Genome Editing of *Oryzias latipes* using CRISPR-Cas9 and CRISPR-Cas12a Technologies

An overview of several experiments performing knockouts and knock-ins targeting a variety of genes in the *Oryzias latipes* genome using CRISPR-Cas9 and CRISPR-Cas12a protein systems.

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Abstract

As part of an international scientific research collaboration made possible by the generous funding from the National Science Foundation (NSF Project #1952513), we conducted many experiments for genomic exploration on *Oryzias latipes,* also known as medaka or Japanese rice fish. We were able to pursue these multiple experiments at the National Institute of Basic Biology within the National Institutes of Natural Science in Okazaki, Aichi Prefecture, Japan. CRISPR stands for clustered regularly interspaced short palindromic repeats and Cas means CRISPR-associated protein. We used CRISPR-Cas proteins 9 and 12a. We conducted a knockout of slc45a2 using CRISPR-Cas9 in *Oryzias latipes*. We inserted fluorescent reporter gene mNeonGreen into the N-terminus of the myosin heavy chain. We designed primers and prepared RNP complex for the microinjection and eventual knockout of relaxin family peptide receptor genes rxfp3.3b and rxfp4 in Oryzias latipes as part of an international collaboration with Canadian scientist Dr. Drake Hetcher. We successfully completed a knockout of slc45a2 using CRISPR-Cas12a in Oryzias latipes. We made significant progress towards three different experiments involving the insertion of different reporter genes to the PER2 region of Oryzias latipes.

Introduction

Medaka, or Japanese rice fish, are a beloved fish known culturally to be representative of the summer season in Japan. Scientifically, they are known as *Oryzias latipes*. Thanks to scientists like Dr. Kiyoshi Naruse, Masato Kinoshita, Kenji Murata, and Minoru Tanaka, Medaka is an exemplary model organism used in genomic research.

This paper describes seven of the many experiments performed concurrently under the supervision and mentorship of Dr. Kiyoshi Naruse. The aim of this project was to research novel genetic elements regulating behavior of Medaka. This international collaboration contributed to the National Bioresource Project at the National Institute of Basic Biology at the National Institute of Natural Sciences in Okazaki, Aichi Prefecture, Japan. This international collaboration was made possible by the National Science Foundation (NSF Project #1952513) and the generous funding provided to embark on this endeavor.

This paper is a partial representation of the extensive research and training that occurred over the course of two and a half months. I am omitting experiments including cryopreservation and artificial insemination because we did not go far into the realm of genetic engineering using those projects and therefore have less significant data on which to report.

Slc45a2 CRISPR-Cas9 Knockout

The first experiment uses CRISPR-Cas9 to knock out gene slc45a2, located in the b locus on chromosome 12. Slc45a2 provides genetic instructions for making a protein in melanocytes. In wild type medaka with functional slc45a2, normal eye pigmentation is entirely black. Without slc45a2, melanocytes cannot develop and as a result, there is no normal eye pigmentation. Successful knockout of slc45a2 shows white eye mutation. Partial success of the

knockout shows a mosaic phenotype of partially white and partially black eyes. Phenotypic variation is apparent during embryonic development. An advantage of this is that it does not require the ongoing maintenance of adult fish beyond embryonic development. The aim of this experiment is to practice microinjection in a way that is easily observable, and to make RNP complex.

Myosin Heavy Chain mNeonGreen CRISPR-Cas9 Knock-in

The second experiment uses CRISPR-Cas9 to knock fluorescent reporter gene mNeonGreen into the N-Terminus of the Myosin Heavy Chain locus, just downstream of the untranslated 5'UTR. This insertion occurs directly following the ATG start codon at the beginning of the open reading frame. The goal is to insert mNeonGreen directly after codon that creates the first methionine at the N-terminus so that the first coded-for region of the ORF is this fluorescent reporter gene mNeonGreen. For this purpose, we select a PAM site that is closest to the desired insertion site at the start of the open reading frame.

To assist this insertion, we are inserting the HA tag and 3x FLAG alongside the mNeonGreen gene. HA tag and 3X FLAG are both used for detection, but are recognized by different antibodies. We made a DNA fragment consisting of an initial homology region + mNeonGreen + HA tag + a second homology region. The template plasmid structure is Linker-Flag(tag)-mNeonGreen-HA(tag)-Linker, in which both linker sequences are distinct from one another. The linker sequences are flexible sequences that maintain the conformation of the protein. The homology regions are each 35 base pairs long. The end product of the inserted DNA fragment is a 35 bp Homology Region + Linker + 3X FLAG + mNeonGreen + HA tag + Linker + 35 bp Homology Region, in which each linker sequence is distinct and both Homology Regions are distinct from each other despite both being 35 base pairs in length. The 5' end of

the primer is biotin. The 3’ end of the primer is also biotin. The purpose of the biotinylation is to prevent degradation and protect the integrity of the plasmid.

Rxfp CRISPR-Cas9 Knock-in

This project is a collaboration with Dr. Drake Hetcher at Winnipeg University in Canada. He emailed Dr. Kiyoshi Naruse to request a knockout of two genes: rxfp 3.3b and rxfp 4. These genes are relaxin family peptide receptors. This functions as a protective energy sensor involved in regulation of metabolism, inflammation, and several kinds of homeostasis. He asked us to perform two knockouts, one of the rxfp3.3b gene and another for the rxfp4 gene, in medaka. After these two sets of knockouts were complete, he asked that we send him the resulting eggs so that he may observe phenotypic variation in development. With nothing but two target genes, we began by designing primers for this region.

INTRODUCTION TO CRISPR-Cas12A PROJECTS

Cpf1 stands for CRISPR from Prevotella and Francisella1. It is also known as CRISPR-Cas12a. This is a protein with a CRISPR-Cas nuclease. Specifically, we are using Lachnospiraceae bacterium ND2006, LbCpf1. Thus far, the slc45a2 knockout and the mNeonGreen knock-in to the myosin heavy chain complex were both conducted using CRISPR-Cas9. CRISPR-Cas9 is another CRISPR-Cas nuclease with a PAM sequence of NGG. PAM sequences for CRISPR-Cas12a is TTTV.

It is good to have more than one option for a PAM sequence because sometimes it is advantageous to use one or the other, depending on the specific sequence of the target region. CRSPR-Cas12a also has a molecular weight that is less than that of CRISPR-Cas9. Developing

CRISPR-Cas12a as an alternative genome editing tool would be beneficial to expand the molecular toolbox used in medaka. The broader implications of these experiments are to assist further research in the realm of medaka genomics.

Another difference between these two CRISPR-Cas proteins is precisely where they cut in relation to their PAM sequences. CRISPR-Cas9 cuts 3 base pairs upstream from the PAM sequence. CRISPR-Cas12a cuts 18 base pairs downstream from the PAM sequence. Additionally, Lb cpf1 cuts with a sticky end, whereas CRISPR-Cas9 cuts with a blunt end. CRISPR-Cas12a has a cutting site that is further away from the PAM sequence than in CRISPR-Cas9. The CRISPR-Cas9 protein is located very close to the cutting site, which sometimes prevents or inhibits the function of the homologous recombination repairing system.

The distance provided by CRISPR-Cas12a may enhance the knock-in efficiency.CRISPR-Cas9. We suspect that this may affect knock-in efficiency, but would have no effect on knockout (Moreno-Mateos et al. 2017). Compared to CRISPR-Cas9, the structure of CRISPR-Cas12a guide RNA is simple. Cas12a guide RNA doesn't use tracrRNA. The crRNA alone functions as the guide RNA. Guide RNA means it guides the CRISPR-Cas protein to a specific region of the genome. Cas9 guide RNA uses a combination of crRNA and tracrRNA.

Slc45a2 CRISPR-Cas12a Knockout

This experiment was designed as a test of CRISPR-Cas12a knockout efficacy in medaka. Using CRISPR-Cas12a, we designed this experiment to knockout slc45a2, a gene that controls melanophore cell development in the eyes. We took inspiration from a previously published paper, *Optimized CRISPR-Cpf1 system for genome editing in zebrafish* by Fernandeza et al. in 2018. This paper uses CRISPR-Cas12a to knockout slc45a2 in zebrafish. This paper expects 90-95% of injected embryos to exhibit mosaicism and 5-10% with albino eyes. Albino eyes indicate a successful biallelic knockout.

At the National Institute of Basic Biology in Okazaki, previous assistant Professor Satoshi was testing the activity of LbCpf1 CRISPR-Cas12a mRNA on medaka using the SLC45a2 genes. He did not use the CRISPR-Cas12a protein. He found that some embryos have white eyes but he only obtained the efficiency of some having mosaicism and some having white eyes. There was no detailed analysis. With Dr. Naruse, we want to analyze this more deeply using the CRISPR-Cas12a protein instead of the CRISPR-Cas12a mRNA.

PER2

PER2 is a region of genes involved in circadian rhythms. We want to attach a reporter gene to this region. There are three fluorescent proteins in question that we may attach to the PER2 Region: Luciferase from fireflies, nLucP from shrimp, and mNeonGreen from Branchiostoma lanceolatum. Attaching a reporter gene would improve the ability of downstream experiments to monitor the development of this region and observe phenotype differentiation of edited genotypes as for how they pertain to these genes.

Luciferase CRISPR-Cas9 Knock-in to PER2

Preceding this experiment, our knock-in for the n-terminal ends were successful using mNeonGreen and CRISPR-Cas9. We used mNeonGreen with the Myosin Heavy Chain locus. We observed green fluorescence in some of the bodies of our microinjected embryos. That indicated that the N-terminal end was successful. We then went on to test the insertion efficacy at the C-terminal end with microinjection of crRNA targeting the per2 C-terminal region. The MultiNA assay that we performed on those products showed successful cutting by the smear pattern with multiple bands The heteroduplex mobility assay is an assay that checks for

heterozygosity. This detects nuclease-induced indels because homozygous dsDNA moves faster than heterozygous ssDNA, causing a multiple band pattern due to the ssDNA being cut and the dsDNA is left uncut.

In this experiment, we attempt to insert a reporter gene into the c-terminus of the PER2 locus by a knock-in using luciferase. The luciferase is a fluorescent protein used as a reporter gene. It comes from the firefly genome. It is roughly 1.8 kb. There are two types of luciferase reporter genes that we are using. This experiment uses firefly luciferase, which is roughly 1800 base pairs or 1.8 kb in length.

nLucP CRISPR-Cas9 Knock-in to PER2

Separate from the firefly luciferase insertion described above, nLucP is the name of the shrimp luciferase gene. nLucP is roughly 600 base pairs in length, about ⅓ the size of firefly luciferase. Once we have the insertion of nLucP, the PER2 locus at the C-terminus of the gene should emit fluorescence. We are also attempting to knock-in this gene to observe differences and compare the knock-in of nLucP to the knock-in of the much larger luciferase gene.

mNeonGreen CRISPR-Cas9 Knock-in to PER2

The idea behind this experiment is similar to the luciferase and nLucP knock-ins using the same CRISPR-Cas12a protein. mNeonGreen is inserted into the C-terminus of the PER2 region. The greater impact of this experiment is to equip scientists with a way to study the development of the PER2 region in medaka. The broader effect of our work here is to help the exploration of circadian rhythm research.

NSF

With generous funding from the National Science Foundation (NSF Project #1952513), we were able to pursue these multiple experiments at the National Institute of Basic Biology within the National Institutes of Natural Science in Okazaki, Aichi Prefecture, Japan. We conducted a knockout of slc45a2 using CRISPR-Cas9 in *Oryzias latipes*. We inserted fluorescent reporter gene mNeonGreen into the N-terminus of the myosin heavy chain. We designed primers and prepared RNP complex for the microinjection and eventual knockout of relaxin family peptide receptor genes rxfp3.3b and rxfp4 in *Oryzias latipes* as part of an international collaboration with Canadian scientist Dr. Drake Hetcher. We successfully completed a knockout of slc45a2 using CRISPR-Cas12a in *Oryzias latipes*. We made significant progress towards three different experiments involving the insertion of different reporter genes to the PER2 region of *Oryzias latipes*.

Experimental Method

Slc45a2 CRISPR-Cas9 Knockout

For this experiment, we used primers that have already been designed and tested in previous experiments at the National Institute of Basic Biology, and did not design novel primers as we did for other experiments. We made sgRNA by combining 1.2 microliters of 100 microMolar crRNA with 1.2 microliters of 100 microMolar tracrRNA. After vortexing, we centrifuged this mixture. We then put it into the Thermocycler at 95 degrees celsius for 5 minutes. We then dropped the temperature down to 24 degrees celsius at a rate of 0.10 degrees celsius per second. Once the mixture reached 24 degrees celsius, we held it at this temperature for 5 minutes. Then we cooled it to a hold at 4 degrees celsius.

Once the thermocycler program was complete, we diluted 2.4 microliters of this sgRNA with 2.4 microliters of nuclease-free duplex buffer. Then we made RNA Protein Complex (RNP) by combining 2 microliters of diluted sgRNA with 0.8 microliters of CRISPR-Cas9 protein, which comes at 62 microMolar concentration, and 7.2 microliters of water. We incubated this solution at 37 degrees celsius for 5 minutes. After incubation, we centrifuged the RNP complex at 13,000 RPM for 1 minute. This creates 10 microliters of RNP complex for microinjection.

We kept fish in tanks of two with one male and one female. Before the experiment, we separated them with a clear barrier so that they could see one another, but could not make physical contact. This is because medaka fish prefer to breed with familiar partners. Upon separation of the barrier, the pairs of fish quickly mated within a few minutes. We then collected the freshly fertilized eggs that were attached to the abdomen of the female using a glass pipette without harming the fish. Using this RNP complex, we performed microinjection of the freshly fertilized eggs while they remain in the single-cell stage of development. After embryonic

development, we performed crude DNA extraction with Proteinase K digest and then performed PCR on that extracted crude DNA using DNA polymerase KOD-FX-Neo.

Myosin Heavy Chain mNeonGreen CRISPR-Cas9 Knock-in

For this experiment, we began by making a donor DNA fragment using PCR. PAP27 is a plasmid containing mNeonGreen, gifted to us from France by Alexandre Paix. The first PCR added homology regions and biotin to create the sequence [mNeonGreen + HA tag + linker]. The second PCR had a final product of [Biotin + Homology Region + mNeonGreen + HA tag + linker + HR + biotin]. Annealing of the primers happens after the linker and flag sequences, leaving only mNeonGreen + HA + linker to be amplified by PCR. This is how the [homology region - biotin] piece is added to the sequence.

We then used a high fidelity enzyme KOD PLUS to perform PCR. This amplified this sequence. The high fidelity enzyme KOD PLUS is necessary to prevent mutations. High-fidelity PCR enzymes are used for applications requiring high accuracy during DNA amplification such as cloning, sequencing or mutagenesis. After high fidelity PCR, the products are purified by column to ensure that all of the primers have been removed. This is to remove the residual primers and dNTPs to avoid the problem of primer dimers, remove proteins and other contaminants that would interfere with downstream experiments. The purification protocol is provided with the kit that we used for this process: NucleoSpin® Gel & PCR Clean-up Kit.

After PCR Clean-up, we Nanodropped our purified PCR products to check concentration. We wanted to ensure that amplification was successful. After Nanodropping, We checked that all of the desired parts were there using electrophoresis to see the expected DNA size. Upon confirmation, we adjusted the concentration of our donor DNA by diluting it to 50 nanograms per microliter. We used this diluted donor DNA to create the RNP complex for our microinjection solution. We combined the RNP Complex and the donor DNA to make the

microinjection solution. We used this microinjected solution to microinject 93 eggs, collected in the same manner described in the slc45a2 methods above.

Rxfp CRISPR-Cas9 Knock-in

We began our process of primer design for these two regions by copying and pasting the top strand of the sequence for which this primer will be designed from the snapgene file into Primer3 online primer development tool. The target sequence is the PAM + crRNA because we want to use these primers to amplify the sequences around that sequence. The target sequence itself is the cutting site, so we want to know the mutations around that cutting site. We designed the crRNA based on the PAM sequence using the ccTop database website for designing crRNA.

First we selected the PAM sequence and crRNA sequence in the snapgene file. We then designed forward and reverse primers to amplify the surrounding region. We put brackets around the target sequence to exclude primers from being designed within that region. The T(m) melting point is the annealing temperature. We set the parameters for the T(m) to have an optimal temperature of 60 degrees celsius, a maximum temperature of 63 degrees celsius, and a minimum temperature of 57 degrees celsius. We set the size parameters to a minimum of 20 base pairs, a maximum of 27 base pairs, and an optimal size of 25 base pairs. We set the primer GC percentage to have a minimum of 20% and a maximum percentage of 80%. We set the number to return as 5. We set the maximum repeat mispriming to 12 and the maximum template mispriming to 12 as well. We set the maximum 3’ stability to 9 and the pair max template mispriming value to 24. We then set the product size range to a range of 100-150 base pairs. The following table represents our primer output from this primer design process. This table represents the four different primers for each of the two different genes.

TABLE 1: Primer design for genes rxfp3.3b and rxfp4

Gene	Primer Set	Forward Primer	Reverse Primer
ENSORLG00000019204 rxfp3.3b	1	GAACTGGTCTGCGGAGGAGA	GACGCATACCAGCGCATACA
ENSORLG00000019204 rxfp3.3b	2	GTGCCTTGCGTACACCAACA	CGGCTCCACTCCACTTGTCC
ENSORLG00000003213 rxfp4	1	TGAGGTTTTTCAGGGAGACAAAA	GTCTCCAAGCCACTGCCATT
ENSORLG00000003213 rxfp4	2	CAACTGCCTGGCTTTTACCA	GCCTCCTCCGAGTGCATCTT

We then conducted a PCR using KOD-FX-Neo to test these primers. We made the RNP complex using crRNA + tracrRNA and proceeded to microinjection. After microinjection, we waited a few days for development and then extracted crude DNA and conducted another PCR. We then performed a hetero duplex mobility assay to monitor the cutting efficiency using electrophoresis.

Slc45a2 CRISPR-Cas12a Knockout

We designed a crRNA that would cut the slc45a2 locus. We designed primers using the Primer3 website. The methods for this portion were generally the same as the rxfp project, with the exception that this process required no tracrRNA. The crRNA is the sgRNA. This is an

advantage of CRISPR-Cas12a because you don't have to purchase additional reagent tracrRNA, which saves money.

Luciferase CRISPR-Cas9 Knock-in to PER2

This experiment began with the designing of a donor plasmid for firefly luciferase. This donor plasmid should contain the elements [linker - 3X FLAG tag - luciferase - HA tag - linker]. This creates the donor DNA fragment for insertion using CRISPR-Cas9. We also designed a primer for the insertion to the region containing the period genes. We designed the crRNA to cut right before the stop codon. The length of homology arms are 35 base pairs in each direction. On primers btn-per2-luc-F & btn-per2-luc-R, the homology arm is towards the outside of the sequence, with the annealing region on the edge towards the inside of the plasmid sequence. The annealing region overlaps with the luciferase gene.

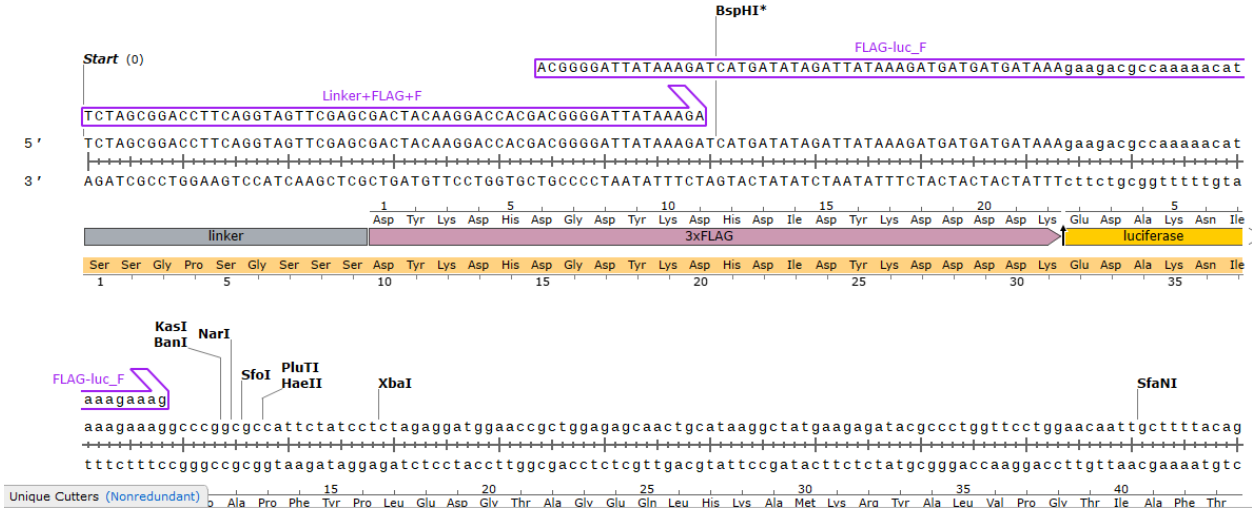
This luciferase experiment used a line of competent e coli cells called DH5 alpha. LB broth is added to the agar to prevent the growth of unwanted bacteria. The physical agarose medium has LB broth to feed the growing e coli on the plate. The mixture of LB broth, agar, and MilliQ water is poured into a plate on which to grow colonies. We took LB agar powder and added milliQ water then added ampicillin into that mixture. The ampicillin plate has a concentration of 50 milligrams per microliter.

We synthesized FLAG-luc-F primer and linker-HA-luc primer. As the result of PCR with these two primers, linkers and both epitope tags are added to the flanking region of the luciferase. We needed to do 2 PCRs because the linker+FLAG+F primer and the FLAG-luc-F primers were too big to be ordered as one primer. We instead had to order them as two separate primers and add them on individually in two separate PCRs.

We performed xbaI digestion as one step of the luciferase plasmid construction. The first PCR that we conducted doesn't add the linker regions to the plasmid, just the 3X FLAG and HA

tag. The second PCR has the linker regions attached. This is why we needed to do digestion with xbai, a restriction enzyme. Restriction enzymes recognize certain sites and cut them. Different sites (sequences) are specific to different restriction enzymes. The total size of the second PCR's expected amplicon is 1794 base pairs. The expected total size of the amplicon from the first PCR is about 1751 base pairs for just the luciferase gene. The difference between the two fragments is small, only 43 base pairs, so it is difficult to distinguish between the first and second PCR products by using simple DNA gel electrophoresis. That's why we will use xbal to cut it. The 43 bp difference is too small to see with the resolution of the ladder we use. We need to make it all smaller so we can use a better scale. We use restriction enzyme xbal digestion to distinguish the success of the second PCR before moving forward. We used restriction enzymes to cut up our PCR products to better see the difference between PCR products 1 and 2. This is to distinguish the success of the second PCR. This process is to distinguish between the first and second pcr products. Once this successful ligation is confirmed, we can sequence and confirm that we have the new template plasmid for the donor DNA fragment.

FIGURE 1: SNAPGENE SEQUENCE OF XBAI SITE



nLucP CRISPR-Cas9 Knock-in to PER2

We grew the shrimp luciferase plasmid in the E. coli to create the donor fragments containing nLucP luciferase genes. We tested the infusion to make sure he had the right size. We performed an inverse PCR to remove mNeonGreen. The idea was to then insert nLucp into the plasmid in mNeonGreen's place. We ran a 1% agarose gel electrophoresis on the open plasmid (linear, not currently circular) sequence that has no mNeonGreen and no nLucP. We ordered the nLucP with 15 base pairs of homologous regions, also called homology arms, on either side of the fragment. The nLucP sequence is about 600 base pairs, so this fragment should be about 630 base pairs. We intended to amplify this nLucP region with the homology arms and then anneal it to the linker and 3x flag tag and HA tag regions.

We have colonies that we have attached the big dye to in the thermocycler by cycle sequencing. They then go to the sanger sequencing machine after ethanol precipitation. We then infused the [15 base pair homology arm - nLucP - 15 base pair homology arm] sequence into the vector which contains the complete [puc19 - linker - 3X FLAG - mNeonGreen - HA Tag - linker - loop of puc19] sequence without the mNeonGreen region. The idea was to remove mNeonGreen by reverse PCR. In this process, the PCR is positioned by our primers to start such in a way that excludes the region that is desired to be removed.

After infusing the nLucP into the vector, we transform it into an E. coli. Then we plate the E. coli so that it may multiply and give us more of this desired sequence. We then cleaned the vector and fragment which were both produced by inverse PCR. We purified both of them with NucleoSpin Gel and PCR Clean-Up. Using the NucleoSpin Gel and PCR Clean-Up kit, we purified by column to remove dNTPs, primers, primer dimers, and other components of the PCR to prevent disturbance of the downstream parts of the experiment, such as transformation and ligation. We purify only the amplified fragments and dilute them with TE buffer or water.

We then performed transformation on our bacterial cultures of E. coli. We put 10

microliters on one plate and and 100 microliters on the other of a strain called Top 10 E. coli transformed with puc19 containing nLucP genes. After ligation, there is a knick where one strand is combined but the other strand is not. E. coli must repair this process and then transformation happens. The rapid method has incubation time using SOC medium. For the plasmid backbone, we use puc19, which contains ampicillin resistance genes. This is why we use the ampicillin resistant plates in this experiment. To determine the success of the transformation, we check the size and then pick up the colonies and grow the E.coli. We will then isolate the plasmid from the e coli. To do this, we used a kit called QIAprep spin Miniprep kit.

After isolation, we sequence the plasmid using the sanger sequencing machine. Inoculation of puc19 vector containing nLucP into circlegrow. Inoculation is introducing the E. coli into a new medium. We are growing the colony. The E.coli is in the LB broth that I dipped a toothpick into. Circlegrow is a broth that promotes the growth of the E. coli. When I introduce my puc19 E. coli that contains nLucP into circlegrow, it will grow and amplify, while ampicillin added into the broth kills any other bacteria that may have contaminated the tube.

Now we are inoculating in circlegrow. Take 3 milliliters of circlegrow. Add 3 microliters of ampicillin. Add 5 microliters of LB broth containing puc19 E. coli containing nLucP. Combining circlegrow and ampicillin at a 1 milliliter circlegrow : 1 microliter ampicillin ratio. Here we are adding 3 milliliter of circlegrow to 3 microliter of ampicillin, then adding 5 microliters of LB broth containing the colony. I am using 4: colonies J K L and M. These samples then go into shaking incubation overnight. Then we extract plasmid DNA from nLucP plasmid.

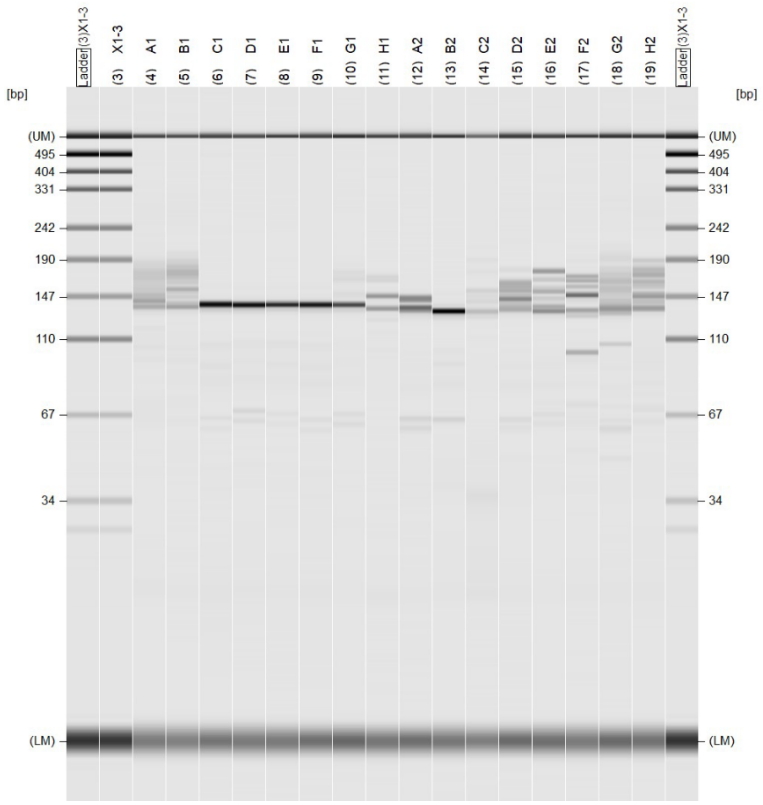
mNeonGreen CRISPR-Cas9 Knock-in to PER2

The methods for this experiment were similar to those from the Myosin Heavy Chain mNeonGreen CRISPR-Cas9 Knockin. The key difference was the target insertion site, which required different primers and homology regions in order to better suit the PER2 region and not the myosin heavy chain region.

Results

Slc45a2 CRISPR-Cas9 Knockout

FIGURE 2: Heteroduplex mobility assay electrophoresis conducted on the MultiNA



These results depict nuclease activity. A1, B1, C1, D1, and E1 represent DNA extracted from embryos expressing the black eye phenotype. A1 and B1 show a smear pattern. C1, D1, and E1 show a solid band at around 140 base pairs. F1 is a wild-type control from the CAB line of medaka. F1 shows a solid band around 140 base pairs. G1, H1, A2, B2, and C2 represent DNA extracted from embryos expressing mosaicism. G1 and B2 show solid banding. The band in column G1 lines up with those of C1-E1, which are from black eyed embryos, and F1, which is a CAB wild-type control. B2 shows a solid black band, but at a slightly lower position on the

gel. H1, A2, and C2 have a multiple banding pattern.

Columns D2, E2, F2, G2, and H2 represent DNA extracted from slc45a2 knockout mutants in which the embryos expressed a phenotype lacking melanophores, resulting in completely colorless eyes. D2, E2, F2, G2, and H2 all show multiple banding patterns. The first and last columns, labeled (3)X1-3 represent the DNA ladder, LD1, Ladder1.

From my microinjections, there were 59 surviving microinjected embryos. 5 of the 59 embryos had white eyes. This accounts for about 8.5% of surviving embryos. 26 of the 59 embryos had white eyes. This accounts for about 44% of surviving embryos. 5 of the 59 embryos had white eyes. This accounts for about 44% of surviving embryos.

TABLE 2: Phenotypic Distribution of surviving embryos after microinjection with Slc45a2 CRISPR-Cas9 Knockout solution

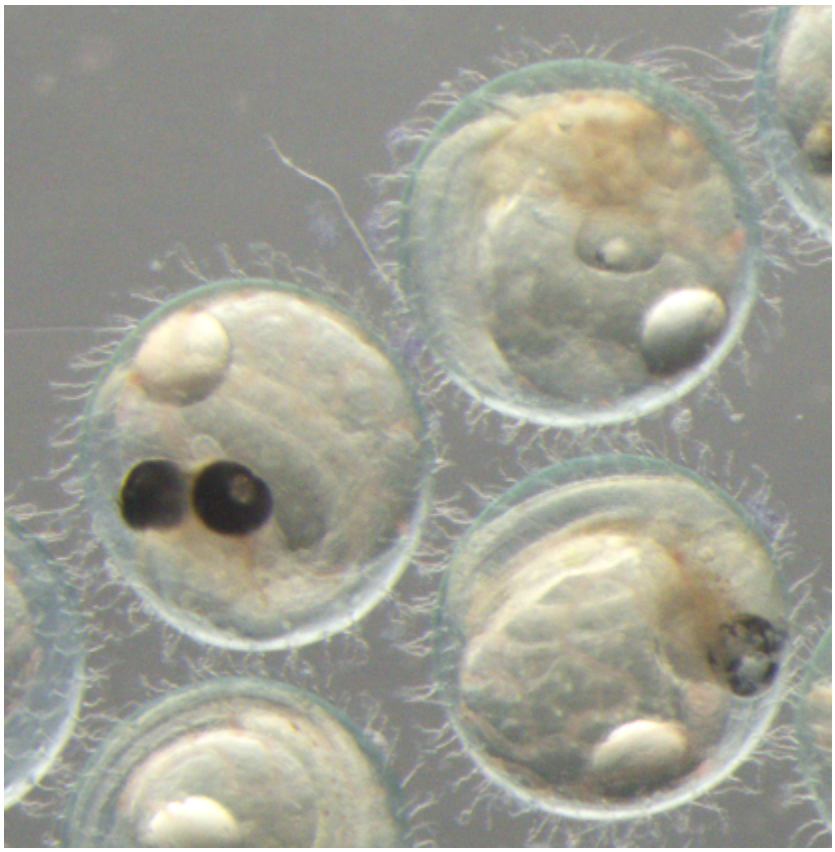
Eye Color (Phenotype)	Number of Embryos	Percentage
White Eyes	5	~ 8.5%
Mosaic	26	~ 44%
Black Eyes	26	~ 44%

About 52.5% of microinjected embryos showed partial or full mutation success by phenotype of either white eyes or mosaicism. Roughly 44% of embryos showed no phenotypic difference from the wild type, indicating that the mutation was unsuccessful and therefore slc45a2 was not knocked out. It is however possible that some nuclease activity did occur and

that slc45a2 may have been deleted in some cells, but was not deleted in enough cells to cause the phenotype to change from wild type pigmentation to white eyes resulting from slc45a2 knockout using CRISPR-Cas9.

FIGURE 3: Phenotypic Variation in slc45a2 Knockout using CRISPR-Cas9.

On the left is a black-eyed embryo showing a phenotype that appears the same as in wildtype embryos at this stage of development. The top right embryo shows the white-eye phenotype. The bottom right embryo shows mosaicism, a patchwork mix of both aforementioned phenotypes.



Myosin Heavy Chain mNeonGreen CRISPR-Cas9 Knock-in

Out of 93 injected embryos, 3 survivors showed green fluorescence. The knock-in for the C-terminal and N-terminal ends were successful using mNeonGreen and CRISPR-Cas9. We used mNeonGreen with the Myosin Heavy Chain locus. We observed green fluorescence in the body. That indicated that the N-terminal end insertion was successful.

FIGURE 4: Microscopic photography of Fluorescent Embryos.

Figure 4a and b depict two different embryos exhibiting fluorescence at the myosin heavy chain as a result of microinjection with mNeonGreen CRISPR-Cas9 Knock-in solution. Figure 4c shows the three successfully fluorescent embryos together.

Figure 4a:

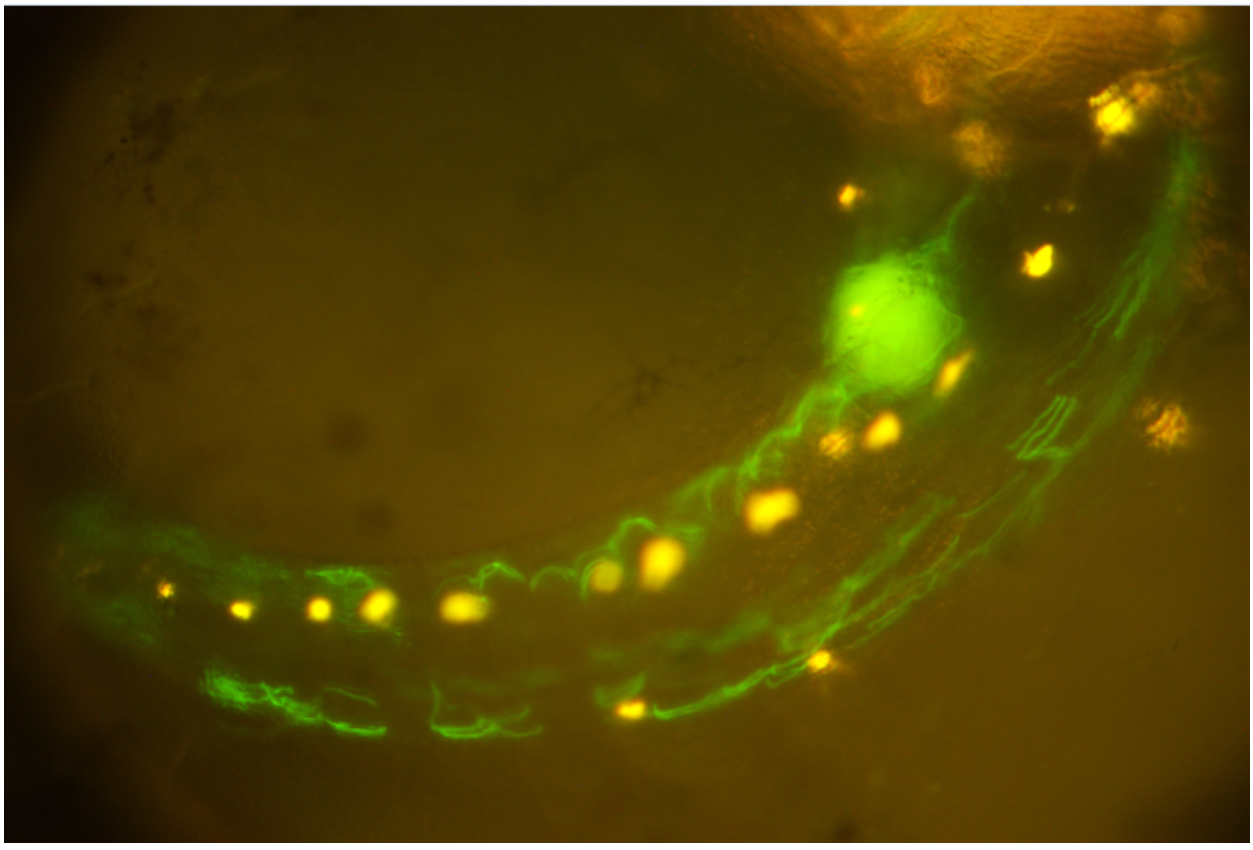


Figure 4b:

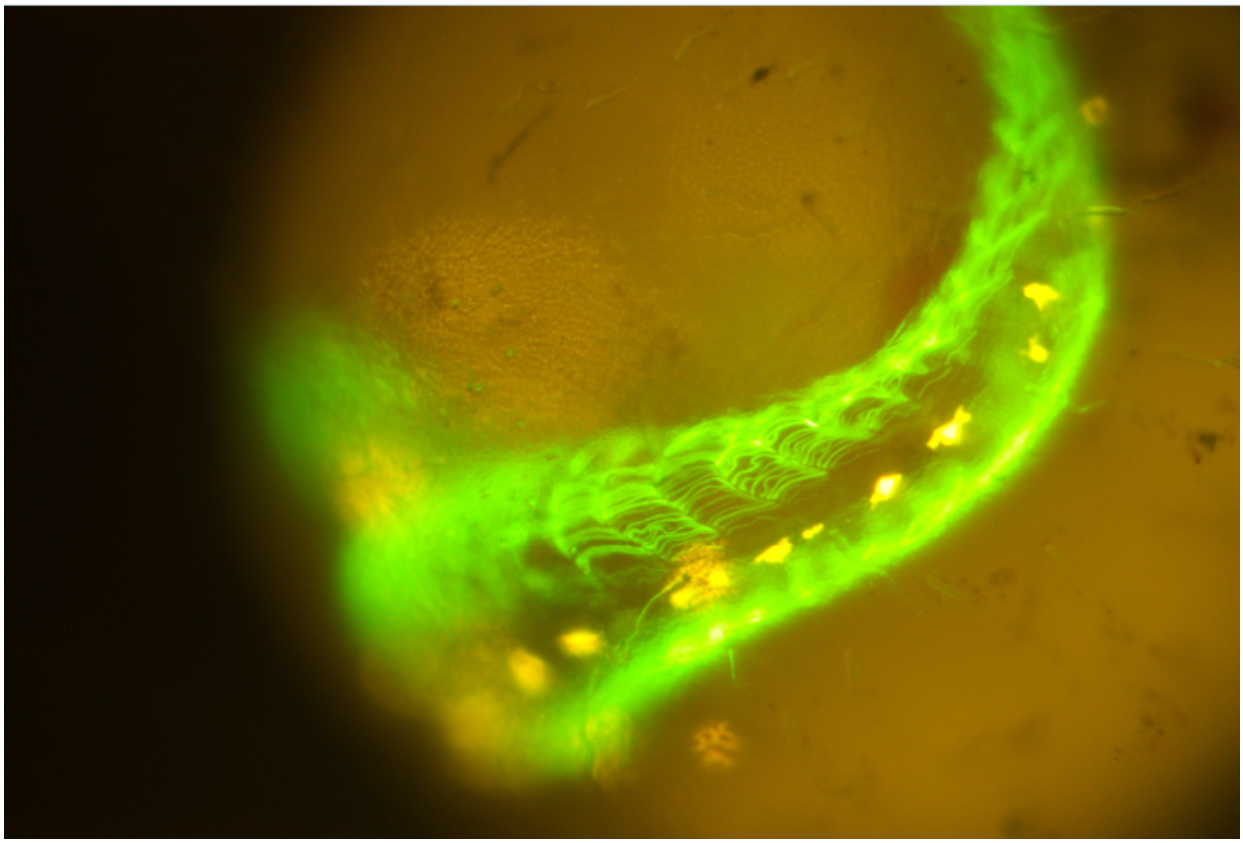
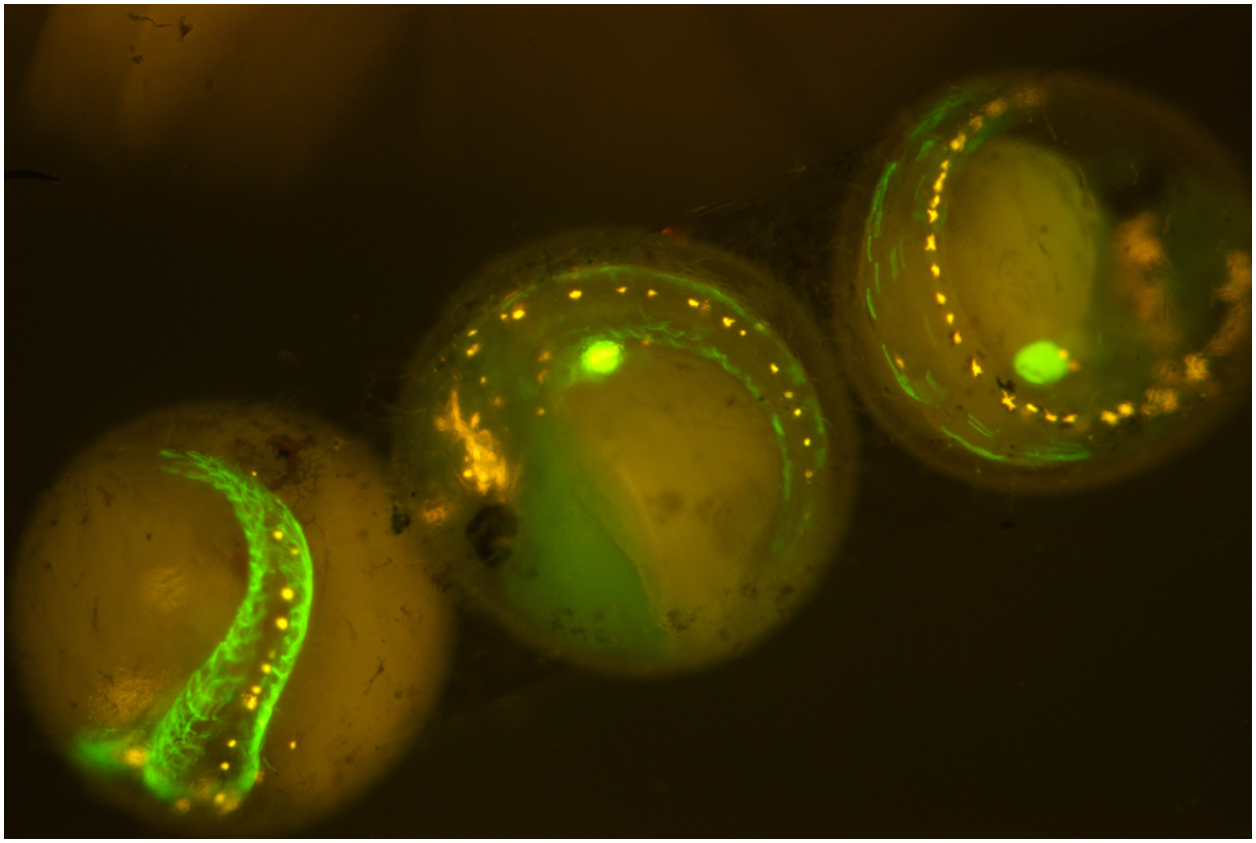


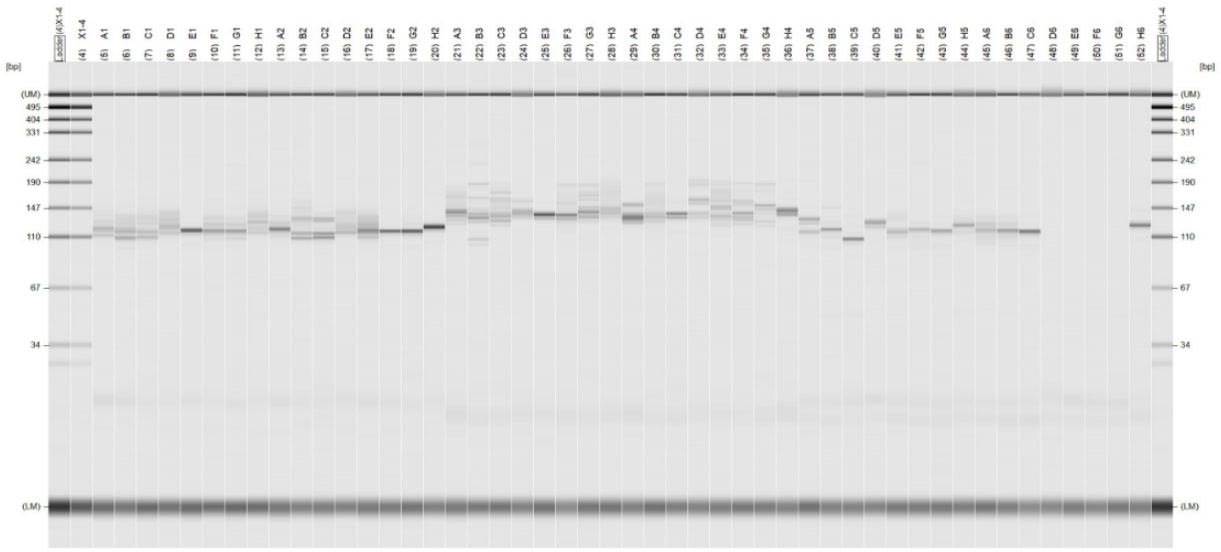
Figure 4c:



Rxfp CRISPR-Cas9 Knock-in

FIGURE 5: Heteroduplex Mobility Assay

Result of heteroduplex mobility assay electrophoresis performed on MultiNA device using DNA500 separation buffer and LD1 DNA ladder. SYBR (R) Gold dye used. This is the gel image-analysis data.



Slc45a2 CRISPR-Cas12a Knockout

TABLE 3: Survivorship data from three different dates of microinjection.

Table 3a: Survivorship from June 21, 2022

Concentration of Microinjection Solution	Survived/Injected	Survived/Day2PM	WES/Inj	WES/Day2PM
20 microMolar	0.1818181818	0.4	0	0
10 microMolar	0.3846153846	0.6666666667	0	0
5 microMolar	0.5483870968	0.8095238095	0.1612903226	0.2380952381
Total	0.3924050633	0.6739130435	0.06329113924	0.1086956522

Table 3b: Survivorship from June 22, 2022

Concentration of Microinjection Solution	Survived/Injected	Survived/Day2PM	WES/Inj	WES/Day2PM
20 microMolar	0.5277777778	0.8636363636	0.3611111111	0.5909090909
10 microMolar	0.5609756098	0.9583333333	0.1951219512	0.3333333333
5 microMolar	0.3142857143	0.7333333333	0.08571428571	0.2
Total	0.4732142857	0.868852459	0.2142857143	0.393442623

Table 3c: Survivorship from June 23, 2022

Concentration of Microinjection Solution	Survived/Injected	Survived/Day2PM	WES/Inj	WES/Day2PM
20 microMolar	0.393442623	0.9230769231	0.2459016393	0.5769230769
10 microMolar	0.2931034483	0.7727272727	0.06896551724	0.1818181818
5 microMolar	0.5714285714	0.7407407407	0.08571428571	0.1111111111
Total	0.3961038961	0.8133333333	0.1428571429	0.2933333333

TABLE 4: Phenotypic Distribution of Surviving Embryos

	Black Eyes	Mosaic	White Eyes	Mosaic + White	Total	Ratio of MW/Total
2x Concentration	6	13	28	41	47	0.8723404255
1x Concentration	26	12	12	24	50	0.48
0.5x Concretration	18	19	11	30	48	0.625
Total	50	44	51	95	145	0.6551724138

FIGURE 6: Microscopic photography of White Eyed Embryos.

Figure 6a: White-eyed embryo at 6 days after microinjection with 10 microMolar concentration of Cas12a slc45a2 knockout solution

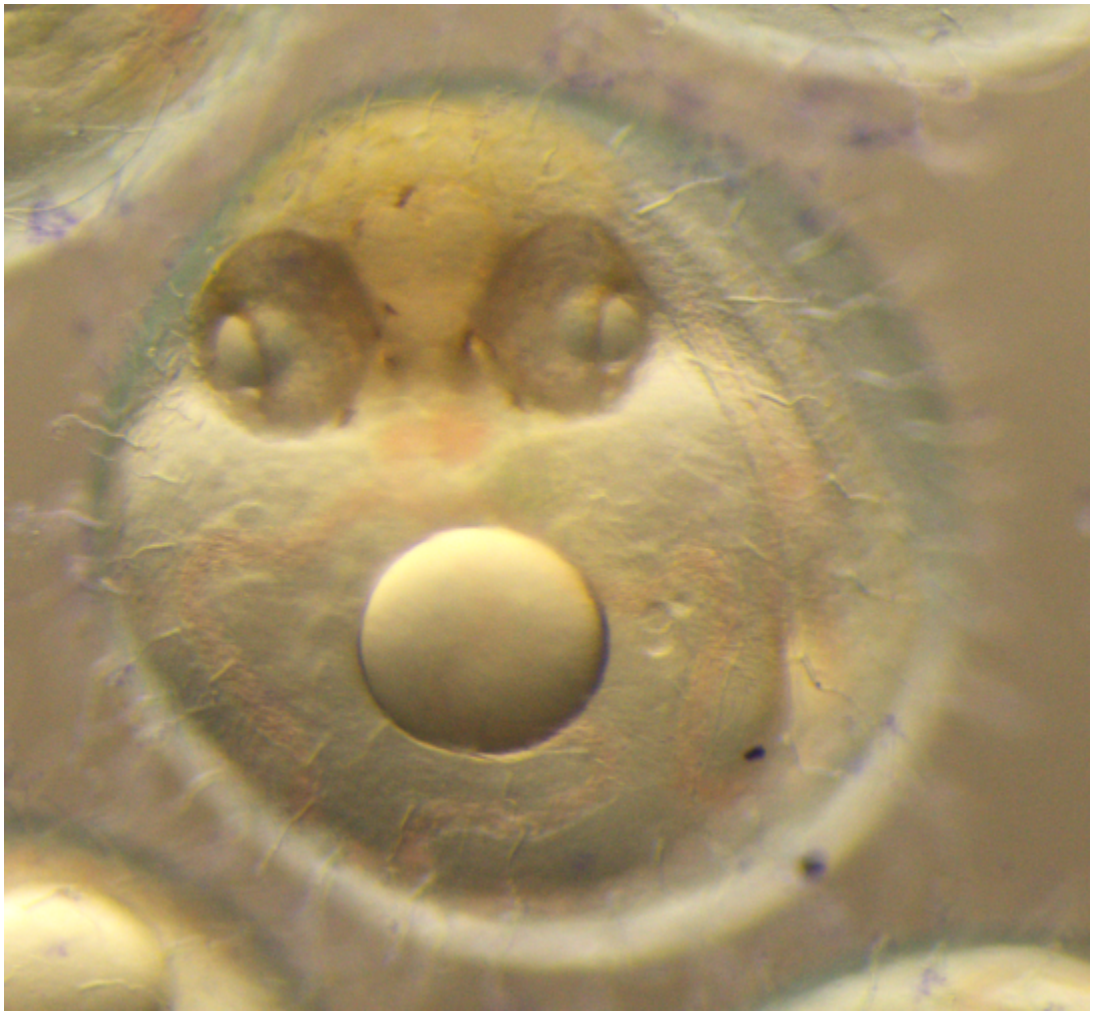


Figure 6b: White-eyed embryos at 5 days after microinjection with 10 microMolar concentration of Cas12a slc45a2 knockout solution

Figure 6c: White-eyed embryos at 5 days after microinjection with 10 microMolar concentration of Cas12a slc45a2 knockout solution

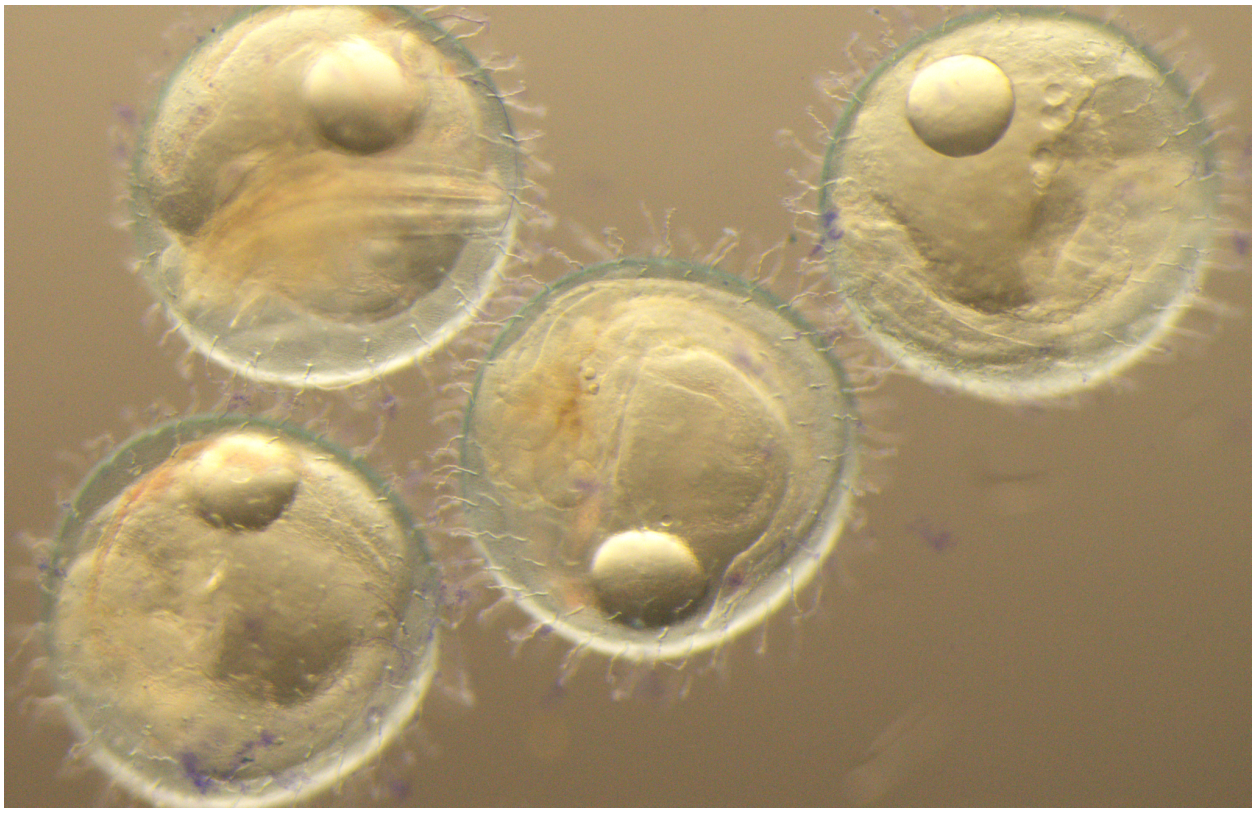


Figure 6d: White-eyed embryos at 5 days after microinjection with 10 microMolar concentration of Cas12a slc45a2 knockout solution

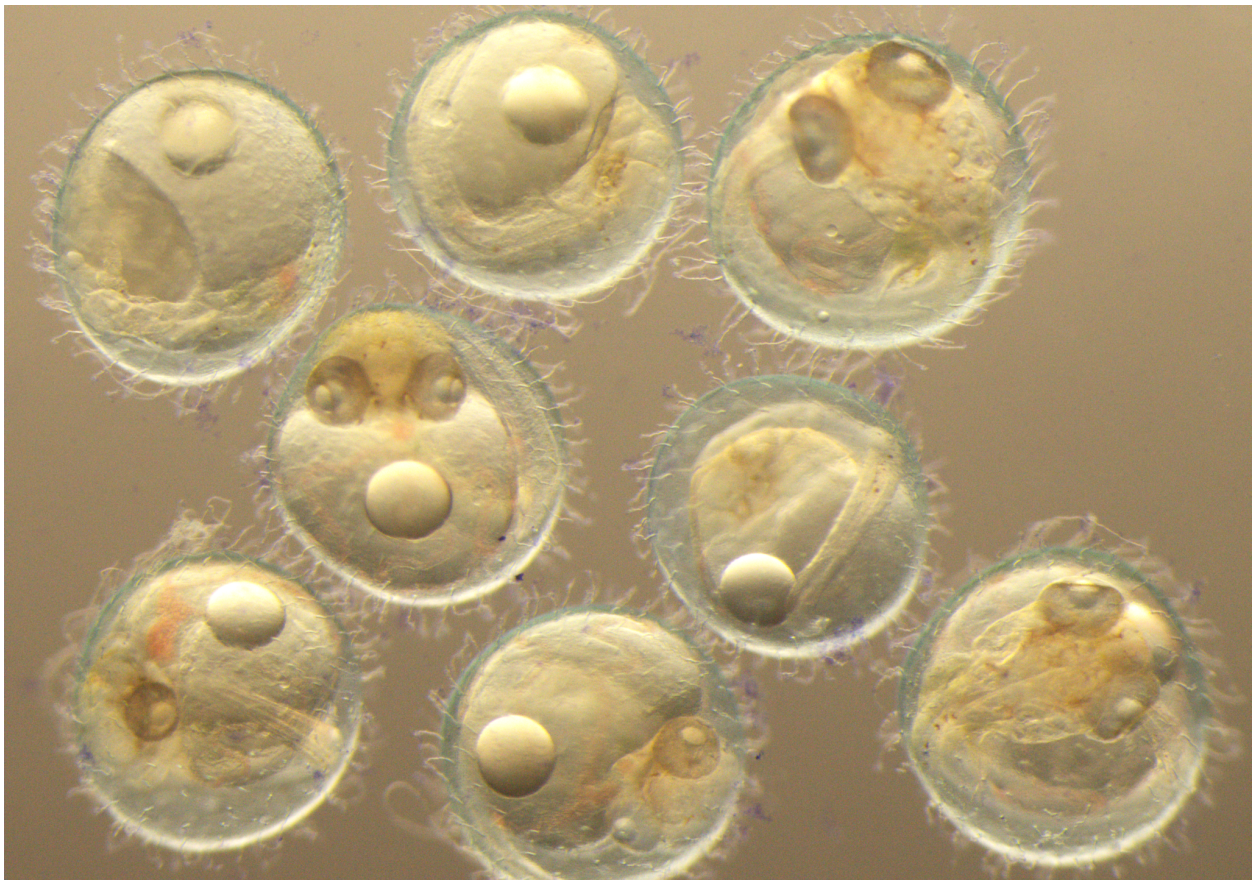


FIGURE 7: Microscopic Photography of Mosaic-eyed embryos

Figure 7a: Mosaic-eyed embryos at 7 days after microinjection with 5 microMolar concentration of Cas12a slc45a2 knockout solution.

Figure 7b: Mosaic-eyed embryos at 7 days after microinjection with 5 microMolar concentration of Cas12a slc45a2 knockout solution

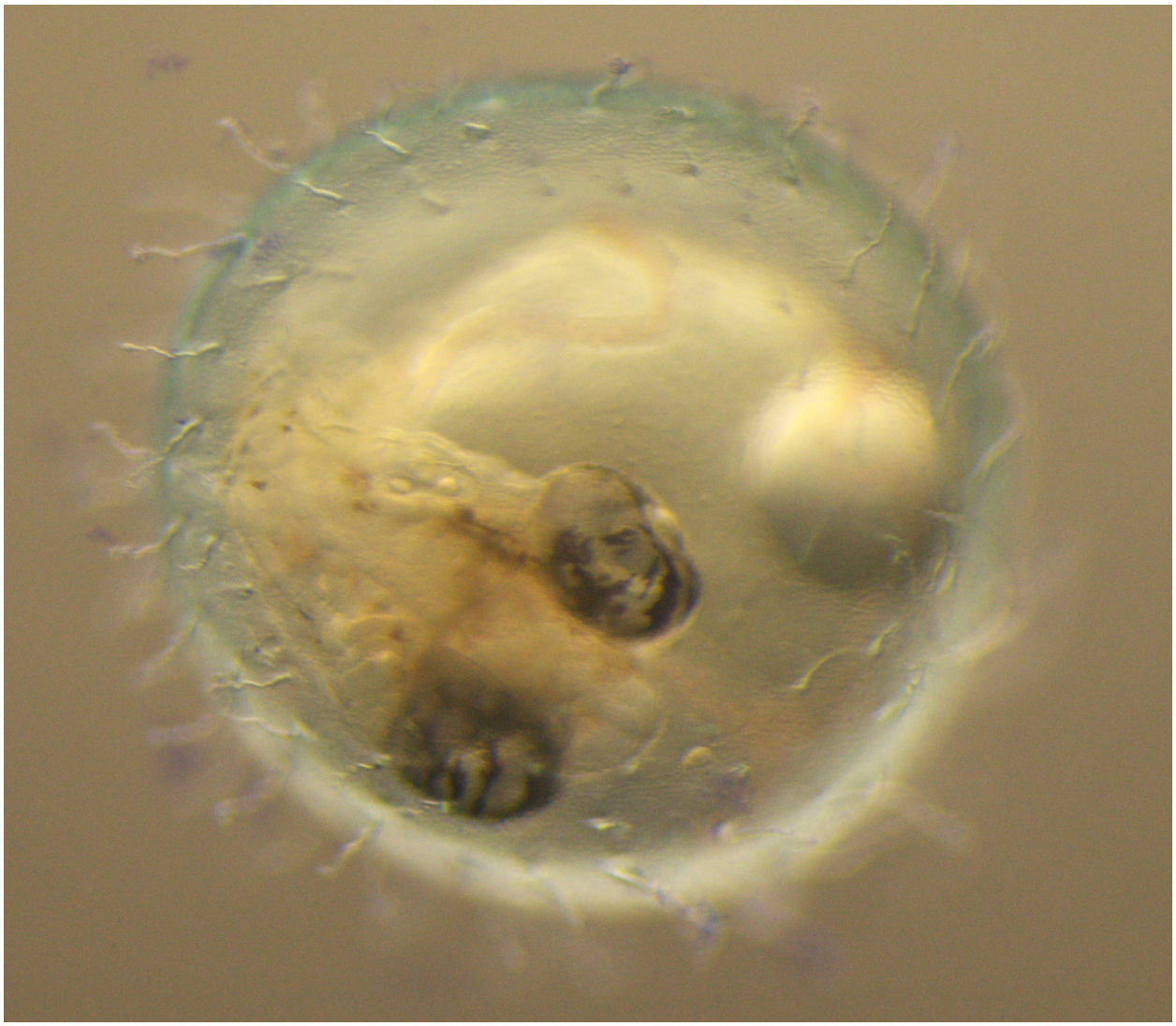


Figure 7c: Mosaic-eyed embryos at 7 days after microinjection with 5 microMolar concentration of Cas12a slc45a2 knockout solution

Figure 7d: Mosaic-eyed embryos at 7 days after microinjection with 5 microMolar concentration of Cas12a slc45a2 knockout solution

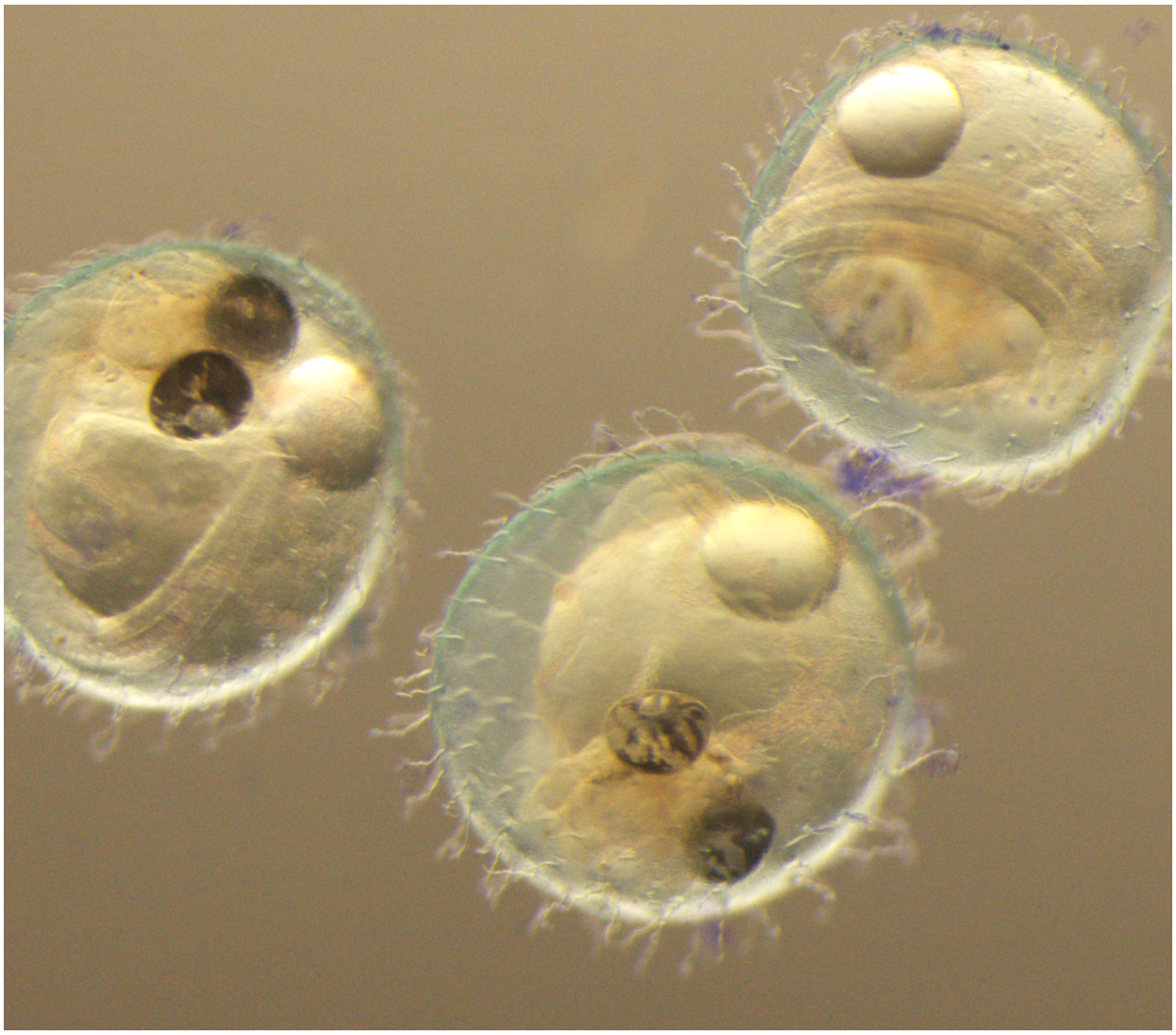


FIGURE 8: Microscopic photography of black-eyed embryos.

Figure 8a: Black-eyed embryos at 5 days after microinjection with 10 microMolar concentration of Cas12a slc45a2 knockout solution

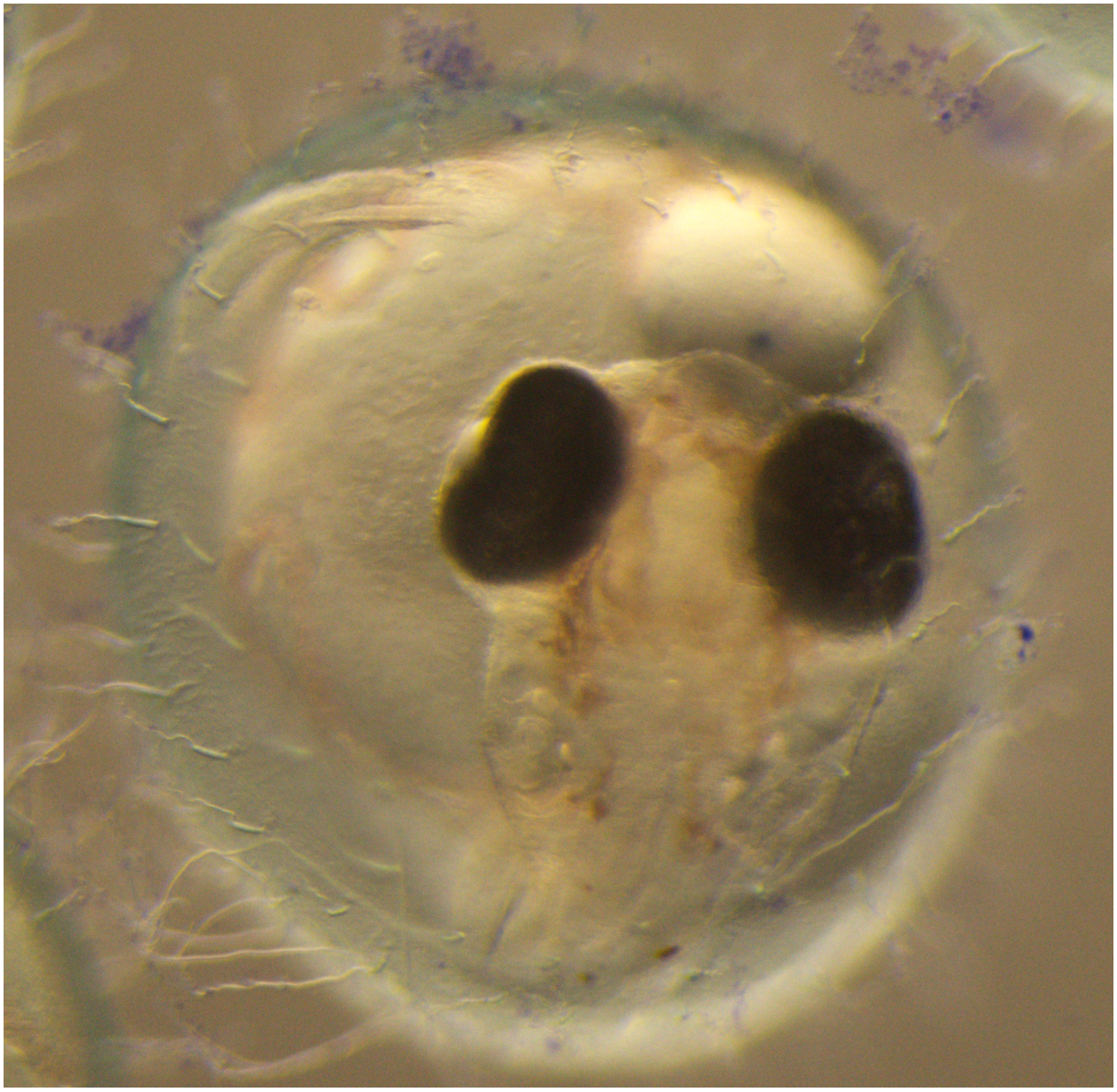


Figure 8b: Black-eyed embryos at 5 days after microinjection with 10 microMolar concentration of Cas12a slc45a2 knockout solution

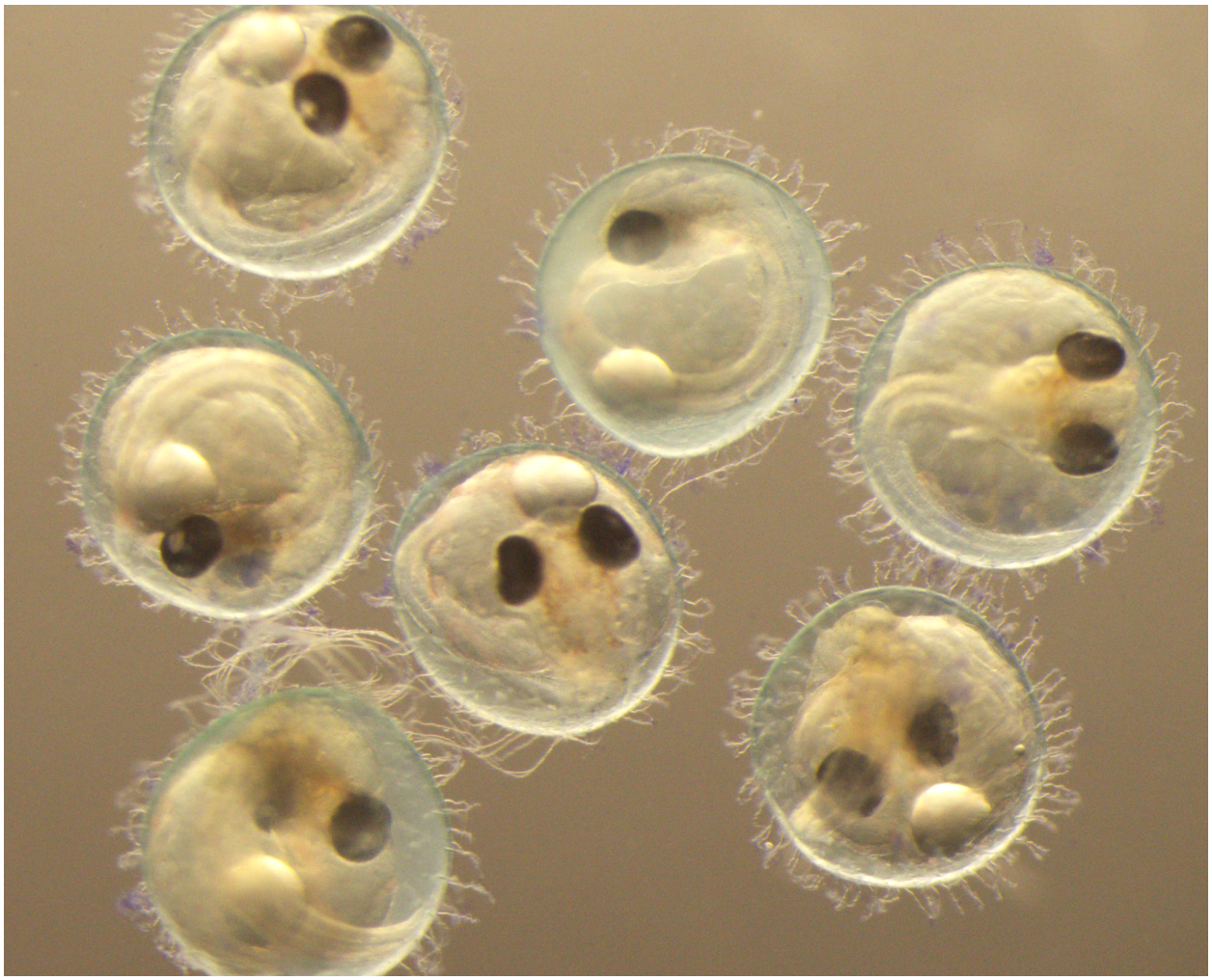


Figure 8c: Black-eyed embryos at 5 days after microinjection with 10 microMolar concentration of Cas12a slc45a2 knockout solution

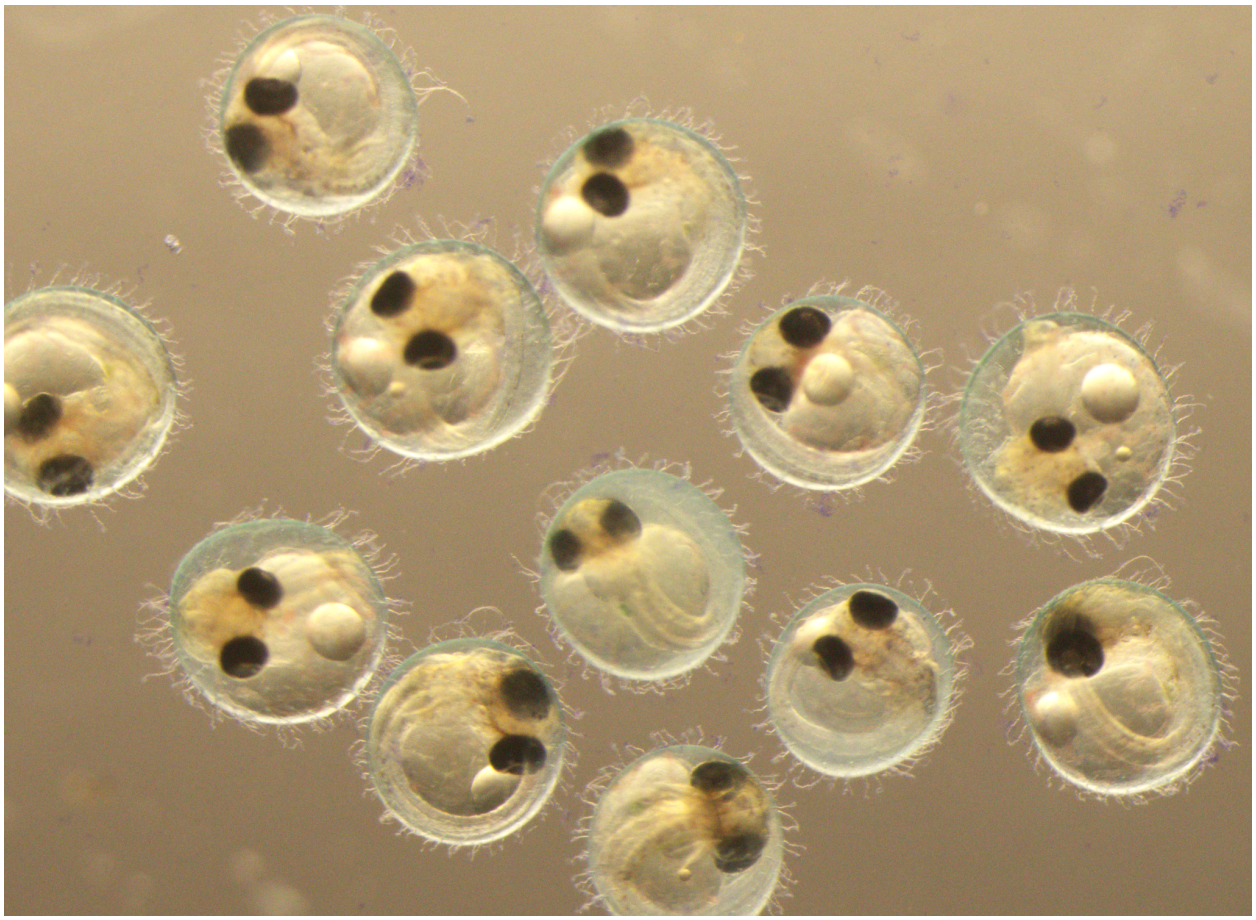


Figure 8d: Black-eyed embryo at 7 days after microinjection with 5 microMolar concentration of Cas12a slc45a2 knockout solution

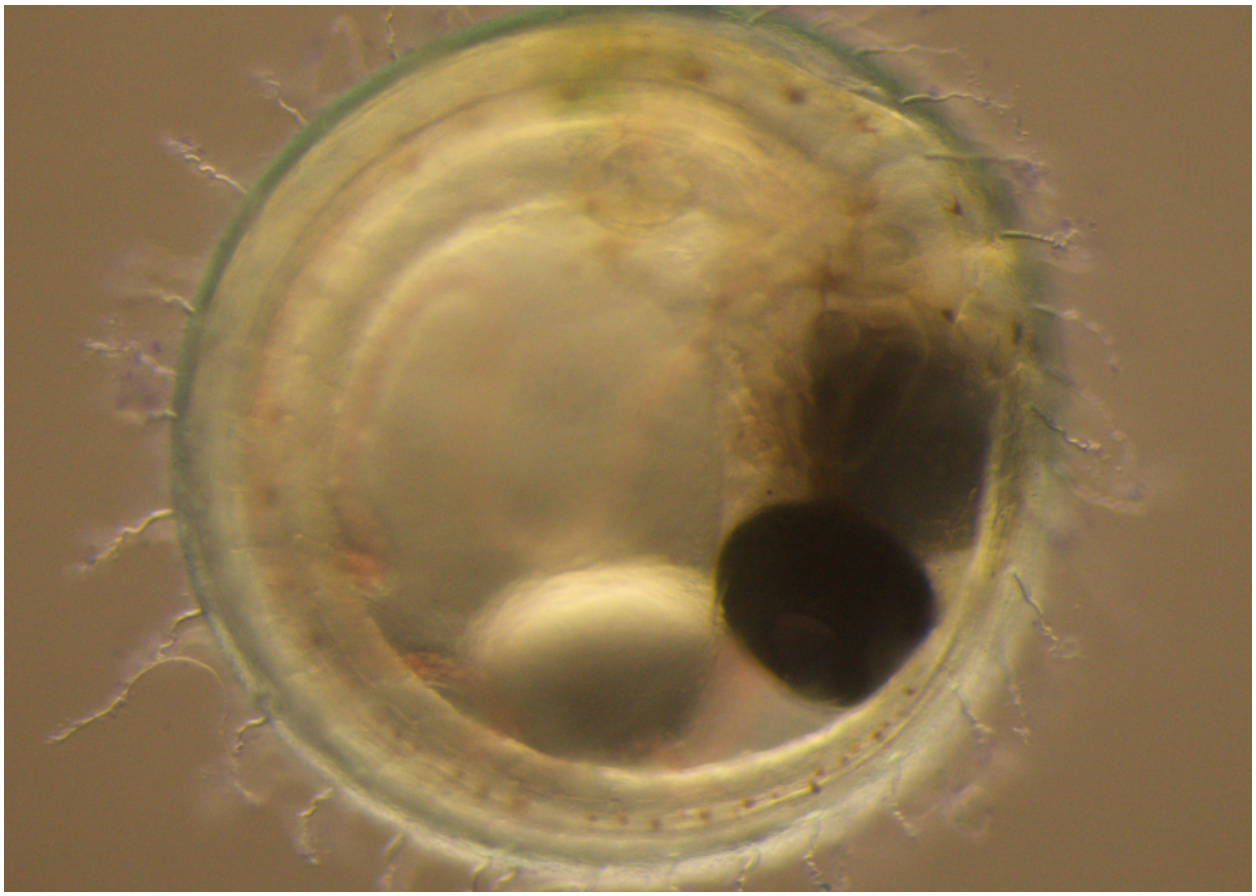
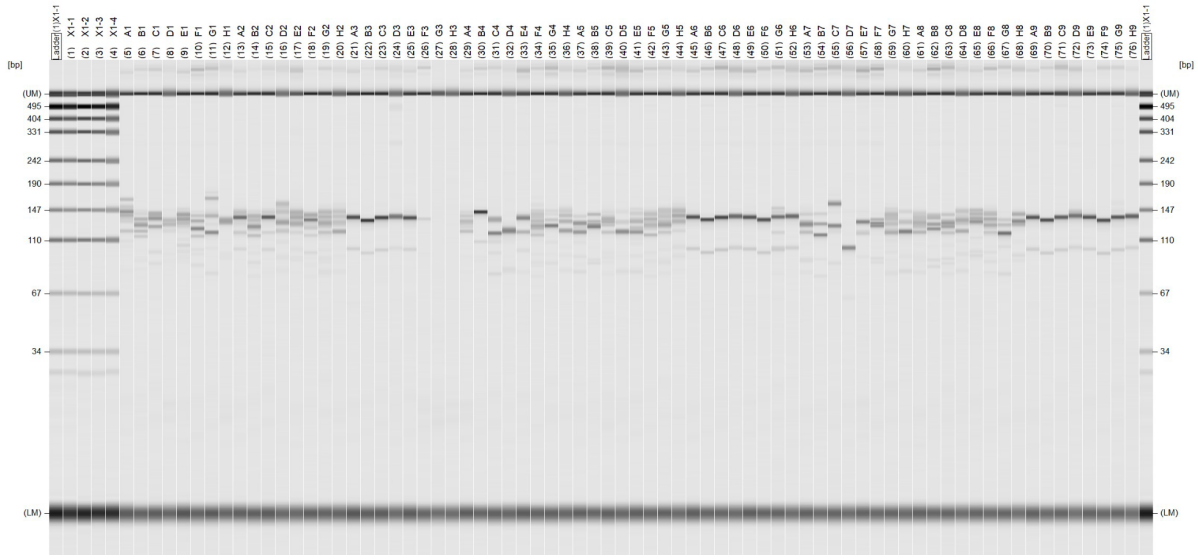


FIGURE 9: Heteroduplex Mobility Assay Microchip Electrophoresis

Result of heteroduplex mobility assay electrophoresis performed on MultiNA device using DNA500 separation buffer and LD1 DNA ladder. SYBR (R) Gold dye used. This is the gel image-analysis data.



Luciferase CRISPR-Cas9 Knock-in to PER2

The limited time allotted for this international collaborative effort was not ample enough for us to complete the knock-in luciferase to the C-terminus of PER2. We ran out of time before designing new primers for amplification of this successfully created donor DNA plasmid. We created a donor DNA fragment but did not have enough time to insert it.

FIGURE 10: MultiNA microchip electrophoresis

MultiNA microchip electrophoresis depicting double stranded breaks made at the C-terminal end. The gel shows a band at 1.5 kilobase pairs (1.5kb).

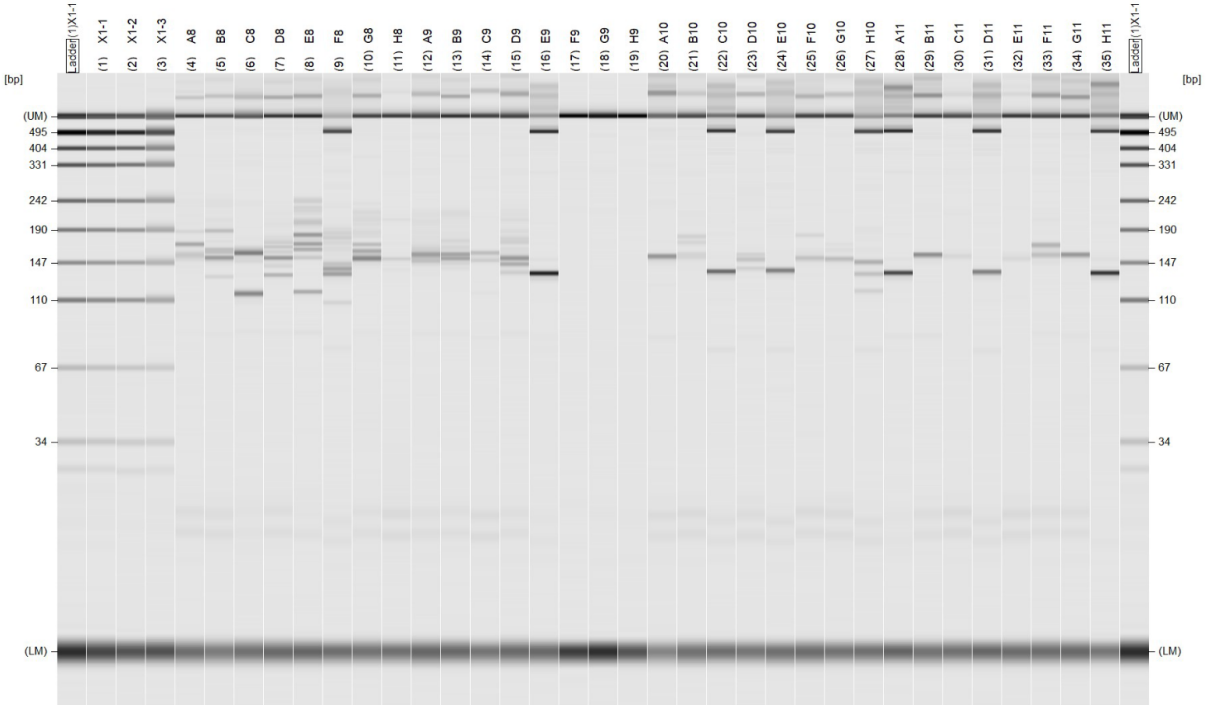
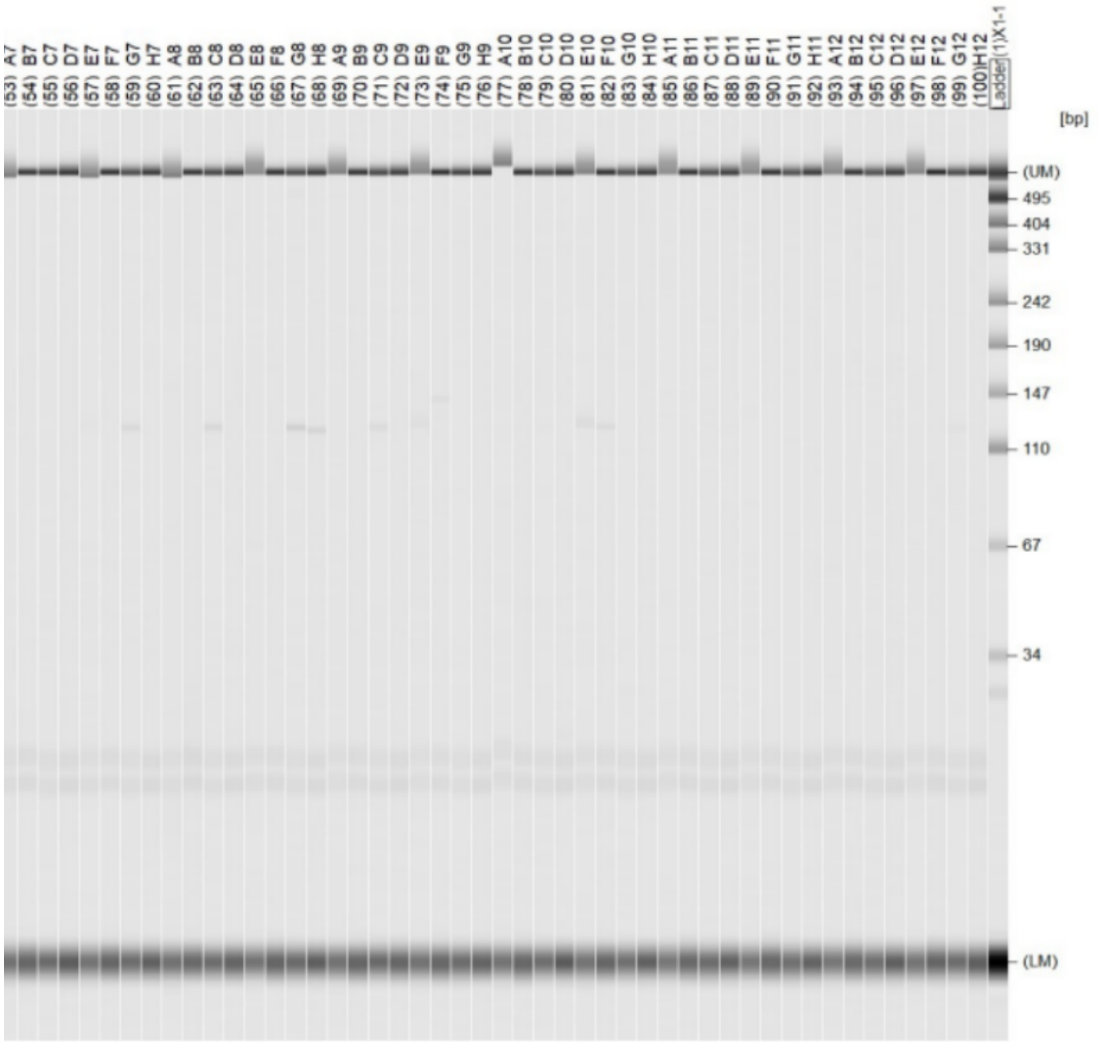


FIGURE 11: Heteroduplex Mobility Assay

Result of heteroduplex mobility assay electrophoresis performed on MultiNA device using DNA500 separation buffer and LD1 DNA ladder. SYBR (R) Gold dye used. This is the gel image-analysis data.



Discussion/Conclusion

Slc45a2 CRISPR-Cas9 Knockout

A1, B1, C1, D1, and E1 represent DNA extracted from embryos expressing the black eye phenotype. A1 and B1 show a smear pattern. This indicates that successful nuclease cutting did in fact occur in these embryos. The rest of the black eyed embryos should have given data that looks like these. The retinal epithelial cells had no mutations. We are able to determine this by looking at the phenotype, which is presented as the same as wild type, with pigmented black eyes. We are able to observe the wild type retinal epithelial pigmentation during embryonic development. This is a major advantage of doing proof of concept work with this slc45a2 gene. The quick phenotypic differentiation saves time and costs of raising fish to adulthood.

C1, D1, and E1 show a solid band at around 140 base pairs. F1 is a wild-type control from the CAB line of medaka. F1 shows a solid band around 140 base pairs. This is the expected result for this control, however the injected cells that show black eyed phenotype, similar to the wild type phenotype, should not have banding patterns that match the cab. One possible explanation as to why these results occurred is technical error during the process of microinjection. It is possible that the micropyle was not directly pierced during microinjection of the RNP complex containing CRISPR-Cas9 proteins and the single guide RNA. Improper injection can lead to failed incorporation of the CRISPR-Cas nuclease.

G1, H1, A2, B2, and C2 represent DNA extracted from embryos expressing mosaicism. G1 and B2 show solid banding. The band in column G1 lines up with those of C1-E1, which are from black eyed embryos, and F1, which is a CAB wild-type control. B2 shows a solid black band, but at a slightly lower position on the gel. This indicates that the size of this DNA fragment is shorter, perhaps closer, perhaps closer to 130 base pairs long. The solid banding despite

mosaic phenotype is not expected. This may signify that there was a low proportion of cells which were properly mutated by the experiment, however these few cells were enough to cause the phenotypic change that resulted in a mosaic pattern of pigmentation, as represented in Figure 3.

H1, A2, and C2 have a multiple banding pattern. The combination of mosaic phenotype and multiple banding pattern on the MultiNA result suggest that some nuclease activity occurred, but a perfect knockout was not achieved. Partial knockout of slc45a2 led to a partial loss of pigmentation.

Columns D2, E2, F2, G2, and H2 represent DNA extracted from slc45a2 knockout mutants in which the embryos expressed a phenotype lacking melanophores, resulting in completely colorless eyes.

D2, E2, F2, G2, and H2 all show multiple banding patterns. This indicates that nuclease activity was successful. Cutting by the CRISPR-Cas9 protein functionally occurred, resulting in a multiple banding pattern that looks like a smear. The smears are not uniform because the cutting left fragments of different sizes in each of the embryos. The sizes are variable due to a variety in deletion locations.

White eyes indicate a fully successful mutation, where the knockout of slc45a2 did occur enough to make an observable impact on phenotype. Black Eyes indicate an unsuccessful mutation were the knockout of slc45a2 did not occur enough to make an observable impact on phenotype. Mosaicism indicates a partially successful mutation so that the knockout of slc45a2 did occur enough to make an observable impact on phenotype but not enough to entirely change the eyes from black to white.

Myosin Heavy Chain mNeonGreen CRISPR-Cas9 Knock-in

Out of 93 injected embryos, 3 survivors showed green fluorescence, indicating that mNeonGreen was successfully incorporated into the myosin-heavy chain region. The knock-in for the c terminal and n terminal ends were successful using mNeonGreen and CRISPR-Cas9. We used mNeonGreen with the Myosin Heavy Chain locus. We observed green fluorescence in the body. That indicated that the N terminal end insertion was successful. The N-terminal end insertion was successful. Biotinylation prevents concatemerization, where ligation of two donor DNAs causes an elongated repetitive sequence. Leaving the fragment without biotinylated ends could result in a string of consecutive donor DNA fragments. (Hoshijima et al, 2016, 2019)

Rxfp CRISPR-Cas9 Knock-in

Primer design and testing were successful. Microinjection was successful. Observing a smear pattern (multiple banding) on our hetero duplex mobiilty assay shows that cutting is occurring. On Drake Hetcher's word, we are ready to microinject and send him KO eggs. As of now, this project is awaiting correspondence from Drake Hetcher to move forward/

Slc45a2 CRISPR-Cas12a Knockout

Only Biallelic knockout shows the mutant phenotype because in most CRISPR-Cases the mutation is recessive. Mosaicism means some cells have knockout and some don't. It's a mixing of the wild type and mutant cells. Mosaicism does not equal heterozygous. Heterozygous means one allele has WT phenotype of black eyes and one allete has knockout phenotype white eye. Mutant phenotype in the G0 general shows that CRISPR-Cas12a can introduce biallelic mutations.

Luciferase CRISPR-Cas9 Knock-in to PER2

We successfully synthesized this donor plasmid: [linker - flag - luciferase - HA tag - linker] . We ran out of time before we were able to knock-in luciferase to the C-terminus of PER2. All sequences were confirmed by sanger sequencing. We did not need to make the last part/ the HA tag-linker end, part because we were inserting at the C-terminus, just before the stop codon. For this reason, we only needed to make the linker-FLAG-luciferase part. We ordered the pieces, and we mixed the fragments using PCR. This is different from what we did with our N-terminus insertion, which required the [HA tag - linker] sequence to be inserted at the 3' end. We were able to synthesize the donor plasmid but were not able to amplify the fragment due to some suspected error with our primer design. We ran out of time before designing new primers for amplification of this successfully created donor DNA plasmid. We Created a donor DNA fragment but did not insert it. We have the plasmid with the linker sequences and the antigen tags, however we could not attach the biotin or the homology regions due to some error with the primers. We were attempting to attach homology regions that corresponded to a crRNA that Dr. Naruse designed for use with CRISPR-Cas9. After the template plasmid is successfully created, you can choose whether to design it for CRISPR-Cas9 or CRISPR-Cas12a, and that depends on the design of the crRNA. The choice of crRNA affects the choice of homology regions. The last step of creating the donor DNA is to attach the homology regions, which will be specific to the CRISPR-Cas protein chosen for the experiment. Then knock-in luciferase to PER2 with either CRISPR-Cas12a or CRISPR-Cas9.

nLucP CRISPR-Cas9 Knock-in to PER2

The goal accomplishment of this project was to use either CRISPR-Cas12a or CRISPR-Cas9 to knock-in nLucP to the PER2 region. This would attach a reporter gene to PER2, allowing for further experiments to better understand this region's effect on circadian rhythms.The broader impact of this experiment would be that this work would aid in the study of circadian rhythms. Test the insertion efficacy at the C-terminal end with microinjection of crRNA targeting the per2 C-terminal region.

Establishing a reporter gene for the PER2 region helps scientists to monitor its development. The heteroduplex mobility assay performed on the MultiNA by microchip electrophoresis showed successful cutting by the smear pattern with multiple bands. We then went on to make the donor DNA, which is a biotinylated product with homology regions attached to it ready to be injected alongside a CRISPR-Cas protein. For this we would use either CRISPR-Cas9 or CRISPR-Cas12a and this prepared donor DNA fragment. We attempted to make this donor DNA fragment, but ultimately the reverse PCR failed and we could not remove mNeonGreen to insert nLucP into the plasmid. Our sanger sequencing revealed that we did not successfully remove mNeonGreen. We searched for the region on NCBI BLAST and confirmed that it was in fact remaining mNeonGreen.

Before this experiment regarding CRISPR-Cas12a, we wanted to test it by doing a knockout and knock-in. The knockout test was slc45a2, which was successful. The knock-in test was supposed to be inserting mNeonGreen into the middle of the slc45a2 gene, rendering slc45a2 gene ineffective and no longer functional. This would result in a phenotype of white eyes with mNeonGreen fluorescence under blue light. Excitation of mNeonGreen is under blue light but fluorescence is green. One advantage of mNeonGreen is that emission energy is high. It is a bright fluorescent protein when compared to GFP. m stands for monomeric, meaning

there is a single protein that emits fluorescence. Some fluorescence proteins are dimeric or trimeric.

The 1% agarose gel electrophoresis results showed a band at 1.5 kilobase pairs (1.5kb).which is expected, but the amplification efficiency was low. This may be because it is a long fragment, or that there was not a lot of template added before PCR.

We made the donor DNA for this using the PAP27 template from Alexander Paix. We have the donor DNA ready to go for inserting mNeonGreen into the middle of slc45a2, which includes biotin, homology regions, and epitope tags successfully attached to the DNA fragment containing mNeonGreen. The only thing stopping us is time. The only thing that went wrong within the progress of this experiment is that we ran out of CRISPR-Cas12a protein. We ordered it, but it did not come in time before I left the lab in Japan.

The question we are addressing is: can you do a knock-in with CRISPR-Cas12a? We would explore this question by testing it with inserting mNeonGreen into slc45a2. This is a good test because it is easy to see the phenotype in embryonic development. This would precede the PER2 knock-in because we don't know when exactly development of the PER2 region would come to a point that allows fluorescence to be visible. We need to do this slc45a2 experiment successfully before moving on to PER2 because the circadian rhythm doesn't show observable phenotypic variation until much later in development.

mNeonGreen CRISPR-Cas9 Knock-in to PER2

The MultiNa microchip electrophoresis for this experiment showed no amplification at expected base pair lengths. There may have been contamination or improper measurements in the master mix used for the PCR preceding this electrophoresis. I kept aside 24 surviving microinjected embryos to monitor their development, but at the time of leaving the lab fluorescence was not yet visible. This does not indicate that the experiment was unsuccessful,

because we did not expect to see advanced development in the PER2 at the early embryonic stage. As these embryos hatch into fish and continue to develop, they will be monitored for fluorescence.

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Naoko Torii (Animal Caretaking), National Institute of Basic Biology, Okazaki, Aichi Prefecture, Japan.

Emiko Tsuruta (Animal Caretaking), National Institute of Basic Biology, Okazaki, Aichi Prefecture, Japan.

References

pUC19 was a gift from Joachim Messing (Addgene plasmid # 50005 ; http://n2t.net/addgene:50005 ; RRID:Addgene_50005)

pLV6-Bmal-luc was a gift from Steven Brown (Addgene plasmid # 68833 ; http://n2t.net/addgene:68833 ; RRID:Addgene_68833)

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