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Developing New Research Skills Using Transgenic Zebrafish in an International Setting

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Abstract

During the summer of 2022 I had the amazing opportunity to visit Dr. K. Kawakami Lab at the National Institute of Genetics in Japan. His lab has different projects some focused on optimizing gene editing tool others on characterizing neuron and determining their behavior roles. My aim during this summer was to learn new skill using the zebrafish as the experimental model. The idea was to expose me to a different set of technique common to the Kawakami lab like transgenic line screening, embryo microinjections, inverse PCR, in situ hybridization chain reaction (HCR), confocal microscopy, and in vitro mRNA transcription to diversify my skills and broaden my research perspective. Additionally, Dr. Kawakami focused my experience so they could relate to my thesis project back at my home institution at the University of Puerto Rico. I was able to synthetize transposase mRNA which was used to microinject embryos. These embryos were assayed, and plasmid integration were determined via plasmid excision assay finding that 25% of my injected embryos integrated the donor plasmid. Moreover, HCR experiments provided the opportunity to assay larvae zebrafish expression of glial fibrillary acidic protein (GFAP) a radial glial cell marker in transgenic zebrafish to assess colocalization between GFP expressing cells and GFAP probes which unfortunate seem to have not been colocalized. The overall results of my experiment facilitated me to further narrow my future aims, grow as a researcher while providing an experience with techniques I will implement in my thesis project further increasing the quality of m project results.

Introduction

The scientific community has vastly expanded from select niches to almost all regions of the earth in just a few decades. Unarguably, the science field has moved forward evolving and adjusting to cultures. With the advancements of technology, the research community began to connect and collaborate outside their national community. International research collaborations are highly valued and almost expected for this new generation of scientist. It is an opportunity to learn from other research cultures, broaden your skill, share, and receive new

perspectives, as well as to interact with a diverse scientific community. International collaborations have been argute to be an indicator of high-quality research (Freshwater et al., 2006). As discussed by Rolfe et al. (2004) "*International research collaboration presents health researchers with opportunities to share experiences, data and methods that can provide the basis for new and important perspectives on existing practices*". However, these benefits are not limited to health care researcher.

During the summer of 2022 I decided to join the International Research Experience for Student program (IRES) held in Japan. My experience was hosted by Dr. Kawakami's Laboratory at the National Institute of Genetics (NIG). The main objective of this experience was to expose myself to a different laboratory setting to learn new techniques, research perspectives, and further develop my critical thinking capabilities. The rational is to further develop skills that would help me develop as a researcher while also providing me with experience I can implement at my home institution thesis project.

The research scope of the Kawakami lab is very broad. Their work focuses on understanding the neuronal network circuitry for behaviors, optimization of genetic modification techniques by establishing effective double knock outs and knock ins, neuronal characterization, and further understanding the roles of the TDP-43 protein in amyotrophic lateral sclerosis zebrafish models. However, what binds all these projects toguether is the use of transgenic zebrafish models. The zebrafish has risen as a potent model in many fields, this in part was achive by the stable generation of transgenic lines. The Kawakami lab are pioneers on developing stable and cost-effective transgenic zebrafish models having developed the Tol2 Mediated Gal 4-UAS Gene Trap System and generated over 3000 different transgenic lines over the course of 20 years. By providing such tool, the zebrafish organism has become a powerful model in the field of neuroscience, molecular genetics, and developmental biology.

Tol2 Mediated Gal 4-UAS Gene Trap System

To understand the transgenic generation system, we must first understand the system protagonists and

their roles. *Tol*2 is a transposable element which researchers have taken advantage to make what they call the *Tol*2 transposition system (Kawakami et al., 2016). The transposition system is composed of a donor plasmid which has two *Tol*2 element sites between a gene of interest and an *in vitro* synthesized transposase mRNA (Figure 1A). The two *Tol*2 element sites are critical for sequence transposition. These *Tol*2 elements position at the left and right of the gene of interest and it is done so because there are the sequence regions that the transposase uses to excise the region from the donor plasmid and later integrate into the genomic DNA. Although the original *Tol*2 element is a larger DNA sequence researchers at the Kawakami lab were able to show that 200-bp from the left end and 150-bp from the right end are enough for transposition (Kawakami, 2007; Kawakami et al., 2016). To generate transgenic lines, both donor plasmid and transcriptase mRNA are mixed in a solution and injected into a fertilized egg (Figure1B). Because the *Tol*2 system is active in all vertebrate cells the sequence region between the *Tol*2 recognition site of the plasmid can be excised and randomly integrated into the host egg genome during the germ cell lineage (Kawakami, 2007). Hence, this procedure is normally done at the single cell stage roughly 0-15 minutes after embryos are fertilized. These injected zebrafish adults are called founders and although they do not express green fluorescent protein (GFP) or other gene of interest they do possess the genetic material to generate transgenic zebrafish lines (Figure1C). Two different founders need to be created to develop stable transgenic

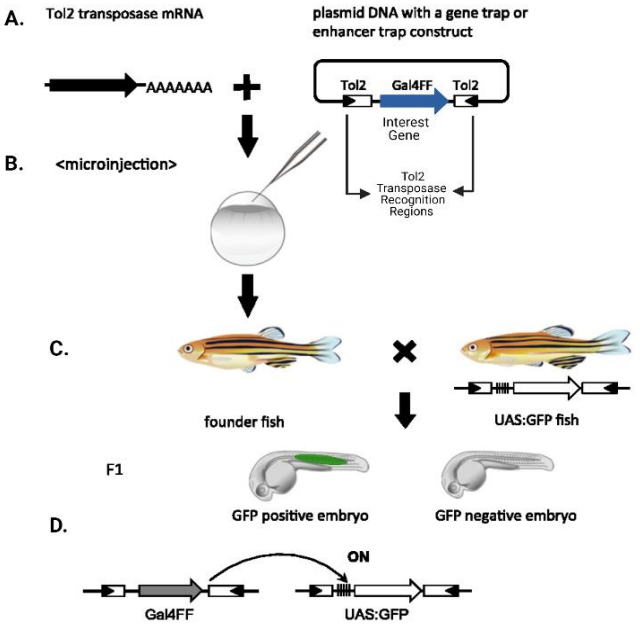

Figure 1: General workflow for generation of stable transgenic zebrafish lines. This image illustrated the immediate components necessary to generate a transgenic effector gene using as example GFP. Image was used from (Kawakami et al., 2016)

positive lines. One founder needs to integrate Gal4 which is a yeast transcription factor consisting of 881 amino acids, and although many GAL4 variants have been created over the years

Table 1. Upstream activating sequence (UAS) reporter and effector lines

UAS-transgene	Effector or reporter	Reference
UAS:EGFP	Green fluorescent protein	Asakawa *et al.* (2008)
UAS:mRFP1	Red fluorescent protein	Asakawa *et al.* (2008)
UAS:Kaede	Photoconvertible fluorescent protein	Scott *et al.* (2007); Davison *et al.* (2007)
UAS:KikGR	Photoconvertible fluorescent protein	Hatta *et al.* (2006)
UAS:Dronpa	reversible fluorescent protein	Aramaki & Hatta (2006)
UAS:*myc-notch1a-intra*	An activated form of Notch receptor	Scheer & Campos-Ortega (1999)
UAS:*fezl*	Fezl protein	Jeong *et al.* (2007)
UAS:*nfsB-mCherry*	Nitroreductase tagged with mCherry	Davison *et al.* (2007)
UAS:iGluR6(L439C)	Light gated iGluR6 receptor	Szobota *et al.* (2007)
UAS:TeTxLC	Tetanus toxin light chain	Asakawa *et al.* (2008)
UAS:TeTxLC:CFP	Tetanus toxin light chain tagged with CFP	Asakawa *et al.* (2008)

CFP, cyan fluorescent protein.

Table 1: Table illustrates some of the transgenic lines established using the *Tol*2 transposase mediated GAL4: UAS system in the zebrafish. Image was taken by (Kawakami, 2007)

researchers use the one more adaptable to their research objectives (Asakawa and Kawakami, 2008). The second founder need to integrate the gene of interest (for simplification lets assume it is GFP) mediated by the (upstream activating sequence) UAS promoter. Because Gal4 specifically binds to this UAS sequence it activates GFP and has proved to be a robust system to generate tissue specific transgenic lines. It is important to state that this system is not limited to GFP expression. Over time researchers have used this system to express many other genes of interest in specific regions. Regardless of the gene, they are mediated by the GAL4: UAS system.

Using this system, the Kawakami lab has been able to develop unique transgenic lines that expressed tissue specific GFP, GCaMP calcium indicators, tetanus toxin light chain among many others effector which have facilitated an array of projects and consequently reliable information (Table 1). During my 10 weeks stay at the NIG the Kawakami lab has decided to give me a hands-on opportunity to work with the model, by learning the general workflow of the zebrafish model, develop transgenic GFP lines, determine their sequence insertion sites, and determining specific gene expression patter by implementing in situ hybridization chain reaction experiments. Hence, in this report I will be providing a detail insight of the experiments I conducted during my experience as well as the results and implication. However, I also have to say that some of these experiments are not

continuous. Meaning that there are different non-related objectives between the experiments. To facilitate the articles reading and comprehension

Results

In vitro synthesis of transposase mRNA

Over the course of 20 years the Kawakami Laboratory has consistently generated several transgenic zebrafish lines. They have achieved this by employing a six-step process that includes (A) Plasmid design for transposase mRNA (B) Plasmid processing and transposase mRNA *in vitro* transcription (C) Effector or reporter gene plasmid design (donor plasmid) (D) Solution preparation (E) Embryo injection (F) founder fish crossing and screening. For details on this procedure please address the methodology seccion. From steps A to D we would be enable to have a viable solution with all the components of the *Tol*2 transposition system (transposase mRNA and donor plasmid). When injected into the embryo at the one cell stage transposition of the donor plasmid should occure and integration into the genomic DNA is expected.

The aim of this experiment was to microinject wild type zebrafish embryos with a solution of *in vitro* synthetized transposase mRNA and a donor plasmid which ubiquitously expressed GFP. If successful microinjected WT larvae would

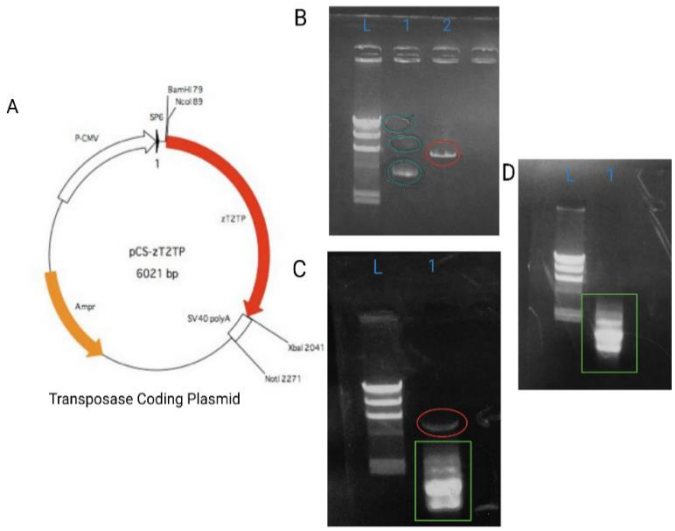

Figure 2: Results for *in vitro* synthesis of transposase mRNA. **2A.** Show pCS-zTP transposase coding plasmid map. **2B.** Demonstrates gel electrophoresis for plasmid enzymatic digestion with NotI restriction enzyme. Well 1 shows undigested plasmid well 2 shows NotI digested plasmid. **2C.** Gel electrophoresis after mRNA in vitro transcription using linearized pCS-zTP plasmid as template. Red circle shows linearized plasmid (DNA template) Green rectangle shows mRNA products. **2D.** Demonstrated final mRNA product once isolated and purified using DNAse and phenol: chloroform treatment.

continue to express GFP thought their life cycle. This process would develop skill that can be translated to electrophysiology experiments as well

as general knowledge from generation of other transgenic models (i.e., Xenopus).

I first started by processing the transposase encoding plasmid and synthetizing the *in vitro* mRNA for transposase. The plasmid that has de cDNA sequence for transposase is called pCS-zT2TP (Figure 2A). To synthesize the *in vitro* mRNA, I had to linearized plasmid via NotI restriction enzyme digestion. To confirm plasmid linearization, I did a gel electrophoresis where I were able to conclude that plasmid linearization was achieved successfully (Figure 2B). After this I did a Phenol: Chloroform isolation to isolate only linearized plasmid. This was followed by measurement of the DNA concentration which provided a concentration value of 299.5ng/ul. This ensured me that indeed I was able to isolate linearized DNA plasmid encoding for transposase mRNA. Having these results, I proceeded the *in vitro* synthesis of transposase mRNA using linearized plasmid as DNA template. At the end of the reaction, I did a gel electrophoresis to determine if mRNA product was synthesized. As seen in Figure 2C I had to major bands, the smaller molecular weight (green rectangle) being the transposase mRNA while the higher molecular weight band (red circle) being my DNA template. After this reaction I proceeded to do DNAse digestion and a Phenol: Chloroform purification to get rid of our template DNA and purify my synthesized mRNA. This step was also evaluated with a gel electrophoresis that demonstrated no DNA template marks (Figure 2D). I also tested this by determining the mRNA concentration on a nanodrop which gave me a mRNA concentration of 1690.9ng/ul and a 260/280 value of 2.09. Taking all

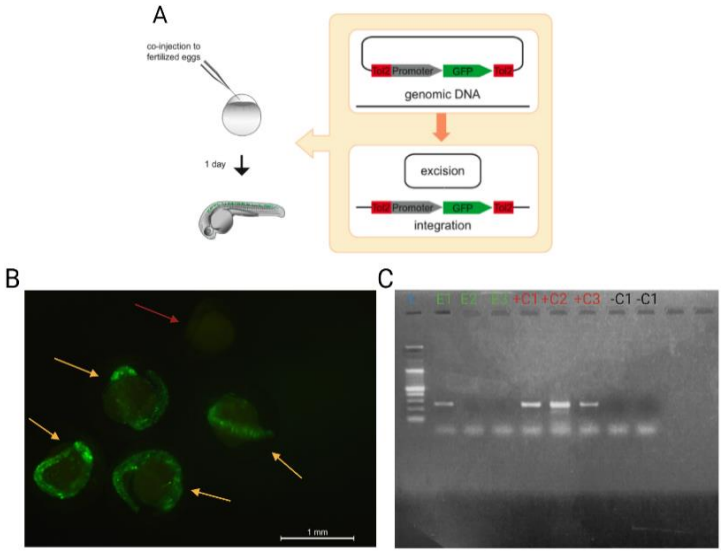

Figure 3: Microinjection results. **3A.** demonstrates microinjection pipeline and excitation diagram. **3B.** demonstrates embryo GFP expression after 24h post-microinjection. **3C.** Demonstrates gel electrophoresis of microinjection excision assay 24h post-microinjection. (Green E1-E3) my samples; (Red +C1-C3) experienced researcher; (-C1-C2) non-injected WT zebrafish. Image 3A was obtain from (Kawakami, 2007)

these result into consideration I can say that I was able to obtain synthesized and isolated mRNA product from the transposase coding plasmid pCS-zT2TP.

Microinjection of WT Zebrafish Embryos

After successfully synthetizing mRNA from the transposase coding pCS-zT2TP plasmid all that is left to select the donor plasmid and microinject the embryos with the *Tol*2 injection solution. Details on microinjection solution and overall experiment are found in the methodology seccion. The donor plasmid selected was the pT2GgSAIzGFFD capable of self-expressing GFP while also having a *Tol*2 recognition region to integrate to the genomic DNA in the presence of transposase enzyme. The rational to use this plasmid is that it enables me to determine if microinjection was success by simply observing GFP expressing over time. Successful plasmid integration in the genome is observed by continues GFP expression after 48 hours. If microinjection is abrupt (i.e., damaged cell or small solution injection,) GFP would only be seen until 24h post injection given its ubiquitous GFP expression. However, lack of integration would suggest poor microinjection technique.

After microinjecting embryos using a modified microinjection syringe, they are left in culture for 24 hours and two confirmation assays are executed. The first assay is to visualize GFP expression under the fluorescent microscope 24h post microinjection. This would enable me to determine if embryos survived microinjection procedure and the donor plasmid functionality. Figure 3B demonstrate that most of the injected embryos (4 out of 5) expressed GFP patterns in the developing central nervous system (CNS). This indicated that plasmid is being expressed and that embryos seem to have survived the microinjection procedure 24h post injection.

The second assay employ is called the excision assay. This assay would help me establish whether the plasmid integrated or not into the zebrafish genome. The principle behind this assay is that when a plasmid is cut by the transposase one seccion is integrated into the genome (in this case containing GFP gene) while the other seccion called the excision strand is re-circularized

eventually degrading in the cell's cytoplasm (Figure 3B). Using primers that specifically target the sequence in the excitation strand I can amplify the excision DNA using it as an indicator of transposase activity. Given the nature of this experiment amplification of the excision site would only be seen if plasmid is cut. This is achived by doing short elongation PCR cycles. It is important to highlight that this assay does not provide direct information regarding DNA integration into the genome. However, it does provide direct information of the synthesized transposase activity which we can correlate to genome integration. As seen in Figure 3C only one of the three samples taken for this experiment demonstrated exciton site amplification. Transposase inactivity was discarded by providing the same microinjection solution to an experimented microinjection researcher (lane +C1 to +C3) as we can see all demonstrated excision site amplification suggesting that my microinjection technique requires further practice. Regardless, this experiment demonstrated a successful *in vitro* transposase mRNA synthesis as well as microinjection. These embryos were further

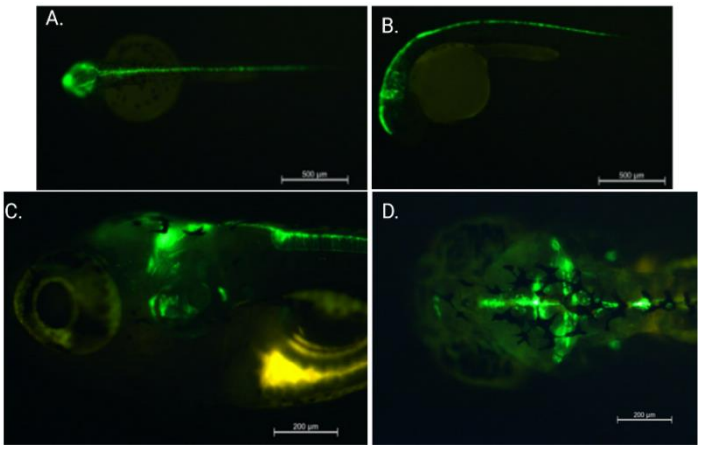

Figure 4: Tissue expression patterns on gSAIzGFFD3336A line. **4A-B** illustrate tissue expression pattern on 1-day post-fertilization 3336A embryos. **4C-D** illustrate 5-days post-fertilization GFP expression patterns on 3336A line. ***Images are property of the Kawakami Lab and should not be reprinted***

evaluated 5 days post injection to indeed confirm genome integration by continues GFP expression demonstrating that 25% of the injected embryos continued to express GFP (data not shown).

Inverse PCR Procedure

The Kawakami lab has generated new transgenic zebrafish lines implementing the GAL4: UAS system. Once Gal4 founder's zebrafish are crossed with UAS: GFP founders we have random Gal4 integrations hence, distinct, and unique GFP patterns are displayed by the F1 progeny (Figure 1). A new transgenic line called the gSAIzGFFD3336A was recently generated and

discovered by the Kawakami lab. This GFP expressing F1 progeny was assay and observed for GFP expression from day 1 until day 5 of development. During this time GFP expression patterns are observed and GFP expressing tissues are identified as being CNS tissues (Figure 4A-D).

Regardless, this is still not enough to determine where the exact GAL4 integration site is. Therefore, the purpose of this experiment was to determine the precise Gal4 integration site. To achieve this, I employed the use of inverse PCR method which has already been shown to be a powerful method for this objective (Urasaki and Kawakami, 2009). Inverse PCR is a method that is employ when sequence information is known only on one regions of the target DNA (Clark and Pazdernik, 2016). The principle behind this technique is that if we know the sequence of one DNA region (in this case our integrated *Tol*2 sequences) we can isolate that region, use it to amplify the vicinity sequences, and then via sanger sequencing we can obtain the specific insertion site in the

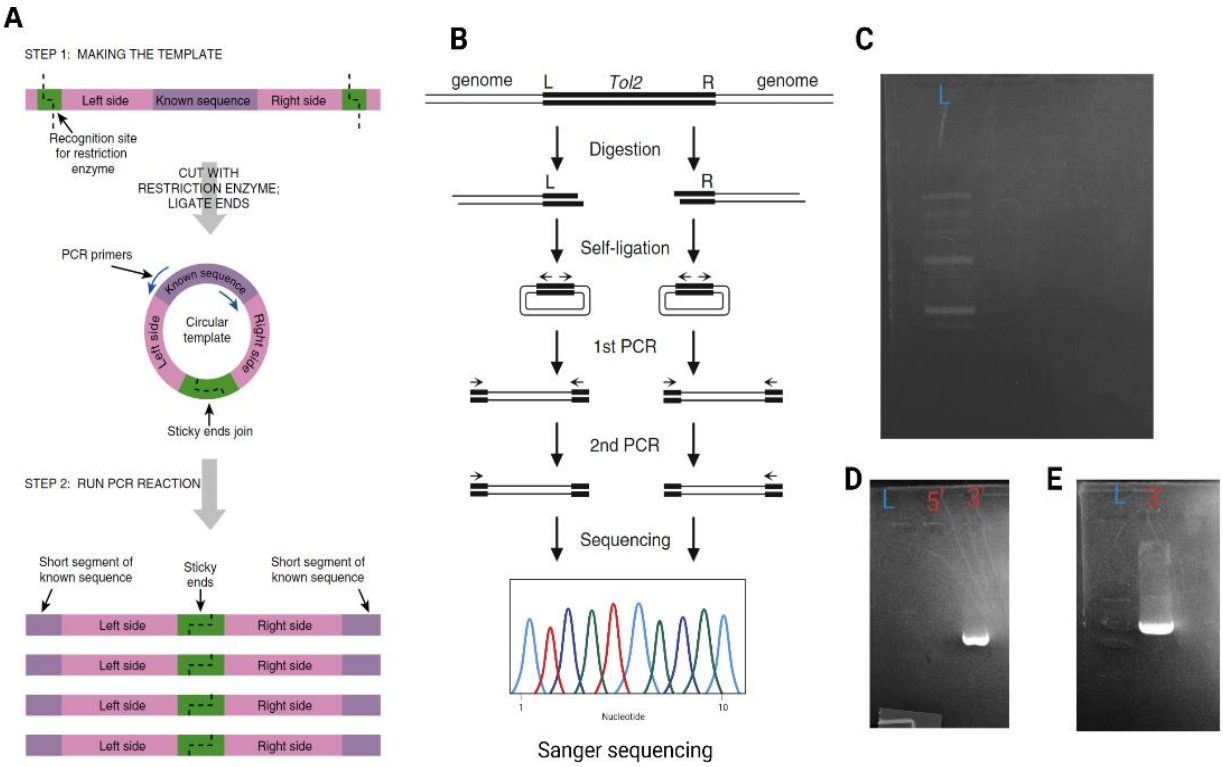

Figure 5: Inverse PCR results. **5A.** Illustrates inverse PCR principal workflow. **5B.** Illustrates inverse PCR adapted to the Tol2 insertion region. **5C.** Gel electrophoresis after DNA enzyme digestion treatment. 4D. Gel electrophoresis after ligation and first PCR amplification. **5E.** Gel electrophoresis after 2nd PCR amplification. Figure 4A and 4B were obtain from (Clark and Pazdernik, 2016; Urasaki and Kawakami, 2009)

genomic region (Figure 5A and 5B). To do this, we first isolated genomic DNA from F1 progeny which is then treated with restriction enzymes that will not cut within the known sequence but sequences near it while also generating sticky ends. This process is essential to isolate our integrated strand in the unknown genomic DNA. Figure 5C demonstrates gel electrophoresis of the digested DNA. As expected, no bands besides the ladder are seen, mainly this is because when digested, sequences are not long or abundant enough to see on a gel. Digested samples are then treated with ligase generating circular DNA which are then selectively amplified using outward pointing primers for the *Tol*2 sequence region. These "outwards pointing" primers would start amplification in the *Tol*2 sequence region however extending and amplifying the unknow genomic DNA in the process. As seen in Figure 5D selective

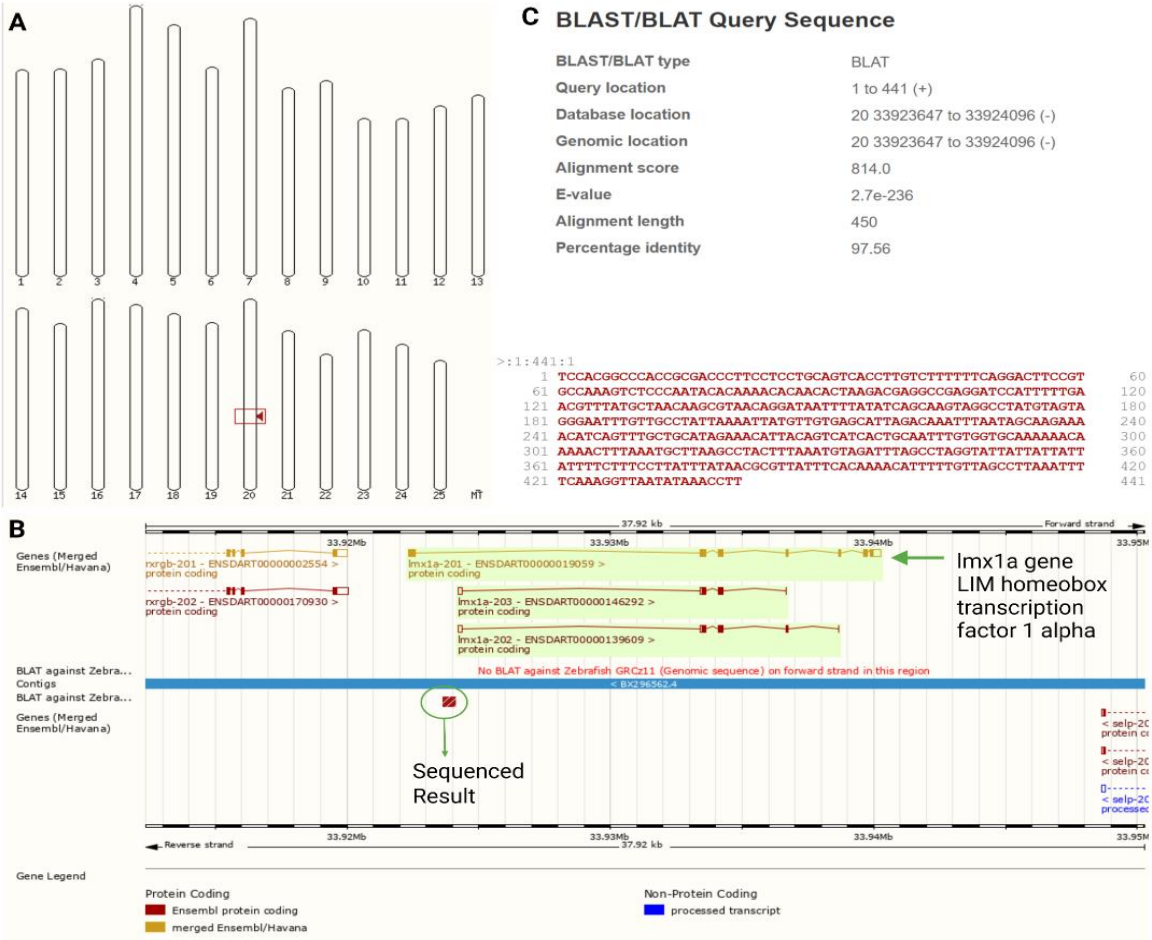

Figure 6: Sanger sequencing results. **6A.** illustrated insertion site at the chromosome level. **6B.** Demonstrates sequence structure aligment displaying lmx1a gene exons and introns as well as our sequence. **6C.** Shows blast results E- value, sequence length and overall sequence.

amplification was achieved although unfortunately only of the 3' prime end of the *Tol*2 sequence. Let me remind that when Gal4 insertion is achieved, this sequence DNA is embedded between two *Tol*2 sequences the 5' and 3' ends. Hence, in theory we could have amplified both ends. Unfortunately, I was only able to amplify the 3' end only. I continued the downstream analysis with the 3' end doing a second amplification and doing another gel electrophoresis of all the samples (Figure 5E). I isolated the band by removing it from the electrophoresis gel and doing a DNA isolation procedure. I measured the sample concentration having a value of 22.0ng/ul and prepared it for sanger sequencing. From the gel isolated DNA, I did sanger sequencing and got the whole sequence of my insertion site as well as the unknown genomic region of insertion. I Blast this sequence with the whole zebrafish genome using the ensemble webpage to determine the exact insertion site. Blast results provided an aligment score of 814 and a E-value of 2.7e-236 with a percentage of identity of 97.56 (Figure 6C). As seen from Figure 6A-C the insertion site was identified to be in chromosome 20 more specifically starting in sequence 33924096 and ending in 33923647 at the intronic region of lmx1a gene. lmx1a gene is a curated gene also known to be a homeobox gene transcription factor 1 known to be highly expressed in the CNS structures during development. As shown above, the overall results confirm GAL4 insertion to the genomic region of the zebrafish while also demonstrating the exact insertion sequence.

In Situ Hybridization Chain Reaction

To further understand the new transgenic line gSAIzGFFD3336A we decided to implement third generation in situ hybridization chain reaction experiment (HCR). In situ hybridization has been historically an invaluable tool to study and understand gene expression on a tissue section (Wilcox, 1993). The principle behind this technique is that nucleic acid probes are specifically designed to hybridize via base pair complementary to a desired mRNA sequence. Probe hybridization to targeted sequence would facilitate researchers to reach conclusions at the single cell level given its high sensitivity in comparison to northern blot which at the time also used for mRNA studies.

However, in situ hybridization technique over the years became more popular enabling scientist to reach conclusions that would help then understand gene expression changes, special temporal gene activation or inhibition, gene interaction and cell identification via anatomical and genetic profiling. Regardless of these advantages, this technique also had some disadvantages that would render impossible to use in some studies. (1) Designing specific target mRNA probes was labor intensive and time consuming given that they had to be validated to ensure their effectiveness. (2) Even when probes were validated, the technique sometimes generated significant background noise which led to unconclusive results. (3) slight mRNA concentration was sometimes hard to detect and hence, was impossible to study gene effects on slightly overexpressed genes.

Researchers took notices of these set back and began their search to enhance the technique overall output and hence, the third generation in situ hybridization chain reaction was born. HCR technique beautifully addressed its predecessor disadvantages by including an amplification step and off target silencing mechanism its reaction (Choi et al., 2018). HCR works by employing two types of molecules. (a) Two HCR initiators which are responsible of hybridizing and identifying a target mRNA. (b) and two DNA HCR hairpins modified with fluorophores that would conduct an amplifying response to identify a target and increase it signal (Figure 7A-B). One of the most important aspects of this technique is the need of the double initiator bind to target mRNA. Two initiator probes are design for one target sequence.

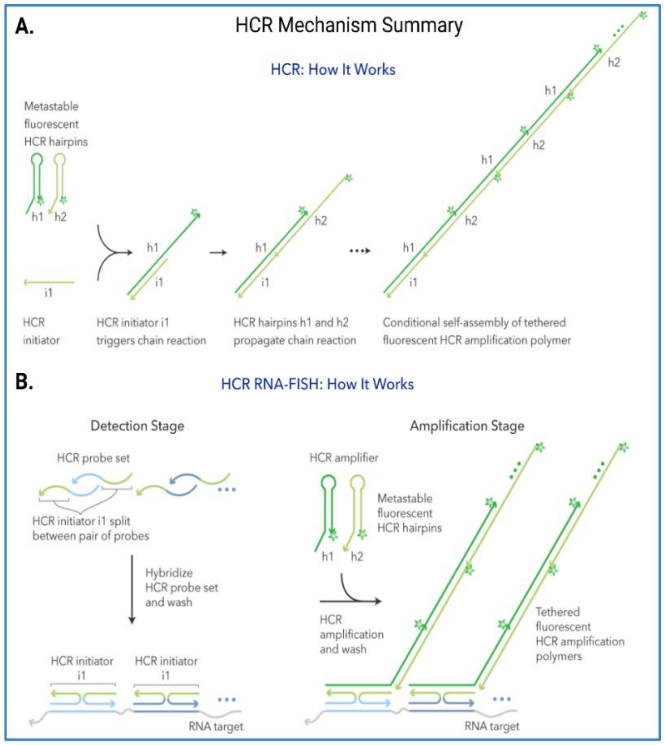

Figure 7: HCR mechanism understanding. **7A.** describe how each component of the HCR mechanism as well as its contribution. **7B.** illustrates HCR mechanism in the presence of a mRNA desired target. Image was obtained from molecular instruments web

If both probes can bind to the target sequence (mRNA) then and only then it is recognized by one of the hairpins modified fluorophore DNAs and the amplification reaction begins (Figure 7A-B). This distinct feature facilitates a self-silencing characteristic that subsequently facilitates increase target detection and decrease noise recognition. Additionally, this technique enables scientist to lable up to five different target genes in the same experiment providing a unique possibility of analyzing special temporal gene interactions (Choi et al., 2018). Finally, one of the most attractive features is that HCR probes are not required to be validated. Given its non-specific silencing mechanism, researchers design at least 24 probes per gene target this ensured biding to target sequence.

Using this technique, I decided to explore whether there were radial glial cells (RGC) of gSAIzGFFD3336A transgenic line expressing GFP. To do this I design 24 probes (12 sets) specifically to Glial Fibrillary Acidic Protein (GFAP) gene which is an accepted RGC marker and has been used to validate RGC transgenic lines (Bernardos and Raymond, 2006). As a control probe, I used several glutamate receptors probes

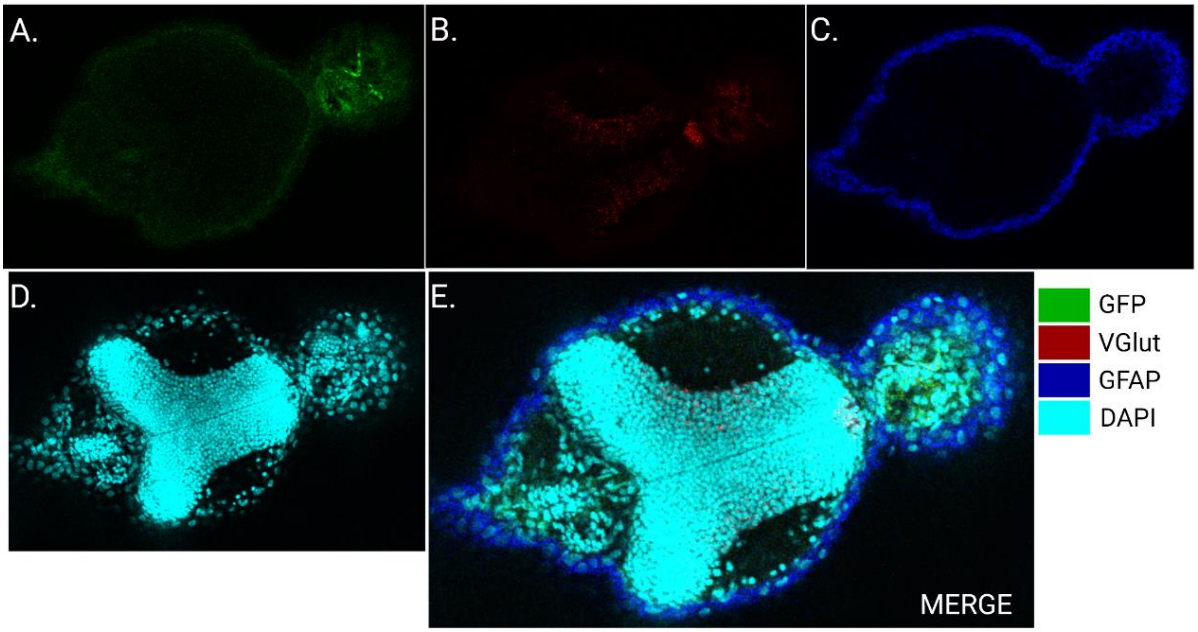

Figure 8: First set of HCR experiment using of the lower brain region of gSAIzGFFD3336A transgenic line at 512x512 pixel resolution. **8A.** Exposure to GFP filter **8B.** Target probe for vglut mRNA using Alexa 546 **8C.** Target probe for GFAP mRNA using Alexa 647 **8D.** DAPI **8E.** Filter merge

vglut1 (slc17a7a), vglut2a (slc17a6b), and vglut2b (slc17a6a) already validated to work by other members of the Kawakami laboratory. In case there is no GFAP detection the use of vlgut probes will serve as positive controls to rule out experimental errors.

Given that this was my first experience with both the HCR experiment and confocal imaging. The main objective for my first set of experiments was to (1) learn the basics of confocal imaging (2) learn to use ImageJ software (3) test my GFAP design probes (4) and determine if HCR experiment worked by analyzing fluorescence for GFAP, vglut, GFP, and DAPI. Given the nature of my experiment, I decided to not invest time in having high resolution images (at least for this first set) hence my first confocal images were taken at a resolution level of 512x512 pixelation. As we can see in Figure 8A-E I was able to detect all probes which include the already validated ones for vglut

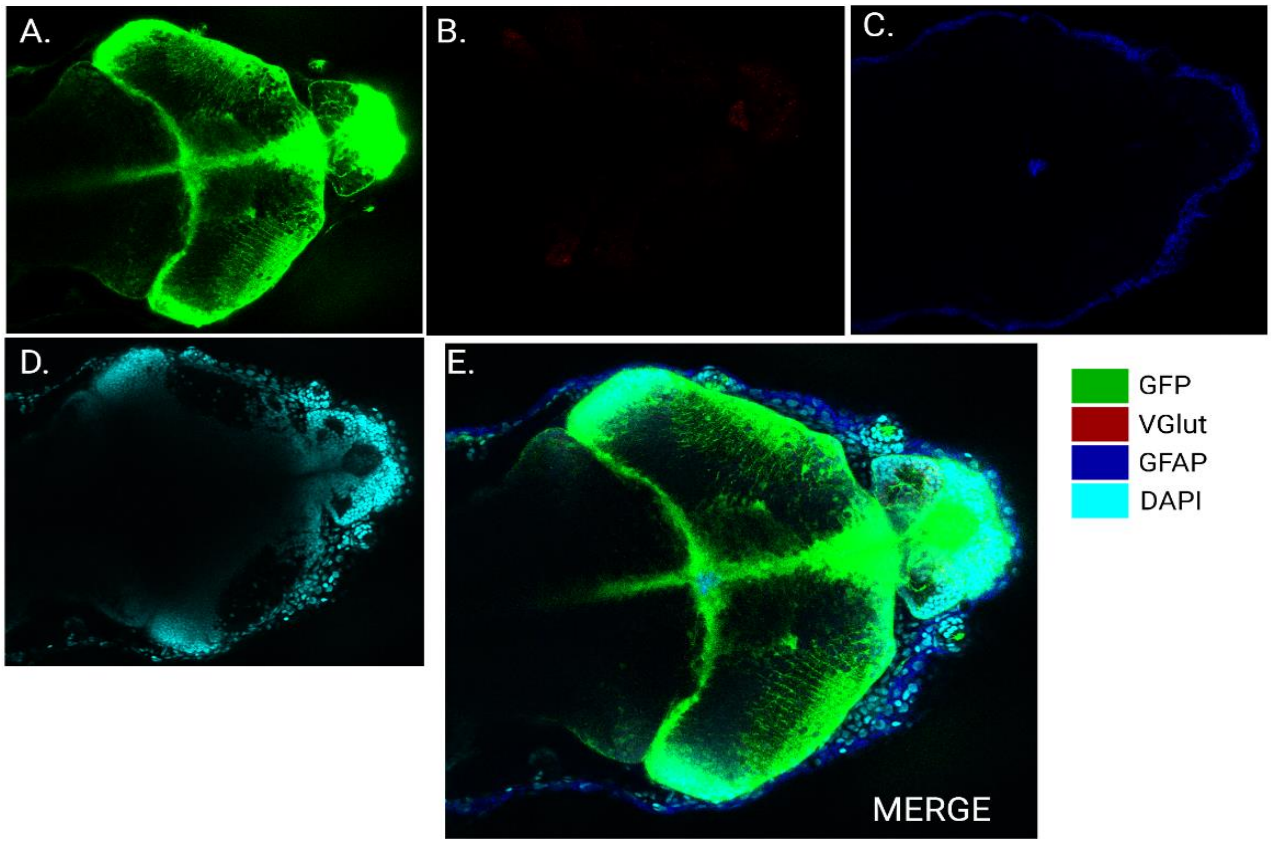

Figure 9: First set of HCR experiment using of the lower brain region of gSAIzGFFD889A transgenic line at 512x512 pixel resolution. **9A.** Exposure to GFP filter **9B.** Target probe for vglut mRNA using Alexa 546 **9C.** Target probe for GFAP mRNA using Alexa 647 **9D.** DAPI **9E.** Filter merge

as well as the one I design for GFAP. Unfortunately, given the resolution of this confocal experiment it was hard to determine the certainty of colocalized GFP and GFAP. To see Figure 8 complete Z stack video please visit:

https://drive.google.com/file/d/1d9NRJlqN7F_RL8HIF8m8FaDutQQFyH6y/view?usp=sharing

Given that one of my objectives was to determine if there is colocalization between GFP and GFAP cells it was only prudent that I would select another transgenic zebrafish line as a method of comparison and result validation. Hence, I selected the gSAIzGFFD889A transgenic line. This line gave me other distinct GFP pattern visualization which would help me validate my HCR experiment by adding biological replicates while also help me visualized the experiment using different GFP expression patterns within the CNS structures (Figure 9A-E). gSAIzGFFD889A transgenic line had strong GFP expression patterns making probe

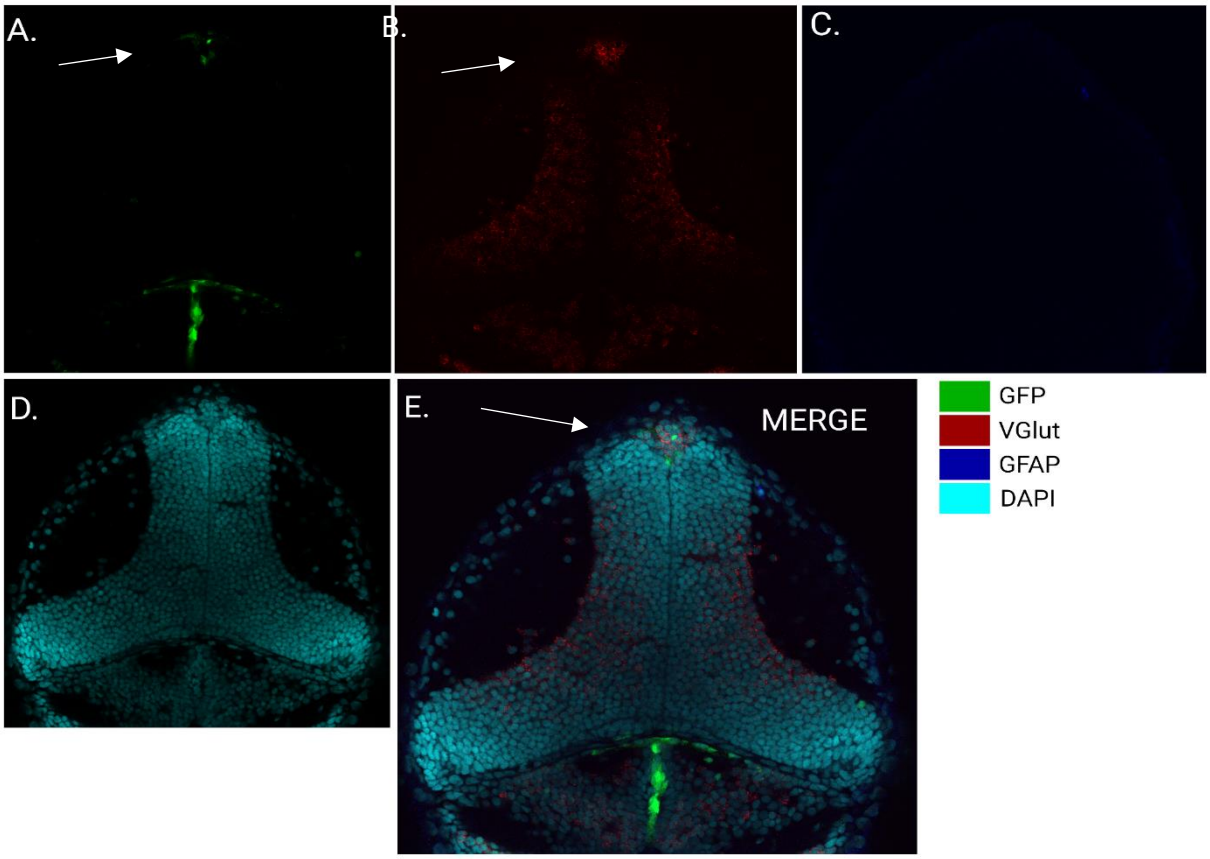

Figure 10: Second set of HCR experiment using of the lower brain region of gSAIzGFFD3336A transgenic line at 2012x2012 pixel resolution. **10A.** Exposure to GFP filter **10B.** Target probe for vglut mRNA using Alexa 546 **10C.** Target probe for GFAP mRNA using Alexa 647 **10D.** DAPI **10E.** Filter merge

visualization dificult (Figure 9E). We can also add to this my lack of experience in the confocal making it dificult to provide conclusive images demonstrating probe functionality with this transgenic line. On the bright side, I was able to increase resolution results to 1020x1020 pixelation. To visualize whole z stack video please visit: https://drive.google.com/file/d/1lhKByiUlWs64jeluPOCVQUOS_x-TQauu/view?usp=sharing

Regardless of the above results (Figure 8 and 9) I decided to try again this time with the aim of determining GFP/GFAP colocalization in gSAIzGFFD3336A line and by decreasing GFP fluorescence intensity levels in gSAIzGFFD889A line increasing resolution imagery. Moreover, in the process I noted that GFAP/GFP colocalization has not been addressed in gSAIzGFFD889A either hence, I decided to aim at determining the possibility of GFP/GFAP co-localization in both transgenic lines.

I started by analyzing gSAIzGFFD3336A line. First, I aimed at adjusting confocal parameters to get better resolution imagery. I started by increasing pixelation to 2010x2010 pixelation and adjusting the laser transmission values and decrease z tack range to facilitate downstream software analisis. As seen in Figure 10 I was able to significantly increase image resolution to a point where we can observe the end barriers between one fluorophore and another. By observing this set of images, I was able to visualize GFP^+ / $Vglut^+$ cells in the upper larvae brain region (white arrows Figure 10A,10B and 10E). Moreover, this image also provided data to show that there was in fact no colocalization between GFAP and GFP. Suggesting that this transgenic zebrafish line does not express GFP in its RGC. This does not exclude the possibility that other glial cells could be expressing GFP, but it does for RGC at least in the larvae stage of the organims. To see the whole z stack video for figure 10 please access the following link:

https://drive.google.com/file/d/1cwqO3vBkDbfZFdxPKB9m3IZCq_H_SLCG/view?usp=sharing

Also, another z stack video was generated however this one was had been magnified and focused to the regions that had higher GFP expression to ensure and visualize the colocalization between fluorescent dyes. Although at the beginning of the video (seconds 1 to 4) it seems to be colocalized cells we are not convinced given that the GFP expression is higher, and it could just be cells from another plane of the z stack. Please access video through this link:

https://drive.google.com/file/d/165WNUOUc6jOHumeqdXYG0yno1CtXYLiI/view?usp=sharing

I then decided to shift my efforts to increase picture quality resolution as well as to try and determine GFAP/GFP florescent colocalization using the gSAIzGFFD889A transgenic line. I decided to decrease GFP laser transmissivity from 0.50% to 0.15% to counter the high GFP expression the transgenic line expresses. I also increase GFAP laser transmissivity to 5.00%. the overall image

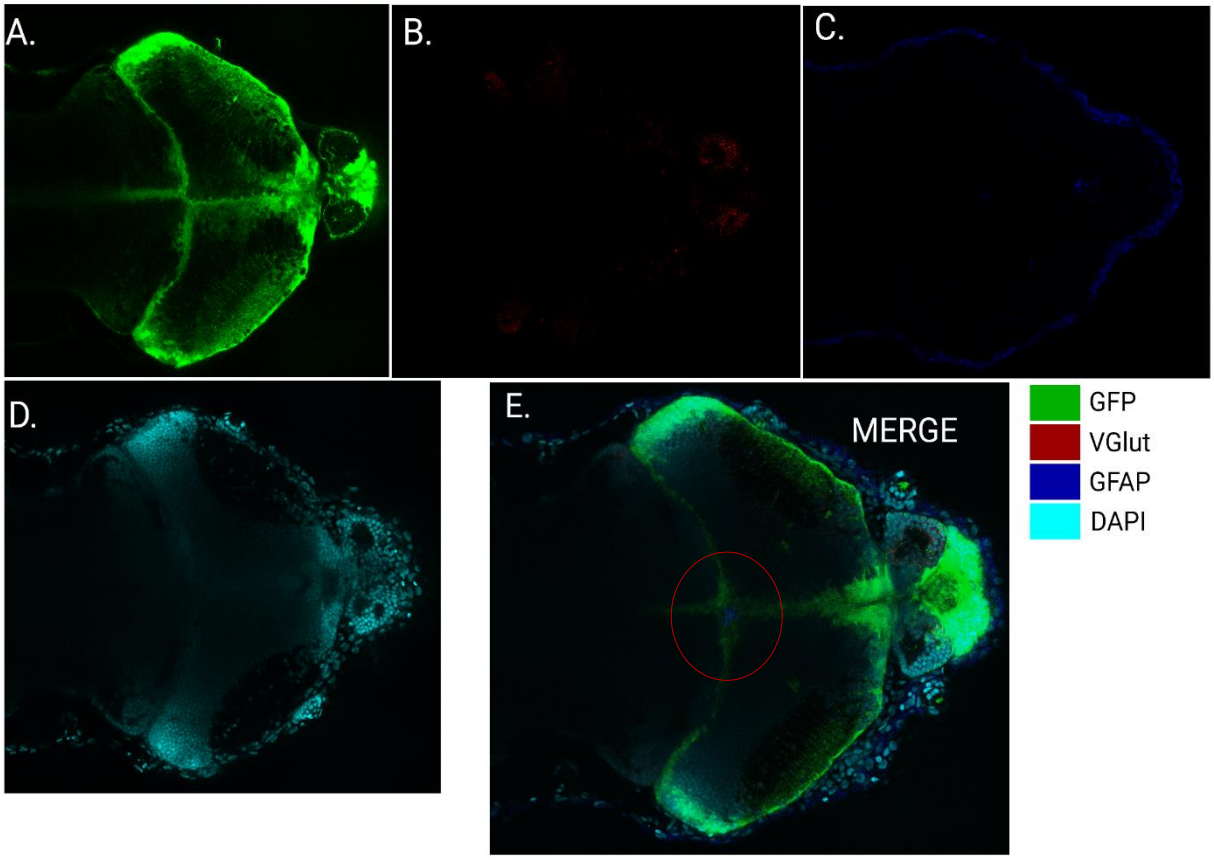

Figure 11: Second set of HCR experiment using of the lower brain region of gSAIzGFFD889A transgenic line at 2012x2012 pixel resolution. **11A.** Exposure to GFP filter **11B.** Target probe for vglut mRNA using Alexa 546 **11C.** Target probe for GFAP mRNA using Alexa 647 **11D.** DAPI **11E.** Filter merge

pixelation was also increased to 2010x2010 resolution. As seen in Figure 11 image quality did increase significantly facilitating and giving a better insight into the possible colocalization used in the in situs. In terms of GFP/GFAP colocalization, it was hard to determine. In Figure 11E there are clear GFAP stains however GFP does not appear to be cells rather fluorescence from vicinity GFP positive cell (inside the red circle). Just by this experiment it was dificult to determine given that it is hard to distinguish between cells when DAPI is not visible. Hence the overall results of this experiment are not enough to conclude that RGC express GFP in either of the transgenic lines assayed. To access the whole z stack video for this experiment please visit the following link. https://drive.google.com/file/d/1rQ-7n-cKr6j4hPKxgeIgz4pQ_fW-O/view?usp=sharing

Discussion

During my 10-week experience at the Kawakami Lab I was addressed several experiments all design to enhance my research and analytical skill. I first started by doing an *in vitro* mRNA synthesis of transposase mRNA. As illustrated above, I was able to synthesize mRNA using a cDNA which was generated by digesting pCS-zT2TP plasmid. Although the used cDNA template codes for transposase mRNA it is fair to say that this still not enough prove to say that the synthesis indeed led to transposase mRNA. To prove this, we used this synthesized mRNA in our microinjection experiment which definitively proved to have caused integration of our GFP donor plasmid. As seen by figure 3C samples E1-E3 it could suggest an inefficient transposase translation given to deficient *in vitro* transcription. However, samples +C1 -+C3 disprove this idea. These microinjections were done using the same microinjection solution which had the same *in vitro* synthesized mRNA. The difference was that the +C1-3 samples were done by “Akiito San” one with the most microinjection experience in the Kawakami lab. After analyzing my samples, it became clear that most of them were microinjected in the yolk instead of in the single cell. This obviously radically changed the results prohibiting *Tol*2 transposition system interaction with genomic DNA.

Given the time limit, I was not provided with a donor plasmid that only expressed Gal4 transcription factor to do the microinjections. If I would have microinjected with only a Gal4 plasmid it would had taken at least 5 months to get conclusive results. Hence, that's the reason I microinjected with a ubiquitous GFP expressing plasmid. Regardless, I continued the general pipeline by using microinjected adult fish that had been injected with Gal4 months before. This was the gSAIzGFFD3336A transgenic line which I subsequently continue to screen on the subsequent experiments. By doing inverse PCR I was able to determine the insertion Gal4 insertion site via inverse PCR isolation and sanger sequencing. This experiment however showed that I was not able to amplify the 5' end. A possibility of not isolating the 5' end could have been in the initial restriction enzyme digestion. It could have happened that the Gal4 was inserted relatively close to a NotI restriction site in the genome and hence could have inhibited amplification and visualization of our 5' end. Regardless, using only the 3' end it is possible to determine the insertion site. Normally is it well accepted to use one site as sample to determine the insertion site. The Kawakami lab however uses both 3' and 5' end as a method to confirm and validate their findings which is a better practice.

Finally, HCR experiments were done on the gSAIzGFFD3336A transgenic line. However, I also opt to use the gSAIzGFFD899A line to establish a comparative analysis. Because the difference in GFP expression the fluorescent patters would vary facilitating a tool to validate our findings while also increasing our chances of finding RGC expressing GFP. It took quite a while to obtain results. Something worth mentioning is that I did not consider the computer processing power needed to obtain high quality results. As I increased the amount of pixelation and resolution it also increases the file size which got to a point where I was not able to analyze it in my computer. This however, helped me better understand ImageJ software. This software has different mechanism that help you counter big file size which I eventually used to my advantage enabling data analisis on my computer. My main objective was to determine if RGC expressed or not GFP in any of these assayed transgenic lines. Unfortunately, my

data is not enough to conclusively answer this question. For the transgenic line gSAIzGFFD3336A it appears that RGC do not express GFP. Supplemental videos on google drive demonstrate GFP/GFAP expressing regions highly suggesting no colocalization between probes. All z stacks done using this line demonstrated close GFP/GFAP expression however, it is impossible to determine if they are the same cell. DAPI transmissivity in deeper tissue is very low hence, using DAPI to give each cell a boundary and better analyze it is not a good strategy. A better strategy would be to use house keeping gene in the HCR experiment, this would add further contrast to deeper located cells, facilitating us to better determine if GFAP cells do in fact express GFP. Another alternative we could implement is to cut the tissue to increase DAPI wavelength absorbance. The concern with this strategy is that by cutting the dorsal plane of the larvae might damaged the brain structure anatomy enough to a point where the imaging results might be misleading.

Analyzing gSAIzGFFD899A transgenic line was far more dificult given its overpowering GFP expression. Even lowering the laser transmission power to 0.15% was not enough to get rid of undesired GFP expression on vicinity cells that do not express GFP. These results were most ambiguous showing some frame that might have GFP/GFAP colocalization. A strategy employed was to confirm these results by is anatomical region using already described RGC in zebrafish. The main issue that inhibited us to do this was that these are larvae (6-day post fertilization) and most of RGC describing articles are focused on adult's zebrafish. However, I think both transgenic lines should be further explored implementing experiment to get conclusive results regarding possible GFP/GFAP expressing cells. Something that could be further done is the implementation of flow cytometry sorting by fluorescence. Flow cytometry in HCR marked cells is an already validated experiment. Using this experiment, we might be able to sort GFP^+ from $GFAP^+$ and both $GFP/GFAP^+$ cells if any. All things consider, this experiment clearly demonstrates the potential of HCR experiment as well as its limitation. Suggesting further experiments to support and validate our results.

Conclusion

Given my overall results I can say I was able to efficiently synthesized transposase mRNA which was used to integrate GFP using a donor plasmid into a genomic DNA. Also, I was able to identify gSAIzGFFD3336A insertion site using inverse PCR providing conclusive results that integration occurred in chromosome 20 at the intronic region of lmx1a gene. The overall conclusions of the HCR experiments for both gSAIzGFFD3336A and gSAIzGFFD899A lines should be further explored and better characterized. Although its GFP expression is highly unlikely to be RGC judging by its expression patterns it does appear to be glial line oriented. Hence, if fully identified these zebrafish expressing GFP lines could serve as a novel model to study glial cells, their interactions, and roles.

Behind each experiment the overall aim was to increase my skills and knowledge as a researcher and without a doubt this is something I was able to achive. I did grow as a new and innovative researcher learning new techniques that diversified my skill. More importantly, I learned techniques translatable to my project and will definitely implement in my PhD thesis project back at my home institution.

Materials and Methods:

In vitro synthesis of transposase mRNA

a. Digest pCS-zT2TP plasmid with NotI Restriction enzyme overnight at 37°C.
b. Do a Phenol: Chloroform DNA extraction and purification. Using this as your DNA template.
c. Synthesize the mRNA doing an in vitro transcription reaction (see recipe below).
d. Purify mRNA using the mini quick spin column: Roche 11 814 427 001.
e. Finally measure mRNA concentration and look at its integrity using a gel electrophoresis.

Restriction enzyme Recipe:

a. 10ul of 10x buffer
b. 10ul of BSA
c. 10ul of triton
d. 5ug of pCS-zT2TP plasmid
e. Adjust to 100ul with H2O

Restriction enzyme Recipe:

a. 3.3ug of Linear Plasmid
b. 2.7ul of RNAase free water
c. 10ul of 2x NTP/cap
d. 2ul of 10x Reaction Buffer

e. 2ul of SP6 Polymerase

Microinjection of WT Zebrafish Embryos

a. Place male and female zebrafish in matting boxes during the evening. (Zebrafish mate and lay eggs in the next morning) Microinjection should be done at the one cell level (0-30min post fertilization)
b. Make injection ramp at 1% agarose with the glass plate
c. Prepare injection needle using glass capillary puller.
d. Fill microinjection with microinjection solution (see solution recipe below). Then attached capillary to needle holder and connect it to 20ml syringe with Teflon tube modify to make microinjections.
e. Align eggs with chorions at the edge of the agarose ramp
f. Inject eggs with 1nl of mixture in the cell cytoplasm
g. Incubate injected embryos in a dish at 28℃

Microinjection Solution Recipe

a. 2ul of 250ng/ul of zT2TP mRNA
b. 10ul of 0.4M of KCL
c. 2 ul of 250ng/ul donor plasmid
d. 1ul of 0.5% phenol red
e. 5ul of RNAase free water

Excision Assay

To determine if microinjected embryos processed of not the administer plasmid (indicator sequence incorporation into genome)

a. Select 3 GFP positive embryos and 3 GFP negative embryos and 3 WT non-injected embryos.
b. Lyse using proteinase K at 50℃ for 5hours
c. Amplify plasmid excision site via PCR (see recipe below)
d. Do a 1.5% gel electrophoresis to visualize excision bands

PCR Amplification recipe

a. 2.5ul of 10x PCR buffer
b. 2.5ul of dNTPs
c. 1.25ul of forwards and revers primers
d. 0.25ul of HiFi Taq
e. 16.25ul of H2O
f. 1ul of template DNA

PCR Cycles: 35

94℃ 2min

94℃ 15sec

55℃ 15sec

72℃ 15min

4℃ continuous

Inverse PCR Procedure

DNA digestion and ligation

a. Isolate genomic DNA from adult fish.
b. Digest genomic DNA with MspI and AluI restriction enzymes for the 5' and the 3' respectively (in different tubes) each using1ug of DNA and 2ul of each enzymes buffer overnight at 37℃.
c. Ligate digested DNA by doing a ligation reaction. (1) 70℃ incubations for 10min (2) add 430ul of helix water (3) 70℃ incubation for 10min (4) incubation at 16℃ (5) add 50ul of ligase buffer and 2ul of T4 DNA ligase to each tube (6) incubate at 16℃ overnight. (7) Inactivate ligase by incubating at 70℃ for 10min.
d. Do DNA purification by isopropanol precipitation.

1st and 2nd PCR Reaction:

Amplify isolated regions. By doing first inverse PCR reaction adding HIFI taq and 10ul of the template product.

1st PCR Amplification recipe

g. 10ul of DNA template
h. 5ul of 10x HIFI Buffer
i. 4ul of dNTPs
j. 1ul of forwards and reverse primers
k. 1ul of HIFI taq
l. 28ul of molecular grade H2O

PCR cycles: 30 cycles

94℃ 2min

94℃ 30sec

55℃ 30sec

72℃ 2min

72℃ 5min

4℃ continuous

2nd PCR Amplification recipe

a. 1ul of DNA template (from 1st PCR)
b. 5ul of 10x HIFI Buffer
c. 4ul of dNTPs
d. 2.5ul of forwards and reverse primers
e. 1ul of HIFI taq
f. 34ul of molecular grade H2O

After 2nd PCR check product on gel electrophoresis adding all the sample. Do a electrophoresis cut out using QIAquick gel extraction kit. Measure DNA concentration and send sample for sanger sequencing.

In Situ Hybridization Chain Reaction

Important notes:

- All sample washes should be don in shaker movement at their respective temperatures.

Sample preparation

a. Cross gSAIzGFFD3336A and WT zebrafish to obtain eggs.
b. At 12-hour post-fertilization start adding phenylthiourea (PTU) at 0.003% diluted in E3 buffer treatment until 6 days post fertilization.
c. At this time, fix samples by adding 4%PFA diluted using PBST (0.1%Tween20) at 4℃ overnight.
d. Add samples to a 2ml tube and wash two times using PBST (0.1%Tween20) for 5 minutes.

Sample Dehydration

a. Wash sample solution with 25% MeOH diluted in PBST for 5 minutes at room temperature.
b. Repeat this step using 50% MeOH/PBST, and 75% MeOH/PBST.
c. After words do two more washes with 100% MeOH (no Tween) change to new 100% MeOH
d. Store in -20℃ overnight. At this step, you can leave samples in -20℃ for more time if needed.

Sample Rehydration

a. Wash samples in 75% MeOH/PBST for 5 minutes.
b. Repeat this step using 50% and 25% MeOH/PBST.
c. Do two more washes with only PBST for 5minutes.

HCR Hybridization Step

a. Change PBST with 2xSSCT. Do three washes with 2xSSCT for 5 minutes.
b. Start Prehybridization step by replacing to hybridization buffer (no probe) for 30minutes at 37℃.

During prehybridization step, prepare hybridization probe solution by adding 1/1000volum of the probe stock solution (1000x) to hybridization buffer. Keep this solution at 37℃ before commencing the hybridization step.

c. Commence hybridization reaction by adding hybridization probe buffer to all the samples.
d. Leave samples at 37°C in this buffer overnight.
e. Wash samples three times with 2xSSCT_30% formamide, for 20 minutes at 37°C.
f. Wash samples two times with 2xSSCT for 20minutes at room temperature.

HCR Amplification Step

a. Wash samples with amplification buffer for 30minutes at room temperature.

At this prepare dye conjugation solution:

a. Heat 9pmol (3ul) h1, h2 in separate tubes (90sec, 95°C)
b. Snaped-cool to room temperature in dark conditions
c. Add h1, h2 solution in 150ul of amplification buffer

-This is the volume amount for one probe only.

b. Add amplification buffer (with conjugation solution) to sampled to begin amplification reaction overnight at room temperature under dark conditions.

Final Wash

a. Wash sample three times with 5xSSCT wash x3 for 20 minutes at room temperature. Samples can be maintained in these conditions at 4°C until confocal image is needed to be done.
b. Just before mounting samples, wash with 2xSSCT one time for 5 minutes and then wash two times with PBST for 5 minutes.
c. Add DAPI staining in PBST and leave for 20 minutes.
d. After this, remove to new PBST and mount samples on 2% low melting agarose in a petri dish (adjust the sample orientation to were needed to).

Solutions:

For 50ml of hybridization buffer add:

a. 25ml of 4xSSC,
b. 500ul of 10% Tween20,
c. 10ml of 50% dextran sulfate
d. 5ml of 100% (v/v) formamide
e. 9.5ml of Helix Water

For 40 ml of Amplification buffer add:

a. 10ml of 20xSSC
b. 400ul of 10% Tween20
c. 8ml of 50% (v/v) dextran sulfate

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