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We use this protocol and it's working

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Cichlid genome modification

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ABSTRACT

Here we provide a microinjection protocol for the modification of cichlid fish via CRISPR or transgenesis.

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General considerations

1 Obtaining viable embryos can be the most trying part of generating transgenic fish. Putting in time up front to maximize survival, and to be able to get embryos on demand will be well worth your time!
If your cichlid species of choice is not a year-round spawner, get to know the conditions that will elicit egg laying.
Even year-round spawners have ovarian cycles that are weeks-long (*A. burtoni's* is ~4 weeks). With such long cycles, any given female is unlikely to be gravid at the time you are ready to inject the embryos. Therefore, I have a large cohort of females isolated from males, so that I increase the odds of getting a spawning female. Additionally, being able to identify those females that are ready to spawn allows me to collect eggs when I see signs that a female will lay eggs. For *A. burtoni*, this includes a distended abdomen packed with eggs, a protruding genital papilla, and aggressive behavior toward other females.
Before doing any injecting, ensure that you can collect fertilized eggs just after spawning and raise them to juvenile stage. This can allow you to see whether fertilization is efficient, and whether there are any other factors necessary to get the eggs to survive. Luckily for us, *A. burtoni* appears to survive just fine outside the female's mouth.

Fish housing

2 House a single stud male and a cohort of females separately by sex. We keep one male and 7-15 females in a 168 x 46 x 30 cm tank (~120 liters), separated by a barrier.

Microinjection needle production

3 Use a Sutter P-97 micropipette puller (or similar) to make needles from borosilicate capillary tubes (1.0 mm O.D., 0.58 mm I.D.; GC100F-10, Harvard Apparatus).
Puller settings: heat, 515; pull, 60; velocity, 100; time, 170.
You may need to adjust these settings based on chorion hardness, egg size, etc. This pulls a closed-tip pipette, which must be broken open.

Alternative settings if needle breakage during injection is high. These settings produce a smaller tip reducing the needle tip breakage.
Puller settings: heat, 505; pull, 45; velocity, 100; time, 205.

4 Break by gently tapping onto a taut Kimwipe.

Screen under microscope equipped with an ocular micrometer for needles of width 7.5-12.5 µm. Needles with >12.5 µm diameter in our experience have low survival rates (Li et al, 2021), while narrow diameters will lead to small volume delivery.
Alternatively, image analysis software may be used to measure the diameter of needles under microscope equipped with a camera.

5 Repeat to create tens of needles ready for use.

Injection station setup

6 Microinjections require a standard stereomicroscope (Nikon, SMZ745) equipped with a 3D micromanipulator (Narishige, M-152), and a source of pressurized air (Airgas, compressed dry air, size 200, adapter CGA-590) equipped with a Milli-Pulse Pressure Injector (Applied Scientific Instrumentation; MPPI-3).
https://www.asiimaging.com/manipulation-and-injection/mppi-3-pressure-injector/
Set pressure = 22 psi; Pulse duration = 2.5 ms. Set back pressure such that a small amount of injection fluid leaks out into the water, rather than water moving up into the needle by capillary action.

7 Confirm appropriate volume delivery.
In our experience, a 10 µm diameter needle delivers ~1 nL of solution when a 2.5 ms air pulse at 22 psi is delivered. We directly measure this volume by injecting 0.7% Phenol Red solution into a small petri dish of mineral oil (Sigma, M3516). The diameter of the resultant sphere of red solution can be measured under a microscope, and its volume calculated by $V = (4/3)*\pi r^2$. If the injection volume differs significantly from 1 nL, we recommend adjusting the air pulse duration.

Prepare molecular reagents for injection

8 **CRISPR guide RNAs**
- For details on synthesis of crRNA and tracrRNA plus Cas9 protein, see Li et al, 2023.

Tol2 transgenesis plasmids and transposase mRNA
- For details see Ma et al, 2015.

Prepare Embryo injection holder

9 *A. burtoni* embryos and maximizing the number of eggs that can fit securely within a single plate. The mold is placed inverted into 2% agarose solution (in tank water, boiled in the manner of a molecular biology agarose gel), poured into an appropriate container (we use the lid of a 6-well plate). The gel should be prepared at the beginning of the 30-minute window post-fertilization to

give it time to solidify prior to injections. It may also be stored at 4ºC for ~1 week in a sealed plastic bag.

9.1 3D-print file for embryo holding mold. The 3D mould will create different size indentations on the agarose gel allowing to fit broods and eggs of different sizes.
Variable_Mold_sketch2.stl

Obtain fertilized embryos

10 Remove barrier separating stud male and females.
Watch for spawning activity. In *A. burtoni* this typically occurs within 20 minutes of barrier removal. If no interest from females is observed, we replace the barrier and try on a subsequent day.
When egg laying is noted, we wait for **30** minutes for additional eggs to be laid and fertilized.

11 During egg laying and fertilization, prepare mixture to be injected.
CRISPR:
1.0 µL 25 µM Cas9 protein (final conc. 5 µM),
1.0 µL 25 µM crRNA:tracrRNA duplex (final conc. 5 µM),
2.5 µL ultrapure deionized H2O,
0.5 µL 2.5% Texas Red conjugated dextran (final conc. 0.25%)
- For details see Li et al, 2023.

Tol2 transgenics
1.0 µL 225 ng/µL Tol2 mRNA (final conc. 75 ng/µL),
1.0 µL 225 ng/µL Tol2-flanked construct plasmid (final conc. 75 ng/µL),
1.0 µL 2.5% Texas Red conjugated dextran (final conc. 0.75%)
- For details see Ma et al, 2015.

12 Collect water from spawning female's tank into a 500 mL beaker, and add 1:1000 Methylene Blue (stock concentration 1 mg/mL).
Net mouthbrooding female, and use a narrow-tip transfer pipet to deliver water into female's mouth, flushing fertilized embryos into beaker.

13 Fill 6 mL into each well of a sufficient number of 6-well plates to house all embryos individually. Wells can be sharpie-labeled with number for tracking and notes regarding each embryo's treatment and/or outcomes.

14 Cover agarose embryo holder with methylene blue-containing tank water, and transfer in embryos.

15 Using a paintbrush, insert the eggs into the injection holder by pressing the embryo gently into the individual indentations created by the 3D mould. Try to choose an indentation slightly narrower than the embryo but not too tight. Too tight can damage the embryo, resulting in white spots on the surface of the yolk or high internal pressure can lead to leakage from the injection site. Orient the germinal disc up or slightly angled towards the needle tip so that you deliver the injection construct in the disc and not the yolk.

Inject and raise embryos

16 Using 3D micromanipulator, advance needle to germinal disc of embryo. After contact with chorion, the edge of the embryo will flex (chorion is tough). When in contact with the edge of the embryo the needle is prone to bend, with the help of the 3D micromanipulator adjust the angle to reduce bend and possible needle tip breakage before applying more pressure to the chorion. Look for signs of the chorion springing back as the needle penetrates the chorion. Withdraw the needle slightly, pulse the injection mix once, withdraw slightly again, and pulse again. We find that 2-4 injection pulses deliver sufficient material and increase the likelihood of it reaching the nucleus of the developing embryo.

16.1 In between embryos, confirm that injection material flows through the needle tip by a) visualising injection material flowing through the needle tip, b) adjusting the back pressure, c) pulsing the injection mix. If the needle is blocked and no injection material is coming out, switch the Milli-Pulse Pressure Injector to continuous flow momentarily to unblock the needle tip. If this procedure does not work, switch to a new needle.

17 Repeat for each embryo, making notes about those embryos that received more/less injection material, those that burst open, those for which the needle readily penetrated chorion, etc. Identification of correlations between these differences and outcomes will help to identify key steps and features of the workflow that should be improved.

18 After injecting all the embryos, remove them one by one from the agarose and put them in individual wells of a 6-well plate. Using a metal spatula, break the agarose a few millimetres under the injected embryo and push the embryo up. Do not apply too much pressure since it might cause spillage from the injection site. Examine the embryo for signs of damage, such as some discolouration of the yolk (white spots), since it affects the survival of the embryos.

Raise embryos

19 During the first 10 days of development, put injected embryos on an orbital shaker to loosely mimic the tumbling provided by mouthbrooding females.

20 Track the survival of embryos regularly over the first ~10 days of life. Remove dead embryos from the wells to prevent microbial contamination of the remaining wells. Note the survival rates and the emergence of any phenotype, including transgenic fluorescence.

21 At ~10 days post-fertilization, cichlids will have consumed most of their yolk and should be rehoused, and feeding should begin. Move fry to ~1 liter tanks for grow-out. In our lab we feed flake food ground with a mortar and pestle.

By this stage, you may choose to select specific fish to transfer, based on expression of linked transgene (eg, coexpressed myl7:RFP red heart marker) or other phenotype.
However for most CRISPR gene editing, we transfer all injected embryos and screen for efficiency of mutation at ~4 weeks of age.

Genotyping injected fish

4w

22 We typically assess mutation rate by size analysis of PCR from finclip DNA. We do this at ~4 weeks post fertilization because we find relatively low death rates at this age. (We have also performed this under a stereomicroscope at 14 dpf with success, this it is more laborious.) 4w

23 We design PCR primers ~150 bp 5' and 3' from the targeted cut site, in order to amplify this region and analyze variation in amplicon size. To the 5' end of the forward primer, we add the M13 sequence (5'- TGT AAA ACG ACG GCC AGT -3'), and a "pigtail" sequence to the 5' end of the reverse PCR primer (5'- GTGTCTT-3') to ensure terminal adenylation.
We use a common fluorescent M13 primer (5'-/56-FAM/TGT AAA ACG ACG GCC AGT-3', IDT) that tags PCR amplicons with fluorescein that can be detected after size separation using capillary electrophoresis (3730xl DNA Analyzer, Applied Biosystems).

24 DNA extraction (modified HotSHOT).
- Transfer tissue samples to 0.2 mL tubes
- Add 180 µL of NaOH (50 mM) to each sample
- Heat at 95ºC for 15 min, then allow to cool to room temperature
- Add 20 µL of Tris buffer (1 M, pH 8.0) to neutralize pH.
We include as negative controls ~4 uninjected embryos and HotSHOT components without tissue. We dilute the extract 1:10 by adding 10 µL of the DNA sample to 90 µL of Tris 8.0 (100 mM) prior to PCR.

25 PCR mix for target amplification
- 14.1 µl Water (ultrapure)
- 2.0 µl 10x Standard Taq Polymerase Buffer (NEB)
- 0.5 µl Forward primer (10 µM)
- 0.5 µl Reverse primer (10 µM)

- 0.5 µl FAM-M13 primer (10 µM)
- 0.2 µl dNTP (10 mM)
- 0.2 µl Taq polymerase (NEB)
- 2.0 µL Embryo template DNA

- Amplify samples on a thermocycler: 94°C, 2 min; 94°C, 15 s, 55°C, 15 s, 72°C, 30 s, repeated for 35 cycles; 72°C, 7 min; cool to 4°C.
- Mix 7 µL of PCR product with 2 µL loading buffer and run on a 2% agarose gel to confirm successful amplification.

Gel electrophoresis may occasionally reveal additional bands with sizes that deviate from WT by ~20 bp or more, but most indels are <15 bp in size and cannot be resolved by this method.

26 Indel mutation detection. To quantify mutation prevalence by size analysis, vortex 20 µL of GeneScan 500 ROX Size Standard (ThermoFisher, 4310361) and add to 1 mL Hi-Di Formamide (ThermoFisher, 4311320). Add 1 µL of each PCR product to a well in a 96-well plate, followed by 9 µL of the ROX-Formamide solution, while empty wells receiving only 10 µL ROX-Formamide to serve as controls. Seal the plate with a rubber septa seal and analyze by capillary electrophoresis on the 3730xl DNA Analyzer (Applied Biosystems) with a default protocol module (GeneMapper50_POP7). We analyze amplicon sizes using Peak Scanner 2 (Thermo Fisher, freely available at resource.thermofisher.com/page/WE28396_2). Peak Scanner enables visualization of PCR amplicon sizes present. Uninjected embryos should have a single peak. Additional peaks in samples from injected embryos are indicative of indels: difference from wildtype size indicates size of insertion or deletion while the height of peaks correlates with its prevalence in the embryo.

27 To quantify mutation efficiency, we use an online tool, Fragment Analysis (fragmentanalysis.com). We output .csv files from Peak Scanner that contain the size and prevalence of each PCR size variant, which permits the quantification of the fraction of each allele present in each embryo. The website tool is used to establish a “Fragment Analysis Set” and conduct “Standard Fragment Analysis” to access the percentage of DNA fragments that fall into the ranges established by the set. After setting up analysis parameters, the .csv file from Peak Scanner can be uploaded to create a new fragment analysis report. The program quantifies the area under each peak, which is proportional to allele frequency within the mosaic tissue. The ratio of the area under the wildtype peak to sum of areas under all peaks represents the fraction of unmodified alleles. This approach can also be used to identify offspring of injected fish that carry a mutation. When analyzing these animals, it is expected that heterozygotes will display two comparably sized peaks.

28 Select animals for breeding. Identify those fish most likely to transmit mutations to F1 offspring. Typically these are those animals with the highest prevalence of mutations. However, care should be taken to consider whether there are likely to be fertility or lethality phenotypes associated with mutations, and then ensure sufficient numbers are bred.

Notes on breeding and analyzing mutant fish

29 Set up matings between selected fish carrying mosaic mutations (CRISPR) or transgenes with wild-type fish.
It is also possible to cross injected individuals, but see below for a discussion of limitations of this approach.

30 Collect offspring from mouthbrooding females, and raise them in 6-well plates or small aquaria. Screen these animals for evidence of successful genome editing.
- Dominant transgenic markers may be observed early in life
- CRISPR mutations or difficult to detect transgenes may require screening by PCR at age ~4 weeks of age.

31 PCR products from heterozygotes should be sequenced from each side of the cut site to identify indel location. The sequence of the mutant allele may be inferred from the overlapping sequences: additional chromatogram peaks are found where sequences diverge. The wild-type sequence can be masked and the mutated sequence determined, a process assisted by programs such as Poly Peak Parser. Alternately, TA cloning of PCR products can unambiguously reveal the alternate allele sequence. Animals carrying mutations of likely large effect (e.g., frameshift) should be maintained and bred. Furthermore, insertions or deletions of ≥15 bp can be detected through agarose gel electrophoresis. Using lines carrying such mutations will significantly speed the genotyping process for future generations, as mutant and wildtype amplicons can be readily separated on an agarose gel. If a mutation of small size is recovered, carriers can be detected using PCR primers that bind specifically to either the mutant or wildtype allele. Alternatively, if a restriction enzyme recognition site is created or destroyed by the mutation, these size differences may be detected after PCR and cleavage.

32 Experimenters may analyze animals at any generation of this process, though caveats apply to each. As CRISPR/Cas is a robust technology that often leads to high mutation rates, the injected (F0) fish often exhibit a phenotype. However, this founder analysis suffers from potential confounds. First, though CRISPR/Cas is reported to have a low rate of off-target gene modification, it may induce phenotypes due to mutation, particularly at homologous sites in the genome. Second, this approach is also prone to false negatives, as some cells of the mosaic animal carry cells with unaltered DNA, or mutations of weak effect. Third, the variety of mutations created lead to reduced reproducibility. Despite these concerns, we regularly analyze injected animals to obtain preliminary data, refine phenotype tests, and observe unanticipated phenotypes. Further crosses are warranted to obtain animals carrying mutations in all cells. One may intercross F0 fish, which will result in offspring carrying mutations in all cells, thereby increasing the likelihood of observing a phenotypic effect. Since CRISPR/Cas generates a variety of mutation sizes and sequences even within the germline, each offspring will likely carry a different pair of alleles, complicating interpretations. Furthermore, each parent may contribute mutation(s) in off-target genes to the offspring. Thus, it is important to outcross F0 animals to wildtypes in order to dilute the effect of off-target effects. Assuming that the on- and off-target loci are unlinked, each generation of outcrossing leads to a reduction by half of the co-inheritance of off-target mutations. Furthermore, unlinked off-target mutations will be inherited by siblings at equivalent rates, providing important controls for effects of this genetic

background. Ideally, therefore, crosses of heterozygous F1 (or later) generation fish will yield control genotypes in addition to homozygous animals for analysis, while controlling for off-target effects. We also recommend analyzing ≥2 independent mutant lines for analysis to ensure that phenotypes are reproducible.