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Wholemount In Situ Hybridization for Astyanax Embryos

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TITLE:

Wholemount In Situ Hybridization for *Astyanax* Embryos

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SUMMARY:

This protocol enables visualization of gene expression in embryonic *Astyanax* cavefish. This approach has been developed with the goal of maximizing gene expression signal, while minimizing non-specific background staining.

ABSTRACT:

In recent years, a draft genome for the blind Mexican cavefish (*Astyanax mexicanus*) has been released, revealing the sequence identities for thousands of genes. Prior research into this emerging model system capitalized on comprehensive genome-wide investigations that have identified numerous quantitative trait loci (QTL) associated with various cave-associated phenotypes. However, the ability to connect genes of interest to the heritable basis for phenotypic change remains a significant challenge. One technique that can facilitate deeper understanding of the role of development in troglomorphic evolution is whole-mount in situ hybridization. This technique can be implemented to directly compare gene expression between cave- and surface-dwelling forms, nominate candidate genes underlying established QTL, identify genes of interest from next-generation sequencing studies, or develop other discovery-based approaches. In this report, we present a simple protocol, supported by a flexible checklist, that can be widely adapted for use well beyond the presented study system. It is hoped that this protocol can serve as a broad resource for the *Astyanax* community and beyond.

INTRODUCTION:

In situ hybridization is a common method for staining fixed tissues to visualize gene expression patterns[1]. This technique has been performed for years in other traditional[2] and non-traditional[3] model systems, for a variety of biological studies. However, several steps and reagents are necessary to successfully perform this procedure. For investigators who have never performed

this technique, initiating the process can be intimidating owing to the many steps involved. Further, the lengthy nature of this procedure lends itself to technical errors, which can be challenging to troubleshoot.

The overall goal of this article is to present a simple and straightforward method that will render this hybridization technique accessible to a wide audience. To reduce the introduction of errors, we present a straightforward approach that yields high quality gene expression staining and minimizes non-specific background signal. This procedure is similar to other approaches developed in traditional model systems, such as *Danio rerio*[4]. Here, we aim to facilitate careful implementation of each step using a downloadable checklist (**Supplemental File 1**), to promote careful implementation of the protocol. The rationale for doing this is to facilitate organization through the many steps involved in this procedure. This article is appropriate for researchers interested in performing whole-mount in situ hybridization in developing embryos, but have not yet performed the procedure. The advantage of the chosen approach for *Astyanax* researchers is that it has been tested and proven in both cavefish and surface fish morphs, thereby facilitating comparative expression analyses. The presented method can be used by researchers in studies on *Astyanax* and other systems.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati (Protocol #10-01-21-01).

1. Fixation

1.1. Isolate desired number of *Astyanax mexicanus* embryos from a breeding tank and fix ~50 embryos at a time. If embryos are large and old, it may be necessary to fix 25 at a time to ensure even fixation.

1.2. Depending on the age of the embryo, utilize the IACUC-approved method of anesthesia. For older embryos with a functioning nervous system, sacrifice embryos via anesthetic overdose. Accordingly, place embryos in a solution of ~1% tricaine (buffered to pH 7.4) to minimize pain and discomfort for the organism.

1.3. Once the embryos are unresponsive to touch, replace system water containing tricaine, and add ~1 mL of 1x phosphate-buffered saline (PBS, pH 7.4).

1.4. Remove the PBS solution, and add 1 mL of 4% paraformaldehyde (PFA). Fix embryos overnight at 4 °C.

CAUTION: PFA is hazardous (i.e., it is flammable and is a skin and lung irritant), handle with care.

2. Dehydration

2.1. To dehydrate the embryos, remove the fixative solution and rinse with 1 mL of PBS. Place embryo-containing vials at an angle (between 30° and 45°), on a platform shaker during rinse. Continue to wash the embryos twice, 5 min per wash.

2.2. If embryos still have a chorion, place all 50 embryos into a 100 mm x 25 mm Petri dish, and carefully isolate them from the chorions using two sets of Watchmaker's forceps (e.g., #5 forceps) under a microscope.

NOTE: During the steps described below, and for the remainder of the protocol, carefully remove all liquid from the previous step using clean, glass Pasteur pipettes before adding the next solution

2.3. Dehydrate the embryos in a series of increasingly concentrated washes of methanol (MeOH) described below. The dilutions are based on a 1 mL total volume with 500 µL of solution going into each 4 mL glass vial. Perform all dehydration steps at room temperature (RT) on platform shaker.

2.4.1. Carefully remove the PBS solution. Add a 25% MeOH solution (250 µL of MeOH + 750 µL of PBS). Gently shake on a platform shaker for 5 min.

2.4.2. Carefully remove the 25% MeOH solution. Add a 50% MeOH solution (500 µL of MeOH + 500 µL of PBS). Gently shake on a platform shaker for 5 min.

2.4.3. Carefully remove the 50% MeOH solution. Add a 75% MeOH solution (750 µL of MeOH + 250 µL of PBS). Gently shake on a platform shaker for 5 minutes.

2.4.4 Carefully remove the 75% MeOH solution. Add a 100% MeOH solution (1 mL of MeOH). Gently shake on a platform shaker for 5 min. Repeat this step 3 times.

2.5 At this point, store dehydrated embryos, as needed, in their glass vials at -20 °C (long term). Alternatively, proceed directly to day 1 of the protocol.

3. Day 1: Rehydration

3.1. Obtain dehydrated embryos from -20 °C freezer (or proceed directly from step 2.5).

3.2. Sort the embryos using a Pasteur pipette. One can sort based on morphotype (i.e., cave and/or surface), and the number of genes assessed in each experiment. There are usually no more than 12 embryos per vial once sorted. To maintain organization, use colored lab tape to designate vials and pipettes for each gene. Embryos will stay in the same vial throughout the entire protocol.

NOTE: Place the tip of the Pasteur pipette in 100% EtOH to sterilize it between uses.

3.3. Set a shaking water bath to 70 °C to be used in a later step. Carefully, draw out the MeOH in vials of sorted embryos and replace with 500 µL of new 100% MeOH. Wash briefly (~1 min) on platform shaker.

3.4. Rehydrate embryos in an increasing concentration of 1x PBS with Tween 20 (PBT, see below) on platform shaker. The dilutions are based on a 1 mL final dilution volume with 500 µL going into each vial.

3.4.1. Add a 25% PBT solution (250 µL of PBT, 750 µL of MeOH). Gently shake on a platform shaker for 5 min.

3.4.2. Carefully remove the 25% PBT solution. Add a 50% PBT solution (500 µL of PBT, 500 µL of MeOH). Gently shake on a platform shaker for 5 min.

3.4.3. Carefully remove the 50% PBT solution. Add a 75% PBT solution (750 µL of PBT, 250 µL of MeOH). Gently shake on a platform shaker for 5 min.

3.4.4. Carefully remove the 75% PBT solution. Add a 100% PBT solution (1 mL of PBT). Gently shake on a platform shaker for 5 min. Repeat this step 3 times.

4. Day 1: Digestion and fixation

4.1. Prepare a proteinase K (PK) solution by adding 1 µL of PK (20 mg/mL) to 2 mL of PBT.

4.2. In anticipation of subsequent steps, obtain frozen aliquots of hybridization buffers (Hyb- and Hyb+; see **Supplemental File 2** and **Supplemental File 3**) and PFA from -20 °C storage.

4.2.1. Allow PFA to thaw at RT.

4.2.2. Place aliquots of Hyb- and Hyb+ in a rotating 70 °C water bath. Place all reagents and vials inside a small "gasket" with a mesh bottom, inside the floating water bath apparatus. This enables simple addition and removal of tubes and vials from the rotating 70 °C water bath.

4.3. Gently add PK solution to the vial(s) of embryos ensuring all tissues are completely covered with solution. Digest embryos for ~12 min in PK working solution on the platform shaker.

NOTE: The length of digestion can be varied by the investigator to ensure optimal results.

4.4. Gently draw off the PK solution, and briefly flood the vial with PBT to dilute any remaining PK.

4.5. Draw off the PBT solution and replace with 500 µL of new PBT. Allow the solution to rinse on the platform shaker for 5 min.

4.6. Draw off PBT and replace with 500 µL of thawed 4% PFA. Allow the embryos to incubate for 20 min on the platform shaker at RT.

4.7. Draw off the 4% PFA, and briefly flood the vial with PBT to dilute any remaining PFA. Draw off the PBT, and replace with 500 µL of fresh PBT. Allow embryos to rinse for 5 min on the platform shaker. Repeat this step 4 more times.

5. Day 1: Prehybridization

5.1. Place 500 µL of pre-warmed Hyb- solution into the vial. Carefully place the vial in the 70 °C water bath (inside gaskets) **without** shaking, for 5 min.

5.2. Draw off the Hyb- solution and flood the vial with 500 µL of pre-warmed Hyb+ solution. Place the vial back into the 70 °C water bath with shaking (40 rpm). Incubate for either 4 h, or overnight.

NOTE: A 4 h incubation will yield a complete in situ protocol that will last for 4 days in total. Here, this step is presented as an overnight incubation, which will yield a protocol lasting 5 days in total.

6. Day 2: Hybridization

6.1. Place an aliquot of Hyb+ from the -20 °C freezer into the shaking hot water bath for 5 min.

6.2. Draw off the Hyb+ from the vial and replace with 500 µL of pre-warmed Hyb+. To this solution, carefully add 2 µL of RNA probe to each vial. Gently swirl the vial to ensure even distribution of the probe.

6.3. Incubate the Hyb+ (with added probe) solution in the 70 °C hot water bath overnight while shaking at 40 rpm.

NOTE: One can re-use Hyb+ (with probe) solution. For this, take Hyb+ with probe from the first run from the -20 °C freezer and place it in a hot water bath for 5 min. Replace Hyb+ from the day 1 protocol with Hyb+ with probe and allow incubating overnight in hot water bath.

7. Day 3: Solution preparation

7.1. Prepare microcentrifuge tubes labeled Hyb+ with the “gene-of-interest” RNA probe. Prepare the series of dilutions that will be used during day 3.

7.1.1. Using 6 separate tubes, prepare the following series of dilutions of Hyb- and saline sodium citrate (SSC, 0 to 100%) in a 1 mL volume, and place them in the 70 °C shaking water bath: Tube 1 = 100% Hyb- (1 mL of Hyb-): Tube 2 = 25% 2x SSC (250 µL of 2x SSC, 750 µL of Hyb-); Tube 3 = 50% 2x SSC (500 µL of 2x SSC, 500 µL of Hyb-); Tube 4 = 75% 2x SSC (750 µL of 2x SSC, 250 µL of Hyb-); Tube 5 = 100% 2x SSC (1 mL of 2x SSC); Tube 6 = 100% 0.2x SSC (2 mL of 0.2x SSC).

NOTE: Be vigilant of the concentration of SSC, as it changes from 2x to 0.2x.

7.1.2. Using 4 separate tubes, prepare the following series of dilutions of PBT and SSC in a 1 mL volume, and place at RT: Tube 1 = 25% PBT (250 µL of PBT, 750 µL of 0.2x SSC); Tube 2 = 50% PBT (500 µL of PBT, 500 µL of 0.2x SSC); Tube 3 = 75% PBT (750 µL of PBT, 250 µL of 0.2x SSC); Tube 4 = 100% PBT (1mL of PBT).

7.1.3. Prepare a tube with 2 mL of maleic acid buffer containing Tween 20 (MABT) working solution.

7.1.4. Prepare two 15 mL conical tubes of blocking solution. In each tube, add 0.2 g of blocking reagent to 10 mL of MABT (see **Supplemental File 4**). Place both tubes on a nutating mixer (or platform shaker) until completely dissolved in solution (up to 3 h).

8. Day 3: Probe removal

8.1. Draw off Hyb+ (with probe) solution with a glass Pasteur pipette and place it into a sterile, labeled microcentrifuge tube. Retain this tube in the -20 °C freezer for future use (if probe-labeling is successful).

8.2. Carefully add 500 µL of the warm SSC/Hyb- dilutions (indicated below). Incubate in each of the following solutions for 10 min each in the 70 °C shaking water bath.

8.2.1. Incubate sequentially with 100% Hyb- (1 mL of Hyb-), 25% 2x SSC (250 µL of 2x SSC, 750 µL of Hyb-), 50% 2x SSC (500 µL of 2x SSC, 500 µL of Hyb-), 75% 2x SSC (750 µL of 2x SSC, 250 µL of Hyb-), 100% 2x SSC (1 mL of 2x SSC), 100% 0.2x SSC (2 mL of 0.2x SSC).

8.3. Following the last step, incubate in each of the following solutions for 10 min each. All of the following incubations take place at RT on the platform shaker: 25% PBT (250 µL of PBT, 750 µL of 0.2x SSC), 50% PBT (500 µL of PBT, 500 µL of 0.2x SSC), 75% PBT (750 µL of PBT, 250 µL of 0.2x SSC), 100% PBT (1 mL of PBT).

8.4. After a 10 min incubation, remove the 100% PBT, and add 500 µL of MABT into each vial. Repeat this step twice for 5 min.

9. Day 3: Blocking

9.1. Remove MABT from each vial and flood with premixed blocking solution from one of the tubes (prepared in step 7.1.4). Place vial on a nutating mixer for ~4 h at RT.

9.2. Add 2 µL of Anti-DIG-AP Fab fragments to the second vial of 10 mL blocking solution (prepared in step 7.1.4) and briefly vortex.

9.3. Fill each vial almost completely with blocking solution (~5 mL) and place on nutating mixer overnight in a refrigerator at 4 °C.

10. Day 4: MABT Rinses

10.1. Prepare a stock vial of 10% normal goat serum (NGS) in MABT (add 100 µL of NGS to 900 µL of MABT).

10.2. Draw off the blocking solution in each vial and add 500 µL of NGS/MABT mixture into each vial. Allow the embryos to incubate for 25 min at RT on the platform shaker.

10.3. Replace the NGS/MABT mixture with 500 µL of 100% MABT. Incubate for 30 min at RT on the platform shaker. Perform this rinse 11 more times throughout the day every 30 min.

10.4. Fill the vial with 100% MABT, and place on a nutating mixer overnight in a refrigerator or walk-in chamber at 4 °C.

11. Day 5: Probe visualization

11.1. Prepare a 50 mL aliquot of alkaline phosphatase (AP) buffer (see **Supplemental File 5**). Combine the following in a 50 mL conical tube wrapped in aluminum foil to limit light exposure: 5 mL of 1 M Tris (pH 9.5), 5 mL of 50 mM $MgCl_2$, 5 mL of 1% Tween 20, 5 mL of 1 M NaCl, 30 mL of ddH2O.

11.2. Remove MABT and replace with 1 mL of AP buffer (tube wrapped in foil). Let it wash for 5 min. Do this twice to ensure complete removal of MABT.

11.3. Remove AP buffer and replace with 1 mL of AP buffer with 3.5 µL 5-bromo-4-chloro-3'-indolyphosphate (BCIP) and 4.5 µL of nitro-blue tetrazolium (NBT). Replace with freshly prepared AP buffer/NBT/BCIP once every hour until reaction is complete. Monitor closely, checking every 15 min, to allow the coloration reaction to take place until the desired level of staining has been achieved. If precipitate begins to form, replace the solution sooner.

11.4. Stop the coloration reaction by rinsing the embryos in fresh 100% AP buffer (without NBT/BCIP) for 5 min. Continue rinses in PBT until optimal levels of signal (with minimal amounts of background staining) are achieved. Continue to rinse specimens with increasing dilutions of PBT in AP Buffer as follows: 25% PBT (250 µL of PBT, 750 µL of AP Buffer), rinse for 5 min; 50% PBT (500 µL of PBT, 500 µL of AP Buffer), rinse for 5 min; 75% PBT (750 µL of PBT, 250 µL of AP buffer), rinse for 5 min.

11.5. Rinse embryos in ~5 mL of 100% PBT on nutating mixer until desired minimum background staining is reached. Switch out with fresh PBT several times. This could take up to several days.

11.6. When rinsing is complete, wash embryos in 500 µL of sterile PBS on a platform shaker. Perform this rinse twice for 5 min. After PBS washes, post-fix the specimens in 500 µL of 4% PFA for 1 h at RT on a platform shaker. Alternatively, fix overnight in 1 mL of 4% PFA in the refrigerator at 4 °C.

11.7. Replace the fixative with fresh, sterile PBS. Perform this rinse at least twice for 5 min. Place the embryos in ~4 mL of 100% sterile PBS, and store long term at 4 °C.

12. Imaging

12.1. Make up an imaging plate in a Petri dish using 3% agarose and TAE buffer.

NOTE: Quantities depend on how many plates are needed. Plates can be reused several times. It is recommended that a shallow rectangular mold is placed in the Petri dish while the gel is cooling in order to create a depression for containing the embryos on the plate.

12.2. Place the embryos on the plate in the PBS.

NOTE: It is best to gently pour embryos onto the plate instead of pipetting them out because it has been found that they will stick to the inside of plastic pipettes.

12.3. Use light microscopy in order to visualize each embryo. Use a blunt probe to maneuver embryos to desired position.

12.4. Take an image when the embryo is in desired position. Note that it is important to take images of embryos within a couple weeks of completed staining to avoid potential degradation of stain.

REPRESENTATIVE RESULTS:

In this report, we provide a simple and straightforward approach to perform labeling of embryonic *Astyanax* specimens for high-quality gene expression analysis. This technique can be carried out in either four or five days, and each principal step in the procedure is represented in a color-coded flowchart (**Figure 1**). Once completed, stained embryos should harbor a dark purple chromatic label in tissues expressing the particular gene of interest. We have successfully implemented this protocol in both Pachón cavefish (**Figure 2A–B,E**) and surface fish (**Figure 2C–D,F**) embryos.

The cavefish embryos were stained for two transcription factors that label early neural crest tissues, *Sox9* and *Tfap2a*[5,6]. Embryos labeled for *Sox9* expression demonstrate clear labeling in the developing branchial arches and pectoral fin (yellow arrowheads, **Figure 2A**). Note that staining is virtually absent in the yolk sac or the developing somites on the flank (**Figure 2A**). Similarly, *Tfap2a* expression is evident in the portions of the developing head, as well as early migration neural crest cells (**Figure 2B**, arrowhead) along the dorsal flank region of the embryo. The third representative gene presented for cavefish embryos is *Phf20a*, a marker of osteoblast

differentiation[7]. Note the positive staining in the portions of the somitic mesoderm and posterior head that are destined to give rise to bony tissue (**Figure 2E**, arrowheads).

In surface fish embryos, we probed for the genes *CXCR*, *Adcyap1a*, and *Sox10*. *CXCR* encodes a G-protein membrane-bound receptor that binds CXC chemokines[8]. Positive labeling is present in isolated regions of the head and flank (**Figure 2F**, arrowheads), as well as a few individual cells overlying the yolk sac. The gene *adenylate cyclase-activating polypeptide*, *Adcyap1a*, is expressed in regions of the central nervous system, including pituitary cells. Note the highly specific expression in paired, bilateral clusters of cells on the dorsal aspect of the embryo; as well as a larger region of midline expression (**Figure 2C**, arrowheads). Finally, we present the expression of *Sox10*, a transcription factor which labels early neural crest and oligodendrocyte cells[10]. Highly specific positive staining is evident as an early marker of neural crest on the left and right sides of the dorsal embryo (**Figure 2D**, arrowheads).

We present each of the two types of confounding issues other investigators may encounter. The first issue one periodically encounters is punctate specks of non-specific labeling. These specks may arise as precipitate from the final MABT rinses, or the AP buffer during the coloration reactions. An example of this non-specific labeling is evident on the yolk sac of a surface fish embryo stained for expression of *Pnp4a*. This gene encodes an enzyme (purine nucleoside phosphorylase) that facilitates production of iridescent pigmentation[11]. This gene is first evident in the developing eye and the swim bladder. The punctate specks observed in some surface specimens (**Figure 3A**), were eliminated by frequent washes and replacement of the AP Buffer + NBT/BCIP in the final stages of the protocol (**Figure 3B**). A second issue that one periodically encounters is the diffuse, largely non-specific expression of genes that would otherwise produce a distinct expression pattern. One example is the gene *BMP4*, which appears as a largely diffuse pattern with low levels of chromogen present throughout the specimen (**Figure 3C**). In cases such as these, we generally identify a different region of the gene, amplify into a vector and perform a new probe synthesis (see **Supplemental File 6**). The example of a control (no probe) specimen (**Figure 3D**) is provided to illustrate the diffuse and non-specific nature of our failed *BMP4* probe.

FIGURE AND TABLE LEGENDS:

Figure 1: A simple flowchart for whole-mount in situ hybridization. This flowchart utilizes color-coding to illustrate the principal steps of in situ hybridization.

Figure 2: Representative staining for six genes, using both cave and surface morphs of *Astyanax*. (**A**) Image shows the specific staining (yellow arrows) of *Sox9* on the right lateral side of a 72 h post-fertilization (hpf) Pachón cavefish (45x). (**B**) Specific staining for *Tfap2a* is evident on the right lateral side of a 36 hpf Pachón cavefish (45x). (**C**) Bilateral and midline staining (yellow arrowheads) of *Adcyap1a* are labeled in the dorsal region of a 72 hpf surface fish (100x). (**D**) Staining of *Sox10* in neural crest tissues of a 24 hpf surface fish (100x). (**E**) *Phf20a* demonstrates a faint, but clear, pattern of expression in the dorsal region of a 24 hpf Pachón cavefish (100x). (**F**) The cytokine receptor, *CXCR*, is expressed in distinct regions of the right lateral side of a 24 hpf surface fish (100x). Scale bars in A, B, E, F = 0.5 mm; scale bars in C, D = 2.5 mm.

Figure 3: Representative examples of sub-optimal results for whole-mount in situ hybridization. (**A**) Specific staining is evident alongside non-specific precipitate and/or debris (red arrowhead) on the right lateral aspect of a 72 hpf Pachón cavefish (100x). (**B**) The same probe visualized in A, depicting the same staining patterns without precipitate or background in a 72 hpf Pachón cavefish (100x). (**C**) The right lateral flank of a 72 hpf Pachón cavefish demonstrates diffuse, non-specific staining for *BMP4* at 45x magnification. (**D**) A 72 hpf Pachón cavefish subjected to this protocol, without the addition of probe (45x). Scale bars = 0.5 mm.

DISCUSSION:

Owing to the vulnerability of RNA to degradation, one of the most critical steps in the protocol concerns the sterile synthesis of the RNA probe. However, if a probe is carefully generated, and provides good results, it can be reused in subsequent staining reactions. A second crucial step is the careful production of all reagents used throughout the protocol. Since this protocol involves several days and many small steps, it is essential that all reagents are accurately produced, and stored in a sterile manner. Further, it is fundamentally important that the investigator keeps careful track of each step in the protocol. We have found that the provided checklist of steps can be extremely useful in ensuring accurate and precise completion of each aspect of this protocol.

We do not often modify the protocol we presented here. However, investigators may perform probe incubations at different temperatures than the ones suggested (i.e., 70 °C). Slight changes in hybridization temperatures will impact binding of RNA probes, and therefore, seeking the optimal hybridization temperature can positively impact the quality of staining. With respect to troubleshooting, we strongly encourage other investigators to utilize the checklist provided with this article (**Supplemental File 1**). Maintaining careful records is a necessary first step in ensuring high quality staining. A second minor modification is suggested is to perform the final coloration reaction without rocking (e.g., without placing on a platform shaker or nutator). The reason for this is that periodically we note the production of precipitate, that presumably arises from the AP buffer solution. This precipitate usually overstains to a dark color and creates punctate (non-specific) background on the stained tissue. To minimize the production of this precipitate, we prepare sterilized AP buffer just prior to each reaction. Further, once NBT and BCIP have been added to the buffer, we replace it with fresh buffer and NBT/BCIP every hour until the coloration reaction is completed.

A limitation to the presented method is that a chromatic stain was used for gene expression visualization. We prefer this approach since it is cost-effective, and only requires light microscopy to visualize. If one were interested in evaluating quantitative differences, we suggest they use a fluorescent coloration reaction. This will enable semi-quantitation, for example, through comparison of relative fluorescent units of expression between experiments.

Protocols for in situ hybridization are available widely on the web[12,13], as well as in scientific publications. The protocol that we present was developed specifically for our model system, *Astyanax mexicanus*. We have used this protocol to stain the expression of several dozens of genes, and feel it consistently provides high-quality results. A significant advantage of this

protocol is the step-by-step checklist of items to enable the investigator to perform multiple tasks while ensuring accurate completion of each of the steps of this protocol. We hope that this protocol will serve as a helpful resource to other investigators in the field and beyond, and anticipate that this common laboratory technique will support future discoveries linking genotype to phenotype in the blind Mexican cavefish.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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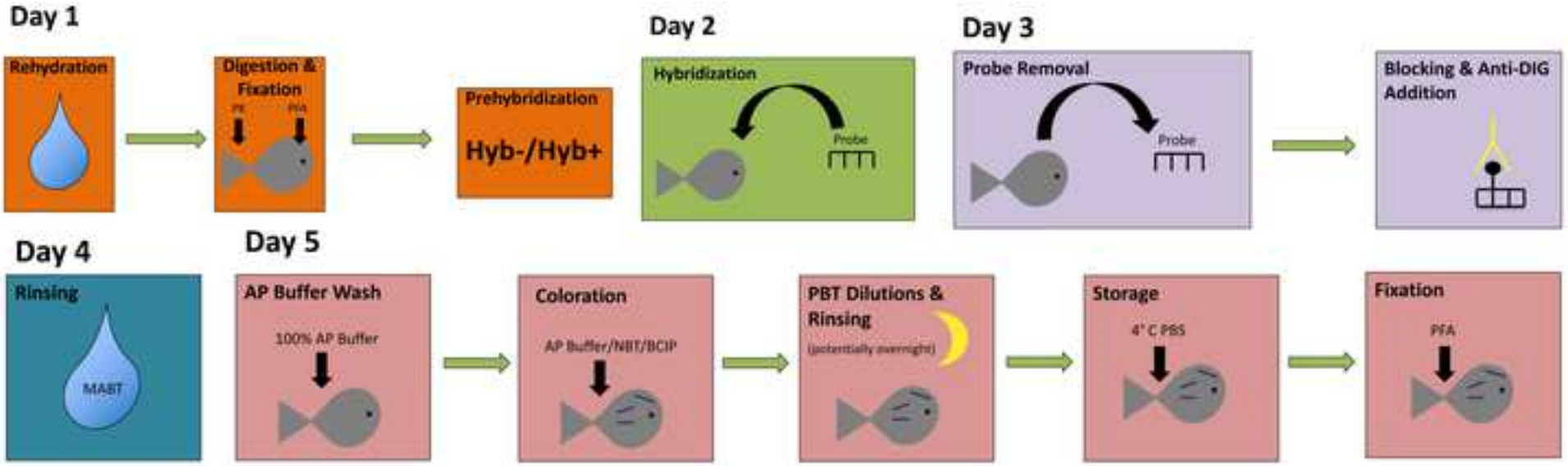

Figure 2

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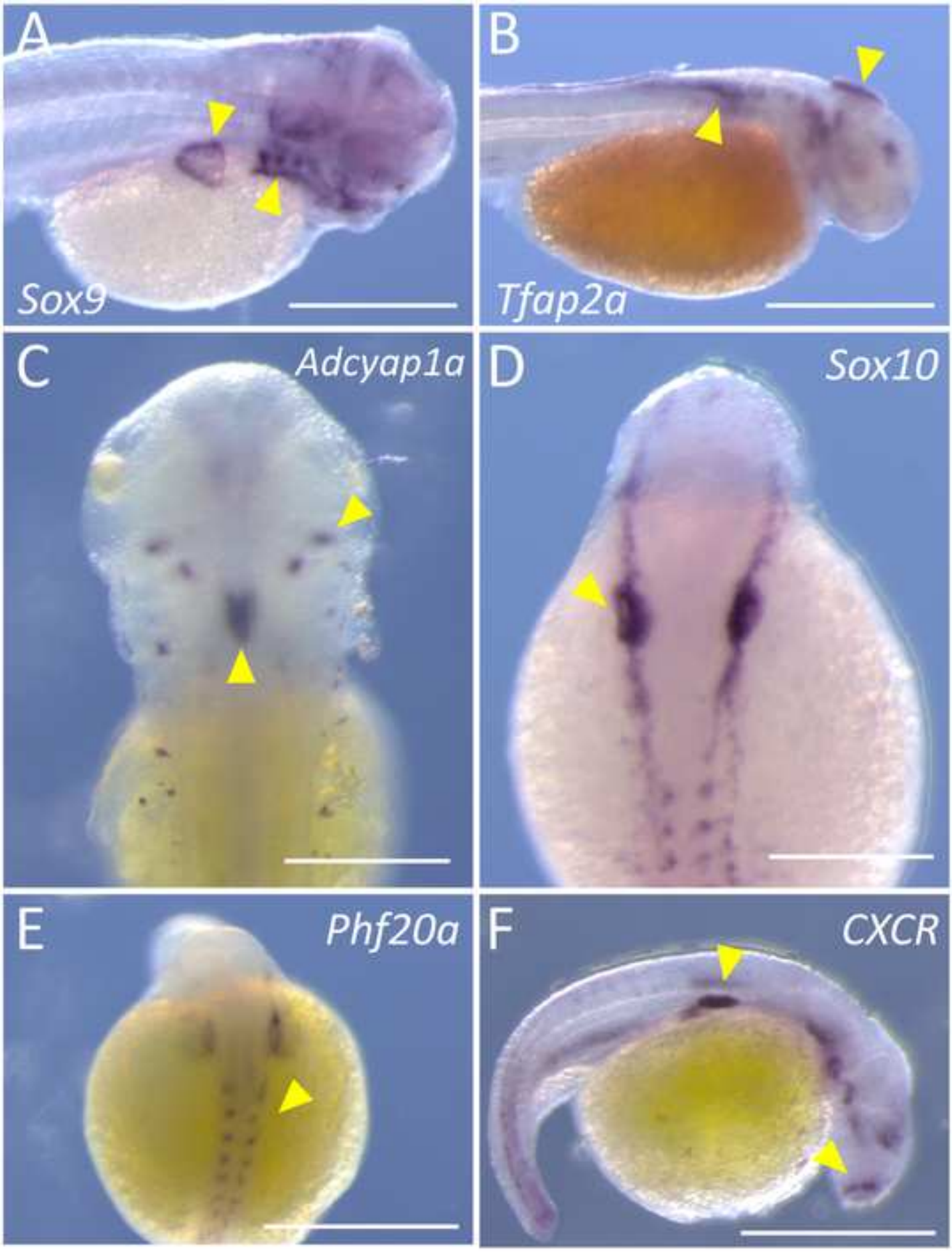
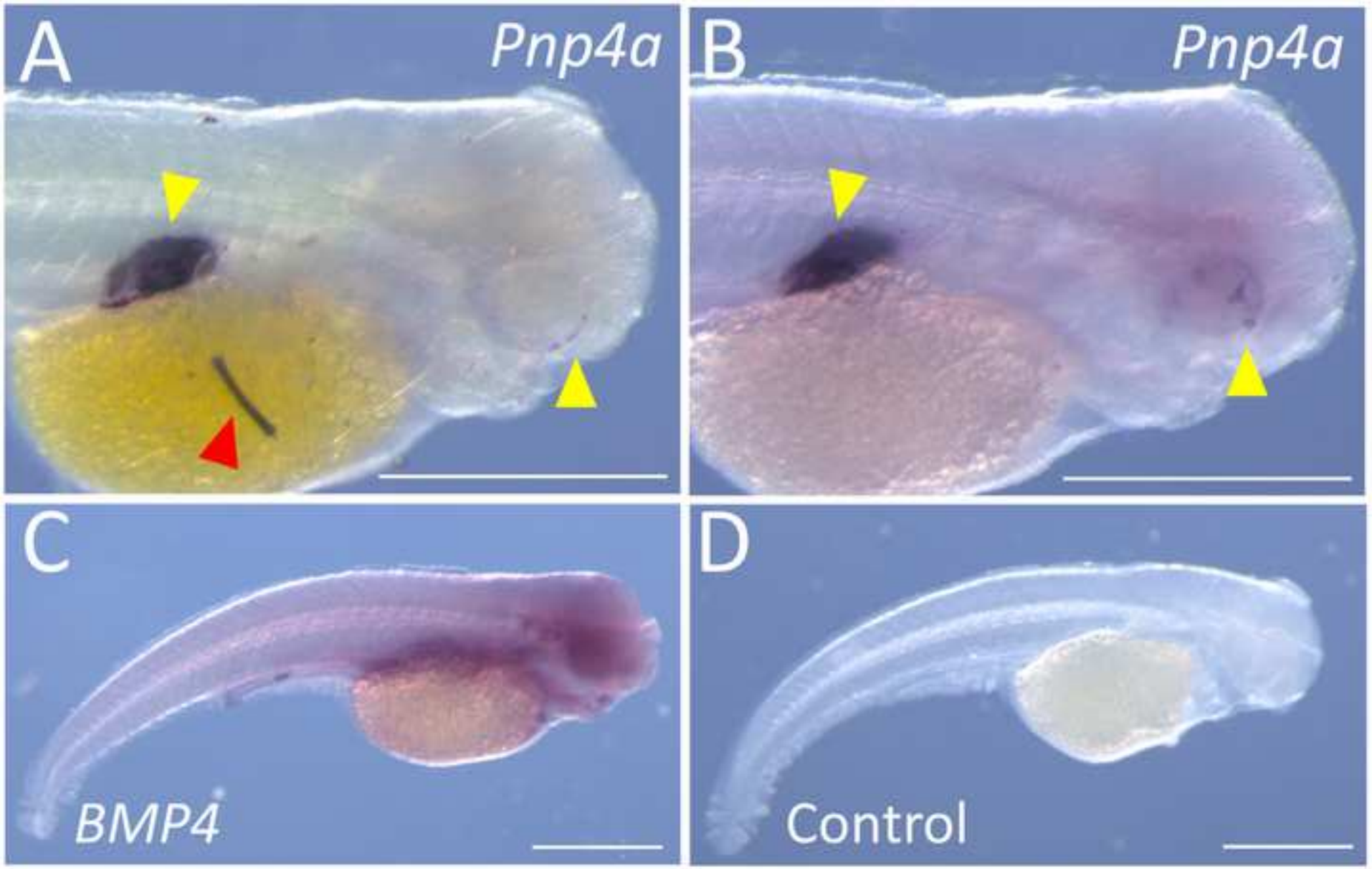


Figure 3



Materials List

Click here to access/download;Table of Materials;Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10 mL Serological Pipette	VWR	89130-888	
1000 mL Filtration Unit	VWR	89220-698	
15 mL Conical	VWR-Greiner	82050-278	
25 mL Serological Pipette	VWR	89130-890	
250 mL Filtration Unit	VWR	89220-694	
5 mL Serological Pipette	VWR	89130-886	
50 mL Conical	VWR-Falcon	21008-940	
500 mL Filtration Unit	VWR	89220-696	
Anti-Digoxigenin-AP, Fab fragı	Sigma-Roche	11093274910	
BCIP	Sigma-Aldrich	B8503-1G	1 g
Blocking Solution	Sigma-Roche	11 096 176 001	50 g
Citric Acid	Fisher Scientific	A104-500	500 g
DIG RNA Labeling Kit (SP6/T7)	Sigma-Roche	11175025910	
Eppendorf Tubes	VWR	20170-577	
Ethanol	Fisher-Decon	04-355-223	1 Gal
Formamide	Thermo Fisher Scientific	17899	100 mL
Glass dram vials	VWR	66011-041	1 Dr
Glass Pipettes	Fisher Scientific	13-678-8A	
HCl	Thermal-ScientificPharmco-AAPER	284000ACS	500 mL
Heparin	Sigma	H3393-25KU	
Magnesium Chloride-crystallir	Fisher Scientific	M33-500	500 g
Maleic Acid	Sigma	M0375-100g	100g
Methanol	Fisher Scientific	A452-4	4L
Molecular-grade Water (RNas	VWR	7732-18-5	500 mL
NaCl	Fisher Scientific	S271-3	3 kg
NaOH pellets	Fisher Scientific	S318-500	500 g
NBT Substrate powder	ThermoFisher Scientific	34035	1 g
Normal Goat Serum	Fisher-Invitrogen	31873	
Nutating Mixer	VWR	82007-202	

Paraformaldehyde	Sigma	158127-500g	500 g
PBS 10x	Fisher Scientific	BP399-20	20L
Proteinase K (200mg/10ml)	Qiagen	19133	10 mL
Plastic Pipettes	VWR-Samco	14670-147	
RNAse	Sigma	R2020-250mL	250 mL
Shaking Water Bath 12 L	VWR	10128-126	12 L
Standard Analog Shaker	VWR	89032-092	
Tris	Sigma Millipore-OmniPur	9210-500GM	500 g
tRNA Yeast	Fisher-Invitrogen	15401011	25 mg
Tween 20	Sigma	P9416-50mL	50 mL
Vortex-Genie 2	Fisher Scientific-Scientific Industries, Inc	50-728-002	
Lithium Chloride (LiCl)	Sigma-Aldrich	203637-10G	10 g

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Author(s): Heidi Luc, Connor Seape, Andrew Raczka, Joshua B. Gross

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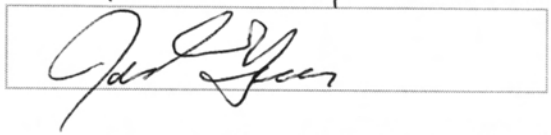
CORRESPONDING AUTHOR:

Name: Joshua Gross

Department: Biological Sciences

Institution: University of Cincinnati

Article Title: A simple & flexible protocol for in situ hybridization for Astyanax embryos.

Signature:  Date: 9/17/18

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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).

DAY ONE		
Embryos divided		
Water bath: **70°C no shaking**		
Rehydrate *5min/step*	100% MeOH	
	25% PBT	
	50% PBT	
	75% PBT	
	100% PBT	
1µl of PK added to 2 ml of PBT		
Aliquots of 4% PFA thawed		
Aliquots of HYB+ and HYB- placed in water bath		
Embryos digested in PK for ***12 minutes***		
Brief PBT Rinse		
PBT for 5 min		
Post-fix with 4% PFA for 20 min		
Brief PBT Rinse		
PBT Washes (5 min/wash)		
Warm HYB- added (5 min) ***NO SHAKING***		
HYB- removed		
Warm HYB+ added (4 hr or O/N)		

DAY ONE or TWO	
Aliquots of HYB+ placed in water bath (if not done previously)	
HYB+ removed	
HYB+ added	
Probe added	
Probe made contact with all embryos	
Incubate in water bath O/N	

Gene 1: Label and tape color

Gene 2: Label and tape color

Initials and date:

DAY THREE

Water bath turned on **70°C, 40rpm**		
Blocking solutions prepared		
HYB+ with probe removed and saved		
HYB+ with probe stored and Label:		
Solutions prepared: ***Place in water bath**	**HYB-***	
	25% SSC*	
	50% SSC*	
	75% SSC*	
	2xSSC*	
	0.2xSSC*	
	25% PBT	
	50% PBT	
	75% PBT	
	100% PBT	
Decreasing HYB- rinse series (10 min/step)	HYB- (briefly)	
	25% SSC	
	50% SSC	
	75% SSC	
2x SSC for 10 min		
0.2x SSC for 30 min		
Water bath turned off		
Increasing PBT rinse series (10 min/step)	25% PBT	
	50% PBT	
	75% PBT	
	100% PBT	
Wash with MABT (5 min/step)		
Blocking solution fully dissolved		
Incubate in blocking solution for 4 hours		
Add 2µL Anti-DIG to blocking solution		
Incubate in BS w/ Anti-DIG O/N at 4°C		

DAY FOUR

NGS thawed	
Add 100µl of NGS to 900µl of MABT	
MABT Washes (30 min/step) AT LEAST 12 washes	
Incubate O/N in 4°C in walk-in	

DAY FIVE		
Make up AP Buffer		
Pull off MABT		
Wrap drams in foil		
100% AP Buffer for 5 minutes		
Incubate in 1mL of AP Buffer/NBT/BCIP for 1 hour. Switch out every hour.		
Pull off AP Buffer/NBT/BCIP		
100% AP Buffer for 5 minutes		
Increasing PBT dilutions for 5 minutes each	25% PBT	
	50% PBT	
	75% PBT	
	100% PBT	
Terminate reaction by rinsing in PBT until clear (5mL). Switch out every hour.		
Rinse in PBS (5min/step)		
Thaw aliquots of 4% PFA		
Fix embryos either for 1 hour at RT or O/N in 4°C		
100% PBS for 5 minutes		
Store embryos in 100% PBS at 4°C		

Exposure time gene 1:

Exposure time gene 2:

Hybridization Buffer (Hyb-) for *in situ* Hybridization

Reagents	**Volume Added**	**Final Concentration**
Formamide	25 mL	50% Formamide
20x SSC buffer	12.5 mL	5x SSC
Tween-20	50 µL	0.10%
1M Citric Acid	460 µL	→ pH 6
RNase-free H_2O	11.99 µL	→ 50 mL

1. Collect all materials necessary to make Hybridization buffer (+):
   Formamide
   20x SSC
   Tween-20
   1M Citric Acid
   RNase-free H_2O
   Bucket of ice (*Formamide must remain cold!*)
   Automated pipettor w/ three 10 mL and two 25 mL serological pipettes
   Sterile 300 mL glass beaker
   P1000 pipet w/ sterile-filter tips
   P200 pipet w/ sterile-filter tips
   Two sterile 50mL conical vials w/ holders
   Repeat pipettor w/ 10 mL tips
   50 sterile Eppendorf tubes

Make up these reagents prior to Step 2:

- Pour roughly 250 mL 20x SSC buffer into the sterile 300 mL glass beaker. Sterile filter and label "Sterile 20x SSC buffer, name, date."
- Take a new 50 mL conical and add 50 mL RNase-free H_2O, then add 10.51 g Citric Acid Monohydrate (F.W. 210.14) to make 1M Citric Acid. Sterile filter and label "Sterile 1M Citric Acid, name, date."

2. Clean inside the fume hood. Bring all necessary items into the fume hood once they are wiped down with 70% ethanol. Close the hood and UV sterilize for 15 seconds.

3. Using a serological pipette with three 10 mL tips, add 25 mL formamide to a new sterile 50 mL conical vial.

4. Using a serological pipette with one 25 mL tip and a p1000 set to 500 µL, add 12.5 mL 20x SSC buffer to the 50 mL conical vial.

5. Using a p200 – over the course of one minute – slowly pipet up 50 µL of Tween-20 and dispense it into the 50 mL conical vial.

6. Using a p1000, add 460 µL Citric Acid to the 50 conical vial.

7. Using a serological pipette with one 25 mL tip and a p1000 set to 990 µL, add 11.99 mL RNase-free H_2O to the 50 mL conical vial.

8. 50 sterile Eppendorf tubes should be placed in a clean rack with the lids open prior to pipetting.

9. Insert a 10 mL tip into the repeat pipettor and set the dial to 1 mL. Aliquot 1 mL of HYB- into 50 sterile Eppendorf tubes. *(Be sure to mix the solution between each few aliquots, this is important for equal concentrations between each tube.)*

10. Label and parafilm each tube. Store in a box labeled “HYB-” in -20°C freezer.

Hybridization Buffer (Hyb+) for *in situ* Hybridization

Reagents	**Volume Added**	**Final Concentration**
Formamide	25 mL	50% Formamide
20x SSC buffer	12.5 mL	5x SSC
5 mg/mL Heparin	500 µl	50 µg/mL
25 mg/mL tRNA	1 mL	500 µg/mL
Tween-20	50 µL	0.10%
1M Citric Acid	460 µL	→ pH 6
RNase-free H_2O	10.49 µL	→ 50 mL

1. Collect all materials necessary to make Hybridization buffer (+):
   Formamide
   20x SSC
   5 mg/mL Heparin
   25 mg/mL tRNA
   Tween-20
   1M Citric Acid
   RNase-free H_2O
   Bucket of ice (*Formamide must remain cold!*)
   Automated pipettor w/ three 10 mL and two 25 mL serological pipettes
   Sterile 300 mL glass beaker
   P1000 pipet w/ sterile-filter tips
   P200 pipet w/ sterile-filter tips
   Two sterile 50mL conical vials w/ holders
   Repeat pipettor w/ 10 mL tips
   50 sterile Eppendorf tubes

Make up these reagents prior to Step 2:

- Pour roughly 250 mL 20x SSC buffer into a sterile 300 mL glass beaker. Sterile filter and label "Sterile 20x SSC buffer, name, date."
- Take a new 50 mL conical and add 50 mL RNase-free H_2O, then add 10.51 g Citric Acid Monohydrate (F.W. 210.14) to make 1M Citric Acid. Sterile filter and label "Sterile 1M Citric Acid, name, date."

2. Clean inside the fume hood. Bring all necessary items into the fume hood once they are wiped down with 70% ethanol. Close the hood and UV sterilize for 15 seconds.

3. Using a serological pipette with three 10 mL tips, add 25 mL formamide to a new sterile 50 mL conical vial.

4. Using a serological pipette with one 25 mL tip and a p1000 set to 500 µL, add 12.5 mL 20x SSC buffer to the 50 mL conical vial.

5. The concentration of heparin in the -20°C freezer is 5 mg/mL. Using a p1000, add 500 µL Heparin to the 50 mL conical vial.

6. Use a p1000 for this step. Add 1 mL RNase-free H_2O to a new vial of 25 mg yeast tRNA to bring the concentration in the vial to 25 mg/mL. Add the full amount (1 mL) to the 50 mL conical vial.

7. Using a p200 – over the course of ~1 minute – slowly pipet up 50 µL of Tween-20 and dispense it into the 50 mL conical vial.

8. Using a p1000, add 460 µL Citric Acid to the 50 conical vial.

9. Using a serological pipette with one 25 mL tip and a p1000 set to 490 µL, add 10.49 mL RNase-free H_2O to the 50 mL conical vial.

10. 50 sterile Eppendorf tubes should be placed in a clean rack with the lids open prior to pipetting.

11. Insert a 10 mL tip into the repeat pipettor and set the dial to 1 mL. Aliquot 1 mL of HYB+ into 50 sterile Eppendorf tubes. *(Be sure to mix the solution between each few aliquots, this is important for ensure equal concentrations will be distributed to each tube.)*

12. Label and parafilm each tube. Store in a box labeled “HYB+” in -20°C freezer.

MABT for *in situ* Hybridization

Reagents	**Volume Added**	**Final Concentration**
Maleic Acid (M.W. 116.07)	4.35 g	150 mM
NaCl (F.W. 58.44)	1.46 g	100 mM

1. Collect items as follows:
   - Bottle of **RNase-free H_2O**
   - 250 mL graduated cylinder
   - 300 mL glass beaker
   - Weigh boats
   - **Maleic acid** (M.W. 116.07)
   - **NaOH pellets**
   - 50 mL conical vial of **NaOH, 1M**
   - Bottle of **HCl** located in fume hood
   - P200 pipette w/ adjoining sterile-filter tips
   - **NaCl** (F.W. 58.44)
   - 50 mL bottle of undiluted **Tween-20**
   - P1000 pipette with sterile-filter tips
   - Sterile filter 250 mL bottle
   - Magnetic stir bar

2. Add 200 ml of RNase-free H_2O to the 300 mL glass beaker.

3. Weigh out 4.35 g of maleic acid on a weigh boat and pour its contents into the 300mL glass beaker. Use the probe in order to record its pH. It should read approximately 1.65 pH. Stir with magnetic stir bar.

4. Adjust the pH w/ NaOH pellet-wise to 7.5. It will take approximately 25 pellets. Add them slowly and allow them to suspend into solution before adding more. If the pH reaches a value > 7.5, add HCl dropwise to the solution (10 µl ≈ .1 pH decrease).

5. Add 1.46 g of NaCl to the 300 mL glass beaker.

6. Using a P1000, slowly (over the course of several minutes) pull up 250 µl of Tween-20 and add it to the 300 mL glass beaker. (*Be sure not to incorporate bubbles or residual Tween-20 dripping off the pipette tip.)*

7. Add the contents of the 300 mL glass beaker to the 250 mL graduated cylinder. Top off the solution with RNase-free H_2O for an end volume of 250mL.

8. Add the contents to a sterile filter 250 mL bottle. Pull the contents through the sterile filter using a vacuum. Wrap the bottle in aluminum foil, label it “MABT’ and store in the 4°C fridge.

Alkaline Phosphatase (AP) Buffer Recipe and Protocol

1. **Make up stock solutions**. Sterile filter all solutions into 50 mL conicals. Wrap conicals in foil to limit light exposure. Store in 4°C refrigerator until needed.

a. 1 M Tris (pH 9.5)
-add 6.06g of Tris to 50 mL of ddH_2O
-add HCl in 100µl increments until reaching pH 9.5. This take approximately 600µl.

b. 50mM $MgCl_2$
-add 0.51g of $MgCl_2$ to 50 mL of ddH_2O

c. 1% Tween-20
-add 500µl of Tween-20 to 50 mL of ddH_2O

d. 1 M NaCl
-add 2.92g of NaCl to 50 mL of ddH_2O

e. Sterile filter 50 mL of ddH_2O (does not need to be stored at 4°C in foil)

2. **Make up working solution of AP Buffer** the day of the coloration reaction. Work in the laminar flow hood, using a sterile 50 mL conical. Wrap conical in foil to limit light exposure. Add the following components in order, using serological pipets.

5mL of 1 M Tris
5mL of 50 mM $MgCl_2$
5mL of 1% Tween-20
5mL of 1 M NaCl
30mL of ddH_2O

RNA Probe Synthesis for *in situ* Hybridization

In addition to the reagents below, prepare sterile aliquots of the following: molecular grade water, 70% EtOH, and 100% EtOH.

Each reagent of the following reagents from the DIG RNA labeling Kit (SP6/T7) should be stored in the -20°C freezer:

8 – Transcription Buffer, 10X
7 – Nucleotide Mix (DIG), 10X
Gene-of-interest, Purified PCR fragment
10 – RNase Inhibitor
11 – SP6 RNA polymerase OR **12** – T7 RNA polymerase, (depending on fragment orientation)
9 – DNase

1. Set thermocycler or heat block to incubate at 37°C. If using thermocycler, we recommend that the lid temperature is set to 105°C.

2. Place purified PCR reaction tube on ice and add the following:
   10.5 µl H_2O for Probe Synthesis
   8 – 2 µl Transcription Buffer, 10X
   7 – 2 µl Nucleotide Mix (DIG), 10X
   For 'SOX10' – 4 µl Purified 'SOX10' PCR Fragment
   10 – 0.5 µl RNase Inhibitor
   11 – 1 µl SP6 RNA polymerase OR **12** – T7 RNA polymerase
   = Total volume of 20 µl in a 800 µL reaction tube.

7. Place PCR reaction tube in the thermocycler for 2 hours.

8. Add 1 µl DNase to the PCR tube. Thoroughly mix reaction, and spin down using a microfuge before returning to the thermocycler for another 15 minutes @ 37°C.

9. Add the following to an Eppendorf tube placed on ice:
   a. 80 µl dH_2O for Probe Synthesis
   b. 10 µl 4m LiCl
   c. 330 µl 100% EtOH

10. Using a p200, transfer the 21 µL total volume from the PCR reaction tube to the Eppendorf tube.

11. Vortex and centrifuge, then incubate at -20°C for at least 30 minutes, or overnight (for best results).

12. Balance and spin the Eppendorf tube in a refrigerated centrifuge (set to 4°C) for 10 minutes at 14,000K.

13. Decant liquid, but make certain to retain pellet. Check by simply tilting the Eppendorf tube with liquid in it while the cap is on.

14. Wash pellet with 200 µl 70% EtOH. Be certain to resuspend the pellet before placing the Eppendorf tube back into the centrifuge.

15. Place the Eppendorf tube in the refrigerated centrifuge (set to 4°C) for 10 minutes at 14,000K.

16. Repeat Steps 13 through 15.

17. **Do NOT decant the supernatant!** The pellet is most likely floating freely in the Eppendorf tube. Use a p200 pipet tip to pipette off ~200 µl of 70% EtOH.

18. Allow the pellet to dry for 10 minutes and dissolve the sample in 10 µl H_2O.

19. Run 1 µl of the probe on a 1.5% gel at 70V for ~1.5 hours. We use ~5 µl of Marker VIII MW Ladder Roche) to estimate size and quantity of the synthesized probe.

20. Probe can now be used for whole-mount *in situ* hybridization.