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**Developing a Novel Means of Observing the Intracellular Activities of
Heterogeneous Nuclear Ribonucleoprotein K within *Xenopus laevis***

An honors thesis presented to the
Department of Biological Sciences
University at Albany
State University at New York
In partial fulfillment of the
Honors Program Requirements

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May 2015

Committee Recommendation

The Honors College
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Abstract

Research into the regeneration of optic nerves in *Xenopus laevis* has determined that heterogeneous nuclear ribonucleoprotein K (hnRNP K) plays a crucial role in regulating the trafficking and translation of mRNAs essential for the organization of the axonal cytoskeleton. To further explore this role, our lab has turned to tools that can definitively elucidate hnRNP K's translocation in-and-out of the nucleus, as well as directly quantitate its degradation rate, *in vivo*. An appropriate tool for such experiments is the monomeric Eos fluorescent protein (mEosFP), which can be stably and irreversibly photo-converted. This fluorescent protein naturally emits green light (~516nm) and upon photo-conversion with UV (~405nm), it stably emits red light (~581nm). Using traditional cloning techniques, we are fusing mEosFP (*Xenopus* codon specific) to *Xenopus* hnRNP K in a plasmid designed to generate 5' capped mRNA through *in vitro* transcription. The resultant RNA will be microinjected into 2-cell stage *Xenopus* embryos, and these embryos will be allowed to mature to stage 22 (24 hrs. post-fertilization). From these animals we will create dissociated, embryonic spinal cord / myotome cultures. We will then photo-convert mEosFP using a Zeiss 710 confocal microscope and monitor the subcellular location as well as quantitate the resulting fluorescence. To achieve this, we will use time-lapse photography and ImageJ software, respectively. A series of controls will be included to ensure the incorporation of the fluorescent protein does not interfere with hnRNP K's biological activity. This technique will enable us to track hnRNP K's movements through the cell more accurately and quantify its turnover rate *in vivo*.

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I would like to thank Dr. Szaro for giving me the opportunity to work in his lab for the past two years, for telling me interesting stories, and giving me advice about school. I can definitely say I had a well-rounded experience in his lab. I would also like to thank Jamie for being extremely patient with me as well as making my lab experience fun and taking the bite away from lab work. I think it is also necessary to thank Dr. Hutchins, Chen, Rupa, and Megan for answering the many questions I had for them over the years.

I would like to take this time to express my appreciation towards Dr. Wulff for agreeing to be a part of my thesis committee- it meant a lot that he took the time out of his busy schedule to read my paper.

Last, but not least, I would like to thank my parents, Kay and Fabian and my other family members for their support throughout my undergraduate career. I would like to thank Mary for being my Honors college buddy and helping me stay on track for the past four years. And I would like to thank Najua for baring through all the nights I kept our room light on so I could complete my school work.

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Introduction

Axonogenesis- the growth of axons- is an essential component of development for all organisms with a functioning nervous system. Once the process is complete, the axons, or nerve fibers, allow signals to be transmitted from one part of the body to the next in response to a series of chemical changes. As important as these structures are, many organisms, including mammals, lack the ability to mend their axons or to reinitiate axonogenesis upon neuronal damage. However, the African clawed frog *Xenopus laevis* has the ability to regenerate its optic axons due to its cells' ability to sustain cytoskeletal organization, gene transcription, and post-transcriptional control of its cytoskeletal composition (Hutchins and Szaro, 2013) through a series of protein-kinase interactions. In the Szaro lab, my project focused on heterogeneous nuclear ribonucleoprotein K (hnRNP K), an integral RNA-binding protein responsible for the post-transcriptional control of a specific range of mRNAs (Liu & Szaro, 2011). During neural development, the protein helps to coordinate the synthesis of structural proteins used to organize microtubules, microfilaments, and NFs [neurofilaments] into an axon through its export out of the nucleus. Humans and the African clawed frog are separated by a great evolutionary distance, but are genetically joined in the fact that they express homologous hnRNP K proteins (Dreyfuss, 1997). Thus, studying *X. laevis*' hnRNP K protein may provide insight into the mechanisms that allow amphibians to regenerate axons and prevent humans from recreating such events.

Several hypotheses have been raised to explain what regulates the transport of hnRNP K in and out of the nucleus (Adolph, Flach, Mueller, Ostareck & Ostareck-Lederer, 2007; Michael, Eder, and Dreyfuss, 1997), but research is ongoing. Prior experiments have provided a copious amount of information regarding the most efficient ways to study the protein. Previously in the Szaro lab, various cDNAs, of interest, have been inserted into a modified pGEM-3Z plasmid

containing a promoter for the SP6 RNA polymerase vector (Lin and Szaro, 1996; Liu, Gervasi and Szaro, 2008; Hutchins and Szaro, 2013). Furthermore, based on the works of Paul Krieg and Doug Melton (1984), synthetic mRNAs produced by the SP6 RNA polymerase by *in vitro* transcription of cDNA clones from such plasmids are translated just as efficiently as native mRNAs in injected embryos. Thus, the study of a laboratory-manipulated endogenous protein is possible due the vector's capability of producing protein expression in intact embryos comparable to proteins left untampered by scientists.

Prior to the revolutionary experiments and studies performed using Green Fluorescent Protein (GFP) (Chalfie *et al.*, 1994), scientists wanting to monitor gene expression were limited to using methods that required them to observe the cellular localizations of a particular target protein only after the death of the organism. Use of the commercially available markers such as protein-specific antibodies and β -galactosidase required the animals to be fixed and permeabilized to allow the reagents used for their detection to enter the tissue. In addition, several pictures of the organism had to be compared in order to effectively monitor the target protein (Chalfie, 2008). Thus, after the discovery of the fluorescent protein naturally produced by the jellyfish *Aequorea victoria*, Martin Chalfie recognized its potential as a less invasive biological marker.

The dimeric protein is composed of a unique 3-dimensional structure in which β strands surround and protect a chromophore, (consisting of the residues Serine-Tyrosine-Glycine), located towards the center of a "beta-can" configuration. To further protect the reactive chromophore, the "can" is capped by α helices and short loop regions, allowing the protein to fluoresce even while submerged in fluorescence quenching agents such as acrylamide and molecular oxygen (Chalfie and Kain, 1998). Furthermore, there are two methods in which GFP

can be induced to emit a similar green light that are identical in spectral properties. The first, natural mechanism, the Förster-type, occurs through a radiation-less energy transfer when the excitation energy is transferred from the donor molecule to the acceptor without the emission of a photon. The second method involves the donor molecule emitting light that is consequently absorbed and reemitted by the acceptor (Chalfie and Kain, 1998). Consequently, by stimulating fluorescence of the protein by illuminating it with blue light, it was possible to harness the fluorescent capabilities of the protein to visualize it without the use of any additional reagents. Chalfie took advantage of additional qualities that made the protein an ideal biological marker such as its relatively small size with only 238 amino acids, its maintained functionality after modification to a monomeric protein, its fluorescence in the absence of cofactors or other small molecules, and its resistance to photobleaching (Chalfie, 2008). Advancements in technology have since allowed Martin Chalfie, Roger Tsien, and Osamu Shimomura to genetically modify the GFP gene so that it can fluoresce brighter within an organism, in addition to working efficiently in many different model systems.

Following the commercialization of GFP, other naturally occurring proteins have been discovered and genetically enhanced to develop fluorescent variants that better serve the purposes of the various types of studies done in the laboratory. Originating from a range of jellyfish, coral reefs, and sea anemone species, each product has features that makes it better suited for certain experiments over other protein types. Proteins such as Kaede, tdTomato, and EosFP are attractive to scientists around the world due to their relative brightness (compared to enhanced GFP [EGFP]) and their molecular structures. However, when choosing an appropriate fluorescent protein to be included in an experiment, there are several factors that must be taken into consideration. Properties such as excitation/ emission peaks, optimal temperature for protein

folding, whether it forms oligomers, and photostability all have the potential to negatively affect the outcome of an experiment (Haimovich, 2014). While Kaede and tdTomato offer some of the highest levels of relative brightness, their tetrameric and tandem dimeric structures, respectively, have the potential to impair proper protein functions *in vivo* and can lead to the creation of artifacts that may mislead scientists and skew data. This is because larger molecular structures tend to be driven towards oligomer formation *in vivo* (Chalfie and Kain, 1998) especially when expressed with proteins, such as tubulin, that naturally oligomerize within the organism. Furthermore, while tdTomato's tandem dimeric structure signifies that the protein is essentially monomeric, but has twice the molecular weight and size, it may still restrict the cellular localizations of the target protein due to the larger size of the fusion tag (Shaner, Steinbach, & Tsien, 2005).

Originating from the stony coral *Lobophyllia hemprichii*, EosFP is a tetrameric fluorescent protein similar to Kaede. Upon the introduction of two amino acid substitutions, the monomeric derivative (mEosFP) is produced. Although the two proteins have virtually similar spectroscopic properties, the point mutations allow the expressed fusion proteins to be functionally expressed at temperatures below 30°C- a key element while working with the cold-blooded *Xenopus laevis* whose natural body temperatures fall between 20-23°C (Piston *et al.*). A naturally green- fluorescing protein (~516nm), it can be easily photo-converted to emit red light (~581nm) following violet light excitation at ~405nm. The irreversible photoconversion is possible due to a chromogenic triad of amino acids (Histidine-Tyrosine-Glycine) (Wiedenmann *et al.*,2004) that acts as a chromophore in which the energy difference between two different molecular orbitals of the region falls within the range of the visible spectrum. Once this region in

the molecule is excited by UV or violet (405 nm) light, a series of conformational changes occurs allowing the molecule to emit light of a longer wavelength.

Consequently, we decided to utilize mEosFP's fluorescent capabilities to track hnRNP K's movements through the cell by initially creating a recombinant fusion protein (between mEosFP and hnRNP K). To create a cDNA construct that could be used to express the fusion protein in embryos, traditional cloning methods were used. The cDNA encoding the mEosFP/hnRNP K complex (referred within this paper as the insert) was then ligated into a modified pGEM-3Z vector, which can be used to transcribe RNA, *in vitro* (a feature that is necessary for its later use in an intact organism) ("Instructions for Use", 2009). To propagate the plasmid, I transformed it into *E. coli* competent cells, using the recombinant resistant strain DH5 α . As the competent cells replicate, they will make copies of our plasmid of interest.

Materials & Methods

mEosFP Fragment Acquisition: The amino acid sequence of mEosFP was obtained from the Wiedenmann *et al.* (2004) paper. Using the *Xenopus* codon usage table published by J. Michael Cherry (1991) as a reference, we translated the known amino acid sequence of mammalian mEosFP into a nucleotide sequence that is more compatible with translation in *Xenopus laevis* sequence by using the most prevalent codon for each amino acid within *Xenopus laevis*. In addition, we introduced two amino acid substitutions to make the protein more thermostable below 37°C (AA 160 C→D & AA 194 E→H) (Piston *et al.*). For cloning purposes, restriction sites and additional coding sequences were also added. A Kozak sequence, GCCACCATGG(GA) was added in order to increase the translation efficiency and GA was added to the 3' end to maintain the correct reading frame throughout translation of the sequence so that it could be fused, in frame, with hnRNP K. The resulting codon of GGA translates as a

glycine which is the most abundant amino acid within *Xenopus laevis*. 5' to the Kozak sequence, a NotI restriction site, GCGGCCGC, was added to the mEosFP sequence to provide the front-flanking restriction site of the mEosFP/hnRNP K insert. At the 3' end of the mEosFP amino acid sequence, a linker region was placed by Dr. Szaro and Linn von Pein as a means to join the mEosFP and the hnRNP K proteins. Included in the linker region was a SalI (GTCGAC) restriction site as well as a BsrGI (TGTACA) site for orientation and cloning purposes, respectively (**Figure 1**). The above described construct was ordered as a GeneArt® String from Invitrogen.

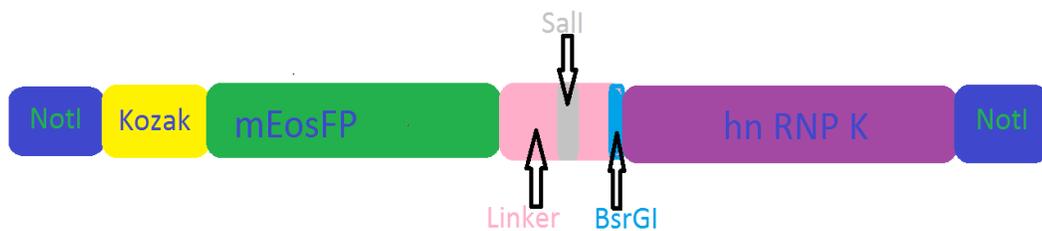


Figure 1: Illustrates the finished ligation product of the mEosFP and hnRNP K inserts with their respective restriction sites.

hnRNP K Fragment Acquisition: The hnRNP K fragment was already cloned into a pGEM-3Z plasmid created by a former member of the Szaro lab, Yuanyuan Liu (Liu & Szaro, 2008). Located at both the 5' and 3' ends of the hnRNP K sequence were NotI restriction sites. To remove the hnRNP K fragment, the vector was digested with NotI enzyme and the product was purified using Promega's Wizard SV Gel and PCR Cleanup System (#A9282). The vector was then CIP-treated to prevent self-ligation.

Fragmented Insert Amplification: Using Invitrogen's Platinum Pfx DNA Polymerase (#11708-013), I amplified the mEosFP and the hnRNP K fragments, separately, using the Polymerase Chain Reaction (PCR). The enzyme's high fidelity and 3' proofreading capabilities helped ensure there would be no mutations in the resulting amplicon. I performed the amplification using three different reaction conditions to determine which resulted in the highest product yield. The three conditions included (1) the manufacturer's recommendations; (2) the manufacturer's recommendations with PCRx Enhancer Solution; and (3) Enhancer Solution with increased template concentrations (**Table I**). The cycling parameters (**Table II**) were based on the manufacturer's recommendations and the reactions were performed in the AB 2720 Thermal Cycler. Samples were run on a genetic technology grade (GTG) 1% agarose/Tris borate EDTA (TBE) gel (Lonza SeaKem® GTG agarose, #50070) and the separated bands were visualized using 5µL of ethidium bromide added to the agarose solution prior to polymerization (EtBr; 10mg/mL). The agarose containing the desired bands were excised from the gel, pooled together, and the DNA was purified using Promega's Wizard Kit (#A9282).

Reagents	Cond. 1		Cond. 2		Cond. 3	
	Vol. (μL)	Conc.	Vol. (μL)	Conc.	Vol. (μL)	Conc.
10X Buffer	5.0	1X	5.0	1X	5.0	1X
10mM dNTPs	1.5	0.3mM ea.	1.5	0.3mM ea.	1.5	0.3mM ea.
50mM MgSO₄	1.0	1mM	1.0	1mM	1.0	1mM
10μM Primer (F&R)	3.0 (1.5 ea.)	0.3 μM ea.	3.0 (1.5 ea.)	0.3 μM ea.	3.0 (1.5 ea.)	0.3 μM ea.
Template 80pg/μL	2.0	160pg/L	2.0	160pg/L	4.0	320pg/L
10X enhancer	0	0	5.0	1X	5.0	1X
Pfx 2.5U/μL	0.4	1 unit	0.4	1 unit	0.4	1 unit
H₂O	37.1		32.1		30.1	
Total	50		50		50	

Table I: Demonstrates the reagent volumes and concentrations for the three reaction conditions used for the Pfx PCR to amplify the hnRNP K and mEosFP amplicons.

Cycling Parameters		
	Temp. ($^{\circ}\text{C}$)	Time
30X	94	5min
	94	15sec
	55	30sec
	68	1.5min
	4	∞

Table II: The cycling parameters used for all of the conditions in the Pfx PCR to amplify the hnRNP K and mEosFP amplicons.

mEosFP & hnRNP K Ligation: The two fragments of our prospective insert were digested with BsrGI (NEB, #R0575S) at 37°C following the manufacturer's recommendations, and the products were run on a 1% agarose/TBE gel (Invitrogen). The DNA was then quantified (A_{260} ; ND-1000; NanoDrop/ Thermo Scientific).

The two PCR products were ligated in an equimolar ratio (150 fm) using T4 DNA Ligase (NEB, #M0202S) according to the manufacturer's recommendations (16°C, overnight). In this procedure, λ bacteriophage DNA that had been digested with HindIII (USB, #70061) was ligated in a parallel reaction to confirm that the ligation reaction ran to completion. Once it was confirmed that the reaction had run to completion, the ligase was heat inactivated at 65°C for 10 minutes. The ligation product was then electrophoresed on a GTG 1% agarose gel (Lonza SeaKem® GTG agarose, #50070) at 100V for 130 minutes, and the appropriate band; (~2Kb), was excised from the gel, and the DNA purified, and quantified on the spectrophotometer as before. Because we did not obtain enough product to proceed, we performed a second PCR reaction, this time using Promega's Go Taq Green Master Mix (#M712B) in a 25 μ L reaction (denature 1 min at 95°C, anneal 1 min at 60°C, and extend at 72°C for 2.5 min for 30 cycles). The product was electrophoresed through a 1% TBE agarose gel purified and quantified as before.

Insert & Vector Ligation: To prepare the insert for ligation within the pGEM-3Z vector, we performed an overnight NotI-HF (NEB, #R3189S) digestion according to the manufacturer's recommendations. After inactivating the reaction, we purified the product after a 1% TBE agarose gel electrophoresis and quantified the product as before.

The mEosFP/hnRNP K insert and CIP-treated vector were ligated together using a 3:1 molar ratio (150:50 fm, respectively) using NEB's T4 Ligase (#M0202S) and 5X T4 ligase

buffer with polyethylene glycol (Invitrogen, #Y90001)- a macromolecular crowder- to further increase the chances of a successful ligation. Following confirmation that the ligation reaction was complete, the ligation product and controls were Phenol-Chloroform extracted and ethanol precipitated to remove the polyethylene glycol, which is toxic to *E. coli*. The final DNA products were resuspended in 20 μL of nuclease-free water for transformation.

Transformation: The purified ligation products were then transformed into Subcloning Efficiency DH5 α Competent Cells (Invitrogen, #18265-017). Following the manufacturer's protocol, 5 μL of the ligated DNA was added to the DH5 α cells in addition to 2.5 μL of the pUC19 positive control plasmid (100pg/ μL) provided by the company. The transformed cells were plated onto agarose plates containing LB medium supplemented with ampicillin (50 $\mu\text{g}/\text{mL}$), added to select for cells transformed by the plasmid, which contained an ampicillin resistance gene on the original pGEM-3Z parent vector. Super Optimal Broth with Catabolite repression (SOC) was used in conjunction with the culture to facilitate recovery from the transformation prior to spreading the cells onto the agarose plates. The plates were incubated overnight at 37°C.

Miniprep & Sequencing: This section has yet to be performed. Assuming a successful ligation, and sequencing from GeneWiz, **Figure 4** demonstrates the DNA construct we will produce.

Results

The yields from the Platinum Pfx PCR reaction to amplify mEosFP and hnRNP K fragments (**Figure 2**) were 87.9 and 229.8 ng/ μL , respectively. Following the BsrGI digestion of the fragments, we had 11.1 ng/ μL of mEosFP and 12.3 ng/ μL of hnRNP K. After ligating the two PCR products (**Figure 3**), we had 3.13 ng/ μL in 31 μL (97ng). We felt that 97 ng wasn't enough

material to continue with the experiment, so a second GoTaq Green PCR reaction was performed to produce 40.02 ng/ μ L in 38 μ L, for a final yield of 1520 ng. After the newly-ligated insert was NotI-HF digested, we arrived at a final concentration of 9.84 ng/ μ L in 34 μ L. Since this was too dilute for the vector/insert ligation, the digested product was dried down in a Heto-SpinVac, and the pellet resuspended in 5 μ L for a final concentration of 66.6 ng/ μ L. *The transformation performed was not successful and we are still reviewing the results.* Figure 4 demonstrates the DNA construct that would have been produced assuming a successful transformation which includes the ligated mEosFP/hnRNP K insert (~2Kb) and the pGEM-3Z vector (without the depiction of the 3'UTR rabbit β -globin fragment) (~5Kb).



Figure 2: Demonstrates the results on the 1% GTG gel after the Invitrogen Platinum Pfx PCR reaction using the three different conditions. The results were as expected with the mEosFP fragment at ~0.7Kb and the hnRNP K fragment at ~1.2Kb. The gel was imaged on a UV light box on the analytical setting.

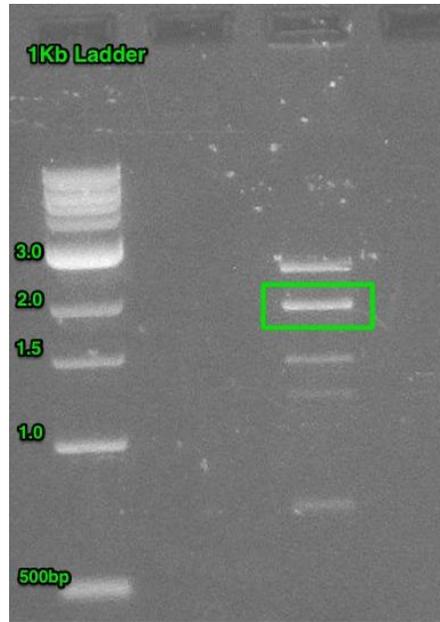


Figure 3: Demonstrates the 1% GTG gel used to purify the DNA after the mEosFP/hnRNP K ligation using NEB's T4 DNA Ligase. The squared fragment band represents our product of interest at ~2Kb. The gel was imaged on a UV light box on the analytical setting.

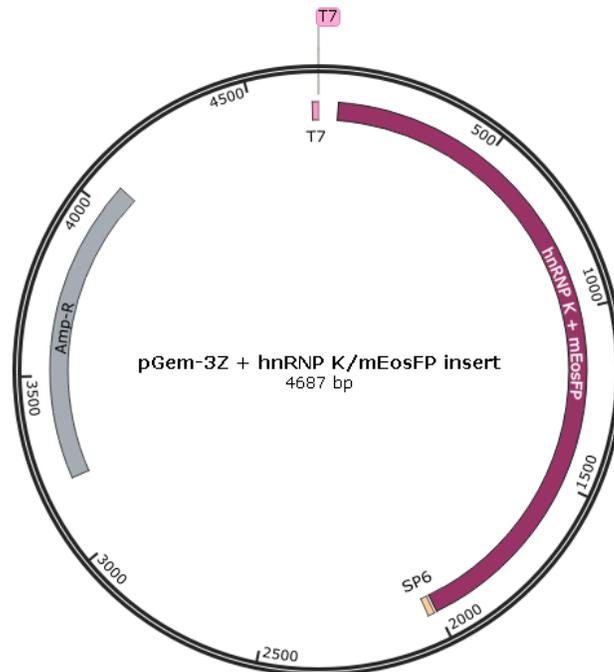


Figure 4: Image of the completed map that was created in the lab, without the 3'UTR rabbit β -globin that would be between the T7 promoter and the hnRNP K portion of the insert.

**Note: The missing 3'UTR rabbit β -globin would add ~2.2 kb to the depicted map.*

Discussion

To conclude my time working on this project, agarose gels determined that I successfully amplified and ligated mEosFP and hnRNP K to create a cDNA encoding an mEosFP/hnRNP K fusion protein. For efficient transcription and expression of the resulting protein, however, the insert had to be ligated within the bacterial SP6 vector, pGEM-3Z. Unfortunately, upon transformation, no colonies were produced. Under more successful circumstances, colonies from the transformation would have been mini-prepped and sent out for sequencing by the company GeneWiz. After sequencing has confirmed the introduction of the insert in the appropriate orientation within the plasmid, with no mutations that will interfere with subsequent experiments, it would then be possible to move on to *in vitro* transcription of the DNA into 5' capped mRNA and injecting it into the 2-cell stage of *Xenopus laevis* to observe the processes and localizations of hnRNP K *in vivo*. Transcribing the DNA into 5' capped RNA is essential for stabilizing the injected RNA once it is inside of the embryo. In addition to the 5' cap, the 3'UTR of rabbit β -globin also acts as a stabilizer of the RNA due to an intrinsic cytoplasmic polyadenylation element (Gervasi & Szaro, 2004).

The frog's expression of a photoconvertible protein presents an innovative way in which to circumnavigate the issue of observing natural processes within the organism without the disturbances of invasive procedures. As mentioned earlier, hnRNP K acts as a scaffolding protein for other proteins that are integral in the proper growth and synapse formation in optic neurons. However, to control such processes, hnRNP K's activation in the nucleus and export towards the cytoplasm has to be carefully regulated. We plan to excite the photoconvertible protein while observing cells through a Zeiss LSM 710 confocal microscope. Then, using time-lapse photography and the ImageJ program, we plan to observe the subcellular localization of the

protein and quantitate the resulting fluorescence. Photoconverting the protein will enable us to monitor the decay of the red fluorescent form of the protein to measure hnRNP K's turnover rate in different regions of the cell.

Prior to the collection of any noteworthy data while using the fluorescent protein to observe hnRNP K, a preliminary experiment would have to be conducted by the lab to confirm its efficiency for continued lab use. Currently in the Szaro lab, fluorescent proteins such as EGFP are used to observe the intracellular processes of hnRNP K (Hutchins and Szaro, 2013). However, the GFP used for the fusion protein is not *Xenopus* codon-optimized like the mEosFP protein created for this experiment. Additionally, mEosFP is reportedly brighter than EGFP (Piston *et al.*). Thus, we would have to perform a comparative experiment in which we determine whether more hnRNP K protein is made with the mEosFP fusion (using western blots) which could be attributed to its codon optimization for our model organism. Furthermore, a higher expression of hnRNP K will increase the brightness of the signal intensity.

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