November 22nd, 2018

Dear Editors of JOURNAL of VISUALIZED EXPERIMENTS (JoVE),

We thank you for giving us a chance to revise our manuscript JoVE59147 and for all the valuable comments from the reviewers. Following the comments by the reviewers, we extensively revised the manuscript including the quality of the language. Herewith, we are submitting our revised manuscript entitled, “A simple and versatile mounting method of living Arabidopsis leaves for intravital time-lapse imaging”.

Our re-submission includes:

3 figures,

2 tables,

1 movie

Followings are the list of our responses to the editorial and peer review comments.

**Editorial comments:**  
Changes to be made by the author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We proofread the manuscript, and then sent it for proof-reading company.

2. Please provide an email address for each author.

We added e-mail addresses for all the authors.

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. 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: Tanaka sangyo, Berger, Terumo, 3M, Micropore, Yamato, Leica, etc.

We made changes accordingly.

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

We revised the protocol text accordingly.

5. Please revise the protocol to contain only action items that direct the reader to do something (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.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

We revised the protocol text accordingly. Some parts of “Note” in the previous version are now integrated into “Discussion” part.

6. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

We revised the protocol text accordingly.

7. 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.

We revised the protocol text accordingly.

8. Please include single-line spaces between all paragraphs, headings, steps, etc.

We revised the protocol text accordingly.

9. 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.

We did it.

10. 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.

We did it.

11. 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.

We did it.

12. Table 1: Please change the time units “sec” to “s”, “mins” to “min”, and “hrs” to “h”.

We made changes accordingly.

13. Movie: Please add a title and a short description of the data presented in the movie in the Figure Legends after the Representative Results.

We added the Figure Legends for the movie.

14. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

We revised “Discussion” part extensively to include the points raised here.

15. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

We did it.

**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
The manuscript entitled "A simple and versatile method for mounting Arabidopsis leaves for intravital time-lapse imaging of the immunity-related promoter-reporter activity" describes a method for live imaging of fluorescent markers in leaves, with a goal to gain better understanding of the dynamics of plants' immune responses. This method has value even for researchers outside of the plant immunity field and some of the suggested changes (mostly minor) reflect this opinion. I support publishing this article after addressing the following concerns:  
  
Major Concerns:  
The language needs to be improved and some parts re-written for clarification. For example "swinging" should be replaced with "sleep movement circadian response" (Introduction, line 76) or with "moving" (step 2.6, line 136). Another example is the second part of the first paragraph of the Discussion (line 228) where the authors should clearly specify what kind of a method of leaf imaging is lacking and explain why.

We have improved the language by ourselves as well as sent the manuscript for a professional English-editing service. In addition, we changed the words accordingly.

Minor Concerns:  
1. The title could be shortened and made more general. For example ""A simple and versatile method for mounting Arabidopsis leaves for intravital time-lapse imaging".

We thank you for a nice suggestion. We changed the title accordingly.

2. Step 2.1: Add "out" in line 98 after "cut".

We did accordingly.

3. Step 2.2: Explain which side of the leaf is supposed to be infiltrated (abaxial/adaxial). The reference to Figure 1D is out of place. It should be moved at the end of the first sentence in the paragraph.

We clarified as “abaxial” in the text and placed the reference to Figure 1D accordingly.

4. Step 2.4: Add references in the text to the arrows pictured in Figures 1H and 1I. Explain that a bridge formed from a plastic tape is supposed to be placed over the petiole (line 126).

We did accordingly.

5. Step 2.5: Explain that a tip of a leaf blade is fixed on the slide (line 130). Is the last sentence in this paragraph supposed to mean, "Do not fold the tape"?

We did accordingly. The sentence was also rephrased clearly.

6. Step 2.6: Is the last sentence in this paragraph supposed to mean, "Do not fold the tape"?

We rephrased the sentence clearly.

7. Step 3.3: Note needs to be re-written to better explain the preliminary experiment. Abbreviations (e.g., ROI) need to be defined.

We explained more clearly about the preliminary experiment in Discussion part. ROI is now clearly defined in the text.

8. Figure 1 Legend should not repeat the procedure.

Thank you for your suggestion. We changed the legend extensively.

9. Figure 1E: The slide is barely visible.

We changed the figure design. Figure 1 was splitted into two figures. The previous Figure 1E became more visible in the current version.

10. Figure 1F: double-headed arrows are not visible.

We changed the size of arrows now.

11. Figure 1: the resolution is low.  
  
We prepared Figure 1 with a better resolution. If the figure appears to be low resolution in the PDF file, please click the link in the PDF file to download the original figure image.

**Reviewer #2:**  
Manuscript Summary:  
The manuscript ´"A simple and versatile method for mounting Arabidopsis leaves for intravital time-lapse imaging of the immunity-related promoter-reporter activity" by authors Shigeyuki Betsuyaku, Hiroo Fukuda and Nobuhiko Nomura, describes a method for a live-imaging/long-term/time-laps analysis of the whole plant leaf expression pattern of the fluorescently labeled reporter, in this case nuclearly localized YFP applied in the study of a spatiotemporal regulation of an immunity-related promoter of pathogenesis related 1 gene. The manuscript is nicely written and all the steps of the procedure are nicely explained. However, there are several items that should be matter of further improvement or better explanation.  
  
Major Concerns:  
The main weakness of the manuscript is that it describes a long term observation of the leaf/plant kept in conditions suitable for fluorescent microscopy, with a bright field light set as the only source of light (if I understood well; if not please explain additionally light conditions during the imaging) in comparison to previous cultivation of the whole plant under constant white light. It would be good to show that this does not impose stress by monitoring eg. chlorophyll fluorescence in mock infiltrated plant (several time points would be sufficient). The method also allows monitoring of the adaxial side of the leaf only. Thus, the described method is more suitable for live-imaging of gene expression which is not sensitive to light conditions and also for adaxially expressed genes.

We thank you for the comment. We measure the power of the epi-white light (LED) of our microscope system and adjust it to the level of our plant growth room. Therefore, our lighting condition of our microscopic system used in this protocol almost mimics our normal plant growth condition, since we use 24 h continuous light condition in our lab. We added a “Note” to explain clearly this point in 4.3.3.

As we published in our previous manuscript (Betsuyaku et al, PCP, 2018), our continuous light exposure condition in our time-lapse imaging does not weaken/activate chlorophyll autofluorescence and does not cause ectopic *pPR1* activation. We incorporate a new figure (Figure 3C) from the previous manuscript into the revised manuscript to show the point.

Minor Concerns:  
Even though it is obvious from figure that the infiltration was performed abaxial, it would be good to mention it in the text, for other leaves/species adaxial can be possible as well and it can give different patterns.

Thank you for the comment. We explained clearly by adding the word “abaxial’ in the text.

Is there a time delay between 2.2 and 2.3, a time given to leaf to „rest" from infiltration?

No, there is no delay between 2.2 and 2.3. We added a sentence to explain this point clearly at the beginning of 3.1.

Could a plastic tape Yamato been substitute by any other pressure-sensitive insulating tape or it has some special features?

Yes, any plastic/vinyl tape with a reasonable stiffness can be substituted with the Yamato tape. We explained the point clearly in the text.

Figure 1 - part E requires better resolution (glass is poorly visible); F should be enlarged, so that arrowheads are more obvious.

We changed the figure design. Figure 1 was splitted into two figures. The previous Figure 1 E and F became more visible in the current version. If the figures appear to be low resolution in the PDF file, please click the link in the PDF file to download the original figure images.  
  
**Reviewer #3:**  
Manuscript Summary:  
The authors describe a method to reliably monitor a fluorescent reporter gene over an extended period.  
  
Major Concerns:  
While it is possible to understand the main points and direction of the manuscript, the authors need to extensively revise the text so that it is written by someone with a higher level of English proficiency. In its current form, the manuscript has are far too many errors to be unambiguous.

We have improved the language by ourselves as well as sent the manuscript for a professional English-editing service.

Minor Concerns:  
Figures 1 and 2 are low resolution and some of the important details are lost.

We changed the figure design. Figure 1 was splitted into two figures. The previous Figure 1 E and F became more visible in the current version. We also improved the resolution of Figures 1 and 2. If the figures appear to be low resolution in the PDF file, please click the link in the PDF file to download the original figure images.

Again, we thank the reviewers for giving us an opportunity to revise our manuscript for possible publication in JOURNAL of VISUALIZED EXPERIMENTS. These comments improve the quality of our manuscript. We believe that the revised manuscript will be widely cited. We hope very much that you will find it suitable for publication in JOURNAL of VISUALIZED EXPERIMENTS.

Sincerely yours,

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