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Genome editing in Astyanax mexicanus using transcription activator-like effector nucleases (TALENs)

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Abstract:	Identifying alleles of genes underlying evolutionary change is essential to understanding how and why evolution occurs. Towards this end, much recent work has focused on identifying candidate genes for the evolution of traits in a variety of species. However, until recently it has been challenging to functionally validate interesting candidate genes. Recently developed tools for genetic engineering make it possible to manipulate specific genes in a wide range of organisms. Application of this technology in evolutionarily relevant organisms will allow for unprecedented insight into the role of candidate genes in evolution. Astyanax mexicanus (A. mexicanus) is a species of fish with both surface-dwelling and cave-dwelling forms. Multiple independent lines of cave-dwelling forms have evolved from ancestral surface fish, which are interfertile with one another and with surface fish, allowing elucidation of the genetic basis of cave traits. A. mexicanus has been used for a number of evolutionary studies, including linkage analysis to identify candidate genes responsible for a number of traits. Thus, A. mexicanus is an ideal system for the application of genome editing to test the role of candidate genes. Here we report a method for using transcription activator-like effector nucleases (TALENs) to mutate genes in surface A. mexicanus. Genome editing using TALENs in A. mexicanus has been utilized to generate mutations in pigmentation genes. This technique can also be utilized to evaluate the role of candidate genes for a number of other traits that have evolved in cave forms of A. mexicanus.
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TITLE: Genome editing in *Astyanax mexicanus* using transcription activator-like effector nucleases (TALENs)

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SHORT ABSTRACT:

Gene-targeting mutagenesis is now possible in a wide range of organisms using genome editing techniques. Here, we demonstrate a protocol for targeted gene mutagenesis using transcription activator like effector nucleases (TALENs) in *Astyanax mexicanus*, a species of fish that includes surface fish and cavefish.

LONG ABSTRACT:

Identifying alleles of genes underlying evolutionary change is essential to understanding how and why evolution occurs. Towards this end, much recent work has focused on identifying candidate genes for the evolution of traits in a variety of species. However, until recently it has been challenging to functionally validate interesting candidate genes. Recently developed tools for genetic engineering make it possible to manipulate specific genes in a wide range of organisms. Application of this technology in evolutionarily relevant organisms will allow for unprecedented insight into the role of candidate genes in evolution. *Astyanax mexicanus* (*A. mexicanus*) is a species of fish with both surface-dwelling and cave-dwelling forms. Multiple independent lines of cave-dwelling forms have evolved from ancestral surface fish, which are interfertile with one another and with surface fish, allowing elucidation of the genetic basis of cave traits. *A. mexicanus* has been used for a number of evolutionary studies, including linkage analysis to identify candidate genes responsible for a number of traits. Thus, *A. mexicanus* is an ideal system for the application of genome editing to test the role of candidate genes. Here we report a method for using transcription activator-like effector nucleases (TALENs) to mutate genes in surface *A. mexicanus*. Genome editing using TALENs in *A. mexicanus* has been utilized to generate mutations in pigmentation genes. This technique can also be utilized to evaluate the role of candidate genes for a number of other traits that have evolved in cave forms of *A. mexicanus*.

INTRODUCTION:

Understanding the genetic basis of trait evolution is a critical research goal of evolutionary biologists. Considerable progress has been made in identifying loci underlying the evolution of traits and pinpointing candidate genes within these loci (for example[1-3]). However, functionally testing the role of these genes has remained challenging as many organisms used for studying the evolution of traits are not currently genetically tractable. The advent of genome editing technologies has greatly increased genetic manipulability of a wide range of organisms. Transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPRs) have been used to generate targeted mutations in genes in a number of organisms (for example[4-11]). These tools, applied to an evolutionarily relevant system, have the potential to revolutionize the way evolutionary biologists study the genetic basis of evolution.

Astyanax mexicanus is a species of fish that exists in two forms: a river-dwelling surface form (surface fish) and multiple cave-dwelling forms (cavefish). *A. mexicanus* cavefish evolved from surface fish ancestors (reviewed in[12]). Populations of cavefish have evolved a number of traits including loss of eyes, decrease or loss of pigmentation, increased numbers of taste buds and cranial neuromasts, and changes in behavior such as loss of schooling behavior, increased aggression, changes in feeding posture and hyperphagia[13-19]. Cavefish and surface fish are interfertile, and genetic mapping experiments have been performed to identify loci and candidate genes for cave traits[1,20-26]. Some candidate genes have been tested for a functional role in contributing to cave traits in cell culture[1,19], in model organisms of other species[21] or by overexpression[27] or transient knockdown using morpholinos[28] in *A. mexicanus*. However, each of these methods has limitations. The ability to generate mutant alleles of these genes in *A.*

mexicanus is critical for understanding their function in the evolution of cavefish. Thus, *A. mexicanus* is an ideal candidate organism for application of genome editing technologies.

Here we outline a method for genome editing in *A. mexicanus* using TALENs. This method can be used to evaluate mosaic injected founder fish for phenotypes and for isolating lines of fish with stable mutations in genes of interest[29].

PROTOCOL:

All animal procedures were in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Iowa State University and the University of Maryland.

1. TALEN Design

1.1. Input desired target sequence to a TALEN design website. (For example: https://tale-nt.cac.cornell.edu/node/add/talen). Input chosen spacer/repeat array lengths.

1.1.1. Copy the genomic sequence into the box labeled "Sequence."

1.1.2. Within the "Provide Custom Spacer/RVD Lengths" tab select the spacer length and array length. Note: Spacer lengths of 15 base pairs and repeat array lengths of 15-17 work well and make assembly less complex.

1.2. Select a TALEN pair. TALEN pairs designed around a unique restriction enzyme site allow for genotyping by restriction enzyme digesting a PCR product.

1.3. Design primers to amplify the genomic region surrounding the TALEN target site using a website such as Primer3[30-32]. When genotyping with a restriction enzyme, design primers to amplify a region that contains the restriction enzyme site only once. It is recommended that this region is amplified and sequenced prior to TALEN construction to identify any polymorphisms present in the *A. mexicanus* lab population to be used for microinjection.

2. **TALEN assembly (Modified from the TALEN kit protocol)[33].** For additional details and troubleshooting, see the protocol[34].

2.1. Prepare and sequence necessary plasmids from the TALEN kit according to the manufacturer's instructions.

2.2. Set up the #1 reactions for reactions A and B. Include repeat-variable diresidues (RVDs) 1-10 and the destination vector pFUS_A in Reaction A. Include RVDs 11-(N-1) and the destination vector pFUS_B(N-1) in Reaction B where N is the total number of RVDs in the TALEN.

2.2.1. Add to each reaction: 1 µL of each plasmid containing each RVD (100 ng/µL), the destination vector (100 ng/µL), 1 µL *Bsa*I restriction enzyme, 1 µL Bovine Serum Albumin (BSA) (2 mg/mL), 1 µL ligase, 2 µL of 10x ligase buffer and X µL of water for a total volume of 20 µL. For example, see Table 1. Note: Half reactions can also be used.

2.2.2. Place reactions in a thermocycler and run the cycle: 10x(37°C/5 min + 16°C/10 min) + 50°C/5 min + 80°C/5 min.

2.3. Incubate the reactions with nuclease. To each reaction add 1 µL 25 mM ATP and 1 µL nuclease. Incubate reactions at 37°C for 1 hour.

2.4. Transform reactions.

2.4.1. Transform 2.5 µL of each reaction into 25 µL chemically competent cells. Note: Homemade competent cells can be used. However, cells with low competence can result in lack of colonies.

2.4.1.1. Mix 2.5 µL of the reaction with 25 µL of chemically competent cells. Incubate on ice for five minutes. Incubate the cells for 30 seconds at 42°C.

2.4.1.2. Place the tubes on ice for 2 minutes. Add 125 µL of Super Optimal broth with Catabolite repression (SOC). Shake the tubes at 37°C for 1 hour.

2.4.2. Plate 100 µL of the transformed cells onto LB plates with spectinomycin (50 µg/mL), X-gal and Isopropyl β-D-1-thiogalactopyranoside (IPTG). Grow overnight at 37°C.

2.5. Pick 2-3 white colonies for each reaction and check by colony PCR using primers pCR8_F1 and pCR8_R1 (Table 2).

2.5.1. Make a master mix of the reagents. For example, see Table 3.

2.5.2. Pick a colony and smear it into the bottom of a PCR tube, and then put the remains of the colony into 2 mL LB with spectinomycin (50 µg/mL). Place 15 µL of the master mix into the tube with the colony.

2.5.3. Run the following PCR program (Table 4)

2.5.4. Check the PCR (run the entire volume) on a 1.5% agarose gel by electrophoresis. The correct clones will have a band at the expected size as well as a smear and a ladder of bands. For an example of the appropriate smear, see[34,33].

2.5.5. Grow 2 mL cultures of the correct clones in LB media overnight at 37°C in a shaking incubator.

2.6. Miniprep the plasmids according to the manufacturer's instructions.

2.7. Sequence to check TALEN sequence with pCR8_F1 and pCR8_R1. Follow previously described methods[35].

2.8. Using the correct clones verified by sequencing, set up the reaction #2 (TALEN kit), which will place the RVDs from the A and B vectors and the final RVD into the destination vector.

2.8.1. Prepare mix for reaction 2 (Table 5). Note: Half reactions can be used.

2.8.2. Place the reactions in a thermocycler and run the following program: 37°C/10 min + 16°C/15 min + 37°C/15 min + 80°C/5 min.

2.9. Transform reactions.

2.9.1. Transform 2.5 µL of each reaction into 25 µL chemically competent cells.

2.9.1.1. Mix 2.5 µL of the reaction with 25 µL of chemically competent cells. Incubate on ice for five minutes. Heat shock the cells for 30 seconds at 42°C.

2.9.1.2. Place the tubes on ice. Add 125 µL of SOC. Place the tubes in a shaking incubator at 37°C for 1 hour.

2.9.2. Plate 100 µL of the transformed cells onto LB plates with ampicillin (100 µg/mL), X-gal and IPTG. Grow overnight at 37°C.

2.10. Pick 1-3 white colonies for each reaction and check by colony PCR using primers TAL_F1 and TAL_R2 (Table 2).

2.10.1. Make a solution of the polymerase mastermix, water and primers as described in Table 3. Pick a colony and smear it into the bottom of a PCR tube, and then put the remains of the colony into 2 mL LB with ampicillin (100 µg/mL). Place 15 µL of the solution into the tube with the colony.

2.10.2. Run the PCR program (Table 6).

2.10.3. Check the PCR on a 1.5% agarose gel by electrophoresis. The correct clones will have a smearing and a ladder of bands. For an example of the appropriate smear, see[34,33].

2.10.4. Grow 2 mL cultures of the correct clones in LB media overnight at 37°C in a shaking incubator.

2.11. Miniprep the plasmids according to the manufacturer's instructions.

2.12. Sequence to check TALEN sequence with TAL_F1 and TAL_R2 following previously described methods[35].

3. mRNA transcription of TALENs

3.1. Digest 4 µg of sequence-verified template with 2 µL *Sac*I for 2 hours at 37°C.

3.2. Run 2 µL of the *Sac*I-digested plasmid on a 1.5% agarose gel by electrophoresis. Plasmids that are correctly digested will display a single band.

3.3. Purify the remaining *Sac*I-digested plasmid following the PCR purification kit protocol. Wash twice with the wash solution prior to elution. Elute into 30 µL of nuclease-free water.

3.4. Follow the standard protocol for T3 mRNA production.

3.4.1. Set up half reactions using 0.5 µg of linearized template (prepared above). Incubate at 37°C for 2 hours.

3.4.2. Add 0.5 µL of DNase (included in kit) and incubate at 37°C for 15 minutes.

3.5. Purify the mRNA, following the manufacturer's instructions. Elute into 30 µL nuclease-free water.

3.6. Run a 1.5% agarose gel by electrophoresis to check the mRNA.

3.6.1. Clean the gel apparatus with a product to eliminate RNase contamination and prepare a 1.2% gel.

3.6.2. Mix 1 µL mRNA + 4 µL nuclease-free water + 5 µL glyoxyl loading dye. Incubate samples at 50°C for 30 minutes. Centrifuge the tubes briefly and place on ice before running the gel.

3.7. Check the concentration using a method of quantifying nucleic acid concentrations and store the RNA in aliquots at -80°C. Choose an aliquot size such that RNA is not frozen/thawed more than once.

Note: Concentrations of 500-1000 ng/nL are typically obtained. RNA with a lower concentration can be used as long as RNA integrity is maintained (as assessed by a band rather than a smear on the gel).

4. Inject *Astyanax mexicanus* embryos with TALEN mRNA

4.1. Prepare tools for injection.

4.1.1. Pour injection plates by pouring 1.2% agarose in fish water (water conditioned with sodium bicarbonate and sea salt to pH 7.4 and conductivity 700 µS) into a petri dish. Place a mold (plastic piece with projections to make wells for fish eggs) inside the dish. Remove the mold when the agarose has hardened and store at 4°C. For details on the mold see[36].

4.1.2. Pull needles for injection using a needle puller according to manufacturer's instructions.

Note: Appropriate needle length is important for injections. Needles that are too long will be too flexible for injections. The protocol for pulling needles will vary with the equipment used to pull needles. An example of an appropriate needle is shown in Figure 1. The approximate needle diameter can be determined using the scale bar provided.

4.1.3. Prepare glass pipettes for embryo transfer by breaking the pipette so that the opening is large enough for an egg to pass through. Flame the broken end until it is no longer sharp. Note: It is important to use glass pipettes and glass bowls when working with *A. mexicanus* eggs and embryos as they are sticky and will adhere to plastic.

4.2. Collect 1-cell stage eggs.

4.2.1 Breed *A. mexicanus* following standard protocols[37]. Note: For example, if fish are maintained on a 14 light:10 dark cycle and using Zeitgerber time (ZT) with ZT0 as lights on and ZT14 as lights off, our surface fish spawn between ZT15 and ZT19. Exact spawning time must be determined for each individual lab.

4.2.2 Induce spawning by overfeeding fish for 3-4 days prior to mating and placing fish into fresh water. Raise the temperature 2°F. Note: Our initial water temperature is approximately 72°F.

4.2.3 Collect surface fish eggs in the dark, checking every 15 minutes to obtain eggs at the 1 cell stage. Hundreds of eggs can be obtained from a single pair of surface fish.

4.2.4 Collect eggs in glass bowls to prevent sticking to plastic surfaces and sort to isolate embryos at the 1 cell stage prior to injection by observing eggs under the microscope and collecting eggs that are a single cell. Keep eggs in fresh system water (tank water in which adult fish are housed, which has been treated for pH and conductivity).

4.3. Inject TALEN mRNA.

4.3.1. Inject different amounts of total mRNA (equal amounts of each TALEN in the pair) to determine the optimal concentration for injection as toxicity and efficiency vary by TALEN pair. Start by injecting concentrations of total mRNA that are 400-800 pg. Dilute and combine mRNA to desired concentrations for injecting 1.5 nL.

4.3.2. Load diluted mRNA into the back of the needle and attach the needle to a micro-

injector.

4.3.3. Break the needle using forceps.

4.3.4. Calibrate the needle. For example, eject 10 times and collect the resulting drop in a micro capillary. For 10x100 disposable 1.0 µL, 32 mm micro capillary, the drop should fill the micro capillary to 0.5 millimeters for 1.5 nL/1 injection. Adjust the injection time and pressure as needed.

4.3.5. Insert the needle into the single cell and inject the mRNA. Inject the mRNA directly into the cell, not into the yolk.

4.3.6. Collect injected embryos in glass bowls. Keep embryos at 23-25°C. Remove dead embryos (embryos that become cloudy and irregularly shaped) regularly for the first few days following injection. Record numbers of dead and deformed embryos from control (uninjected) and injected plates.

Note: Increased mRNA concentration can lead to increased toxicity and deformity/death of embryos. Thus, toxicity versus efficiency must be balanced to determine the best concentration of mRNA to inject.

5. Phenotype founder fish and evaluate TALEN efficiency

5.1. Sacrifice embryos according to institutional animal protocol. Note: We euthanize embryos by rapid chilling on ice.

5.2. Collect embryos into 0.8 µL PCR strip tubes using a transfer pipette. Note: Genotyping can be performed on individual embryos or pools of embryos.

5.3. Extract DNA.

5.3.1. Place embryos into 100 µL 50 mM sodium hydroxide (NaOH) and incubate at 95°C for 30 minutes, then cool to 4°C.

5.3.2. Add 1/10th volume (10 µL) of 1 M Tris-HCl pH 8.

5.4. Perform a PCR on the region using the primers designed in step 1.3 (see Tables 7 and 8 for sample protocols). For individual embryos 1 µL of DNA is sufficient for the PCR reaction.

5.5. Digest the resulting PCR product with the appropriate restriction enzyme and run a 1.5% agarose gel by electrophoresis.

5.5.1 For example, for genotyping the *oculocutaneous albinism 2 (oca2)* locus in injected embryos digest the PCR product using the restriction enzyme *Bsr*I by adding 0.5 µL of *Bsr*I

directly to 12.5 µL of the completed PCR reaction, incubating at 65°C for 2 hours. Run the undigested and the digested product on a gel. Restriction enzyme resistant bands (i.e., bands that do not digest) indicate that TALEN-induced mutations are present (Figure 2).

5.6. (Optional) Calculate percentage mutation rate by determining the percentage of uncut product by analyzing images of gels in Fiji[38] using the gel analysis tool to calculate the intensity value of each band as described previously[29].

5.7. Determine the sequence of mutant alleles by TA cloning the gel purified restriction enzyme resistant mutant band and sequencing clones.

5.7.1. Gel purify the restriction enzyme resistant mutant band following the manufacturer's instructions. TA clone the band following the manufacturer's instructions. Pick colonies and grow in 1.5 mL LB overnight at 37°C in a shaking incubator.

5.7.2. Miniprep cultures following the manufacturer's instructions. Send the DNA for sequencing.

5.7.3. Using a program such as ApE, align the mutant sequences to the wildtype sequence.

5.7.3.1. Copy and paste both sequences into ApE files. Choose the "Align Two Sequences" tool from the "Tools" menu.

5.7.3.2. Specify the two DNA sequences using the dropdown menus. Note: The reverse complement of the cloned sequence may need to be used depending on the direction the PCR went into the cloning vector. If the sequences do not align, repeat steps checking the "Rev-Com" box in the "Align DNA" box.

5.8. Evaluate founder fish for phenotypes at appropriate stage and using appropriate methods for the expected phenotype[29] (For example, Figure 3). Methods of phenotyping will be based on the expected phenotype for the gene targeted by the researcher using the protocol.

6. Screen for germline transmission

Note: *A. mexicanus* reach sexual maturity at 4-8 months.

6.1. Cross sexually mature founder fish to wild type fish.

6.2. Screen embryos or adult fish using methods in steps 5.1-5.7 (Figure 4). For adult fish, a piece of the tail can be clipped following anesthetization.

6.2.1 Anesthetize fish by submerging fish in a solution of tricaine (3-aminobenzoic acid ethyl ester) to reduce stress during fin clipping. Following fin clipping, allow fish to recover in fresh water. Fish recover rapidly; observe until they have recovered (are swimming normally).

REPRESENTATIVE RESULTS:

TALEN pair injections result in binding of the RVDs to specific DNA nucleotides and thus dimerization of *FokI* domains, resulting in double stranded breaks[39] which can be repaired through non-homologous end joining (NHEJ). NHEJ often introduces errors that result in insertions or deletions (indels). Indels can be identified by amplifying the region surrounding the TALEN target site and digesting the resulting amplicon with a restriction enzyme that cuts within the TALEN spacer region. Alleles without an indel will digest while alleles containing indels that change the restriction enzyme target sequence will not digest, producing a restriction enzyme resistant band (Figure 2).

TALEN injections can likely result in biallelic gene mutations in *A. mexicanus*[29]. Thus, some phenotypes may be assessed in founder fish. For example, we evaluated pigmentation in surface fish injected with TALENs targeting *oca2*, the gene hypothesized to be responsible for albinism in multiple albino populations of cavefish[1,28]. We found albino patches in *oca2* TALEN-injected fish not present in uninjected fish[29] (Figure 3).

For many experiments, it is desirable to have mutant lines of fish for evaluating phenotypes. Founder fish with transmitted mutant alleles can be identified by genotyping progeny from crosses of founder fish to wild type fish (Figure 4).

Figure and Table Legends:

Figure 1: Needle for injecting mRNA
Photograph of a micropipette prior to being broken used for injecting TALEN mRNA into single celled embryos.

Figure 2: TALEN efficiency for Oca2
306 bp PCR products from exon 9 of *oca2* in *Astyanax mexicanus* were examined for loss of the restriction enzyme site when different amounts of TALEN mRNA were injected[29]. The amplicon from a control embryo was digested while a portion of the amplicon was resistant to restriction digest in the pools of 10 TALEN injected embryos. Restriction enzyme digest resistant bands from embryos injected with TALEN mRNA targeted *oca2* have been shown to contain indels[29]. Note that increasing concentrations of mRNA injected results in increased TALEN efficiency (more undigested DNA). Lanes with "-" are undigested, lanes with "+" are digested with restriction enzyme.

Figure 3: Phenotyping founder fish for changes in pigmentation
A. Control uninjected surface *A. mexicanus*. B. Patch lacking melanophores in a founder surface fish injected with 400 pg TALEN mRNA targeting *oca2* (arrow).

Figure 4: Germline transmission of TALEN induced mutations
306 bp PCR products from exon 9 of *oca2* in *A. mexicanus* were examined for loss of the

restriction enzyme site in pools of 10 F_1 fish from an injected founder fish. The amplicon from a control embryo was digested while a portion of the amplicon was resistant to restriction digest in the pools of 10 F_1 embryos. Restriction enzyme digest resistant bands from *oca2* F_1s have been shown to contain indels[29]. Lanes with "-" are undigested, lanes with "+" are digested with restriction enzyme.

Table 1: Example reaction assembly A and B for a TALEN containing RVDs NH-NH-NG-HD-HD-HD-NG-HD-NG-HD-NG-HD-NH-NI-NG.

Table 2: PCR primers for colony PCR, from[34].

Table 3: Master mix for 100 µL (15 µL/reaction) for colony PCR 1.

Table 4: PCR program for colony PCR 1.

Table 5: Protocol for second assembly reactions.

Table 6: PCR program for colony PCR 2.

Table 7: Sample protocol for gene specific PCR.

Table 8: Sample PCR program for gene specific PCR.

DISCUSSION:

Great strides have been made in recent years towards understanding the genetic basis of the evolution of traits. While candidate genes underlying the evolution of a number of traits have been identified, it has remained challenging to test these genes *in vivo* due to the lack of genetic tractability of most evolutionarily interesting species. Here we report a method for genome editing in *A. mexicanus*, a species used to study the evolution of cave animals. Genetic mapping studies[1,21,23] and candidate gene approaches[19,40] have identified a number of candidate genes for the evolution of traits in the cave form of *A. mexicanus*. The recent publication of the cavefish genome[41] provides an additional powerful tool for identifying candidate genes for the evolution of cave traits. Testing the function of many of these candidate genes requires techniques to reduce gene expression. The only current option for studying reduced gene expression in *A. mexicanus* is by the use of morpholinos. However, morpholino gene knockdown is transient, limited to a few days post fertilization, and is not useful for studying traits in adult animals, such as behavioral differences between adult cave and surface fish like schooling[17], hyperphagia[19] and vibration attraction behavior[42]. Generation of loss of function alleles of genes, such as those that can be made using TALENs, will be critical for testing the role of candidate genes for these traits.

Methods have been developed for easy assembly of TALEN pairs[33] and the detailed protocol for this method is available[34]. This protocol was optimized for zebrafish use by Bedell *et al.*[7], using

a different final destination vector, pT3Ts-goldyTALEN (pT3TS-gT). This vector allows for transcription of TALEN mRNA for injection into single celled zebrafish embryos. We have used this modified assembly method, explained in detail here, to assemble and transcribe TALENs for injection into *A. mexicanus*. We found that when injected into single-celled surface *A. mexicanus* embryos, as described within this protocol, we could mutate *A. mexicanus* genes[29]. For future research on candidate genes not described in this protocol, sequences can be found in the cavefish genome[41] and used to identify TALEN target sites.

Critical for successful injections is high quality TALEN mRNA. Thus, checking for RNA quality by running a small amount of RNA on a gel prior to injection is important (Step 3.6). Other precautions for maintaining RNA integrity, such as freezing aliquots to avoid freeze thaws (Step 3.7), and maintaining sterile conditions by using clean water and RNAse-free tubes and tips during injections (Step 4), should be taken. An additional critical step to raising injected fish is cleaning out dead embryos following injection, as dead embryos can rapidly affect water quality. Thus, we remove dead embryos the morning following injections, and periodically for the next few days following injections to maintain healthy live embryos (Step 4.3.6).

TALEN mutagenesis in *Astyanax mexicanus* can be highly efficient; however, efficiency varies depending on the TALEN pair injected[29]. Increased mRNA concentrations can lead to increased toxicity and deformity and death of injected embryos. Thus, toxicity versus efficiency must be tested and balanced to determine the optimal concentration of mRNA to inject. For highly efficient TALEN pairs, phenotypes may be assessed in injected founder fish. For example, injection of TALENs targeting *oca2* resulted in albino patches in surface fish[29] (Figure 3). For other traits or genes, however, assessment of phenotypes in founder fish may be challenging due to subtly of the phenotype or low efficiency of the TALEN pair injected. Thus, for many applications it will be desirable to generate germline mutations in a gene of interest for analysis of the role of a candidate gene in a non-mosaic animal. Obtaining germline transmission of TALEN-induced mutations in *A. mexicanus* is possible[29] (Figure 4). Thus, this technique can be applied to evaluate other candidate genes.

A few limitations to performing genetic manipulations in *Astyanax mexicanus* exist at this time. Surface and cavefish breed in the dark, late in the night. In a laboratory where it is not possible to reverse the light dark cycle, researchers must come in late at night to perform injections, as it is critical to inject immediately after spawning. Additionally, it is important to collect surface fish embryos in the dark, as light will affect spawning.

Other techniques, in particular the CRISPR/Cas system (reviewed in[43]), exist for genome editing and will likely be applicable to *A. mexicanus.* Indeed, protocols for guide RNA assembly exist that are rapid and easy[44] and the protocol reported here can be modified for CRISPR/Cas injection. Additionally, new applications for genome editing are rapidly being developed, and many of these may prove useful for *A. mexicanus* researchers. For example, in zebrafish precise mutations have been made in a gene of interest by coinjecting TALENs with a single stranded oligo containing a mutation[7]. This technique could be useful for evaluating the role of cave alleles in generating cave phenotypes, such as the role of a missense mutation in certain cave

alleles of *mc4r* in differences in metabolism observed between cavefish and surface fish[19]. TALENs have also been used to generate alleles of genes via homologous recombination that express fluorescent markers in patterns similar to the endogenous loci[45]. These methods could be used in *A. mexicanus* to evaluate subsets of cells or expression of candidate genes. The CRISPR/Cas system has been used in zebrafish to obtain tissue-specific gene knockout[46]. These techniques, applied to *A. mexicanus*, could be useful to evaluate the genetic basis of processes such as eye loss in cavefish. The lens plays a critical role in the process of eye loss in cavefish[47] and the tissue-specific CRISPR/Cas system could be used to evaluate the role of candidate genes for eye loss specifically in the lens versus other tissues of the eye. These and other genome-editing techniques can be utilized in *A. mexicanus* in future studies to answer critical questions about the evolution of cave traits.

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DISCLOSURES:

The authors have nothing to disclose.

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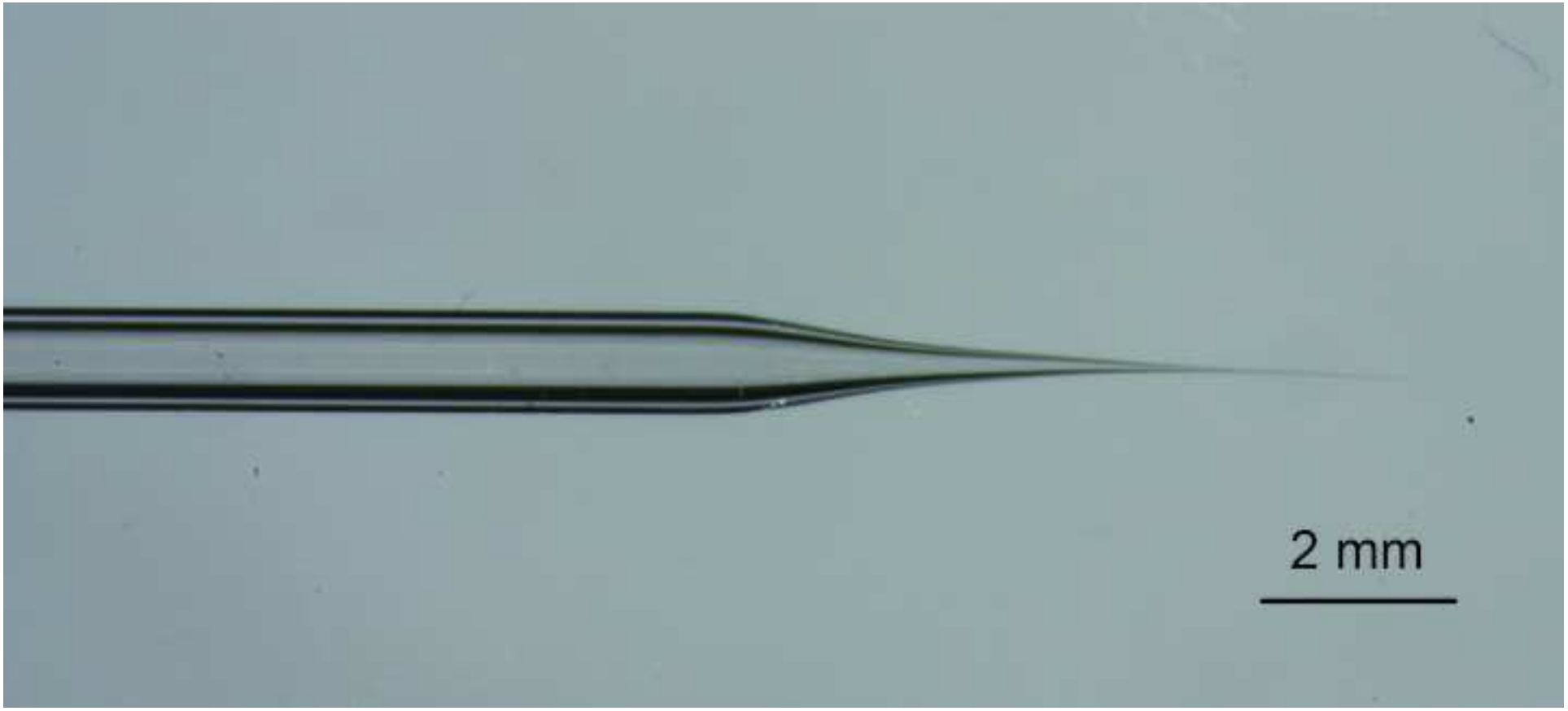

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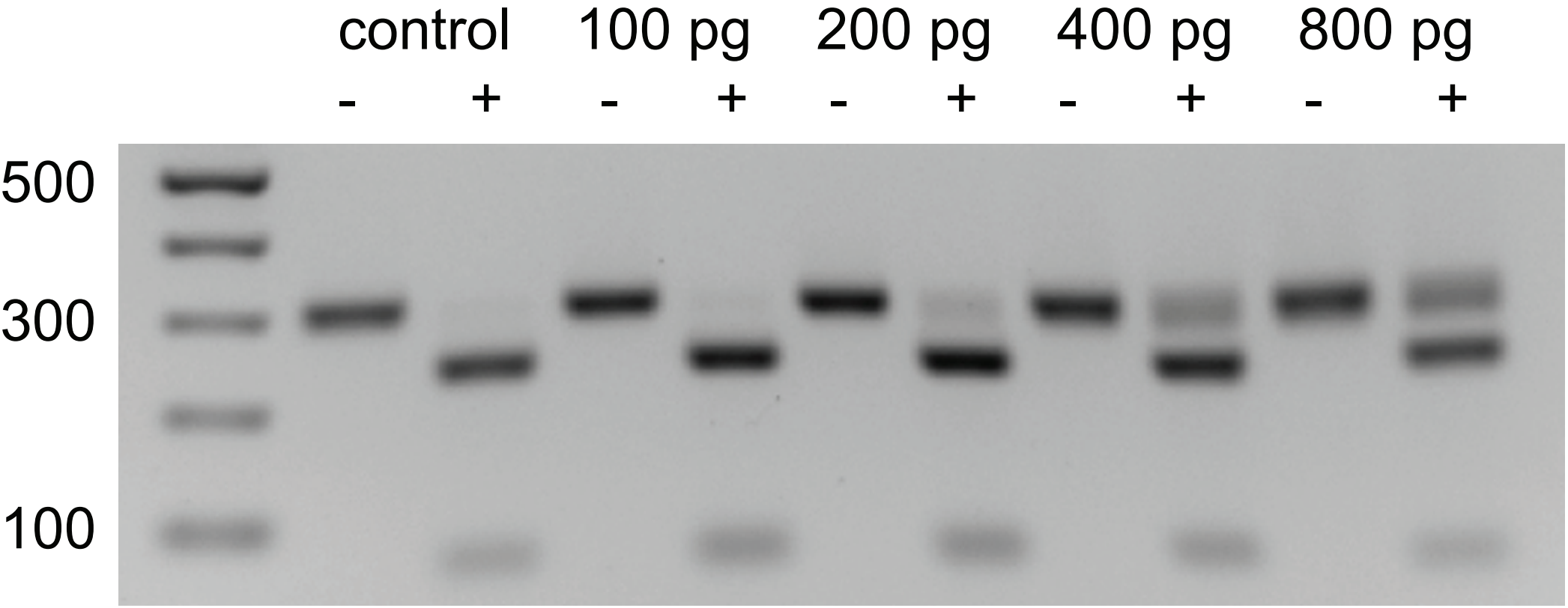

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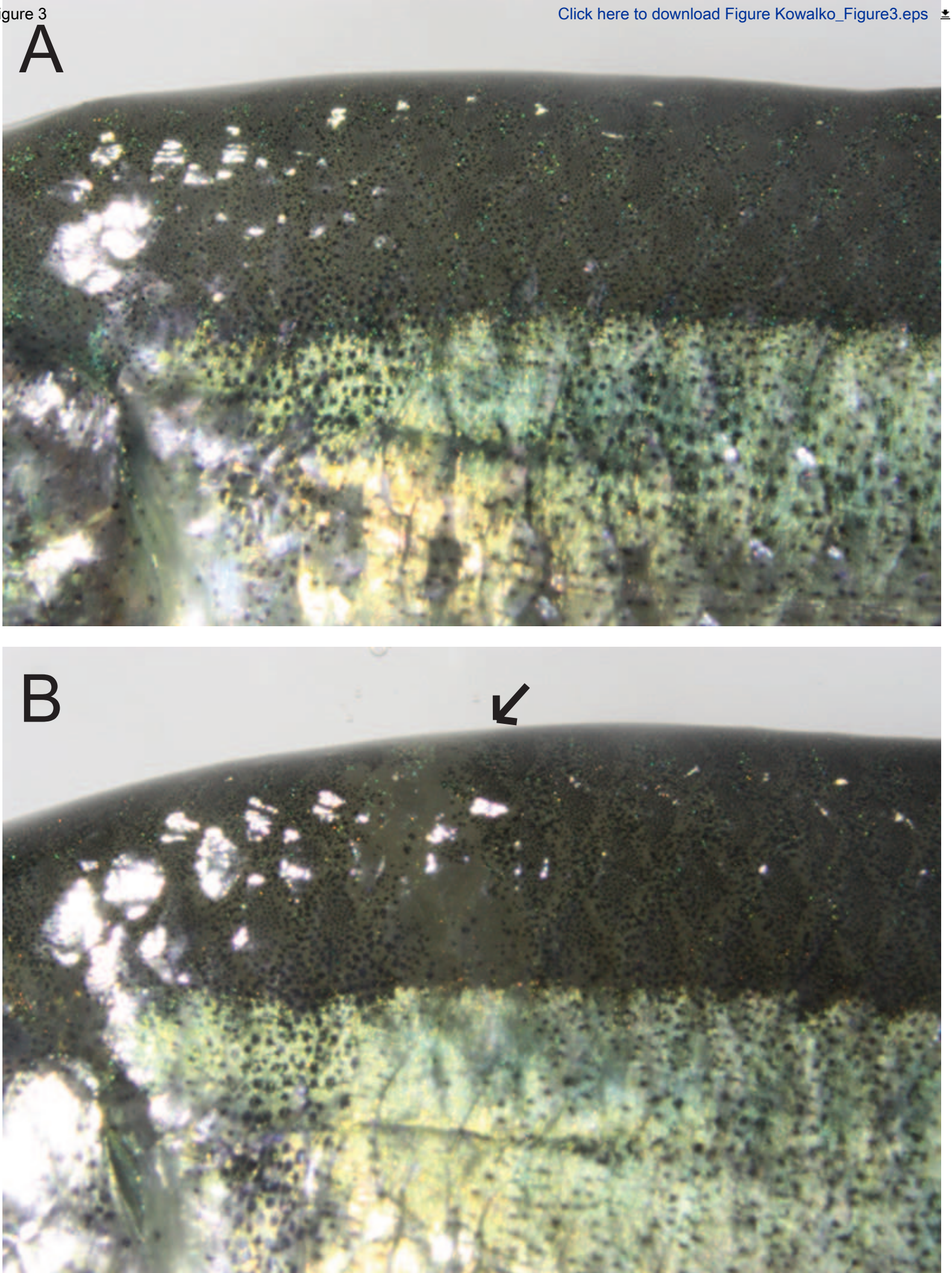

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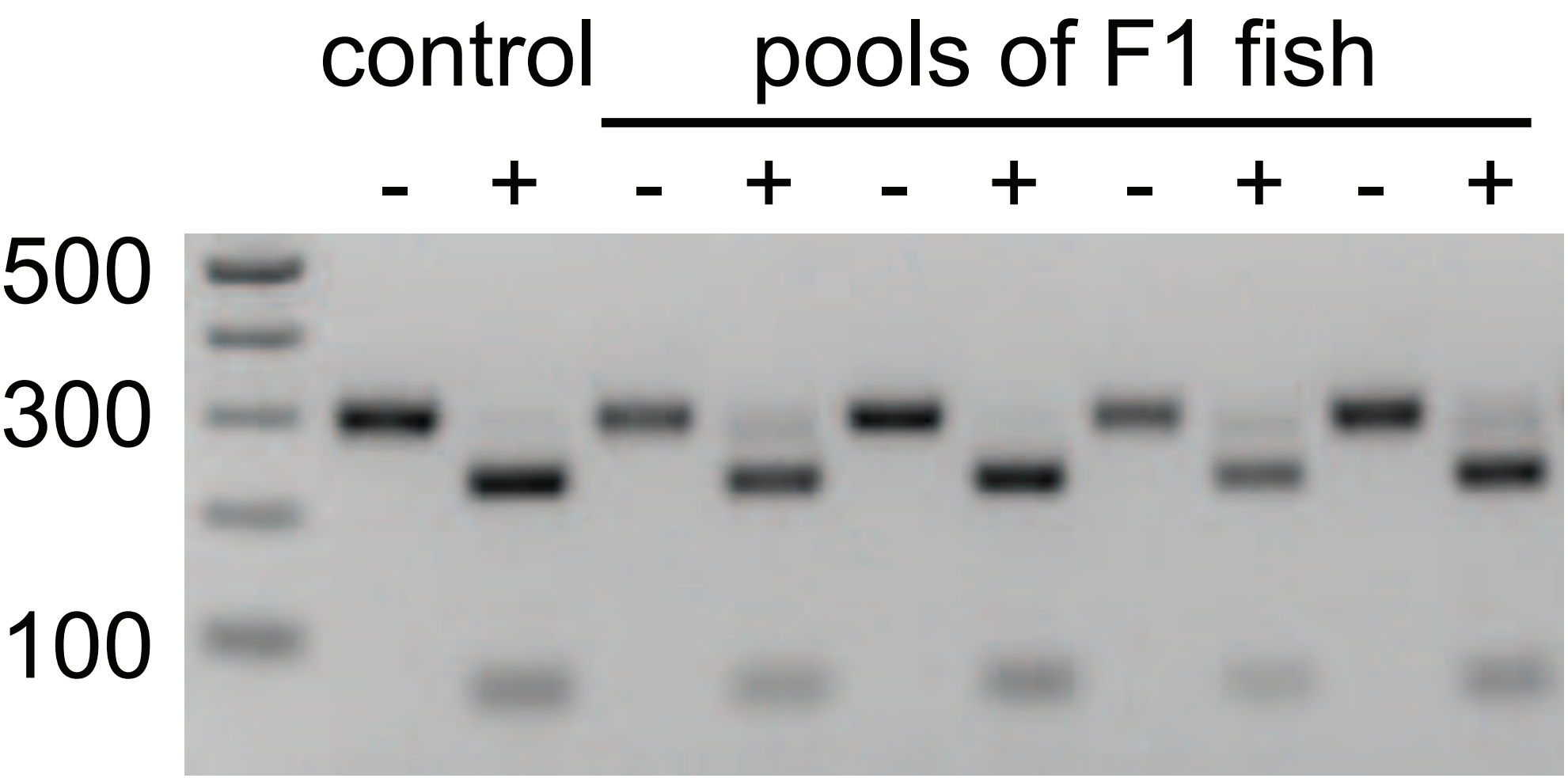

Table 1

Reaction A	
amount	reagent
4 uL	water
1 uL	pFUS_A
1 uL	BsaI
1 uL	BSA
1 uL	Ligase
2 uL	10x Ligase buffer
1 uL	pNH1
1 uL	pNH2
1 uL	pNG3
1 uL	pHD4
1 uL	pHD5
1 uL	pHD6
1 uL	pNG7
1 uL	pHD8
1 uL	pNG9
1 uL	pHD10

Reaction B	
amount	reagent
10 uL	water
1 uL	pFUS_B4
1 uL	BsaI
1 uL	BSA
1 uL	Ligase
1 uL	10x Ligase buffer
1 uL	pNG1
1 uL	pHD2
1 uL	pNH3
1 uL	pNI4

Table 2

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primer name	sequence (5'-3')
pCR8_F1	ttgatgcctggcagttccct
pCR8_R1	cgaaccgaacaggcttatgt
TAL_F1	ttggcgtcggcaaacagtgg
TAL_R2	ggcgacgaggtggtcgttgg

Table 3

reagent	amount
Taq masterm	50 uL
pCR8_F1 prin	4 uL
pCR8_R1 prin	4 uL
Nuclease-free	42 uL

*Adjust master mix if a different taq is used

Table 4

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step	temperature	time (seconds)
1	95	120
2	95	30
3	55	30
4	72	105
5	Go to step 2 for 30 cycles	
6	72	300

Table 5

amount	reagent	concentration
12 uL	water	
1 uL	vector A	100 ng/uL
1 uL	vector B	100 ng/uL
1 uL	destination v	50 ng/uL
1 uL	final RVD (pLI	100 ng/uL
1 uL	Esp3I	
1 uL	ligase	
2 uL	10x Ligase buffer	

Table 6

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step	temperature	time (seconds)
1	95	120
2	95	30
3	55	30
4	72	180
5	Go to step 2 for 30 cycles	
6	72	300

Table 7

reagent	amount
Taq masterm	12.5 uL
gene specific	1 uL
gene specific	1 uL
Nuclease-free	9.5 uL
DNA	1 uL

*Adjust master mix if a different taq is used

Table 8

step	temperature	time (seconds)
1	95	120
2	95	30
3	56	30
4	72	60
5	Go to step 2 for 35 cycles	
6	72	300

*adjust annealing temperature and extension time for specific primers and PCR product size

Excel Spreadsheet- Table of Materials/Equipment

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kowalko_materials_revised.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Equipment			
Thermocycler			
Injection station			
Gel apparatus			
Needle puller			
Nanodrop			
Supplies			
Note: As far as we know, supplies from different companies can be used unless otherwise indicated			
Golden Gate TALEN and TAL Effector Kit 2.0	Addgene	Kit #1000000024	
Fisher BioReagents LB Agar, Miller (Granulated)	Fisher	BP9724-500	
Fisher BioReagents Microbiology Media: LB Broth, Miller	Fisher	BP1426-500	
Teknova TET-15 in 50% EtOH	Teknova (ordered through Fisher)	50-843-314	
Spectinomycin Dihydrochloride, Fisher BioReagents	Fisher	BP2957-1	
Super Ampicillin (1000x solution)	DNA Technologies	6060-1	

ThermoScientific X-Gal Solution, ready-to-use	Thermo Sci Fermentas Inc (Ordered through Fisher)	FERR0941	
IPTG, Fisher BioReagents	Fisher	BP1620-1	
Petri dishes	Fisher	08-757-13	
Bsa I	New England Biolabs (ordered through Fisher)	50-812-203	Use *Bsa* I, not *Bsa* I-HF (as described in the Golden Gate TALEN and T
BSA	New England Biolabs	provided with restriction enzymes	
10x T4 ligase buffer	Promega (ordered through Fisher)	PR-C1263	
GoTaq Green Master mix	Promega (ordered through Fisher)	PRM7123	Other Taq can be used, but the reaction should be adjusted accordir
Quick ligation kit	New England Biolabs (ordered through Fisher)	50-811-728	We use Quick Ligase for all TALEN assembly reactions

One Shot TOP10 Chemically Competent E.coli	Invitrogen	C4040-06		Other chemically competent cells or homemade competent cells car
	Thermo Sci Fermentas Inc (Ordered through			
Esp 3I	Fisher)	FERER0451		
	Epicentre (Ordered through			
Plasmid-Safe ATP-dependent DNase	Fisher)	NC9046399		
QIAprep Spin Miniprep Kit	Qiagen		27106	The Qiagen kit should be used for the initial plasmid preparation (as
QIAquick PCR Purification Kit	Qiagen		28104	
GeneMate LE Quick Dissolve Agaraose	BioExpress	E-3119-125		
	Promega (Ordered through			
Sac I	Fisher)	PR-R6061		
mMESSAGE mMACHINE T3 Transcription kit	Ambion	AM1348M		
Rneasy MinElute Cleanup Kit	Qiagen		74204	
	Ambion (ordered through			
NorthernMax-Gly Sample Loading D	Fisher)	AM8551		
	Decon (ordered through			
Eliminase	Fisher)	04-355-32		

Fisherbrand Disposable Soda-Lime Glass Pasteur Pipets	Fisher	13-678-6B
Standard Glass Capillaries	World Precision Instruments	1B100-4
Microcaps	Drummond Scientific Company	1-000-0010
Eppendorf Femtotips Microloader Tips for Femtojet Microinjector	Eppendorf (ordered through Fisher)	E5242956003
Sodium hydroxide	Fisher	S318-500
Tris base	Fisher	BP152-1

TAL Effector Kit protocol)

ngly

ı be used

described in the Golden Gate TALEN and TAL Effector Kit protocol)

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Article Title: Genome editing in Astyanax mexicanus using transcription activator like effector nuclease (TALEN)

Signature: [signature] Date: 12/3/15

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Editorial comments:

1) All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor may have made minor copy edits to your manuscript and formatting changes to comply with the JoVE format. Please maintain these changes. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. ***Please use this updated version for any future revisions and track all changes using the track changes function in Microsoft Word****.*

2) In steps 2.5.5 and 2.10.4 - please specify what size cultures are grown and what media is used.

We have revised step 2.5.5 to "Grow 2 mL cultures of the correct clones in LB media overnight at 37°C in a shaking incubator." And step 2.10.4 to "Grow 2 mL cultures of the correct clones in LB media overnight at 37°C in a shaking incubator."

3) In step 5.3, please specify the PCR conditions and reaction set up used for your experiments.

We have included this information in Tables 7 and 8, and updated the protocol text to reflect this: "Perform a PCR on the region using the primers designed in step 1.3 (see Tables 7 and 8 for sample protocols). For individual embryos 1 µL of DNA is sufficient for the PCR reaction."

4) The highlighting was removed from the protocol section as several steps (e.g. 4.1.1, 4.1.2, 4.2 etc) were highlighted that do not contain sufficient detail for filming. All steps that will be filmed must have detailed step-wise instructions on how to perform the step - it is NOT sufficient to cite manufacturer's instructions or provide references for steps that will be filmed (it is ok to provide references/cite manufacturer's protocols for steps that will NOT be filmed). For example, Section 1 (TALEN design) does not need to be highlighted. Similarly, since portions of Section 2 (TALEN Assembly) rely on manufacturer's instructions/referenced sources, it is recommended not to film this section. Keeping these points in mind, please highlight 2.75 pages or less of text (which includes headings and spaces) to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

We have highlighted a portion of the protocol for filming. If the editors believe that additional sections should be filmed, we are happy to take suggestions on what is recommended for filming.

5) In the Discussion, please explicitly discuss the steps in the protocol that are critical to the success of your technique. Please also expand on the limitations of, and troubleshooting for, this technique.

We have included a discussion of critical steps, limitations and troubleshooting in the discussion, as outlined below.

"Critical for successful injections is high quality TALEN mRNA. Thus, checking for RNA quality by running a small amount of RNA out on a gel prior to injection is important (Step 3.6). Other precautions for maintaining RNA integrity, such as freezing aliquots to avoid freeze thaws (Step 3.7), and maintaining sterile conditions by using clean water and RNAse-free tubes and tips during injections (Step 4), should be taken. An additional critical step to raising injected fish is cleaning out dead embryos following injection, as dead embryos can rapidly affect water quality. Thus, we remove dead embryos the morning following injections, and periodically for the next few days following injections to maintain healthy live embryos (Step 4.3.6)."

"A few limitations to performing genetic manipulations in *Astyanax mexicanus* exist at this time. Surface and cavefish breed in the dark, late in the night. In a laboratory where it is not possible to reverse the light dark cycle, researchers must come in late at night to perform injections, as it is critical to inject immediately after spawning. Additionally, it is important to collect surface fish embryos in the dark, as light will affect spawning."

"Increased mRNA concentrations can lead to increased toxicity and deformity and death of injected embryos. Thus, toxicity versus efficiency must be tested and balanced to determine the optimal concentration of mRNA to inject."

6) Please print and sign the attached Author License Agreement, then scan and upload it with your manuscript files.

We have printed and included this in the manuscript files.

7) Formatting:
-Please define all abbreviations at their first use in the manuscript (RVD/BSA).

We have defined RVD in Step 2.2 and BSA in Step 2.2.1.

-Please include company/catalog number information for all items in the Materials/Equipment list (e.g., thermocycler). In addition, please check that the TALEN kit has been included in this table.

We have added the TALEN kit to the table. As any thermocycler should work for this protocol, we have not included ordering information for this product.

8) Grammar: 4.3.4-"fill the needle" or "fill the microcapillary"?

We have updated this to fill the micro capillary.

9) Additional detail is required:
-2.5.4-What volume of PCR product is used?

We have updated this step to include this information "Check the PCR (run the entire volume) on a 1.5% agarose gel by gel electrophoresis. The correct clones will have a band at the expected size as well as a smear and a ladder of bands. For an example of the appropriate smear, see[34,33]."

-4.1.1-What is the composition for fish water? In addition, if this step is to be filmed, please include a brief description of the mold.

We have updated this step to include the information:

*"*Pour injection plates by pouring 1.2% agarose in fish water (water conditioned with sodium bicarbonate and sea salt to pH 7.4 and conductivity 700 µS) into a petri dish. Place a mold (plastic piece with projections to make wells for fish eggs) inside the dish. Remove the mold when the agarose has hardened and store at 4°C. For details on the mold see[36]."

-4.1.2/4.3.3-What is the (approximate) needle diameter?

We have included a photograph of a needle in Figure 1 that should provide this information to readers. The back end diameter of the needle is based on the size of the glass capillaries purchased. We have provided the product number for this in the supplies table.

-4.2.2-What is the initial temperature (before the 2°F raise)?

We have included this information in this step: "Induce spawning by overfeeding fish for 3-4 days prior to mating and placing fish into fresh water. Raise the temperature 2°F. Our initial water temperature is approximately 72°F. Collect surface fish eggs in the dark."

-4.3.6-Are injected embryos kept at room temperature during this period?

We have included this information at this step: "Embryos are kept at 23-25°C."

-5.7.3.2-Is this step is to be filmed, please indicate which "drop down menus" must be clicked/how the two sequences are specified.

We have unhighlighted this step. If the editor suggests including it in filming, we will include this information.

-5.8-What is meant by "appropriate methods…phenotype"? Please clarify.

Methods of phenotyping will be based on the expected phenotype for the gene targeted by the researcher using the protocol.

10) Branding should be removed:
-2.2.1-BsaI-HF (registered trademark of NEB)
-Table 1/Table 5-Quick ligase

We have removed this from the text and the tables.

11) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

12) Please disregard the comment below if all of your figures are original. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

We are not reusing figures from a previous publication.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This is an important and timely manuscript on genome editing in A. mexicanus. This protocol will likely be of interest to those working in diverse species of fish, as well as the growing community studying Mexican cavefish. The authors have pioneered this approach, providing a method to manipulate the genome of this model system. I have minimal concerns about this manuscript and believe it is ready for publication. Minor suggestions/concerns listed below.

Major Concerns:
N/A

Minor Concerns:
-Is the format of the website references correct?

We have updated the format of the website references.

1.1. If multiple talen design websites are available, it may be useful to add a second.

We have only used the cited TALEN design website, and as such, cannot comment on other design websites.

4.2 More detail about the circadian time fish usually lay eggs would be helpful. Additionally, the number of animals one may expect.

We have included the time that our surface fish spawn as a reference, and noted that spawning time can vary between labs and should be determined. We have also updated the protocol to indicate number of eggs.

4.2.1 Breed *Astyanax mexicanus* following standard protocols[37]. For example, if fish are maintained on a 14 light:10 dark cycle and using Zeitgerber time with ZT0 as lights on and ZT14 as lights off, our surface fish spawn between ZT15 and ZT19. Exact spawning time must be determined for each individual lab.

4.2.2 Induce spawning by overfeeding fish for 3-4 days prior to mating and placing fish into fresh water. Raise the temperature 2°F. Our initial water temperature is approximately 72°F. Collect surface fish eggs in the dark. Hundreds of eggs can be obtained from a single pair of fish.

*-Some discussion of the sequenced genome (McGaugh et al, 2014) and how this could be used to facilitate the generation of TALENS may be helpful. [**Editorial recommendation:** The above comment may be addressed in the Discussion.]*

We have included this in our discussion in two places:

"The recent publication of the cavefish genome[41] provides an additional powerful tool for identifying candidate genes for the evolution of cave traits."

"For future research on candidate genes not described in this protocol, sequences in candidate genes can be found in the cavefish genome[41] and used to identify TALEN target sites."

5.1. My understanding is that fish embryos are not covered under IACUC protocols. If this is the case, the part about sacrificing the embryos should be removed.

We have included this line as some universities IACUCs cover embryos.

Additional Comments to Authors:
N/A

Reviewer #2:

Manuscript Summary:
I have read over the manuscript and felt that it was clearly written, that the background information is sufficient and that the steps were explained in detail. The figures help to understand some of the key steps and aid in the interpretation of gels. I think that the paper nicely provides a step by step guide of how the TALENs CRISPR procedures could be carried out on the Mexican cavefish.

Major Concerns:
N/A

Minor Concerns:
Page 3, 2., bracket missing after '_assembly_v7.pdf'

We could not identify this on page 3.

Page 8, 4.2: What is meant by 'fish system water'? Should it be aerated?

We have addressed this comment:

4.2.3 Collect eggs in glass bowls to prevent sticking to plastic surfaces and sort to isolate embryos at the 1 cell stage prior to injection by observing eggs under the microscope and collecting eggs that are a single cell. Keep eggs in fresh system water (tank water in which adult fish are housed, that has been treated for pH and conductivity).

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
The manuscript entitled "Genome editing in Astyanax mexicanus using transcription activator-like effector nucleases (TALENs)" by Kowalko, J., Ma, L. and Jeffery W.R. presented a new method to generate a genome-edited Mexican tetra, Astyanax mexicanus. It covers the design of TALEN targeting constructs, mRNA synthesis, microinjection with the embryos of A. mexicanus, and the genotyping method of the TALEN targeted site. These methods are significantly beneficial to the A. mexicanus community as well as the researchers who plan to use this new model animal. As for each procedure, it was well written and clear to understand except that I have some concerns in the following parts.

Major Concerns:
There is no major concern.

Minor Concerns:

1) at Line 270-273
Embryos can adhere to the fresh glass but not to the glass which is coated by proteins such as bovine serum albumin (BSA) or just gunk in the fish aquarium. Authors may change the sentence as read or similar:
"… Note: It is important to use the protein- or silicone-coated pipettes and bowls when working with Astyanax mexicanus eggs and embryos as they are sticky and will adhere to non-coated glass or plastic. For convenience, the protein-coated pipettes and bowls can be ready by pipetting or rubbing with the gunk containing water from fish aquarium."

We have not found that embryos stick to non coated glass. As such, we have not changed this line in the manuscript.

2) Line 284-286
For my experience of micro-injection, it would be difficult to correct 1 cell-stage embryos in the indicated technique. You might have to perform in vitro fertilization to acquire 1 cell-stage embryos. Alternatively, please provide the more details of how to find the surface fish embryos at 1-cell stage around Line 282 at the sentence "Collect surface fish eggs in the dark." For example, "Surface fish aquarium may be checked every 15 min and embryos should be recovered immediately after deposited.

We have not found it necessary to perform in vitro fertilization, but instead obtain eggs at the one cell stage by checking frequently. Thus, we have included this in the protocol step:

4.2.4 "Induce spawning by overfeeding fish for 3-4 days prior to mating and placing fish into fresh water. Raise the temperature 2°F. Our initial water temperature is approximately 72°F. Collect surface fish eggs in the dark, checking every 15 minutes to obtain eggs at the 1 cell stage. Hundreds of eggs can be obtained from a single pair of fish."

3) Line 301
"microcaps" should be "micro capillary"

We have updated this in the text in Step 4.3.4.