Dear Editor,

We have revised the manuscript and responded to all comments of the reviewers. We were somewhat surprised by the first reviewer who suggests to revise the manuscript so that it exclusively works with Leica microscopes, as a consequence of the technical improvements he/she demanded. However, we have considered his/her points of criticism, but kept the protocol in a general, brand-independent way. We hope we can convince you with the revised manuscript.

Best regards,

Thorsten and Sonja

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. Please define all abbreviations at first use.

Hopefully we defined them all now.

2. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Done.

3. Why is RET the abbreviation for Förster resonance energy transfer in line 37?

We have added an “F” in brackets. It is RET for the basic process, FRET for fluorescence and BRET for bioluminescence.

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

Done.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have revised the manuscript, eliminated all “should” and “could”.

6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol (lines 69-88) to the introduction or Discussion.

The discussion has been moved and follows directly the introduction.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. 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. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Checked and revised.

8. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

Checked. To not exceed three pages, the calculations were omitted from the video.

9. Some of your references are incomplete; please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names.

Has been revised.

10. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend (e.g., Fig 4).

Scale bars has been defined in the legend (Fig. 4).

11. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Please make sure that decimals appear as periods and not commas in the tables and figures and anywhere else in the manuscript.

Done.

12. Please ensure that the Table of Materials contains a comprehensive list of essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

Revised.

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We are grateful to all reviewers for their valuable and helpful comments. Please find our response in red next to the comments.:

Reviewers' comments:

Reviewer #1:

Journal of Visualized Experiments
FRET-measurements in living plant cells
Sonja Schmidtpott
Thorsten Seidel

This paper describes a protocol for intensity-based FRET measurements in living plant cells. I reject this paper as I disagree with many aspects listed in this paper. The authors state to calibrate laser lines and photomultipier tubes for FRET measurements. In my opinion to determine FRET using sensitised emission it is important to adjust the microscope as such that reliable signals are obtained, which requires identical imaging settings. In this paper, the authors use 2 microscopes and these two microscopes have either GasP of PMTs. For quantitative FRET measurements one should use detectors like Hybrid detectors that are able to read signal in photon counting mode. The whole calibration with detectors is truly not necessary as FRET is a ratiometric method. Furthermore, detailed description on how the measurements were done is not provided; image properties, mode of recording, scan speed etc.

We have included a recommendation for 12- or 16-bit scanning, line-by-line scanning and moderate scan speed. “moderate”, because setting the scan speed is different for the various LSM-programs and depends on the manufacturer.

Hybrid detectors are specific for Leica LSMs such as the SP8 or later models. We do not want to suggest a protocol, which is brand-specific. Photon counting is also not available for all confocal laser-scanning microscopes.

Remarks
Line 57: The brightness of a fluorophore is given by its absorption co-efficient and its quantum yield. I would say it is the product of molar extinction coefficient and quantum yield.

We have revised the sentence as suggested.

Line 58 For FRET-measurements, it is of advantage to choose fluorophores of nearly similar brightness. Again, to me it is not necessary to have this, for intensity based FRET it is handy to have an acceptor that has bright like YFP, but you can use mCherry as well as is shown in publications. I wonder why the authors show in their paper the FRET using ECFP and EYFP as a FRET pair. This is definitely not a very good FRET pair as many new variants have appeared, yielding higher R0 and thereby obtaining higher FRET.

It has not been our intention to go for the best FRET-pair. We wanted to suggest a protocol that even works for less optimal pairs, the more since ECFP and EYFP are still frequently used.

Line 62 On the other hand, the lifetime of the acceptor should be lower than the lifetime of the donor, ensuring the availability of the acceptor for energy transfer. Maybe the authors can explain this to me. It is important to have a donor with long lifetime, so it is longer in the excited state and possibly undergo FRET. Acceptors need to have high molar extinction coefficient.

Two points of view: 1) The donor increases the probability of energy transfer by a long lifetime. 2) in a 1:1 ratio of donor and acceptor or an excess of donor the donor would already be ready to emit or transfer energy, when a long-lifetime acceptor is still in the excited state. This prevents energy transfer and decreases the probability.

Line 146: Define the detection range, we recommend 470 - 510 nm for donor detection and 530 - 600 nm for acceptor/FRET detection in case of ECFP/EYFP. I disagree to select donor emission up to 510 nm as a small fraction of YFP is captured at that wavelength as well. I recommend using 440 nm laser and select a window for cyan coloured proteins between 450-495 nm.

Looks again like you are used to a Leica SP8. This is true for systems with an AOBS and the pulsed 440 nm laser typical for Leica, but again it is not our intention to suggest the optimal equipment and a protocol that only works with the system of one manufacturer. We want to suggest a protocol for a standard confocal with dichroic mirrors and argon-ion lasers. However, we have included a recommendation that pays attention to the now sold 445 (440) nm diode laser instead of the argon-ion laser and to the usage of an AOBS. With dichroic mirrors you will not get any detectable acceptor signal at 500 – 510 nm, since spectral properties point to less than 1% emission of the maximum peak, considering both less effective excitation and low emission. Furthermore, the filter gap is much wider with the dichroic mirror than with an AOBS, resulting in an additional suppression of acceptor crosstalk.

Line 152: keep laser intensities and detector gains constant. Use the pinhole diameter for fine-tuning. Adjusting pinhole for getting similar intensities is new for me, lose significant resolution….

We have included “keep in mind that changes of the pinhole diameter affect spatial resolution”. Using the pinhole for fine-tuning, you do not lose much of resolution and still ensure separation of organelles and subcellular structures. Furthermore, we strictly separate high-resolution co-localization experiments from interaction studies by FRET.

Figure 2 shows the emission vs laser percentage, but usually micro-Watt is plotted on X-axis. This shows that the AOTF has linear dependency.

Thank you for this more precise suggestion. The legend has been revised.

Line 224 The spectral bleedthrough is shown. With LSM2 the DSBT is 1.6, meaning the donor only has a higher signal in acceptor channel compared to donor channel. This observation you obtain if you keep all the laser and detector setting similar. As mentioned before, use photon counting for reliable read-out.

Please read the sentence again. This DSBT is valid for measurements in cells, but not for the recombinant fluorescent protein. This discrepancy is already addressed in the manuscript. We moved this comment closer to the statement, so that it becomes clearer.

Reviewer #2:
Manuscript Summary:
This paper describes a protocol on confocal-based sensitised-emission FRET in living plant cells. The protocol provides plenty of detail and explanations which will enable readers / users with a basic microscopy background to apply the described techniques themselves in a successful manner. I endorse this submission for publication.

Major Concerns:
no major concerns

Minor Concerns:
The labelling of the figures, graphs and tables is insufficient. Readers would appreciate if some of the information from the legends was presented in the figures/tables/graphs as well (such as Figure 3A; also state "detector 1" and in Fig 3B "detector 2).

Thank you for this suggestion, we have revised the figures.

Or in Fig 4C, the columns are labelled with LSM5 or LSM780, but these instruments are always referred to as LSM1 or LSM2 in the protocol. It would make sense not to mix the names.

Sorry for that, we missed to correct the names in the figure. It is revised now.

Authors should not use the term "spectra" as singular; please use the term "spectrum" instead (line 301: "The emission spectrum of EYFP...").

Revised.

In table 1, please highlight the intensity values/laser powers that were used in the protocol (see line 212-213).

Done.

In the section 6 "Data evaluation", please describe the parameters more precisely, such as: alpha-values describe acceptor bleedthrough caused by direct excitation of the acceptor at the donor excitation wavelength or beta-values describe emission cross-talk by the donor (that is just an example; I am sure the authors can do better);

We have revised the protocol.

describe IA=fluorescence intensity of the acceptor, ID=fluorescence intensity of the donor, et cetera.

The definition is available in 6.4.

In line 220, I would say that ..."increasing the laser power is cytotoxic and promotes photobleaching". In line 246, I personally think that the standard deviation (1.525+/-1.844) reveals the outliers.

Both have been revised, thank you!

Reviewer #3:
Manuscript Summary:
FRET-measurements in living plant cells is a detailed protocol that provides a worked example of how to measure emission-based FRET between ECFP-EYFP tagged proteins transiently expressed in Arabidopsis mesophyll protoplasts. This method has been successfully utilized by the authors in number of studies whose results are well-published. Therefore, according to the intent, the authors have written a biologist-friendly procedure of high quality. The adjustment of the confocal microscope setup before the routine acquisition, representative results, calculations and data evaluation are clearly described in a step-by step manner. The introduction provides also an overview of the palette of currently used fluorescent proteins (FPs) for FRET measurements including characterization of fluorescence lifetime for each FP. An advantage of this protocol is that can be adapted to the available standard confocal laser-scanning microscope and for other fluorophore pair then ECFP-EYFP. From my point of view, this protocol will be of great interest for plant biologists working in the area of protein-protein interaction in living plant cells.

Minor comment:
Please, unify Figures referencing style (Figure or Fig.) in the text main body.

Done.

Thank you for your support!