The Editorial Office of JoVE Ulm, 09/30/16

Dear Sir/Madame,

please find enclosed an extensively revised version of our manuscript JoVE55368 "Detection and quantitative analysis of protein interactions in large multi-protein complexes by acceptor-photobleach-FRET" by Sroka et al. to be considered for publication in JoVE. We provide a concise protocol for the quantitative analysis of protein interactions in multi-protein complexes by acceptor-photobleach Fluorescence resonance energy transfer (AB-FRET). We list advantages of the method, describe limitations, caveats and provide experimental approaches to control AB-FRET experiments. Utilizing published direct protein interactions in the E-cadherin adhesion complex, we subsequently verify our experimental protocol.

We are grateful for the Reviewer suggestions. In our extensively revised manuscript we have addressed all the Editor's and Reviewer's comments. The changes made to our manuscript considerably improve our work. Please find below a point-by-point discussion of comments and suggestions.

Editorial comments:

The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55368_R0_081816.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.

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.

- *We have extensively proofread our manuscript, corrected typos and errors.*

2. In the Figures, please use the decimals with all numbers correctly instead of commas: 2C ,2D, 3B, 3D

- *We have replaced commas with periods for all decimals as requested.*

3. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results.

- *Figure legends were adapted, accordingly.*

4. The manuscript should receive copyediting for grammar and formatting issues. Some examples are provided here (this is not a comprehensive list):

-Short abstract: "At the example of the E-cadherin adhesion complex we demonstrate the application of AB-FRET..." This sentence must be corrected.

- *Sentence has been corrected.*

-Line 103, odd comma at the end of the line.

- *Comma has been removed.*

-Line 230, there should be a comma in 150,000

- *Comma has been placed.*

-Line 274, "at the example of"?

- *Sentence has been corrected.*

-Line 295, "Set detection intensities by laser power"

- *Sentence has been corrected.*

-Line 339, should be "right clicking" rather than "right click."

- *Wording has been changed accordingly.*

-Line 533, there is a missing space typo.

- *Has been corrected.*

-Line 534 should probably be "methodological" rather than "methodical."

- *Has been corrected accordingly.*

-Line 570, a sentence should not start with "E.g." "For example" is fine.

- *Has been change to “For example”.*

-Lines 599-601 - This final paragraph reads more like an abstract or intro paragraph than a conclusion.

- *The final paragraph has been re-written.*

5. Additional detail is needed:

-Steps in Section 3 should be re-written to provide sequential and step-wise descriptions of the manipulation of the software. For instance, in step 3.5, where should the experimenter be making these adjustments? In what panel(s) of the software? By entering values, adjusting a slider, clicking a button? Steps 3.6 require similar clarification.

- *Steps 3.5 and 3.6 have been re-written and a step-by-step description with details has been added. Due to additional reformatting these steps are now 4.3.5 to 4.3.7.*

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This paper describes the process of measuring the relative proximity of proteins in an intracellular complex under different physiological conditions using acceptor photobleach FRET. This is an extremely valuable assay that cannot be conducted using any other optical technique and which can only be tested biochemically with some difficulty, and without any spatial resolution. The specific proteins in question are members of the E-cadherin cell adhesion complex (and relevant controls) and the explanation is generalizable to most protein complexes of interest. The step by step guide details how this can be done using the Leica SP8 confocal microscope using a mix of proprietary software (Leica) and open-source analysis tools (ImageJ/Fiji).

This guide is well tailored for intermediate microscope users who are interested in using FRET for the first time. The specific benefits of FRET are clearly explained and it gives appropriate attention to choosing which FRET technique is most appropriate in which circumstance. It is especially nice to see a warning against measuring absolute distance using FRET.

Aside from mostly minor concerns listed below, this manuscript will be an excellent resource for a broad range of researchers whose work will benefit from rigorous FRET analysis.

- *First, we would like to thank the Reviewer for finding our guide well tailored and an excellent resource for a broad range of researchers.*

Major Concerns:

My primary concern has to do with the choice of using a Leica SP8 confocal microscope. This is a high quality instrument and a great choice for sharp and sensitive images. However it potentially limits the protocol's usefulness.

* A widefield scope is certainly sufficient for quantitative FRET and vastly more readers will have one routinely available. Proprietary software strikes me as a particularly unnecessary barrier here since all appropriate corrections from the raw data are easily done in ImageJ/Fiji or similar. Treating the SP8 as one of many ways to acquire raw data followed by a more generalizable processing pipeline may be helpful.

The above point is most important and IMO relatively easy to address. I would suggest mentioning when a widefield would be preferable or at least sufficient.

- *We agree with the Reviewer that this is an important issue. As suggested, we have now mentioned that it is possible to perform AB-FRET experiments with widefield fluorescence microscopes. However, we have also pointed out that confocal line scanner systems allow for a superior control of the bleaching process, due to a bleach-ROI-based setup and controllable laser power (lines 99-108). The bleaching of defined areas using a widefield microscope would require at least fixed aperture systems to direct the excitation light. Also, bleaching times may increase and more stable dyes, such as Alexa-Fluor-FRET pairs, that can be readily bleached by lasers may be problematic in a widefied system with lamp-based bleaching. Thus, we still prefer using confocal laser scanning systems to perform AB-FRET experiments, but experiments in principle are possible with widefield microscopes.*

Generally regarding confocals and FRET:

* The high precision of Z-axis sampling could possibly work against you here, by limiting the measurements to an arbitrarily thin slice that potentially leaves out regions of interest in even slightly different focal planes. The solutions to this potential concern include opening the pinhole, using a lower NA objective or taking Z sections. The point in lines 530-531 about thermal drift underscores this potential concern.

- *We agree that high precision Z-axis sampling could be an issue, if structures of interest are not fully present within a single confocal slice. In our representative experiments we study protein interactions in the E-cadherin adhesion complex. These membrane proximal structures fully fit within a single confocal section. If structures are bigger, we now have added the suggestion under 'general considerations' step 1.4 that the confocal pinhole may be opened as an easy solution and the microscope may effectively be operated as a widefield system.*

* CCD detectors tended to have a somewhat more linear response curve than earlier generations of confocal PMT, making the confocal less ideal for wide dynamic range intensity measurements. This is usually offset by the confocal's higher spatial precision, which is less important when measuring FRET. It is possible that Leica HyD detectors are better than traditional PMT detectors for linearity of detection.

- *We agree with the Reviewer that CCD detectors may have a somewhat more linear response than early generation PMTs, but the newer systems with PMTs, such as the LSM 510[1], 710[2,3] or even more recent systems from Zeiss as well as the SP5[4] to SP8[3] series from Leica, that are nowadays broadly available in Imaging Core Facilities, work perfectly well for the detection of AB-FRET with the protocol presented in this manuscript[1-5]. However, HyD hybrid detectors are even better suited to detect signals with a good linear response and a high dynamic range. HyDs also posess a superior signal-to-noise ratio due to a high voltage electron acceleration in the first step that results in a 500-times higher signal amplification compared to PMTs, where the variance during low level first-step amplification significantly contributes to signal noise. Thus, HyD detectors have drastically minimized dark current values compared to PMTs[6].*

* I did not understand the explanation for detecting in 8-bit mode.

- *We have opted to detect images in 8-bit mode to set a baseline threshold for detection of specific FRET signals. In 8-bit images intensities are coded with 256 gray-levels, which in our hands produced relevant and reproducible FRET values[2-4,7]. When 16-bit images are acquired, changes are encoded in 65,536 gray-levels that display even the smallest intensity fluctuations. We have also rephrased the explanation in step 4.4.3 to make it more easily accessible.*

Minor Concerns:

I suppose that _Fluorescent_ Resonant Energy Transfer has become standard through ubiquitous use, but the 'F' in FRET originally stands for Förster. This is somewhat important in line 83 where naming the R0 variable 'Förster radius' will help readers to consult outside reference materials. Speaking of R0, perhaps mention in line 80 that the needed d/a distance can vary significantly depending on the pair.

- *We have added the term 'Foerster energy transfer' to the 'Introduction' section and have renamed the R0 distance 'Foerster radius' as requested (lines 72/83). We have also mentioned dependence of the FRET distance on the R0 value for the utilized FRET pair (lines 79-80).*

Use of an overexpressed acceptor would make it possible to perform an acceptor titration curve. This is a highly valuable control that is not specifically necessary for the experiments described here but should probably be on readers' radar.

- *As suggested, we have proposed acceptor titration as an additional control for FRET systems with overexpressed Acceptor (1.5.3).*

In lines 111-112, stochasticity from a single fluor is integrated over several cycles of excitation and emission and thus not _quite_ as binary as suggested. This only becomes a factor in fairly arcane single molecule techniques.

- W*e have mentioned integration of binary FRET signals in lines 124-126.*

Typos: lines 92, 164 (comma), 293/294/315 (AOTF/AOBS), 430 (were), 431 (compared)

- *Typos have been corrected*.

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

The technique of acceptor bleaching as a means to determine protein interaction is a powerful method although not new, it has been used e.g. by the Jovin and Arndt - Jovin years ago.

Nevertheless a detailed description how to perform this method reliably may be valuable regarding the broad distribution of confocal microscopes in combination with a large variety of proper fluorochromes. These allow for easy control adjustments of excitation and emission spectra used for imaging.

The introduction describes the possibilities and limitations of acceptor bleaching FRET very well. In particular the « caveats « are scholarly written. I would like to recommend to include permeabilized cells (see e.g. V. Saks et al.) in addition to fixed cells as examples for immobilized specimen which are very close to the living state. The method is exemplified using E-cadherin / ß-catenin binding, which is a well known pair of interacting proteins, which in addition, have further binding partners. α-catenin interaction serves as a negative control. The demonstration of the method using this model system is well done and convincing

- *We would like to thank the Reviewer for describing our protocol as valuable contribution. As suggested we have added the interesting model of permeabilized cells to our introduction (lines 112-114).*

Major Concerns:

N/A

Minor Concerns:

The „results section" is a bit confusing. The authors should consider to integrate the content of line 415-459 in the figure legends which outline the real results.

- *We have removed content describing experiments from the results section. Descriptions are now only present in the Figure legends.*

If any separate description would be desirable then a general list of the steps to be done in advance of the example. General would mean - not related to a specific instrument (here TCS8 SP), an only about 1 line each step.1.3: the general list of good donor-acceptor pairs should be separated from the example which is going to be presented (195-197).

- *As requested we have separated 'general considerations before performing the experiment' (section 1 to 1.8) from the 'representative experiments' (section 2 to 2.6.1).*

Paragraph 2 - immunofluorescence staining - describes a well known method which is not specific for this method and should be eliminated.

- *We agree that the protocol for immunofluorescence staining is not specific for the FRET method. However, since JoVE publishes methods papers with representative results, we are required to provide a detailed step-by-step description of experiments. We do however suggest that in the video part only microscopy and quantitative evaluation of results should be shown (yellow markup).*

Fig. 2, 3C : marking the bleached areas with small white rings covers the region of interest and does not allow an estimation of the intensity distribution. The black rectangle alone might be sufficient to show the ROI, probably an arrow could be used to draw the observer´s attention to the right position. The increase in donor fluorescence after acceptor

- *We have removed the sub-ROIs from Donor pre- and post-bleach images to allow for an estimation of the intensity distribution. The placement of sub-ROIs is now only shown in Acceptor pre-and post-bleach images of Fig.2, 3C.*

Line 490 : Fig. 2 at wrong place

- *Has been corrected, accordingly.*

Line 493 : ns would be important to explain for Fig. 3 and not for Fig. 2. - Because of wrong arrangement of the figures the descriptions are confusing.

- *Ns, explanation is now only present in the relevant Figure legend.*

Additional Comments to Authors:

N/A

Reviewer #3:

Manuscript Summary:

Detection and quantitative analysis of protein interactions in large multi-protein complexes by acceptor-photobleach-FRET
Sroka, Seufferlein, and Eiseler

The authors present a step-by-step procedure for measuring protein-protein interactions in fixed cells by acceptor photobleaching FRET. The protocol is detailed and the authors have included the relevant controls to quantify the FRET efficiency. There are several areas in the protocol and results that could use clarification to aid the manuscript for application by the larger audience.

Major Concerns:

-The background indicates that authors are only aware of a portion of the pre-existing work in this area. They seem to be citing many less well-read manuscripts while neglecting major on photobleaching FRET from labs such as the Jovins, Hoppe and Zal groups.

- *First, we would like to thank the Reviewer for his comments. We apologize for not citing all the important work done by other groups regarding the development of new FRET methods. The recent work of the Jovin group, Hoppe and Zal has focused more of the development of FRET live imaging applications or 3-way FRET systems. Nevertheless, we have now added additional Review articles by the pioneering Jovin group[8,9] and the work of Hoppe and Zal was cited on FRET imaging in living cells[10-12].*

Citation 5 is heavily referenced, often in cases were it is being used to refer to more of an opinion than fact. For example, 160-162 states that AB-FRET cannot be used to determine molecular distances. This is purely an opinion that originates from that work's lack of ability to fully quantify the relevant parameters such as bound versus free probe.

- *We would like to clarify that we do not recommend the determination of absolute distances in multi-Donor and Acceptor systems when studying protein-protein interactions in cells. While we do agree that it is possible to determine absolute distances with AB-FRET[13], we also suggest in line with Snapp et al. 2006[14] it is much more sensible to determine changes in relative distances by FRET when the same set of proteins at different end points are investigated. This is in particular the case when one or both interaction partners are detected with antibodies, which further complicate the relationship between a FRET signal and the absolute distance of the FRET pair. We have therefore adapted our phrasing to clarify the statement in the manuscript (lines 134-140)*

-The description of the controls for the experiments could be stronger. Many of the FRET signals detected in the data provided are very weak. What criteria are used to distinguish instrument or experimental error from giving a false positive FRET signal? What about using isotype control antibodies?

- *To control our experiments we have suggested to investigate FRET negative controls with a protein that resides at the same subcellular compartment or is present in the same protein complex, but does not interact with one of the partners. Using alpha-catenin (negative control), which is a member of the E-cadherin adhesion complex and binds to beta-catenin, but not E-cadherin directly, we have verified the specific detection of FRET signals for the binding of beta-catenin to E-cadherin under different experimental conditions. We have also tested 'Donor only' experiments, where samples have been incubated with the respective non-specific secondary antibody for the Acceptor. Both, control experiments indicated that FRET detected during our experiments was specific. For the acquisition of AB-FRET experiments we have used a highly sensitive HyD hybrid detector. As stated above, these detectors have very low dark currents and display excellent signal-to-noise ratios. Nevertheless, we have now added an additional experimental control to Figure 3E that determines the 'aggregated instrumental error' (dark current and additional instrumental factors). To this end, we have acquired 'instrumental error' images with settings similar to FRET studies, but without laser excitation. Subsequently, intensity values were similarly extracted by sub-ROI analysis. The instrumental error in 8-bit images amounted to an intensity of 0.01002 ± 0,001953 (mean and SEM, n=15 experiments and 6 sub-ROIs), which is negligible compared to the more then 100-times higher intensities of Donor signals displayed in Figure 3C, D.*

Minor Concerns:

-Some of the concepts seem to run together in the text. For example in lines 135-139 the discussion meanders from choosing donor fluorophore to stoichiometry without making a clear point about what the actual concern is.

- *With our remarks in this section we wanted to clarify that choice of Donors and Acceptors does impact AB-FRET efficiency. Unbound donors critically lower % FRET values, whereas unbound acceptors do not impact overall FRET efficiency due to Acceptor bleaching in AB-FRET experiments. Thus experiments should be planned in a way that the Donor labeling is assigned to the protein with the lower stoichiometry to reduce the amount of unbound Donors in experiments.*

-The control experiments steps 1.4 are repeated as additional step in the representative experiments, Steps 1.5.3 and 1.5.4.

- *In line with suggestions made by Reviewer 2, we have now separated 'general considerations before performing the experiment' (section 1 to 1.8) from the 'representative experiments' (section 2 to 2.6.1) to avoid unnecessary repetition.*

-Step 3.3.2. The dynamic range of the image does not detect only robust intensity changes. This would be a function of gain and scan speed. The limited bit depth will lower the dynamic range of the measurement, and reduce the linear range of the measurement.

- *We have rephrased our statement to make it more accessible (see step 4.3.3).*

-Step 3.9. It is unclear how long the bleach cycle is. This is an important parameter for bleaching and should be noted, especially if it can be changed.

- *Using the AB-FRET macro at the TCS SP8 system, bleaching is performed with scan speed settings that are similar to the acquisition of pre- and post-bleach images (scan speed: 200Hz; pixel dwell time: 1,2µs). These data has been added to the respective step (4.3.10).*

-Step 3.9.1. This is written as note to the user and not as a step, but it is a very important step in the setup for a well-controlled experiment. It would be helpful to indicate that this step is where the donor only controls are used to determine. Additionally, the use of an acceptor only sample will aid in this process to determine the optimal bleaching parameters. This may be more appropriate in step 1.4.

- *As requested, we have now added a step to the 'general considerations before performing experiments' section stating that 'Acceptor only' samples will aid in the optimal setup of bleaching parameters (step 1.5.4). The use of 'Acceptor and Donor only' samples during setup of bleaching conditions is further indicated in the experimental protocol step 4.3.10.*

-Step 4.2. It is unclear if the random selection of sub-roi's is done on the pre or post bleached image. Additionally, this seems to be a place where the user may -introduce bias in selection of the ROI. How do the results compare with calculating the average intensity of the bleach region divided by area for an roi drawn tightly outlining the membrane in the bleached roi?

- *We have now described in detail how placement of sub-ROIs was done in step 5.5. We agree, that placement of smaller Sub-ROIs at this step may introduce bias, if selection is*

not performed randomly. However, we have found the results to be reliable and reproducible in previous studies [2,3,7]. Also, results after normalized sub-ROI analysis readily follow relative distribution of FRET signals extracted from the entire bleach-ROI. We have also analyzed the intensity distribution of donor and acceptor pre-bleach signals to demonstrate that cells and sub-ROIs were not placed purposely on bright or less bright areas to alter the FRET signals. We also found in our previous studies that circular ROIs can be more effectively placed on discontinuous subcellular structures, such as Golgi stacks[2]. Of course, in the context of the FRET studies at adherens junctions bigger ROIs may be used, when FRET intensities are normalized by the area of the ROI.

-Step 4.8. Setting the values that are less than zero will skew the variance in the measurement, and will have an impact on the statistical differences, especially when the FRET efficiency is very small. This will include the impact of incidental photobleaching as well.

- *When calculating AB-FRET efficiency by the formula given in step 5.9, there are by definition no 'negative' FRET values. Thus, either there is a positive % FRET value or 'no FRET' has been detected. We therefore have to set values to '0', when they show no increase in post-bleach donor intensities or exclude these cells from evaluation, which also would skew results.*

-Line 439. Figure 2D shows FRET efficiency, this does not show reduced E-cadherin levels. The comparison made in Figure 3 A and B, should be a post-bleach intensity of the donor, as the presence of FRET will reduce the donor intensity.

- *We have compared donor and acceptor pre-bleach intensities during AB-FRET analysis to verify a random sampling of cells and bleach-ROIs, especially when Donors are overexpressed (Figure 3A). For native proteins, the evaluation of pre-bleach intensities may also provide information on localized protein levels of Donors and Acceptors. Indeed, evaluation of Donor post-bleach signals would counter reduced intensities as a consequence of FRET, but these changes are comparatively small in respect to the overall intensity signal. Also, Acceptors can not be evaluated at the post-bleach state. Since, in literature, evaluation of protein expression at subcellular compartments is generally done without correcting for putative energy transfer by FRET, we have opted to compare pre-bleach images.*

-Line 557. What is meant by "change in protein proximity that result in exceeding FRET distances."

- *The statement has been rephrased to make it more accessible (line 654-655).*

-Typographical errors are prevalent

-FRET the acronym is defined twice in the abstract.

-Line 294, acronym should be AOBS.

-Line 309, should read saturated pixels

-Line 388. The xml file is referenced in step 3.11.1

-Line 563, should read these kinds of experiments

- *Typos have been corrected.*

Additional Comments to Authors:

N/A

In conclusion, we would like to thank again the Reviewers and the Editor for their valuable comments. We believe that following this extensive revision we now convincingly present a concise protocol verified by our representative results. We think that the present manuscript will be of interest to the broad readership of JoVE and beyond. The data contained in this manuscript are not under simultaneous consideration by any other journal. All authors have seen and agreed with the content of the manuscript and declare no competing interests.

Thank you very much for considering the revised version of our manuscript. We are looking forward to hearing from you.

Sincerely yours,

Tim Eiseler

(corresponding author)

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