**POINT-BY-POINT RESPONSE TO REVIEWER 1**

Editor and Reviewer in black

Responses in purple

**Editorial comments:**  
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.  
  
2. Please be consistent with access type selected: You have chosen standard access in Editorial Manager but checked off open access in the ALA.  
  
3. Please provide an email address for each author (missing email address of Paola Lamperti).  
We will add her email address.

4. Please refrain from using personal pronouns (we, our, etc.) throughout the manuscript.  
We have removed personal pronouns from the protocol section, but kept some in the discussion. We looked at a number of other published JoVE articles and they all contained personal pronouns, so we hope this is acceptable. We found that their use in the discussion helps to distinguish which parts of the protocol are designed the way they are due to theory, and which parts are designed the way they are because of first-hand experience.  
  
5. Please use SI units, e.g. use “μL” instead of “μl”.

Done  
  
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.

The specific 3D printer and resin we used to make the mold been removed from the main text and moved to the materials and reagents table. The other two specific references to a company were the name of the microscope we used (Leica SP8), and the software we used (Matlab). We have noted that other JoVE articles also refer to specific microscopes and software. Removing the reference to Leica SP8 would mean that we cannot give step-by-step instructions, which are specific to the Leica graphical user interface. We also didn't delete the reference to Matlab, since our script is written in Matlab and would not work in other software.

7. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Some of the Notes have been moved to the discussion section or incorporated into the protocol steps.  
  
8. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 35-38, 48-50, 157-159, 217-220 and etc. Please also see the attached iThenticate report and revise accordingly.

This has been changed.  
  
9. Short abstract should not contain more than 50 words, current version has 56! Please shorten the short abstract to maximum 50 words.  
The short abstract is now 35 words.  
  
10. The current Long Abstract is the Introduction. Please include a Long Abstract that clearly states the goal of the protocol in 150-300 words. In addition, Abstracts cannot contain any citation.

We have added a long abstract. It is 278 words.  
  
11. 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Done  
  
12. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. 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.

Done  
  
13. 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  
  
14. Protocol: 1.2: What is the approximate temperature of the fridge  
The text has been changed to “4'C”  
  
15. Protocol: 2.1: How is the incross done? How many embryos are grown?

The section now reads:

1.     Incross the *Tg(fli1a:Gal4FFubs, UAS:Kaede)* line and grow approximately 200 embryos in egg water in the dark at 28.5 °C. For more details on how to cross zebrafish lines, please refer to this article in the JoVE Science Education Database11.  
  
16. Protocol: 3.2: How much time is approximately needed?

The section now reads:

1.     Add 2 mL of mounting medium to the mounting dish and allow 15-30 minutes for the solution to diffuse into the agarose.  
  
17. Protocol: 3.3: How much time is needed for melting and cooling processes?  
The section now reads:

In the meantime, melt a 1 mL aliquot of 0.7 % low melting point (LMP) agarose by placing the tube in a 70 °C heat block for about 5 minutes.   
  
18. Protocol 5: How is the heart stopped? How are the embryos re-embedded? There are two section 3's of the protocol.

The numbering problem has been corrected.

19. Protocol: 6: Is any special toolbox required? Please provide more information about the software and explicitly explain the steps of using it. We need explicit step wise details in a graphical user interface. I would recommend un-highlighting step 6 of the protocol.  
The toolboxes required is now listed in the Materials and Reagents spreadsheet. The Matlab script does not come with a graphical user interface. Considering that the script is not user friendly, we prefer not to make the Matlab script freely available at this point, and so explicit step-wise details of how to use the script are not possible.   
  
20. Figure 1: Please include the units for scale. Please label the panels and the parts.

This has been done.

21. Figure 3: Please provide the unit for all the axes.  
They have been added.

22. Please provide a more descriptive title for the two movie files.  
This has been done.  
  
23. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.  
This has been done.   
  
  
**Reviewers' comments:**  
**Reviewer #1:**  
Summary:  
The article is a nice protocol that illustrates the use of photoconvertible proteins to understand developmental processes in vivo. One can follow the steps in the protocol easily and it gives sufficient information to reproduce the procedure. The results are representative and sufficient to illustrate the power of the method.  
I only have some minor comments, which could help to make the manuscript even more useful for the readers.  
  
General comments:  
- Can the authors also make a statement on how convertible this protocol is in case other microscopes than the Leica SP8 is used?

This is now discussed in the *Microscope*section.

Introduction  
- It should already be mentioned in the intro that the experiment will lead to a temporary stop of the heart.

This is now mentioned in the introduction and in the abstract.

- AVC is used as abbreviation before it is defined.

This has been fixed.

- The protein Kaede should be spelled with capital K throughout the manuscript.

This has been done. We also changed *Tg(fli1a:Gal4FFubs, UAS:Kaede)* to *Tg(fli1a:gal4FFubs, UAS:kaede).*   
   
Protocol  
- Step 1.4 The authors probably mean 0.7% agarose and not 0.07% agarose

Yes, thank you. This has been fixed.

- Step 2.1 I don't think this step is necessary to show in the video. It is a very standard procedure. It would be better to dedicate more time to the photoconversion itself and how to troubleshoot it.

We agree. We’ve removed this step from the video.

- Step 2.2 The authors probably mean fluorescent stereoscope, not fluorescent microscope

Yes, thank you. This has been changed.

- Step 3 There are two steps 3

This has been fixed.

- Step 3.4 I don't think this step is necessary to show in the video as again it is a very standard procedure.

We agree. We’ve removed this step from the video.

- Step 3.5 It is not clear how the embryo should be positioned, say explicitly that it lies on its side or on its belly. Did the authors experiment with mounting only the tails of the fish in the agarose and keeping the heads and trunks completely outside of the agarose? This would be something that could be tried in order to improve the imaging conditions. If the authors tried and it did not work, it would be still an interesting piece of information.

We have now specified that the yolk side faces up.

Regarding mounting only the tails of the embryos - we tried and it didn't quite work. Embryos tend to become dislodged when we had to walk from our lab to the room containing the microscope located in a separate building. This could potentially be avoided if we had a mounting station directly next to the microscope. We are developing new molds that would allow us to either embed only the tails or to not use LMP agarose at all, but we haven't managed to do so yet.

The following sentence has been added to the discussion:

Adding sufficient LMP agarose to cover the entire embryo is advised to ensure that the embryos do not become dislodged between mounting and photoconversion/imaging, especially if the microscope is not located directly adjacent to the mounting station.

- Step 3.1 The objective magnification should be given with the × sign instead of letter X

This has been fixed.

- Step 3.1 'We have experience with mild conversion of Dendra2 also with 488 nm light.' Is this the case also for Kaede? In that case it would be beneficial to add a note that the red channel should be imaged before the green channel for taking the "before" picture.

We have not noticed mild conversion of red Kaede with 488 nm light, but imaging the red channel first is better practice and we have made changes in the text.

- Step 3.3 The details of the photoconversion settings could be discussed in more detail, either here as a note or in the discussion. How did the authors conclude 25% laser power and scanning 3 times? Why is this better than e.g. 35% laser and scanning 2 times etc.  
- Is there a danger of killing the cells with this laser power? How is phototoxicity in the converted cells identified? In our experience the newly converted red version of the protein can be easily bleached with the intense 405 nm illumination, so longer photoconversion does not yield more red fluorescence. The authors could comment on that as well.

Discussion of these two points have now been added to the *Photobleaching and phototoxicity*section. Re: killing of cells - we've never been able to kill cells directly using this method.

Discussion  
- Mounting section: It is not currently clear what the authors mean by "embryo facing a little to the left".

This part has been made more specific:

In mounting, the tilt of the embryo in the left-right direction needs to be adjusted according to the embryo’s developmental stage and the area one wishes to photoconvert. For example, to photoconvert the AVC at 36 hpf, the embryo should face roughly 15° to the left, while at 55 hpf, the embryo should face roughly 10° to the left.

- Future directions: The information that one can detect the converted cells for up to five days is not really a future direction. However, this information should definitely be included in the manuscript either as a note in the protocol part or early in the discussion.

This information has been moved to the introduction.  
  
Language:  
- The last sentence in the abstract is very long and hard to understand, should be rephrased.  
- Line 47, is 'morph' a real word?

Yes, it is a word. But we agree that it is better to use simpler words unless necessary. We changed the word to 'change'.

- Line 62 lack a 'by'.

This has been added.

- line 136 lack a 'the' in front of photoconversion.

This has been added.

- Line 68, I would rephrase to 'taking care of avoiding bubbles'

We are worried that 'taking care of avoiding bubbles' would imply that bubbles are already there, and that you should avoid touching them. What we mean is that one should avoid trapping air when they place the mold into the melted agarose. We changed the wording to "Place the plastic mold in the liquid agarose, taking care to avoid trapping air between the mold and the agarose. " Hope that makes the section clearer.

- Line 159 lack a 'the' embryos.

This has been added:

1.     At the desired stage, stop the heart and re-embed **the**embryos like under Step 3 of this protocol, with the important difference that embryos must be treated with BDM in separate, marked dishes to keep track of embryos.

- Line 258- 260, this is a very weird sentence.

We have changed it to:

At embryonic stages later than 55 hpf, the ballooning of the ventricle and the atrium also means that the violet laser beam used for photoconversion cannot reach AVC cells without first passing through either the atrium or ventricle. This means that beyond 55 hpf, in order to photoconvert EdCs of the AVC, one must also photoconvert additional EdCs in the atrium and ventricle.

- Is there a more scientific term for 'egg water'?  
We changed 'egg water' to 'embryo medium' throughout the text.  
  
**Reviewer #2:**  
Manuscript Summary:  
The manuscript addresses a major challenge in cardiac development, namely tracing at cellular resolution single atrioventricular valve cells. This will be particularly interesting for a number of researchers trying to analyze and interpret valvular phenotypes. The manuscript is well - written and detailed enough.  
  
Minor Concerns:  
A minor concern, although touched upon very briefly in the text (Discussion, 249) is the area of labeling and the challenges when trying to photoconvert at later stages. It will be very helpful if the authors give a little bit more details on photoconverting beyond the 48hours post-fertilization and some guidelines on how to achieve this in the most efficient way.

Unfortunately, we have no special tips for photoconverting embryonic hearts older than 55 hpf beyond what we have already written. We have tried specifically photoconverting atrioventricular valve cells at 72 hpf, but found that the light diffuses too much for the images to be useful.  Thus, we have found it simplest to 'guess' where cells visualised at 48-55 hpf would end up, and image later to see if we are right. Beyond 55 hpf, it may be possible to label AVC cells via exclusion (ie. photoconvert all cells around the AVC cells). One can potentially access ventricular/atrial EdCs without photoconverting AVC EdCs by mounting the embryo at different angles. However, we have never tried this ourselves. We think that photoconversion between 28 and 36 hpf is possible, though we haven't tried it ourselves. At 28 hpf, we mount the fish embryo on its side for imaging.

We realised we may have misled the reader into thinking that we typically photoconvert embryos all the way up to 96 hpf. We have reworded the section on mounting embryos such that we clarify that the mold is used for imaging embryonic hearts up to 96 hpf, but only up to 55 hpf for photoconversion. We have also clarified the stages we have tried in the Introduction.  
  
**Reviewer #3:**  
Manuscript Summary:  
In this manuscript Chow et al., the authors describe a method to photoconvert Kaede for lineage tracing of atrioventricular canal (AVC) cells in the developing zebrafish heart using a transgenic line that restricts expression of Kaede to the endocardium. The authors describe a workflow that includes the mounting, photoconversion, image and image analysis of the linear heart tube. The step-by-step protocol is informative and has the potential for phenotyping in various mutant background, however, there are some points that should be address before publication.  
  
Major Concerns:  
1. A potential use of this approach is for phenotyping mutants or embryos that have been treated with agents to disrupt AVC development. It would be good for the authors to point out this potential usage of their methods.

This is a very important point and we now discuss it in the abstract and introduction.

2. In the experimental pipeline, it will be important to keep track of each embryo that is photoconverted to correlate the position of the photoconverted cells at each development stage examined. This information is not noted in the text.

We have made changes to the text to make this point:

"After photoconversion, use a glass pipette to press down slightly just above the head of the embryo to break the LMP agarose, then gently suck up the embryo.  Eject the embryo from the glass pipette into a 35 mm petri dish containing egg-water with PTU (to wash away BDM containing medium), then transfer the embryo to a well in a 6 well plate containing embryo medium with PTU. During this step, make sure to keep note of which embryo goes into which well, as this is essential to correlate the position of photoconverted cells at later developmental stages."

3. For the image analysis, the authors describe the use of Matlab to segment and project the 3D data into 2D space. The Matlab script should be provided.  
Unfortunately, at this stage the Matlab script is not user-friendly enough to be included in the manuscript. We are happy to provide the Matlab script to anyone who requests it, and to go through it with them over Skype.

4. This may have been a technical problem with the upload but the two movies were not available for viewing  
The movies are in .avi format.... We are not sure why they were not available for viewing.  
  
Minor Concerns:  
1. Authors discuss the use of 2P for imaging of later stage embryos put only provide parameters for exciting green Kaede. What wavelength should one use for exciting red Kaede?

In theory, red Kaede should have a two photon excitation spectra similar to that of DsRed, and could be imaged at 950 nm. This is now mentioned in the discussion section. We are not sure why, but we have not been able to perform 2P imaging of red fluorescent proteins on our microscope set up.

2. Line 74 describes preparing "0.07% low melting point agarose". Is this a typo as the rest of the text "0.7%"?  
Yes, it is a typo. It has now been fixed.  
  
**Reviewer #4:**  
Manuscript Summary:  
This is a timely protocol that will help the community performing in vivo cell tracking in the specific case of endocardial cells.  
  
Major Concerns:  
None  
  
Minor Concerns:  
-Line 62: The authors should mention also the following detailed protocol for performing near UV photoconversion of single cells using live zebrafish: Dempsey, Qin, and Pantazis, (2014) Methods Mol Biol 1148:217-28.

Reference has been added in Future Directions.

-The authors rightfully mention that photoconversion using nearUV light illumination is not confined, yet miss to mention its potentially cytotoxic effect, as photoconversion typically requires intense and extended nearUV illumination. Please include a note.

This has been added.

-Line 321: Please cite also the original publication of primed conversion: Dempsey et al., (2015) Nature Methods 12:645-648.

This reference has now been added.

-Recently, Kaede has been engineered to be primed convertible - please mention: Mohr et al., (2017) Angewandte Chemie International Edition 56:11628.

This is very interesting and has been added.