Journal of Visualized Experiments

A method to visualize cellular gibberellin levels using the nlsGPS1 FRET biosensor

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58739R1
Full Title:	A method to visualize cellular gibberellin levels using the nlsGPS1 FRET biosensor
Keywords:	gibberellin; Phytohormone; Spatiotemporal; Plant development; Cell growth; Biosensor; FRET
Corresponding Author:	Alexander M Jones University of Cambridge Cambridge, UNITED KINGDOM
Corresponding Author's Institution:	University of Cambridge
Corresponding Author E-Mail:	alexander.jones@slcu.cam.ac.uk
Order of Authors:	Annalisa Rizza
	Ankit Walia
	Bijun Tang
	Alexander M Jones
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Cambridge, United Kingdom

Cover letter for

"A method to visualize cellular gibberellin levels using the nlsGPS1 FRET biosensor"

Rizza A, Walia A, Tang B, Jones AM, 3 July 2018 JoVE 2018

Dear Editors,

We would like to submit for evaluation a protocol focused on gibberellin patterning in plant development. This manuscript was prepared as the documentary support for a video article in JoVE as invited by editors Indrani Muhkerjee and Lyndsay Troyer.

Thank you kindly for the invitation and for consideration of this manuscript.

Best regards,

Alexander Jones
Group Leader and Gatsby Fellow
Sainsbury Laboratory, Cambridge University
Cambridge, CB2 1LR
United Kingdom

TITLE:

Visualizing Cellular Gibberellin Levels Using the nlsGPS1 Förster Resonance Energy Transfer (FRET) Biosensor

AUTHORS AND AFFILIATIONS:

Annalisa Rizza[1], Ankit Walia[1], Bijun Tang[1], Alexander M. Jones[1]

[1]Sainsbury Laboratory, University of Cambridge, Cambridge, United Kingdom

Corresponding Author:

Alexander M. Jones (alexander.jones@slcu.cam.ac.uk)

Email Addresses of Co-authors:

Annalisa Rizza (annalisa.rizza@slcu.cam.ac.uk)
Ankit Walia (ankit.walia@slcu.cam.ac.uk)
Bijun Tang (bijun.tang@slcu.cam.ac.uk)

SUMMARY:

Gibberellin Perception Sensor 1 (GPS1) is the first Förster resonance energy transfer-based biosensor for measuring the cellular levels of gibberellin phytohormones with a high spatiotemporal resolution. This protocol reports on the method to visualize and quantify cellular gibberellin levels using the genetically encoded nlsGPS1 biosensor in *Arabidopsis* hypocotyls and root tips.

ABSTRACT:

The phytohormone gibberellin (GA) is a small, mobile signaling molecule that plays a key role in seed germination, cellular elongation, and developmental transitions in plants. Gibberellin Perception Sensor 1 (GPS1) is the first Förster resonance energy transfer (FRET)-based biosensor that allows monitoring of cellular GA levels *in vivo*. By measuring a fluorescence emission ratio of nuclear localized-GPS1 (nlsGPS1), spatiotemporal mapping of endogenously and exogenously supplied GA gradients in different tissue types is feasible at a cellular scale. This protocol will describe how to image nlsGPS1 emission ratios in three example experiments: steady-state, before-and-after exogenous gibberellin A_4 (GA_4) treatments, and over a treatment time-course. We also provide methods to analyze nlsGPS1 emission ratios using both Fiji and a commercial three-dimensional (3-D) micrograph visualization and analysis software and explain the limitations and likely pitfalls of using nlsGPS1 to quantify gibberellin levels.

INTRODUCTION:

Plant hormones play a fundamental role in plant growth and development. These small, mobile signaling molecules are typically regulated at several levels, such as biosynthesis, catabolism, and short- and long-distance transport[1-4]. The understanding of hormone signaling pathways and downstream transcriptional responses has sharpened over the years. However, to link the diverse cellular responses of the hormone signaling pathways with the regulatory inputs directing hormone distributions, we require a spatiotemporal quantification of hormone levels at a cellular

scale. FRET-based biosensors that can detect phytohormones can advance scientists' ability to quantify hormone levels at a cellular scale. FRET-based biosensors consist of a FRET pair (donor and acceptor fluorescent proteins) linked to a sensory domain that binds a specific ligand or responds to a biological stimulus. For small molecule biosensors, ligand binding triggers a conformational change of the sensory domain that results in a change of distance and/or orientation between the two fluorescent proteins of the FRET pair. A ratiometric analysis of a FRET biosensor is accomplished by exciting the donor and measuring the fluorescence emission ratio of acceptor over donor[5,6]. Ligand binding is detectable as a change in this emission ratio[7].

We recently developed a FRET-based biosensor for the plant hormone GA. GAs are a class of hormones that can promote seed germination, cellular elongation, and the developmental transition from vegetative to flowering phases. The nlsGPS1 biosensor is nuclear localized and provides spatiotemporal insights into GA dynamics in diverse plant tissues. In *Arabidopsis* cells, GA binds to soluble receptors, gibberellin-insensitive dwarf (GID), and the complex induces the degradation of DELLA proteins that act as negative regulators of GA signalling[2]. The GA sensory domain of nlsGPS1 consists of the *Arabidopsis* GA receptor (AtGID1C) linked to a 74-amino acid truncation of a DELLA protein (AtGAI) and a FRET pair consisting of enhanced dimerization variants of Cerulean as the donor fluorescent protein and Aphrodite (a codon-diversified Venus) as the acceptor fluorescent protein[8]. The nlsGPS1 biosensor is a high-affinity sensor for the bioactive GA_4 (K_d = 24 nM for GA_4) and it can be utilized in diverse tissue-types to map and quantify GA gradients. To avoid misinterpretation of the *Arabidopsis* GA levels *in vivo*, we have also developed a nonresponsive variant of nlsGPS1 (nlsGPS1-NR) to use as a negative control. The nlsGPS1-NR protein carries mutations in the GA-binding pocket that disrupt the binding of GA and mutations in the DELLA protein that disrupt the interaction with GID receptor proteins[7,9]. Emission ratio patterns or changes observed in both nlsGPS1 and nlsGPS1-NR lines can be considered artefacts not directly related to GA-binding events. It is also important to note that nlsGPS1 binding to GA_4 is not rapidly reversible, and therefore, cellular nlsGPS1 emission ratios should be interpreted as representing the highest recent concentration of GA in a given nucleus rather than the real-time steady-state levels. As a consequence, an analysis of falling GA levels is not possible with nlsGPS1.

Here we provide a detailed protocol for utilizing a nlsGPS1 biosensor in cells of the model plant *Arabidopsis*, using confocal imaging-based approaches at a high-resolution. The protocol provides information on imaging plant roots and hypocotyls both at steady state and over time-courses. The nlsGPS1 sensor could potentially be utilized in diverse tissue-types, as well as across plant species, to map and quantify GA distributions.

PROTOCOL:

1. Preparations

1.1. Prepare ½ Murashige and Skoog agar pH 5.7 with no sucrose (1 L).

1.1.1. Dissolve 2.2 g of Murashige and Skoog (MS) basal medium in 950 mL of ultrapure water. Adjust pH to 5.7 with 5 M KOH.

1.1.2. Make up the solution to a final volume of 1 L with ultrapure water. Divide it into two 500 mL bottles, each containing 1% plant agar. Autoclave at 121 °C for 20 min.

1.2. Prepare ¼ MS liquid at pH 5.7 with no sucrose (1 L).

1.2.1. Dissolve 1.1 g of MS in 950 mL of ultrapure water. Adjust pH to 5.7 with 5 M KOH. Make up the solution to a final volume of 1 L with ultrapure water. Divide it into two 500 mL bottles. Autoclave at 121 °C for 20 min.

1.3. Prepare GA_4.

1.3.1. Dissolve the GA_4 in ethanol 70% to make a final concentration of 100 mM GA_4 stock.

1.3.2. To prepare working solution, dilute the GA_4 stock to 0.1 - 1 µM in ¼ MS liquid pH 5.7.

2. Plant Growth

2.1. Sterilize the *Arabidopsis* seeds.

2.1.1. Work using a chemical hood. Aliquot seeds (approximately 200 seeds) into 2 mL microcentrifuge tubes. Use a marker with chlorine-resistant ink and place the tubes with open lids inside the sterilization vessel (a large box that can be sealed).

2.1.2. Place a 250 mL beaker containing 50 mL of ultrapure water and 50 mL of sodium hypochlorite solution inside the sterilization vessel.

2.1.3. Add 3 mL of concentrated hydrochloric acid (HCl) to the beaker. Immediately seal the vessel and allow sterilization by chlorine gas for 6 - 16 h.

2.1.4. After unsealing the vessel, close the lids of all the microcentrifuge tubes in the seed rack. Sterilized seeds can be stored dry until the time of plating.

2.2. Sow approximately 20 sterilized *Arabidopsis* seeds on ½ MS agar square plates. Seal the plates with porous surgical tape and wrap them with aluminum foil. Incubate them at 4 °C for 1 - 3 d for stratification.

2.3. For root imaging, transfer the plates to the growth chamber in a vertical position for 3 d with the following growth conditions: long-day conditions (LD, 16 h of light/8 h of dark); intensity light of 120 µmol/m^2s; temperature of 22 °C (during the light cycle) or 18 °C (during the dark cycle), 65% relative humidity (RH).

2.4. For dark-grown hypocotyl: transfer the plates to the growth chamber for a light pulse of 1 - 4 h to synchronize the germination. Wrap the plates with aluminum foil and place them into the growth chamber in a vertical position for 3 d.

3. Sample Preparation

3.1. Steady-state measurements (Figure 1A)

3.1.1. On a clean microscope slide, add 50 µL of ¼ MS liquid (from now on termed as mock solution). Gently transfer seedlings expressing nlsGPS1 from the plate to the slide.

3.1.2. Take a clean coverslip and spot a drop of vacuum grease on each corner of the coverslip. Gently place the coverslip over the seedlings and carefully add extra mock solution to remove any air bubbles.

3.2. GA_4 treatment: chemical exchange experiment (Figure 1B).

3.2.1. Before the GA_4 treatment, use a 20 mL syringe filled with vacuum grease (attached to a pipet tip) to draw a rectangle (length: 3.5 cm, height: 2.5 cm) with a uniform layer of vacuum grease on a clean glass slide (**Figure 1B**). To allow for a fine line of vacuum grease, the pipet tip should be cut to have an opening of 1 mm in diameter.

3.2.2. Add 50 µL of mock solution to the glass slide. With clean forceps, pick the nlsGPS1 seedlings and gently place them on the mock solution. To prevent any damage, transfer the seedlings by supporting the undersides of the cotyledons without grasping the seedling with the forceps.

3.2.3. Take a clean coverslip and spot a drop of vacuum grease on each corner of the coverslip. Using the forceps, place the coverslip in the center of the vacuum grease rectangle. Now the coverslip is sealed on the two sides corresponding to the long edges of the vacuum grease rectangle.

3.2.4. Carefully add extra mock solution to fill up the reservoir and remove any air bubbles within the reservoir without disturbing the seedling(s) inside.

3.2.5. Acquire the images before the GA_4 treatment using a confocal microscope. Follow the instructions as described in section 4 of this protocol.

3.2.6. For the GA_4 treatment, remove the microscope slide from the confocal stage. Set a timer for 20 min.

3.2.7. After starting the timer, exchange the buffer solution with ¼ MS liquid containing 1 µM GA_4. Add the GA_4 solution (approximately 50 µL) from the left side of the coverslip and remove the previous (mock) solution from the right side. Keep on exchanging the solution to replace the mock solution completely, which takes approximately 10 min (**Figure 1B**).

3.2.8. Place the glass slide back on the microscope stage. Wait another 10 min before acquiring the after-GA image.

3.3. **Set up of a time course for the tissue of interest**

Note: The tissue of interest might be, for instance, hypocotyls or roots. We routinely perform time courses for imaging nlsGPS1 biosensor using a commercial perfusion system (**Figure 1C**, see **Table of Materials**) or a RootChip[7,10,11].

3.3.1. Add 200 µL of mock solution to the center of the perfusion channel of the sticky-slide (see **Table of Materials**). Gently pick nlsGPS1 seedlings and place them on the mock solution in the sticky-slide.

3.3.2. Using forceps, gently place the glass coverslip and, using the backside of the forceps, press gently on the outer edges of the coverslip so that it forms a strong bond with the sticky material on the periphery of the sticky-slide.

3.3.3. Using two elbow Luer connectors (with an inner diameter [ID] of 0.8 mm) and the silicone tubing (with an ID of 0.8 mm), connect the sticky-slide to a 20 mL syringe and to an outlet container collecting the outflow solution. Use the Luer lock connector (with an ID of 0.8 mm) to connect the syringe to the silicone tubing.

3.3.4. Gently press the syringe containing the mock solution manually to let enough solution pass through the chamber so that there are no air bubbles left in the chamber. Place and hold the syringe on the programmable syringe pump.

3.3.5. Set the pump parameters specific to the syringe used (*i.e.*, diameter and volume) along with the flow rate according to the manual provided with the pump.

Note: In this protocol, a flow rate of 1 - 3 mL/h was used, and this can be changed according to the experimental demands. Initiate the time course by starting the pump.

3.3.6. For GA_4 treatment during a time course (**Figure 1C**), stop the perfusion by pausing the pump and change the syringe with a new one containing ¼ MS liquid supplemented with GA_4.

4. Microscopy

Note: We perform confocal laser microscopy.

4.1. Acquire images using a confocal microscope equipped with lasers to perform FRET imaging.

Note: For nlsGPS1, variants of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are imaged. In this protocol, commercial microscopes (see **Table of Materials**, listed as microscope 1 and 2) are used with 10X or 20X dry 0.70 harmonic compound PLAN APO objectives.

4.2. For microscope 1, use 448 nm and 514 nm wavelength lasers to excite CFP and YFP, respectively. Acquire sequential scans. Set detectors (*e.g.*, HyD SMD) to detect 460 - 500 nm for CFP (donor emission) and 525 - 560 nm for YFP (FRET emission) after the excitation of CFP. Using a second sequence, set a detector to detect 525 - 560 nm for YFP (YFP emission) after the excitation of YFP.

Note: This YFP fluorescence is used as an expression control, as well as in segmenting nuclei, to generate surfaces using a commercial 3-D micrograph visualization and analysis software.

4.3. For microscope 2, use 440 nm and 514 nm wavelength laser lines to excite CFP and YFP, respectively. Acquire two tracks. For track 1, set detectors (*e.g.*, ChS) to detect 464 - 500 nm for CFP (donor emission) and 526 - 562 nm for YFP (FRET emission) after the excitation of CFP. For track 2, set a detector to detect 526 - 562 nm for YFP (YFP emission) after the excitation of YFP.

Note: This YFP fluorescence is used as an expression control, as well as in segmenting nuclei, to generate surfaces in the 3-D visualization and analysis software.

4.4. Acquire images using a format of 512 x 512 pixels and a resolution of 12 bits.

4.5. The gain needs to be adjusted to an empirically determined value that allows for a good signal while not saturating pixels. The gain should not be changed between CFP and FRET emission over the experiment. Set the pinhole to 1 airy unit (AU).

4.6. In the microscope 1 software, while in an active live scan, utilize **Glow over/under** located on the top left side of the image screen to determine the underexposure and saturation of the region of interest. In the microscope 2 software, utilize **Range Indicator** located on the bottom side of the image screen to determine the underexposure and saturation of the region of interest. For any quantitative image analysis, there should be no pixel saturation.

4.7. Set the z-stacks with a step size of 1 µm. The step size can be reduced for an increased z-resolution or increased for an increased speed of acquisition or to increase the number of positions/samples that can be imaged. For the automated time course, set the time along with the z-stacks (xyzt mode from the acquisition mode tab) to acquire the images at set time intervals.

5. Image Analysis Using Fiji

Note: Using ImageJ (Fiji) it is possible to process imaging data and produce two-dimensional (2-D) images of the nlsGPS1 emission ratio in *Arabidopsis* seedlings. For examples of images, see

Figures 2A, **2C**, **2E**, **2G**, and **3A**. In ImageJ, it is possible to find each command of this protocol using the search function. Press the **space bar and L** on the computer keyboard. A new window will open; type the required command in the search field.

5.1. Drag the file (either .lif or .lsm files) into Fiji (Image J) and open the images as hyper-stack.

5.2. From the main menu, select **Image** > **Stack** > **Z project** and select **Sum slices** to capture all pixels rather than only the brightest pixels as in **Max projection**.

5.3. From the main menu, select **Process** > **Subtract background** and set the rolling ball radius to 50 pixels. Unselect any other options and process all three images. This step removes background using the "rolling ball" algorithm.

Note: 50 pixels was empirically determined to include nuclei as foreground.

5.4. From the main menu, select **Image** > **Color** > **Split channels**. Three new windows will open: **C1-SUM** (CFP channel); **C2-SUM** (FRET channel), and **C3-SUM** (YFP channel).

5.5. Select the **C3-SUM** window and, from the main menu, select **Process** > **Filters** > **Gaussian Blur** and apply a Gaussian Blur of 1 to reduce the image noise.

5.6. From the main menu, select **Image** > **Adjust** > **Brightness/Contrast** and select auto.

5.7. From the main menu, select **Process** > **Enhance Contrast**, and set **saturated** = 0.35.

5.8. From the main menu, select **Image** > **Type** > **8-bit** and convert the stacks into an 8-bit image.

5.9. From the main menu, select **Image** > **Adjust** > **Auto-Local Threshold**. In the **Auto-Local Threshold**, select the following parameters: **Phansalkar method**, **radius = 15**, **parameter 1 = 0**, **parameter 2 = 0**, and **white stack**. In this step, YFP stacks are used to make a binary mask.

5.10. Select the **C2-SUM** window and, from the main menu, select **Process** > **Filters** > **Gaussian Blur** and apply a Gaussian Blur of 1.

5.11. Select the **C1-SUM** window and, from the main menu, select **Process** > **Filters** > **Gaussian Blur** and apply a Gaussian Blur of 1.

5.12. To create the emission ratio stack from the two channels, from the main menu, select **Process** > **Image Calculator**. In the new window that will appear, select **C2-SUM** as image 1, select **Divide** as operator, and select **C1 sum** as image 2.

5.13. Make sure to select **Create a new window** and the 32-bit format. A new window will appear named **Result of C2-SUM**.

5.14. From the main menu, select **Image** > **Lookup table** > **LUT 16_colors**.

5.15. From the main menu, select **Process** > **Image Calculator**. In the new window that will appear, select **Result of C2-SUM** as image 1, select **Multiply** as operator, and select **C3-SUM** as image 2. In this step, the YFP binary mask is multiplied with the YFP/CFP stack to show only pixels present in the YFP control channel.

5.16. From the main menu, select **Process** > **Math** > **Divide** and set the value to 255. In the new window that opens, select **Yes** to analyze all images in the stack. A new image will appear named **Result of Result of C2-SUM**.

5.17. From the main menu, select **Image** > **Adjust** > **Brightness/Contrast** and select **Auto**.

5.18. From the main menu, select **Process** > **Enhance Contrast**, and select **Type saturated = 0.35**.

5.19. From the main menu, select **Image** > **Adjust** > **Brightness/Contrast**, and set minimum and maximum values to capture the GA distribution. Optional step: from the main menu, select **Analyze** > **Tools** > **Calibration Bar** and **Show LUT-calibration bar**, and save the **Result of Result of C2-SUM** as a .tiff file.

5.20. To obtain values of the emission ratios, from the main menu, select **Analyze** > **Set measurement** and select **Mean gray value** and **Standard deviation**.

5.21. Select the rectangle shape from the toolbar and draw a region of interest (ROI). From the main menu, select **Analyze** > **Measure**. A new window will report the mean value from the selected ROI.

5.22. Copy and paste the obtained values into spreadsheet software (*e.g.*, Excel or OriginPro) and make either a histogram for a before-and-after GA_4 treatment experiment or a line graph for a time course experiment.

6. Image Analysis Using 3-D Visualization and Analysis Software

Note: The advantage of using the selected software (see **Table of Materials**) is to segment objects (*e.g.*, nuclei) and create 3-D images from a confocal z-stack. For examples of images, see **Figures 2B**, **2D**, **2F**, **2H**, and **3B**.

6.1. Open the software and import the file (either .lif or .lsm files).

6.2. Segment nuclei based on the control YFP emission channel using the **Surfaces wizard**. The segmented objects are termed “surfaces”. This segmentation step permits the analysis of all voxels in a nucleus as one object while eliminating background from voxels outside of the nucleus.

6.3. In the **Surfaces wizard**, set the background subtraction (local contrast) to 3 µm and the thresholding to default. Mask the CFP emission (donor excitation donor emission [DxDm]) and FRET emission (donor excitation acceptor emission [DxAm]) channels based on the surfaces created using the YFP emission (acceptor excitation acceptor emission [AxAm]) channel.

6.4. Use the extension **XT Mean Intensity Ratio** to compute a ratio of donor excitation acceptor emission divided by donor excitation donor emission (DxAm/DxDm) between the mean intensity values of the individual surfaces in the two channels.

Note: This extension is available online for download.

6.5. To color the individual surfaces with the nlsGPS1 emission ratio, select **Color coding with statistics**, which is represented as a color wheel icon. Select **Mean intensity ratio** as statistics type.

6.6. Export the ratios of individual values from the table, which is found under the **Statistics** icon.

6.7. Copy and paste the values into a spreadsheet and make either a histogram for before-and-after GA_4 treatment experiments or a linear graph for time course experiments.

7. Statistical Analysis

Note: See **Figure 3D** for a beeswarm and box plot of nlsGPS1 emission ratios.

7.1. Open the software and paste the emission ratio of the nuclei surfaces as Y columns.

7.2. Select the columns of interest and select **Statistics** > **Statistics Description** > **Normality test** to know whether the samples are normally distributed. Run the Normality test and a new window will open and report the results.

7.3. If the samples are normally distributed, use the *t*-test as statistical test. Select **Statistics** > **Hypothesis testing** > **Two-Sample t-test on Rows** and run the *t*-test.

7.4. If the samples are not normally distributed, select **Statistics** > **Statistics Hypothesis testing** > **Two-Sample test for variance** to know whether the variance between the samples is not significantly different.

7.5. If the variance is not significantly different, use the **Mann-Whitney U test** as statistical test. Select **Statistics** > **Nonparametric Test** > **Mann-Whitney test** and run the test.

7.6. If the variance is significantly different, use **Kruskal Wallis ANOVA test** as statistical test. Select **Statistics** > **Nonparametric Test** > **Kruskal Wallis ANOVA test** and run the test.

REPRESENTATIVE RESULTS:

Using nlsGPS1, it is possible to measure cellular GA_4 levels in tissues amenable to fluorescence imaging, including root tips and dark-grown hypocotyls (**Figure 2**). In the *Arabidopsis* root, the nlsGPS1 emission ratio gradient is indicative of low GA levels in the meristematic and division zones and high GA levels in the late elongation zone (**Figures 2A** and **2B**). In contrast, an emission ratio gradient was not observed in nlsGPS1-NR roots, suggesting that the endogenous GA gradient is not an artefact (**Figures 2C** and **2D**). A nlsGPS1 emission ratio gradient was also formed in dark-grown hypocotyls, with low levels in the cotyledons and the apical hook and high levels in the rapidly elongating basal region of the hypocotyl (**Figures 2E** and **2F**). In contrast, an emission ratio gradient was not observed in the nlsGPS1-NR hypocotyls (**Figures 2G** and **2H**). In both *Arabidopsis* roots and dark-grown hypocotyl cells, endogenous GA accumulation correlated with cellular elongation rate.

Furthermore, exogenously supplied GA_4 accumulates preferentially in the elongation zone compared to the division zone of the *Arabidopsis* root (**Figure 3**), indicating that nlsGPS1 can be used to study endogenous and exogenous GA patterning.

During time course experiments, nlsGPS1 seedlings were placed in sticky-slide chambers and perfused with ¼ MS liquid, followed by a treatment with 0.1 µM GA_4 for 30 min. The video shows a faster accumulation of exogenous GA_4 in the root elongation zone compared to the division zone (**Video 1**).

FIGURE LEGENDS:

Figure 1: Sample preparation for confocal imaging. These panels show a schematic representation of the sample preparation for (**A**) a steady-state experiment, (**B**) before-and-after exogenous GA_4 treatments, and for (**C**) a treatment time course experiment using sticky-slides (C).

Figure 2: The GA gradient in *Arabidopsis* roots and dark-grown hypocotyls. Two-dimensional images of (**A**) nlsGPS1 and (**C**) nlsGPS1-NR roots were analyzed using ImageJ software, and three-dimensional images of (**B**) nlsGPS1 and (**D**) nlsGPS1-NR were analyzed using a commercial three-dimensional image analysis software. Both analyses showed an endogenous GA_4 gradient in *Arabidopsis* roots. Two-dimensional images of (**E**) nlsGPS1 and (**G**) nlsGPS1-NR dark-grown hypocotyl were analyzed using ImageJ software, and three-dimensional images of (**F**) nlsGPS1 and (**H**) nlsGPS1-NR were analyzed using the commercial three-dimensional image analysis software. Both analyses showed an endogenous GA_4 gradient in dark-grown hypocotyls. The LUT bar displays the false coloration of nlsGPS1 emission ratios. YFP images are reported as expression controls. Hypocotyl images were acquired using two stage positions.

Figure 3: The exogenous GA gradient in roots. The first two panels show (**A**) two-dimensional and (**B**) three-dimensional images of a nlsGPS1 root before and 20 min after the treatment of exogenous GA_4 (1 µM). YFP images are reported as expression controls. The last two panels show (**C**) the mean and standard deviation and (**D**) beeswarm and box plot of nlsGPS1 emission ratios for nuclei of the elongation zone (the region which is defined with a white frame). In the

elongation zone, the nlsGPS1 emission ratio was significantly higher after GA_4 treatment (Mann-Whitney U test, *** *P*-value < 0.0001).

Video 1: Perfusion experiment of nlsGPS1 root using sticky-slide. This video shows three-dimensional images of nlsGPS1 perfused with ¼ MS liquid and treated with 0.1 µM GA_4 for 30 min. In the time course, imaging was acquired every 10 min for 3 h with the following intervals: 30 min of mock solution (frame *t* = 1, *t* = 2, *t* = 3), 30 min of GA_4 treatment (frame *t* = 4, *t* = 5, *t* = 6), 2 h of mock solution (frame *t* = 7 to *t* = 18) solution. Prior to the acquisition, the sample was perfused with mock solution for 2 h.

DISCUSSION:

The FRET-based GA biosensor nlsGPS1 provides a quantitative method to report and measure GA hormone gradients in multicellular plants. FRET-based biosensors can quantify dynamics with an improved spatiotemporal resolution over direct detection by mass spectrometry and indirect measurement by transcriptional reporters or signaling-protein-degradation-based methods[12,13]. High-resolution cellular imaging in diverse tissue-types can yield meaningful insights into GA biology and spark new hypotheses regarding the regulation and function of GA accumulations in a multicellular context. For example, monitoring changes in the nlsGPS1 biosensor in specific GA biosynthetic, catabolic, and transport mutants, as well as during spatiotemporally induced perturbations, could be very informative to test specifically how GA gradients are established in the root and address root cell responses to GA gradients. The sensor could be used in other model and crop species to test the conservation of the mechanisms that control the GA-mediated control of seed germination, cellular elongation, and flowering.

The critical steps in the FRET-based imaging of the nlsGPS1 biosensor are that, 1) the pixels should not be saturated during the quantitative FRET analysis, 2) imaging parameters such as “detector gain” should be kept constant for the donor emission (DxDm) and acceptor emission (DxAm) acquisitions, 3) control nlsGPS1-NR lines should be used to rule out artefacts, and 4) samples should be prepared to minimize drift and focal-change issues. Additionally, the environmental conditions in which samples are grown are important to control since GA levels are sensitive to environmental conditions such as light duration and light intensity[14-17]. A key limitation of this type of analysis is that a high signal-to-noise ratio is required for imaging due to the increase in noise inherent in ratiometric imaging. Thus, nlsGPS1 imaging will not be useful for tissues and organs that are not amenable to ratiometric fluorescence microscopy using cyan and yellow fluorescent proteins—for example, deeper tissues where fluorescent proteins are poorly detected. On the other hand, ratiometric readouts are often preferred over intensiometric readouts, because an internal control is helpful to rule out artefacts stemming from changes in biosensor expression, stability, brightness, or detectability in a given cell, tissue, or condition. For example, FRET biosensor imaging and image analyses have also been used to study a variety of ligands in a variety of tissues[5,6,18,19,20,]. The imaging experiments and image analyses reported here can be modified to suit new imaging methods, such as light sheet microscopy, that could yield novel insights in, for example, deeper root tissue-types.

The first-generation nlsGPS1 biosensor is a high-affinity sensor that provides a high-resolution map of GA gradients that can also report on intracellular increases in GA following exogenous GA treatments. One of the current limitations of nlsGPS1 is that the sensor is not rapidly reversible and, thus, reports not on steady-state GA levels but, likely, on the maximum recent GA concentration in the solution of interest. The precise turnover rate for the sensor is also not known and this, combined with low reversibility, precludes detection of endogenous GA depletions that might be happening within a minutes to few a hours in some tissue-types. It is also important to note that nlsGPS1 has a high affinity for GA_4 (K_d = 24 nM) compared to other GA forms (GA_3 K_d = 240 nM, GA_1 K_d = 110 nM) when imaging other bioactive GAs[7]. Future generations of GA biosensors can be engineered to increase reversibility while maintaining high affinity or to exhibit different specificities for the various precursor, bioactive, or catabolite GAs.

ACKNOWLEDGMENTS:

This work has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement n° 759282).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Binenbaum, J., Weinstain, R., Shani, E. Gibberellin Localization and Transport in Plants. *Trends in Plant Science*. **23** (5), 410-421 (2018).

2. Sun, T. Gibberellin Metabolism, Perception and Signaling Pathways in Arabidopsis. *The Arabidopsis Book*. **6**, e0103 (2008).

3. Rieu, I. *et al.* Genetic Analysis Reveals That C19-GA 2-Oxidation Is a Major Gibberellin Inactivation Pathway in Arabidopsis. *The Plant Cell Online*. **20** (9), 2420-2436 (2008).

4. Regnault, T. *et al.* The gibberellin precursor GA12 acts as a long-distance growth signal in Arabidopsis. *Nature Plants*. **1**, 15073 (2015).

5. Okumoto, S., Jones, A., Frommer, W. B. Quantitative Imaging with Fluorescent Biosensors. *Annual Review of Plant Biology*. **63** (1), 663-706 (2012).

6. Walia, A., Waadt, R., Jones, A.M. Genetically Encoded Biosensors in Plants: Pathways to Discovery. *Annual Review of Plant Biology*. **69** (1), 497-524 (2018).

7. Rizza, A., Walia, A., Lanquar, V., Frommer, W.B., Jones, A.M. In vivo gibberellin gradients visualized in rapidly elongating tissues. *Nature Plants*. **3** (10), 803-813 (2017).

8. Rizzo, M.A., Springer, G., Segawa, K., Zipfel, W. R., Piston, D. W. Optimization of pairings and detection conditions for measurement of FRET between cyan and yellow fluorescent proteins. *Microscopy and Microanalysis*. **12** (3), 238-254 (2006).

9. Ueguchi-Tanaka, M. *et al.* GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. *Nature*. **437**, 693-698 (2005).

10. Grossmann, G. *et al.* Time-lapse Fluorescence Imaging of Arabidopsis Root Growth with Rapid Manipulation of The Root Environment Using The RootChip. *Journal of Visualized Experiments*. (65), 34290 (2012).

11. Grossmann, G. *et al.* The RootChip: an integrated microfluidic chip for plant science. *Plant Cell*. **23** (12), 4234-4240 (2011).

12. Brunoud, G. *et al.* A novel sensor to map auxin response and distribution at high spatio-temporal resolution. *Nature*. **482**, 103-106 (2012).

13. Wang, N.N., Shin, M.C., Li, N. The GUS reporter-aided analysis of the promoter activities of Arabidopsis ACC synthase genes AtACS4, AtACS5, and AtACS7 induced by hormones and stresses. *Journal of Experimental Botany*. **56** (413), 909-920 (2005).

14. Alabadi, D. Gibberellins Repress Photomorphogenesis in Darkness. *Plant Physiology*. **134** (3), 1050-1057 (2004).

15. De Lucas, M. *et al.* A molecular framework for light and gibberellin control of cell elongation. *Nature*. **451** (7177), 480-484 (2008).

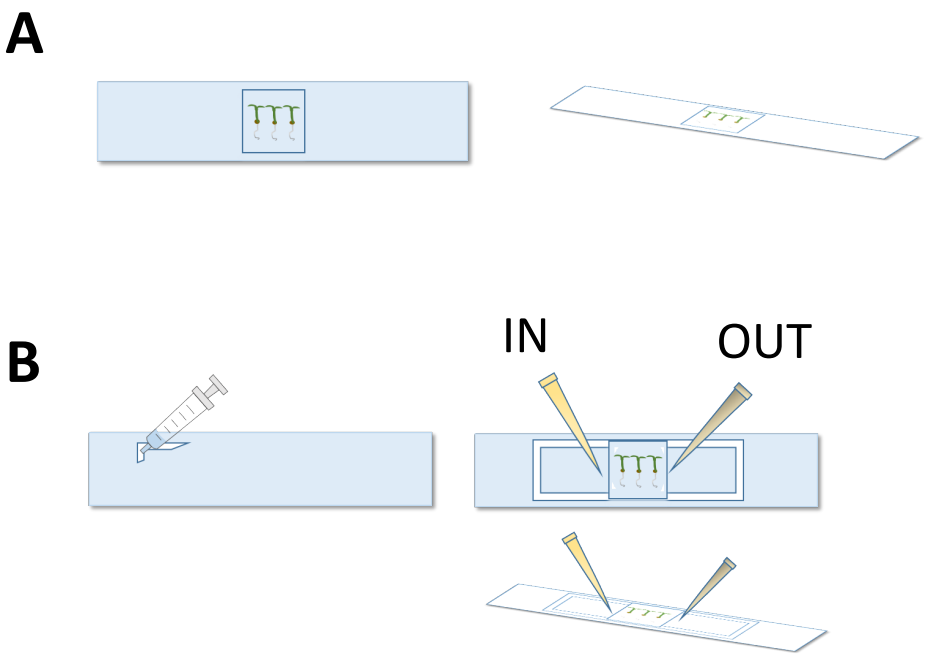
16. Strasser, B., Sanchez-Lamas, M., Yanovsky, M.J., Casal, J.J., Cerdan, P.D. Arabidopsis thaliana life without phytochromes. *Proceedings of the National Academy of Sciences*. **107** (10), 4776-4781 (2010).

17. Feng, S. *et al.* Coordinated regulation of Arabidopsis thaliana development by light and gibberellins. *Nature*. **451**, 475-479 (2008).

18. Choi, W. G., Swanson, S. J., Gilroy, S. High-resolution imaging of Ca2+, redox status, ROS and pH using GFP biosensors. *Plant Journal*. **70** (1), 118-128 (2012).

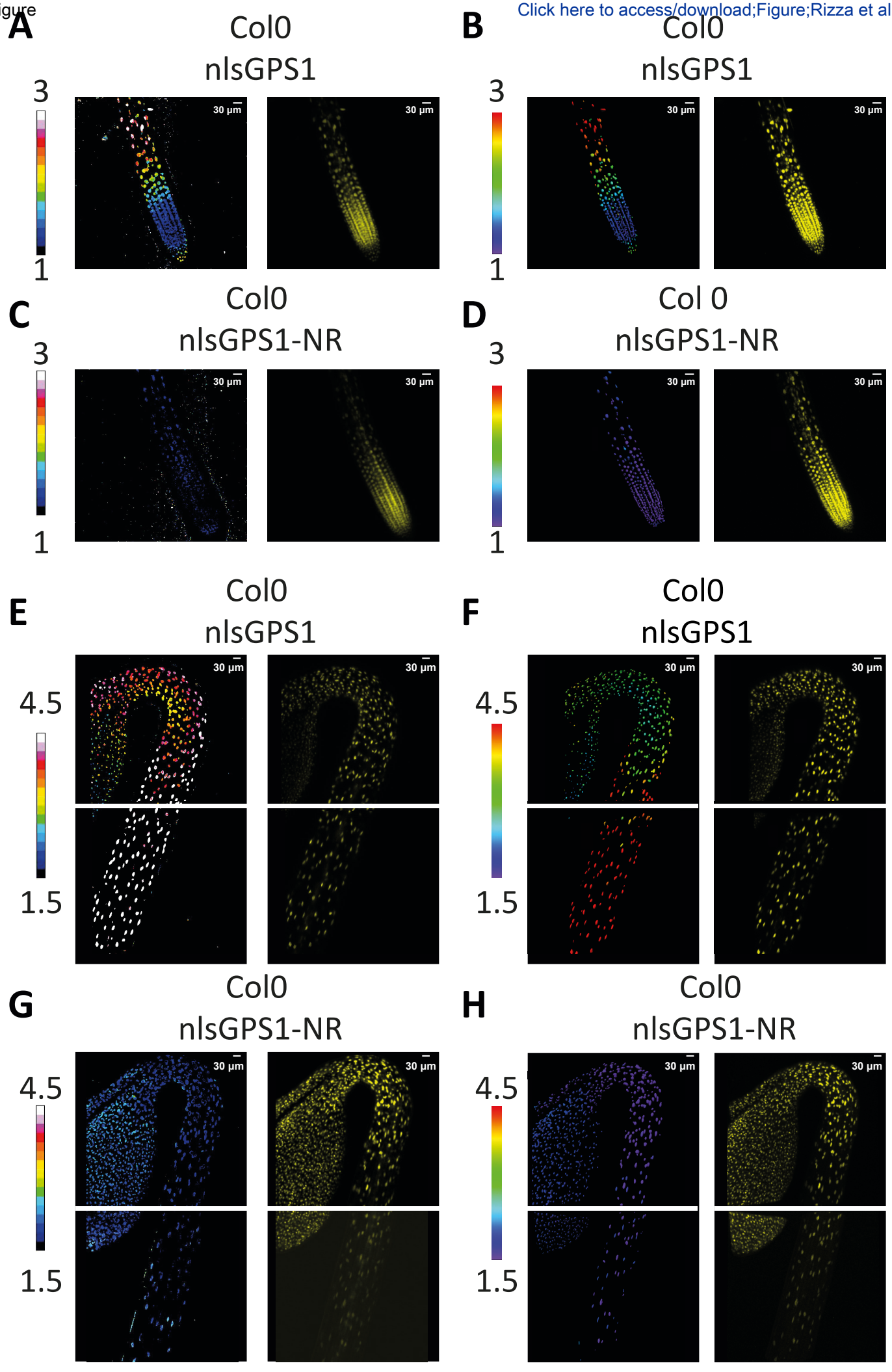
19. Lanquar, V. *et al*. Dynamic imaging of cytosolic zinc in Arabidopsis roots combining FRET sensors and RootChip technology. *New Phytologist*. **202** (1), 198-208 (2014).

20. Krebs, M., Schumacher, K. Live cell imaging of cytoplasmic and nuclear Ca2+ dynamics in Arabidopsis roots. *Cold Spring Harbor Protocols*. **2013** (8), 776-780 (2013).



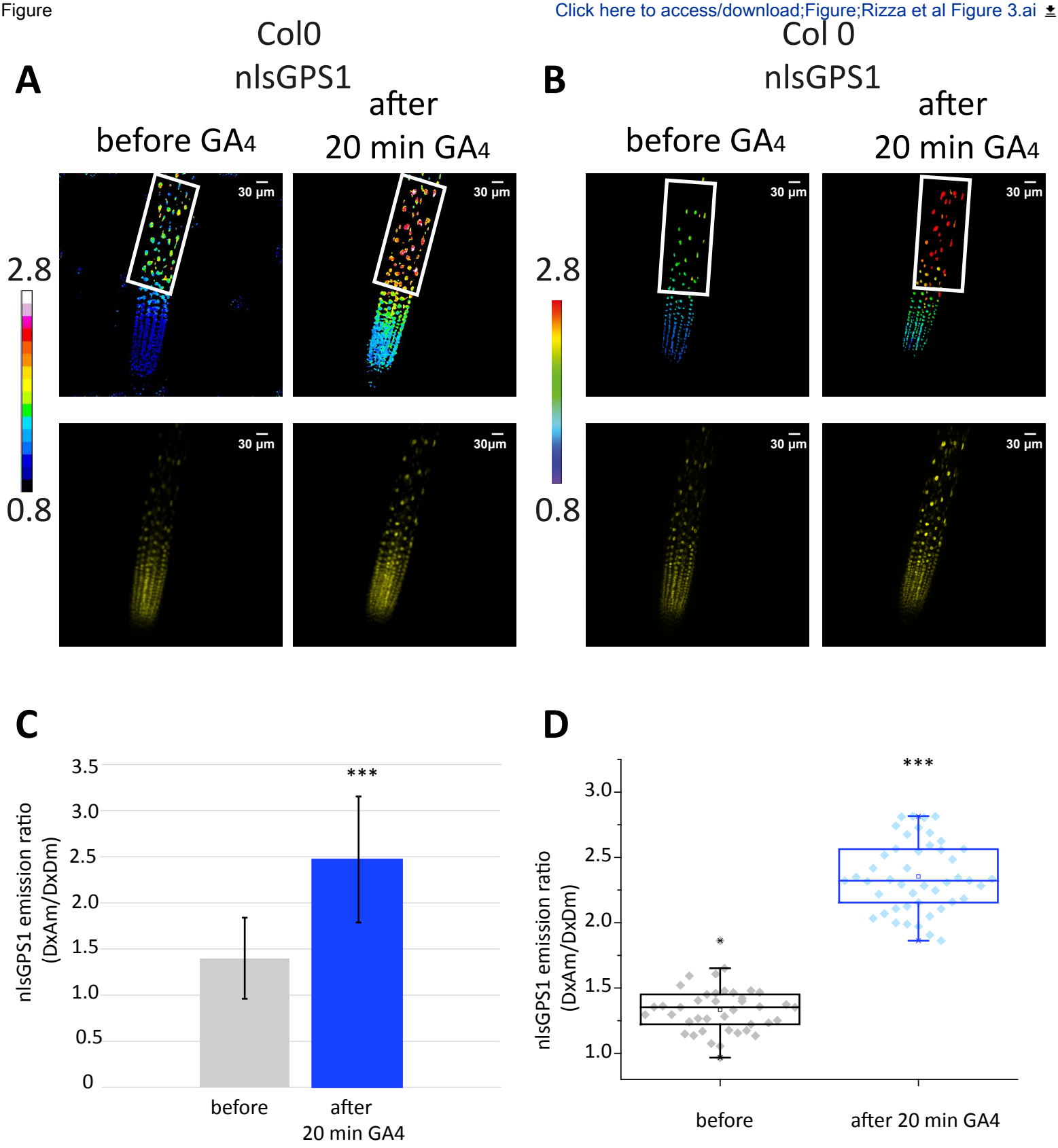
Figure

Click here to access/download;Figure;Rizza et al Figure 2.ai



Figure

Click here to access/download;Figure;Rizza et al Figure 3.ai



Click here to access/download
Video or Animated Figure
Rizza et al Movie 1.mov

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
nlsGPS1 Col0 Arabidopsis seeds	NASC	N2107734	
nlsGPS-NR Col0 Arabidopsis seeds	NASC	N2107735	
Gibberellin A_4 (GA_4)	Sigma	G7276	dissolve in EtH70 % , and keep at -20°
sodium hypochlorite solution (Bleach)	Fisher S/5040	HSRA 064	
Hydrogen cloride HCl	Sigma	31434	
Micropore tape	3M	1530-1	
ibidi sticky-slide	Ibidi	81128	Luer 0.1 for root imaging
ibidi sticky-slide	Ibidi	80168	Luer 0.2 for hypocotyl imaging
glass coverslip for sticky slides	Ibidi	10812	
Elbow Luer Connectors	Ibidi	10802	
silicone tubing	Ibidi	108401	
Luer Lock Connector	Ibidi	10826	
programmable syringe pump	World Precision Instruments	AL-1000	
Vacuum grease	Sigma	18405	
Murashige and Skoog Basal Salts	Duchefa	M0221	
Agar plant, 1kg	Melford	P1001	
Microscope slide ground edges, 76mm x 26mm, 1.0mm to 1.2mm thick	Fisher Scientific	12383118	
Cover slip No.1 1/2 glass 22mm x 22mm	Fisher Scientific	12363138	
Luer-slip Syringe 2o ml	Fisher Scientific	10785126	
3M Micropor Surgical Paper Tape	Fisher Scientific	12787597	
Potassium Hydroxide, 500g	Sigma Aldrich	221473-500G-D	
Absolute Ethanol	Fisher Scientific	10428671	
Forceps Watchmaker 5 StSteel	Scientific Laboratory Supplies	INS4340	
Scissors, 125mm, stainless steel	Fisher Scientific	12338099	
Fitting reducer 0.5 to 1.6	Ibidi	10829	
Leica SP8			Confocal laser microscope 1
Zeiss LSM 780			Confocal laser microscope 2

Imaris	Bitplane	3D visualization and Analysis software
Fiji		image analysis software
OriginPro	Origin Lab	Statistical Analysis Software

C

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

Title of Article: A Method to visualize cellular gibberellin levels using the nlsGPS1 FRET biosensor

Author(s): Annalisa Rizza, Ankit Walia, Bjun Tang, and Alexander Jones

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☒ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution 3.0 Agreement (also known as CC-BY), the terms and conditions of which can be found at: http://creativecommons.org/licenses/by/3.0/us/legalcode ; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video - Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video - Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties**. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

discretion andwithout giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contaminationdue to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, Jo VE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Alexander M. Jones
Department:	Sainsbury Laboratory, Cambridge University
Institution:	University of Cambridge
Title:	Group Leader
Signature:	Alexander M Jones
Date:	3 JULY 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

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