoresce.[32]

Following anesthetizing by urethane, the *Nematostella* are placed on slides and bleached using an Olympus confocal microscope with the 63X objective lens. The pre-activation time was 3000 milliseconds (three frames, with the activation time of each frame set for 1000 milliseconds). The laser then bleached the proximal axon using a wavelength of 405 nanometers at full laser power for 500 milliseconds. The area adjacent to the cell body is chosen as it is where the membrane diffusion barrier is located on axons containing the axon initial segment. The signal was then allowed to recover for ten frames (activation time 1000 milliseconds). Following the bleaching, the fluorescent protein is then unable to fluoresce, and the targeted region appears black due to the loss of fluorescence. As seen in Figure 7 below, the signal will eventually diffuse back into the bleached region of the neurite. The intensity of the signal in the bleached region approaches the initial value; however, it is somewhat darker due to the loss of fluorescence in the photobleached molecules. The amount of time that it takes for the signal to re-enter the proximal axon is dependent on the presence or absence of the membrane diffusion barrier. Here we measure the amount of time it takes for the molecules to migrate into the photobleached portion of the neurite. If no diffusion barrier is present, within seconds the green mCD8-mCitrine reporter will re-enter by means of

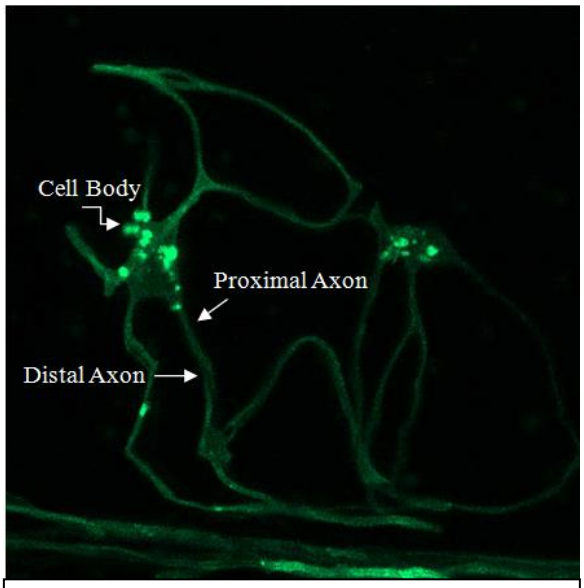

Figure 6: FRAP performed on a neurite labeled with mCD8-mCitrine

diffusing through the soma. If there is a diffusion barrier, the bleached region next to the cell body will remain dark for minutes to hours.

Figure 7: Bleaching and recovery of neurite tagged with mCD8-mCitrine

Chapter 3 : Results

NvElav1-mCD8-mCitrine Reporter

The NvElav1-mCD8-mCitrine reporter was successfully created using molecular cloning. This was the first construct that we created, and it provided the opportunity to better understand ideal injection conditions. We found that between 200 and 400 polyps could be injected following the fertilization of *Nematostella* oocytes. These injected polyps could then be viewed under a macroscope three days later to visualize which polyps fluoresced to better understand the success of the injections. Two weeks after the polyps were injected the animals were inspected under the fluorescent macroscope and screened for mosaic animals. There was difficulty visualizing the mCD8-mCitrine reporter, which kept many of the mosaic animals from being sorted from the animals that did not integrate the reporter. mCitrine is difficult to visualize during screening due to the mobile fluorescent particles in the gut cavity. Of the two to four hundred animals that were injected with the construct, around five to ten were recovered during sorting for mosaic animals. With the limited ability to sort these animals, fewer animals were available for FRAP analysis; however, the mCitrine fluorescent protein was easy to visualize using the confocal microscope.

NvElav1-mCD8-mCherry Reporter

Following increased screening success in the lab using the mCherry fluorescent protein, we then created the NvElav1-mCD8-mCherry construct anticipating that the screening process would allow for an increased percentage of the mosaic animals to be visualized, screened, and analyzed by FRAP. After the creation of the NvElav1-mCD8-mCitrine construct, the molecular cloning of the NvElav1-mCD8-mCherry construct was relatively simple. After the construct was sequence verified, the transgenic animals were made using the same techniques as were used with the NvElav1-mCD8-mCitrine construct. We believe that the number of mosaic animals that were created using the red reporter were approximately equivalent to the green reporter; however, this construct allowed for an increased number of the mosaic animals to be recovered for FRAP analysis due to the lack of mobile particles in the red channel. This lack of autofluorescence at this wavelength allowed for better visualization of the mCherry fluorescent protein over mCitrine. The number of animals that were recovered from the screening process following a typical injection (200-400 injected polyps) ranged from around ten to twenty. This was around twice the number of recovered animals of injections with the construct containing mCitrine. These animals were then visualized under the confocal bleaching microscope from the Rolls lab. While the mosaic animals made with the mCherry fluorescent protein were easier to screen, they were more difficult to visualize for photobleaching than the brighter, easier to distinguish on the black background, mCitrine green construct. FRAP was successfully conducted on thirty-three different neurites.

NvElav1-mCD8-mCitrine-2A-mCherry Reporter

After discovering that mCherry was a better fluorophore for screening as it has a lower background signal, allowing for an increased recovery of the transgenic animals and that mCitrine was a better fluorophore for photobleaching as it does not bleach as readily, we created the NvElav1-mCD8-mCitrine-2A-mCherry construct. This construct allowed for the same expression of mCD8 by the Elav1 promoter, but it also allowed for screening by mCherry and photobleaching by mCitrine. The 2A self-cleaving peptide sequence allows for multiple gene products from the same construct.[33] The construct was created using the same molecular cloning technique using NvElav1-mCD8-mCitrine and NvElav1-EB1-GFP-2A-mCherry as the template DNA.

Fluorescence Recovery After Photobleaching

The bleaching experiments were performed on mosaic animals created from each of the three constructs: NvElav1-mCD8-mCitrine, NvElav1-mCD8-mCherry, and NvElav1-mCD8-mCitrine-2A-mCherry. During the experiment, the *Nematostella* were placed on slides to be viewed and bleached using the Olympus confocal microscope at a 63X objective lens. When performing the fluorescence recovery after photobleaching experiments on a neuron, every neurite extending from that neuron was bleached using a single bleach scan at 405 nanometers at full laser power and integration time of 500 milliseconds.[34]

In total, there were thirty-three successful photobleaching experiments recorded. Images of the bleached neuron were collected every second for a total of ten frames over ten seconds. Of the thirty-three photobleaching experiments, thirteen were unable to be analyzed. The results of

some of the FRAP experiments could not be quantitated due to movement of the neurite or a very weak signal.

In order to quantitate the photobleaching experiment results, the ImageJ program was used to determine the intensity of the fluorescence before, during, and after the photobleaching. Total pixel intensity was summed in a 1 um by 1 um area of the background region of the image, the bleached region of the proximal neurite, and a part of the unbleached region of the distal axon for each time point.[35] The background signal of each frame was then subtracted from both the proximal axon and distal axon values, and then the ratio of the bleached to unbleached intensities was taken for each time point and divided by the initial pre-bleach ratio to correct for difference in intensity between regions of the cell.[34] This method allows for the correction of the change in the background intensity due to general bleaching by the laser.

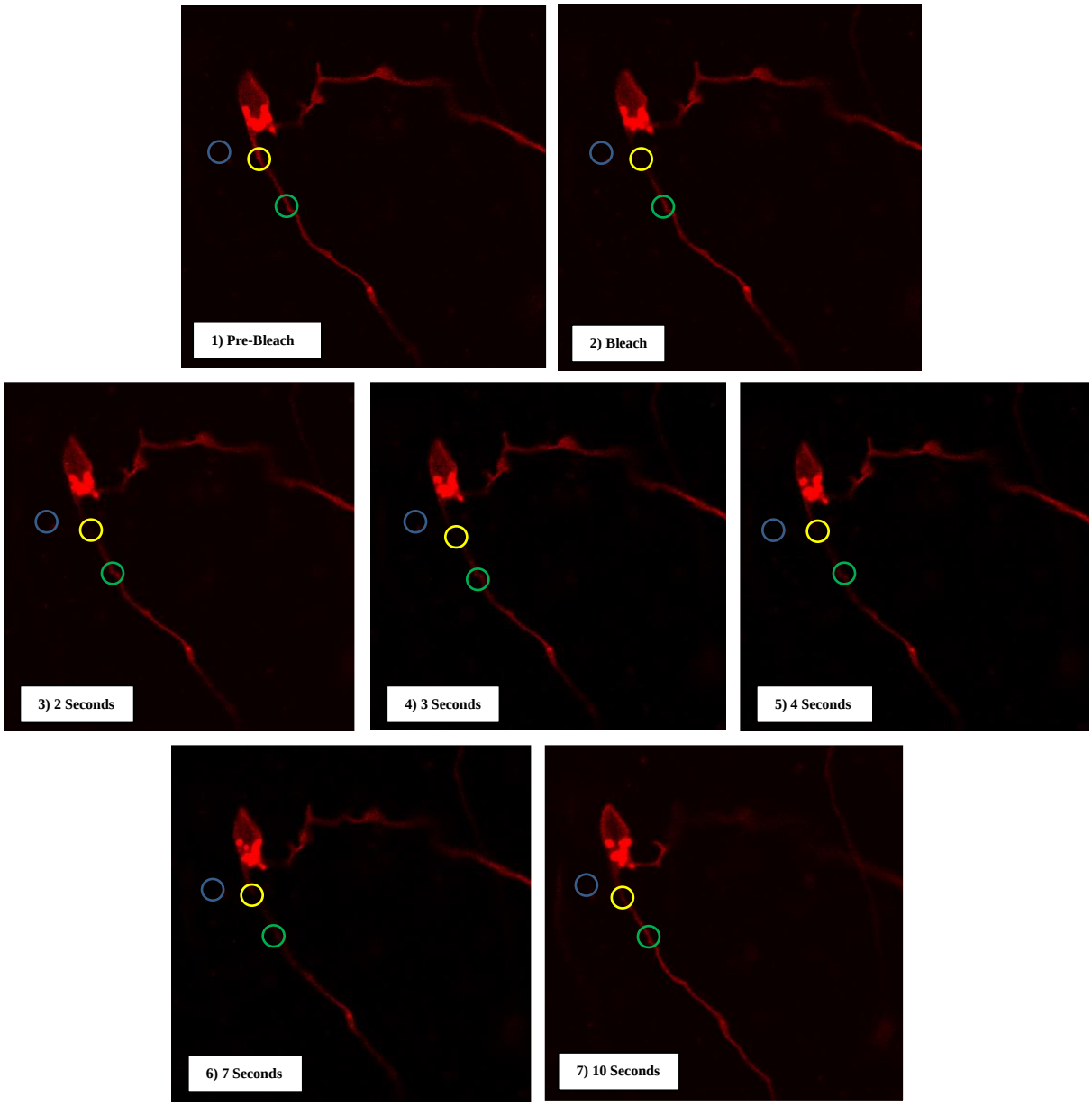

Figure 8: Succession of photobleaching measurements

In Figure 8 above, the bleaching experiment and data collection is depicted as it was performed. The blue regions show the initial background measurement, the yellow regions show the proximal axon measurements, and the green regions show the distal axon measurements in each of seven frames. Twenty-one fluorescence intensity measurements were completed per neurite.

Following the collection of twenty-one intensity measurements for each of the twenty neurites, the following ratio was calculated for each of the seven frames shown in Figure 8.

$$\frac{\dfrac{\text{Proximal Axon} - \text{Background Signal}}{\text{Distal Axon} - \text{Background Signal}}}{\dfrac{\text{Pre-Bleached Proximal Axon} - \text{Background Signal}}{\text{Distal Axon Before Bleaching} - \text{Background Signal}}}$$

After calculating this ratio for each of the seven frames of the twenty bleached neurites, the ratios for each of the twenty neurites were averaged. These averages are depicted in the graph below.

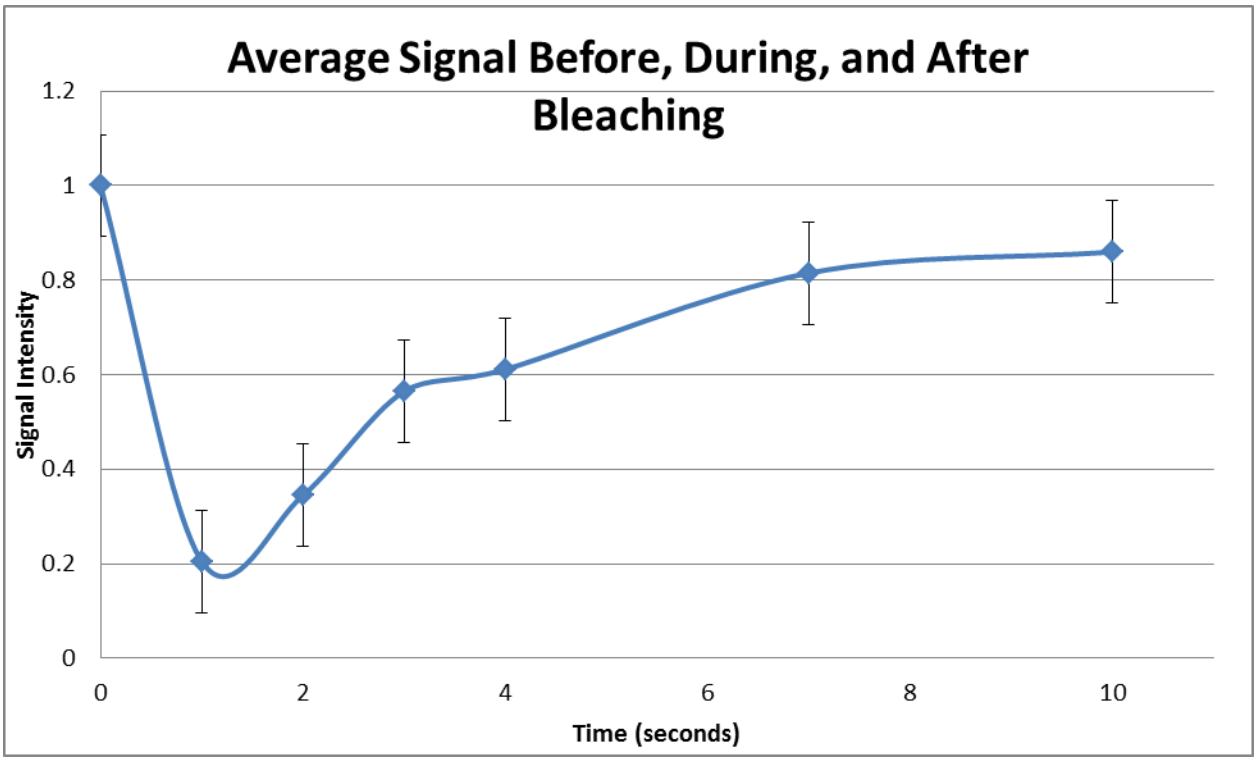

Figure 9: Average signal before, during, and after photobleaching

Graph contains error bars indicating vertical standard error

The graph in Figure 9 shows that the pre-bleach fluorescence was set at 100%. Immediately following the bleach the intensity was reduced to 20.5% of the pre-bleach intensity, accounting for a change in the background intensity due to bleaching of the mCD8-mCherry signal during image recording. The signal then recovered for nine seconds following the bleaching, and the signal had 85.6% of the intensity of the initial fluorescence. This indicates that within seconds the mCD8 signal returned to the region where the proximal axon had been bleached. The P values of each of the steps seven were calculated to show that the return of the fluorescent signal was statistically significant.

Time	Average	Standard Error	Two-Tailed P Value	Statistical Significance
0 seconds (Pre-Bleach)	1	0		
1 second (Bleach)	0.205255	0.041629		
2 seconds (Post-Bleach)	0.344737	0.057762	P = 0.0575	"not quite statistically significant"
3 seconds (Post-Bleach)	0.564804	0.090996	P = 0.0009	"extremely statistically significant"
4 seconds (Post-Bleach)	0.611372	0.087644	P = 0.0002	"extremely statistically significant"
7 seconds (Post-Bleach)	0.815054	0.087704	P = less than 0.0001	"extremely statistically significant"
10 seconds (Post-Bleach)	0.859694	0.117384	P = less than 0.0001	"extremely statistically significant"

Table 1: FRAP experiment error analysis

(P values and statistical significance calculated from http://www.graphpad.com/quickcalcs/ttest2/)

Error analysis compared the data of each post-bleach timepoint with the initial bleach values in order to determine whether or not the return of the signal was significant. The calculated P values indicated that one second after the initial bleach the signal recovery was not quite statistically significant. After two seconds the signal recovery was already extremely statistically significant. Increasing statistical significance was seen at every timepoint following the bleach. This error analysis indicates that the recovery of the signal occurs within seconds and is therefore uninhibited by a membrane diffusion barrier.

Using FRAP thirty-three neurites were successfully bleached, and twenty neurites were able to be analyzed; however, no membrane diffusion barriers were found. Upon further analysis it became clear that this was the expected result as predominately unbranched neurons were bleached due to the lack of specificity of the Elav1 general promoter. We anticipate that experimentation using constructs driven by neural subtype specific promoters will allow for a better understanding of branched neurons with multiple neurites.

Chapter 4 : Discussion

Molecular Cloning Further Applications

The creation of the NvElav1-mCD8-mCitrine, NvElav1-mCD8-mCherry, and NvElav1-mCD8-mCitrine-2A-mCherry constructs allowed for a more comprehensive understanding of whether or not *Nematostella* have the same mechanisms for establishing functional polarity as higher animals. These constructs allowed for a heightened understanding of which fluorophores were most effective with certain microscopic techniques including that mCherry is most preferred for performing FRAP due to lack of autofluorescence allowing for better visualization. mCitrine is imperfect as the laser is at off-peak power, which we were not aware of initially. The mCherry fluorescent protein is effective for tens of seconds, but it would bleach too quickly to detect a barrier, which requires the signal to be maintained for minutes. For this reason, eGFP or mCitrine is a better fluorophore as they are more bleach resistant during the imaging phase. This understanding will allow for more effective sorting and bleaching of other constructs in the lab as well as for any further applications of this project. It was also demonstrated that the 2A linker peptide sequence can be placed in between two fluorescent proteins (in this case mCitrine and mCherry) to allow for effective sorting by mCherry and bleaching by mCitrine. This sequence has already been used with different constructs in the lab to more successfully conduct the bleaching experiments.

FRAP Analysis

After performing FRAP, it was evident that the neurites that we labeled with the marker and photobleached did not contain membrane diffusion barriers as 85.6% of the initial intensity of the signal diffused back into the bleached region of the distal axon within seconds. As seen in the experiments where membrane diffusion barriers were detected in *Drosophila*, if a barrier had been present the signal would likely not have recovered past 40% in 400 seconds.[35] In all of the photobleaching experiments that were performed, we visualized bipolar and tripolar unbranched neurons. These experiments did not allow for the isolation and experimentation on branched *Nematostella* neurons as the Elav1 promoter is a general neuronl promoter and does not specifically select for branched neurons. As anticipated, it was found that the photobleached unbranched neurons did not have a barrier as they are not polarized. It is possible that these neurons have homogenous membrane properties and therefore do not require a membrane diffusion barrier. We expect that the polar branched neurons may contain the membrane diffusion barriers indicative of axon initial segments.

The Elav1 promoter that was used in each of the three constructs is a general neuronal promoter. Since it allows for the tagging of all neurons, it is very difficult to specifically target the branched neurons. For this reason, the unbranched neurons were likely the only neurons that were tested using FRAP. In order to further explore the existence of membrane diffusion barriers in branched neurons, additional experimentation where the branched neurons are selected for is required. It is possible to use the Elav1 promoter and screen a substantially larger quantity of neurons in order to find the branched neurons; however, this method is wasteful in both the amount of money and time that it requires. Alternatively, we are looking to use specific neuronal cloning promoters to explicitly label the branched neurons. We anticipate that branched neurons

having more than one neurite may contain membrane diffusion barriers. The neurons that most probably contain membrane diffusion barriers are the neurons that contain many minus-end-out neurites. In these neurons, it is likely that one of the neurites may be a dendrite with mixed polarity. The unbranched neurons are all plus-end-out and therefore all equivalent, indicating that a barrier for segregation is not essential to the function of the neurite. After performing the FRAP protocol, we saw this to be true, finding no membrane diffusion barriers in any of the neurites of an unbranched neuron.

In determining the presence or absence of a membrane diffusion barrier in branched neurons containing more than one neurite, we will be able to gain insight about the evolution of nervous systems. If the membrane diffusion barrier is present, we will see that the axon initial segment is a conserved domain all the way back to Cnidarians. However, it is also possible that membrane diffusion barriers are not an integral part of initiation and therefore will not be present in any *Nematostella* neurons.

Future Steps

The initial photobleaching experiments using the NvElav1-mCD8-mCitrine, NvElav1-mCD8-mCherry, and NvElav1-mCD8-mCitrine-2A-mCherry constructs have allowed for further questioning of the classification of Nematostella neurons. It is evident that most of the neurons present in *Nematostella* do not contain membrane diffusion barriers and instead function as an unpolarized nerve net as current phylogeny trees predict. However, in an effort to better understand and classify these neurons, the lab has begun extensive analysis on their polarities. By creating an EB1-GFP reporter, the microtubules of the neurons may be tagged, allowing for

determination of which neurons are plus end or minus end out. While it was previously postulated that there are three different types of neurons present in *Nematostella*, the lab has potentially identified greater than eight types. Some of the classifications of neurons identified occur relatively infrequently, so more quantitation of results is required. While this experimentation has demonstrated that unbranched *Nematostella* neurons do not feature membrane diffusion barriers, we believe that the barrier may be present in one or more of the other classifications of neurons.

In order to determine whether or not membrane diffusion barriers exist in the branched neurons with multiple neurites in Cnidarians, we are looking to create a construct with a promoter that specifically targets branched neurons upstream of the reporter. As we have not yet indicated an effective promoter, we are currently still working with the EB1-GFP construct to sort branched neurons from unbranched neurons. The *elav* gene in *Drosophila melanogaster* is designated as neuronal during early embryogenesis, causing it to be an effective molecular marker of neural differentiation.[36] It is likely that a specific promoter from the Elav family would allow for the neuronal specificity required to selectively target branched neurons.

This analysis would allow for a more comprehensive understanding of the evolution of the central nervous system. If there is no membrane diffusion barrier present, we predict the evolution of polarity to have occurred more recently with the development of the central nervous system. Alternatively, if there is a membrane diffusion barrier present, the diffusion barrier could be a fundamental feature of an axon identity rather than a specialization for segregated signaling in central nervous systems. Following the finding of the diffusion barrier, more useful studies could be done to better understand the establishment of polarity. We could then create a knock-out construct without the ankyrin G ortholog to see whether or not ankyrin truly is essential in

the formation of the axon initial segment and subsequently the membrane diffusion barrier. The determination of the presence or the absence of the membrane diffusion barrier will allow for a better understanding of the evolution of the central nervous system and functionality of the *Nematostella* nervous system.

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ACADEMIC VITA

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EDUCATION

The Pennsylvania State University, University Park, PA
B.S. in Biochemistry and Molecular Biology, **Schreyer Honors College**
Minors in Spanish and Chemistry

Seville, Spain Study Abroad *Summer 2013*
- Enrolled in Spanish grammar as well as culture classes
- Interacted with native speakers through the homestay and school activities

London Study Tour
- Travelled to London for ten days with a theatre class with Schreyer

RELATED EXPERIENCE

Externship with Johnson & Johnson *May 2012*
- Toured research and development, production, and packaging facilities
- Attended presentations and met with the heads of the laboratories

Dr. Timothy Jegla's Neuroscience Lab *August 2012- Present*
- Condust molecular cloning and photobleaching experiments to better understand whether the nervous systems of Cnidarians are axon-like of dendritic
- Exploring applications to human and human diseases including epilepsy

LEADERSHIP ACTIVITIES

Association of Residence Hall Students (President) *August 2012- Present*
- Elected president of the on-campus student government in the spring
- Determine how to allocate monies to programs and organizations
- Plan events for almost 15,000 residents and advocate for students
- Supervise other officers to ensure duties are fulfilled

Science LionPride *Spring 2014-Present*
- Act as an ambassador for the Eberly College of Science
- Coordinate events with alumni, give tours, and work to benefit the community

Schreyer Orientation Leader *February 2012-Present*

- Mentor incoming Schreyer scholars to ease the college transition
- Plan events for the incoming freshmen class including a service project

HONORS AND AWARDS

Summer Discovery Grant *Summer 2014*

- Awarded $4,000 to spend the summer researching in State College for my thesis
- Contributing author on a paper sent to be published with two more up and coming

Eberly College of Science Research Scholarship *October 2013*

- Awarded $1,500 for the Jegla laboratory to continue to thesis research project
- Record and present results at poster presentation of undergraduate research

National Resident Hall Honorary (NRHH) *May 2013*

- Recognized as part of the top 1% of residence hall students

PROFESSIONAL PRESENTATIONS

- **Undergraduate Poster Exhibition, Penn State University Park** *Spring 2014*
- **Summer Discovery Grant Poster Session, Penn State University Park** *Fall 2015*

PUBLICATIONS

Li, Xiaofan, Hansi Liu, Jose Chu Luo, Sarah A. Rhodes, Liana M. Trigg, Damian B. van Rossum, Andriy Anishkin et al. "Major diversification of voltage-gated K+ channels occurred in ancestral parahoxozoans." *Proceedings of the National Academy of Sciences* 112, no. 9 (2015): E1010-E1019.