Reviewer response letter, Cook et al.

We are grateful to the reviewers for the time they have taken in reviewing our work, and for their thoughtful summaries and evaluation. Below we respond to each of their requests point-by-point. Our revised version of the manuscript includes track changes that display all edits that have been made since the initial submission.

Reviewer #1 comments & responses:

Minor Concerns:

1) It is unclear to me whether the yellow highlighting in the submitted manuscript was intended, or was left over from an earlier draft.

We wish to thank this reviewer for the careful eye they used in going through our manuscript, and for their suggestions that have helped us to make key improvements.

The yellow highlighting is requested by JoVE to indicate the sections of the protocol that will be filmed.

2) In point 1.1, I think it would be useful to either say more about setting up fish, or at least provide a reference, so novices know where to find additional information.

We have added references.

3) In point 1.3 need to be clear that the "DNA solution" is the one from point 1.2 Calling it the "DNA solution" in 1.2, or calling it the "injection mix" in 1.3 would clarify this point.

We have clarified this ambiguity by using the reviewer's suggestion to call it "DNA solution" in both spots.

4) In 1.4 it might be helpful to say how long the embryos are maintained in E3.

Done.

5) In point 2.1, does the "group" refer to the embryos from 1.4?

Yes. We have added a clarification in the text for 2.1.

6) In point 2.3 it would be useful to reiterate the incubator temperature. Also, the word embryos is missing after the word transfer.

Thank you for this catch. Temperature has been added and misspelling corrected – in what is now numbered 2.4.

7) The Note at the bottom of section 2 is the first time the hsp70 promoter is mentioned. This should be brought up earlier. Also, it isn't clear what immediately refers to. After what step?

We have now added text about the heat shock promoter in the Introduction, and we have also moved this Note to the beginning of section 2.

8) In point 3.1, it isn't clear whether embryos can be examined two to four hours after the beginning of the heat shock, or after the end of the heat shock. It might also be useful to mention magnification here, although it is covered in more detail below.

We have clarified point 3.1 to indicate that imaging can begin 2-4 hours after the completion of the heat shock step.

9) What type of fishing line?

We have added more information about the fishing line to clarify this point. The specific brand information is listed in the table of materials.

10) I didn't understand what line refers to in 5.2.1

We have removed this detail for simplicity. Line referred to the type of sequential imaging that can be done.

11) The legend to Figure 1 needs to be proof read. For example, there are no B1, B2 or H in the figure.

Thank you - we have corrected the typos in the figure legend.

Also, the Ds and M in the inset are very difficult to see. The inset should be blown up and cropped so the cell representations are larger.

We have enlarged the inset as suggested.

12) I felt that some of the information in the Discussion should be redistributed to the Introduction and the Protocol. For example, the different brainbow constructs could be brought up much earlier. I also wonder whether it wouldn't be more useful to put some of the troubleshooting into the protocol at the appropriate step, rather than saving it all up for the end.

We have addressed the first suggestion by listing the different Brainbow constructs earlier in the manuscript. We have left several troubleshooting points in the Discussion, however, as guided by the JoVE protocol.

13) There is a table called Comments/Description at the end. It was not at all clear what this is for.

This was a hanging column from the required materials table, and has been removed.

Reviewer #2 comments & responses:

Major Concerns:

1) There is very little explanation about the post-imaging stages of time-lapse analysis. For example, the authors present quantification of relative channel weight in ternary plots but do not explain their value and how to generate them, but simply refer to the software. More information should be provided on analysis tools for the unique properties of Brainbow imaging.

We wish to thank this reviewer for their time in reviewing our manuscript carefully, and for their extremely useful suggestions that have helped us to improve the manuscript.

We have added a new section (Section 7) that describes our quantitative analysis of Brainbow images.

2) When it comes to long time lapse imaging, it is common to use a heated stage so that a fixed pre-determined temperature is maintained and in very long time lapse imaging even an apparatus that allows circulation of embryo medium. The authors should at least mention these considerations and if they think these are unnecessary explain why.

Since the room temperature of our imaging room (~75F) is within about one degree of 28C we do not use a heated stage and we have found that it is not necessary for fish survival. Some users may wish to use a heated stage to control temperature and avoid evaporation. We have now included a note about this in the manuscript.

Minor Concerns:

1) Please specify which kind of glue is used for preparing the imaging chamber (line 174).

We have added this information. The specific brand and catalog number are listed in the table of materials.

2) Figure legend 1 refers to B1 and B2 but in the figure only B is marked. Also, there are no G or H panels in the image but these are referred to in the legend text (lines 346, 347).

Thank you for this catch. The figure legend has been corrected.