RE: Response to review of manuscript ID JoVE59344

**We appreciate that the editor and reviewers found our manuscript to be of interest. We have revised our manuscript according to the comments and recommendations provided by the editor and reviewers, and feel that these changes have improved the quality and clarity of this work.**

**Editorial comments:**

Changes to be made by the author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**We have proofread the document as requested.**

2. Please define all abbreviations before use.

**We have defined abbreviations as requested.**

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

**We have made this change.**

4. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

**We have made this change.**

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

**We have made this change.**

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Fisher Superfrost, Styrofoam, parafilm, Vectashield, NovaRed, Oregon Green, etc.

**We have made this change.**

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. Please move the discussion about the protocol to the Discussion.

**We have made these changes.**

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

**We have made these changes.**

9. Line 101: Please provide the composition of PBSt.

**We have made this change.**

10. Lines 170, 179: Please specify the primary/secondary antibody used. What is the dilution factor?

**We have made this change.**

11. Lines 180, 186: Please specify incubation time.

**We have made this change.**

12. Line 202: Please specify incubation temperature.

**We have made this change.**

13. Line 203: Please list an approximate volume to prepare.

**We have made this change.**

14. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

**We have made this change.**

15. Please include single-line spaces between all paragraphs, headings, steps, etc.

**We have made this change.**

16. 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 made this change.**

17. 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.

**We have made this change.**

18. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

**We have made this change.**

19. Figure 5: The scale bars in panels A and B are difficult to read. Please revise. The unit should be µm instead of µM.

**We have made this change.**

20. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol. Data from both successful and sub-optimal experiments can be included.

**We have made this change.**

21. References: Please do not abbreviate journal titles.

**We have made this change.**

22. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

**We have made this change.**

**Reviewers' comments:**  
  
Reviewer #1:  
  
Manuscript Summary:  
The manuscript is clearly and concisely written, but with sufficient detail to allow replication of this rather complex protocol. The use of fish gelatin in embedding embryos for frozen sections is an advance over the standard protocol provided in the Zebrafish Book. Producing excellent frozen sections of zebrafish embryos is not trivial. Given the wide use of this model organism for many types of biomedical research and the limited number of antibodies available as research tools, the optimization of such procedures is valuable to the community.  
  
Major Concerns:  
No major concerns  
  
Minor Concerns:  
Negative controls are needed for primary antibody such as nonimmune serum from same species for immunofluorescence as well as immunohistochem. Did do secondary only control.  
  
**We appreciate the reviewer’s comments and suggestions. We have added images from our negative controls (secondary only) for IF and IHC to Figure 5 as requested.**  
Reviewer #2:  
  
Manuscript Summary:  
The methods paper by Ferguson and Shive describes a method for sequential immunofluorescence and immunohistochemistry on cryosectioned zebrafish embryos. It is useful technique that will help in specific experimental situations to show co-localization of specific antigens.  
  
Minor Concerns:  
  
The paper is generally clear. However, there are some clarification that might help the reader and additional limitations for the applicability of the method that could be addressed.  
  
1. The paper discusses the use of IHC but it is not clear what immunohistochemistry they are using and why. The paper simply says "Immunohistochemistry was performed for 298 Oregon Green to detect donor cells". While there is nothing in the actual text about what histochemistry is associated with the secondary used to detect Oregon Green, the legend for Figure 5 mentions Nova Red and the reagent list mentions HRP. This was the only clue in the paper that the secondary antibody used to detect the Oregon Green Primary was associated with HRP. The text should contain a simple description of the reagents used sequentially in the immunofluorescence and immunohistochemistry protocols to make the actual protocols and choice of reagents easier to understand.

**We appreciate the reviewer’s comments and suggestions. We have provided more clarification with regard to the purpose of the IF and IHC we performed in the Introduction and Discussion. We have described the reagents used more clearly in the Protocol as recommended.**   
  
2. The paper illustrates the use of this technique for co-localization with co-labelled nuclei. These are relatively large cellular structures well suited to this protocol. However, I was concerned about how easy registration of sequentially collected images is how easily it might be effective in determining whether two structures are close or overlapping. What is the practical resolution of this approach?

**We have discussed the resolution of this technique in the Discussion.**

3. A concern with cryosections is the degree to which cellular morphology is retained. I was wondering how well cell membranes are preserved ? How does this approach compare with sections made with JB4 for example. In which situations would it be optimal to use one versus the other technique for embedding?

**We have addressed these questions in the Discussion.**