**Response to editor and reviewer 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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Thanks for your suggestion, we improved the manuscript based on your comments.  
2. Please provide an email address for each author.

Email of all authors are provided.  
3. Abstract: Please do not include references here.

We have corrected it.  
4. Please spell out each abbreviation the first time it is used.

We have corrected it.

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

We have corrected it.

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

We have corrected it.  
7. 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: Duchefa, MilliQ, Micropore, ibidi, World Precision Instruments, Leica, Zeiss, etc.

We have corrected it.  
8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

We have corrected it following your suggestion.  
9. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have corrected it.  
10. 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. Please move the discussion about the protocol to the Discussion.

We have revised the protocol following your instructions.  
11. Please add more details 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. 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. Some examples:  
Line 69: Please specify the final volume.  
Line 84: How many seeds are sown per plate?  
Line 98: How many seedlings are placed on each slide? Please specify throughout.  
Line 107: Please specify the growth conditions.

We have revised the protocol following your instructions.  
12. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

We have formatted the protocol following your instruction.  
13. Please include single-line spaces between all paragraphs, headings, steps, etc.

We have corrected it.  
14. 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 have formatted the protocol following your instruction.  
15. 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 have formatted the protocol following your instruction.  
16. 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 have formatted the protocol following your instruction.  
17. Figures: Please include a space between numbers and their units of the scale bar.

We have corrected it.  
18. Figure 1E and F: Please line up panel labels better.

We have corrected it.  
19. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

We have corrected it.  
20. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

We have corrected it.  
21. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

We have corrected it.  
22. A minimum of 10 references should be cited in the manuscript.

We have corrected it.  
23. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

We have corrected it.  
  
**Reviewers' comments:**  
  
  
  
**Reviewer #1:**  
Manuscript Summary:  
The article "A method to visualize cellular gibberellin levels using the nlsGPS1 FRET biosensor" by Rizza et al. describes protocols for the use of the GA biosensor GPS1. The protocol to analyze steady state GA levels and GA uptake and distribution in vivo using confocal microscopy are comprehensively described, and controls and critical steps are included.  
  
Major Concerns:  
There are three major points that could be improved  
1) Improve the description of the sample mounting by adding a new figure with images or schematic presentation of the different sample mountings.

We thank the reviewer for the suggestion. We have now included a new Figure (Figure 1) with schematic presentation of the different sample mountings.  
3) Improve the description of the microscopy settings.

We have added the improved description now.  
3) Besides image processing, also include image data analyses. How to get quantitative data from the images, and how to present them in a graph?

We thank the reviewer for the suggestion. We have now included the quantitative analysis of the images.  
  
Minor Concerns:  
Minor points: typing errors in ()

We have corrected it.  
line 18) locali(s)ed

We have corrected it.  
line 52) The nlsGPS1-NR carries mutations in the GA binding pocket (mutations) that disrupt...

We have corrected it.  
line 54) Maybe cite here also original publication about GA receptor structure ...

We have corrected it.  
lines 66 & 72) maybe spell out MS

We have corrected it.  
line 79) E(T)OH

We have corrected it.  
line 81) Working solution: Dilute the GA4 stock in 1/4 MS liquid pH:5.7 to 1 µM as working concentration

We have corrected it.  
lines 86-89) add relative humidity conditions for the growth chamber

We have corrected it.  
line 91) Wrap the plates in aluminum foil...

We have corrected it.  
line 100) add schematic picture for glass slide preparation or cite [4]?

We have corrected it.  
line 106) Maybe cite original publication for root chip, or cite [4]

We have corrected it.  
line 138) Using (your) forceps

We have corrected it.  
lines 148-150) Explain how to remove the mock solution from the left side

We have corrected it.  
line 169) SP8-FLIM(an) ?

We have corrected it.  
line 163) better separate microscope settings for LEICA SP8 and Zeiss LSM 780. Better to use new headers for each microscope

We have clarified the different settings for the different microscopes.  
line 176) and separate excitation/emission scan?

We have corrected it.  
lines 171-189) Add more microscope settings: Objective, pixel resolution, scan frequency, bit-depth of images, bidirectional scan? zoom? HyD mode? How many z-steps? Sequential scanning for Ex CFP / Em, CFP/FRET and Ex YFP / Em YFP? pinhole size? Inverted or upright microscope?

We have included more details following your instructions   
line 194: with the; the detector?

We have corrected it.  
line 200) add more microscope settings?

We have corrected it.  
line 202) add Fiji WWW link  
 We have added it.

line 205) explain why Sum is better to use than Max

We have corrected it. The “Sum” function is better because it includes all the pixels

while the “Max” function only takes the brightest pixel.

lines 224-226) not sure what this means and if this is correct?

We have improved the text of the protocol to explain these steps of the Fiji analysis. line 249) Description of quantitative data analyses is missing (Extraction of numbers/values from the pictures)

We have now included the quantitative data analyses.  
line 260) seedlings were placed (into?) "Ibidi sticky slide"

We have corrected it.  
Video 1: scale bar, calibration bar and time scale are missing  
Comments/Description: 70% EtOH? For what is this description good for?

We have corrected it.  
  
  
**Reviewer #2:**  
Manuscript Summary:  
The ability to measure hormone levels in situ at the cellular level is an important advance: the very high resolution enabled by these methods will undoubtedly improve understanding of hormone-regulated processes. The development and application of a FRET-based sensor for gibberellin A4 was described in a recent publication in Nature Plants and has stimulated considerable interest. Although the scope for the method is at present quite limited, the detailed protocols for its use in Arabidopsis seedlings under defined conditions will be helpful for those wishing to follow up this work and have access to the plants or the constructs. Some of the protocols, for example the perfusion method, have more general application. The methods are clearly described and accessible to most plant scientists with access to a confocal fluorescence microscope.  
  
Major Concerns:  
There is no discussion of calibrating the method for absolute quantitation. Can the units on the LUT bars be translated into GA concentration?

The emission ratio of the sensor cannot be directly translated into GA concentration. Since the range of the nlsGPS1 biosensor is between ~2 nM to 200 nM *in vitro*, the range of nlsGPS1 emission ratio values *in vivo* likely falls in the same range. However, as it is not currently possible to establish the *in vivo* minimum ratio for apo-sensor and maximum ratio for saturated sensor, we cannot confidently translate ratios to exact concentrations. Nevertheless, we can be more confident that strong biosensor ratio changes are indicative of GA concentration changes around the Kd of 24 nM.

Minor Concerns:  
Line 84: For imaging roots on vertical plates, should square plates be specified?

We have corrected it.

Line 106: For the RootChip, why not refer to the original publication, Grossmann et al Plant Cell 2012

We have added the reference now.  
Line 288: sample prep (jargon)

We have corrected it.  
The figure legends to do not mention the second of the pairs of images in each subfigure. Are these of YFP emission?

We have corrected it.

**Reviewer #3:**  
Manuscript Summary:  
Clear and concise.

We thank the reviewer.  
  
Major Concerns:  
No major concerns  
  
Minor Concerns:  
\*Line 41. The authors may specify already at the beginning of the protocol which is the bioactive GA primarily sensed by the sensor.

We have corrected it  
\*Line 47. It would be more consistent with the rest of the paper to report "edCerulean" instead of "Cerulean".

We have corrected it  
\*Line 52. I would slightly modified the following sentence: "The nlsGPS1-NR carries mutations in the GA binding pocket mutations that disrupt….." by removing the word "mutations" after "pocket".

We have corrected it  
\*Line 67. From Duchefa there are several MS media with or without Vitamins. For the sake of clarity, the authors might also report the code, e.g. M0221.

We have added the Cat N.   
\*Line 70. Also, in this case if the authors want be really precise, they can indicate from which company they order the "Plant Agar" or specify if any agar can be used.

We have added the Cat N

\*Line 84. The authors may also report that the seeds need to be sterilized before being plated on agar plates. They could add a sentence in which report the method of sterilization they use.

We have now added the sterilization method.  
\*Line 166. The authors might report if the microscope they use is inverted or upright.

We have corrected. In this protocol we used upright confocal microscope.  
\*Line 172. Please, revise the following sentence: "A 3% laser power is used to excite the 448nm and 1-2% power to excite 514nm laser." It does not sounds really good.

We have corrected it following your suggestion.  
\*Line 179. The authors report: "The detector gain is set to 110 to capture CFP (excited donor) and YFP (FRET emission). For the FRET-imaging, it is very important that the gain is kept constant while collecting the donor and FRET-emission. We use a line average of 4 for the majority of our experiments".  
The authors may just report that the gain needs to be adjusted to a given value and not changed. Indeed, the gain value depends on the used objective, the transgenic line (e.g. the nlsGPS1 expressed in a mutant could have a lower or higher expression level) and other parameters. They can report that in their case the gain used was 110 but I would avoid to provide a fixed number.

Thank you for your suggestion. We have now corrected the text accordingly.  
\*Lines 183-185. To avoid pixel saturation the operator has to change parameters such as laser power and gain, thus I would avoid being so strict in reporting the settings routinely used by the authors.

Thank you for your suggestion. We have now corrected the text accordingly.  
\*Line 194. There are two "the".

We have corrected it.  
\*Line 218. A technical issue comes from how the authors have suggested to calculate the ratio. The authors calculate the maximum projection of the stack (#2. Select "Z project" and run Z projection, selection type Sum slices) and, later, the fluorescence ratio obtained from the stack. However Maximum Intensity Projection (MIP) is a non-linear process: for each pixel of the image, the maximum value within the stack is visualized. This means that the ratio is calculated between voxels that are randomly chosen within the stack. Hence, to be precise they should have first calculated the ratio and eventually later show the MIP. The introduced error it is probably not dramatic, but for the sake of clarity I wanted to mention it.

Thank you for your suggestion. However, we did not use the MIP in our calculation. We have used the Sum slice projection method that incorporates all the pixels in the stack.  
\*Line 243. Please explain the meaning of "(DxAm/DxDm)".

We have included the explanation. DxAm/DxDm means: Dx is donor excitation, Am is the acceptor emission and Dm is donor emission.   
  
  
**Reviewer #4:**  
This protocol is Important and timely as GPS1 represents the first GA responsive biosensor that has been made. Aiding the community in its effective use is an important goal. The protocol is well written but I have a few suggestions that might make it easier to follow for the non-specialist reader.  
  
In the introduction, some more references might be useful in helping the reader understand the background to the work. For example:  
Line 41: Should there be a reference to the original GPS1 paper here?

We have now included the reference.

Line 43: Referencing some recent reviews on GA here would greatly help the reader who is not an expert in the GA field.

We thank the reviewer. We have now included more references.  
Line 45-46: Add a short description of normal GA sensing and how this makes the GID1C/GAI partnership a key part of the GPS1 sensor.

We have now added a short description of GA signalling.  
Line 47: A reference to the enhanced dimerization variant of cerulean, to Aphrodite and to the nls used would be helpful.  
 We have now included more references  
In the protocol:  
Line 84: Are these seeds surface sterilized? If they are, the protocol should be added.

We have now added the sterilization method.  
  
Line 84: "Microspore paper tape", Micropore tape?

We have corrected it  
Line 98: In this section, I think a non-specialist reader will find it hard to picture the setup. Perhaps add a picture/diagram?  
Line 129: Similarly, for the section on chemical exchange, a diagram or picture might help understand the setup

We thank the reviewer for the suggestion. We have now included a new Figure (Figure 1) with schematic presentation of the different sample mountings  
Line 151: adding the approximate total volume of GA4 that flows through over the 10 minutes would help researchers know if they were reproducing the required exchange.

We have added the approximate volume of GA to use during the exchange.

For the section on image J processing (line 201-), it would help immensely to have notes explaining what each step is accomplishing. For example, Line 206: What is the rolling ball local background subtraction designed to do? What are the 3 images being processed in this step? Line 216: Why is the Gaussian blur being applied? Without the explanation of this step it sounds like this will change pixel values prior to ratio calculation. Some annotations for the processing steps might help clear up these kinds of questions.

We thank for the suggestion, we have now included more details.   
Line 276: It would be helpful here to provide references to examples of the other techniques being compared to in order for the reader to be able to see the kinds of resolutions made by these alternative methods.

We have added more references  
Line 290: Some references showing environmental sensitivity of GA levels would be helpful here for the interested reader to get an idea of how variable the GA levels are likely to be.

We have added more references  
  
Line 293: Providing some examples of what kinds of things will make a tissue not amenable to ratiometric imaging would be very helpful to those planning these kinds of experiments.  
 Thank you for the suggestion, a clarifying example was provided.  
Line 306: Could the GA affinities be listed to help the reader make the comparison?  
 We added more information on affinities.  
Figure 1. Are the yellow images in each pair presented the YFP Aphrodite signal? If so, it would be good to label the panels, especially as the order of the images is different in A-D vs E and F. Presenting an example of quantitiative data extracted from these images would also be very useful.  
 We have corrected it and add the quantitative analysis.  
Figure 2. These look like tiled images of the cotyledons and the hypocotyl section below. It would be useful to describe this in the legend.

We have included a short description in the figure legend. The images were acquired as two stage position and we did not make the stitching.  
Line 325: GA is noted as 1uM here but 0.1 uM in line 261.  
 We have corrected it  
Last page comments and description: There is an orphan sentence fragment "dissolve in EtH70 % , and keep at -20°C"

We have corrected it