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TITLE:

Whole Mount Immunohistochemistry in Zebrafish Embryos and Larvae

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KEYWORDS:

zebrafish, *Danio rerio*, antibodies, immunohistochemistry, protein expression, glutamate receptors

SUMMARY:

Here, we present a protocol for fluorescent antibody-mediated detection of proteins in whole preparations of zebrafish embryos and larvae.

ABSTRACT:

Immunohistochemistry is a widely used technique to explore protein expression and localization during both normal developmental and disease states. Although many immunohistochemistry protocols have been optimized for mammalian tissue and tissue sections, these protocols often require modification and optimization for non-mammalian model organisms. Zebrafish are increasingly used as a model system in basic, biomedical, and translational research to investigate the molecular, genetic, and cell biological mechanisms of developmental processes. Zebrafish offer many advantages as a model system but also require modified techniques for optimal protein detection. Here, we provide our protocol for whole-mount fluorescence immunohistochemistry in zebrafish embryos and larvae. This protocol additionally describes several different mounting strategies that can be employed and an overview of the advantages and disadvantages each strategy provides. We also describe modifications to this protocol to allow detection of chromogenic substrates in whole mount tissue and fluorescence detection in sectioned larval tissue. This protocol is broadly applicable to the study of many developmental stages and embryonic structures.

INTRODUCTION:

The zebrafish (*Danio rerio*) has emerged as a powerful model for the study of biological processes for several reasons including short generation time, rapid development, and amenability to genetic techniques. As a result, zebrafish are commonly used in high throughput small molecule screens for toxicological research and drug discovery. Zebrafish are also an attractive model for the study of developmental processes given that a single female can routinely produce 50-300

eggs at a time and the optically clear embryos develop externally allowing for efficient visualization of developmental processes. However, early research relied mostly on forward genetic screens using N-ethyl-N-nitrosourea (ENU) or other mutagens due to challenges in establishing reverse genetic techniques. Roughly two decades ago, morpholinos were first used in zebrafish to knockdown targeted genes[1]. Morpholinos are small antisense oligonucleotides that inhibit translation of target mRNA following microinjection into an embryo at an early developmental stage. A major weakness of morpholinos is that they are diluted as the cells divide and generally lose effectiveness by 72 hours post-fertilization (hpf). While morpholinos remain a powerful tool for zebrafish gene disruption, transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats (CRISPRs) are more recently being used to directly target the zebrafish genome[2,3]. These reverse genetic strategies, in combination with forward genetics and high throughput screens, have established the zebrafish as a powerful model to study gene expression and function.

The ability to study gene function generally requires an evaluation of the spatio-temporal distribution of gene or gene product expression. The two most commonly used techniques to visualize such expression patterns during early development are in situ hybridization (ISH) and whole mount immunohistochemistry (IHC). In situ hybridization was first developed in 1969 and relies on the use of labeled antisense RNA probes to detect mRNA expression in an organism[4]. In contrast, labeled antibodies are used in immunohistochemistry to visualize protein expression. The idea of labeling proteins for detection dates back to the 1930's[5] and the first IHC experiment was published in 1941 when FITC-labeled antibodies were used to detect pathogenic bacteria in infected tissues[6]. ISH and IHC have evolved and improved significantly over the subsequent decades and are now both routinely used in the molecular and diagnostic research laboratory[7-11]. While both techniques have advantages and disadvantages, IHC offers several benefits over ISH. Practically, IHC is much less time consuming than ISH and is generally less expensive depending on the cost of the primary antibody. In addition, mRNA expression is not always a reliable metric of protein expression as it has been demonstrated in mice and humans that only about a third of protein abundance variation can be explained by mRNA abundance[12]. For this reason, IHC is an important supplement to confirm ISH data, when possible. Finally, IHC can provide subcellular and co-localization data that cannot be determined by ISH[13-15]. Here, we describe a step-by-step method to reliably detect proteins by immunohistochemistry in whole mount zebrafish embryos and larvae. The goal of this technique is to determine the spatial and temporal expression of a protein of interest in the whole embryo. This technology utilizes antigen-specific primary antibodies and fluorescently tagged secondary antibodies. The protocol is readily adaptable to use on slide-mounted tissue sections and for use with chromogenic substrates in lieu of fluorescence. Using this protocol, we demonstrate that developing zebrafish skeletal muscle expresses ionotropic glutamate receptors, in addition to acetylcholine receptors. NMDA-type glutamate receptor subunits are detectable on the longitudinal muscle at 23 hpf.

PROTOCOL:

The procedures for working with zebrafish breeding adults and embryos described in this protocol were approved by the Institutional Animal Care and Use Committee at Murray State University.

1. Embryo collection and fixation

1.1. Prepare spawning tanks by placing adult zebrafish mixed sex pairs or groups in tanks with a mesh or slotted liner filled with system water overnight.

1.2. At lights on, change the spawning tank water for fresh system water to remove feces. Use a 14 h/10 h light dark cycle with lights coming on at 9 am.

1.3. Once eggs are laid, return the adults to home tanks.

1.4. Collect eggs by drawing them up using a transfer pipet or pouring them into a mesh strainer.

1.5. Transfer the eggs to Petri dishes filled halfway with embryo medium (such as 30% Danieau or E2 embryo medium with 0.5 mg/L methylene blue), limiting the number of embryos per dish to 50.

1.6. Remove any eggs that are dead or fail to divide.

NOTE: Dead embryos can be easily identified as they become opaque and often appear “cloudy”. If methylene blue is added to embryo medium, the dead embryos take on a dark blue appearance.

1.7. Incubate dishes of eggs at 28.5 °C until they reach the desired stage. For this experiment, raise the embryos until 23 hpf.

1.8. (Optional) Transfer the embryos at 24 hpf to 200 µM 1-phenyl 2-thiourea (PTU) in embryo medium to prevent melanogenesis[16,17]. Alternatively, bleach embryos post-fixation (see optional section 5).

1.9. Change embryo medium or PTU medium daily.

1.10. Dechorionate unhatched embryos using ultra-fine-tip forceps under a stereomicroscope. Alternatively, chemically dechorionate embryos by incubating in 1 mg/mL Pronase in embryo medium for several minutes at room temperature. Remove the embryos from Pronase and wash three times with embryo medium.

1.11. Dechorionated embryos will stick to plastic. Keep them in glass or plastic Petri dishes coated with 1-2% agarose dissolved in embryo medium. Move dechorionated embryos using fire-polished Pasteur pipets to minimize damage.

1.12. Transfer the embryos to 1.5 mL centrifuge tubes using a plastic or fire-polished pipet.

1.13. Remove embryo medium with a micropipette. Leave only enough liquid to just cover the embryos after each fluid change.

1.14. Prepare 4% paraformaldehyde (PFA) in 1x phosphate-buffered saline (PBS) in a chemical fume hood.

CAUTION: PFA is a hazardous material. Wear gloves and dispose of contaminated liquids and solids in designated areas.

1.15. Fix the embryos in 4% PFA for 1-2 h with gentle rocking at room temperature. Alternatively, fix the embryos 4 h to overnight at 4 °C.

1.16. Wash three times in 1x PBS + 1% Triton-X (PBTriton) for 5 min.

1.17. Use the embryos immediately or store at 4 °C for up to 1 week.

1.18. For long term storage, dehydrate the embryos in 100% methanol (MeOH) 2 h or overnight at -20 °C. Store the embryos at -20 °C in MeOH for several months.

CAUTION: MeOH is a hazardous material. Wear gloves and dispose of contaminated liquids and solids in designated areas.

2. Embryo preparation

2.1. Rehydrate the embryos through serial incubations at room temperature.

2.1.1. Incubate in 75% MeOH/25% 1x PBS for 5 min, rocking.

2.1.2. Incubate in 50% MeOH/50% 1x PBS for 5 min, rocking.

2.1.3. Incubate in 25% MeOH/75% 1x PBS for 5 min, rocking.

2.1.4. Incubate in 100% PBTriton for 5 min, rocking.

2.2. (Optional) Prepare a fresh proteinase K working solution (10 µg/mL in PBTriton) on ice by adding 10 µL of freshly thawed proteinase K stock (10 mg/mL).

2.2.1. Permeabilize the embryos by digesting up to 30 min in Proteinase K.

NOTE: Suggested timing is < 24 hpf: no digestion; 24 hpf: 15 min digestion; and 7 days old: 30 min digestion.

2.2.2. Rinse permeabilized embryos in PBTriton and re-fix in 4% PFA for 20 min at room temperature.

2.2.3. Wash the embryos three times in PBTriton for 5 min at room temperature with gentle rocking.

3. Primary antibody incubation

3.1. Select a commercial blocking solution or serum matching the secondary antibody host species (ex. 10% goat serum in PBTriton) with or without 2 mg/mL Bovine Serum Albumin (BSA).

3.2. Block the embryos in blocking solution for 1-3 h at room temperature or overnight at 4 °C while rocking.

3.3. Incubate in primary antibody diluted in blocking solution or 1% serum in PBTriton overnight at 4 °C while rocking. In this experiment, the primary antibodies used were anti-NMDAR1, anti-pan-AMPA receptor, and anti-phospho-Histone H3, each diluted to a final concentration of 1:500 in 1% goat serum in PBTriton.

3.4. Wash five times in PBTriton for 10 min at room temperature while rocking.

4. Secondary antibody incubation

4.1. Select a secondary antibody based on the host species of the primary antibody and the desired wavelength.

4.2. Incubate in secondary antibody diluted in blocking solution or 1% serum for 2 h at room temperature (or overnight at 4 °C) while rocking.

NOTE: Fluorescent secondary antibodies are light sensitive. We used 1:500 goat-anti-mouse Alexa488 diluted in 1% goat serum in PBTriton.

4.3. Cover the tubes with aluminum foil or cover with a light-blocking box for this and all subsequent steps.

4.4. Wash three times in PBTriton for 10 min at room temperature while rocking.

4.5. Transfer the embryos to a 50% glycerol solution in PBS over a bed of 2% agarose in embryo medium and proceed to documentation or proceed to further processing steps below.

Optional Steps

5. Bleaching

5.1. Prepare bleach solution in a 1.5 mL tube by adding 810.7 µL of ddH_2O, 89.3 µL of 2 M KOH, and 100 µL of 30% H_2O_2.

5.2. Invert the tube three times to mix.

5.3. Pipette 1 mL of bleach solution direction to the embryos.

5.4. Open the embryo tube cap to allow gas to escape. Gently tap the tube on the bench to dislodge bubbles.

5.5. Monitor the bleaching process (use a microscope if necessary) and stop the reaction when pigment is sufficiently removed (approximately 5 min for 24 hpf or 10 min for 72 hpf).

5.6. Carefully remove the bleaching solution with a micropipette and rinse embryos three times in 1 mL of PBTriton.

NOTE: Embryos are sticky in this step.

5.7. Proceed to documentation or further processing steps below.

6. Embryo dissection and deyolking

6.1. To remove the yolk, transfer a small amount (~ 200 µL or enough to completely cover the embryo but restrictive enough to limit where it can float) of 1x PBS to a depression slide or a plain glass slide.

6.2. Use a plastic transfer pipet to move 1 or more embryos to the PBS droplet.

6.3. Use ultra-fine forceps and 00 insect pins to break apart the yolk and very gently scrape yolk granules from the ventral surface of the embryo (see also Cheng et al., 2014)[18].

6.4. Remove yolk granules and replenish PBS as needed.

6.5. Repeat until embryo is sufficiently free of yolk.

7. Flat mounting on slides

7.1. Transfer deyolked embryos to a charged glass slide with a plastic pipette or a 1 mL micropipette with a trimmed tip (to reduce shear stress). Orient as desired with a 200 µL micropipette tip or insect pin.

7.2. Wick away excess PBS with a Kim wipe or paper towel.

7.3. Add 2-3 drops of mounting media to the slide and coverslip.

7.4. Air dry for approximately 5 to 10 min.

7.5. Seal the cover glass onto the slide with clear nail polish.

NOTE: The edges of the cover glass must be completely covered with a thin, continuous layer of nail polish.

7.6. Allow to dry approximately 10 min before imaging.

8. **Mounting in agarose**

8.1. Prepare 1% agarose in embryo medium by adding 0.5 g of agarose to 50 mL of embryo medium in a microwave-safe flask or beaker of at least 3x greater volume than desired.

8.2. Heat in a microwave, swirling every 30 s, until agarose is completely dissolved.

8.3. Make 1 mL aliquots in 1.5 mL centrifuge tubes. Store the aliquots at room temperature.

8.4. Cover tube caps with cap locks before heating.

8.5. Place agarose tubes in a floating tube holder in a beaker half-filled with water.

8.6. Microwave the beaker with floating tubes for 2-3 min, or until agarose is completely melted.

8.7. Transfer an embryo to the bridged slide with a plastic pipette or a 200 µL micropipette with a trimmed tip (to reduce shear stress).

8.8. Position the embryo on a rectangular coverslip using insect pins and add approximately 20 µL melted agarose directly to the embryo.

8.9. Quickly orient the region of interest closest to the coverslip using 00 insect pins.

NOTE: This is an upside-down mount.

8.10. Return the agarose tube to the hot water tube float between each use and microwave as needed.

8.11. Image using a microscope when the agarose hardens. Keep the mounted embryo upside-down for use on an inverted microscope. Flip the coverslip over (so the agarose is under the coverslip) for use on upright microscopes.

9. **Mounting on bridged slides**

9.1. To make bridged slides, glue square coverslips to the glass slide using a small dot of

superglue.

NOTE: There should be a trough at least 5 mm wide between the coverslips. Two #1 coverslips high is typically appropriate for 24-48 hpf embryos while three coverslips high may be necessary for 72 hpf.

9.2. Transfer 1-2 deyolked embryos to the bridged slide with a plastic pipette or a 200 µL micropipette with a trimmed tip (to reduce shear stress).

9.3. Wick away excess fluid with a Kim wipe or paper towel.

9.4. Add a drop of ≥ 80% glycerol directly to the embryo.

9.5. Cover with a rectangular cover glass. The droplet of glycerol should touch the cover glass.

9.6. Add more glycerol to the space between the cover glass and slide as needed to completely cover the embryo with a margin of glycerol on the sides of the embryo.

9.7. Slide the rectangular cover glass gently to roll the embryo into position for imaging.

10. DAB staining

NOTE: This section begins after step 4.2 above and replaces the rest of step 4.

10.1. Incubate the embryos in a blocking solution with a peroxidase-conjugated secondary antibody for 2 h at room temperature or overnight at 4 °C while rocking.

10.2. Wash three times in PBTriton for 10 min at room temperature.

10.3. Transfer the embryos to a culture plate or depression slide with a transfer pipette.

10.4. Mix 50 µL of 1% DAB (3,3'-diaminobenzidine) dissolved in ddH_2O and 50 µL of 0.3% hydrogen peroxide and bring to 1 mL with PBS.

CAUTION: DAB is a hazardous material. Wear gloves and dispose of DAB contaminated liquids and solids in designated areas.

10.5. Cover HRP-stained embryos with the DAB solution prepared above and monitor for color development (typically 1-5 min) under a microscope.

10.6. After reaching the desired level of color development, rinse the embryos briefly in PBS.

10.7. Transfer the embryos back to a 1.5 mL tube before fixation.

10.8. Re-fix the embryos for 15-20 min in 4% PFA at room temperature.

10.9. Wash the embryos three times in PBTriton for 5 min.

10.10. Proceed to documentation.

11. **Modified protocol for staining sectioned tissue that is mounted on slides**

11.1. Encircle tissue to be stained with a pap pen.

11.2. Transfer the slides to a humid chamber.

11.3. Add 1 mL ofPBS directly to the slide.

11.4. Incubate 7 min at room temperature to remove embedding medium.

11.5. Pour off PBS by inverting slide.

11.6. Rehydrate 1 min in up to 1 mL of TNT buffer (100 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween20).

11.7. Block in up to 1 mL of blocking solution (commercial or 10% serum + 2% BSA) for 1 h at room temperature.

11.8. Incubate overnight in primary antibody diluted in 1% serum or blocking solution at 4 °C.

11.9. Wash five times in up to 1 mL of TNT buffer at room temperature.

11.10. Incubate in secondary antibody for 2 h at room temperature or overnight at 4 °C. Cover the chamber with foil or use a dark lid.

11.11. Wash five times in TNT at room temperature. Pour off last wash.

11.12. Mount with 2-3 drops of mounting medium and coverslip. Let sit 5-10 min.

11.13. Seal the cover glass onto the slide using clear nail polish. Allow to dry completely before imaging.

12. Documentation

12.1. Record the full procedure and any deviations in a lab notebook.

12.2. Record the concentration, name, catalog number, manufacturer, and lot number of the primary antibody.

12.3. Place appropriately mounted sample on the microscope stage. Locate the region of interest.

12.4. Select a relatively bright example. Set camera exposure and gain so that signal is sufficiently bright without saturating.

12.5. Compare staining intensity of the same region of interest using the same exposure settings when comparing between experimental antibody-labeled embryos and control antibody (ex. IgG) embryos.

REPRESENTATIVE RESULTS:

Whole mount immunohistochemistry uses antibodies to detect the spatial pattern of protein expression in the intact animal. The basic workflow of immunohistochemistry (depicted in **Figure 1**) involves breeding zebrafish, raising and preparing embryos, blocking non-specific antigens, using an antigen-specific primary antibody to target the protein of interest, detecting that primary antibody with a labeled secondary antibody, mounting the specimen, and documenting expression.

Whole mount immunohistochemistry is a valuable tool for the study of spatial and temporal protein expression during zebrafish development. Zebrafish exhibit spontaneous contractions mediated by gap junctions beginning at before 19 hpf - before motor neuron contact[19]. The zebrafish neuromuscular junction, like other vertebrates, is mediated by acetylcholine acting at nicotinic acetylcholine receptors. These assembled receptors are first detected at approximately 16 hpf and expression expands and remodels as neurons form contacts[20]. Studies in frogs[21] and rats[22] suggest that the skeletal muscle of vertebrates can also express ionotropic glutamate receptors. Whole mount immunohistochemistry for the GluN1 subunit of the NMDA-type glutamate receptor reveals expression of glutamate receptor subunits throughout developing zebrafish muscle at 23 hpf (**Figure 2**). This corresponds approximately with the timing of motor neuron innervation. Expression was compared to no primary control embryos to determine the background fluorescence of the fish and the secondary antibody and to a 2 µg/mL mouse IgG to determine the relative contributions of nonspecific antigen binding. AMPA type glutamate receptors were not detected in the muscles at this stage of development. Antibody concentrations are listed in **Table 1**. To generate these images, these embryos were processed as described in this protocol with none of the optional steps except for deyolking. Embryos were flat mounted and coverslipped (**Figure 3B**).

Dividing cells express different histone modifications from quiescent cells that can be detected by immunohistochemistry using antibodies that recognize specific modifications, such as protein phosphorylation. Phosphorylation of histone 3 at serine 10 is associated with cell division[23]. The modifications presented to this protocol for adaptation of immunohistochemistry to sectioned tissue that is mounted on slides was used to detect proliferating cells in the larval zebrafish brain. Frozen sections of 72 hpf embryos were mounted on slides and immunostained for p-H3 (**Figure 4**). Several cells express p-H3, and expression is most notable at the ventricular zones. Expression

was compared to no primary control embryos and to a 2 µg/mL mouse IgG to determine the relative contributions of nonspecific antigen binding.

FIGURE AND TABLE LEGENDS:

Table 1: List of antibodies and concentrations used.

Figure 1: Flowchart of whole mount immunohistochemistry procedure. The basic workflow of the procedure is to breed fish; collect and prepare embryos; block non-specific antigens; incubate in primary and secondary antibodies in series; mount tissue; and document. Optional steps are indicated with small arrows at the appropriate point in the workflow.

Figure 2: Larvae schematic and NMDA receptor IHC representative results. The use of whole mount immunohistochemistry tested glutamate receptor expression in developing muscle. The orientation and region of interest at 23 hpf is indicated. No signal was detectable when primary antibodies were not included. Mouse IgG control antibody shows the low level of non-specific expression. GluN1 subunit of the NMDA-type glutamate receptor (NMDAR) is expressed across the developing muscle, with higher concentrations at somite boundaries (arrowheads). AMPA-type glutamate receptors (AMPAR) are not expressed at this stage.

Figure 3: Schematic of mounting schemes. (A) An embryo sunk in 50% glycerol can be easily repositioned. **(B)** An embryo flat-mounted on a slide in mounting medium can be preserved and imaged at a later date. **(C)** An embryo mounted in a droplet of 1% agarose can be fixed in position to view a difficult region. **(D)** An embryo mounted in glycerol on a bridged slide can be rolled and repositioned.

Figure 4: Representative results of IHC in sectioned tissue. Using the protocol modifications in the optional steps, immunohistochemistry tested for proliferating cells in the zebrafish larval brain at 72 hpf. Mouse IgG control antibody and excluding primary antibodies reveal a low level of non-specific expression. As a marker of proliferating cells, p-H3 is expressed in discrete locations, including the ventricular zones (arrowhead).

DISCUSSION:

Immunohistochemistry is a versatile tool that can be used to characterize the spatio-temporal expression of virtually any protein of interest in an organism. Immunohistochemistry is used on a wide variety of tissues and model organisms. This protocol has been optimized for use in zebrafish. Immunohistochemistry in different species may require different fixation and handling techniques, blocking solutions depending on species and the presence of endogenous peroxidases, and incubation times due to the thickness and composition of tissues. IHC in zebrafish has been integral in advancing our understanding of cancer[24] , metabolic disease[25], neurological disorders[26], and numerous other areas of great relevance to human health. One major advantage to IHC is that the procedure is relatively short compared to other techniques such as ISH and is not technically demanding. There are, however, numerous steps that require optimization based on the age of the specimens, the antigen being targeted, and the antibodies being used.

The duration of several steps in this protocol is flexible. Durations given for flexible steps as noted represent minimal times generally required. In general, whenever embryos are washed three or more times in PBTriton, they can be kept overnight at 4 °C in the last wash if needed. Permeabilization and fixation times are less flexible and should only be adjusted with deliberate intention as part of a troubleshooting strategy. We noted several points in the protocol that are optional to show how these steps can be integrated in the workflow as is experimentally relevant. For example, if pigmentation interferes with signal detection, prevent melanogensis by PTU treatment or bleach fixed embryos. Bleaching can damage tissue, so care must be taken to minimize the time embryos spend in bleach. However, bleaching may be preferable to PTU treatment, which can affect certain aspects of development[27-30]. We also present options for fluorescent and chromogenic detection. If fluorescence is not desired or if the antigen produces a signal that is too weak to be adequately detected by fluorescence microscopy, chromogenic detection can be achieved using a horseradish peroxidase (HRP)-conjugated antibody and DAB.

The biggest challenge of IHC in zebrafish is finding suitable antibodies. Indeed, ISH is often used as a proxy for protein expression when commercial antibodies are not available for the desired protein of interest. Many commercial antibodies are designed to target mammalian targets and epitopes are not always conserved in zebrafish. When available, select commercially available antibodies that have been tested in zebrafish. We have found that antibodies that recognize antigens that are >80% conserved between zebrafish and the target species generally work in zebrafish. We have also found that antibodies that are demonstrated to work in either birds and/or amphibians in addition to mammals generally also work in zebrafish, even when efficacy in zebrafish has not been tested. Typically, polyclonal antibodies developed against a mammalian antigen are more likely to detect zebrafish homologs than monoclonal antibodies due to their lower specificity. Whenever testing a new antibody, it is beneficial to run a positive control experiment using a cross-linked target peptide, mammalian tissue or cells that are known to express the protein, or cells that express a reporter construct. Antibodies can also be tested by western blot to verify the size of the target antigen.

While most commercial antibodies provide a suggested dilution range for IHC, it is important to empirically determine the dilution that works best. Antibody concentrations that are too high often result in non-specific staining and increased background, while too little antibody fails to provide a discernible signal. Depending on the antibody and the antigen, it may be advantageous to first permeabilize the embryos as described in step 3.2 above, however, this step may not be necessary and in some instances can result in reduced signal. This protocol uses Triton-X-100 as a detergent that permeabilizes cells, which may be sufficient for thin tissue or superficial expression. Deep or thick tissue, such as deep brain regions or older larvae, may require proteinase permeabilization. Conversely, Triton-X-100 should be excluded from all steps when immunostaining only proteins at the cell surface is desired over labeling intracellular proteins. The duration of the blocking step as well as the choice of a commercial blocking solution versus using serum and BSA can also be adjusted to correct for antibody sensitivity and background staining. High concentrations of serum used in blocking (10% in this protocol) can reduce background staining, though should be diluted when antibody is present to minimize masking

antibody binding sites. Plant-based blocking solutions may be beneficial if background signal is consistently high, even at low antibody dilutions (< 1:1000). Finally, sensitivity can be fine-tuned by the stringency of the washes. It may be necessary to increase or decrease the duration of the post-antibody wash steps as well as adjust the amount of Triton-X-100 in the PBTriton. Typical working ranges of PBTriton span 0.2% to 1.0% Triton-X-100. It is good practice when using a new antibody to stain negative control embryos with primary IgG and labeled secondary antibodies to determine antibody specificity and identify potential false positive signals.

If antibody optimization fails to produce a positive signal, it may be due to the fixative masking the antigen. Generally, fixation in 4% PFA does not mask antigen sites to prevent antibody binding, although antigen masking does occasionally occur with some antibodies. Antigen retrieval can result in damage to the embryo but a working protocol has been described by Inoue and Wittbrodt[31]. Alternatively, if the selected antibody is incompatible with 4% PFA or if PFA results in cellular morphology changes, methanol, 2% trichloroacetic acid, or glyoaxal can be used to fix the samples[27]. Compatibility of an antibody with formaldehyde fixation must be determined empirically. If a positive control signal cannot be obtained following PFA fixation, it may be worth trying an alternative fixative such as methanol. Alternative fixation protocols may also be necessary for primary antibodies that were raised against conjugated antigens (such as GABA-BSA).

There are several effective options for mounting immunostained zebrafish embryos for imaging. Embryos can be transferred to a Petri dish of glycerol, positioned with pins or forceps, and imaged either with a widefield view from above or from below imaging through the dish with an inverted microscope (**Figure 3A**). This method is simple, quick, and temporary. Drawbacks include the propensity for embryos to roll out of focus in the fluid glycerol, potential reflections off the surface of the glycerol in the dish, and the difficulty of imaging through the thick dish. Embryos can be mounted flat on glass sides (**Figure 3B**) with mounting medium, coverslips, and nail polish. These mounts can be viewed on an upright or inverted microscope. Embryos prepared in this way can be prepared ahead of time and the slides stored at 4 °C until imaging or can be stored and reimaged later. This can be especially advantageous when imaging time is limited. The disadvantages of this mount include the limited options for embryo position and tissue thickness, and the inability to reposition or recover embryos. Embryos mounted in this way often need deyolking, as the yolk granules are autofluorescent in most commonly used fluorescence channels and cannot be moved or removed after mounting. When the region of interest requires difficult positioning of the embryo, it can be most advantageous to mount the embryos in 1% agarose on a cover glass (**Figure 3C**). The embryo can be held in position with insect pins with the region of interest closest to the cover glass until the agarose cools. The agarose will hold the embryo in position without the embryo rolling for at least several minutes. Agarose mounting is best for inverted microscopes, though the cover glass can be inverted carefully for use on an upright microscope. This mount can be time consuming and embryos are generally not repositionable or recoverable. Mounting embryos on bridged slides (**Figure 3D**) offers somewhat of a compromise between these methods. The bridged mount is quick, and embryos can be repositioned and recovered. The height of the bridge can allow greater flexibility in tissue thickness and embryo position than flat mounts, while maintaining the ability to image from

above or below. This method requires preparing the bridged slides ahead of time. The thickness of the tissue to be mounted dictates how many coverslips high the bridge needs to be. Bridged slides can be reused several times for one day but should be disposed of after the imaging session because the glycerol is difficult to clean off the slides and it will slowly dislodge the glue holding the bridge.

IHC is an effective method for determining the timing and pattern of protein expression in an organism or tissue. IHC has several advantages over ISH in that is relatively low cost and can be completed in a fraction of the time typically necessary for ISH (2-3 days versus 5-8 days). In addition, IHC is a better indicator of gene product expression as detection of mRNA levels do not predict posttranscriptional and posttranslational processes that can affect protein expression. IHC is also capable of providing subcellular localization data (although this is not true when using DAB staining), which is not afforded by ISH. It is possible to perform a dual ISH/IHC in zebrafish.

IHC also has some drawbacks, foremost amongst them being antibody availability. While there are numerous antibodies available that are of suitable quality for IHC, finding antibodies that specifically work in zebrafish is more challenging and often requires testing and troubleshooting antibodies generated against mammalian antigens. However, there are increasing numbers of zebrafish validated antibodies on the market and the emergence of CRISPR/Cas9 technology has made it possible to epitope tag endogenous proteins through genome engineering. These processes are time consuming and challenging, however, and require validation of protein function.

The protocol described in this report can be used broadly on zebrafish embryos and larvae at any stage and can be applied to tissues from adult animals as well. In addition, this protocol also allows for staining of frozen or paraffin sectioned samples with little modification. Whole mount immunohistochemistry was used to examine neurotransmitter receptor populations in muscle, revealing expression of the ionotropic NMDA glutamate receptor obligatory subunit 1 in developing zebrafish muscle. This rather widespread and diffuse staining across the developing muscle is consistent with the developmental expression of the same receptor subunit in developing Xenopus larvae[21]. This suggests that the expression of glutamate receptors in developing muscle is evolutionarily conserved. Altogether, this protocol is valuable for gaining a better understanding of gene expression through the use of IHC and provides a powerful tool for determining the spatio-temporal distribution of protein expression in zebrafish.

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The authors have no information to disclose.

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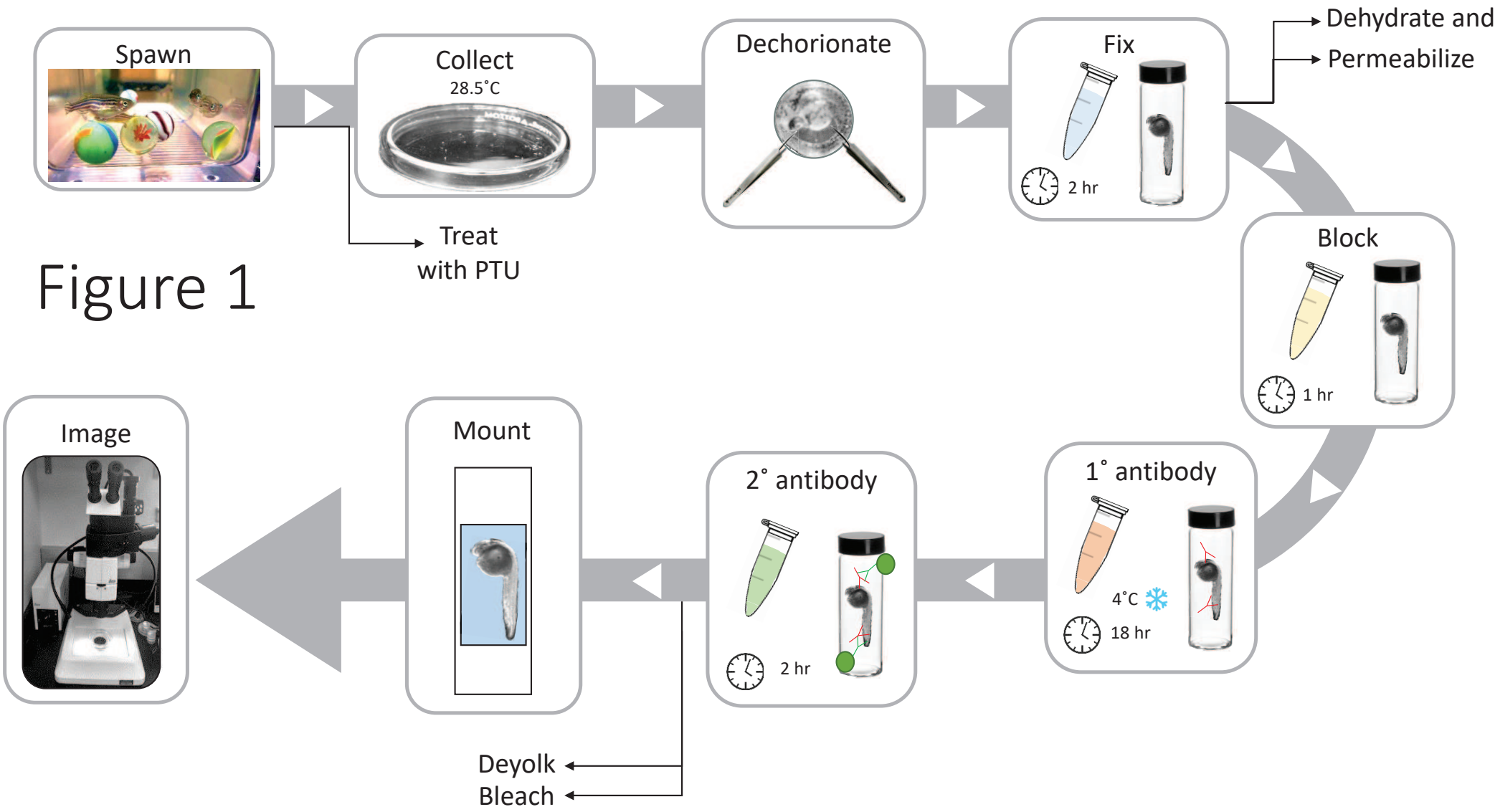


Figure 1

Figure 2

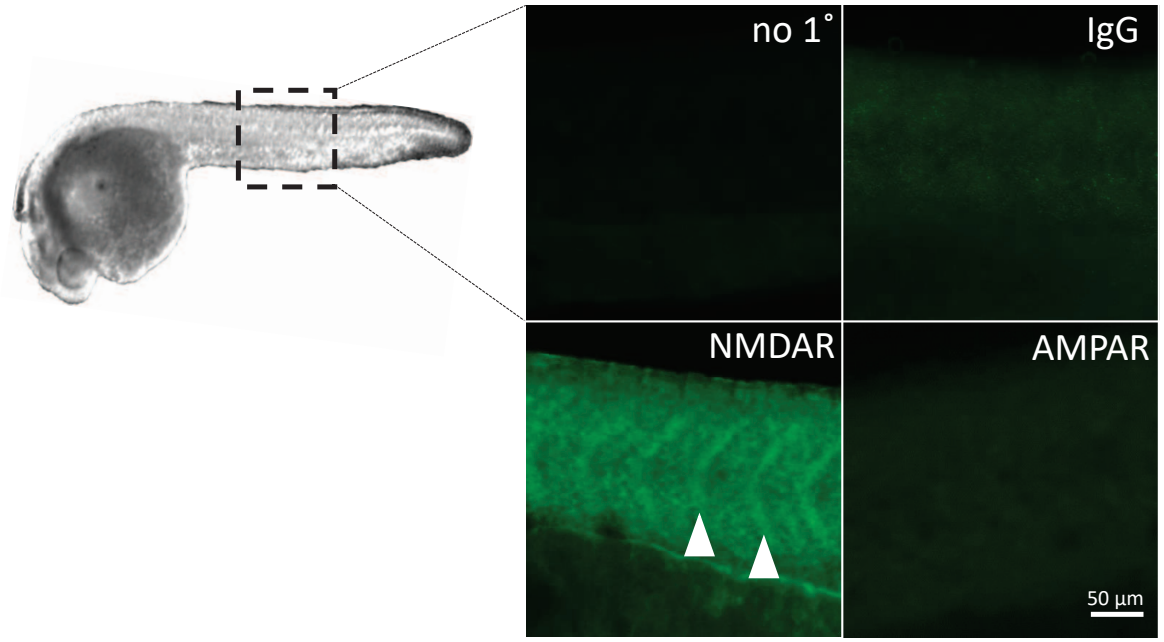


Figure 3

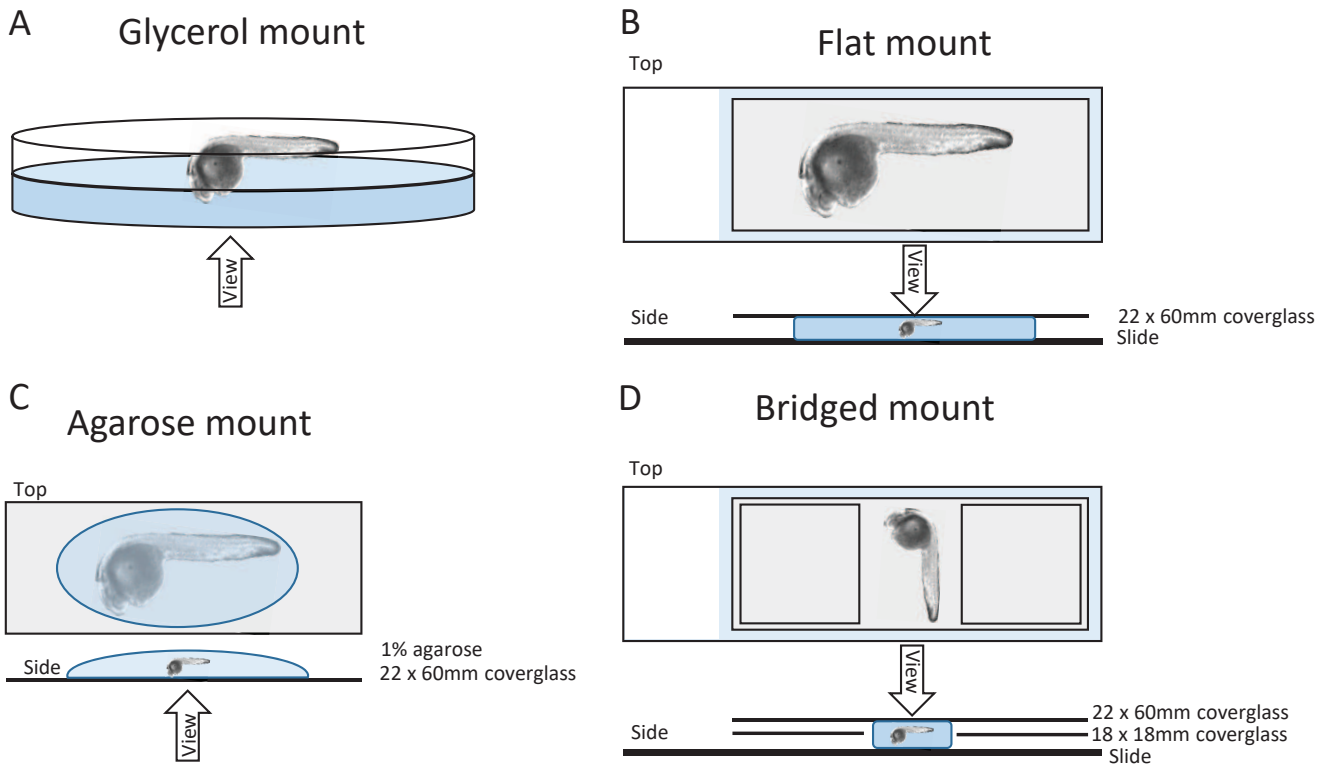
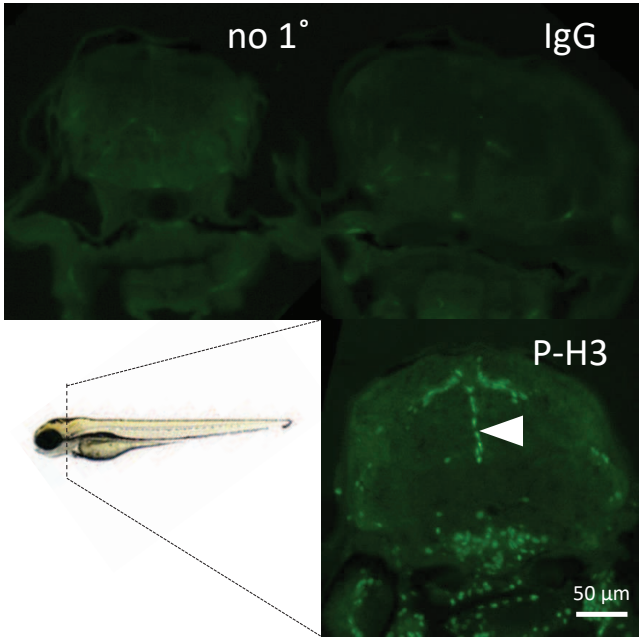


Figure 4



Table

Antibody	Target	Concentration
Mouse IgG Isotype Control	Non-specific antigens	2 µg/mL
Mouse anti-NMDAR1	GluN1 subunit	1:1,000
Goat anti-mouse Alexa488	Mouse IgG	1:500
Mouse Anti-phospho-H3	phosphorylated Histone H3	1:500
Mouse Anti-pan-AMPA receptor	GluR1-4	1:500

Table of Materials

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Name of Material/Equipment	Company	Catalog Number
Agarose	Fisher Scientific	BP160-100
Aluminum foil, heavy duty	Kirkland	
Anti-NMDA antibody	Millipore Sigma	MAB363
Anti-phospho-Histone H3 (Ser10), clone RR002	Millipore Sigma	05-598
Anti-pan-AMPA receptor (GluR1-4)	Millipore Sigma	MABN832
Bovine serum albumin (BSA)	Fisher Scientific	BP1600-100
Calcium Nitrate [$Ca(NO_3)_2$]	Sigma Aldrich	C4955
Centrifuge tubes, 1.5 mL	Axygen	MCT150C
Clear nail polish	Sally Hanson	
Depression (concavity) slide	Electron Miscroscopy Sciences	71878-01
Diaminobenzidine	Thermo Scientific	1855920
Embryo medium, Danieau, 30%		
Embryo medium, E2		
Floating tube holder	Thermo Scientific	59744015
Fluorescence compound microscope	Leica Biosystems	DMi8
Fluorescence stereomicroscope	Leica Biosystems	M165-FC
Glass coverslips 18 x 18	Corning	284518
Glass coverslips 22 x 60	Thermo Scientific	22-050-222
Glass slides	Fisher Scientific	12-544-4
Glycerol	Fisher Scientific	BP229-1
Goat anti-mouse IgG Alexa 488	Invitrogen	A11001
HEPES solution	Sigma Aldrich	H0887
Humid chamber with lid	Simport	M920-2
Hydrogen peroxide, 30%	Fisher Scientific	H325-500
Immunedge pap pen	Vector labs	H-4000
Insect pins, size 00	Stoelting	5213323
Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$)	Sigma Aldrich	63138
Mesh strainer	Oneida	
Methanol	Sigma Aldrich	34860
Methylene blue	Sigma Aldrich	M9140
Micro-tube cap lock	Research Products International	145062

Microwave oven	Toastmaster	
Mouse IgG	Sigma Aldrich	I8765
Normal goat serum	Millipore Sigma	S02L1ML
Nutating mixer	Fisher Scientific	88-861-044
Paraformaldehyde	Fisher Scientific	04042-500
Pasteur pipettes	Fisher Scientific	13-678-20C
PBTriton		
Permount mounting medium	Fisher Chemical	SP15-500
Petri dish (glass)	Pyrex	3160100
Petri dish (plastic)	Fisher Scientific	FB0875713
1-phenyl 2-thiourea	Acros Organics	207250250
Phosphate buffered saline (PBS), 10x, pH 7.4	Gibco	70011-044
Phosphate buffered saline (PBS), 1x		
Potassium Chloride (KCl)	Sigma Aldrich	P9333
Potassium Hydroxide (KOH)	Fisher	P250-500
Potassium Phosphate Monobasic (KH_2PO_4)	Sigma Aldrich	P5655
Pronase	Sigma Aldrich	10165921001
Proteinase K	Invitrogen	AM2544
Sodium Chloride (NaCl)	Sigma Aldrich	S7653
Sodium Phosphate Dibasic (Na_2HPO_4)	Sigma Aldrich	S7907
Spawning tank with lid and insert	Aquaneering	ZHCT100
SuperBlock PBS	Thermo Scientific	37515
Superfrost + slides	Fisher Scientific	12-550-15
Superglue gel	3M Scotch	
TNT		
Transfer pipette	Fisher	13-711-7M
Trichloracetic Acid (Cl_3CCOOH)	Sigma Aldrich	T6399
Tris Base	Fisher Scientific	S374-500
TritonX-100	Sigma Aldrich	T9284
Tween20	Fisher Scientific	BP337-500
Ultrafine forceps	Fisher Scientific	16-100-121
Water, ultrapure/double distilled	Fisher Scientific	W2-20

Comments/Description

Any brand may be substituted

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17.4 mM NaCl, 0.21 mM KCl, 0.12 mM MgS04, 0.18 mM Ca(NO3)2, 1.5 mM HEPES in ultrapure water.
7.5 mM NaCl, 0.25 mM KCl, 0.5 mM $MgSO_4$, 75 uM KH_2PO_4, 25 uM Na_2HPO_4, 0.5 mM $CaCl_2$, 0.35 mM $NaHCO_3$, 0.5 mg/L methylene blue

Any brand may be substituted

1% TritonX-100 in 1x PBS

1x made from 10x stock diluted in dH_2O

100 mM Tris, pH 8.0; 150 mM NaCl; 0.1% Tween20; made in dH_2O

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Aug. 31, 2019

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Dear Editor:

Herewith I am re-submitting the manuscript entitled “Whole mount immunohistochemistry in zebrafish embryos and larvae” on behalf of the authors, Dena Weinberger and Gary ZeRuth to be considered for publication in the *Journal of Visualized Experiments*.

We thank the editor and the reviewers for the insightful suggestions given regarding our manuscript. We have used the comments provided to revise the manuscript and feel that it has subsequently been significantly improved. The editorial and reviewer comments are listed below along with the corresponding revision or rebuttal. All changes to the manuscript have been tracked using Track Changes.

We hope the protocol described in this manuscript as well as the novel findings presented therein would be suitable for publication in *JoVE*. Please feel free to contact me if there is anything else I can provide.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been proofread and any spelling or grammar errors have been corrected, to the best of our knowledge.

2. Please provide an email address for each author.

Email addresses can now be found in line 6.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

The summary was re-written in complete sentences and contains 19 words.

4. Please ensure that the long Abstract is within 150-300 word limit and clearly states the goal of the protocol. Presently it is less.

The Abstract was expanded to provide a more detailed summary of the protocol and the intended objectives. The Abstract now contains 162 words.

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

The overall goal of this method is stated in lines 69-72.

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

The rationale for the development of IHC is provided in line 52-53. Its history and development are now mentioned in lines 58-61.

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

The advantages of IHC over ISH is described in lines 62-69

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

A description of the technique within the wider body of literature can be found in lines 52-68

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

Information to help readers determine applicability is provided in lines 70-74.

6. Please include more citations in the introduction section.

Additional references have been added to the Introduction.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly.

We have revised wording and added "notes" where necessary throughout the protocol section.

8. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Non-action items have been moved to the Discussion

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Steps have been shortened or broken into multiple steps. Some content has been moved to the discussion as appropriate.

10. Please use complete sentences throughout the protocol section.

Protocol has been updated to ensure that only complete sentences are used

11. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

We have added details to the protocol steps. For example, 11.5. pour off PBS by inverting slide; 6.2 use a plastic transfer pipet to move 1 or more embryos to the pbs droplet; 1.12. Remove embryo medium with a micropipette. Leave only enough liquid to just cover the embryos should remain after each fluid change.

12. 1.1: If this needs to be a step, please use imperative tense, else some of the details can be moved to the intro/discussion section and details specific to the step below can be converted to a note and placed wherever applicable.

This paragraph has been moved to the discussion.

13. 2.2: When is the light turned on?

The light cycle details are now described in line 89

14. 2.4: How do you check for the dead eggs?

Identification of dead embryos is described in lines 100-102.

15. 2.5 What is the desired stage in your experiment?

The desired stage for the representative experiment is now stated in lines 104-105.

16. 3: Please use complete sentences for this section.

The section has been revised to only include complete sentences.

17. 4.2: What is the primary antibody of choice for your experiment?

The primary antibodies of choice are now stated in lines 179-182 and listed in the resources table.

18. 6.1: This is not an action step but a note instead.

This line has been changed to a note

19. 6.6.: Please include the step number where documentation is being performed.

A Documentation step (12) has been added (lines 371-386)

20. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. 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.

The highlighted steps are now < 2.75 pages.

21. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

No copyright protected material is used in this manuscript.

22. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The Discussion has been extensively revised and now covers a-e above.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript is generally well-written, with a few errors. The egg collection and handling of embryos is described. How to perform immunohistochemistry on fixed embryos is also described. The procedure is specific, and will prove a valuable source for researchers that are using zebrafish embryos and immunohistochemistry for

the first time.

Major Concerns:
Two controls are critical and need to be described and included: no primary control (to determine non-target binding of the secondary) AND an isotype control (to determine non-target binding of the primary immunoglobulin type to the tissue. Isotype control should be used at the same concentration as the primary antibody.

> *We have included no primary controls and IgG controls. These steps are described in Representative results and shown in Fig 2*

Also, the authors need to correctly use the terms pipet and pipette, as both could be used at different places in the procedures in this manuscript. A pipet is a tool used to transfer liquids in chemistry or serology, and involves a bulb to draw liquid into the pipet. A pipette tip is used with micropipettors. Both of these come in many different sizes. The sizes of zebrafish embryos and larvae can vary depending on the strain, so the size of the pipette tip used at different places in the procedure needs to be specified.

> *Sizes and volumes have been noted where applicable in the text. The specifics of the pipets and micropipettes used are listed in the resource table*

Minor Concerns:
errors: line 107: bleached post-fixation is optional step #6, NOT #9; line 194: 'when pigment is sufficiently removed' instead of sufficiently gone; line 211: 'gently scrape', not gently scrap

> *These concerns have been corrected. The bleaching step is now correctly referenced as optional step 5. The word "gone" has been changed to "removed" in line 219. "Scrap" has been changed to "scrape"in line 235.*

Reviewer #2:
Manuscript Summary:
Review of JoVE Article. Manuscript number JoVE60575
"Whole mount immunohistochemistry in zebrafish embryos and larvae"

Overview:
This protocol outlines the general method for performing an immunostaining experiment using zebrafish embryos or larvae. The protocol is basic and straight forward and includes variations that may be applicable to variety of interests and needs in the field. The procedural steps are clearly outlined and generally easy to follow. However, the introduction and discussion of the protocol requires some significant revision. As it currently stands, the introduction leads the reader to think the protocol is going to be focused on zebrafish genome editing and genetic techniques rather than immunostaining. There is repetition throughout the text and incorrect reference to "gene expression" comparing it to "protein expression". These two processes are inherently different and should not be compared as providing the same information. In addition, there are a places throughout the protocol that are written as the opinion of the authors without factual basis. Detailed comments are listed below.

Major Concerns:
1. The introduction to the protocol is written in a way that makes the reader think the protocol is going to be about zebrafish genetics and generation of knockout lines. There is a lack of focus on the immunostaining procedure itself and its importance. Instead, this has more focus on the zebrafish as a model system. This is a bit misleading and distracting from the point of the protocol. In particular, lines 35-52 are all about mutants and knockdowns. How is the relevant? Instead, focus on the importance of immunostaining rather than the different types of genetic manipulations used in zebrafish. Since the title of the article is "Whole mount immunostaining in zebrafish embryos and larvae" it does not seem necessary to "sell" the zebrafish as a model system. The protocol will be used by zebrafish researchers.

The Introduction has been revised to minimize discussion of genome editing and to redirect focus to IHC.

2. There are some significant terminology issues throughout the manuscript.
* A "knockout" fish is not the same as a mutant. A more appropriate term would be "loss-of-function" unless the gene has truly been "knocked out". (lines 44-45). The gene editing section should be revised.

We agree with the reviewer on the terminology. References to knockout and loss-of-function mutants have been removed from the Introduction.

There is a significant issue with the use of the term "gene expression" and its comparison to "protein expression". These are not the same and should not be compared as such. They are regulated by vary different processes and should be considered independently. Therefore, it is not possible to directly compare gene expression techniques (in situ hybridization) with protein expression techniques (here IHC), as they are addressing two different biological questions. These references and comparisons should be removed throughout as a mentioned in regard to the benefit of IHC over ISH. One specific example is line 55-59.
* Similarly, the authors indicate "IHC is a better indicator of gene expression". First, IHC cannot be used to study gene expression and this is an opinion statement. (Lines 437-441). This needs to be revised.

While we understand what the reviewer is attempting to explain, there is disagreement on this point. Gene expression is the process of expressing a functional gene product from a gene. While it may involve the production of an RNA product (as in the case of tRNA, rRNA, miRNA, lncRNA, etc.), it typically refers to the production of a protein product. Certainly, in the context provided in this manuscript, it is clearly referring to the production of a protein product from a gene. Indeed, there are many steps involved in gene regulation starting with transcriptional regulation and ending with posttranslational regulation. Thus, gene expression can be measured by ISH (postranscriptionally) or by IHC (posttranslationally). These techniques for determining gene expression in situ are commonly compared in the literature (Morimoto, et al. 1996; Newton, et al. 2002; Lopez, 2014; Corthell, 2014). We have added references to the manuscript to reinforce this convention.

The statements referred to as "opinions" have citations indicating that mRNA levels are not reliable metrics of gene output. We have reworded the lines mentioned (now lines 560-561) and hope that this helps alleviate any remaining concerns.

3. There are several additional "opinion based" statements in the manuscript. Are there data to support the comments in section 1 "selecting antibodies" stating that "antibodies that recognize antigens that are >80% conserved between zebrafish and the target species will work in zebrafish". Section discussed in lines 82-86. Is there data to show this is always true? A reference and/or actual data should be provided to make such a statement. Other examples: Line 384-386, 435-437.

The wording of these statements has been changed to reflect that an opinion is being given based on anecdotal evidence and personal correspondences.

4. With regard to the protocol, it is highly recommended that the authors also include a peptide competition control for their experiments. Currently, to demonstrate antibody specificity in a zebrafish using a different model system antibody, the controls need to be quite rigorous. A no-antibody control and IgG control are important, but sometimes not enough for reviewers and the current expectations in the field.

We have not yet performed a peptide competition or a Western blot using the anti-NMDAR1 antibody but we agree with the reviewer on the necessity for stringent controls when optimizing a new antibody. We

have included a section in the discussion suggesting recommended controls that could be used prior to the publication of original research findings (lines 492-495).

5. The data presented by the authors only includes one antibody staining example using immunofluorescence, at one time point. It is important to include additional data with additional antibodies, additional time points, and flat mount versus whole mount images. The same antibody with the use of the chromogenic substrate should also be presented.

Additional antibodies were added (anti-AMPA and anti-pH3). Further, stained sections from an additional timepoint (72 hpf) were added in Fig 4.

6. Images of deyolked embryos should be included. All figures of embryos have the yolk and this is likely to be a critical step for most antibodies.

The yolk has been removed from all imaged samples in Figures 2 and 4.

Minor Concerns:
1. Can you provide a reference for the use of PTU (line 106).

References are now provided in line 108

2. Can you provide additional guidance for section 2.7 as to the best type of fix and the timing? Does it depend on the type of protein you are trying to detect? Membrane bound or not? Nuclear? (line 117-121). How do you know if the antibody is incompatible with a certain fix? (Line 406-407). Is there a way to predict antigen masking? (Line 402-404).

Additional details have been provided in lines 519-528.

3. Would it be more beneficial to perform serial dehydration steps in 2.9 as shown in 3.1 to prevent shrinking the embryos too quickly? (line 126).

We have tried dehydrating embryos both serially and by adding 100% MeOH and haven't discerned any difference between the methods.

4. How do you decide when to permeabilize? Section 3.2. Can you provide a guideline? (line 140).

Additional details have been added in lines 502-506.

5. Are there additional guidelines for blocking times? Does it depend on the type of antibody? Protein? (Section 4.1, line 154).

We have provided additional guidelines for blocking solutions and blocking times in lines 512-517

6. Please clarify throughout what is meant by "documentation". Do you mean imaging? Or notes? Or ?? (line 200, line 295,). The way it is written, it seems that there should be a section "11. Documentation" to explain what this step includes.

We have now included a section, 12. Documentation.

7. In line 331 it states that "This protocol has been optimized for use in zebrafish." Are there particular parts that should be pointed out here for the non-zebrafish researcher?

Additional details have been provided in lines 457-460.

8. It would be helpful for the authors to indicate what some of the "most critical" steps include to help with troubleshooting.

We have noted the steps that are time sensitive in the discussion and reported antibody as most challenging part of the protocol

Best regards,

Gary T. ZeRuth, Ph.D.
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