**Dear Dr. Jaydev Upponi,**

**We thank the editorial board and the reviewer for their constructive feedback. Please find below our detailed response to these comments.**

**We have highlighted our answers in this document and any changes that we made in the original manuscript in green for your convenience.**

**Please do not hesitate to contact me if you have any further questions.**

**With best regards,**

**Marco Morsch (on behalf of the authors)**

**Editorial comments:**

•***NOTE: Please download this version of the Microsoft word document (File name: 54983\_R1\_060116) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review.***   
  
•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

•Formatting:  
-Middle initials should appear after the author first name.

The middle initials have now been moved behind the author’s first name.

-Zebrafish rearing conditions should be a separate note or step in the protocol rather than a part of the ethics statement.

The sentence addressing the zebrafish handling has now been moved to protocol step 1.1.1.

-Please include spaces between all paragraphs and bullet points.

Spaces have been added where appropriate.

-Please define the abbreviation “FRAP” at first occurrence.

Fluorescence recovery after photobleaching has been introduced at its first appearance on page 6, line 253-254.

-Please include a space between all numbers and units.

Spaces have been added where applicable.

-References – Please abbreviate all journal titles.

We have now abbreviated the journal titles.

•Grammar:  
-1.1.3 – “placed in an incubator”  
-1.3.3 – “majority of embryos has”

These typos have been rectified.

•Additional detail is required:  
-2.1 – What magnification?

We have highlighted to the reader to choose the appropriate magnification (page 6, line 230) and added a statement at the end that we generally perform our ablations with a 40x objective (page 6, line 232-233).

-2.3.1 – How does one know the center of the cell is reached?

The sentence before describes to manually determine the upper and lower limit of the cell soma. That should give the researcher a clear indication of where the middle of the cell is. We have now changed the structure of the sentence to suggest that the middle of the cells soma is a good starting point for doing this kind of ablation.  
  
•Branding should be removed:  
-Section 3 note – Please remove the name and version of the software, and instead include them in the materials table.

We understand this comment, but because of the specialised nature of the image analysis and ablation software we think it is valuable for readers to have this information in this section. We have added the information that most conventional microscopes with the UV laser and an equivalent FRAP module can perform this type of ablation.

-Please remove trademark symbols from the materials table.

TM symbols have been removed.

•Discussion: Please discuss the critical steps and limitations of the protocol. Please also discuss any modifications/troubleshooting that can be performed.

We have added a paragraph in the discussion that highlights the limitation of this approach in terms of triggering ‘physiological’ cell death (page 11, lines 457-460).

We have also added a paragraph explaining a limitation of the protocol, such as that the embedding of the fish in agarose might affect the efficiency of the approach (page 11, lines 462-466).

• If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

•JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

We have included the dois to the references (besides reference 22 & 34 where we couldn’t identify a doi).

•IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.                                               

We have double checked the manuscript for grammatical errors and typos.

•NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

**Reviewers' comments:**

**Reviewer #1:**  
*Manuscript Summary:*  
In the manuscript entitled Triggering cell stress and death using conventional UV laser confocal microscopy, Morsch et al describe the use of the confocal auto bleaching tool to specifically ablate fluorescent cells in live zebrafish. This technique could be of use to those in the zebrafish community who are interested in understanding the in vivo consequences of targeted cell ablation.  
  
*Major Concerns:*  
none  
  
*Minor Concerns:*  
1.1 Since the protocol only works for fluorescent cells, the section titled "generation of transgenic lines" should mention that the targeted cells should fluoresce.

The title has been changed to ‘Generation of zebrafish (*Danio rerio*) expressing fluorescent proteins’.

1.42. What is the agarose prepared in?

We have clarified this by stating that it is prepared in egg water (page 5, line 184).

1.4.3 I have a hard time understanding how you create the agarose mold for longer term imaging. Perhaps include an image of this too.

We have now added a Supplementary Figure to illustrate the cast for longer term imaging (Supplementary Figure 1) and have added the reference where appropriate (page 5, lines 189 & 195)

*Additional Comments to Authors:*  
N/A

**Reviewer #2:**  
*Manuscript Summary:*  
103: "highly evolutionary conserved model organism" to this reviewer seems incorrect and a bit to general statement. Conserved is always relative to something else, e.g. conserved between species, in this case probably the conservation with mammals is indicated. If so, what is conserved genetics? Cells? Organs? Behavior? Biology?

We have removed the term ‘highly evolutionarily conserved’.

126: Unclear why it says compound transgenic fish, why would these be required? I understand it would be beneficial to label multiple cell types, but might be a good to add a phrase that says so.

We have changed the protocol point to ‘Generation of zebrafish expressing fluorescent proteins” (page 3, line 132) and have added a sentence to outline the options to achieve this, including the options of plasmid injections and crossing transgenic lines (page 4, lines 134-136),

134: please define embryo medium

The confounding terms embryo medium and E3 have been removed and the term egg water has been used throughout the manuscript (detailed in the material list).

152: Screen the animals and select for… What is meant by screening? Screening for fluorescence? Please indicate

Fluorescence screening has been added to this protocol step (page 4, line 163).

153: egg water is the same as embryo medium?

We have consolidated this term to egg water as stated above.

162: Pasture should be Pasteur

This typo has been corrected.

164: egg water embryo medium? Please use single term consistently

Changed throughout the manuscript to egg water as stated above.

180 a few 100 ul? Not sure if this is an accepted notation

We have specified this to the amount of ~300 µl we routinely use in our experiments.

195: what type of tip is used, 200 ul?

It is correct that a 200 µl tip was used and we have added this to the protocol.

203: brush? What type of brush?

We have given more detail on what type of brush we use for the positioning of the embryo (page 5, line 216).

235: "modern confocal" might be good to refer to this in more objective term

We changed this term to ‘conventional’ confocal microscopes that is equipped with the laser line and FRAP module (page 6, lines 252-254).

263: Intensities 60-80%; if this reviewer understands it correctly, these values may have no meaning at all with regard to absolute intensities as these differ between individual lasers and depend on the wear-and-tear/age of the laser. So 60% laser power in our hands could be 20% in yours?

The reviewer is correct that these values are not absolute and differ for each imaging setup and laboratory. We feel on the other hand that it is important to give the reader an indication of what settings we are using in our experiments (e.g. that the percentages can be quite high) and that is why we included that detail as a NOTE. To clarify this a bit more we have added a sentence to the paragraph pointing out this caveat (page 7, lines 280-281).

285: What fluorescence in the ROI would be high after FRAP; Kaede? most fluorescent proteins aren't excited at 405 nm? Please explain.

In this protocol point we are referring to the emission of the ‘normal’ fluorescence emission (e.g. GFP or Cherry) after excitation with the appropriate laser lines (e.g. 488 or 555 respectively). With our setup, a picture of the structure of interest with these ‘appropriate’ laser lines (not the 405 nm excitation) will be taken before and after the ablation process. This allows the researcher to quickly judge their success during that process. We are not referring to an emission after an excitation with a 405nm laser line.

We have now clarified this process in the protocol (point 3.6, page 7, lines 300-302).

310: Succesful… fluorescence fades: Fading is likely caused by leakage from the cell because it's dead? Please comment

That is correct. If fluorescence never resumes after the ablation process the most likely explanation is that it leaks out of the cell because of its death. Sometimes this process is accompanied by small vesicle formation (most likely remnants of the cell membrane) around the ablation site or faint fluorescence in the periphery. However, as most people do not look for these phenomena we have not incorporated this into this technical protocol.

311: Ablation…. : this is then simply photobleaching which is measured typically using FRAP, and not ablation.

We have clarified this by stating that these were ‘attempts of ablation’ to confirm the 405nm specificity (page 8, line 332).

313: characteristic features: such as?

We have added additional information of neuronal blebbing and detection of AnnexinV, (page 8, lines 335-336).

320: is the any evidence the cells are stressed?

Very good point and most likely yes. We are currently investigating this by using stress reporters such as XBP1, although we would like to emphasize that this is not the major point of this manuscript.

340: E3 solution = embryo medium?

Changed throughout the manuscript as outlined above.

431: -3MNX1: please indicate what is meant here, a selected region of the Mnx1 promoter?

The ‘-3’ in front of the mnx1 promoter refers to a 3kb fragment upstream of the mnx1 start codon. We have now included the references for all the promoter used in this manuscript at the first mention, including the one for -3mnx1 (page 4, lines 157).

433-434: "Single-cell …..ablation." It is unclear why this sentence is included. Generally, the word apoptosis is mentioned only once in the manuscript? Would it make sense to include the use of an apoptotic marker, which would suggest the type of cell death? Annexin is mentioned and seems to be used in previous work by the authors, but is unclear why this is not used here; annexin labeling of spinal cord neurons can be detected in annexin transgenic zebrafish starting before fragmentation (van Ham et al., 2010) and acridine orange has been used in vivo to label cell death in the spinal cord of Tau expressing Alzheimer's zebrafish (Paquet et al., 2010).

We feel it is important to highlight the degeneration along the axon as a result of our approach (please see lines 453-455 leading up to this sentence). Importantly, this process is accompanied by labelling with the apoptotic marker AnnexinV as we reported previously. Without restating these findings, we have now specified the figure and video in our previous publication followed by the caveat of a potential accelerated cell death the reviewer points out later in his peer-review.

442-444: "We recently….remnants" Other groups have looked in detail at engulfment of apoptotic neurons in vivo before; would be fair to include original references (e.g. van Ham et al., 2012; Mazaheri et al., 2014).

We have included these references now in this paragraph (page 11, line 473).

For photoconversion it is shown that the intensity used for conversion does not convert kaede in neighboring cells. However, for the ablation it is not clear if neighboring cells are not injured, where a much higher laser power is used. Could the authors comment on this?

We hope that Figure 5 (panel C & D) will help to clarify this concern. When we ablate with nearly the highest laser power in our setting (95%) we can see an immediate disappearance of the Kaede labelled cell. In a proximity of ~20 µm around the ablation site we consequently see the conversion of green Kaede labelled neurons to red. Following up these neurons over longer periods of time (up to 5 hours) we have never observed any death or obvious signs of disturbance, such as blebbing or fading of fluorescence, in these converted ‘by-standers’.

The Kaede conversion experiments have been thoroughly explained elsewhere, and seem somewhat unrelated to this story, as there is no "measure" that these converted cells are actually stressed, although this could be expected.

We have changed the order of the paragraphs in the results as we can see how it read before as if the kaede experiments demonstrated cellular stress to individual cells. Overall, we believe it is important to emphasize the Kaede conversions in this protocol to highlight the specificity of this approach.

Other points: GFP lines are used for ablation. Does the fact that it's GFP influence the ablation? I.o.w. if another fluorescent protein is used RFP or CFP does ablation work -equally- well?

The ablation approach is fluorescence independent and has been performed by us for BFP, GFP, mKO2 and mCherry expressing cells.

FPs are known to release ROS upon excitation, is this mechanisms involved in the ablation used here or is it DNA damage related? Please comment.

It is an important question and we are currently investigating this process in more detail by using fluorescent ROS probes and Caspase markers in AnnexinV-labelled transgenic fish. While not the focus of this study, a real-time representation of these presumably apoptotic events will help us to verify this process and moreover visualize the order of events *in-vivo*.

As well, I'd appreciate if the authors can comment on a limitation that laser-killling of cells could be very un-physiological and therefore elicit a complete different response of the surrounding tissue than in disease such as ALS where cells slowly (?) degenerate.

We report the activation of AnnexinV in dying neurons after our UV laser ablation approach, demonstrating that the specific ablated neuron is undergoing a programmed series of cell death. This accords with the apoptotic neuronal death that occurs during neurodegeneration, although we recognise that our ablation technique may temporally accelerate the apoptotic process that occurs normally.

We have now included this caveat in the discussion (page 11, lines 457-460).

*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
N/A  
  
*Additional Comments to Authors:*  
N/A