**Response to Reviewers**

**Author responses shown in red font**

**Editorial comments:**  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done  
2. Please provide an email address for each author.

Done  
3. Please define all abbreviations before use.  
Defined PBT and MeOH

4. Please expand the Introduction to include all of the following:  
a) A clear statement of the overall goal of this method

Done  
b) The rationale behind the development and/or use of this technique

Done  
c) The advantages over alternative techniques with applicable references to previous studies

Done  
d) A description of the context of the technique in the wider body of literature

Done  
e) Information to help readers to determine whether the method is appropriate for their application  
Done

5. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.  
Moved above step 1: Fixation.

6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).  
Made changes throughout protocol text.

7. 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.  
 We have changed to imperative tense where necessary.

8. 1.1: Please specify the type of animal used.  
 Added and highlighted in red.

9. 1.2: How many embryos are placed in one container?  
Typically, we fix, dehydrate and store the embryos in groups of 50 per vial. We generally add no more than 12 individuals per vial during the ISH protocol, however it is likely that additional embryos can be stained in the same vial. This information has been added to the revised manuscript.

10. 2.1: Please specify the angle applied.  
Now specified in protocol. The angle was chosen because it made sure that all specimens were able to be thoroughly and gently washed in the solution.

11. 2.2: How many embryos are placed in one petri dish? How large is the petri dish?  
We place all 50 embryos from the storage vial in a 100mm x 25 mm petri dish to remove the chorions although any size will most likely work.

12. 2.3: Please specify the concentrations of methanol used and how long the embryos are placed in each solvent. If such details are mentioned in 2.4.1-2.4.4. Please combine these steps.  
Switched steps 2.3 and 2.4 and indicated that the concentrations mentions are described in steps 2.4.1-2.4.4.

13. 3.2: Please describe how to sort embryos in the imperative tense.  
Done

14. 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.  
We have gone through and highlighted headings and essential steps.

15. 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.  
We have highlighted complete sentences.

16. 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

17. Figure 1: Please include a space between the number and the temperature unit (4 °C).  
Done

18. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

Changes made.   
**Reviewers' comments:**

**Reviewer #1:**  
Manuscript Summary:  
The authors present a comprehensive, clear, and well-documented protocol for in situ hybridization in Astyanax. This will serve as an excellent and invaluable guide. Wonderful work!  
Thank you!

Major Concerns:  
There are no major concerns.  
  
Minor Concerns:  
Unclear why some steps are highlighted in yellow?

Went back through and changed some of the highlighting. Since many of the steps are repetitive, we tried to highlight any steps where there was a major change in solution being added or steps that are challenging to visualize (e.g. the use of the water bath apparatus).  
L394. That->than

Changed.   
The only suggestion I could make is to start a forum or other list-serv for trouble shooting.  
We appreciate the suggestion, although it is beyond the scope of this manuscript. We hope to recruit community members to develop a list-serv, similar to that used in the zebrafish field, to further assist colleagues.

**Reviewer #2:**  
Manuscript Summary:  
This protocol will be of interest to scientists using this model system but also to others working in different teleost fishes.  
  
Major Concerns:  
Clarity is required in numerous steps. The materials list, check list and text need to be carefully cross-referenced as not all chemicals/equipment needed is listed in the materials list and some catalogue numbers are incorrect.  
Done  
Minor Concerns:  
What age embryos does the protocol work for? Does it work for larvae that have hatched. Some of the images shown look like larvae and not embryos.  
The youngest embryos that we use are 24 hours post fertilization and the oldest we use are 72 hours post fertilization. This protocol would most likely work on older embryos keeping in mind that the digestion time may need to be altered.

Step 1.3 What is the 4% PFA made up in?  
We make up the PFA in a beaker and then aliquot it into Eppendorf tubes for storage.

Step 2.3 says to dilute the samples, but this is described in steps 2.4.1 through 2.4.4, so step 2.3 should be part of step 2.4

Addressed in editorial comment #12.  
3.3 says to set the shaking water bath but to place the embryos on a platform shaker. If the shaking water bath is for a later step, please indicate this in the instructions for clarity.

The water bath is set early in the day because it takes an hour or so to heat up all the way. This is now clarified in protocol.   
4.3 is the 12 minute digestion appropriate for any age embryo?

12 minutes works well for the ages that we typically work with (24 hpf-72 hpf) but increased digestion may be necessary as embryos get older. For example, we’ve recently tried to do ISH on fin clips and the digestion period was increased to 30 minutes.   
6.2 what concentration is the RNA probe or how does one determine that 2ul is the right amount.  
6.3 the second and third sentences are confusing. "replace Hyb+ from day 1 protocol" - do you mean form the first run protocol? In this step you replace the hyb+ from the end of the day 1 protocol with previously stored hyb+ with probe added (because you save the hyb+ with probe during the probe removal step of day 3). This is just an alternative way of adding the probe instead of using another 2µl of concentrated probe.   
In the protocol presented, step 5.2 is an overnight step. Therefore why is hyb+ thawed in step 4.2.2?

The hyb+ used for the overnight step needs to be warm before added just to keep the temperature consistent. Hyb+ can be added to the hot water bath ~ 5 minutes before adding it to the embryos for the overnight step but we just find it easier to save a trip to the freezer by grabbing it with the PFA in step 4.2.2.  
In step 6.2 probe is added, but it appears tubes are only labelled with gene of interest in step 7.1

Probe is added to the embryos in the glass vials in step 6.2. Step 7.1 refers to labeling an eppendorf tube with your gene of interest because this is the tube that you will save your hyb+ with probe in so that you can use it for future in situ runs.   
8.2 does warm mean at room temperature?

The warm hyb- refers to the 100% hyb- used in the dilution series for 8.2. All of these solutions are warmed in the water bath to 70 ° C. Changes made to protocol to clarify this.   
In steps 8.2,8.3 and 8.4 - is the solution removed each time or the embryos transferred to new tubes?

The embryos stay in the glass vials throughout the whole protocol (3.2).   
In 7.1.4. blocking agent is added to MABT (two tubes are prepared). In step 8.4 however, only MABT is used. It is unclear why the tubes in 7.1.4 were prepared (perhaps in step 9.1?). Please clarify.

The blocking reagent can take a few hours to go into solution so it is important to let it vortex throughout the protocol until needed. This is now clarified in the protocol.   
In step 9.1, is "remixed blocking solution" referring to the blocking agent in MABT prepared in step 7.1.4?

Yes this is the blocking solution made in 7.1.4. This is now clarified in the protocol.   
In step 9.2, again is the "blocking solution" referring to the blocking agent in MABT prepared in step 7.1.4?

See above comment.   
Anti-DIG-AP fab is listed differently in the materials list

This has been fixed.   
Steps 9.2 to 9.3: In 9.2, the antibody is added to the blocking solution giving a total of 10 ml but in step 9.3 it says to put 5ml to each vial. Is the vial in 9.3, the vial with the embryos? Is the blocking solution mentioned in 9.3 the one to which the antibody is added?

Please clarify what size vials are used for the samples throughout the method? Are these glass vials or plastic?

The vials we use to make up the blocking solution are the 15 mL VWR plastic conicals just because they fit well in the vortex-genie. This is now clarified in the protocol. In step 9.1 each of the two glass vials of embryos gets ~5 mL of blocking solution without the antibody and it incubates for 4 hours. In step 9.3 you would use the second 15 mL conical of blocking solution to add your 2µl of antibody and again adding ~5 mL of this to each of the vials of embryos.   
10.1 what is the concentration of the goat serum (either the stock concentration or the final diluted concentration)

We buy our normal goat serum from ThermoFisher Scientific and then reconstitute with 10 mL of water as indicated in the directions. We then aliquot this into Eppendorf tubes and store at –20 ° C until needed.   
10.3 does it matter if these washes are done in quick succession or is the idea to do them about 30 minutes apart?  
Line 269 - by walk in chamber do you mean a walk in refrigerator?

The idea is to do them 30 minutes apart but if you find yourself short on time we have found that the quality of staining does not suffer if you happen to do a couple 15 minute washes instead of 30 minute. The walk-in chamber is a walk in refrigerator.   
11.1 why is 50ml of AP solution made - how many sample vials is this good for?

Since you switch out the AP Buffer/NBT/BCIP solution every hour it is preferable to have 50 mL made. Depending on the gene you may only use a few milliliters but there have been times where we’ve used all 50 mL of the AP Buffer. We’ve found that it is best to make up the AP Buffer the day of the coloration reaction and to only use it that day. We advise against storing the AP Buffer for long term use because it may contribute to precipitate formation.   
11.3 what concentration are BCIP and NBT? Please provide correct catalogue numbers and include in materials list. How are these solutions made up. If they are purchased in liquid form then please provide the concentrations.

In 1ml of AP buffer, we add 3.5 µl of BCIP and 4.5 µl of NBT. Corrected catalog numbers have been added to the materials list.   
Does sterile PBS, mean autoclaved PBS

Sterile PBS just means that we sterile filter it before use.   
Are any solutions in the protocol depc-treated?

No, our water is RNase-free and most solutions are sterile filtered after being made (indicated in supplemental protocols when necessary).   
Please add RNAse-free water to the check list

Added to materials list.  
Day three checklist - please clarify what "w/ probe" means? This is an American abbreviation not recognised by all countries.

This has been changed to say “with probe”.   
Citric acid is listed differently in the materials list and the check sheets

We buy the citric acid listed on the materials list and then make up 1M Citric acid solution.   
What concentration is the PK ?

We order proteinase K (200mg/10 ml) from qiagen  
There are various chemicals used in the supplementary files, that are not listed in the materials list -eg. MgCl2 etc.  
Also various pieces of equipment are also not listed in the materials list yet are required to do the protocol.

Added platform shaker, vortex, water bath, Tris, and MgCl2 to materials list.   
Supplemental file s6 - please list which kit is utilized here. Concentrations of reagents and/or contents are not provided.

The kit we use is the sigma-roche DIG RNA Labeling Kit (SP6/T7) now specified in the protocol and on materials sheet.   
Please double check the materials list: The correct name for the company Fisher is Fisher Scientific. Some of the catalogue numbers listed are incorrect. Eg. NBT and formamide - there may be others.  
Please double check the checklist corresponds to the methods in the text.

We reviewed and corrected the catalog numbers.   
  
  
**Reviewer #3:**  
Manuscript Summary:  
My name is Damian Moran. This is a fantastic, step-by-step, walk through of the in situ hybridization process. I say this not as a skilled molecular biologist or histologist, but as a physiologist who sees the type of work described in this manuscript as a natural direction researchers such as myself need to extend ourselves. I cannot critique the methodology in any capacity, I defer to my more informed peers on any problems with the approach outlined. I confess that I have also not carefully checked the volumes and quantities reported for accuracy and consistency. However, the clarity of explanation is totally sufficient from my reading of it. Particularly useful are the check lists, the clear explanations of the reagent and buffer recipes, the pitfalls and suggested optimisations.  
  
This manuscript is fine in its current form. The only extra I would like to see (although I feel a little greedy for asking for it) are some tips on taking good images. For example, how can the embryos be held or mounted to visualise the different surfaces? Also, can these embryos be stored after visualisation, or is there a decay of stain over time? These questions are almost certainly addressed in other literature and I'm sure the authors know about this. A sentence or two pointing novice researchers would be a cherry on the top!  
We added in a 12th section with a few details on imaging.   
Thank you to the authors for making this resource available, and thanks to JoVE for creating a journal format so very useful.  
  
Minor Concerns:  
There are some minor grammatical errors that will be picked up at the proofing stages.  
Thank you, we have reviewed the manuscript for errors.

**Reviewer #4:**  
Manuscript Summary:  
The manuscript will be useful to the cavefish and possibly other scientific communities.  
  
Minor Concerns:  
-Ratio of fix to tissues should be noted. Is this 50 embryos per 4 mls? Too many embryos per vial can lead to uneven fixation.

Sentence added in 1.1 to clarify that if embryos are older, e.g. 72 hpf, it may be necessary to drop the embryo count to 25.   
-We find fixing embryos while rocking helps.  
-Addition of instructions for making paraformaldehyde would be helpful. We emphasize not breathing the fumes when weighing out or putting PFA into solution. We also find it has a short half-life, and the best results are obtained when it is made fresh. Our zebrafish protocol suggests wearing a mask when weighing out the powder.

We have ensured appropriate warnings are present in the revised manuscript (e.g., when working with it in the powdered form). We usually aliquot it into Eppendorf tubes and then keep it frozen until needed.  
-I recommend removing chorions in PBST to avoid breathing fumes from paraformaldehyde. 0.1% Tween 20 also prevents the embryos from sticking to everything.  
-"Dilutions based on 4 ml volume" makes little sense until you read further. Then the total volume is 1 ml. Should this be total volume per vial?

Wording changed in section 2.4 to hopefully make a bit more sense.  
-Headings would help organize individual steps.  
-Sorting embryos in methanol is cumbersome. I recommend sorting prior to fixation.  
-Recommended to avoid using the first person in the protocol (page 3 line 172).

This has been changed.  
-It is difficult to understand what is meant by the mesh, apparatus and gaskets for hybridization. This could use a figure and/or a drawing.

Thank you for the suggestion, we will ensure these are included in the video of this manuscript.   
-Amount of probe is not designated, nor how it is made in the main protocol. Having this be the first thing on the checklist and in the protocol would help.

We always add 2µl of probe to the hyb+ indicated in section 6.2. Supplement File S6 is a detailed protocol on how to make the probe.   
-Final concentrations should be included for all solutions.

Done  
-Embryos will likely look better if the tissue is cleared in 70% glycerol/30% PBST and photographed using DIC optics with minimal polarization. Just a suggestion. We photograph in depression well slides. Adding some comments on microscopy would also add to the protocol.

Comments on microscopy have been added to the protocol (section 12).