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Dr Nam Nguyen
Manager of Review
JoVE

30th November 2020

Dear Dr Nguyen,

We would like to thank the reviewers for their comments and suggestions and have modified the manuscript to address all of the issues raised. Please see below for a detailed point-by-point response to the feedback with the reviewers' comments in *italics* and our responses as normal text.

Editorial comments:

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.*

   We have proofread the manuscript and corrected all spelling and grammatical issues identified. Additionally, we confirm that that all abbreviations have been defined at first use.

2. *Introduction lines 73-78: Here, the text reads as though your protocol addresses the involvement of muscle stem cells in skeletal muscle regeneration in zebrafish. However, in the discussion (lines 324-326), you have stated that this protocol does not reflect the functional status of the muscle stem cells. Please modify the text in the introduction to account for this fact.*

   We have amended the text to reflect that the protocol described examines muscle regeneration, which a complex process involving multiple cell types.

3. 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. For example: Zebrafish Embryo Genotyper (ZEG), FluoroDish, etc

   The commercial names Zebrafish Embryo Genotyper (ZEG) and FluoroDish have been removed from the manuscript.

4. *Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:*

   a) If you are starting with zebrafish for your study instead of the embryos directly, please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

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We have now added the following ethics statement: “Zebrafish maintenance was carried out as per the standard operating procedures approved by the Monash University Animal Ethics Committee under breeding colony license ERM14481.”.

b) Please specify the euthanasia method, but do not highlight it.

We have added an additional step, 3.11, detailing the euthanasia method used.

c) Please mention how animals are anesthetized and (step 2.1) how proper anesthetization is confirmed.

We have added the following statement to highlight how proper anesthetization is confirmed: “Wait for 10 minutes to ensure that the fish are completely anesthetized, evident when the fish stop swimming.”.

d) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

This is not applicable to the protocol we have described.

e) Discuss maintenance of sterile conditions during survival surgery.

This is not applicable to the protocol we have described.

f) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

This is not applicable to the protocol we have described.

5. 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. Please include all safety procedures and use of hoods, etc.

We confirm that the protocol section has been written in imperative tense.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. 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. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) 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.

We have added the following details to ensure all users can replicate the described protocol:

1.6: What is the DNA extraction protocol used here? Is it just vibrating at 2.4 V for 8 min?

The DNA protocol is indeed the vibration at 2.4V. However, we have added more information to clarify this: "Set the base unit to 2.4 volts, 0.051 A, and 0.12 W and start the DNA extraction protocol by pressing the "ON/OFF" button" and "NOTE: The vigorous vibration results in shedding of epidermal cells, from which genomic DNA is extracted".

1.13: Please quantitate the volume of medium used.

The volume used is two drops, which is sufficient volume to be able to collect the embryo and move it to the first well of a 24 well plate.

1.15: What downstream assays are done here? Please provide a citation.

The downstream genotyping assay will depend on the zebrafish strain used. To clarify this, we have amended this step to:

"Perform appropriate downstream genotyping assays to determine the genotype of the embryos.
NOTE: The DNA obtained from the embryo genotyping platform can be successfully amplified by PCR and can be used for subsequent analysis including sequencing, gel electrophoresis, or high-resolution melt-analysis. To genotype the *lama2* strain described in the representative results, PCR, restriction digests and gel electrophoresis was used. Given that the concentration of DNA obtained from each embryo is low, it is suggested to utilize most, if not all, of the genetic material obtained for the downstream assay."

1.16: What volume of embryo medium is used?

A volume of 25 ml has been added.

7. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

We have highlighted sections of the protocol to clarify which parts need to be sections.

8. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Scale bars have now been added for all images taken with a microscope.

9. 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

We have revised the discussion to detail the points highlighted above.

10. Please sort the Materials Table alphabetically by the name of the material.

The table is now alphabetically sorted.

Reviewer 1:

1. The biorefringence in lama2 mutants appear uneven and disconnected, reflecting defects in muscle integrity. While using uninjured area to normalize the biorefringence, lama2 mutants showed increased regenerative capacity at 3 dpi. However, biorefringence is stronger in wild type embryos than in the lama2 mutant at 1 dpi. Does this indicate that wild type embryos regenerate better by 1dpi? Can the authors image at day 0 right after the stab injury to test this?

The reason why the extent of injury is examined at 1 dpi, instead of 0 dpi, is because by this point cells of the innate immune system have infiltrated the wound site and cleared away debris resulting from the injury. Imaging at 0 dpi therefore has no benefit as the presence of debris within the wound site prevents accurate quantification of the extent of injury. We appreciate that we had not justified why imaging is performed at 1 dpi, and as such, we have now added a statement to clarify this point: "At 0 dpi, the wound site contains a large amount of cellular debris, which makes it difficult to quantify the extent of muscle injury (Supplementary Figure 1A-B). It takes approximately 18-20 hours for this debris to be cleared from the wound site, and as such it is more reliable to image larvae at 1 dpi, rather than 0 dpi, to determine the extent of injury elicited". To demonstrate this point, we have also added birefringence images for 0 dpi *lama2*$^{+/+}$ and *lama2*$^{-/-}$ fish, which is now included in Supplementary Figure 1.

Consistent with this argument, although this has not been experimentally validated, we hypothesize that the reduced birefringence within the wound site of *lama2*$^{-/-}$ mutants at 1 dpi maybe due to increased clearance of the debris, compared to *lama2*$^{+/+}$. Indeed, in Duchene muscular dystrophy, increased macrophage infiltration has been observed (Villalta *et al*., (2009)), and this may explain the increased clearance of the debris within the wound site and subsequent reduction in birefringence compared to controls.

Reviewer 2:

1. Line 149-151: Step 2.5. Using a 30-gauge needle, perform a quick but precise stab in the epaxial muscle located above the horizontal myoseptum. Was this done under a dissecting scope?

This step, along with the previous step on orientating the fish, is performed under a dissecting scope, which we have now clarified in the text: "Carefully remove excess embryo medium with a pipette, and under a stereomicroscope, orientate the fish such that the head is on the left, tail on the right, dorsal region up and ventral region down" and "Working under a stereomicroscope, use a 30-gauge needle to perform a quick but precise stab in the epaxial muscle located above the horizontal myoseptum".

2. Line 219-221: The authors stated that at 3 dpi, wildtype larvae typically show a normalized birefringence of 70 +/- 15.3% at the wound site, indicating that in 3 days, the wound has recovered by

approximately 30%. I am not sure if I can follow the logic of the calculation. If the wildtype larvae show a normalized birefringence of 70 +/- 15.3% at the wound site compared with the uninjured somites, does this suggest a 70% recovery?

We acknowledge that we had made an error in highlighting the mean normalized birefringence in wildtype larvae at 3 dpi, which should have been 60 +/- 15.3%. Based on this, the recovery in 3 days is 11.5%. This is because, normalized birefringence is the birefringence intensity within the wound site relative to two uninjured areas (step 4.4), and not to the birefringence at 1 dpi. Given that wildtype larvae typically show a normalized birefringence of 48.5 +/- 14.3% at the wound site at 1 dpi, and that at 3 dpi, the normalized birefringence increases to 60 +/- 15.3%, the recovery is approximately 11.5% (60 - 48.5). We have corrected this recovery value in the text, and also added details on how this recovery was calculated: "Given that the normalized birefringence within the wound site at 1 dpi was 48.5 +/- 14.3% (step 4.6), the increase in birefringence at 3 dpi to 60 +/- 15.3% indicates a recovery of approximately 11.5%.".

3. Line 264-267: To compare the regenerative potential of larvae in each genotype, the regenerative index was determined, and we reveal that lama2-/- larvae displayed a striking increase in muscle regeneration compared to lama2 +/+ larvae (Figure 2D; mean in lama2-/- = 1.30 +/- 0.251; mean in lama2-/- = 1.65 +/- 0.497), indicative of an increase in muscle stem cell function. Given that the normalized birefringence (%) is much lower in the 1 dpi lama2-/- mutant compared with 1dpi lama2 +/+ larvae, a small increase of normalized birefringence (%) at 3 dpi could translate into a bigger increase of the regenerative index. Is the comparison of regenerative index between wt and mutant scientifically meaningful? Have the authors performed any antibody staining (such as myopsin, a-actin and myomesin) to evaluate and compare the muscle regeneration in wt and lama2-/- mutant?

The approach we have outlined takes into account the extent of injury at 1 dpi and examines the recovery of the same fish at 3 dpi. Since the rate of recovery is linear, this comparison is valid, and allows the examination of regeneration relative to the size of the injury inflicted. While every effort is made to induce the same size of injury, the reality is that every injury is slightly different. It is therefore important to determine the recovery relative to the size of injury. Additionally, some mutants may inherently display a more severe injury, as seen in the *lama2*$^{-/-}$ mutant, and in such situations, it is imperative to examine recovery relative to the starting point. In the representative results presented, wildtype larvae, display a normalized birefringence of 48.5% and 60% at 1 dpi and 3 dpi respectively, which is a 123% recovery (60/48.5). In the same duration, *lama2*$^{-/-}$ mutants display an improvement of 214% (30/14). This highlights a greater recovery of *lama2*$^{-/-}$ larvae, and this is reflected by the regenerative index. As pointed out in the discussion, the lower recovery of the *lama2*$^{+/+}$ larvae does not indicate that they are unable to regenerate, but in this case it highlights that lama2$^{-/-}$ larvae are quicker at recovering that their siblings.

To further validate the improved regeneration in *lama2*$^{-/-}$ larvae, we have also stained the muscle with an antibody against F-Actin, and this data is included in Supplementary Figure 1. While these results confirm that *lama2*$^{-/-}$ mutants do indeed regenerate, evident by the presence of differentiated muscle fibres within the wound site, the inability to examine the same fish at 1 dpi and 3 dpi limits the ability to quantify and compare the regenerative response between *lama2*$^{+/+}$ and *lama2*$^{-/-}$ fish. Nevertheless, this data has been included in the representative results to support the validity of the protocol described. Since this experiment is not part of the protocol, it has not been detailed in the protocol section.

4. In Fig. 2C, one wt larvae showed a regenerative index of less than 1, indicating muscle regeneration was impaired. Any explanation?

Unfortunately, we have observed this on rare occasions but we have not been able to explain it. While every effort is made to avoid injuring the neural tube and notochord while performing the needle stab injury, it is possible that in this instance these tissues were also damaged thus preventing appropriate muscle regeneration. However, since we observed no evidence of inappropriate injury, we had no scientific basis from excluding this unexpected value from the analyses.

Sincerely,

Dr Avnika Ruparelia