**Response to editorial comments and reviewers' comments**

**Editorial comments:**  
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.

* We have proofread the manuscript thoroughly and also asked an English native speaker to correct grammar and spelling issues.

2. Note that, in the signed Author License Agreement (ALA), two boxes are checked in item 2. Please check one box only, sign and upload the revised ALA to your Editorial Manager account.

* We have corrected this mistake and uploaded the revised ALA.

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

* We have done accordingly.

4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

* We thank the editors for this comment, but are convinced that we have stated the purpose of the method clearly in the introduction. In L63/64 we write: " Here, we present a protocol enabling researchers to investigate glucocorticoid-mediated effects in adult zebrafish bones undergoing regeneration. " In L73-76 we furthermore add: " The methods presented here will help to further address underlying mechanisms of glucocorticoid action in rapidly regenerating bone and may also be employed in other settings of systemic drug administration in the context of zebrafish tissue regeneration."

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

* We have corrected the abbreviations to SI standards wherever necessary.

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

* We have included spaces accordingly.

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

* We have replaced personal pronouns in the protocol section and restricted their limited use to the introduction and discussion part.

8. 2.1: Please specify what level 4 of anesthesia is.

* We have added the following sentence in section 2.1 (L131-133): "At this stage, the rate of opercular movement is decreased, muscle tone is lost and the fish does not move upon touch 12."

9. Lines 226-230: Please write the text in the imperative tense and include it in step 4.6.

* In the edited version of the manuscript (Lines 233 to 236) we have included the text in step 4.6 and have written it in the imperative tense:

" **Feeding**

**4.6** During short treatments (for example 2 days) do not feed zebrafish. During longer experiments feed zebrafish. Take special care to avoid pollution of the incubation solution. Thus, feed with *Artemia ssp.* rather than with flake food, and only on every second day. *Artemia* are regularly used to feed young zebrafish, and should be available in your zebrafish haltering unit."

10. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. See lines 240-245, 329-332.

* We thank the editors for this suggestion. We have changed these paragraphs:

The section in lines 329-332 (original version of the manuscript) we have now included in protocol step 5.17 in the imperative tense (L339-341):

**"Live imaging of zebrafish**

**5.17** Use this approach to aquire images of regenerating bone tissue such as in the fin or skull throughout the experiment without sacrificing the zebrafish. Anaesthetize the zebrafish according to section 2.1."

The paragraph in lines 240-245 (original version of the manuscript) is necessary to inform the reader on the 2 following types of protocols that are possible to do. We have written this paragraph in the imperative tense (L244-249):

"**5. Analyses of samples**

After incubation of injured zebrafish in prednisolone and DMSO containing fish water, respectively, either perform bone mineralization/calcification analyses on fixed tissue (5.1 to 5.16) or carry out live imaging of zebrafish under the dissection microscope (5.17 to 5.21) 10, 11, 15. Use live imaging to determine fin regenerate length and to detect differences in reporter gene expression in transgenic zebrafish."

Similarly, we have instructed the reader to either perform fin resection (amputation) or fin fracture to injure bone in the fin (L120-123):

**"2. Generation of injuries in zebrafish fins**

To injure bone in zebrafish fins, perform resection of the fin (amputation, usually in the caudal fin) or fracture individual bony fin rays (fracture model). To this end anaesthetize adult zebrafish first."

11. 5.10: Please explain what RT O/N and dH2O mean.

* We have replaced the term RT O/N, which is an abbreviation, with the full phrase 'room temperature over night' in the revised manuscript, and the abbreviation O/N with 'over night'. We have also replaced the term dH2O by 'deionized water'.

12. 5.14: Please specify how to adjust pH if it is not neutral.

* We have added the sentence "Use 1 M NaOH to adjust the pH." to section 5.14 (L328-329).

13. 5.19: Should be Figure 1D instead of Figure 1C.

* We have corrected the mistake.

14. There is a 2.75 page limit for filmable content. 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.

* We have reduced the filmable content to 2.75 pages.

15. 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. Please do not highlight any steps describing euthanasia.

* We have done accordingly.

16. Please revise 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.

* We have revised the representative results accordingly, i.e. we made clear which types of analyses have been performed in/for the respective figure. We have also suggested further alternative analyses, e.g. regarding cell proliferation. The paragraph now reads the following (L355-370):

**"REPRESENTATIVE RESULTS:**

The protocol presented here has been used repeatedly to induce rapid bone formation in the course of regeneration of the zebrafish fin and skull 10, 11, 15. In combination with the presented method of prednisolone administration, studies on prednisolone's effects during bone regeneration can be pursued. For example, studies on bone formation and mineralization in the regenerate can be performed. Prednisolone, as other glucocorticoids 18, 19, leads to overall inhibition of fin regeneration, including bone formation, as detected by alizarin red staining on fixed caudal fin tissue (**Figure 3A**). Similarly, prednisolone has a delaying effect on (calvarial) skull injury closure, which can be illustrated by alizarin red (**Figure 3B**) or in vivo calcein staining (not shown). In addition, prednisolone exerts a profound anti-inflammatory effect in both fin and skull tissue, by triggering apoptosis in the monocyte/macrophage lineage. Reduced macrophage numbers can be detected by immunohistochemistry on frozen tissue sections, e.g. by using an anti-mcherry-antibody in transgenic *mpeg1*:mCherry zebrafish (**Figure 3C)** 10, 20. Similarly, the number, distribution, proliferation and apoptosis of other cell types of interest both in the exo- and the endoskeleton (e.g. vertebrae) can be analyzed with the help of immunohistochemistry."

17. 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

* Critical steps within the protocol are discussed in the paragraphs "Reproducibility of injury assays" and "Considerations regarding drug treatments" of the discussion. The critical steps include the resection of fins at always the same level (L425-428; "During fin regeneration, regeneration speed (i.e. regrowth of fin tissue, including bone, per time unit) depends very much on the amount of fin tissue that is being resected 13. In order to avoid unwanted variability of fin regeneration, make sure to always resect equivalent amounts of fin tissue in all specimens."), the fact that the brain can be damaged during trepanation, which should be avoided (L434-437; " Calvarial injuries of the skull by microdrilling should be performed cautiously. If damage is done to the brain (i.e. the cerebellum) postural and locomotory deficits will become apparent by erratic swimming of the specimen 15. Zebrafish with cerebellar injury may show adverse reactions to drug treatment and should not be used to study bone regeneration."), and vulnerability of immunosuppressed zebrafish (L447-453; " Immunosuppressive treatment predisposes treated specimens to microbial infections. Therefore, single housing in autoclaved beakers containing autoclaved fish water is important. Although these conditions are more cumbersome to carry out and are not sterile, they help to minimize infection in treated zebrafish. We did not pretreat ('sterilize') Artemia eggs. However, this might be an additional measure to prevent infection in zebrafish, if necessary. Furthermore, antiseptic substances such as methylene blue (1 %) can be added to fish water before use. "

b) Any modifications and troubleshooting of the technique

* We discuss troubleshooting of immunosuppressive treatment in the paragraph "Considerations regarding drug treatments" of the discussion (L447-453, see above). Increased mortality due to infections was the main occasion on which we needed to troubleshoot.

c) Any limitations of the technique

* We mention in the discussion that long term treatment by immersion in individual beakers is cumbersome and that alternative methods, such as prednisolone pellet implantation might be superior to our technique (L454-460; "Experiments with prednisolone, both short- and long-term, require daily changes of fish water. We have treated adult zebrafish for up to 8 weeks. Treatment of a large number of individuals for such a long time can lead to a certain experimental 'burden', and should be planned carefully. It is pivotal to always have the required amounts of autoclaved fish water and glass ware ready. Although this has not been tested in zebrafish (to our knowledge), implantation of slow release pellets for drugs of interest might represent a valuable alternative for long term drug exposure in zebrafish.")

d) The significance with respect to existing methods

* In the new version of the manuscript we have included a phrase in the discussion (L417-420) emphasizing the fact that our protocol can be used to study adult endoskeletal bones: "This protocol has succesfully been used to induce immunosuppressive and bone inhibitory effects in regenerative tissues of zebrafish, and can also be adapted for studies on the impact of prednisolone and other drugs in adult endoskeletal bones such as the spine." We have put this into context in the introduction part, stating (L54-62):

"Glucocorticoid-mediated bone loss has been induced in zebrafish larvae, for example, which led to the identifcation of counteractive compounds increasing bone mass in a drug screen 7. Furthermore, glucocorticoid-induced bone inhibitory effects have been mimicked in zebrafish scales both in vitro and in vivo 8,9. These assays are very convincing approaches, especially when it comes to the identification of novel immunosuppressive and bone anabolic drugs. However, they only partly take into account the endoskeleton and do not work in a regenerative context. Thus, they do not allow the investigation of glucocorticoid-mediated effects during rapid modes of adult, regenerative bone formation."

We very much value other existing methods to study the effects of drugs on the zebrafish skeleton, and would not like to emphasize the significance of our method beyond what we have written.

e) Any future applications of the technique

* We mention the fact that our protocol can be used to study other anti-inflammatory drugs as well as other drugs of interest on 3 different occasions. These are:

i. at the end of the introduction (L73-76): "The methods presented here will help to further address underlying mechanisms of glucocorticoid action in rapidly regenerating bone and may also be employed in other settings of systemic drug administration in the context of zebrafish tissue regeneration.",

ii. in the section 'Considerations regarding drug treatments' in the discussion (L441-444): "Thus, dose-response experiments should be carried out to identify the required dose of other immunosuppressive agents that might be applied.", and

iii. at the very end of the discussion (L476-479): "In sum, the protocol presented here can be used to study effects of immunosuppressive agents and other drugs after systemic administration in zebrafish that are undergoing bone regeneration either in the fin or skull. This will be useful to delineate the pathogenesis of GIO and to investigate the mechanisms underlying successful bone regeneration.".

18. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

* We have changed the formating style of the bibliography to Jove.ens style and hope that this style fulfills the requirements.

**Reviewers' comments:**  
  
**Reviewer #1:**  
JoVE manuscript review JoVE58429  
Title: Adult zebrafish injury models to study the effects of prednisolone in regenerating bone tissue  
Authors: Karina Geurtzen, Franziska Knopf  
  
Zebrafish is able to regenerate various organs, including tissues of the appendages (fins) after amputation. Regeneration of appendages and skull accompanies the regeneration of bones, and thus this type of tissue regeneration is a good model for investigating the underlying molecular and cellular mechanism. In this article, the authors describe the use of these 3 injury models, fin amputation, fin ray bone fracture and calvarial skull injury, using adult zebrafish and their combined application with systemic chemical treatment to dissect bone inhibitory and immunosuppressive effects.  
  
Overall, they described the respective injury models and their step-by-step protocols in sufficient detail, so the article is useful for people who would like to follow and/or newly start the similar studies. I do not have any special comments on the manuscript. However, if they could provide videos that show the actual procedures for making respective injuries, it would be very helpful for people who are not familiar to these models.

Response:

* We thank the reviewer for his/her positive response. Of course, we are going to demonstrate the injury procedures in the video version of the article.

**Reviewer #2:**  
Manuscript Summary:  
This manuscript is based on two articles previously published by the authors, where damages of the zebrafish skeleton are performed to examine intramembraneous bone regeneration under normal conditions (Development 2014) or upon immune system suppression (JBMR 2017).  
Here, the authors present a series of detailed protocols allowing three types of bone damages to be performed (fin amputation, trepanation of calvarial bones, and fin fractures) and the subsequent examination of bone regeneration using in vivo staining techniques (including transgenic lines) and histological procedure.  
They further show that their protocol can be used to study the effect of drugs inhibiting the function of the immune system, such as prednisolone.  
  
Major Concerns:  
I have no major concerns regarding the described protocol.

Response:

* We thank the reviewer for his/her positive evaluation and valuable comments. We have addressed each of the comments below.

Minor Concerns:  
While the text reads fluidly, there are many grammatical mistakes and unusual phrasing that should be corrected. I recommend the text to be edited by a native English speaker. Some examples: L34 "In here"; L45 "genetically highly amenable", L61 "do not perform" etc...

* We have corrected the mistakes and unusual phrasing to our best knowledge with the help of a native English speaker and a person with a long residence time in the UK. We hope that the text now reads well.

L148: Replace "by heating 2% agarose" with "by heating a 2% agarose solution prepared in fish water or E3".

* We thank the reviewer for pointing out this mistake to us. We have replaced the phrase accordingly (L152).

L179: calvaria is classified as exoskeleton. Please correct and cite: Zoological Lett. 2015 Jan 13;1:2. doi: 10.1186/s40851-014-0007-7. Hirasawa & Kuratani.

* We apologize for the confusion of terms. We have corrected the mistake in protocol section 3 and cite Hirasawa & Kuratani 2015 Zoological Letters (L186).

L277 Perhaps "body" is better suited than "corpse"?

* We have replaced the word "corpse" by the word "body" in section 5.6 of the protocol (L283).

L379: Replace "Cryosection view of uninjured skull and brain tissue of treated" by "Cryosection view of uninjured skull and brain tissue of treated (Pred.) and untreated (DMSO)..."

* We thank the reviewer for this suggestion and have changed the sentence accordingly (L397-398).

Fig3: indicate that BF means "bright field" in the legend. Indicate the position of the bone and brain tissues on the bright field image.

* We have changed the legend accordingly (L402). We have also changed Figure 3C and indicated the position of epidermis (epid) and bone (bn) in the bright field image. The brain is not visible on the images since it usually detaches from the bone during fixation and cryosectioning.
* Again, we would like to thank the reviewers and editors for their positive and constructive feedback.