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DEVELOPMENT OF A NOVEL CRISPR-BASED TOOL FOR INSTANTANEOUS VISUAL GENOTYPING OF ZEBRAFISH

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Introduction

Zebrafish have proven to be a valuable model system to study human genetic diseases. This is because they are genetically and physiologically similar to humans, and well-suited to large-scale experimental manipulation.[1] In addition, they have high fecundity, are cheap to maintain, and their embryos are nearly transparent which allows researchers to study embryogenesis in great detail.[4]

For effective modeling of human genetic disease, it is important to understand the extent to which zebrafish genes and gene structures are related to orthologous human genes.[2] Comparison to the human genome shows that approximately 70% of human genes have at least one obvious zebrafish orthologue. In addition, the high quality of the zebrafish genome assembly provides a clearer understanding of key genomic features in zebrafish.[2] Genome editing techniques such as Zinc Finger Nucleases (ZFNs), Transcription Activation-Like Effector Nucleases (TALENs) and the CRISPR-Cas9 system have simplified the ability to introduce targeted mutations in specific zebrafish orthologues of interest and, therefore, allow the efficient generation of stable models of human genetic disease.[1]

Zebrafish also allow *in vivo* studies to be performed at a scale usually restricted to *in vitro* studies and are particularly well suited to *in vivo* screens, in which thousands of small molecules are tested for their ability to modify disease phenotypes in zebrafish disease models.[3] This is due to their high fecundity and the fact that the resulting embryos and larvae are sufficiently small that they can be housed in 96-well or 384-well plates, allowing phenotypic analysis using high-throughput microscopy.[1] Soluble compounds can also be dissolved directly into the medium in which the embryos are housed, providing a convenient platform for rapid and efficient screening of small molecule phenotype modifiers.[1]

As a whole, zebrafish serve as an efficient model organism for studying human genetic diseases and for creating screenable disease models. The most obvious approach for creating such disease models is a genetic approach[3] via targeted gene knockouts. Though many homozygous mutants are viable and fertile, many are lethal, resulting in infertile or inviable offspring that can only be propagated through heterozygotes. In-crossing heterozygotes yields 25% homozygotes. However, the issue with this method is the extensive amount of genotyping required for identifying the homozygotes. A method of instantaneous visual genotyping of

zebrafish embryos will be extremely useful in disease modeling and quickly identifying homozygous mutants needed for large-scale chemical screening, and that is what we propose.

Description of the Method Development

The development of this method of visual genotyping involves integrating a fluorescent protein within the target gene-of-interest via CRISPR-Cas9 mediated gene knock-in. A targeted knock-in approach is utilized, as opposed to random integration of fluorescent proteins via transposases, for instance, so that it serves a dual purpose – knocking out the target gene *and* introducing fluorescence within the same target gene. However, targeted gene knock-in itself has its challenges, one being the low efficiency of insertion of large sequences into a target gene via homology directed repair (HDR). Most generally used fluorescent proteins, such as *mCherry* and *eGFP*, have large sequences (~710-720 nt long) and the efficiency of HDR drops with increasing sequence length. In addition, doing this twice for mCherry and eGFP is even more challenging. To overcome this challenge, the *attP-attB* site-specific recombinase system is used, wherein the fluorescent protein of interest is integrated into the target gene-of-interest via a two-step process.

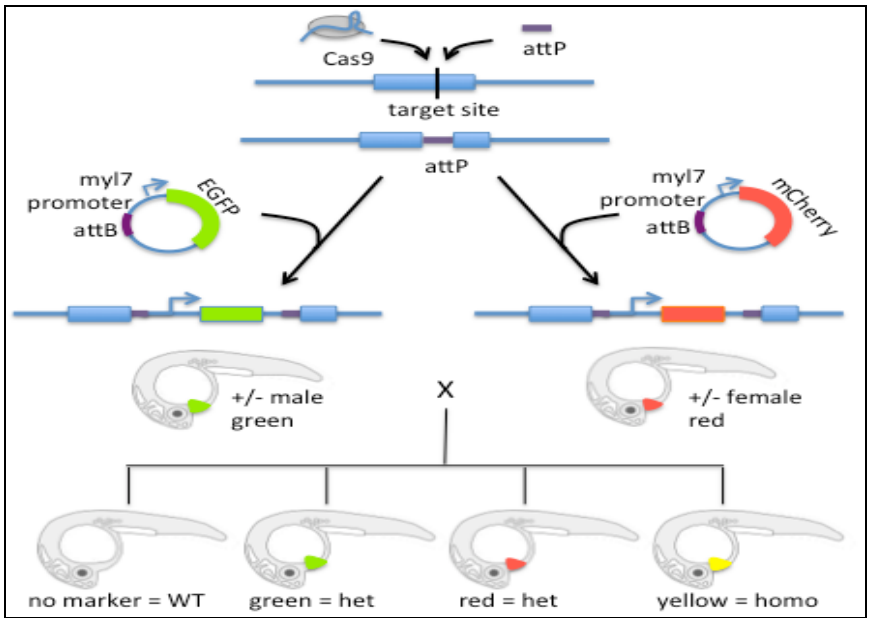

Figure 1 (Courtesy of Dr. Randall T. Peterson): Schematic of the Method *– CRISPR-Cas9 is used to insert the attP sequence into the target gene locus via asymmetric ssDNA-mediated HDR. Donor plasmids containing the attB sequence are modified to incorporate mCherry and eGFP and are then cloned. The modified attB plasmids are then integrated into the target gene via site-specific transgenesis using the phiC31 Integrase System. With the target gene in the zebrafish embryo now expressing fluorescence, they can be crossed to yield four potential types of embryos in terms of fluorescence.*

Figure 1 above shows the schematic of the method. The first step involves the insertion of a sequence known as *attP* into the target gene-of-interest in the zebrafish via asymmetric ssDNA-mediated homology-directed-repair utilizing CRISPR-Cas9. This is done by single-needle microinjection of single-cell-stage zebrafish embryos. The inserted ~50 base pair *attP* site functions as a binding site for another sequence known as *attB*. Embryos are screened to identify germline transmission of the *attP* site insertion and potential founders are isolated.

The next step is to modify donor plasmids containing the *attB* sequence (*pDestattB*) via restriction enzyme digestion to insert fluorescent tags, namely *mCherry* and *eGFP*, followed by cloning these modified plasmids to generate two sets of modified *attB* plasmids corresponding to their fluorescent tag. Once these plasmids are generated, they are then co-injected with the *phiC31 Integrase mRNA* into the F1 embryos of the heterozygous *attP* founders, which will cause site-specific transgenesis and, therefore, integration of the fluorescent tags (*mCherry* and *eGFP*) into the target gene. Embryos are further screened to identify germline transmission of the *mCherry* and *eGFP* integration and potential founders are isolated. F1 embryos from these founders are then screened under a fluorescence microscope and the fluorescent ones are isolated.

Once the fluorescent markers are inserted into the target gene-of-interest, the zebrafish can then be utilized for visual genotyping in the following manner. An *mCherry* containing male heterozygote zebrafish is crossed with an *eGFP* containing female heterozygote zebrafish (or vice-versa). The embryos obtained will be of four types, in terms of fluorescence, as illustrated in Figure 1 above. If the embryo shows no fluorescence under the fluorescence microscope, it can be characterized as a wild-type (WT or +/+, in terms of presence of the normal, unmodified wild-type alleles of the target gene). If the embryo shows one type of fluorescence (red due to *mCherry*, or green due to *eGFP*), then it can be characterized as a heterozygote (+/- or -/+). Finally, if the embryo shows both types of fluorescence (both red and green), then it can be characterized as a homozygote (-/-, in terms of absence of the normal wild-type gene due to both alleles containing the inserted fluorescent markers).

Proof of Concept and Future Directions

We have been working on performing a proof-of-concept of this visual genotyping tool by targeting the *SURF1* gene locus in zebrafish, as shown in Figure 2 below. *SURF1* is important for cytochrome c oxidase assembly in the mitochondria and defects in the *SURF1* gene cause Leigh Syndrome and Charcot-Marie-Tooth disease. Our tool will be vital for mitochondrial disease modeling in zebrafish.

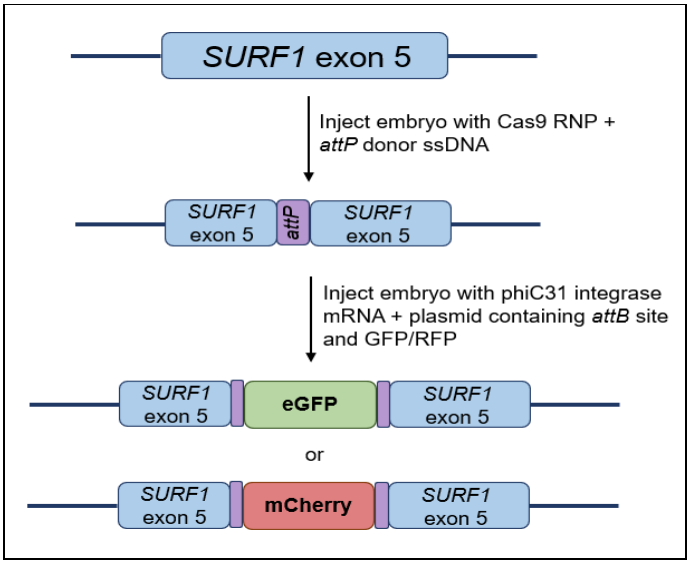

Figure 2 (Courtesy of Dr. Saba Parvez): Schematic of our Proof-of-Concept *– Targeting the SURF1 gene locus for site-specific transgenesis of eGFP and mCherry into exon 5 of the SURF1 gene*

Based on my work in the lab so far, I have successfully been able to clone the *attB* donor plasmids with *mCherry* and *eGFP* reporter proteins using a restriction enzyme-based cloning method. The reporter protein expression is driven by a *cmlc2* promoter, as it is a heart-specific promoter and the zebrafish embryonic heart is very easy to identify under the microscope. I have also generated transgenic lines containing the *attP* sequence at the *SURF1* locus by microinjecting ~1,000 zebrafish embryos with Cas9 protein, a guide RNA targeting exon 5 of the *SURF1* gene, and a single-stranded donor oligo that inserts the *attP* sequence through HDR.

Currently, we are genotyping the embryos and screening for germline transmission of the *attP* site insertion. Once we isolate potential founders, we will proceed to inject the modified *attB* reporter plasmids into the F1 embryos of the potential *attP* founders, as described above. Following the injection of the plasmids, we will screen these embryos for germline transmission of the *mCherry* and *eGFP* insertion and isolate founders, based on their fluorescence under a fluorescence microscope. These founder embryos will then be validated via PCR amplification and Sanger Sequencing to check the integration of the *mCherry* and *eGFP* within the *SURF1* gene. Finally, individual founders will be crossed with each other and the obtained embryos will be screened under a fluorescence microscope to demonstrate our method of visual genotyping. Upon establishing the protocol, we will use the homozygous mutants (showing both red and green fluorescence in the heart) for chemical screens to identify novel therapeutics for the treatment of mitochondrial diseases.

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