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Cost-Efficient and Effective Mutagenesis in Zebrafish with CRISPR/Cas9

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Abstract - The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system was first identified in bacteria, where it was observed to silence foreign nucleic acids. The system has since been adapted to use short RNA sequences that direct Cas9 endonucleases in order to induce site specific DNA cleavage. The CRISPR system has been shown to be effective in cultured cells and in animal studies. In studies making use of the zebrafish model, CRISPR has been shown to induce mutations at rates similar to ZFNs and TALENs when using Cas9 mRNA. Here, we evaluate the efficacy of the CRISPR/Cas9 system in zebrafish embryos using commercially obtained Cas9 protein. We show that using the CRISPR system with commercially obtained Cas9 protein induces high mutation rates. In combination with a relatively inexpensive microinjector apparatus, commercially obtained Cas9 protein could allow effective zebrafish mutagenesis that is both cost and time-efficient, making it a practical approach for labs with a limited budget, undergraduate students completing an honor's thesis, and undergraduate teaching labs.

Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system was first identified as a mechanism to provide acquired immunity in bacteria (Ishino et al. 1987). Bacteria, as well as archaea, have adaptive immune defenses against plasmids and viruses that use short RNA fragments to direct the degradation of foreign nucleic acids (Jinek et al. 2012, Mali et al. 2013b). It was found that when CRISPR acts against invading phages, bacteria transcribe spacer DNA and palindromic DNA into a single RNA molecule. The cell then cuts this single molecule into short CRISPR RNAs (crRNAs) which act with trans-activating RNAs (tracrRNAs) alongside Cas9 proteins to attack foreign DNA that matches the sequence of the crRNA (Deltcheva et al. 2011). Over the last several years, this system has been utilized to induce precise cleavage at desired endogenous loci by targeting Cas9 endonucleases to these regions via short RNA molecules (Cong et al. 2013).

To better understand the mechanisms associated with this newly identified CRISPR system, a study was performed in *Streptococcus pyogenes* type II (Jinek et al. 2012). Here, the CRISPR system showed that a CRISPR RNA (crRNA) fused to a trans-activating crRNA (tracrRNA) to form a two RNA structure. The structure proved sufficient to direct the CRISPR associated Cas9 protein to a specific DNA sequence, cleaving the target site and ultimately generating a double-stranded break (Jinek et al. 2012). The mechanisms observed in *S. pyogenes* suggested that the CRISPR system might function in eukaryotic organisms. By using RNA sequences to cleave target genomic sites, it was hypothesized that a system like CRISPR could improve the current field of genetic engineering (Mali et al. 2013b).

With the CRISPR system, the Cas9 nuclease can be directed to the target site via one of two different mechanisms. One mechanism makes use of a pair of RNA molecules containing a crRNA and a tracrRNA, as described above. Individually, neither of these molecules can direct DNA cleavage via Cas9; rather, both the crRNA and tracrRNA must be present for Cas9 to cleave target DNA (Jinek et al. 2012). Another mechanism to direct the Cas9

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to a target is with a chimeric single guide RNA (sgRNA). Both the crRNA and the sgRNA include a 20-nucleotide sequence that is complementary to the target DNA sequence. The RNA molecule is thus able to recognize the DNA via Watson-Crick basepairing (Choe et al. 2014, Cong et al. 2013, Jinek et al. 2013, Mali et al. 2013a). This RNA molecule guides the Cas9 protein to the target DNA. Once at the target site, the Cas9 nuclease cleaves DNA.

Recently, two studies (Jinek et al. 2014, Nishimasu et al. 2014) have shed light on the structural conformation of Cas9 and the structural mechanisms of RNA-guided DNA cleavage by Cas9. Through these studies, it was observed that the Cas9 family of proteins is characterized by six nuclease domains. The HNH domain is a single nuclease domain characterized by a ββα metal fold, while the RuvC domain resolves the Holliday structure and contains three subdomains (Nishimasu et al. 2014). Both the HNH and RuvC domains initiate single-stranded DNA cuts. The RuvC I subdomain is near the N-terminal region of Cas9 while the RuvC II and RuvC III subdomains flank the HNH domain near the middle of the protein. All Cas9 proteins also have two recognition "REC" domains (Nishimasu et al. 2014). The REC I domain is responsible for binding sgRNA while the protospacer-adjacent motif (PAM) interacting domain is responsible for initiating binding of target DNA. The arginine-rich bridge helix then contacts between 8 to 10 nucleotides on the 3' end of the target DNA and initiates cleavage. The function of the REC II domain is unknown to-date (Jinek et al. 2014, Nishimasu et al. 2014).

The HNH domain of the Cas9 protein cleaves the complementary strand of DNA, while the RuvC domain cleaves the non-complementary strand. Together, these two cleavages generate a double stranded DNA break with blunt ends (Gasiunas et al. 2012, Jinek et al. 2012, Sapranauskas et al. 2011). Double-stranded breaks then activate a repair mechanism, either through non-homologous end joining (NHEJ), or homology-directed repair (HDR), which are both active in most cell types and organisms (Cong et al. 2013, Maruyama et al. 2015). NHEJ-mediated repair of Cas9-generated breaks is useful if the intent is to make a null allele ("knockout") in the DNA region of interest, as it is prone to generating indel errors. Indel errors generated in the course of repair by NHEJ are typically small (1–10 bp), but extremely varied (Maruyama et al. 2015). NHEJ is not the most precise method to repair DSB due to these indel errors; however, NHEJ occurs much more frequently than HDR, which only occurs during the S and G2 phases of the cell cycle (Maruyama et al. 2015).

The CRISPR/Cas9 system has been used to enable numerous targeted genome engineering applications, including *in vivo* applications. Indeed, the CRISPR/Cas9 system has been shown to be able to induce mutations at rates similar to, or better than, both TALENs and ZFNs in zebrafish embryos. In a study comparing the CRISPR system to TALENs and ZFNs, several sgRNAs were designed to target the *fh* gene of zebrafish (Hwang et al. 2013). The majority of these targets in the zebrafish *fh* gene were successfully mutated and showed robust mutations using the CRISPR/Cas9 system. Two of these successful targets had previously been unable to be mutated through either TALENs or ZFNs. For the other mutations, success rates were comparable to TALENs and ZFNs. Overall, 9 out of 11 targets were successfully mutated and showed robust mutations using the CRISPR/Cas9 system. These results show that using sgRNA to direct site-specific DNA cleavage via Cas9 is a rather fast and highly efficient method for altering genes *in vivo*, opening the door for using the CRISPR/Cas9 system in other organisms (Hwang et al. 2013).

A common standard for targeted mutagenesis in zebrafish uses the golden phenotype. The affected gene encodes the Slc24a5 cation exchanger which has been shown to alter pigmentation in both humans and zebrafish (Lamason et al. 2005). Mutations in the *slc24a5* gene display lighter melanocyte pigmentation that is observable in zebrafish embryos as early

as 48hpf. Zebrafish carrying the homozygous *slc24a5* mutation show hypopigmentation in skin melanophores and retinal pigment epithelium (RPE) (Lamason et al. 2005). Using the CRISPR/Cas9 system to target *slc24a5* in zebrafish, mosaic hypopigmentation was observed in skin melanophores and RPE in 94% of the injected embryos, while mutagenesis rates ranged from 78–98%, indicating that most cells contained biallelic mutations (Jao et al. 2013). Germ-line transmission of the Cas9 induced mutation was also observed, as well as recessive null-like phenotypes suggesting high rates of biallelic gene disruption. These results suggest that CRISPR/Cas9 is an efficient way to induce biallelic gene disruptions in zebrafish (Jao et al. 2013). This rate of germline transmission efficiency is comparable to the 45% that has been previously observed with ZFNs. While this incomplete mutagenesis is necessary for generating germline mutants and permits higher rates of embryo survival, recent reports have shown increased mutagenesis efficiency upon injection of *in vitro* assembled Cas9-sgRNA ribonucleoprotein complexes (RNPs) (Gagnon et al. 2014, Kotani et al. 2015, Sung et al. 2014).

In addition to targeting *slc24a5,* the CRISPR/Cas9 system has also been used in conjunction with ribonucleoproteins (RNPs) to recapitulate other loss of function phenotypes (Burger et al. 2016). *tbx16* is a T-box gene that is regulated by FGF signaling (Griffin et al. 1998). In embryos with a spadetail phenotype, *tbx16* is defective resulting in embryos displaying a broad posterior tail. This mutant phenotype is attributed to the failure to differentiate mesodermal progenitor cells to different lineages (Kimmel et al. 1989). Using RNPs to target *tbx16,* more than 60% of all embryos injected displayed a strong phenotype while almost 20% displayed a mild or partial phenotype. Using MiSeq along with a customized software tool, CrispRVariants, for quantifying and visualizing mutagenesis, it was shown that RNPs allow for efficient biallelic mutagenesis for both *slc24a5* and *tbx16* (Burger et al. 2016).

While Burger et al. (2016) previously maximized zebrafish mutagenesis with solubilized CRISPR/Cas9 RNPs, generating such proteins can be both timely and costly. Furthermore, microinjection equipment can be relatively expensive. Here, we show how labs and institutions with limited resources can induce mutagenesis in both an economic and timely manner using commercially available EnGen® Cas9 NLS (New England Biolab Mo646T). In addition, we describe our microinjection set up, which is cost-efficient, as it does not require the recurring purchase of compressed gas, nor an expensive antivibration microscope table. Together, these substitutions make effective zebrafish mutagenesis cost-efficient for institutions and departments with limited funding.

Materials and Methods

Zebrafish care

Zebrafish were cared for as described in *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)* (Westerfield 2000) and all animal use was approved in accordance with the Appalachian State University Institutional Animal Care and Use Committee (IACUC). Fish were housed in a Marine Biotech Z-mod closed system (Aquatic Habitats, Apopka, FL). Within the system, fish were kept in one-liter tanks with a maximum of 7 adults per tank. Light cycle, temperature, pH, conductivity and water hardness were all maintained according to Regular Care and Maintenance of a Zebrafish (*Danio rerio*) Laboratory: An Introduction (Avdesh et al. 2012).

In order to obtain embryos for injection, male and female fish were separated by a divider in a special breeding tank (Aquatic Habitats) for timed mating. Fertilized zebrafish embryos were collected within 15–30 minutes of mating, ensuring that embryos were still at the single cell stage for injections. Washed embryos were then placed in a petri dish. Excess

water was removed from the dish and replaced with Danieau buffer solution (50x 2.9 M NaCl, 35 mM KCl, 20 mM $MgSO_4$, 30 mM $Ca(NO_3)_2$, 250 mM HEPES pH 7.600, adjusted to 1L with RO water). After injections, embryos were placed in a 1x Danieau/ methylene blue solution (~66 µL 1% methylene blue per liter of 1x Danieau buffer) in order to prevent fungal growth. The embryos were then placed in a 28°C incubator until they reached the appropriate stage for imaging.

Transcription of RNAs

To generate templates for sgRNA transcription, a specific oligonucleotide containing three nucleotide clamp, T7 promoter sequence, large sequence (short guide oligonucleotide), and a complementary sequence were annealed to a guide constant (scaffolding) oligonucleotide (Table 1) through polymerase chain reaction (PCR; as in Talbot and Amacher 2014). Samples were prepared for PCR containing: 5 µL 5x Phusion buffer, 200 µM dNTPs, 3 µL of 10 µM short guide oligonucleotide, 3 µL of 10 µM scaffolding oligonucleotide and 0.25 µL of Phusion (Thermo Scientific #F-530S). The samples were brought to a total volume of 25µL with nuclease free water. Samples were placed in the thermal cycler and the following settings were used to carry out the PCR: initial denaturation at 98°C for 30 seconds followed by 45 cycles of normal denaturation at 98°C for 10 seconds, annealing at 60°C for 10 seconds and extension at 72°C for 15 seconds. Once 45 cycles were completed, the sample underwent a final extension at 72°C for 5 min. The samples were then stored at 4°C. Samples were purified using phenol chloroform extraction and then precipitated. The DNA was resuspended with 10 µL of nuclease free water. After the sample was allowed to rehydrate, the DNA was used as a template to transcribe sgRNAs.

sgRNAs were transcribed using the MAXIscript T7 Transcription Kit (Ambion, Life Technologies, Grand Island, NY) according to the recommended protocol with two exceptions. To ensure that the transcribed sgRNA was not immediately degraded, 1 µL of RNAse inhibitor (Promega N2515) was added to the transcription reaction. Also, to ensure that the majority of the template DNA was transcribed into sgRNA, the reaction was incubated overnight at 37°C rather than one hour. After the overnight incubation period, turbo DNAse (Ambion, Life Technologies, Grand Island, NY) was added and the sample was incubated an additional 15 min at 37°C. The sgRNA was purified and concentrated using the RNA Clean & Concentrator™-5 kit (Zymo Research Corporation) by following the recommended

Table 1: Target sequence for tbx16 sgRNA and slc24a5 sgRNA.

Oligo Name	Sequence
Short guide oligo* targeting tbx16	5'-AATTAATACGACTCACTATAGGGTGCAGGTACGTCC TGTA GTTTTAGAGCTAGAAATAGC -3'
Short guide oligo* targeting slc24a5	5'AATTAATACGACTCACTATAGGTCTCTCGCAGGATGTTGCGTTT-TAGAGCTAGAAATAGC -3'
Guide Constant Oligo	5'AAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGAC-TAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3'

*Guide oligos are made of three main parts: a T7 promoter, shown in blue, a variable targeting sequence, underlined, and an overlap with the guide constant oligo. A short clamp, shown in red, is provided to stabilize the 5' end of the sequence. Using PCR, the guide constant oligo, shown in purple, and short guide oligo are annealed together to generate a full-length template for sgRNA synthesis. The guide constant oligo is used as a Cas9 binding scaffold.

protocol. The sgRNA was then resuspended in DEPC water and stored at -80°C until being coinjected with EnGen® Cas9 NLS (NEB Mo646T) into single cell zebrafish embryos.

Microinjection setup

Injections were done using a Nanoliter 2000 Microinjector (World Precision Instruments Model B203XVY) which was attached to a Marhauser MMJR Micromanipulator (World Precision Instruments). To cut down on costs, we have modified our injection set up in multiple ways. To begin, we use a simple dissecting microscope and an injection setup on a steel slab (Fig. 1), rather than on an antivibration injection table. The 90 cm x 45 cm x 0.5 cm thick steel slab was cut to size by the university machine shop and self-adhesive rubber bumpers were placed at each corner of the underside. An injection needle was pulled from a 3.5 nl capillary tube that had been baked at a temperature of 260°C in order to ensure the inactivity of RNases. The capillary tube was secured in a David Kopf Instruments Vertical Pipette Puller model 700C, where the heater was set to 54 and the solenoid set at 10. The weight on the bottom of the machine was used to pull and divide the needle. The tip of the needle was then beveled using size #5 forceps and filled with mineral oil. The needle was then placed on the Nanoliter 2000 Microinjector which was attached to the Marhauser MMJR Micromanipulator magnetically attached to the steel slab. The Nanoliter 2000 Microinjector uses mechanical force to increase pressure and eject the solution out of the needle (Fig. 1). The use of this microinjector alleviated the requirement for purchasing and using compressed gas, another way in which we have cut down the cost of injections. While we pull our own needles, pre-pulled needles (Tritech Research, Inc MINJ-PP) can be purchased in order to avoid the high cost of a pipette puller.

Microinjections with Cas9 protein

While adult zebrafish were mating, injection solutions were thawed. Once thawed, 50 ng/µl of each sgRNA and 250 ng/µl of EnGen® Cas9 NLS (New England Bio M0646T) were combined and brought to a final volume of 4 µL with nuclease free water. The injection solution was then incubated at 37°C for 10 min to allow the Cas9 and the sgRNAs to interact (Sternberg et al. 2014). The microinjector and needle were prepared as mentioned previously. Using the buttons on the micromanipulator, approximately 20–25% of the mineral oil was pushed out of the beveled needle to ensure that the tip was not clogged. The mineral oil was then replaced with the injection solution.

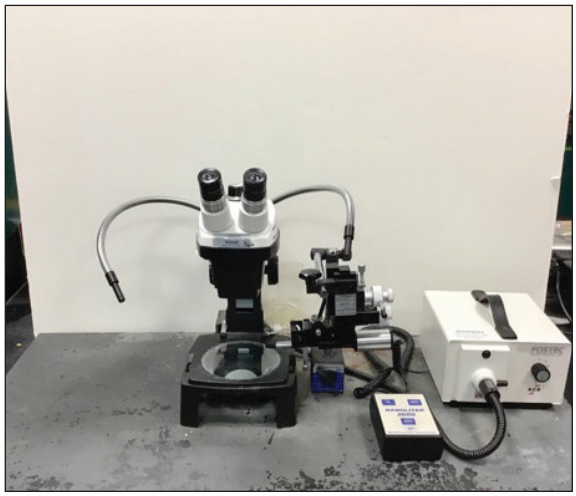

Figure 1. The microinjector system used for the experiments in this manuscript at Appalachian State University. Injections were done using a Nanoliter 2000 Microinjector (World Precision Instruments Model B203XVY), which was attached to a Marhauser MMJR Micromanipulator (World Precision Instruments). Our injection setup is on a steel slab and uses mechanical force rather than compressed gas.

Once the needle was filled with injection solution, between 40 and 60 single cell embryos were placed against a 1.0-millimeter-thick VWR micro slide (VWR international 48300–025) which was taped to the outside of a plastic petri dish to insure stabilization. Single cell embryos were then injected with approximately 1 nl of injection

solution. To ensure that the injection solution was injected directly into the single cell of the developing embryo, a translucent bolus was observed as the injection solution was dispensed into the single cell.

Imaging injected embryos

To examine mutant phenotypes of injected embryos, embryos were collected at specific timepoints (~24 hpf for spadetail and ~48 hpf for golden). Embryos were first dechorionated using size #5 forceps. After dechorionating, between 30 and 50 embryos were placed in an Eppendorf tube. Excess Danieau Buffer was removed from the tube and replaced with approximately 750µL of 4% paraformaldehyde (PFA). Eppendorf tubes were then placed on a shaker (Labrat Gyrotwister) at a speed between 45–50 RPM for 10 min. After 10 min., PFA was removed and replaced with 750µL of fresh PFA. Embryos were then left on the shaker overnight at 4°C or for 4 hours at room temperature.

After fixation, the PFA solution was removed and embryos were placed in a PBS solution on a shaker table at room temperature for 5 min. After 5 min., the PBS was removed and replaced with fresh PBS. After a second 5 min. incubation on the shaker table, embryos were slowly dehydrated in a 50% MeOH/ 50% PBS solution before being transferred into a 100% MeOH solution and stored at -20°C. Previously fixed embryos were rehydrated in accordance with Thisse and Thisse (2008).

Rehydrated embryos were suspended in a 75% glycerol/ 25% PBS solution overnight. The following day, embryos were mounted on a 1.0 mm thick microslide (VWR 48312-004) before covering the sample with a 1-ounce 22x22 mm microscope cover glass (VWR 16004-094). Images were taken using an Olympus IX81 inverted microscope. Images were then processed with Olympus cellSens Software.

Results

In our initial experiments, we faced trials and tribulations in the process of generating mutant fish via CRISPR/Cas9. Originally, we obtained a commercially available plasmid containing a Cas9 insert. The plasmid was linearized and transcribed into mRNA; however, the quality and concentration of the Cas9 mRNA obtained during each experiment varied. This resulted in inconsistent mRNA concentrations in our injections. In order to avoid inconsistencies introduced by in vitro transcription, we obtained a commercially available Cas9 protein (EnGen® Cas9 NLS (New England Biolab Mo646T)). In our initial test using EnGen® Cas9 NLS to assess the efficacy of microinjections using CRISPR, we used a known genomic sequence (Griffin et al. 1998) to design a sgRNA targeting the *tbx16* gene (Table 1). *tbx16* sgRNA and EnGen® Cas9 NLS were injected at a variety of concentrations (Table 2). At approximately 24 hpf, we recorded the number of living embryos and examined them for the spadetail phenotype (Fig. 2A, B). All clutches contained embryos displaying the spadetail phenotype (Table 2). Embryos that were injected with 50 pg/nl of sgRNA and 250 pg/nl of Cas9 protein (n = 195) showed the highest mutation rate (87.2%; Fig. 2G).

To further test the efficacy of commercially obtained Cas9 protein in the CRISPR/Cas9 system, we used a second known genomic sequence (Jao et al. 2013) to design an sgRNA targeting the *slc24a5* pigmentation gene (Table 1). *slc24a5* sgRNA and Cas9 protein were co-injected into single cell embryos at varying concentrations (Table 3). Between 36 and 48 hpf, embryos were examined for hypopigmentation in skin melanophores and retinal pigment epithelium (RPE) (Fig. 2C-F). All clutches contained embryos displaying hypopigmentation (Table 3). Again, embryos (n = 287) that were

injected with 50 pg/nl of sgRNA and 250 pg/nl of Cas9 protein showed the highest mutation rate (82.0%; Fig. 2G). The similar results in terms of efficacy observed using sgRNAs targeting the *tbx16* and *slc24a5* genes suggested that the coinjection of 50 pg/nl sgRNA and 250 pg/nl of commercially obtained Cas9 protein represented optimal conditions in our hands.

Discussion

In order to determine if commercially available Cas9 protein would yield efficient and timely mutagenesis results, we obtained Cas9 protein and in accordance with the zebrafish CRISPR injection protocol by Jeffrey Essner (2016), used 50 ng/ul sgRNA and 250 ng/µl Cas9 protein for CRISPR injections. After targeting *tbx16,* at approximately 24 hpf, the spadetail phenotype was observed (87.2%). The concentration of sgRNA was both increased and decreased in subsequent rounds of injections to determine which concentration resulted in the highest number of embryos exhibiting the mutant phenotype. These concentrations were also tested with *slc24a5* sgRNA injections. For each condition, 50 ng/µl sgRNA and 250 ng/µl Cas9 protein was most effective.

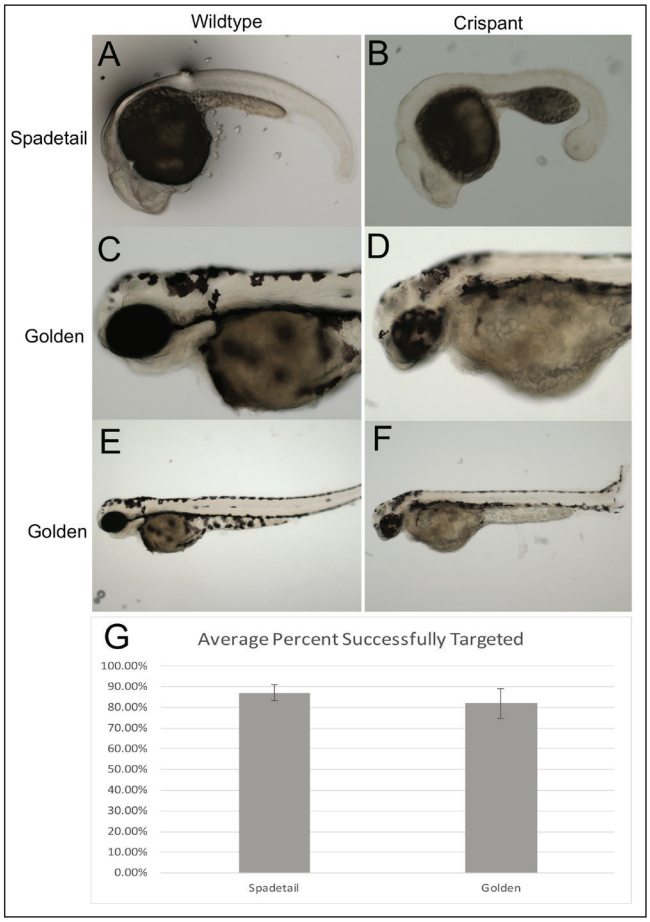

Figure 2. Mutant phenotypes in CRISPR injected embryos. (A, B) Targeting *tbx16* (A) wildtype control (B) targeting *tbx16* with Cas9 protein generates a mutant embryo with a phenotype displaying a broad posterior tail. (C-F) Targeting the pigmentation gene, *slc24a5* (C, E) wildtype control (D, F) targeting *slc24a5* with Cas9 protein generates a mutant embryo with a phenotype displaying mosaicism within the pigmented cells. (G) Observed mutagenesis rates and standard deviation across trials of *slc24a5* and *tbx16* when injecting 50 pg/nl of sgRNA and 250 pg/nl of Cas9 protein into single cell embryos.

In 2016, in order to compare the efficiency of *in vitro* assembled Cas9-sgRNA ribonucleoprotein complexes, Burger et al. (2016) assembled and injected Cas9-sgRNA RNPs into single celled zebrafish embryos. Burger et al. targeted *slc24a5* and compared their results to those results obtained by Jao et al. (2013), who injected single cell embryos with 50 pg of *slc24a5* sgRNA and 150 pg of *nls-zCas9-nls* RNA. At 48 hpf, mosaic hypopigmentation was observed in skin melanophores and RPE in 94% of the injected embryos and mutagenesis rates ranged from 78–98%, indicating that most cells contained bi-

allelic mutations. By comparison, when *slc24a5* was targeted with Cas9-sgRNA RNPs, nearly 80% of successfully injected embryo displayed strong mutant phenotypes. The remaining 20% of embryos displayed different degrees of the mutant phenotype (Burger et al. 2016). When the injected embryos were grown to adulthood, the mutant phenotype was preserved. The fish were then incrossed, resulting in the F1 offspring displaying complete mutant phenotypes, showing that when the *slc24a5* locus was targeted with Cas9-sgRNA RNPs, the germline of injected embryos was mutated (Burger et al. 2016). In addition to targeting *slc24a5,* Burger et al. also used RNPs to recapitulate loss of function phenotypes, like spadetail. Using RNPs to target *tbx16,* more than 60% of all embryos injected displayed a strong phenotype while almost 20% displayed a mild or partial phenotype. Analysis via MiSeq and CrispRVariants revealed that RNPs allow for efficient bi-allelic mutagenesis for both *slc24a5* and *tbx16* (Burger et al. 2016).

Cas9 and sgRNA concentration can be determined using a free online calculator, CrispantCal (available at http://lmweber.github.io/CrispantCal/). Burger et al. (2016) used the CrispantCal system and reported the most efficient concentration of Cas9 protein to be 831 ng/µl, while the optimal sgRNA concentration varied depending on the DNA sequence and the molar mass. Contrary to these results, our results showed the highest efficacy of all sgRNAs occurred at a concentration of 50 ng/ul sgRNA. While we did not test different concentrations of Cas9 protein, we showed that 250 ng/ul Cas9 was efficient. Burger et al. reported that 80% of injected embryos displayed strong mutant phenotypes

Table 2: Embryos Injected with tbx16 sgRNA + Cas9 Protein.

Concentration sgRNA/ Cas9 (pg/nl)	100/250	50/250		25/250		12.5/250	
Trial Number	1	1	2	1	2	1	2
Number injected	73	107	88	68	36	45	67
Number alive	36	92	72	58	28	30	54
Number with phenotype	8	78	65	43	20	13	26
% successfully targeted	22.2%	84.7%	90.3%	74.1%	71.4%	43.3%	48.1%
Average % successfully targeted	22.2%	87.2%		73.3%		46.4%	

Table 3: Embryos Injected with *slc24a5* sgRNA + Cas9 Protein.

Concentration sgRNA/ Cas9 (pg/nl)	50/250			25/250	
Trial Number	1	2	3	1	2
Number injected	93	105	89	58	96
Number alive	81	88	76	44	79
Number with phenotype	71	74	56	25	54
% successfully targeted	87.7%	84.1%	73.7%	56.8%	68.4%
Average % successfully targeted		82.0%		64.2%	

for *slc24a5* injections, while the remaining 20% showed mild phenotypes. In our tests, we saw 82% of embryos exhibiting the *slc24a5* phenotype. Using *tbx16* sgRNA, Burger et al. reported that about 80% of their injected embryos showed the mutant phenotype while our results indicated that 87.2% showed the mutant phenotype. Although our Cas9 protein was different from the protein utilized by Burger et al., our commercially obtained protein was effective at a comparable rate, while also proving to be a cost and time efficient alternative to generating our own Cas9 through *in vitro* translation.

Our results using EnGen® Cas9 NLS (New England Biolab Mo646T) show an average of 84.8% mutant phenotype using Cas9 protein. Combined, these results suggest that protein is not necessarily more efficient than Cas9 mRNA; however, *in vitro* assembled Cas9 provides an effective reagent that can be used for knockout and loss-of-function studies. Further, commercially obtained Cas9 protein eliminates the potential for RNA degradation, a common issue when storing RNA for extended periods.

The efficacy of our experiments suggests that biallelic zebrafish knockouts can be generated with purchased reagents, successfully mating zebrafish, and a general knowledge of injections and molecular biology. While our method efficiently generates biallelic knockouts, mutations of single alleles may be advantageous in some cases, such as when breeding and maintaining stocks with a recessive trait. Although monoallelic mutations were not confirmed in these experiments, it is important to note that a portion of the embryos displaying the wildtype phenotype may have monoallelic disruptions. However, because biallelic knockouts were commonly generated, these primary injected fish could be examined directly for phenotypes during experiments, as only homozygous *slc24a5* and *tbx16* mutants display phenotypes. The ability to generate biallelic knockouts could be beneficial because injection and phenotypic analysis can be completed in just a few days, and could eliminate the need for maintaining stable versions of well characterized transgenic lines.

Although the observation of phenotypes can be a quick process, when there are no known mutant phenotypes, screening injected fish can be a very tedious and laborious process. One potential way in which this process could be expedited is through the use of fluorescently tagged tracRNA. Hamimi et al. (2018) recently showed that by injecting fluorescently tagged tracRNA, sorting fluorescent embryos via stereomicroscope can begin as early as the 512-cell stage. Using this method, the process of identifying embryos with successfully edited genomes using CRISPR/Cas9 was significantly improved (Hamimi et al. 2018). Taken together, this approach, along with the microinjection apparatus described here makes zebrafish mutagenesis practical for labs with a limited budget, undergraduate students completing an honor's thesis and undergraduate teaching labs. Furthermore, the potential to construct a homemade micro-injector (Samaee et al. 2017), could make this even more cost effective.

Additionally, while this approach is efficient in generating mutagenesis and cost effective as described previously, it is time efficient as well. Time considerations are often a challenge in undergraduate teachings labs, institutes where research is done primarily by undergraduates who also have a heavy class load and even master's students who are on a shorter timeline yet often have significant class and teaching responsibilities. In these types of situations, significant troubleshooting can impact the amount of time it takes to complete a project and this can lead to failure to produce results within the allotted timeframe. In addition, normally the economical way to complete projects in a lab is by generating as many materials as possible; however, this does not take into account the cost of labor and the challenges this presents for students on a strict timeline. Furthermore, isolated labs are often unaware of alternative ways to complete experiments that are newer or seem to be

more expensive (i.e., CRISPR), but here we described multiple techniques to save time and cost while still reaching the same final outcome. In conclusion, the approaches we describe can save time and money while still displaying high rates of mutagenesis.

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