**We thank the reviewers for carefully reading the manuscript and for providing constructive edits and suggestions. Below is a point-by-point response to the concerns that were raised.**

*1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.  
2. Line 9: Please provide the address for the affiliation.  
3. Keywords: Please provide at least 6 keywords or phrases.  
4. Please expand the Summary to briefly describe the applications of this protocol.  
5. Please spell out each abbreviation (RO, PBS, DMSO, DAPI, etc.) the first time it is used.  
6. 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: CCS Starter Kit, Reed Mariculture, RGcomplete, Triton, etc.*

**We have made all of the edits (1-6) suggested above.**

**The summary now reads:**

***” In this protocol, we demonstrate how to breed A. mexicanus adults, raise the larvae, and perform whole-mount immunohistochemistry on post-larval fish to compare the phenotypes of surface and cave morphotypes.”***

*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:  
1.1: Please provide more details, e.g., the fish species, number of fish in the tank, size of the breeding tank, etc.*

**We have added more details to the protocol steps. Since the breeding method was brought up by several reviewers, we now include additional steps at the start of our protocol that describe how to breed the fish. We also reference several alternative methods.**

*4.1: Is the rotifer culture prepared in step 3.2?*

**The distributor of the rotifers provides detailed instructions on how to set up the rotifer culture (including a video on their website). We therefore referenced their protocol at this step. Step 3.2 is to create the solution that will be added to the nursery cups. We edited the text from step 3 to 4.1 to make it more clear. It now reads:**

***“3. Prepare the rotifer-based fish food***

***3.1 See table of materials for obtaining rotifers. Follow the referenced protocol to set up, maintain, and harvest rotifers* 1*.***

***3.2 Prepare fish food by adding 3mL algae mixture (see table of materials) to 1 liter of harvested rotifers. This mixture will be added directly to the nursery cups as a food supply.***

***4. Feeding post-larval fish***

***4.1 When the fish are five days post fertilization (dpf), add 3mL fish food to each nursery cup.…”***

*5.4.1: Please list an approximate volume to prepare.*  
*5.4.3, 5.4.5: Please give an example of antibody (including concentration) that will be used in the protocol.*  
*8. 1.6: Please convert temperature to °C instead of °F.*  
*9. Lines 158-159: Please remove the google doc link; instead please upload the file as a Table or Supplemental Information.*  
*10. Please include single-line spaces between all paragraphs, headings, steps, etc.*

*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.*  
*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 do not highlight any steps describing anesthetization and euthanasia.*  
*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.*

**We have addressed the concerns listed above.**

*14. Discussion: Please also discuss any limitations of the technique, the significance with respect to existing methods, and any future applications of the technique.*

**We have added additional text to the discussion to highlight limitations, significance, and future applications. For example:**

***…”Rotifer culture is inexpensive and easy to maintain but requires daily feeding…”***

***“…Fish size may influence penetration; this protocol has not been tested on fish that are greater than 14 days old”***

***…Standard husbandry protocols, both within and among laboratories, are essential to understanding the biological differences between surface fish and cavefish. Our article provides a method….***

***…The protocol described here can be adapted at each step and used to detect antigens in any tissue of interest….***

*15. References 2-5 and 10: Please provide volume (issue) and page numbers. Please provide complete citation information for reference 21.*

**We edited the references.**   
**Reviewers' comments:**  
*Reviewer #1:  
Manuscript Summary:  
This is a clearly written and straightforward MS and I am happy to recommend its acceptance. I have a few suggestions, but they are all minor and should the authors prefer to reject them, that is just fine too.*  
*1) The title might usefully be changed to help the ignorant, like me, know what Astyanax is! (For example "Raising the blind cave fish (Astyanax…) for …; or, "Raising the Mexican tetra Astyanax…) for …).*

**We changed the title to include “Mexican tetra”**

*2) Point 5.3.1 (line 137): I assume the tricane solution is already ice-cold (otherwise the ice bath won't do much in 10 min.). Perhaps clarify?*

**We changed the text to: *Gently submerge the strainer in ice-cold tricane solution and leave on ice for 10 min.***

*3) Line 141: definition of PBS (similar queries apply elsewhere).*

**In the updated protocol, we define PBST at the first use and refer the reader to the materials section.**

*4) Line 143: Personally I prefer such concentrations in grams/L (but if you leave it as it is I think 0.02% is clearer).*  
*5) Line 148. The vial is 5 ml here and 4 ml in the Table of Materials.*

**We changed line 148 to the correct description, 4mL.**

*6) Line 161: be explicit about what "washing" means.*

**We changed the text for clarity. It now reads:**

***5.3.3 Remove the fixative and replace with phosphate buffered saline-triton solution (PBST, see materials). Incubate for 15 minutes at room temperature with rocking.***

***5.3.4 Remove PBST and replace with fresh PBST and incubate for 15 minutes. Repeat this “washing” one additional time.***

7) Line 169: 5.4.7. Maybe explain why you DAPI stain?

**We changed the text to eliminate this optional step.**

*8) Line 212-217. Figure 1.  
a. Identify the (false?) colours in the overlay rather than have us work it out. I assume the blue is DAPI?  
b. Add a reference to Hu expression in fish (and maybe use anti-HuC/HuD in the text to agree with Table 2?).  
c. The mention of elav-4 is a bit confusing to me (I thought HuD was ELAVL-4, for example). Maybe just omit?  
d. Mention tubulin in the legend.  
e. If there are published images from sections that can be compared to your Fig. 1, it would be convincing to reference them.*

**We have added a reference to our article showing Hu expression in *A. mexicanus* and additional panels to figure 1. We have also updated the figure legend using the reviewer’s suggestions. Please see the revised figure and legend.**

*9) Line 232: fungal not fugal.*  
*10) Line 249-250. I'm not clear what the phrase "while BSA similarly competes with the primary antibody for binding" is getting at. Maybe rephrase.*

**We rephrased this paragraph. The text now reads:**

***The blocking step is essential to prevent antibodies from binding to non-target proteins in the tissue. This method uses a combination of normal serum (5%) and BSA (0.2%) in the blocking solution. The blocking solution contains antibodies and proteins that bind to reactive sites on proteins in the tissue, diminishing non-specific binding of the primary and secondary antibodies.***

*11) Add somewhere that reagent penetration is not a problem (and how you know: and also mention how big these post-larval fish are, and what exactly is post-larval?).*

*12) What ages of fish does this method work for?*

**We have added the following text to discuss reagent penetration and fish age/size:**

***Triton and DMSO are included during the blocking and antibody incubation steps at a concentration of 0.5% and 1% respectively. Using this concentration we have observed staining in the brain, pancreas, intestine, and muscle suggesting it is likely effective for penetration of all tissues. Fish size may influence penetration; this protocol has not been tested on fish that are greater than 14 days old (approximately 7mm in length).***

**We have edited the text in several locations to make the distinction between larval (has yolk) and post-larval (no yolk):**

***…”Less is known about how the populations differ at post-larval stages (at the onset of feeding) although it could provide insight into how cavefish survive to adulthood in their natural environment…”***

***“…At 5-days post fertilization, the fish have developed to post-larval stages (no longer having a yolk supply) and are provided algae-fed Brachionus plicatilis (rotifers) as a nutrient rich food source…”***  
  
  
**Reviewer #2:**   
*Manuscript Summary:  
The article by Riddle et al describe methods to grow larval stages and immunohistochemistry procedures to label and visualize different cell types in the absence of huge collection of transgenic lines for Astyanax mexicanus, a fish model system used for evodevo studies. Authors should address below mentioned minor points and a few details in the discussions before it is suitable for publication at JOVE.  
  
Minor Concerns:  
Tricaine/MS222 is misspelled throughout the paper, please correct that.*  
*Please rephrase sentence on line no 227 to fix double use of word therefore*  
*Line 135: Authors suggest place the larvae in Tricaine on ice for 10min, please provide a note why it is necessary to place them on ice.*

**We have corrected the misspelling and added the following note:**

***Note: The euthanasia protocol should be discussed with your Institutional Animal Care and Use Committee. We have noted that tricaine alone does not euthanize post-larval fish; the fish begin to move when transferred from tricaine to fixative if the ice step is not included.***

*Lines 158-160: Antibody details should include Vendor details. End users can determine the optimal dilutions but it is important to know the correct company to order specific antibodies.*

**The antibody details have been included in Table 4 of the revised submission.**   
  
*Line 178 and that para: It was not clear to me whether these are individual pair wise breedings or multiple fish were set up in a single breeding cross. Authors should clarify on how these breedings were set up. They should also mention what are the ages of the breeding fish. It is well known from zebrafish studies that their fecundity decreases as they age, if age is not an issue for cavefish, Authors should highlight that because then it is an important advantage for using cavefish in developmental studies. It is important to show std dev next to average clutch size in table 1 or show data only from age matched populations and if the deviation is huge please add a note on explaining the reasons briefly.*

**We now include additional steps at the start of our protocol that describe the breeding method. We use one female and two males for our breeding set-up. We do not have enough data to confidently state whether fish age influences clutch size. Our breeding fish range in age from 1 year to 4 years old. Other labs have bred fish that are over 10 years old. There is variation in clutch size but it does not appear to be based on age.**

**To address the reviewers concern, we separated the data presented in Table 1 into two tables. Table 1 shows only the success of breeding. Table 2 shows the total number of hatched larvae from individual spawning events with the age of the parent indicated. We cite the average and standard deviation for clutch size in the text. It is currently unclear why there is variation in clutch size.**

**The updated text now reads:**

***“Table one shows the success during one year of breeding surface fish and Tinaja, Molino, and Pachón cavefish using the protocol we described. Surface and Tinaja clutches with fertilized embryos always produced hatched larvae in surface and Pachón, while Molino and Tinaja were unsuccessful some of the time (2/6 and 2/18 clutches did not produce hatched larvae, respectively). There is variation in clutch size that does not appear to be attributed to age of the parent fish. Table 2 shows the total number of hatched larvae resulting from some of the spawning events, and the age of the parent fish. In general, we found that surface fish produce the greatest number of larvae per spawn (average 1,550 ± 894, n=5), followed by Pachón (average 879 ± 680, n = 6), Tinaja (average 570 ± 373, n = 11), and Molino (average 386 ± 276, n =3). The number of larvae produced is typically more than are needed per experiment or for growth into adults. We typically set up 6-18 nursery containers (120-360 larvae) and euthanize the remaining fish.”***

*I have animal care and use committee (ACUC) related concern with cavefish larval rearing protocol. Different institutes have their ACUC committees who decide what is a best practice to grow zebrafish/cavefish/other fish models at their institutes after consultation with their veterinary doctors. This generic protocol might not work for every institute. I know several institutes where zebrafish and cavefish fry has to go on circulating water systems lot before day 14/15 of development. It will be useful if Authors should include a disclaimer or a note at the start of the larval rearing protocol stating researchers should check with their institute's animal care and use committee regarding correct procedures on handling and rearing larval fish. This is important because authors report that three out of four cave populations have only 50% survival with this protocol.*

**We have included the following note at the start of the protocol:**

***“Note: The procedures described throughout this protocol should be discussed with your Institutional Animal Care and Use Committee.”***

**This protocol could be carried out on a recirculating system. We have added this alternative to the discussion.**

**“*This protocol can also be used to raise fish on a recirculating system, however the rotifers must be added daily to compensate for those lost through the tank outflow.”***

***Reviewer #3:*** *Manuscript Summary:  
The current manuscript by Riddle et al. provides details on rearing of Astyanax from larval through 14-day-old juvenile stages. Astyanax is growing as a model organism, and more labs are turning to this system due to the pronounced differences between epigean and hypogean forms. This current issue of JoVE will facilitate the adoption of the model by more labs, and this manuscript is a particularly important contribution, as fish are most precious and fragile at larval and juvenile stages. Riddle et al. present a simple, yet extremely useful approach to care for fry at these stages, based on methods used in zebrafish research. The authors provide additional information for performing immunohistochemistry in fry as old as 12.5 days post fertilization. The manuscript is well written, informative, and extremely useful. I have only minor comments, meant to add clarity to the manuscript; I fully support the manuscript as written, and my comments should be considered mere suggestions.*  
  
*Major Concerns:  
Part 1, Hatching fertilized eggs  
1. Line 81: The reference for the article in this issue describing breeding is Stahl et al. Also, the Borowsky 2008 Cold Spring Harbor Protocols manuscripts should be cited.*

**We have included the references.**

*2. Line 89: Can you give parameters or a reference for "fish-ready water"? It may be clearer to state, "RO or dechlorinated water adjusted to: pH = 7.1 +/- 2, conductivity = 900 +/- 150 µS, and temperature = 76 +/- 2°F", or whatever parameters and/or ranges are typically used in the Tabin laboratory.*

**We have changed this line to include more detail.**   
  
*Part 2, Transferring hatched larvae to nursery cups  
1. Line 96: The authors should define nursery cup. Is this a reference to a literal kitchen cup, or are 1.5 L plastic fish tanks used?*

**We have included more detail to make it clear that this is a 1.5L container. We have replaced the word “cup” with “container”.**

*2. Line 97: Is the type of salt a required parameter, or will any salt do? Is Instant Ocean typically used?*

**We use Instant Ocean. JoVE prohibits use of commercial language in the protocol. We now include a note to direct the reader to the materials list which includes the information.**

*3. Line 104-106: Many OLAW policy, as well as many IACUC's, require addition of sodium hypochlorite, to ensure that development ceases and animals are fully euthanized before discarding. It may be helpful for labs, especially those that do not typically work with fish, if this was included.*

**We now include the guidelines of OLAW:**

***“Dispose of unused larvae at this stage using the guidelines of the Office of Laboratory Welfare (OLAW); add sodium hypochlorite to the tank to achieve a final concentration of 6.15%. Wait at least 5 minutes before pouring down the sink.”***  
*Part 5, Whole mount immunohistochemistry of post-larval fish  
1. Line 158-159: The online excel file is an incredibly useful resource, and that the authors have made this available to users for editing is very attractive. Will the authors add information to the cells that are currently blank? Adding information to missing parameters such as fixation time, protocol details, etc., will not only be helpful, but will inspire others to utilize fully the excel file provided.*

**Unfortunately JoVE does not allow reference to the online excel file. We have included the completed list as Table 4.**   
  
Minor Concerns:  
The authors should check their references, as there are some errors in them

**We have corrected the references.**   
  
***Reviewer #4:*** *Manuscript Summary:  
The manuscript by Riddle et al described methods "how to raise larval and post-larval Astyanax mexicanus" and "how to collect post-larval fish and perform whole mount immunostaining". The manuscript is well written and very informative, particularly for someone starting a new Astyanax research lab. I recommend this manuscript for publication in JoVE.*  
*Major Concerns:  
None  
  
Minor Concerns:  
It would be better to describe more details.  
Line 89 What is the final concentration of methylene blue?   
Line 92 Please add "24.4 degrees Celsius".  
Line 97 How many grams of sea salt? What is the Ph and conductivity of the nursery water?  
Line 146 "(1XPBS, 0.5% Triton X, 0.2% BSA….) "  
Line 166 "Volume of the antibody solution should be enough to… "  
Line 169 What is the concentration of DAPI and phalloidin-fluorophore conjugate?  
Line 172 "perform tissue cryo-sectioning"  
DAPI and phalloidin-fluorophore conjugate should be added to the table of materials.*

**The details and edits suggested by the reviewer have been added.**   
  
  
***Reviewer #5:***  *Manuscript Summary:  
In the manuscript "Raising Astyanax mexicanus for analysis of post-larval phenotypes and wholemount immunohistochemistry" by M. Riddle et al. the authors describe a methodology to optimize the growth of Astyanax mexicanus larvae in laboratory conditions for phenotypic studies and histological processing. A. mexicanus is an original model for evolutionary studies, however as an emergent model it lacks standardized methods for husbandry in comparison to the zebrafish. Importantly, the protocol described by the authors is inexpensive, time saving and most importantly standardizable, in order to compare larvae from different populations, growth under the same conditions of density and food available. I recommend this article for publication in Journal of Visualized Experiments if the comments listed below are addressed.*  
 *Major Concerns:  
1.- Although the authors discussed about their preference to use rotifers, as they are inexpensive and easy to culture, their point on choosing rotifers over Artemia is not clear. It is necessary to give more details in this section in terms of nutritive values of each food source, contamination of the water by decomposition, larval survival, etc. Do the larvae originating from surface versus cave or from different cave populations have different food preference?*

**We have added a lengthy discussion on the benefits of using rotifers as a food source, addressing each of the points made by the reviewer. The text reads:**

***“Newly hatched Artemia nauplii are commonly used as a food source in aquaculture but we found that using rotifers has considerable advantages for raising A. mexicanus, including: reduced price, improved biosecurity, consistent nutrition, and better water quality. We discuss each of these below.***

***Price: weekly cost in consumables is 4 dollars for rotifers compared to 14 dollars for Artemia.***

***Biosecurity: rotifers are raised in the laboratory under controlled conditions while Artemia are collected from the wild and subject to the natural variation in microbial or pathogen content22.***

***Nutrition: the nutrient composition of Artemia nauplii are environmentally determined and therefore inconsistent. Nauplii thrive on their own energy stores after hatching; they quickly loose nutritional value as they develop and should optimally be fed to the fish within several hours. Artemia begin feeding at 12 house post-hatching, representing the first time they could be nutritionally enriched; however at this stage, they have become too large for 5 dpf fish to consume. In comparison, rotifers actively consume marine microalgae resulting in high nutrient content regardless of when the rotifers are harvested. Rotifers are much smaller than Artmeia nauplii (160 microns versus 400 microns) making them easier for the fish to consume. Post-larval surface fish and cavefish consume rotifers in similar quantities suggesting no difference in preference or ability to capture the rotifers10.***

***Water quality: Artemia nauplii begin dying in fresh water several hours after being introduced. Uneaten nauplii will decay, rapidly decreasing the water quality if they are not manually removed. Removing dead Artemia is time consuming and dangerous for post-larval fish that are not much bigger than Artemia and therefore may be accidently removed or injured. Rotifers can live indefinitely in the nursery containers and provide food to the fish at all times without significantly impacting water quality. “***

*2.- Since cavefish are depigmented in contrast to surface fish, the processing for wholemount immunohistochemistry and hence the results obtained may be different at larval stages. I think this must be discussed in the article. In addition, Figure 1 needs to be better explained. A scheme of a larvae to indicate the origin/level of sectioning of this sample and axes for orientation are necessary. Is it a cave or a surface larva? Why is only one morph shown? I think it is important and necessary to show that the method works equally well on both morphs.*

**We have added multiple panels to figure 1 to include the detail requested by the reviewer. In addition, we have added the following text to the discussion:**

***…”The protocol described here can be adapted at each step and used to detect antigens in any tissue of interest. It is important to note however that some tissues may be more difficult to visualize in surface fish due to pigmentation; a barrier that does not exist in unpigmented cavefish, and could influence the interpretation of comparative studies. To address this potential problem, surface fish pigment can be bleached after fixation using 3% hydrogen peroxide….”***

*Minor Concerns:  
Line 81.- "Carry out breeding as described (see Duboue et al, this methods collection)." It is necessary to explain more, i.e. volume of the breeding tanks, number of adults, sex ratios, etc. This information is essential to ensure the obtaining of larvae.*

**We now include additional details on breeding the fish.**

*Line 83.- "4 Inches." Units need to be in cm.  
In Line 92: "79°F" and then in line 143: "4°C." Units need to be systematically on °Celsius.  
Line 97.- What is the meaning of RO water? Which Salt? Instead of "Teaspoons" I think "grams" would be more precise.  
Line 169.- DAPI and/or Phaloidin diluted on PBST? What are the concentrations?*

**These concerns have been addressed in the updated manuscript*.***