Burns responses to the editorial and reviewers’ comments

(replies in red text)

**Editorial comments:**

The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (52514\_R1\_081214.docx) is located in your Editorial Manager account under “File Inventory." Please download the .docx file and use this updated version for any future revisions.

Changes made by the Science Editor:

1. There have been edits made to the manuscript. OK

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. OK

**Reviewers' comments:**

**Reviewer #1:**

*Manuscript Summary:*

This outstanding group of investigators provides an excellent and detailed description of the technique for vagal neural tube grafting and vascular injections in the avian embryo. The text is well-written and the figures are outstanding. With appropriate videos to highlight the method, this will be a very useful addition to the literature.

*Major Concerns:*

1) There is reference to a Figure S1, but I did not find this figure in the pdf. This figure was uploaded along with all the others. I do not know why it was not built into the pdf. I will upload it again.

*Minor Concerns:*

1) Lines 128-129: change the sentence to "Store fertilized chicken eggs, obtained from commercial breeders and from transgenic GFP chicken eggs...." I changed the wording slightly and removed the “obtained from commercial breeders” as the sources of WT eggs and GFP eggs is given in the Table.

2) It would be helpful if the authors provide information on where the GFP chickens can be obtained. See point 1 above.

3) Line 206: the word "micro-pipette" is repeated twice. Deleted.

**Reviewer #2:**

*Manuscript Summary:*

The authors present an interesting combination of marking both migrating neural crest cells and the vascular network in developing chick embryos using transgenic GFP chick- WT chick neural transplants and injection of DiI stain in the vasculature.

Experiments and expected results are clearly described and nicely illustrated.

Comments of the results are useful to emphasize critical points of the methods and the discussion is interesting.

This article should be of great value to developmental biologists working on the avian model as in ovo grafting and injection procedures quite benefit from vizualized and detailed protocols.

*Major Concerns:*

None

*Minor Concerns:*

Description of experiments

- cf 5.7 : can goat serum be replaced by FCS or HS? If probably this is the case, please add a note to text. Yes it can be. This has been added to 5.7.

- cf 6.1: indicated to "keep the orientation of the transplanted GFP+ neural tube". But, how can one keep the anterior-posterior orientation? (by means of some label? or are they anatomical features to identify anterior and posterior parts of the excised neural tube?) I have added the following to 6.1 “The orientation of the neural tube can be identified by leaving a small fragment of ectoderm attached to, or by cutting a small nick in, the dorsal surface”.

- cf 6.3: about the tape: please indicate its width; are any type of tape convenient? as I would guess some could be toxic to the embryo due to glue solvent ? In our experience any type of clear adhesive tape is fine. I have never been aware of any toxicity problems with tape. We use 24mm wide Sellotape so that one strip covers the entire window in the egg. I have added this width to 6.3.

- cf 7.6 and Fig3C: Adaptation of micropipette and injected vein diameter sizes seems critical to DiI labelling of the vascular network. And the choice of the diameter of the vein should be critical too, in order to avoid fatal bleeding after injection into a large vein. It would be thus useful that the authors indicate the range of appropriate diameter sizes of vessels/pipettes. I think Figure 3 reflects the appropriate diameter of vessels and their location, as well as the pipette size needed. It would be very difficult not only to measure the tip diameter, but also to break off a pipette tip to an exact, predetermined size. This aspect is somewhat trial and error, but it soon becomes apparent what is most appropriate and practical.

Figures : All are fine and useful (except that FigS1 was unavailable to this reviewer). This figure was uploaded along with all the others. I do not know why it was not built into the pdf. I will upload it again.

Materials for imaging (eg, camera and software for in ovo imaging) are not mentioned and should be indicated in the Table. These are now given in the Table.

There are some inconsistencies in the abbreviations: NCC appears in the text from line 71 (but would be needed line 38) and thereafter is not always used; similarly ENS is missing line 350. We have now included NCC abbreviations throughout after first use.

In Fig4, ENCC is not mentioned in the legend. Changed to mention ENCC.

Line 440, typing error "embryos" Corrected.

**Reviewer #3:**

*Manuscript Summary:*

The authors describe a technique for dual labeling of neural crest cells and blood vessels within chicken embryos using transgenic GFP chick neural tube grafting and DiI injection of the vasculature.

*Major Concerns:*

none

*Minor Concerns:*

One alternative to India Ink injection that the authors may want to mention is the use of neutral red agar pads to lightly stain embryos for visualization. In our personal experience, this method is preferred because the tissue itself is stained and can be seen during surgery under brightfield, and there is less foreign material introduced into the egg. Agar pads containing neutral red are cut slightly smaller than the embryo, placed on top of the vitelline membrane and embryo for a few minutes until the desired level of staining intensity is reached, and then removed from the egg. Both the donor and host can be stained. This sounds like a very useful method. However, I have never used this approach for embryo visualization, or seen it being used. I would be happy to include a reference for this if the reviewer is willing to provide one.

Line 101: "via" does not need to be italicized Corrected.

Line 105: "bias" does not need to be italicized Corrected.

129: chicken eggs --> chickens I don’t know why this change is suggested. It seems correct as is. We store the chicken eggs in an incubator. We don’t have any chickens. I therefore didn’t make any change.

172: point out which micropipette in Fig 1C Done.

219: refer to figure Done.

222: please describe somewhere how to make or where to buy sylgard base watch glasses The commercial source of sylgard base is now included in the Table.

224: refer to figure Done.

338: "so \_as\_ not" Done.

467: critical steps of DiI --> critical parameters for DiI Done.

**Reviewer #4:**

The protocol presented by Dleanlande et al focuses on dual labeling of neural crest and blood vessels in the chick embryo. This is important because the nervous and vascular systems develop together in many contexts and it is important to be able to visualize both in the same embryo. In general, this is a well written protocol and the images add to the descriptions. However, there are places where more details are required. Below are some suggestions:

1. Please note that the tungsten needle would also need to be electrolitically sharpened or purchased from a source that should be listed (Fine Science tools, pin section). Also can use pulled glass needles, pulled closed. Custom egg holders or watch glasses can be used. The following has been added to 1.2: “Alternatives to the micro-scalpel could be electrolitically sharpened needles, commercially available tungsten needles, or pulled glass needles”

2. Many places do not allow mouth pipetting any longer. One can also use a bent 1cc syringe filled with ink. The following has been added to 4.2.2: “If mouth pipetting is not permitted in your Institution a 1ml syringe can be used to instead”.

3. Add source of pancreatin Done.

4. Again, please state an alternative to mouth injection of DiI. Pressure injection would be one. For these glass needles, please add settings for the use of pulled glass needles. The following has been added to 7.6: “A possible alternative to the mouth tube for DiI delivery could be a pressure injector”. The settings for making pulled glass needles is given in the Table.

5. An important detail that has been left out is how the authors tell dorsal from ventral neural tubes so as not to transplant neural tubes upside down. I have added the following to 6.1 “The orientation of the neural tube can be identified by leaving a small fragment of ectoderm attached, or by cutting a small nick in the dorsal surface”.