Cichlid genome modification - Malawi cichlids

Forked from Cichlid genome modification

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ABSTRACT

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

This is our forked version of the protocol provided by Scott Juntti (https://www.protocols.io/view/cichlid-genome-modification-cj5wuq7e), modified for our Lake Malawi cichlid species for both NHEJ knock-outs and HDR knock-ins

These protocols accompany a review article on cichlid genome editing which contains more general considerations (link will be provided when available).

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We use this protocol and it's working

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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. Compared to *A. burtoni's* ~4 weeks, Malawi species cycles are similar (e.g. *Astatotilapia calliptera*) or longer (e.g. *Tropheops* and *Maylandia*). With such long cycles, any given female is unlikely to be gravid at the time you are ready to inject the embryos. Therefore, we have a large (7-14 individuals) cohort of females isolated from a male, to increase the odds of getting a spawning female. Additionally, being able to identify those females that are ready to spawn allows us to collect eggs when we see signs that a female will lay eggs. For *A. calliptera*, 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 fertilised eggs just after spawning and raise them to juvenile stage. This can allow you to see whether fertilisation is efficient, and whether there are any other factors necessary to get the eggs to survive. So far we have seen that *A. calliptera, Tropheops, Maylandia,* and *Rhamphochromis* survive outside the mother's mouth, though we suspect lower survival rates than those raised inside the mother's mouth especially for the first week or so of development.

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 80, 100, or 200 litre tanks (depending on the age/size of the fish), separated by a transparent barrier.

Microinjection needle production

3 Use a Sutter P-97 micropipette puller (or similar) to make needles from borosilicate capillary tubes GC100F-10, 1.0 mm O.D, 0.58 mm I.D, Harvard Apparatus).
Puller settings: heat, 545; pull, 30; velocity, 50; time, 200**.** Note that the outcome of needle pulling can vary between different individual pullers, even when using the same settings on the same model. Some adjustment of setting may be required to achieve the desired outcome.
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.

4 Repeat to pull tens of needles ready for use. We make needles in bulk in advance but only break as many as we need when when fertilised eggs are obtained (during the extra time required for fertilisation, see "10 obtain fertilised embryos" below)

5 Break needle tip by grinding away the edge tip with a beveller:
Secure needle in a micromanipulator. With two light sources from above but different directions, cast two shadows of the needle on the beveller stone. Focus the microscope on the surface of the stone and start the stone spinning. Using the micromanipulator, slowly bring the needle down towards the stone. The shadows will get closer together. When the shadows touch at the needle tip, the needle is in contact with the stone. Bring the needle a **very** small distance further down and leave for 5-10 minutes for the tip to be smoothly ground away.

We usually judge needle width by eye under the microscope and with test pulses to see volume of injection mix extruded. Needle opening width can be screened more precisely under microscope equipped with an ocular micrometer for needles of width 7.5-12.5 µm. Alternatively, image analysis software may be used to measure the diameter of needles under microscope equipped with a camera.
Needles with >12.5 µm diameter have low survival rates (Li et al, 2021), while narrow diameters will lead to small volume delivery.

Injection station setup

6 Microinjections require a standard stereomicroscope (Leica, MS5) equipped with a 3D micromanipulator (MM33 Micromanipulator, ASI), and a source of pressurized air (BOC, compressed oxygen-free nitrogen, size X, UN1066) 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 = 40 psi; Pulse duration = 1 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.
We aim to inject 1-2 nL of solution. This can be checked by directly measuring the 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 The mould is placed inverted into 1.5% low-melting agarose solution in tap water, boiled in the manner of a molecular biology agarose gel in 10-20 second bursts until dissolved/re-liquefied. Aim to melt without reaching the boiling point because the formation of bubbles while the agarose is setting can mess up the shape of the wells. Pour into an appropriate container (we use a plastic petri dish, and it is fine to re-use washed dishes) and add the mould teeth-down. Use the relevant mould with the correctly sized teeth for each species (for a new species with different egg sizes, a new mould may need to be made using the gradient-sized mould to test best-fitting teeth size).

Formation of large air bubbles can be avoided by adding the mould first at an angle with one end first and then tilting the rest down. After a few moments check there are no small bubbles forming on the teeth of the mould. If there are, the agarose is too hot. Pull the mould out, dragging the small bubbles to the edge of the petri dish, leave it for a few moments to cool down, then try adding the mould again. To speed up the agarose setting, you can put the dish in the fridge for a few minutes, but take it back out with enough time for it to return to room temperature (at least 15 minutes) before injecting.

The gel should be prepared at the beginning of the 40-minute window during fertilisation to give it time to solidify prior to injections. It may also be stored at 4ºC for ~1 week in a sealed plastic bag or covered in water in the petri dish.

9.1 3D-print files for embryo holding mold. We use the variable mould to test which size is best for each species (as egg size varies between species and populations - see Marconi et al., 2023 https://doi.org/10.1111/ede.12429 for examples) and then make a dedicated mould for that species. The 'mbaka' mould files here are designed for our *A. calliptera 'mbaka'* lab population. We currently use the round cone file: it has rounded teeth which gives smoother-edged agarose wells. mbaka_round_cone_2.2mm.stl mbaka_2.1.stl Embryo_mold_1.8-2.8mm_v2_mesh-Body.stl

Obtain fertilized embryos

10 Socialise the cross tanks: remove barrier separating stud male and females.
Watch for spawning activity. In *A. calliptera* this typically occurs within 40 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 40 minutes for additional eggs to be laid and fertilized.

We find egg-laying happens more reliably when fish are **very** well fed and socialised **every** day (we often leave the dividers out over the weekends to keep up reliable egg laying. Even if a clutch is laid over the weekend that wasn't used for injections, for us this is better than potentially losing a week of any egg-laying). After a period apart without frequent socialising, it takes several days (or even weeks) for breeding to resume, and the first clutch(es) after such a break are usually bad quality. This is worse the longer the break is.

Some of the species we have tried do not respond well to breeding on-demand from divided housing like this. *Tropheops* and *Rhamphochromis* species would not breed under these conditions. Instead fertlised single cell embryos can be obtained by frequently checking tanks (with normal mixed housing) during the day for signs of courtship behaviour. This is less efficient and leaves much less control over timing and frequency of possible injections.

11 During egg laying and fertilization, prepare mixture to be injected.

If using two guides for NHEJ

Reagent	Aliquot concentration (ng/ul)	Volume (ul)	Final concentration in 2.5ul mix (ng/ul)
Cas9	500	0.5	100
Guide 1	500	0.75	150
Guide 2	500	0.75	150
Texas Red	2.5%	0.5	0.5%

This gives the same concentration of gRNAs combined as a single guide below

If using one guide for NHEJ

Reagent	Aliquot concentration (ng/ul)	Volume (ul)	Final concentration in 2.5ul mix (ng/ul)
Cas9	500	0.5	100
Guide	500	1.5	300
Texas Red	2.5%	0.5	0.5%

This gives the same concentration of gRNAs as two separate ones above

If using one guide for HDR

Reagent	Aliquot concentration (ng/ul)	Volume (ul)	Final concentration in 2.5ul mix (ng/ul)
Cas9	500	0.5	100
Guide	1000	0.75	300

HDR	166	0.75	50
Texas Red	2.5%	0.5	0.5%

This gives the same concentration of gRNAs as a single guide above

12 Collect water from spawning female's tank into a 500 mL beaker. We do not add Methylene Blue due to allergies (contact dermatitis developed by repeated exposure).
Net mouthbrooding female, and use a plastic pasteur pipette to deliver water into female's mouth, flushing fertilized embryos into beaker.

13 Fill 6 mL of water into each well of a sufficient number of 6-well plates to house all embryos individually. We use tap water with antibiotics added (Sigma P4333 with 10,000 units penicillin and streptomycin 10 mg/mL; diluted 1:1000). We do not use Methylene Blue due to allergies, and in our experience fungal infections would not become an issue until later development i.e. after hatching. Not using methylene blue requires close attention to removing dead embryos (see "20 raise embryos" below)

Wells can be sharpie-labeled with number for tracking and notes regarding each embryo's treatment and/or outcomes.

14 Remove the mould from the set agarose - gently, to avoid bringing away the agarose with the mould. Using a spatula to score (but not cut through to the dish plastic) the edges of the mould where it meets the agarose may help. Cover agarose embryo holder with tank water, and transfer in embryos.

15 Using a small metal spatula, 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.

Embryos that already have signs of damage (e.g. white spots on the yolk, discolouration) before this point are unlikely to survive and probably not worth injecting.

Load the needle using half the injection mix. Reserving the other half means it is quicker to change to a new needle if there is a problem with the first or it breaks.
Put the needle into the micromanipulator. Tighten both grips. Check the range of motion is sufficient to reach the eggs.
Bring the focus higher to see the needle tip out of the water and check that a pedal tap causes a drop of injection mix to come out that is appears the right size. If no liquid comes out, check the needle is in tightly enough and check the pressure. May need to break the tip open further if the first break was too small. Once the needle is loaded, it is better to do this extra breaking using a kimwipe by hand.

Inject and raise embryos

16 Line up the needle with the first egg, with the microscope focus on the chorion.

See Fig 2 of Marconi et al., 2023 https://doi.org/10.1111/ede.12429 for familiairising with egg morphology

Using 3D micromanipulator, advance needle to germinal disc of embryo. Firmly press the needle tip into the chorion over the cell. 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. The aim is to enter the cell without going through to the yolk.

Look for signs of the chorion springing back as the needle penetrates the chorion. Withdraw the needle slightly, pulse the injection mix once with the pedal, 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. A small faint patch of red/purple should be just about visible inside the cell. Avoid the needle penetrating the yolk.

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. Change the water of each well every day by discarding at least 80% of water from the well and replacing with new water (this does not have to be prepared fresh each time, but can come from a bottle of pre-prepared tap+antibiotics water so long as this bottle is not more than a few weeks old).

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. We screen embryos for fluorescence from co-injected Texas Red at 1-3dpf.

21 At ~15-20 days post-fertilization, cichlids will have consumed most of their yolk and should be rehoused, and feeding should begin. Move fry to nets in small tanks for grow-out. In our lab we feed fry artemia (prepared from Hobby Artemix 21100).

For most CRISPR gene editing, we transfer all injected embryos and screen for efficiency of mutation from ~6-12 weeks of age.

Genotyping injected fish

4w

22 **We typically assess mutation rate by Sanger sequencing of PCR from finclip DNA. We do this at ~6-12 weeks post fertilisation because fins are large enough for fish to comfortably withstand fin-clipping and a greater amount of DNA can be extracted from larger fin clips, using fast and easy column-free kits (we use PCRBIO Rapid Extract Lysis Kit, PB15.11).**

4w

We have taken fin clips as early as ~20dpf but this requires careful and precise fin-clipping technique, and the smaller fin tissue volumes limits choice of DNA extraction kit to the more efficient but higher effort column-based methods (we use Zymo Quick DNA/RNA Miniprep D7003).

23 We design PCR primers at least 150 bp 5' and 3' from the targeted cut site, in order to amplify this region and send PCR products for Sanger sequencing

24 DNA extraction

We use PCRBIO Rapid Extract Lysis Kit PB15.11, or Zymo Quick DNA/RNA Miniprep D7003

25 PCR mix for target amplification. We use PCRBIO Taq Mix Red PB10.13.

	A	B	C	D
	Reagent	50µL reaction	Final concentration	Notes
	2x PCRBIO Taq Mix Red	25.0µL	1x	
	Forward primer (10µM)	2.0µL	400nM	see PCRBIO manual for optimal primer design
	Reverse primer (10µM)	2.0µL	400nM	
	Template DNA	<100ng cDNA, <500ng genomicTypically 2-20ul (higher for smaller fin clips or tricky PCRs)	variable	see PCRBIO manual
	PCR grade dH2O	Up to 50µL final volume		

- Amplify samples on a thermocycler:

	A	B	C	D
	Stage	no. of cycles	Temperature °C	Time (seconds)
	Initial denaturation	1	95	60
	Denaturation	40	95	15
	Anneal	55 to 65 depending on primers	15	
	Extension	72	1 to 120 (minimum 20 seconds per kb)	
	Hold	1	12	-

We use extension times longer than the minimum calculation in the PCRBIO manual

- Run 5-10µL 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 Purify by column-based kit, we use QIAquick PCR Purification Kit 28104

Dilute to required concentrations for sequencing and submit to a sequencing facility for Sanger sequencing (we use the facility at the Department of Biochemistry, University of Cambridge https://facilities.bioc.cam.ac.uk/dna-sequencing/sanger-sequencing)

27 Sequencing files are in .ab1 format. Analyse in Synthego ICE and/or Geneious.
We use the two tools in combination.

To use Geneious:

1. Import the files into Geneious
2. Trim the files by highlighting the high-quality areas spanning the approximate length of the PCR product and “Extract Regions”
3. Select all files plus the sequence file for the region of interest annotated with e.g. cut sites and align using Pairwise Align, MAFFT alignment.
4. View the alignment
   If looking for successful CRISPR mutations, look for sudden drops in trace quality and cohesiveness starting at cut sites. Search for the guide predicted cut site to see if it aligns with drops in trace quality.

To use the Synthego CRIPSR edits analysis tool,

1. prepare and upload a spreadsheet of samples using the template file provided for download on the analysis page
2. prepare and upload a zip folder of samples and a WT sample file
3. submit for synthego to analyse.
4. Interpreting the results:

If testing mosaic mutants:

- the percentage mutant reflects the proportion of cells in the individual that are mutated
- the range of mutations are listed in their proportions.

If testing WT x mosaic F1 offspring:

- % close to 0 are WT and % in the region of 50% are heterozygotes.
- Take the most common mutant sequence as the mutant allele sequence
- Check if the mutant sequence causes a frameshift mutation (ie not an indel of a multiple of three)

28 Select mosaic 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.
- heterozygous CRISPR mutations 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. See step 27 for identifying heterozygotes from sequencing files using Synthego ICE.

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.
However, 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.

We breed biallelic mutants via a combination of incrosses and outcrosses.