**Editorial comments:**  
Changes to be made by the author(s) regarding the manuscript:  
*1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

The manuscript has been proof-read, and minor grammatical and other typographical errors have been corrected.

*2. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”*

A summary section has been included.

*3. Abstract (line 39): Please do not include references here.*

References have been removed from the abstract.

*4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.*

The numbering has been adjusted accordingly.

*5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Gene Tools, LLC, Integratred DNA Technologies, Ambion, mMessage mMACHINE, Qiagen, Kim® wipe, etc.*

All commercial names have been removed from the manuscript.

*6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.*

All steps in the protocol section have been revised to state action phrases. We have included notes sparsely, where we felt appropriate. Finally, the discussion has been removed from the protocol section and places in the appropriate place.

*7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.*

The protocol has been revised to include more detail, which we hope better guides the reader. We have also included a citation for the CRISPR/Cas9 generation methods. This citation will point the reviewer to a detailed overview of CRISPR/Cas9 design and implementation.

*8. Please ensure that conditions and primers are listed all PCR procedures.*

We have added the requested details. Primer sequences for gene manipulation using CRISPR/Cas9 are provided in the text, as well as in the main figure.

*9. Line 239: How to record the concentration of the gRNA?*

We have now added details for determining concentration of gRNA. We typically do this using as nanodrop, and this has been explicitly stated in the manuscript.

10. Please include single-line spaces between all paragraphs, headings, steps, etc.

Single-line spaces have been used between all paragraphs, headings and steps.

11. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Done

12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please note that design steps are not appropriate for filming.

Done

13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Done

14. Line 569: For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis).

The references have been reformatted to comply with JoVE guidelines

15. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

Done

16. Please remove the embedded table(s) from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Done

17. Tables: Please change “μl” to “μL”. Please remove commercial language (CutSmart, etc.)

Done

18. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique  
19. References: Please do not abbreviate journal titles.

We have modified the discussion to highlight the above points.

**Reviewers' comments:**  
  
  
  
**Reviewer #1:**  
  
Manuscript Summary:  
The authors provided detailed protocols for manipulation of gene expression in a special cavefish, Mexican tetra (Astyanax mexicanus). These methods are critical for further genetic and functional investigation.

We thank the author for reviewing the manuscript, and for their feedback. Each point below has been addressed.

Major Concerns:  
Lines 254-255: Why didn't use the published genome sequence of this cavefish for designing Tol2 constructs?

We initially decided to use well-established Tol2 vectors, which are widely used in zebrafish research. This allowed us to optimize the protocol using reagents that we knew worked well. However, since establishing this protocol in *Astyanax mexicanus*, subsequent studies can use the published genome sequences to target specific genes of interest.

Lines 252-298: Where is the description of Table #2 in the main text?

We reference the Table in Step #4.2.4

Minor Concerns:  
Please don't use abrreviations for certain normal terms, such as spec on lines 264 & 280.  
The authors are recommended to polish the manuscript carefully, since it is not a note records of experiments.

Abbreviations have been removed, and the manuscript has been proof-read.  
  
**Reviewer #2:**  
  
Manuscript Summary:  
The author described the procedure to generate the gain of function and loss of function in A. mexicanus with detailed parameters and impressive pictures. In overall, the description and protocols were clear, which is ideally to extent the protocols to other fish species. However, there are more data should be provided as the requirement for Journal of Visualized Experiments, the efficacy of the protocol must be demonstrated. Therefore, a minor reversion need be made before accept.

We thank the reviewer for providing thoughtful comments. We have modified the figures and the text to address most of the concerns raised. Unfortunately, we did not systematically keep track of survival for either transgenesis or MO-injection, but we have not observed notable number of deaths when compared to controls. All other points have been addressed fully.

1. In figure 1, the author obtained the morpholino-fish and showed the final effect, how many embryos (n=?) they used for Surface fish and Pachon fish? How many embryos show effect?

We did not systematically track this. On average, the MO does not seem to affect larvae grossly.

2. In Figure 2, the Oca2 gene was disrupted by Crispr/Cas9, and evidently proved by PCR and final mutant fish. Is it possibly and necessary to provide the original sequencing photo/data to show the deletion of 2-bp near PAM? And how many mutants they obtained from the sequenced clones? Another question is: Crispr/Cas9 always give more than one type of deletion in the target region, whether the author observe other type of deletion in the PAM region?

We focused on sequencing of alleles that were transmitted through the germline, and which we could use to establish lines. These sequencing results have been added to the manuscript, notably in Figure 2B-C. Regarding the differences in mutations among individuals: we established 2 lines from two different founder fish, each of which contained a different mutation.

3. In figure 3, they already obtained the transgenic lines and looked great. What is number of injected embryos and the ratio to get the transgenic line?

We also did not track this systematically. We do see that more death following injection in cavefish, but it is not severe. We also expect that this will vary with different constructs.

**Reviewer #3:**  
  
Manuscript Summary:  
The authors describe a protocol for three approaches for studying and manipulating gene functions in A. mexicanus. The protocol included the use of morpholino, tol2 transgenesis and CRISPR-Cas9 gene editing. Morpholinos were used to knock down the neuropeptide hcrt to study its role in sleep regulation. Oca2 gene that is responsible for albinism was knocked out using the CRISPR-Cas9 system. Tol2 transgenic approach was used to generate transgenic fish expressing the GCaMP6s gene.

We thank this reviewer as well for their thoughtful feedback. We have addressed the concerns raised, with in the manuscript or through modified figures. A detailed list of the changes is found below.

Minor Concerns:  
  
Where did the authors microinject the embryos? in the cytoplasm of the one-cell stage or in the yolk? This should be clarified.

We inject into one-cell stage individuals, and we target the yolk. This has been stated explicitly in 5.4.8

Line 187: morpholino

Thanks. This has been corrected.

Line 197-201: It appears that the authors designed their gRNA manually? There are several online tools that are time-saving and usually design more efficient oligos. Those tools generate several oligos for the target genes and rank them by a quality score. Some of those tools also check for potential off targets against some available genomes.

We did design our gRNAs manually, as many of the online tools do not include the cavefish genome. Some online tools now include cavefish, such as <https://crispr.cos.uni-heidelberg.de/>, but we have never used this tool, and cannot assess whether it produces better results.

Line 203-206: the first sentence of the note is confusing to me as it appears that the authors suggest that a gRNA can be designed for a genomic target that doesn't have a PAM sequence (NGG)?

This refers to the GGs at the 5’ end of the gRNA, not the PAM sequence. We have clarified within the text that this refers to the 5’ end.

Line 231: check the sequence of the 3' gRNA primer for mismatches (at least 4 or 5 nucleotides). Is there a reason for this mismatch? Also, why does the 3' gRNA primer begin after 5 nucleotides from the end (the first 5 nucleotides are not included)? Is this a requirement of the primer design, and will not affect the binding of the final oligo with Cas9 protein?

The sequence of the oligo B in the text was incorrect. We have updated this, and thank the reviewer for noticing.

Line 233: what are the PCR conditions? How much do you add of each oligo and other PCR components? How do you check the yield? This should be described, or a reference should be provided.

For PCR, we followed previously reported conditions, Kowalko et al. (2016), and have cited this paper. We have made this clear in the manuscript.

Line 278-283: Step f. is a repeat for step d.

Thank you. We have omitted the duplicated step.

Line 289-290: how long is the incubation time?

For all incubation time throughout the protocol, we follow the guidelines provided by the manufactures of whatever kit we use. For Tol2 step, we use 2 hours, as suggested by the mMessage machine protocol.

Line 329: how much feed do you regularly add so that the 3X can be estimated (% of body weight?)

3x here refers to number of feedings per day, not 3x as much food. We regularly feed fish 1-2x per day. We have tried to make this clear in the manuscript.

Line 334: ZT is defined at line 341 and should be defined at the first time you mention it.

Thank you for bringing this to our attention. The definition has been added earlier.

Line 337: the two statements are confusing regarding the time of check for eggs. If the first one is for surface fish, this should be mentioned.

For reasons that we do not understand, surface fish and cavefish spawn at different times of the day. We have tried to clarify this point in the manuscript.

Line 356: I guess you warm the agarose plates to room temperature after you get them from the refrigerator and before placing the embryos. If so, it is important to mention that here to avoid the harmful effects of temperature fluctuations on embryos.

Yes, the reviewer is correct – plates are warmed. We have added this detail.

Line 363: how do you backfill the injection needles? it should be described.

Needles are backfilled using a standard gel-loading pipet tip. This has been added to section 5.4.1.

Line 374: this step can be deleted.

Thanks. We’ve removed it.

Line 383: how long does the one-cell stage last? This will help researchers to plan a head so that they inject before the embryos reach the two-cell stage.

The one-cell stage lasts for approximately 40 min. We have added this to step 5.4.9.

Line 399: Transposase mRNA not Cas9 mRNA.

Thanks. This has been corrected.

Line 408: what's the size of the glass bowl? Or what's the embryo stocking density, per liter for example?

The size of the glass bowl is 10 x 5 cm. This has been added to step 6.1.1.

Line 423: check the step numbering (2.2.A, 2.2.A)

Thanks. This has been corrected.

Line 444-449: Do you generate lines of fish that are mutant for one or both alleles? The procedures mentioned here can generate mutant fish for one allele but not both. It should be clarified here.

Yes, the reviewer is correct. For the results given here, we have generated a ‘Molino-specific’ allele. We have clarified that this procedure is to establish lines of fish transmitting mutant alleles

Line 452: state the figure number.

This has been fixed.

Line 460-461: a reference for a PCR screening protocol may be provided here.

Whereas this is commonly done in zebrafish, a detailed protocol describing the approach is typically not provided. We have looked for one, but as far as we can see, there is not standard reference for it.

Line 520: "25 pg of gRNA was injected into 1-cell stage surface fish embryos, along with 300 pg Cas9 mRNA" is already mentioned in the methods and can be deleted.

Done.

Line 747: SB should be defined here and in other figures.

Scale bars have been included.  
  
Table of materials  
Morpholino supplies: Standard control oligo.

*In compliance with data protection regulations, please contact the publication office if you would like to have your personal information removed from the database.*