Dear Dr. Dsouza,

Please find the enclosed revised version of the manuscript entitled “**Fluorescence Recovery After Photobleaching of Yellow Fluorescent Protein Tagged p62 in Aggresome-Like Induced Structures**” (JoVE59288 - [EMID: e197ad632e2721c9]) intended for publication in *Journal of Visualized Experiments*.

We have made the appropriate changes to the manuscript as per the suggestions made in the editorial and reviewer comments. Additionally, we have responded to all comments and technical points raised by the reviewers.

**REPLIES TO EDITORIAL COMMENTS:**  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

*Authors’ Reply*: The manuscript has been proofread carefully to ensure that there are no spelling or grammar issues.

2. 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].”

*Authors’ Reply*: We have obtained proper documentation allowing for copyright permission. The manuscript has been modified to indicate an adaptation of figures from previous publications.

3. Please revise the title to be more concise.

*Authors’ Reply***:** The title has been modified and is as follows: “*Fluorescence Recovery After Photobleaching of Yellow Fluorescent Protein Tagged p62 in Aggresome-Like Induced Structures*”

4. Introduction: Please include applicable references to previous studies when describing existing FRAP protocols.

*Authors’ Reply*: The appropriate references have been added to the manuscript. These references are listed as items 5-9 in the references section of the manuscript (page 11; line 444). The in-text citations have been updated accordingly throughout the manuscript.

5. Please define all abbreviations before use.

*Authors’ Reply*: All abbreviations have been defined before use.

6. 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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: MatTek, Polysciences, Hyclone, Sigma, Carl Zeiss MicroImaging, Microsoft Corporation, Excel, etc.

*Authors’ Reply*: All instances of commercial language have been removed from the manuscript.

7. 1.1: What culture medium is used? What are the conditions? Please specify.

*Authors’ Reply*: The type of culture medium as well as required supplements (otherwise known as “complete medium”) and culturing conditions have been defined on page 2; line 78; step 1.1 of the protocol in the manuscript.

8. 1.6: What is complete medium? Serum-free DMEM?

*Authors’ Reply*: “Complete medium” has been defined in page 2; line 78; step 1.1 of the protocol in the manuscript. “Serum-free DMEM” is defined as the base-medium without fetal bovine serum or antibiotics on page 2; line 85, step 1.2.

9. 3.1.2-3.1.5, 3.2.2-3.2.6: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).

*Authors’ Reply*: All software steps have been explicitly explained and more specific details have been provided.

10. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

*Authors’ Reply*: The Protocol has been revised such that individual steps contain 2-3 actions per step and a maximum of 4 sentences per step.

11. After you have made all the recommended changes to your protocol (listed above), 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.

*Authors’ Reply*: The authors have highlighted 2.75 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video.

12. 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.

*Authors’ Reply*: The authors have highlighted complete sentences. The highlighted part of the step includes at least one action written in imperative sense.

13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

*Authors’ Reply*: All relevant details required to perform the highlighted step have been included.

14. Figure 1: Please change the time unit “Min” to “min”.

*Authors’ Reply*: This has been corrected in the revised manuscript.

15. References: Please do not abbreviate journal titles.

*Authors’ Reply*:This has been corrected in the revised manuscript.

16. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.

*Authors’ Reply*: This has been corrected in the revised manuscript.  
  
**REPLIES TO REVIEWERS’ COMMENTS:**

***Reviewer #1***:  
*Manuscript Summary*:  
The manuscript by Cabe et al. describes the procedure for conducting FRAP experiments using live cells. In this manuscript, they have described the procedures for the preparation of cells for analysis, expression of the fluorescently tagged protein p62, setting up of the confocal microscope, and analysis of data. Overall, this protocol is well-written. However, there are a few points that need to be clarified in order to describe a more general protocol for conducting FRAP experiments.  
  
*Minor Concerns*:  
Line 101: The authors should specify the reason why the culture medium was changed to cold Tyrode's buffer containing nocodazole. Would this culture medium be required for FRAP experiments that involve the use of other fluorescently tagged proteins as well?

*Authors’ Reply*: The use of Tyrode’s buffer containing HEPES as the buffering compound allowed us to perform imaging at room temperature and without the requirement of a CO2. The regular culture medium contains bicarbonate as the buffering compound, which requires CO2 to buffer. Performing live cell imaging in culture medium containing bicarbonate while also in the absence of CO2 will change the overall pH. The manuscript has been modified to provide this rationale for using Tyrode’s buffer containing nocodazole in page 3; line 113; after step 2.3.

Line 133: The authors should specify the temperature at which the FRAP experiment was conducted. Moreover, is a CO2 chamber required to maintain incubation conditions during live cell imaging?

*Authors’ Reply*: The manuscript has be modified to reflect the fact that the FRAP experiments were conducted at room temperature. A CO2 chamber for short-term (lasting for ~60 min) live cell experiments such as those reported here but is necessary for longer term (lasting > 60 min) live cell experiments.  
  
***Reviewer #2***:  
*Major Concerns*:  
1. In the representative FRAP images and movie (Figs. 3E and 4, respectively), fluorescence decreased in cell cytosol surrounding the bleach ROI. What is the reason behind this non-specific photobleach?

*Authors’ Reply*: The decrease in fluorescence in the cell cytosol surrounding the bleach ROI is due to repeated imaging of the acquisition ROI, which was scanned 35 times, once every 30 seconds.

2. The authors set the pinhole at 1.95 airy units. We calculated that a pinhole of 1.95 airy units for a 63x/1.4NA objective is 27.5 µm (Airy unit= resolution limit x magnification). Did the thickness of the optical slice lead to loss of image acuity?

*Authors’ Reply*: Please recall that we used a 63x/1.4NA objective with the optical zoom set to 3 and a pinhole set to 1.95 Airy units (Fig. 2C, D). These settings yield an optical slice of less than 1.4 µm, which provides a level of image acuity sufficient for FRAP experiments with ALIS.

3. The authors do not mention whether pre and post-bleach images were taken at different optical depths.

*Authors’ Reply*: This omission has been corrected in the revised manuscript. Pre- and postbleach images were taken at the same optical depth.

4. The authors report that data were excluded in case of image drift > 3µm (line 254). However, the representative video (Fig. 4) shows a drift of more than half the cell length- equal to 4-5µm.

*Authors’ Reply*: With regard to the topic of image drift, the experiment was stopped and the data were discarded when drift of the ALIS (not the bleach ROI) exceeded ~3 µm. We apologize for this error of phrasing. Based on a close examination of the representative video (Fig. 4), we estimate ALIS drift to be less than 1 µm; the fluorescence recovery in the bleach ROI is non-uniform.

5. Line 335 states that the calculated bleach depth from raw fluorescence data presented in Figure. 3. However, we calculated the bleach depth to be 72 (assuming pre and post-bleach fluorescence to be 86 and 24, respectively).

*Authors’ Reply*: Based on the reviewer’s comment, we recalculated the bleach depth for the data presented in Figure 3. The prebleach average of the bleach ROI is equal to 213.08 and the first postbleach value of the bleach ROI is equal to 17.175. Thus, the bleach depth is 91.94 rather than 92.94, the value reported on page 7; line 338 of the manuscript. The manuscript has been corrected accordingly.