Journal of Visualized Experiments

Silencing the spark: CRISPR/Cas9 genome editing in weakly electric fish

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60253R2
Full Title:	Silencing the spark: CRISPR/Cas9 genome editing in weakly electric fish
Section/Category:	JoVE Biology
Keywords:	CRISPR/Cas9, electric fish, single cell microinjection, scn4aa, Electric Organ Discharge, genome modification, mormyrid, gymnotiform
Corresponding Author:	Jason Gallant Michigan State University East Lansing, MI UNITED STATES
Corresponding Author's Institution:	Michigan State University
Corresponding Author E-Mail:	jgallant@msu.edu
Order of Authors:	Jason Gallant
	Savvas J. Constantinou
	Linh Nguyen
	Frank Kirschbaum
	Vielka L. Salazar
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	East Lansing, MI

MICHIGAN STATE
UNIVERSITY

Wednesday, May 13, 2019

Dear Editors:

I am pleased to submit the enclosed manuscript to *The Journal of Visualized Experiments* entitled "*Silencing the spark: CRISPR/Cas9 genome editing in weakly electric fish*". This manuscript represents a significant milestone in our ongoing efforts to characterize the genetic mechanisms underlying electric organ evolution in weakly electric fish, which are an important emerging model in understanding the evolution of phenotypic diversity on our planet.

This manuscript outlines a full protocol for performing CRISPR/Cas9 mutagenesis that utilizes endogenous NHEJ repair mechanisms in weakly electric fish. We demonstrate this protocol is equally effective in both the mormyrid species *Brienomyrus brachyistius* and the gymnotiform *Brachyhypopomus gauderio* by using CRISPR/Cas9 to target indels and point mutations in the first exon of the sodium channel gene *scn4aa*. Using this protocol, we obtained embryos from both species and genotyped them to confirm that the predicted mutations in the first exon of the sodium channel *scn4aa* were present. The knock-out success phenotype was confirmed with recordings showing reduced amplitude electric organ discharge amplitudes when compared to uninjected size-matched controls.

Beyond the obvious impacts to the community of researchers who study electric fish, we feel that these results are worthy of publication in your journal, which has a broad readership, for the following reasons:

Methodological: Numerous researchers are interested in the editing the genomes of 'non-model systems', particularly teleost fishes. We hope that the methods outlined here will help other researchers develop protocols in their chosen model systems.

Broad Applicability of the Model System: Electric fish are a long-established model in systems neurobiology, and the development gene manipulation resources for electric fishes should greatly facilitate expansion of this system into developmental, and cell-biology approaches.

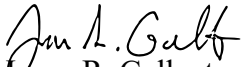
Genome to Phenome Studies in a Charismatic Phenotype: Much of the efforts to date connecting genome to phenome have focused on relatively similar phenotypes: chemicals, colors and morphology. To contrast, electric fish communicate using a modality that is invisible to our sensory system. Second, EODs are very simple *behaviors* with well-known anatomical and physiological substrates-- studies on other communication behaviors are lacking primarily because they are vastly more complex. Our ability to connect genotypic variation to a behavioral phenotype will be compelling to a wide swath of biologists.

For these reasons, I trust that the broad readership of *JOVE* will find this manuscript of great interest.

Department of
Integrative Biology
College of
Natural Science
Michigan State University
Natural Science Building
288 Farm Lane,
Room 203
East Lansing, MI
48824-1115
517/355-4640
FAX: 517/432-2789
integrativebiology@msu.edu
https://integrativebiology.natsci.msu.edu

On behalf of myself and my couauthors, I appreciate your time, dedication and care in reviewing this manuscript, and wish you well for the upcoming summer.

Sincerely,



Jason R. Gallant

Corresponding Author

Assistant Professor, Dept. Integrative Biology, Michigan State University

TITLE:

Silencing the Spark: CRISPR/Cas9 Genome Editing in Weakly Electric Fish

AUTHORS AND AFFILIATIONS:

Savvas J. Constantinou[1], Linh Nguyen[2], Frank Kirschbaum[2], Vielka L. Salazar[3], Jason R. Gallant[1]

[1]Department of Integrative Biology, Michigan State University, East Lansing, MI, USA
[2]Faculty of Life Sciences, Unit of Biology and Ecology of Fishes, Humboldt University, Berlin, Germany
[3]Department of Biology, Cape Breton University, Nova Scotia, Canada

Corresponding Author:

Jason R. Gallant (jgallant@msu.edu)

Email Addresses of Co-authors:

Savvas J. Constantinou (consta64@msu.edu)
Linh Nguyen (nguyenmdlinh12@gmail.com)
Frank Kirschbaum (frank.kirschbaum@staff.hu-berlin.de)
Vielka L. Salazar (Vielka_Salazar@cbu.ca)

KEYWORDS:

CRISPR/Cas9, electric fish, single cell microinjection, *scn4aa*, electric organ discharge, genome modification, mormyrid, gymnotiform

SUMMARY:

Here, a protocol is presented to produce and rear CRISPR/Cas9 genome knockout electric fish. Outlined in detail are the required molecular biology, breeding, and husbandry requirements for both a gymnotiform and a mormyrid, and injection techniques to produce Cas9-induced indel F_0 larvae.

ABSTRACT:

Electroreception and electrogenesis have changed in the evolutionary history of vertebrates. There is a striking degree of convergence in these independently derived phenotypes, which share a common genetic architecture. This is perhaps best exemplified by the numerous convergent features of gymnotiforms and mormyrids, two species-rich teleost clades that produce and detect weak electric fields and are called weakly electric fish. In the 50 years since the discovery that weakly electric fish use electricity to sense their surroundings and communicate, a growing community of scientists has gained tremendous insights into evolution of development, systems and circuits neuroscience, cellular physiology, ecology, evolutionary biology, and behavior. More recently, there has been a proliferation of genomic resources for electric fish. Use of these resources has already facilitated important insights with regards to the connection between genotype and phenotype in these species. A major obstacle to integrating genomics data with phenotypic data of weakly electric fish is a present lack of functional genomics tools. We report here a full protocol for performing CRISPR/Cas9 mutagenesis that

utilizes endogenous DNA repair mechanisms in weakly electric fish. We demonstrate that this protocol is equally effective in both the mormyrid species *Brienomyrus brachyistius* and the gymnotiform *Brachyhypopomus gauderio* by using CRISPR/Cas9 to target indels and point mutations in the first exon of the sodium channel gene *scn4aa*. Using this protocol, embryos from both species were obtained and genotyped to confirm that the predicted mutations in the first exon of the sodium channel *scn4aa* were present. The knock-out success phenotype was confirmed with recordings showing reduced electric organ discharge amplitudes when compared to uninjected size-matched controls.

INTRODUCTION:

Electroreception and electrogenesis have changed in the evolutionary history of vertebrates. Two lineages of teleost fish, osteoglossiformes and siluriformes, evolved electroreception in parallel, and five lineages of teleosts (gymnotiformes, mormyrids, and the genera *Astroscopus*, *Malapterurus,* and *Synodontis*) evolved electrogenesis in parallel. There is a striking degree of convergence in these independently derived phenotypes, which share a common genetic architecture[1-3].

This is perhaps best exemplified by the numerous convergent features of gymnotiforms and mormyrids, two species-rich teleost clades, which produce and detect weak electric fields and are called weakly electric fish. In the 50 years since the discovery that weakly electric fish use electricity to sense their surroundings and communicate[4], a growing community of scientists has gained tremendous insights into evolution of development[1,5,6], systems and circuits neuroscience[7-10], cellular physiology[11,12], ecology and energetics[13-17], behavior[18,19], and macroevolution[3,20,21].

More recently, there has been a proliferation of genomic, transcriptomic, and proteomic resources for electric fish[1,22-28]. Use of these resources has already produced important insights regarding the connection between genotype and phenotype in these species[1-3,28-30]. A major obstacle to integrating genomics data with phenotypic data of weakly electric fish is a present lack of functional genomics tools[31].

One such tool is the recently developed Clustered Regularly Interspaced Short Palindromic Repeats paired with Cas9 endonuclease (CRISPR/Cas9, CRISPR) technique. CRISPR/Cas9 is a genome editing tool that has entered widespread use in both model[32-34] and non-model organisms[35-37] alike. CRISPR/Cas9 technology has progressed to a point where a laboratory capable of basic molecular biology can easily generate gene-specific probes called short guide RNAs (sgRNAs), at a low cost using a non-cloning method[38]. CRISPR has advantages over other knockout/knockdown strategies, such as morpholinos[39,40], transcription activator-like effector nucleases (TALENs), and zinc finger nucleases (ZFNs), which are costly and time-consuming to generate for every target gene.

The CRISPR/Cas9 system functions to create gene knockouts by targeting a specific region of the genome, directed by the sgRNA sequence, and causing a double-stranded break. The double-stranded break is detected by the cell and triggers endogenous DNA repair mechanisms

preferentially using the non-homologous end joining (NHEJ) pathway. This pathway is highly error-prone: during the repair process, the DNA molecule will often incorporate insertions or deletions (indels) at the double-stranded break site. These indels can result in a loss of function due to either (1) shifts in the open reading frame, (2) insertion of a premature stop codon, or (3) shifts in the critical primary structure of the gene product. In this protocol, we utilize CRISPR/Cas9 editing to target point mutations in target genes using the NHEJ in weakly electric fish species. While simpler and more efficient than other techniques, this method of mutagenesis is expected to result in a range of phenotypic severities in F_0, which is attributed to genetic mosaicism[41-44].

Selection of Organisms

For the purposes of facilitating future studies on the comparative genomics of weakly electric fish, a representative species for both gymnotiforms and mormyrids for protocol development needed to be selected. Following discussions during the 2016 Electric Fish meeting in Montevideo, Uruguay, there was community consensus to utilize species that already could be bred in the laboratory and that had genomic resources available. The gymnotiform *Brachyhypopomus gauderio* and the mormyrid *Brienomyrus brachyistius* were selected as species that fit these criteria. In both species, natural cues to induce and maintain breeding conditions are easy to mimic in captivity. *B. gauderio*, a gymnotiform species from South America, has the advantage of low husbandry requirements: fish can be kept at relatively high density in relatively small (4 L) tanks. *B. gauderio* also has fast generational turnover under captive conditions. Under laboratory conditions, *B. gauderio* can develop from egg to adult in about 4 months.

B. brachyistius, a species of mormyrid fish from West-Central Africa, breeds readily in captivity. *B. brachyistius* is readily available through the aquarium trade, has been widely used in many studies, and now has a number of genomic resources available. Their life cycle spans 1−3 years, depending on laboratory conditions. Husbandry requirements are somewhat more intensive for this species, requiring moderately sized tanks (50−100 L) due to their aggression during breeding.

Laboratories studying other species of electric fish should be able to easily adapt this protocol as long as the species can be bred, and single cell embryos can be collected and reared into adulthood. Housing, larval husbandry, and in vitro fertilization (IVF) rates will likely change with other species; however, this protocol can be used as a starting point for breeding attempts in other weakly electric fish.

An Ideal Gene Target for Proof of Concept: scn4aa

Weakly electric mormyrid and gymnotiform fish generate electric fields (electrogenesis) by discharging a specialized organ, called the electric organ. Electric organ discharges (EODs) result from the simultaneous production of action potentials in the electric organ cells called electrocytes. EODs are detected by an array of electroreceptors in the skin to create high-resolution electrical images of the fish's surroundings[45]. Weakly electric fish are also capable of detecting features of their conspecifics' EOD waveforms[18] as well as their discharge rates[46], allowing EODs to function additionally as a social communication signal analogous to birdsong or frog vocalizations[47].

A main component of action potential generation in the electrocytes of both mormyrid and gymnotiform weakly electric fish is the voltage-gated sodium channel NaV1.4[2]. Non-electric teleosts express two paralogous gene copies, *scn4aa* and *scn4ab*, coding for the voltage-gated sodium channel NaV1.4[30]. In both gymnotiform and mormyrid weakly electric fish lineages, *scn4aa* has evolved rapidly and undergone numerous amino acid substitutions that affect its kinetic properties[48]. Most importantly, *scn4aa* has become compartmentalized in both lineages to the electric organ[2,3]. The relatively restricted expression of *scn4aa* to the electric organ, as well as its key role in the generation of EODs, makes it an ideal target for CRISPR/Cas9 knockout experiments, as it has minimal deleterious pleiotropic effects. Because weakly electric fish begin discharging their larval electric organs 6−8 days post fertilization (DPF), targeting of *scn4aa* is ideally suited for rapid phenotyping following embryo microinjection.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University.

1. Selecting sgRNA targets

NOTE: A protocol is provided for manual design of sgRNAs in step 1.1. This was utilized for *scn4aa* target selection. An additional protocol is provided to facilitate this process (step 1.2) using the EFISHGENOMICS web portal. It is advised that users select protocol 1.2, which features several automated 'checks' to ensure success in designing sgRNAs for custom targets.

1.1. Design sgRNA targets.

1.1.1. For generating sgRNA guide oligos, it is best to target exon 1, or other 5' exons. The 5' UTR can be targeted; however, it is best to target the 5' coding sequence. Utilizing genomic information is preferable, but it is possible to develop successful sgRNAs using transcriptome data. Annotations of intron/exon boundaries (genomic data) or exon/exon boundaries is preferable.

1.1.2. Candidate genomic sequences from 1.1 are searched for putative target sequences that match the pattern 5'-N(18)-NGG-3'. This can be automatically performed using desktop sequence analysis software, custom scripts, or through manual inspection of sequences.

1.1.3. Sequences can be prioritized using the method of Doench et al.[49] for on-target activity. Additionally, target sequences may be evaluated by a BLAST search against genomic/transcriptomic databases for off-target binding.

1.1.4. Ensure that standard PCR primers can be generated that flank the target sequence. Primers should be at least 20 bp from either side of the cut site (three bases upstream of the NGG sequence). Ideally, the PCR product should be 150−200 base pairs (bp).

1.1.5. Design oligomers meeting the above criteria using the below template, which includes a T7 promoter (5' of N_{18}) and a complementary region (3' of N_{18}) for annealing to the constant oligomer (1.1.6): 5'-TAATACGACTCACTATAGG-**N_{18}**-GTTTTAGAGCTAGAAATAGCAAG-3'

NOTE: The **N_{18}** sequence does not include the NGG protospacer adjacent motif (PAM) sequence. Do not include this in the oligomer.

1.1.6. Order the constant oligomer (**Table 1**) that will be used to synthesize all sgRNAs, oligomers for sgRNA targets (step 1.1.5), and PCR primers (step 1.1.4) as standard desalted oligomers from an oligonucleotide production service. Oligomers and PCR primers may be ordered as standard desalted oligomers, no additional purification is necessary.

1.2. Perform automated design of sgRNA targets.

1.2.1. Using genomic data, select a target gene. Transcriptome data could be used instead when exon/intron boundaries are known. Targets can be identified using the EFISHGENOMICS portal (http://efishgenomics.integrativebiology.msu.edu). The ideal target will be within exon 1; however, other 5' exons and the 5' UTR can be considered.

1.2.2. Once the target sequence is identified, load the freely available EFISHGENOMICS CRISPR web tool (http://efishgenomics.integrativebiology.msu.edu/crispr_tool/). This web tool uses a customized version of the CRISPOR algorithm for target generation[50,51].

1.2.3. In the box for **Step 1**, enter the name of the sequence and enter the target's sequence into the appropriate box.

1.3. Select the appropriate genome that the sequence derives from. Presently, genomic data is available for *B. brachyistius* and *B. gauderio*. Genome sequences are not required for use of this tool but are useful in assessing potential unwanted off-target effects.

1.4. Select the appropriate PAM. The 20 bp-NGG PAM is recommended, though there are several additional PAM motifs to choose from, depending on the Cas9 protein used.

1.5. A report will be generated with the location of the target sequence in the genome with suggested guide sequences. Select three targets with low predicted off-target effects, high specificity scores, and high efficiency scores. The highest scoring guide sequences are highlighted with green on the left-hand side. Yellow and red highlighted guide sequences should be avoided, if possible.

1.6. Clicking on selected target sequences generates a comprehensive CRISPOR report. Key information from these reports are for "T7 in vitro expression from overlapping oligonucleotides". From this report, extract the following information: recommended sgRNA oligos, PCR primers for the target site, and a constant oligomer that will be used to synthesize all sgRNAs.

1.7. Order the selected oligomers (step 1.6) from an oligonucleotide production service. Oligomers and PCR primers may be ordered as standard desalted oligomers, no additional purification is necessary.

2. Generate sgRNA

2.1. Anneal oligomers in PCR tube: add 1 µL of 100 µM constant oligomer, 1 µL of 100 µM sgRNA specific oligomer, and 8 µL of nuclease-free H_2O

2.2. Anneal oligomers in a thermocycler with the following program: heat at 95 °C for 5 min, cool to 85 °C at -2 °C/s, cool to 25 °C at 0.1 °C/s, and hold at 4 °C.

2.3. To generate sgRNA template, add 2.5 µL of dNTP mix (10 mM), 2 µL of 10x buffer, 0.5 µL of T4 DNA polymerase, and 5 µL of nuclease-free H_2O to the annealed oligomers from step 2.2. Incubate for 20 min at 12 °C.

2.4. Purify template using a PCR clean up column per the manufacturer's instructions.

2.5. Elute in 20–30 µL. Use a spectrophotometer to estimate concentration and purity. The template should be 100–200 ng/µL and 1.8–1.9 $A_{260/280}$.

2.6. Verify via an agarose gel by running 1–5 µL. The dominant band should be 120 bp. See **Figure 1A** for representative results.

2.7. Store gel-verified sgRNA template at -20 °C (long term) or 4 °C (short term, 1–4 weeks).

2.8. Transcribe sgRNA using T7 RNA transcription kit by adding to a 1.5 mL microcentrifuge tube at room temperature 8 µL of dNTP mix (equal volumes of dA, dT, dG, dC), 2 µL of 10x buffer (room temperature), 2 µL of T7 RNA polymerase, 100−200 ng of sgRNA template, and nuclease-free H_2O to a 20 µL total volume. Spin to collect at the bottom. Incubate for 2–4 h at 37 °C.

2.9. Add 1 µL of DNase and incubate an additional 15 min at 37 °C.

2.9.1. Clean up sgRNA by adding 1 µL of glycogen, 30 µL of nuclease-free H_2O, 30 µL of 5 M ammonium acetate and 180 µL of 100% EtOH to transcribed sgRNA. Mix well and incubate at least 20 min at -80 °C (until frozen) or at -20 °C overnight. Centrifuge at 4 °C for 15 min at maximum speed.

2.10. Remove and discard supernatant without disturbing the pellet, and wash with 1 mL of RNase-free 70% EtOH chilled at -20 °C. Spin an additional 5 min at maximum speed at 4 °C.

2.11. Remove and discard the supernatant without disturbing the pellet, and air-dry the pellet for 5 min.

2.12. Resuspend in 30 µL of nuclease-free H_2O and determine purity and yield using UV spectroscopy (minimum yield of 200 ng/uL).

2.13. Verify the presence of sgRNA on an RNase-free gel. The sgRNA appears between 50 and 150 bp as two bands due to the secondary structure (**Figure 1B**).

2.14. Aliquot into 3 µL and store at -80 °C.

3. Validate cutting efficiency in vitro

3.1. Extract genomic DNA from the target species using a commercial DNA extraction kit. Ventral fin clippings can be used without having to sacrifice the fish, because the tissues are regenerated.

3.2. Amplify the target DNA region using the primers designed as described in steps 1.6 and 1.7 using standard PCR. Verify the product by gel electrophoresis. Sequencing the fragment to ensure the proper region is being amplified is suggested, but not necessary. Representative results for the *scn4aa* template are shown in **Figure 2**.

3.3. Clean up the PCR fragment with an established laboratory or company protocol.

3.4. Store the amplified DNA at -20 °C. Consider separating it into aliquots to reduce freeze-thaw cycles.

3.5. Determine the required amounts and volumes of each component. Consider running negative controls (excluding either sgRNA or Cas9) and a positive control (a previously tested sgRNA that cleaves its target PCR amplified DNA) if available, as well as a concentration series of the sgRNA.

3.6. Set up the reaction mixture below in the specified order in a PCR tube at room temperature, adding the sgRNA and Cas9 protein last for best results: 50–100 ng of target DNA PCR product, 1 µL of 10x buffer 3, 1 µL of 10x BSA (0.1 g/mL), 50–200 ng of Cas9 protein (1 mg/mL, 150 ng suggested), and 30–200 of ng sgRNA (100 ng suggested).

3.6.1. Incubate the reaction at 37 °C for 1 h and then at 65 °C for 10 min to inactivate Cas9 protein.

3.6.2. Run the entire sample on a 2%–3% agarose gel (expected band sizes between 30–200 bp) along with positive and negative controls. Successful cleavage may show some of the PCR product but will also have two smaller bands. Representative results are shown in **Figure 2**.

4. Obtaining embryos

NOTE: Obtaining embryos of weakly electric fish can be challenging. Careful monitoring of water

quality, adequate time for fish care, and regular feeding are key to a successful breeding program. Fish must first be conditioned for several weeks for reproduction[52] as described in protocol step 4.1. Following this, a protocol augmenting natural gametogenesis (4.2) for use in natural spawning behavior (4.3), an alternative recently developed in vitro technique for obtaining precisely timed embryos (4.4) are presented. Protocol 4.3 is equally effective for *B. brachyistius* and *B. gauderio*, and protocol 4.4 is superior in *B. gauderio*.

4.1. Conditioning

4.1.1. Keep *B. brachyistius* in couples/small groups (100–150 L tanks) or in very large tanks (2 males and 5–6 females in approximately 475 L tank), as they become very aggressive under breeding conditions. There should be at least 1–2 PVC tubes per fish to be used as shelter (**Figure 3A**,**B**).

4.1.2. *B. gauderio* can be reared at much higher density. Keep up to eight individuals in a 100 L tank (2 males and 6 females). There should be at least 1 PVC tube per fish to be used as shelter. Adding tangled yarn increases enrichment and hiding spots. Add 50 mL centrifuge tubes with 1 cm diameter holes drilled into them to the top of the tank to allow adults to spawn naturally into the tubes (**Figure 3C**).

4.1.3. During the breeding season, feed fish daily fresh blackworms supplemented with frozen bloodworms. The food can be enriched with vitamins and supplements, if desired.

4.1.4. House fish normally in a relatively high conductivity (300–600 µS), pH balanced solution. During the breeding season, gradually lower the conductivity by at least half over the course of 1–3 weeks to induce gonad recrudescence and spawning. Lower conductivity by daily additions of reverse osmosis (RO) water, keeping close attention to pH when conductivity is low (pH <6).

4.1.5. Breeding conditions can be kept for around 3−5 months with egg production tapering off over time. After this time, return fish to high conductivity slowly over 1–3 weeks. Keep another 3 months at high conductivity before being exposed to breeding conditions again.

4.2. Use spawning agent (SGnRHa + Domperidone) injections.

4.2.1. Identify female fish in breeding conditions that appear gravid (**Figure 4**). In *B. gauderio* the female will have swollen gonads just caudal to the vent. *B. brachyistius* females will have swollen bellies and appear deep bodied (**Figure 4A**). Males generally do not have an issue producing sperm. However, larger males are preferred due to the larger sperm volume collected.

NOTE: Spawning agent is a commercial hormone mix that facilitates maturation of gametes and coordinates spawning. If the fish has been injected with spawning agent in the past, allow for >4 weeks of rest and ample feeding between injections. We suggest injecting at least 2 males and 4–5 females to ensure a few clutches of eggs and plenty of sperm.

4.2.2. Anesthetize fish in MS-222 (0.4 g/3 L) for a few minutes, until the fish is not able to keep its posture, is immobile, but continues to have opercular movement (Stage II[53]).

4.2.3. Weigh the fish (g) to calculate spawning agent amount, (0.5 µL/ g) + 0.5 µL. The extra 0.5 µL accounts for pipetting errors.

4.2.4. Add the spawning agent to 4x volumes of buffer (1x PBS or DPBS) in a PCR tube and mix well. The solution will become cloudy. It helps to dispense the spawning agent onto thermoplastic and then use a pipette to measure the calculated dose. The spawning agent is viscous, so be sure to dispense the entire dose and be careful with pipetting.

4.2.5. Pipette the injection solution onto thermoplastic and draw into a precision glass syringe, avoiding air bubbles. We recommend a 28 G, 19–25 mm length, beveled needle.

4.2.6. Inject the solution into the dorsal trunk muscle at a smooth rate. Let the needle sit for 2–4 s and then remove. Immediately put fish into fresh system water for recovery.

4.2.7. After approximately 24 h prepare for collection of embryos (see 4.3 or 4.4). Gather all necessary materials including what is needed for single cell microinjection (see section 5).

4.3. Obtain embryos through natural spawning.

4.3.1. House spawning agent-injected adults (4.2) in a large 450 L tanks. The most effective sex ratios have typically been 1–2 males and 3–4 females with adequate hiding places made from PVC tubes, and dark marble substrate on the tank bottom to prevent egg consumption. Marbles are typically more important for *B. brachyistius* than *B. gauderio*, which prefer to deposit their sticky eggs in crevices or 50 mL centrifuge tubes as described above.

4.3.2. Place fish in a reverse light cycle to match nighttime spawning to regular lab working hours. Using an off-the-shelf consumer grade security system, monitor fish using infrared illumination to minimize disturbance from a remotely connected PC (**Figure 3**).

4.3.3. The fish will spontaneously spawn during the dark photoperiod approximately 24 h post the spawning agent injection.

4.3.4. When spawning begins, collect eggs hourly while the spawning behavior occurs. In *B. brachyistius,* collect eggs using a small-diameter siphon over a fine cotton mesh net to minimize damage to the freshly spawned eggs. Collect *B. gauderio* eggs from 50 mL centrifuge tubes or tank substrate. Work efficiently with minimal disturbance to spawning fish by using a head lamp with a low-intensity red light. As first cleavage occurs approximately 1 h post fertilization (HPF), a large portion of eggs will be suitable for single cell microinjection.

4.3.5. Proceed immediately to microinjection (see section 5).

4.4. Squeeze males for in vitro fertilization of *B. gauderio*.

4.4.1. Prepare the sperm extender solution (SES) as described in The Zebrafish Book[54]: 10 mM HEPES, 80 mM KCl, 45 mM NaCl, 45 mM sodium acetate, 0.4 mM $CaCl_2$, and 0.2 mM $MgCl_2$.

4.4.1.1. Use ddH_2O to bring to volume and adjust pH to 7.7 with 1 M NaOH.

4.4.1.2. Store in fridge.

4.4.1.3. Filter through a 0.22 µm filter before use.

4.4.2. Anesthetize fish in MS-222 (0.4 g/3 L) for a few minutes, until the fish is not able to keep its posture, is immobile, but continues to have opercular movement (Stage II[53]). Place 500 µL aliquots of SES into ice.

4.4.3. Dry hands and fish thoroughly, especially around the head and vent. Place ventral side up, anterior to the left (for right-handed individuals) in a polystyrene foam/sponge holder covered in an MS-222 soaked, damp paper towel. The head should be as parallel with the table as possible.

NOTE: Steps 4.4.4–4.4.6 should be done as quickly as possible, and the fish should only be out of water for 30–60 s.

4.4.4. Apply pressure in the caudal to rostral direction with light squeezing medially over the gonads towards the vent. The fish may expel waste. Be careful to clean the vent with a delicate task wipe if any waste is expelled. Do not collect waste with sperm. Depending on the size of the male, 10-60 µL is common.

4.4.5. Using a micropipetter with a tip, carefully collect the sperm as it is squeezed from the male in 50 µL increments. Place sperm directly into 500 µL aliquots of SES on ice. It can be helpful to have another researcher assist in collecting sperm while the other squeezes. Sperm should be used as soon as possible, but remains viable for at least 1 h.

4.4.6. Immediately after collecting, put fish into fresh system water for recovery. The fish should only be out of water for 30–60 s.

4.5. Squeeze females for in vitro fertilization of *B. gauderio*.

4.5.1. Anesthetize fish in MS-222 (0.4 g/3 L) for a few minutes, until the fish is not able to keep its posture, is immobile, but continues to have opercular movement (Stage II[53]). Place polytetrafluoroethene sheet on workstation.

NOTE: Sections 4.5.1–4.5.5 should be done as quickly as possible and the fish should only be out of water for 30–60 s.

4.5.2. Dry hands, tools, polytetrafluoroethene sheet, and fish thoroughly, especially around the head and vent. Place on side with head facing anterior (for right-handed individuals).

4.5.3. Apply pressure in the caudal to rostral direction with light squeezing medially over the gonads towards the vent. The fish may expel waste. Be careful to clean the vent with a delicate task wipe if any waste is expelled. Depending on the size of the fish, 20–150 eggs is common. Using a polytetrafluoroethene coated spatula/tool carefully collect eggs as they are squeezed from the cloaca. Quickly move eggs to a small Petri dish and cover. Make sure the eggs remain dry during this process. Alternatively, after squeezing, touch the egg mass to the base of a small Petri dish or to the polytetrafluoroethene sheet as they are expelled from the female.

4.5.4. Immediately after collecting, put the fish into fresh system water for recovery.

4.6. Perform egg fertilization for in vitro fertilization of *B. gauderio*.

4.6.1. Add 100 µL of well-mixed sperm in SES (see step 4.4) directly over the egg mass.

4.6.2. Add 1 mL of system water (filtered through a 0.22 µm filter) and mix well for 30–60 s.

4.6.3. Add an additional 1–2 mL system water (leave some space in the Petri dish) and place in incubator. Record time of IVF on Petri dish and move to a 29 °C incubator. Set a 50 min timer to check progress of development (e.g., formation of the single cell at the animal pole, see **Figure 6**). During this time, prepare materials for microinjection.

5. Single cell microinjection

5.1. Pull microinjection needles prior to spawning agent injections (step 4.2) or natural spawning (step 4.3). Use a borosilicate glass capillary with a filament (O.D. 1.0 mm, I.D. 0.58 mm, 10 cm length) and a needle puller to pull microinjection needles. Prepare 2–4 needles per planned treatment injection.

NOTE: Backloading needles with a filament are preferred, because the filament wicks the solution towards the tip and eliminates bubbles. However, needles without a filament can be used and frontloading should have no effect on the outcome. The needle should have a long, but sturdy taper. Use the needle pulling program with the following specifications as a starting point: heat = 500; Pull = 70; Velocity = 70; and Time = 100. Representative needle morphologies are shown in **Figure 5A**.

5.2. Prepare injection solution and prepare needle.

5.2.1. Add 0.9 µL of sgRNA and 0.9 µL of Cas9 enzyme (1 mg/mL) to 0.2 µL of 10% or 100% phenol red (for 1% and 10% final phenol red concentration, respectively) in a PCR tube. Mix well and let sit on ice 2−5 min to allow sgRNA and Cas9 to complex. Calculate the final concentration of

sgRNA, Cas9, and phenol red in the injection solution.

NOTE: If there are issues with needle clogging or cutting efficiency using the above recipe, it may be useful to use a 1x final concentration salt/buffer mix to stabilize Cas9 and prevent needle clogging.

5.2.2. Use a microloader pipette tip to backload the 2 µl of solution into the microinjection needle. Expel the liquid as close to the tip as possible.

5.2.3. Load needle into micromanipulator and set pressure settings (start with 775 p_i, 0.1−0.2 s, and 8-12 p_c).

5.2.4. Under the scope, use a pair of fine forceps to break the needle tip at a beveled angle. Break towards where the taper of the needle starts to have rigidity. It is best to break closer to the tip, test the size of the injection solution and then break further if necessary. The bore of the needle should remain as small as possible while maintaining a rigid taper (**Figure 5A**).

5.2.5. Use mineral oil and a micrometer to measure the size of the injection solution. 1–2 nL is typically used; 0.1 mm diameter is 0.5 nL.

5.2.6. Adjust the needle tip or pressure settings to get an injection volume within specifications.

5.3. Identify developing zygotes and prepare injection stage. Set up the injection stage. Take a 100 mm diameter Petri dish. Invert the bottom and place under the inverted top. Place a glass microscope slide within the inverted top. Depending on how your micromanipulator is mounted to the scope, the added height from the inverted base may be unnecessary.

5.3.1. Look at the developing eggs and identify presumed fertilized zygotes 50–60 min after IVF. The animal pole will begin to form and there will be an excess of small fat droplets around the yolk/animal cell interface (**Figure 6**).

5.3.2. Collect 10–20 eggs with as little water as possible using a plastic transfer pipette (cut the tip slightly to allow eggs to pass) and place onto the edge of the slide. Water will be wicked under the slide and pull eggs against the edge.

5.3.3. Use a delicate task wipe and gently press the slide to remove excess water and allow the eggs to firmly adhere to the edge of the slide. There should be just enough water to keep the eggs moist while maintaining little standing moisture to avoid egg movement during injection.

5.4. Inject directly into single cell.

5.4.1. Align the eggs against the slide vertically, perpendicular to the approaching needle (**Figure 5B**).

5.4.2. Using the micromanipulator, position the needle against the chorion. At roughly a 45° angle, insert the needle into the chorion, and then into the single cell. It can be helpful to enter the single cell through the yolk. Moving both the injection stage with the free hand and the micromanipulator may provide more control over the injection process (**Figure 5B**).

5.4.3. Inject sgRNA/Cas9/phenol red solution into the single cell. Carefully remove the needle. Proceed to the next egg and repeat steps 5.4.1–5.4.3 until all eggs are injected.

5.4.4. Remove any eggs broken during injection with fine forceps. Use 0.22 µm of filtered system water in a squirt bottle to gently remove eggs from the injection stage into a new 100 mm diameter Petri dish.

5.4.5. Repeat steps 5.3.3–5.4.6 until all eggs have been injected or have developed to the two-cell stage. Make sure to set aside approximately 20 presumed fertilized eggs as a no-injection control to determine fertilization rates and injection success. For larger clutches use uninjected embryos that have developed to the two-cell stage, and for smaller clutches set aside presumed fertilized eggs.

5.4.6. Additionally, consider a sgRNA/injection control by injecting 15–25 embryos with Cas9 complexed with an sgRNA against a gene that is not present in the genome. The GFP gene recommended for wild type embryos. Record the number of eggs, parents, IVF time, and date on each Petri dish. Include sgRNA target or label as uninjected. Move to a 29 °C incubator.

6. Animal husbandry

6.1. Care for the eggs.

6.1.1. Check egg viability 4–6 h following injections.

6.1.2. Record the number of dead eggs, as well as any that are unfertilized. The unfertilized eggs will arrest around the eight-cell stage and have a rosette pattern of the cells at the animal pole (**Figure 6**). Depending on number of dead eggs and amount of egg debris in the water, remove at least 50%–80% of the water and replace with filtered system water. It can be helpful to scrub the bottom of the Petri dish if a cellular debris film or biofilm forms.

6.1.3. For the next 2–3 days, check eggs 1–2x daily. Repeat steps 6.1.2–6.1.3 each time the eggs are checked.

6.1.4. After 2−3 days the larvae will hatch. Remove all egg casings as they hatch. Gentle pipetting can help to free half-hatched larvae.

6.2. Care for larvae.

6.2.1. Check eggs 1−2x daily. Repeat steps 6.1.2–6.1.3 each time the larvae are checked.

6.2.2. From 6–14 DPF, feed vinegar eels to the larvae. A slight excess of food is good, because the vinegar eels will remain alive in the dish. Add vinegar eels each time the dish is checked and cleaned.

6.2.3. From 11–14 DPF, add 5–10 freshly hatched *Artemia* per larvae in addition to vinegar eels. During this time the larvae will learn to eat the free swimming *Artemia*.

6.2.4. At 15 DPF, move larvae to egg cups (see section 6.2.5) in a tank with flowing water, filtration, and aeration. There should be no more than approximately 25 embryos per cup. Egg cups are plastic cups with a mesh netting on the bottom. A 100 mm Petri dish top/bottom can be added to the bottom of the egg cup to help stop food from falling through the mesh. Egg cups allow the fish to be housed in a larger volume of water for water quality reasons, while maintaining discrete groups. Continue to add both vinegar eels and 15−30 freshly hatched *Artemia* per larvae from days 15–18. Clean Petri dish piece daily and use a pipette to remove masses of dead *Artemia*.

6.2.5. From days 18–30, feed only freshly hatched *Artemia*. Increase the feeding amount as the fish grow and if tail biting is seen.

6.2.6. After approximately 30 days, move fish to 10 L (2.5 gallon) tanks, approximately 25 individuals per tank. Make sure there is filtration, aeration, and places for hiding. Consider cylindrical biofiltration media and small diameter PVC tubes.

6.2.7. Feed freshly hatched *Artemia* and blackworms from approximately 30–45 days onwards. Maintain standard cleaning and water changes for the tank (i.e., at a minimum ~10%–20% water change per 1 week).

6.2.8. After ~45 DPF, feed only blackworms until ~60 DPF.

6.2.9. After ~60 DPF, move cohorts of approximately 15 fish to 40 L (10 gallon) tanks and begin adult husbandry procedures. Add PVC tubes and a yarn “mop” (a mass of brown yarn tied together around a cork) for hiding places (**Figure 3C**). Fish should be nearing breeding size (10−12 cm) after approximately 3−4 months post fertilization.

7. Adult husbandry

7.1. Feed fish daily with enough blackworms so that a small amount of blackworms are present at the next feeding. This ad libitum feeding allows maximal growth.

7.2. A few times a week, it can be helpful to supplement the blackworm feeding with bloodworms.

7.3. Clean tanks every 2–4 weeks with a 20%–30% water change. If the yarn mop gets full of

biofilm/algae, rub it clean under RO water.

REPRESENTATIVE RESULTS:

The sgRNA target sites were identified within exon 1 of *scn4aa* in both *B. gauderio* and *B. brachyistius* as described in Section 1. The sgRNAs were generated as described in Section 2. Following successful sgRNA selection and synthesis (**Figure 1**), in vitro cleavage was tested (**Figure 2**). The sgRNAs demonstrating in vitro cutting were then selected for single cell microinjections.

Adult fish were conditioned for reproduction (Section 4.1), then injected with a spawning agent (Section 4.2) and subsequently squeezed (*B. gauderio*) for IVF as described in Section 4.4 or allowed to spawn naturally (*B. brachyistius*) as described in Section 4.3. These efforts yielded single cell embryos for microinjection in both species. As described in Section 5, 1.5−2.0 nL of the *scn4aa* sgRNA/Cas9/phenol red complex (65-190 ng/uL sgRNA, 450 ng/uL Cas9, 1%−10% phenol red, final concentrations) was injected at the one-cell stage. Eggs from the same clutch were used as uninjected controls. All embryos were cared for as described in Section 6. Following IVF, 40%–90% of eggs were fertilized, and 70%–90% of embryos survived to hatching following injection.

About 75% of fish survived to 6−11 DPF and were then phenotyped. Larval fish were placed into a 35 mm Petri dish embedded in a larger dish with Sylgard immobilized Ag/Cl recording electrodes (**Figure 7A**). Embryo movement was restricted using 3% agarose molds made with system water and cut to fit the embryo (**Figure 7B**). The same recording chamber was used for both species and the same agarose mold was used among species comparisons. Embryos were recorded for 60 s, which is sufficient to capture hundreds of EODs. Age and size-matched uninjected controls were selected for comparison. At this time point, 10%–30% of surviving embryos show a reduced amplitude EOD. Embryos displaying a reduction in EOD amplitude with no obvious morphological defects and control uninjected whole embryos were digested for DNA extraction and subsequent PCR of the *scn4aa* target site. There was often a range of penetrance of the phenotype, with some individuals having a stronger reduction in EOD amplitude than others.

After PCR clean up and cloning, 30+ clones from each embryo were selected for Sanger sequencing. CRISPR/Cas9 induced mutations were identified in *B. gauderio* (**Figure 8A**,**B**) and *B. brachyistius* (**Figure 9A**,**B**) individuals with strong EOD amplitude reduction (**Figure 8C** and **Figure 9C**, respectively), where uninjected controls had only reference genotypes. Visualization of EOD amplitude between confirmed mutants ("CRISPR") and age/size matched uninjected controls demonstrated that both *scn4aa* mutant *B. brachyistius* (**Figure 10A**) and *B. gauderio* (**Figure 10B**) embryos had significantly lower EOD amplitude than controls ($p < 2.2 \times 10^{-16}$, Welch two-sample t-test). CRISPR/Cas9 targeting of *scn4aa* was successful in both *B. brachyistius* and *B. gauderio* and implicate *scn4aa* in the larval/early electrocyte discharge in both species.

FIGURE CAPTIONS:

Figure 1: sgRNA template synthesis and transcription. (**A**) Gel image of sgRNA template synthesis. Labels correspond to different sgRNAs for *myod* (MYO2, MYO1) and three sgRNAs for *scn4aa* (S1−S3). After annealing the oligomers, a ~120 bp template is produced. (**B**) Gel image of

sgRNA transcription for three sgRNAs for *B. gauderio* (bg2017) and two for *B. brachyistius* (bb2016, 2017). The sgRNA will appear as two bands due to secondary structure and will be between 50−150 bp when using a dsDNA ladder.

Figure 2: Representative gel image of successful (sg1) and unsuccessful (sg2) in vitro CRISPR assays. An equivalent amount of template without CRISPR components is shown in the scn4aa lane. Note the duplicate bands in sg1 that show that cutting has occurred.

Figure 3: Breeding tank setups for weakly electric fish. (**A**) Schematic of the typical setup for wireless video monitoring of spawning behavior. Three commercially available CCTV cameras (Swann, Inc.) capable of producing infrared light are aimed at the top of the water and connected to a digital video recorder (DVR). Video is monitored in real time for spawning behavior in an adjacent room from a network connected computer (PC). (**B**) Spawning behavior captured with such a setup in *B. brachyistius*. (**C**) A typical breeding setup for *B. gauderio* with PVC hiding tubes and yarn mops.

Figure 4: Breeding males and females. (**A**) *B. brachyistius* and (**B**) *B. gauderio.* Both species are sexually dimorphic and easily distinguished visually when sexually mature. Both females are gravid in these photos, exhibiting characteristically swollen bellies that are full of ripe eggs.

Figure 5: Microinjection. (**A**) Glass capillary needle tips must be broken to deliver an appropriate microinjection volume. The tip on the left is unbroken. The middle and right tips are broken with a slightly angled bevel to pierce the egg chorion. (**B**) Eggs are lined against a glass slide (1%–10% phenol red is included as a tracer to visualize the delivery of the injection) and injected with glass capillary needles.

Figure 6: Developmental stages. (**A**) *B. gauderio* and (**B**) *B. brachyistius*. All eggs are assumed fertilized and development is monitored to 24 HPF. Between 12−24 HPF embryos are visible in viable eggs, otherwise eggs exhibit degradation. Several divisions appear to take place on egg activation, regardless of fertilization. Unfertilized eggs exhibit unusual patterns of cleavage that are much more symmetrical in fertilized eggs.

Figure 7: Photograph of larval recording chamber used in this study. (**A**) The electrodes are embedded within Sylguard but extend into the 35mm dish containing an embryo restricted via a 3% agarose mold. (**B**) Higher magnification image highlighting the restricted movement of the embryo due to agarose. Note the pieces of agarose that can be removed as the embryo changes size. *B. gauderio* embryo is facing the positive electrode.

Figure 8: CRISPR/Cas9 induced mutations in *B. gauderio*. (**A**) Thirty-two clone sequences from genomic DNA of Cas9-induced mutations in a single *scn4aa* targeted F_0 *B. gauderio* embryo (11 DPF). The reference sequence is underlined with the sgRNA target site highlighted in gray, the protospacer-adjacent motif (PAM) sequence highlighted in red, and the Cas9 cut site marked with "|". The change from the expected wild type sequence is given and the number of clones for each sequence is given in parenthesis. (Abbreviations: + = insertion, - = deletion, +/- = indel) Any non-

CRISPR associated sequence dissimilarities are bolded. Figure modeled after Jao et al.[60]. (**B**) Amino acid sequence predicted from sequenced clones of *scn4aa* knockdown *B. gauderio* from (**A**). Cas9-induced changes from the wild type sequence are highlighted in red and the nucleotide-induced change number is given. (**C**) Twenty-second electrical recordings from five size-matched larvae, all recorded 6 DPF in the same recording chamber. Gain settings are identical for all traces. Traces in red are from *B. gauderio* larvae with confirmed mutations (one individual shown in **Figure 8A**, **B** above), traces in black are from uninjected *B. gauderio* larvae. Overall, CRISPR/Cas9 editing of *scn4aa* showed a reduction in EOD amplitude, though the effect was heterogeneous.

Figure 9: CRISPR/Cas9 induced mutations in *B. brachyistius*. (**A**) Forty-two clone sequences from genomic DNA of Cas9-induced mutations in a single *scn4aa* targeted F_0 *B. brachyistius* embryo (11 DPF). The reference sequence is underlined with the sgRNA target site highlighted in gray, the protospacer-adjacent motif (PAM) sequence highlighted in red, and the Cas9 cut site marked with "|". The change from the expected wild type sequence is given and the number of clones for each sequence is given in parenthesis. (Abbreviations: + = insertion, - = deletion, +/- = indel) Any non-CRISPR associated sequence dissimilarities are bolded. Figure modeled after Jao et al.[60]. (**B**) Amino acid sequence predicted from sequenced clones from *scn4aa* knockdown *B. brachyistius* in (**A**). Cas9-induced changes from the wild type sequence are highlighted in red and the nucleotide induced change number is given. (**C**) Ten second electrical recordings from four size-matched larvae, all recorded 10 DPF in the same recording chamber. Gain settings are identical for all traces. Traces in red are from *B. brachyistius* larvae with confirmed mutations (one individual shown in **A**, **B** above), traces in black are from uninjected *B. brachyistius* larvae. Overall, CRISPR/Cas9 editing of *scn4aa* showed a reduction in EOD amplitude, though the effect was heterogeneous. Inverted EODs are from the fish changing orientation during the recording. No difference is discernible between experimental fish and controls despite this.

Figure 10: Box plots of average EOD amplitude of CRISPR and uninjected size/age matched siblings. (**A**) EOD amplitude of *B. brachyistius* larvae at 10 DPF. Recorded with a gain of 100, CRISPR n = 56 EODs from two individuals, uninjected n = 114 EODs from three individuals. (**B**) EOD amplitude of *B. gauderio* larvae at 6 DPF. Recorded with a gain of 500, CRISPR n = 34 EODs from two individuals, uninjected n = 148 EODs from three individuals. Amplitude of CRISPR fish was significantly less than uninjected controls ($p < 2.2 \times 10^{-16}$, Welch two-sample t-test). All individuals were recorded with the recording chamber described in **Figure 7**.

Table 1: Oligonucleotides necessary for the protocol.

DISCUSSION:

The phenotypic richness of weakly electric fish, together with a recent proliferation of genomics resources, motivates a strong need for functional genomic tools in the weakly electric fish model. This system is particularly attractive because of the convergent evolution of numerous phenotypic traits in parallel lineages of fish, which are easily kept in the laboratory.

The protocol described here demonstrates the efficacy of the CRISPR/Cas9 technique in lineages of weakly electric fish that evolved electrogenesis and electroreception in parallel, and therefore

represents a major step for this model's promise addressing future work in comparative genomics of phenotypic evolution.

This simple methodological approach requires only basic molecular biology skills and training, following a basic adoption of the Gagnon protocol[38], widely used for zebrafish. It is worth noting that as technology progresses, there are more commercial kits for sgRNA production as well as companies that can synthesize guide RNAs, making this protocol more accessible to laboratories that lack molecular biology experience and equipment. We note first that the high mutagenesis efficiency allows direct phenotyping of injected larvae. However, there appears to be a substantial degree of phenotypic mosaicism, which is not uncommon and is consistent with the literature[41-44]. For example, in this *scn4aa* study, some individuals carrying mutations exhibited much larger amplitude EODs than others that were comparatively silent (**Figure 7**, **Figure 8**). It is presently unclear how many of these mutations are carried into the germline. Immediate future efforts will be directed at creating stable mutant lines.

Utilizing the NHEJ pathway for knockouts is only one of the several potential applications of CRISPR/Cas9 gene editing: the methods outlined here are a stepping stone for more advanced applications[55,56]. Future efforts should be aimed at designing co-injected DNA donor templates with the sgRNA/Cas9 complex. This simple modification would leverage endogenous template-based repair mechanisms (i.e., homology directed repair, or HDR) and allow precise knock-ins. Although HDR occurs at a lower efficiency than NHEJ approaches, progress has been made to increase its efficacy[57,58]. This lower efficiency will require efforts to optimize the design of the DNA donor template/CRISPR/Cas9 construct, make the endogenous repair mechanisms more efficient, and increase embryo production (see below). If this issue of efficiency can be solved, knockins could be utilized to add fluorescent tags, express a mutated form of the gene product, or change promoter or enhancer sequences.

While the molecular biology behind this technique is fairly straightforward, the husbandry requirements are substantial, but not insurmountable. *B. gauderio* are widely available and breed rapidly enough for any research program to have a colony in under a year. In contrast, *B. brachyistius* develop slowly, and anatomical peculiarities have proven attempts at IVF thus far unsuccessful. Other, larger species, such as *Campylomormyrus*[59] may be more conducive to this approach. For *B. brachyistius*, all injected embryos were collected utilizing the natural spawning approach, which is significantly more labor intensive. Future efforts to increase efficiency in *B. brachyistius* IVF will allow for a higher yield of embryos for the efficiency issues described above.

ACKNOWLEDGMENTS:
The authors acknowledge the heroic efforts of Monica Lucas, Katherine Shaw, Ryan Taylor, Jared Thompson, Nicole Robichaud, and Hope Healey for help with fish husbandry, data collection, and early protocol development. We would also like to thank the three reviewers for their suggestions to the manuscript. We believe the final product to be of better quality after addressing their comments. This work was funded by support from the National Science Foundation #1644965 and #1455405 to JRG, and the Natural Sciences and Engineering Research Council DG grant to VLS.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Gallant, J. R. et al. Genomic basis for the convergent evolution of electric organs. *Science.* **344** (6191), 1522–1525 (2014).
2. Zakon, H. H., Lu, Y., Zwickl, D. J., Hillis, D. M. Sodium channel genes and the evolution of diversity in communication signals of electric fishes: convergent molecular evolution. *Proceedings of the National Academy of Sciences of the United States of America.* **103** (10), 3675–3680 (2006).
3. Arnegard, M. E., Zwickl, D. J., Lu, Y., Zakon, H. H. Old gene duplication facilitates origin and diversification of an innovative communication system--twice. *Proceedings of the National Academy of Sciences of the United States of America.* **107**, 22172–22177, (2010).
4. Lissmann, H. W. Continuous electrical signals from the tail of a fish. Gymnarchus niloticus Cuv. *Nature.* **167** (4240), 201–202 (1951).
5. Cuellar, H., Kim, J. A., Unguez, G. A. Evidence of post-transcriptional regulation in the maintenance of a partial muscle phenotype by electrogenic cells of S. macrurus. *FASEB Journal.* **20** (14), 2540 (2006).
6. Modrell, M. S., Baker, C. V. Evolution of electrosensory ampullary organs: conservation of Eya4 expression during lateral line development in jawed vertebrates. *Evolution & Development.* **14** (3), 277–285 (2012).
7. Hopkins, C. D. Design features for electric communication. *Journal of Experimental Biology.* **202** (Pt 10), 1217–1228 (1999).
8. Kawasaki, M. Sensory hyperacuity in the jamming avoidance response of weakly electric fish. *Current Opinion in Neurobiology.* **7** (4), 473–479 (1997).
9. Bell, C. C., Han, V. Z., Sugawara, Y., Grant, K. Synaptic plasticity in a cerebellum-like structure depends on temporal order. *Nature.* **387** (6630), 278–281 (1997).
10. Heiligenberg, W. *Neural Nets in Electric Fish*. (1991).
11. Ban, Y., Smith, B. E., Markham, M. R. A highly polarized excitable cell separates sodium channels from sodium-activated potassium channels by more than a millimeter. *Journal of Neurophysiology.* **114** (1), 520–530 (2015).
12. Markham, M. R., Kaczmarek, L. K., Zakon, H. H. A sodium-activated potassium channel supports high-frequency firing and reduces energetic costs during rapid modulations of action potential amplitude. *Journal of Neurophysiology.* **109** (7), 1713–1723 (2013).
13. Gavassa, S., Stoddard, P. K. Food restriction promotes signaling effort in response to social challenge in a short-lived electric fish. *Hormones and Behavior.* **62** (4), 381–388 (2012).
14. Sinnett, P. M., Markham, M. R. Food deprivation reduces and leptin increases the amplitude of an active sensory and communication signal in a weakly electric fish. *Hormones and Behavior.* **71** 31–40 (2015).
15. Salazar, V. L., Stoddard, P. K. Sex differences in energetic costs explain sexual dimorphism in the circadian rhythm modulation of the electrocommunication signal of the gymnotiform fish *Brachyhypopomus pinnicaudatus*. *Journal of Experimental Biology.* **211**

(Pt 6), 1012–1020 (2008).
16. Lewis, J. E., Gilmour, K. M., Moorhead, M. J., Perry, S. F., Markham, M. R. Action potential energetics at the organismal level reveal a trade-off in efficiency at high firing rates. *Journal of Neuroscience.* **34** (1), 197–201 (2014).
17. Salazar, V. L., Krahe, R., Lewis, J. E. The energetics of electric organ discharge generation in gymnotiform weakly electric fish. *Journal of Experimental Biology.* **216** (13), 2459–2468 (2013).
18. Hopkins, C. D., Bass, A. Temporal coding of species recognition signals in an electric fish. *Science.* **212** (4490), 85–87 (1981).
19. Arnegard, M. E., Jackson, B. S., Hopkins, C. D. Time-domain signal divergence and discrimination without receptor modification in sympatric morphs of electric fishes. *The Journal of Experimental Biology.* **209** (Pt 11), 2182–2198 (2006).
20. Sullivan, J. P., Lavoue, S., Arnegard, M. E., Hopkins, C. D. AFLPs resolve phylogeny and reveal mitochondrial introgression within a species flock of African electric fish (Mormyroidea: Teleostei). *Evolution.* **58** (4), 825–841 (2004).
21. Crampton, W. G. R. Effects of anoxia on the distribution, respiratory strategies and electric signal diversity of gymnotiform fishes. *Journal of Fish Biology.* **53** (sa), 307–330 (1998).
22. Pinch, M., Guth, R., Samanta, M. P., Chaidez, A., Unguez, G. A. The myogenic electric organ of Sternopygus macrurus: a non-contractile tissue with a skeletal muscle transcriptome. *PeerJ.* **4**, e1828 (2016).
23. Lamanna, F., Kirschbaum, F., Waurick, I., Dieterich, C., Tiedemann, R. Cross-tissue and cross-species analysis of gene expression in skeletal muscle and electric organ of African weakly-electric fish (Teleostei; Mormyridae). *BMC Genomics.* **16**, 668 (2015).
24. Traeger, L. L. et al. Unique patterns of transcript and miRNA expression in the South American strong voltage electric eel (Electrophorus electricus). *BMC Genomics.* **16**, 243 (2015).
25. Salisbury, J. P. et al. The central nervous system transcriptome of the weakly electric brown ghost knifefish (Apteronotus leptorhynchus): de novo assembly, annotation, and proteomics validation. *BMC Genomics.* **16**, 166 (2015).
26. Lamanna, F., Kirschbaum, F., Tiedemann, R. De novo assembly and characterization of the skeletal muscle and electric organ transcriptomes of the African weakly electric fish Campylomormyrus compressirostris (Mormyridae, Teleostei). *Molecular Ecology Resources.* **14** (6), 1222–1230 (2014).
27. Mate, S. E., Brown, K. J., Hoffman, E. P. Integrated genomics and proteomics of the Torpedo californica electric organ: concordance with the mammalian neuromuscular junction. *Skeletal Muscle.* **1** (1), 20 (2011).
28. Swapna, I. et al. Electrostatic Tuning of a Potassium Channel in Electric Fish. *bioRxiv.* (2017).
29. Futuyma. *Evolution*. Third Edition (Sinauer Associates Inc., 2013).
30. Thompson, A., Vo, D., Comfort, C., Zakon, H. H. Expression Evolution Facilitated the Convergent Neofunctionalization of a Sodium Channel Gene. *Molecular Biology and Evolution.* **31** (8), 1941–1955 (2014).
31. Pitchers, W. R., Constantinou, S. J., Losilla, M., Gallant, J. R. Electric fish genomics: Progress, prospects, and new tools for neuroethology. *Journal of Physiology Paris.* (2016).

32. Liang, X. et al. Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *Journal of Biotechnology.* **208**, 44–53 (2015).
33. Jung, C. J. et al. Efficient gene targeting in mouse zygotes mediated by CRISPR/Cas9-protein. *Transgenic Research.* **26** (2), 263–277 (2017).
34. Liu, K., Petree, C., Requena, T., Varshney, P., Varshney, G. K. Expanding the CRISPR Toolbox in Zebrafish for Studying Development and Disease. *Frontiers in Cell and Developmental Biology.* **7** (13), (2019).
35. Zu, Y. et al. Biallelic editing of a lamprey genome using the CRISPR/Cas9 system. *Scientific Reports.* **6** 23496 (2016).
36. Crispo, M. et al. Efficient Generation of Myostatin Knock-Out Sheep Using CRISPR/Cas9 Technology and Microinjection into Zygotes. *PLoS One.* **10** (8), e0136690 (2015).
37. Sun, D., Guo, Z., Liu, Y., Zhang, Y. Progress and Prospects of CRISPR/Cas Systems in Insects and Other Arthropods. *Frontiers in Physiology.* **8**, 608 (2017).
38. Gagnon, J. A. et al. Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. *PLoS One.* **9** (5), e98186 (2014).
39. Kok, F. O. et al. Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Developmental Cell.* **32** (1), 97–108 (2015).
40. Morcos, P. A., Vincent, A. C., Moulton, J. D. Gene Editing Versus Morphants. *Zebrafish.* **12** (5), 319 (2015).
41. Mehravar, M., Shirazi, A., Nazari, M., Banan, M. Mosaicism in CRISPR/Cas9-mediated genome editing. *Developmental Biology.* **445** (2), 156–162 (2019).
42. Yen, S. T., et al. Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. *Developmental Biology*. **393** (1), 3–9 (2014).
43. Singh, P., Schimenti, J. C., Bolcun-Filas, E. A Mouse Geneticist's Practical Guide to CRISPR Applications. *Genetics*. **199** (1), 1–15 (2015).
44. Mianné J. et al. Analyzing the outcome of CRISPR-aided genome editing in embryos: screening, genotyping and quality control. *Methods*. **121–122**, 68–76 (2017).
45. van der Emde, G. in *Encyclopedia of Animal Behavior* Vol. 1 (eds Breed, M. D., Moore, J.). 16–23 (Academic Press, 2010).
46. Carlson, B. A. in *Encyclopedia of Neuroscience* (eds Binder M. D., Hirokawa, N., Windhorst, U., Hirsch, M. C.) 4039–4044 (Springer, 2009).
47. Hopkins, C. D. Neruoethology of Electric Communication. *Annual Reviews of Neuroscience.* **11**, 497–535 (1988).
48. Arnegard, M., Zwickl, D., Lu, Y., Zakon, H. H. Old gene duplication facilitates origin and diversification of an innovative communication system- twice. *Proceedings of the National Academy of Sciences of the United States of America.* **107** (51), 22172–22177 (2010).
49. Doench, J. G. et al. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nature Biotechnology.* **32** (12), 1262–1267 (2014).
50. Concordet, J. P., Haeussler, M. CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Resarch.* **46** (W1), W242–W245 (2018).
51. Haeussler, M. et al. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biology.* **17** (1), 148 (2016).

52. Kirschbaum, F. Environmental factors control the periodical reproduction of tropical electric fish. *Experientia.* **31** (10), 1159–1160 (1975).
53. Iwama G. K., McGeer J. C., Pawluk M. P. The effects of five fish anaesthetics on acid-base balance, hematocrit, cortisol and adrenaline in rainbow trout. *Canadian Journal of Zoology* 67:2065–2073 (1989)
54. Westerfield, M. *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio)*. 4th ed. edn (Univ. of Oregon Press, 2000).
55. Barrangou, R., Doudna, J. A. Applications of CRISPR technologies in research and beyond. *Nature Biotechnology.* **34** (9), 933–941 (2016).
56. Adli, M. The CRISPR tool kit for genome editing and beyond. *Nature Communications.* **9** (1), 1911 (2018).
57. Maruyama, T. et al. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nature Biotechnology.* **33** (5), 538–542 (2015).
58. Liu, M. et al. Methodologies for Improving HDR Efficiency. *Frontiers in Genetics.* **9**, 691 (2018).
59. Kirschbaum, F. et al. Intragenus (Campylomormyrus) and intergenus hybrids in mormyrid fish: Physiological and histological investigations of the electric organ ontogeny. *Journal of Physiology Paris.* **110** (3 Pt B), 281–301 (2016).
60. Jao, L. E., Wente, S. R., Chen, W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proceedings of the National Academy of Science of the United States of America.* **110** (34), 13904–13909 (2013).

Figure 1

Click here to access/download;Figure;fig1.ai

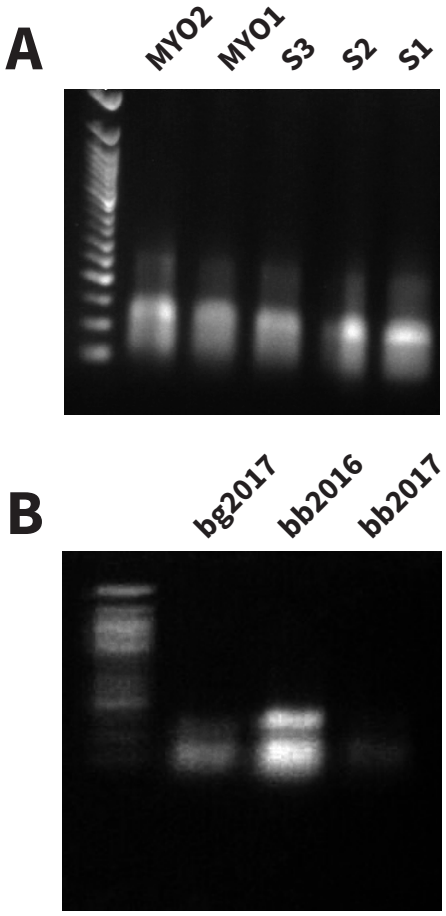


Figure 2

Click here to access/download;Figure;fig2.ai

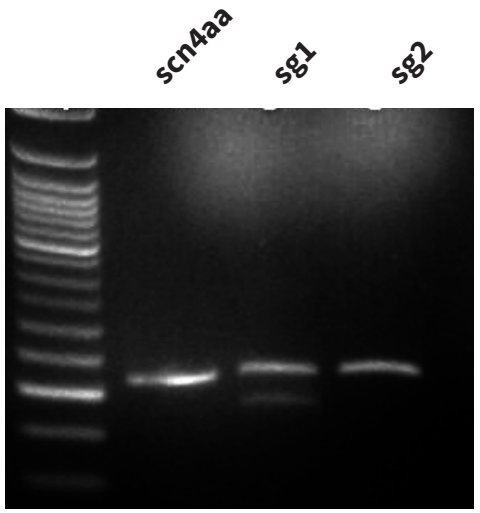


Figure 3

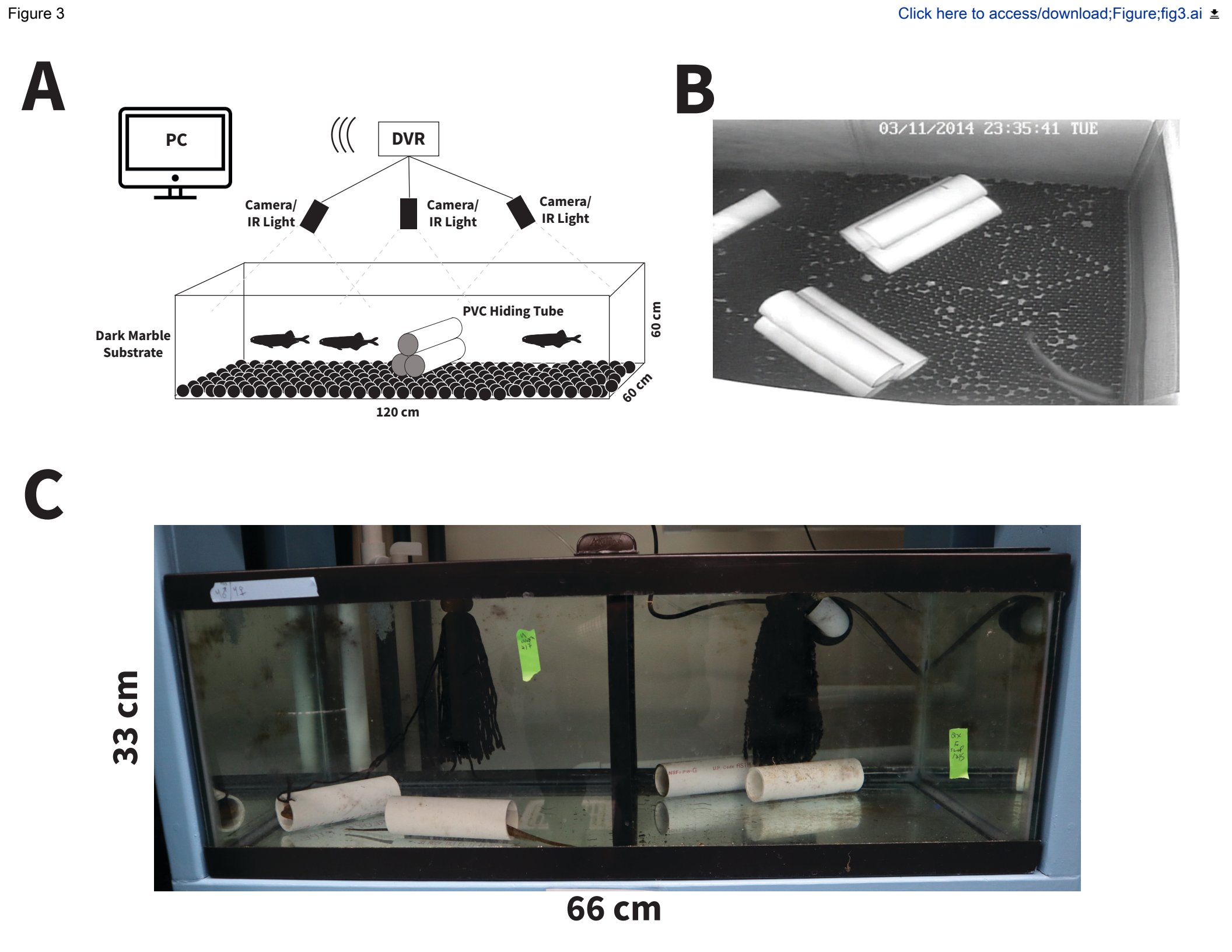


Figure 4

Click here to access/download;Figure;fig4.png

Figure 5

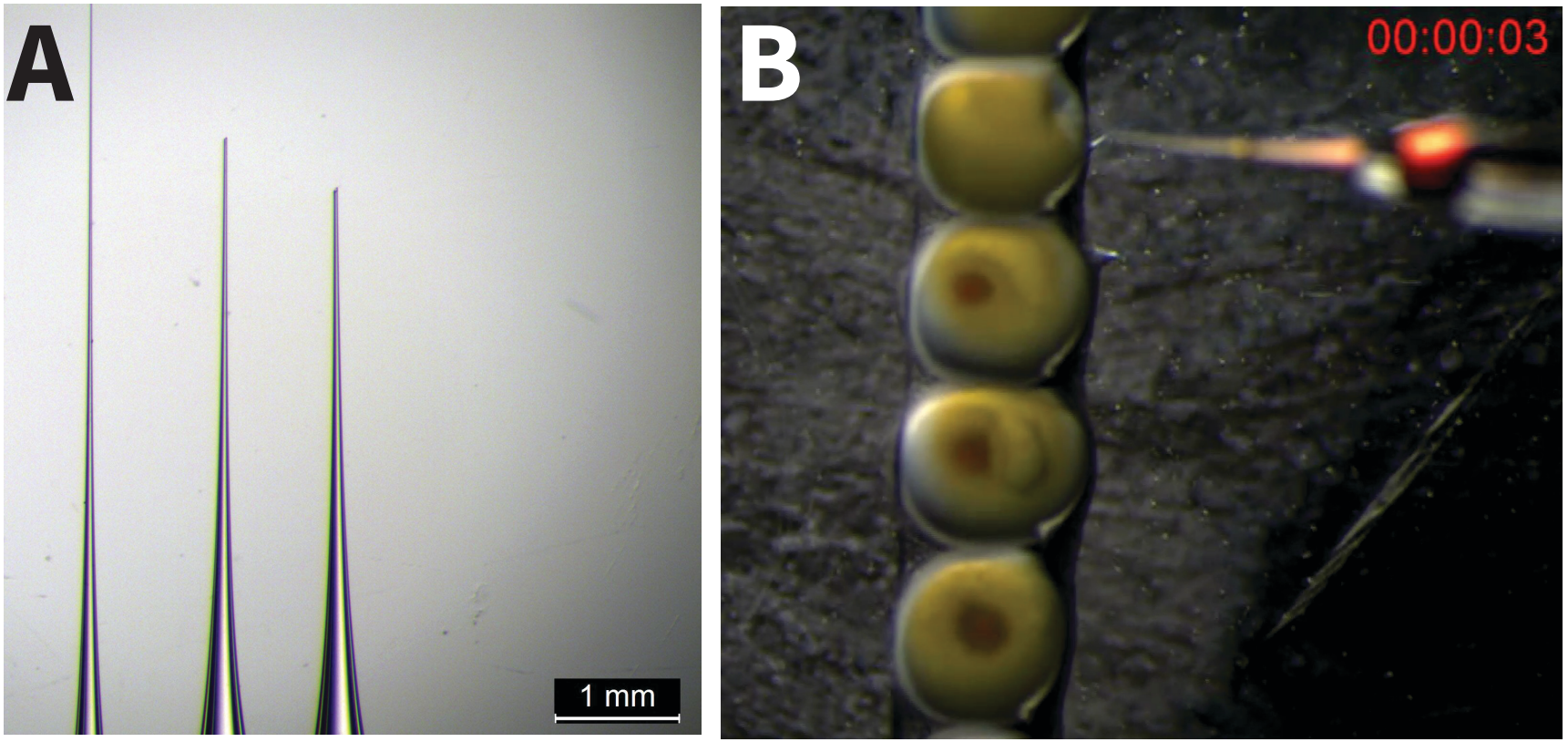


Figure 6

Click here to access/download;Figure;fig6.png

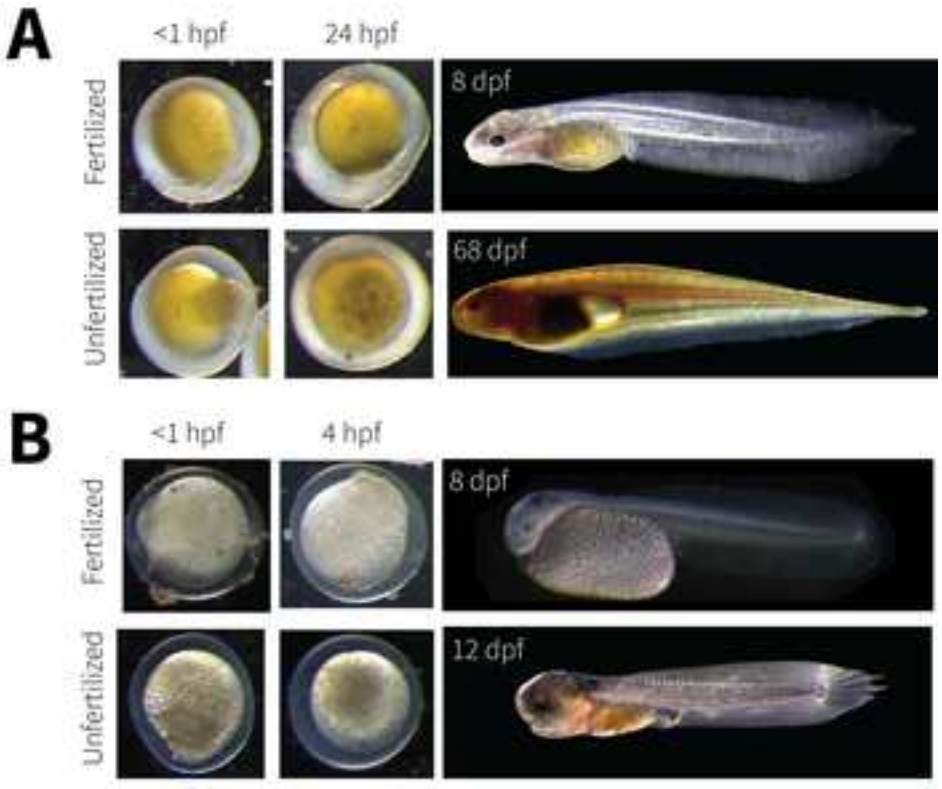


Figure 7

Click here to access/download;Figure;fig_7.ai

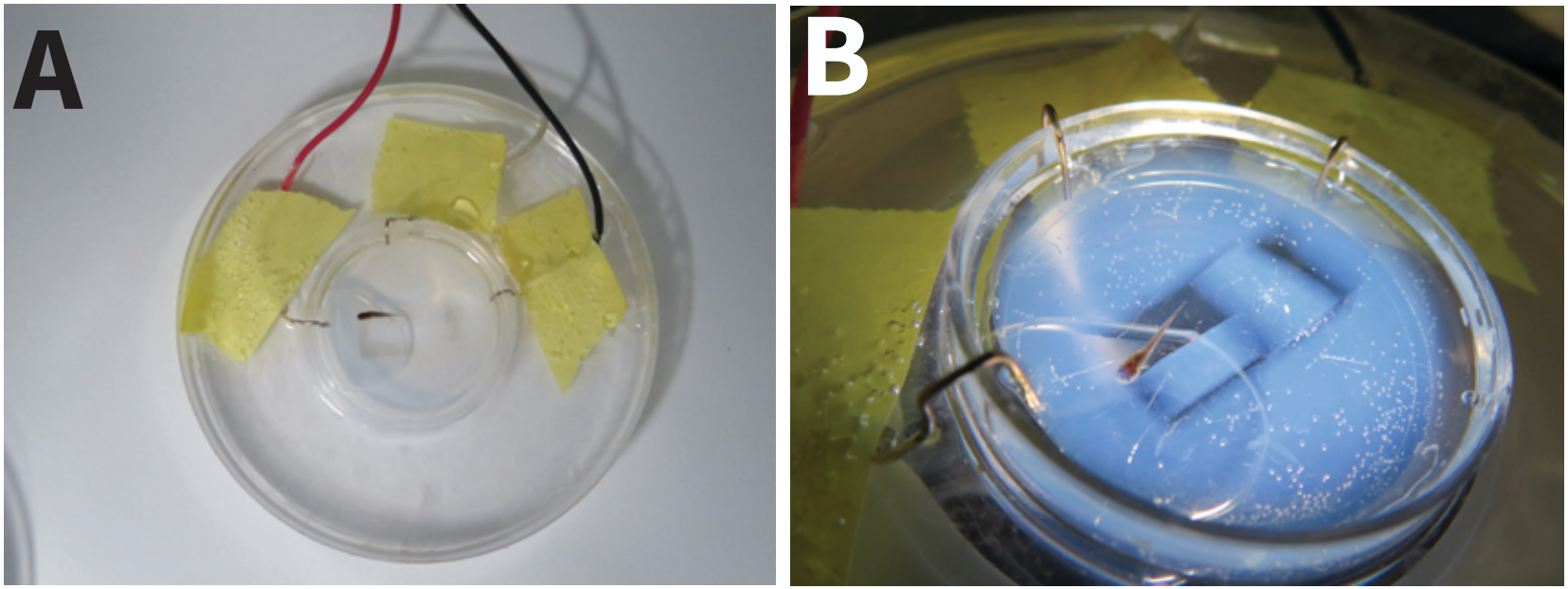


Figure 8

Click here to access/download;Figure;fig_8.ai

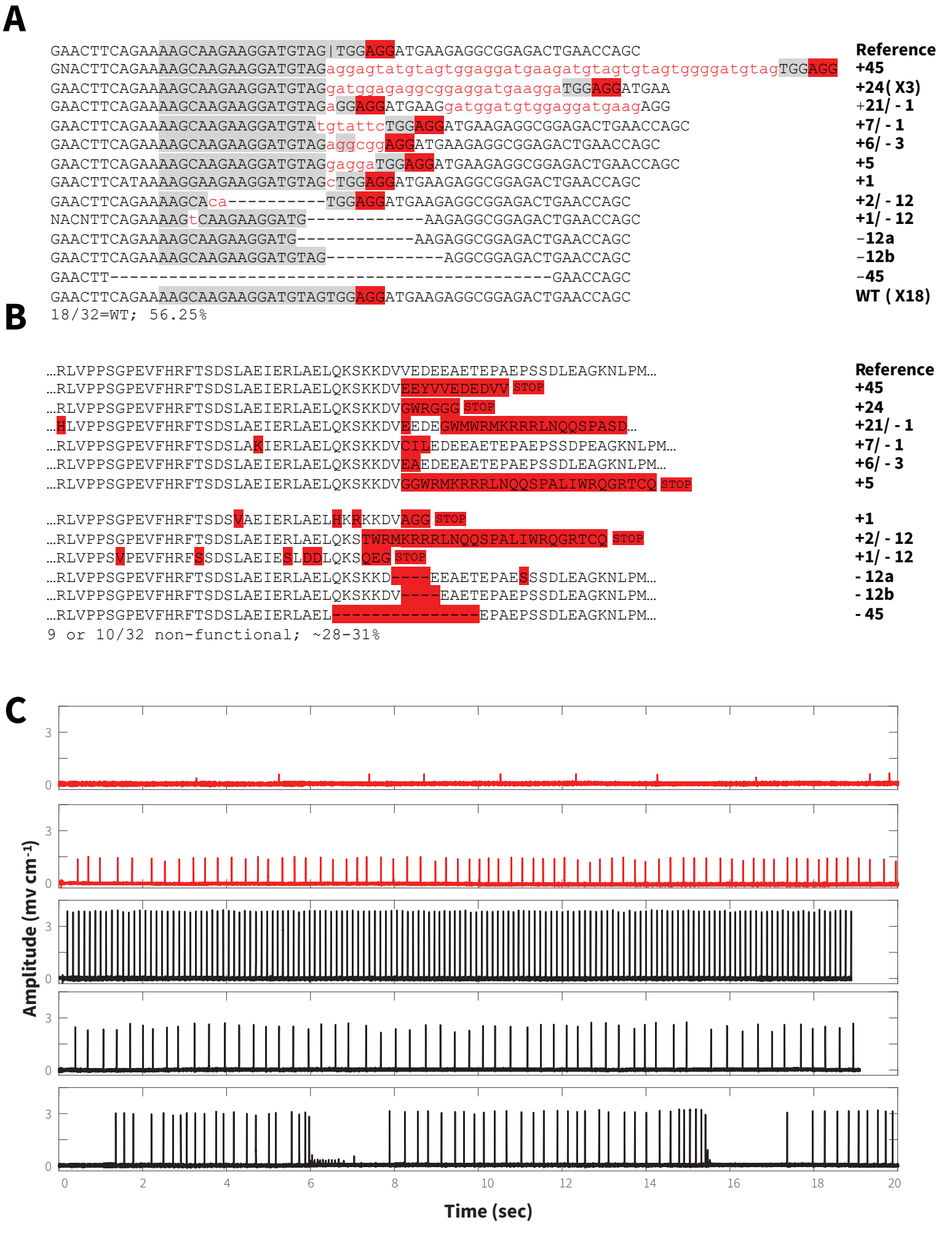


Figure 9

Click here to access/download;Figure;fig_9.ai

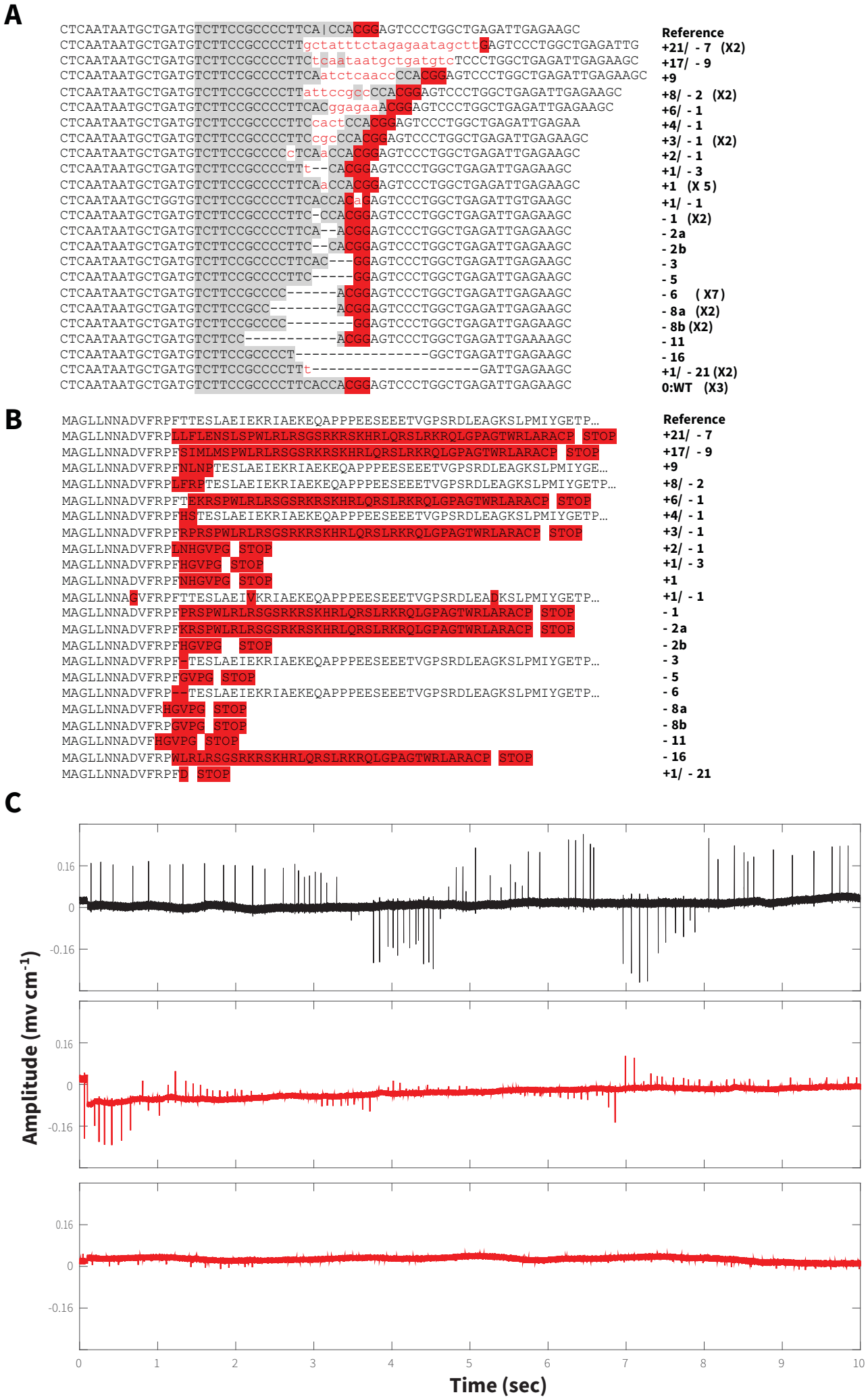
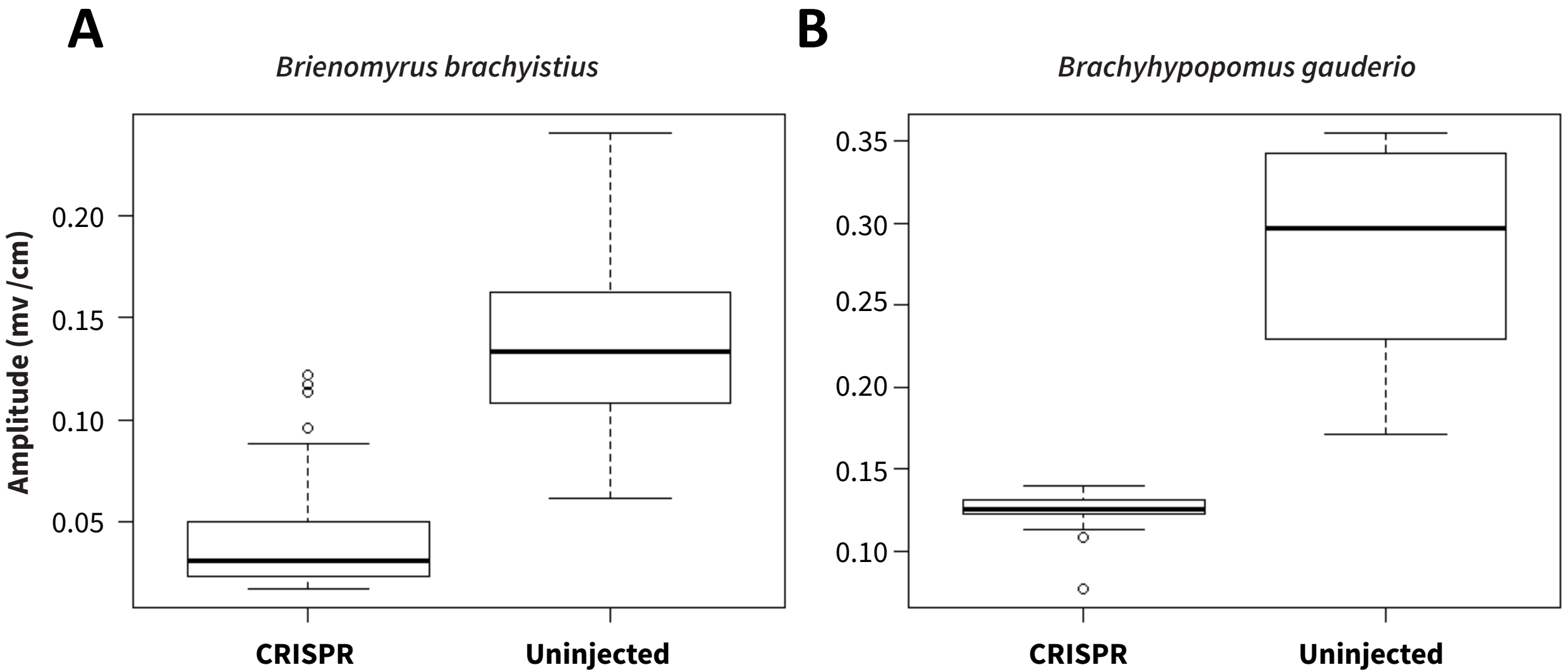


Figure 10

Click here to access/download;Figure;Fig10.ai



Description
Constant oligomer
Target oligomer backbone (GG-N18, no PAM)
Target oligomer backbone (N20, no PAM)
Brienomyrus brachyistius
scn4aa Bb sgRNA target (N18, with PAM):
Scn4aa Bb sgRNA oligomer (GG-N18):
scn4aa Bb PCR primer (218 bp)
Scn4aa_bb_exon1_F:
Scn4aa_bb_exon1_R:
Brachyhypopomus gauderio
Scn4aa Bg sgRNA target (N17, with PAM):
Scn4aa Bg sgRNA oligomer (GG-N17):
Scn4aa Bg PCR primer pair (204 bp)
scn4aa_bg_exon1_F:
scn4aa_bg_exon1_R:

Sequence

5'-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGC TATTTCTAGCTCTAAAAC-3'
5'-TAATACGACTCACTATAGG-N18-GTTTTAGAGCTAGAAATAGCAAG-3'
5'-TAATACGACTCACTATAG-N20-GTTTTAGAGCTAGAAATAGCAAG-3'

5'-TCTTCCGCCCCTTCACCACGG-3'
5'-TAATACGACTCACTATAGG TCTTCCGCCCCTTCACCA GTTTTAGAGCTAGAAATAGCAAG-3'

5'-ATGGCCGGCCTTCTCAATAA-3'
5'-TCTTCCAGGGGAATATTCATAAACT-3'

5’- CAAGAAGGATGTAGTGGAGG-3’
5'-TAATACGACTCACTATAGG CAAGAAGGATGTAGTGG GTTTTAGAGCTAGAAATAGCAAG-3'

5'-CGCCTTGTCCCTCCTTCAG-3'
5'-ATCTTCAGGTGGCTCTCCAT-3'

Name of Material/Equipment	Company	Catalog Number	Comments/Description
20 mg/mL RNA grade Glycogen	Thermo Scientific	R0551	
50 bp DNA ladder	NEB	N3236L	
borosilicate glass capillary with filar	Sutter Instrument	BF100-58-10	(O.D. 1.0mm, I.D. 0.58 mm, 10 cm length)
Cas9 protein with NLS; 1 mg/mL	PNA Biology	CP01	
Dneasy Blood & Tissue Kit	Qiagen	69506	
Eppendorf FemptoJet 4i Microinjector	Fisher Scientific	E5252000021	
Eppendorf Microloader Pipette Tips	Fisher Scientific	10289651	
Hamilton syringe	Fisher Scientific	14-824-654	referred to as "precision glass syringe" in the protocol
Kimwipe	Fisher Scientific	06-666	referred to as "delicate task wipe" in the protocol
MEGAscript T7 Transcription Kit	Invitrogen	AM1334	
NEBuffer 3	NEB	B7003S	used for in vitro cleavage assay
OneTaq DNA kit	NEB	M0480L	
Ovaprim	Syndel USA	https://www.syndel.com/	referred to as "spawning agent" in the protocol
Parafilm	Fisher Scientific	S37440	referred to as "thermoplastic" in the protocol
Pipette puller	WPI	SU-P97	sutter brand
QIAquick PCR Purification Kit	Qiagen	28106	
Reusable needle- requires customization	Fisher Scientific	7803-02	Customize to 0.7 inches long; point style 4 and angle 25
T4 DNA polymerase	NEB	M0203L	Use with the 10X NEB buffer that is included
Teflon coated tools	bonefolder.com	T-SPATULA4PIECE	referred to as "polytetrafluoroethene" in the protocol

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Silencing the spark: CRISPR/Cas9 genome editing in weakly electric fish
Author(s):	Savvas J. Constantinou, Linh Nguyen, Frank Kirschbaum, Vielka L. Salazar, Jason R. Gallant

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

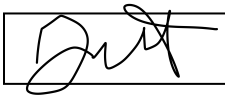
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Jason R. Gallant
Department:	Integrative Biology
Institution:	Michigan State University
Title:	Assistant Professor
Signature:	
Date:	5-July 2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Editorial comments:

We thank the reviewers and editors for their thoughtful comments on this manuscript. We have addressed the issues indicated by the editor and reviewers detailed below. We indicate comments that have been addressed in strikethrough font, and any further information in italic font below the comment/suggestion.

Kind Regards on Behalf of the Authors,

Dr. Jason Gallant

General:
~~1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.~~
2~~. Please ensure that the manuscript is formatted according to JoVE guidelines- letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.~~
~~3. Please print and sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.~~
~~4. Please include email addresses of all authors in the manuscript.~~
consta64@msu.edu; jgallant@msu.edu; Vielka_Salazar@cbu.ca; frank.kirschbaum@staff.hu-berlin.de; nguyenmdlinh12@gmail.com
5. ~~Please include a Summary section of 10-50 words that clearly describes the protocol and its applications in complete sentences between 10¬ and 50 words, e.g.: "Here, we present a protocol to …"~~
6. ~~JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.~~
~~For example: Nanodrop, MEGAscript, QIAgen, OneTaq, Zymo, Falcon, Ovaprim, Parafilm, Kimwipe-, Teflon, Femtojet,~~

Protocol:
~~1. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique.~~
~~2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.~~

~~Figures, Tables, and Figure Legends:~~
~~1. Figures: Please upload each Figure individually to your Editorial Manager account as~~

~~a .png, .tiff, or .pdf file.~~
~~2. Please remove the titles and Figure Legends from the uploaded figures.~~

References:
~~1. Please do not abbreviate journal titles.~~

~~Table of Materials:~~
~~1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.~~

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This is a very important contribution describing the optimization and protocol for gene editing in electric fish, a non-traditional model system for studying evolution and behavior. I have a few comments that I hope will further improve the manuscript prior to publication. I love the title!

Major Concerns:
1. I realize that this is a tool development paper, but I would like to see a little more on why these particular species were chose from a biological perspective, in addition to the practical reasons listed.

In addition to the practical reason listed we also included that there was a community census following the electric fish 2016 meeting due to these species also having genomic resources available.

2. There is mention of phenotypic variability in the discussion and I'm curious whether this was random or related to a protocol variation? For example, did the authors test a range of sgRNA and Cas9 injection ratios/amounts and did this influence the probability and/or severity of the phenotypes that they saw?

Our experiments were performed with the same pools of sgRNA (one for each species) and Cas9 and we kept the concentrations the same throughout. We believe that the variability seen in phenotype is due to variation in cutting efficiency and stochastic nature of indel formation via non homologous end joining pathway.
Mosaicism is very common in F_0 CRISPR/Cas9 injected embryos. We added some citations and a line to the introduction and discussion so that readers know that our representative results showing variation in the mutant phenotype is expected.

3. Can the authors please provide (1) the efficiency of the procedure and (2) average survival and fertilization rates.

We included these in the manuscript in the representative results.

Minor Concerns*: We have addressed those concerns that are crossed off below and appreciate the help of the reviewer.*
~~Line 34: Remove or spell out NHEJ acronym.~~
~~Line 57: Listing some of these genomic resources to show the contrast of what is out there and why this is really new/different would improve this paragraph.~~
~~Line 108: Sometimes electric organs are an acronym and sometimes not throughout the manuscripts. Unless the authors are pressed for word count restrictions, I would prefer they spell it out.~~
~~Line 151: Can the authors describe/annotate the nucleotides that flank the N(18) sequence?~~
~~Section 2: I don't know if it's worth pointing out here or elsewhere that as this technology advances and becomes more widely distributed there are more and more commercially available kits to stream line this process (e.g. NEB EnGen kit for sgRNA synthesis) as well as pre-made guides that can be ordered commercially (e.g. Synthego)~~
Added a note in the discussion about the availability of kits and manufacturers.
~~Step 3.5: I don't understand how you could run a positive control if you don't yet know if this works. What am I missing here?~~
We added a note that that the control would be from previously established guides
~~Section 4.2: Can the authors provide a brief description of what Ovaprim is and what it does?~~
~~Line 418: What is sperm collected in?~~
~~Line 423: How long can the sperm be stored in the refrigerator?~~
~~Line 423: Should this SES solution be made prior to anesthetizing and squeezing the fish in the previous two steps?~~
~~Line 475: What developmental milestones are you looking for here?~~
~~Line 481: Can needles be pulled prior to egg squeezing? Perhaps this can be explicitly stated? What kind (manufacturer and model) is the needle puller used for this?~~
~~Line 490: most people add some salt/buffer to the mix to (1) help stabilize the Cas9 in solution and (2) prevent the needle from clogging.~~
~~Lines 655-659: It would be good to include some estimates of the efficiency of the procedure.~~

Reviewer #2:
Manuscript Summary:
The authors describe a CRISPR/Cas9 approach to introduce targeted manipulations in a sodium channel gene. The success of the mutations has been shown on the sequence level and phenotypically on the level of the electric organ discharge. The latter results might be rather divers and less clear than one might have hoped for but this should be seen as a minor critique.

In my opinion this is a highly relevant contribution to the electric fish community. These genetic tools are really needed and the authors show that they can be applied. The application of the protocol and its similarity to established zebrafish protocols does not

lower its significance. Rather, I find it encouraging that a methodological transfer seems possible.

Major Concerns:
None

Minor Concerns:
** Are the title and abstract appropriate for this methods article?
~~Yes! The first part of the title refers to a classical electric fish paper (Hagedorn 1985), I like it even though the spark apparently has not been completely silenced. The abstract/introduction provides sufficient background.~~

** Are there any other potential applications for the method/protocol the authors could discuss?
~~The authors apply the method to two species and it would be great if the authors could elaborate on the applicability of the methods to the other widely studied electric fish (Eigenmannia virescense, Apteronotus leptorhynchus, Gnathonemus petersii to name a few).~~
We have included a statement in the introduction that this procedure should be applicable to all electric fish as long as researchers are able to collect single cell embryos.

** Are all the materials and equipment needed listed in the table? (Please note that any basic lab materials or equipment do not need to be listed, e.g. pipettes.)
Yes, the list appears to be complete.

** Do you think the steps listed in the procedure would lead to the described outcome?
I have no reason to doubt this.

** Are the steps listed in the procedure clearly explained?
We have addressed those concerns that are crossed off below and appreciate the help of the reviewer.
Yes, I have a few minor comments:
~~1) 1.2.1 For a non-geneticist this is hard to digest. Three "it is best" suggestions in three subsequent sentences are hard.~~
~~2) 2.9 x microliter of H2O, what is x supposed to indicate? An arbitrary amount?~~
~~3) 4.3.1 Ovaprim, I can read from the name what it is supposed to do, but some words for the ignorant would be nice.~~
We added a brief description in section 4.2.1
~~4) Steps 4.4 and 4.5 how long will the the procedures take? Animal respiration is not needed during this time?~~
Only 30-60 seconds, we added a note to move as quickly as possible and included this time frame for fish out of the water.
~~5) 4.5.1 "The fish will be under." I guess it means that the fish is in a certain level of anaesthesia? Which? According to Iwama et al. 1989 there are three levels. In my opinion this should be a little more specific. 4.4.1 states the loss of equilibrium, I~~

~~assume it is the same here.~~
We added a note at all MS-222 steps that it should be Stage II, the fish should not be moving but should still have opercular movement. We also included the reference.
~~6) 4.5.1 why is the Telfon coating important?~~
The polytetrafluoroethene causes less damage to the eggs from friction during handling. The sheet allows for a low friction, water free environment for eggs to land if there are handing errors during squeezing.
~~7) What exactly is the "recovery system water"~~
Untreated water from the tank/system the fish is housed in that the fish can recover from anesthesia in. Made this clearer in manuscript.
~~8) 5.1 the exact glass should not matter, should it? Glass without filament might be even advantageous? If it is important, why?~~
~~9) 5.4.3 Is it correct to speak of embryos in the 1-cell stage? If so, the use should be consistent throughout the procedure.-~~
Used "egg" and "zygote"
~~10) 5.4.6 I guess I understood after reading a few times. You put 2-cell stage eggs that have not been injected (plus some more) aside as "no injection control", correct?~~
Correct, added language to make that more clear.
~~11) 6.1.5 *larvae* will hatch~~
~~12) 6.2.5 Why are these egg cups important?~~
Added a note: "Egg cups allow the fish to be housed in a larger volume of water for water quality reasons, while maintaining discrete groups."
~~13) 6.2.8 and 6.2.12 use of SI units?~~

** Are any important steps missing from the procedure?
As far as I can tell, the procedures appear complete. Actually, animal husbandry descriptions go beyond what is shown in the representative results.

** Are appropriate controls suggested?
Generally yes, I miss a few statements regarding the measurements of the electric organ discharges, though:
~~1. The authors state that the measured animals were matched in size. It was unclear to me whether the same agarose mold, recording chamber was used for measuring all individuals of the same species.~~
Yes the same recording device was used (Fig. 7A) and the same agarose mold was used; note that the agarose mold had the ability to slightly change size (Fig. 7B) to accommodate growing larvae.

2. ~~The geometrical arrangement has a strong effect on the measurements, how was this controlled?~~ How large is the variability in the measurement?
The agarose mold kept fish directly between the electrodes as much as possible, and also restricted the fish from moving much during recordings.

3. In this line I would have appreciated to see some kind of statistical evaluation of the effect. (Boxplots depicting the EOD amplitudes in treated and WT controls)

We have visualized the EOD amplitudes as box plots and performed statistics to help viewers see the significant effect of scn4aa *knockdown in CRISPR injected fish.*

4. Could you provide a comparison of the EOD waveforms of mutations and WT? Relating to figures 8, 9: I am not familiar with B.brachyistus, why are some EODs positive while others are negative defelctions? The larvae were measured in a similar way as in figure 8? Meaning, geometrical effects can be excluded? I assume these figures are mere sketches of what to eventually show, but some labeling would be nice. arrangement of mutations and WT is inverted. ~~Figure caption of fig 9 speaks of larvae.~~
We added a note to the figure legend that any inverted EOD are from the fish changing orientation during recordings and that we see no difference between experimental fish and controls despite this.

~~5. What is the survival rate up to the measurement day? Does the observed effect depend on the dpf?~~
Added survivability percentages. EOD amplitude effect depends on dpf as age is correlated to length. Length is a better indicator of developmental stage than age. The EOD gets stronger in amplitude as the fish grows. Many species of electric fish have larval EODs that differ from those of the adults (monophasic vs bi- or tri- phasic).

** Is there any additional information that would be useful to include?
A little more about the representative results (see above) would strengthen the presentation.

** Are the anticipated results reasonable, and if so, are they useful to readers?
Yes, the authors intended to induce mutations of the sodium channels of the electric organ and observe differences in the EODs of the injected larvae. I do not see a problem in the diversity of the effect.

** Are any important references missing and are the included references useful?
No

Reviewer #3:
Manuscript Summary:
The authors report a full protocol for performing CRISPR/Cas9 mutagenesis in weakly electric fish. They show that the protocol is equally effective in both the mormyrid species Brienomyrus brachyistius and the gymnotiform Brachyhypopomus gauderio. They use CRISPR/Cas9 to target indels and point mutations in the first exon of the sodium channel gene scn4aa that is selectively expressed un the electric organ. As a proof of concept they show that they can selectively alter that gene and alter the electrical behavior of the fish.

Major Concerns:
I am concerned about the brief development on electrical behavior in the Results section, both of the mutated fish and the controls. Although it is said in the work that it is

only a proof of concept and what is shown is basically an example, the success of the genetic manipulation is evident in the electrical alterations of the mutated animals. Also striking is the variability of the results with respect to the DOE in the mutated animals according to the selected examples. That fact is briefly discussed in the discussion but not in the results.
We added a note in the representative results that there is a range of EOD amplitude reduction seen. See also our response to Reviewer 1 #2 concerning how we addressed mosaicism
Ideally a systematic characterization of the electrical behavior would be desirable for a more convincing argument about the success of the tool.
~~Likewise, the figures that show the electrical behavior are untidy: missing the legends of the axes, the calibration bars and some of the records shown are not adjusted to the axes.~~ ~~There is also a mismatch between the number of examples mentioned in the text of figures 8 and nine part C: it is said that there are 4 examples in each case, two corresponding to mutated fish and two to controls but in figure 8 part C there are 5 and in 9 part C there are 4.~~

Minor Concerns:
The selection of citations regarding the development of field of research on the electric fish in the last 50 years in the second paragraph of the introduction is poor and biased but I guess is difficult to be fair in such short space.

Given that this is not a review paper on Efish biology, we feel the references portray the literature well enough for a reader to understand electric fish biology for the purposes of performing CRISPR/Cas9 genomic manipulations in electric fish.