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Editorial Office
JoVE

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,

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