

Video Article

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

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URL: <https://www.jove.com/video/60253>

DOI: [doi:10.3791/60253](https://doi.org/10.3791/60253)

Keywords: Biology, Issue 152, CRISPR/Cas9, electric fish, single cell microinjection, *scn4aa*, electric organ discharge, genome modification, mormyrid, gymnotiform

Date Published: 10/27/2019

Citation: Constantinou, S.J., Nguyen, L., Kirschbaum, F., Salazar, V.L., Gallant, J.R. Silencing the Spark: CRISPR/Cas9 Genome Editing in Weakly Electric Fish. *J. Vis. Exp.* (152), e60253, doi:10.3791/60253 (2019).

## 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.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/60253/>

## 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<sup>1,2,3</sup>.

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<sup>4</sup>, a growing community of scientists has gained tremendous insights into evolution of development<sup>1,5,6</sup>, systems and circuits neuroscience<sup>7,8,9,10</sup>, cellular physiology<sup>11,12</sup>, ecology and energetics<sup>13,14,15,16,17</sup>, behavior<sup>18,19</sup>, and macroevolution<sup>3,20,21</sup>.

More recently, there has been a proliferation of genomic, transcriptomic, and proteomic resources for electric fish<sup>1,22,23,24,25,26,27,28</sup>. Use of these resources has already produced important insights regarding the connection between genotype and phenotype in these species<sup>1,2,3,28,29,30</sup>. A major obstacle to integrating genomics data with phenotypic data of weakly electric fish is a present lack of functional genomics tools<sup>31</sup>.

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<sup>32,33,34</sup> and non-model organisms<sup>35,36,37</sup> 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<sup>38</sup>. CRISPR has advantages over other knockout/knockdown strategies, such as morpholinos<sup>39,40</sup>, 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<sup>41,42,43,44</sup>.

### 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 *Brachyhyppopomus 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<sup>45</sup>. Weakly electric fish are also capable of detecting features of their conspecifics' EOD waveforms<sup>18</sup> as well as their discharge rates<sup>46</sup>, allowing EODs to function additionally as a social communication signal analogous to birdsong or frog vocalizations<sup>47</sup>.

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<sup>2</sup>. Non-electric teleosts express two paralogous gene copies, *scn4aa* and *scn4ab*, coding for the voltage-gated sodium channel NaV1.4<sup>30</sup>. In both gymnotiform and mormyrid weakly electric fish lineages, *scn4aa* has evolved rapidly and undergone numerous amino acid substitutions that affect its kinetic properties<sup>48</sup>. Most importantly, *scn4aa* has become compartmentalized in both lineages to the electric organ<sup>2,3</sup>. 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. Design sgRNA targets.

- 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.
- 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.
- Sequences can be prioritized using the method of Doench et al.<sup>49</sup> for on-target activity. Additionally, target sequences may be evaluated by a BLAST search against genomic/transcriptomic databases for off-target binding.
- 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).
- Design oligomers meeting the above criteria using the below template, which includes a T7 promoter (5' of N<sub>18</sub>) and a complementary region (3' of N<sub>18</sub>) for annealing to the constant oligomer (1.1.6): 5'-TAATACGACTCACTATAGG-N<sub>18</sub>-GTTTGTAGAGCTAGAAATAGCAAG-3'

**NOTE:** The N<sub>18</sub> sequence does not include the NGG protospacer adjacent motif (PAM) sequence. Do not include this in the oligomer.

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.
2. **Perform automated design of sgRNA targets.**
  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.
  2. Once the target sequence is identified, load the freely available EFISHGENOMICS CRISPR web tool ([http://efishgenomics.integrativebiology.msu.edu/crispr\\_tool/](http://efishgenomics.integrativebiology.msu.edu/crispr_tool/)). This web tool uses a customized version of the CRISPOR algorithm for target generation<sup>50,51</sup>.
  3. In the box for **Step 1**, enter the name of the sequence and enter the target's sequence into the appropriate box.
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.
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.
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.
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.
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

1. Anneal oligomers in PCR tube: add 1  $\mu$ L of 100  $\mu$ M constant oligomer, 1  $\mu$ L of 100  $\mu$ M sgRNA specific oligomer, and 8  $\mu$ L of nuclease-free  $H_2O$ .
2. Anneal oligomers in a thermocycler with the following program: heat at 95  $^{\circ}C$  for 5 min, cool to 85  $^{\circ}C$  at -2  $^{\circ}C/s$ , cool to 25  $^{\circ}C$  at 0.1  $^{\circ}C/s$ , and hold at 4  $^{\circ}C$ .
3. To generate sgRNA template, add 2.5  $\mu$ L of dNTP mix (10 mM), 2  $\mu$ L of 10x buffer, 0.5  $\mu$ L of T4 DNA polymerase, and 5  $\mu$ L of nuclease-free  $H_2O$  to the annealed oligomers from step 2.2. Incubate for 20 min at 12  $^{\circ}C$ .
4. Purify template using a PCR clean up column per the manufacturer's instructions.
5. Elute in 20–30  $\mu$ L. Use a spectrophotometer to estimate concentration and purity. The template should be 100–200 ng/ $\mu$ L and 1.8–1.9  $A_{260/280}$ .
6. Verify via an agarose gel by running 1–5  $\mu$ L. The dominant band should be 120 bp. See **Figure 1A** for representative results.
7. Store gel-verified sgRNA template at -20  $^{\circ}C$  (long term) or 4  $^{\circ}C$  (short term, 1–4 weeks).
8. Transcribe sgRNA using T7 RNA transcription kit by adding to a 1.5 mL microcentrifuge tube at room temperature 8  $\mu$ L of dNTP mix (equal volumes of dA, dT, dG, dC), 2  $\mu$ L of 10x buffer (room temperature), 2  $\mu$ L of T7 RNA polymerase, 100–200 ng of sgRNA template, and nuclease-free  $H_2O$  to a 20  $\mu$ L total volume. Spin to collect at the bottom. Incubate for 2–4 h at 37  $^{\circ}C$ .
9. **Add 1  $\mu$ L of DNase and incubate an additional 15 min at 37  $^{\circ}C$ .**
  1. Clean up sgRNA by adding 1  $\mu$ L of glycogen, 30  $\mu$ L of nuclease-free  $H_2O$ , 30  $\mu$ L of 5 M ammonium acetate and 180  $\mu$ L of 100% EtOH to transcribed sgRNA. Mix well and incubate at least 20 min at -80  $^{\circ}C$  (until frozen) or at -20  $^{\circ}C$  overnight. Centrifuge at 4  $^{\circ}C$  for 15 min at maximum speed.
10. Remove and discard supernatant without disturbing the pellet, and wash with 1 mL of RNase-free 70% EtOH chilled at -20  $^{\circ}C$ . Spin an additional 5 min at maximum speed at 4  $^{\circ}C$ .
11. Remove and discard the supernatant without disturbing the pellet, and air-dry the pellet for 5 min.
12. Resuspend in 30  $\mu$ L of nuclease-free  $H_2O$  and determine purity and yield using UV spectroscopy (minimum yield of 200 ng/ $\mu$ L).
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**).
14. Aliquot into 3  $\mu$ L and store at -80  $^{\circ}C$ .

## 3. Validate Cutting Efficiency In Vitro

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.
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. Clean up the PCR fragment with an established laboratory or company protocol.
4. Store the amplified DNA at -20  $^{\circ}C$ . Consider separating it into aliquots to reduce freeze-thaw cycles.
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.

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  $\mu$ L of 10x buffer 3, 1  $\mu$ L of 10x BSA (0.1 g/mL), 50–200 ng of Cas9 protein (1 mg/mL, 150 ng suggested), and 30–200 ng sgRNA (100 ng suggested).**
  1. Incubate the reaction at 37 °C for 1 h and then at 65 °C for 10 min to inactivate Cas9 protein.
  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<sup>52</sup> 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*.

1. **Conditioning**
  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).
  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).
  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. House fish normally in a relatively high conductivity (300–600  $\mu$ S), 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).
  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.
2. **Use spawning agent (SGnRHa + Domperidone) injections.**
  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.
  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<sup>53</sup>).
  3. Weigh the fish (g) to calculate spawning agent amount, (0.5  $\mu$ L/g) + 0.5  $\mu$ L. The extra 0.5  $\mu$ L accounts for pipetting errors.
  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.
  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.
  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.
  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).
3. **Obtain embryos through natural spawning.**
  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.
  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).
  3. The fish will spontaneously spawn during the dark photoperiod approximately 24 h post the spawning agent injection.
  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.
  5. Proceed immediately to microinjection (see section 5).
4. **Squeeze males for in vitro fertilization of *B. gauderio*.**
  1. Prepare the sperm extender solution (SES) as described in The Zebrafish Book<sup>54</sup>: 10 mM HEPES, 80 mM KCl, 45 mM NaCl, 45 mM sodium acetate, 0.4 mM  $\text{CaCl}_2$ , and 0.2 mM  $\text{MgCl}_2$ .

1. Use ddH<sub>2</sub>O to bring to volume and adjust pH to 7.7 with 1 M NaOH.
2. Store in fridge.
3. Filter through a 0.22  $\mu$ m filter before use.

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<sup>53</sup>). Place 500  $\mu$ L aliquots of SES into ice.
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. 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  $\mu$ L is common.
5. Using a micropipetter with a tip, carefully collect the sperm as it is squeezed from the male in 50  $\mu$ L increments. Place sperm directly into 500  $\mu$ L 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.
6. Immediately after collecting, put fish into fresh system water for recovery. The fish should only be out of water for 30–60 s.

#### 5. Squeeze females for in vitro fertilization of *B. gauderio*.

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<sup>53</sup>). 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.

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

#### 6. Perform egg fertilization for in vitro fertilization of *B. gauderio*.

1. Add 100  $\mu$ L of well-mixed sperm in SES (see step 4.4) directly over the egg mass.
2. Add 1 mL of system water (filtered through a 0.22  $\mu$ m filter) and mix well for 30–60 s.
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

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**.

#### 2. Prepare injection solution and prepare needle.

1. Add 0.9  $\mu$ L of sgRNA and 0.9  $\mu$ L of Cas9 enzyme (1 mg/mL) to 0.2  $\mu$ L 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.

2. Use a microloader pipette tip to backload the 2  $\mu$ L of solution into the microinjection needle. Expel the liquid as close to the tip as possible.
3. Load needle into micromanipulator and set pressure settings (start with 775 p<sub>i</sub>, 0.1–0.2 s, and 8–12 p<sub>c</sub>).
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. 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.
6. Adjust the needle tip or pressure settings to get an injection volume within specifications.

#### 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.

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**).
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.



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.
4. **Inject directly into single cell.**
  1. Align the eggs against the slide vertically, perpendicular to the approaching needle (**Figure 5B**).
  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**).
  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.
  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. 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.
  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

1. **Care for the eggs.**
  1. Check egg viability 4–6 h following injections.
  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.
  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.
  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.
2. **Care for larvae.**
  1. Check eggs 1–2x daily. Repeat steps 6.1.2–6.1.3 each time the larvae are checked.
  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.
  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*.
  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*.
  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. 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.
  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).
  8. After ~45 DPF, feed only blackworms until ~60 DPF.
  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

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

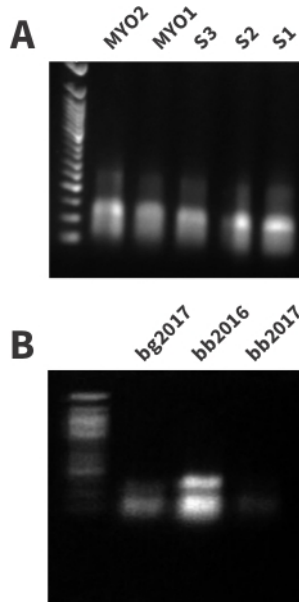
## Representative Results

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.

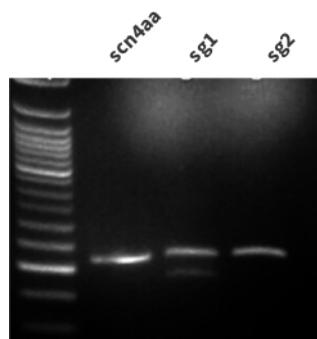
Adult fish were conditioned for reproduction (Section 4.1), then injected with a spawning agent (Section 4.2) and subsequently squeezed (*B. gauderio*) for IVF as described in Section 4.4 or allowed to spawn naturally (*B. brachyistius*) as described in Section 4.3. These efforts yielded single cell 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.

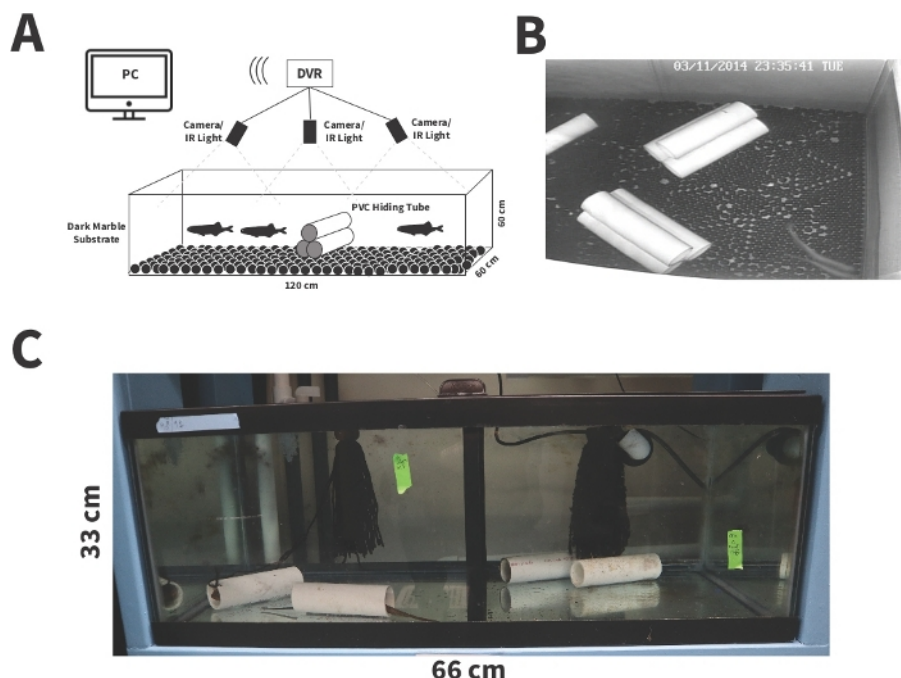
After PCR clean up and cloning, 30+ clones from each embryo were selected for Sanger sequencing. CRISPR/Cas9 induced mutations were identified in *B. gauderio* (**Figure 8A,B**) and *B. brachyistius* (**Figure 9A,B**) individuals with strong EOD amplitude reduction (**Figure 8C** and **Figure 9C**, respectively), 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 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. [Please click here to view a larger version of this figure.](#)



**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. [Please click here to view a larger version of this figure.](#)

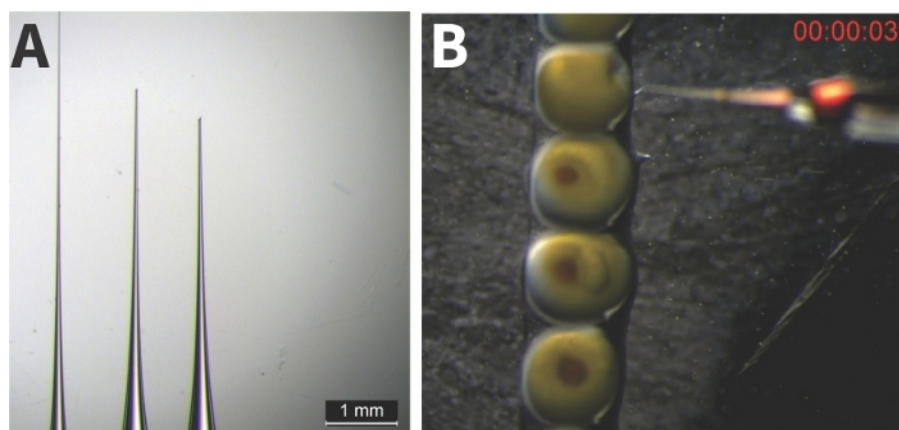


**Figure 3: Breeding tank setups for weakly electric fish.** (A) Schematic of the typical setup for wireless video monitoring of spawning behavior. Three commercially available CCTV cameras (Swann, Inc.) capable of producing infrared light are aimed at the top of the water and connected to a digital 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. [Please click here to view a larger version of this figure.](#)

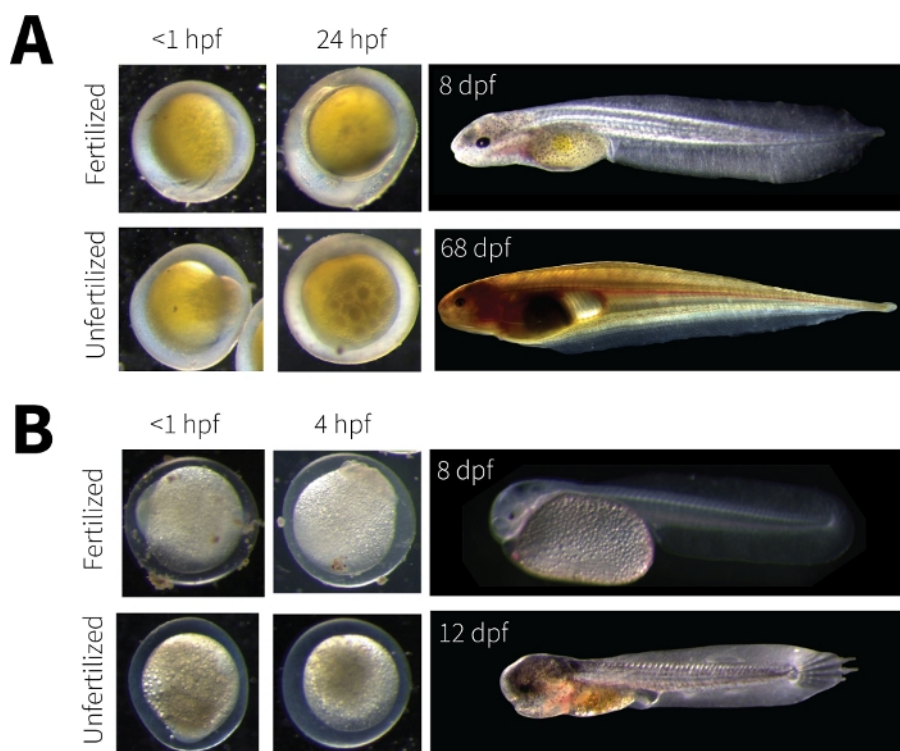


**Figure 4: Breeding males and females.** (A) *B. brachyistius* and (B) *B. gauderio*. Both species are sexually dimorphic and easily distinguished visually when sexually mature. Both females are gravid in these photos, exhibiting characteristically swollen bellies that are full of ripe eggs. [Please click here to view a larger version of this figure.](#)

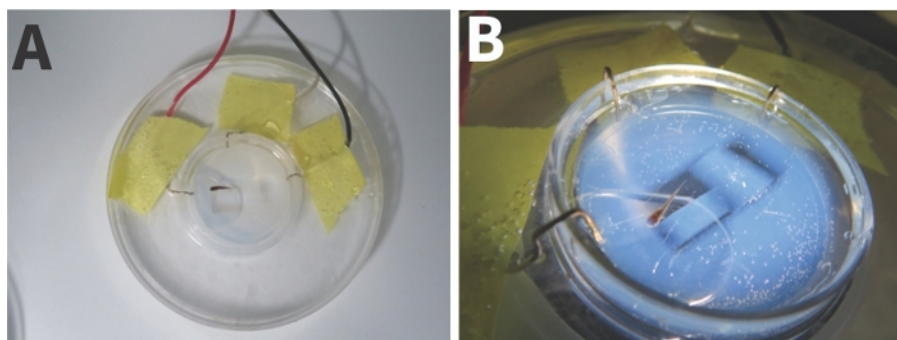




**Figure 5: Microinjection.** (A) Glass capillary needle tips must be broken to deliver an appropriate microinjection volume. The tip on the left is unbroken. The middle and right tips are broken with a slightly angled bevel to pierce the egg chorion. (B) Eggs are lined against a glass slide (1%–10%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



**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. [Please click here to view a larger version of this figure.](#)



**Figure 7: Photograph of larval recording chamber used in this study.** (A) The electrodes are embedded within Sylgard 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. [Please click here to view a larger version of this figure.](#)

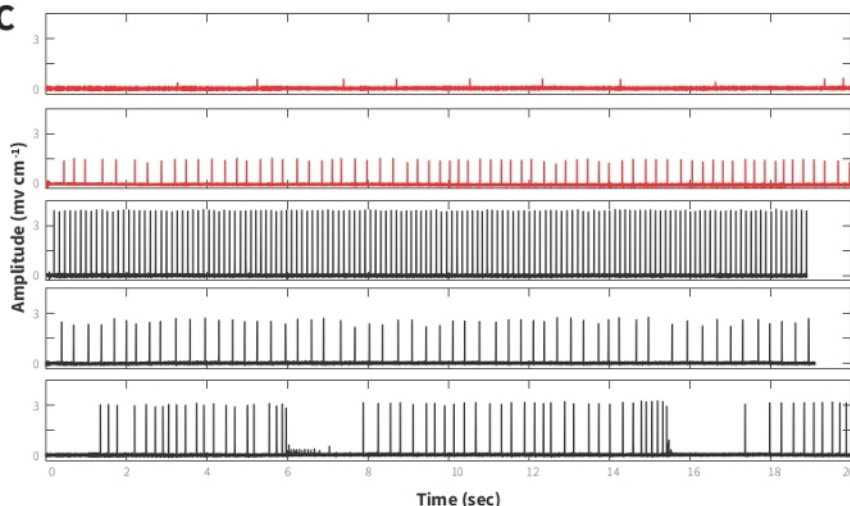
**A**

[illegible]**B**

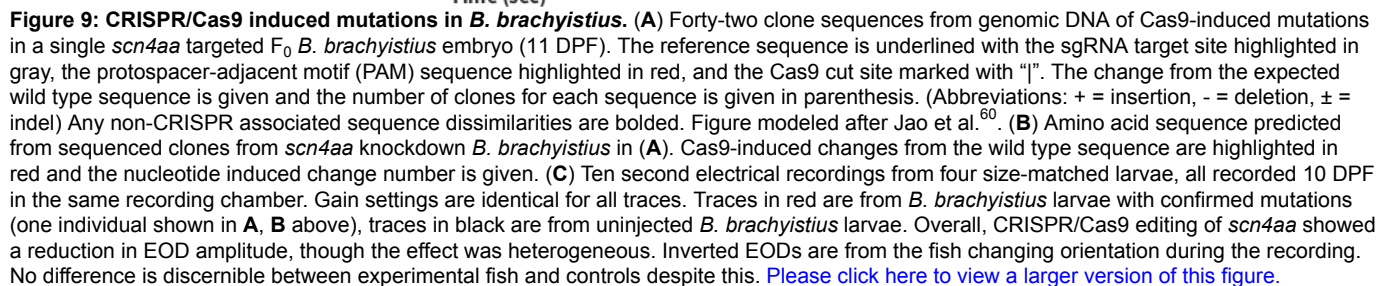
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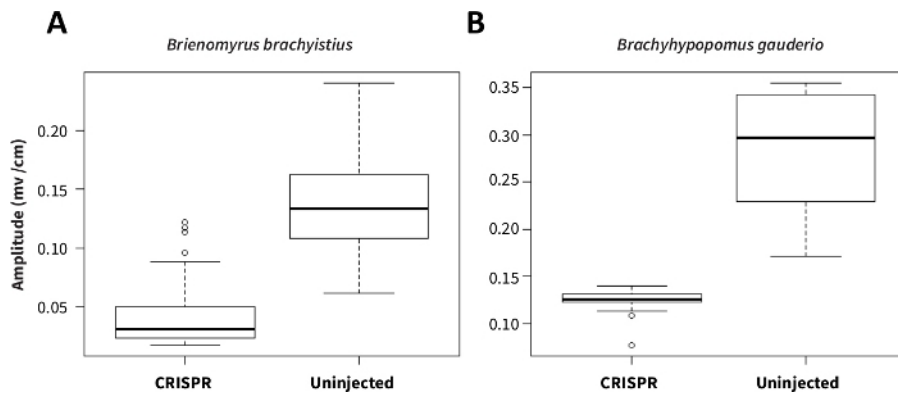
9 or 10/32 non-functional; ~28-31%

C



**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<sub>0</sub> *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.<sup>60</sup>. (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. [Please click here to view a larger version of this figure.](#)





**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. [Please click here to view a larger version of this figure.](#)

Description	Sequence
Constant oligomer	5'-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTTGC TATTTCTAGCTCTAAAC-3'
Target oligomer backbone (GG-N18, no PAM)	5'-TAATACGACTCACTATAGG-N18-GTTTTAGAGCTAGAAATAGCAAG-3'
Target oligomer backbone (N20, no PAM)	5'-TAATACGACTCACTATAG-N20-GTTTTAGAGCTAGAAATAGCAAG-3'
<i>Brienomyrus brachyistius</i>	
scn4aa Bb sgRNA target (N18, with PAM):	5'-TCTTCCGCCCCCTTACCACGG-3'
Scn4aa Bb sgRNA oligomer (GG-N18):	5'-TAATACGACTCACTATAGG TCTTCCGCCCCCTTACCA GTTTTAGAGCTAGAAATAGCAAG-3'
<i>Brachyhypopomus gauderio</i>	
Scn4aa Bg sgRNA target (N17, with PAM):	5'-CAAGAAGGATGTAGTGGAGG-3'
Scn4aa Bg sgRNA oligomer (GG-N17):	5'-TAATACGACTCACTATAGG CAAGAAGGATGTAGTGG GTTTAGAGCTAGAAATAGCAAG-3'
<i>scn4aa Bb PCR primer pair (218 bp)</i>	
Scn4aa_bb_exon1_F:	5'-ATGGCCGGCCTTCTCAATAA-3'
Scn4aa_bb_exon1_R:	5'-TCTTCCAGGGAATATTCATAAACT-3'
<i>scn4aa Bg PCR primer pair (204 bp)</i>	
scn4aa_bg_exon1_F:	5'-CGCCTTGTCCCTCCTTCAG-3'
scn4aa_bg_exon1_R:	5'-ATCTTCAGGTGGCTCTCCAT-3'

**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<sup>38</sup>, 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<sup>41,42,43,44</sup>. 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<sup>55,56</sup>. 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<sup>57,58</sup>. 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*<sup>59</sup> 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.

## Disclosures

The authors have nothing to disclose.

## 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.

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