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Detection and quantitative analysis of protein interactions in large multi-protein complexes by Acceptor-photobleach-FRET --Manuscript Draft--

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Abstract:	<p>In the age of quantitative cell biology, there is strong interest to determine and quantify localized protein-protein interactions in living cells or fixed samples. Fluorescence resonance energy transfer (FRET) is an extremely versatile tool to probe for molecular interaction at sub-nanometer resolution. FRET refers to the non radiative transfer of energy from an excited fluorescent Donor molecule to an Acceptor. Multiple parameters influence the probability of FRET, but for suitable fluorophores the major determinant is the distance separating Donor and Acceptor dyes. One of the techniques used for measuring proximity of interaction partners is Acceptor photobleach-FRET (AB-FRET). AB-FRET has several advantages that render it especially suitable for studying protein interactions in fixed cells. Its detection does not require special instrumentation. Experiments can be performed with commercially available off-the-shelf confocal laser scanning microscopes that are nowadays available at most institutions. AB-FRET is quantitated by a simple arithmetic equation, the technique is unaffected by fluorescence bleed-through, and can be used to gain insights into associated and unassociated protein populations. In experiments, FRET between a Donor and Acceptor is measured upon photobleaching of the Acceptor dye. If the Donor fluorescence is increased after destruction of the Acceptor, Donor and Acceptor molecules were sufficiently close (≤ 10 nm) to allow for energy transfer to occur. Thus, AB-FRET is a very robust method to detect changes in the molecular organization of multi-protein complexes. We here describe a concise protocol to perform quantitative AB-FRET experiments in fixed cells to evaluate protein interactions in multi-protein complexes. Using the example of the E cadherin adhesion complex, we demonstrate the successful application of antibody based AB-FRET to investigate proximity of interaction partners and dedicated approaches to control specificity of experiments.</p>
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TITLE:

The detection and quantitative analysis of protein interactions in large multi-protein complexes by acceptor photobleach FRET

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SHORT ABSTRACT:

Here, we describe a hands-on protocol to perform quantitative acceptor photobleach fluorescence resonance energy transfer (AB-FRET) experiments in fixed cells to evaluate protein-protein interactions in multi-protein complexes. We apply AB-FRET on the E-cadherin adhesion complex to investigate the relative proximity of proteins and to determine approaches to control specificity.

LONG ABSTRACT:

In the age of quantitative cell biology, there is strong interest in determining and quantifying localized protein-protein interactions in living cells or in fixed samples. Fluorescence resonance energy transfer (FRET) is an extremely versatile tool to probe for molecular interactions at a sub-nanometer resolution. FRET refers to the non-radiative transfer of energy from an excited fluorescent donor molecule to an acceptor. Multiple parameters influence the probability of FRET, but for suitable fluorophores, the major determinant is the distance separating the donor and acceptor dyes. One of the techniques used for measuring the proximity of interaction partners is

acceptor photobleach FRET (AB-FRET). AB-FRET has several advantages that render it especially suitable for studying protein interactions in fixed cells. It does not require special instrumentation, and experiments can be performed with commercially available, off-the-shelf confocal laser scanning microscopes that are available at most institutions. AB-FRET is quantitated by a simple arithmetic equation, is unaffected by fluorescence bleed-through, and can be used to gain insight into associated and unassociated protein populations. In experiments, the FRET between a donor and an acceptor is measured upon the photobleaching of the acceptor dye. If the donor fluorescence is increased after the destruction of the acceptor, the donor and acceptor molecules are sufficiently close (≤ 10 nm) to allow for energy transfer to occur. Thus, AB-FRET is a very robust method to detect changes in the molecular organization of multi-protein complexes. Here, we describe a concise protocol to perform quantitative AB-FRET experiments in fixed cells to evaluate protein interactions in multi-protein complexes. Using the example of the E-cadherin adhesion complex, we demonstrate the successful application of antibody-based AB-FRET to investigate the proximity of interaction partners and the dedicated approaches to control the specificity of experiments.

INTRODUCTION:

Studying the molecular organization of protein complexes in subcellular compartments is essential to understanding how cells control vital functions, such as proliferation, protein transport, and cell-cell interactions. Standard microscopy techniques can be used to monitor the co-localization of proteins at subcellular compartments. However, optical resolution is limited by the Abbe Law, and even sub-Abbe optical imaging techniques (e.g., stimulated emission depletion, STED) are currently unable to determine whether proteins are sufficiently close for molecular interaction¹.

Fluorescence resonance energy transfer (Foerster energy transfer, FRET) is a very effective tool for probing proximity and thus molecular interactions in a respective range (≤ 10 nm)²⁻⁸. FRET refers to a process whereby energy is transferred in a non-radiative fashion from an excited fluorescent donor molecule to an acceptor². FRET can occur when a number of conditions are met: (1) The emission spectrum of the donor must display sufficient overlap with the absorption spectrum of the acceptor. (2) The dipole moments of the donor and acceptor have to be properly aligned. (3) Donor and acceptor molecules have to be within a distance of 10 nm or less, which strongly depends on the R_0 -distance of the FRET pair that is used^{2,3,5,9}. For a single donor/acceptor fluorophore pair, the probability of FRET can be calculated by the following equation: $E = 1/[1 + (r/R_0)^6]$, where E is the FRET efficiency, r is the distance separating the fluorophores, and R_0 (Foerster radius) is the distance at which the energy transfer efficiency for the donor-acceptor pair is 50%². Since the FRET efficiency is inversely dependent on the sixth power of the distance between the donor and acceptor, it is highly sensitive to even the smallest sub-nm changes in relative proximities^{6,10-12}.

There are several methods available to measure FRET^{3,6} in cells, which include fluorescence lifetime imaging microscopy (FLIM), sensitized emission^{13,14}, fluorescence anisotropy^{15,16}, and acceptor photobleaching^{5,17-27}. The different methods have certain advantages but also suffer from drawbacks. For example, in the case of FLIM or anisotropy microscopy, special instrumentation and custom-built systems are

required^{5,12}. This manuscript will only focus on a hands-on protocol to quantitate FRET by acceptor photobleaching (AB-FRET). AB-FRET can be quantitated by a simple arithmetic equation⁵. In contrast to sensitized-emission FRET, AB-FRET is unaffected by signal bleed-through and does not require the extensive determination of multiple correction factors for quantitation^{5,12}. AB-FRET measures the energy transfer between a donor (short wavelength) and an acceptor dye (long wavelength) upon photobleaching of the acceptor. If the donor fluorescence is increased after the destruction of the acceptor, the donor and acceptor molecules are sufficiently close (≤ 10 nm) for FRET to occur^{4,5,20,23,28}. AB-FRET can be performed with widefield fluorescence microscopes or confocal laser scanning systems. Widefield microscopes with CCD cameras may have the advantage of more linear response curves during acquisition. However, the bleaching of defined acceptor areas with widefield systems requires customized, fixed apertures with a strong fluorescence excitation lamp or external lasers. For lamp-based systems, longer bleaching times may be necessary, whereas external laser bleaching demands further customization. Overall, confocal line scanner systems allow for superior control of the bleaching process. Regions of interests (ROIs) can be defined, and laser power during bleach cycles is tunable, decreasing unspecific donor bleaching artifacts.

One inherent disadvantage of the AB-FRET method is the destruction of the acceptor dye by photobleaching, which only allows for one measurement in an ROI. Since acceptor fluorescence intensities should be bleached to background levels, AB-FRET works best in fixed samples^{4,5,12,20,23,24}. Alternatively, permeabilized cells with proteins labelled by antibodies may be used as a system that is very close to the “living state” to study the interaction of immobile protein fractions by AB-FRET^{29,30}. AB-FRET may also be used in living samples when the proteins of interest are relatively immobile³¹. However, in live imaging applications, protein dynamics have to be precisely analyzed to exclude “false” FRET signals due to protein migration in and out of the detection ROIs. Thus, AB-FRET is not particularly suitable for most live-cell imaging applications.

When planning AB-FRET experiments, it is vital to consider a number of issues and to adapt the experimental design accordingly. It is important to understand that FRET is a stochastic process. For a given donor-acceptor pair, energy transfer either happens or it does not⁵. However, low FRET efficiency values are often detected in cells, and these values always represent the average probability of energy transfer between a large number of donor and acceptor molecules within a specific region of interest^{5,20,23,24}. Thus, low FRET efficiency values may be the result of a small population of interacting proteins or of highly dynamic processes^{20,23,24}. FRET efficiency can also greatly vary between different cells. Therefore, the sensible determination of the appropriate sample size is very important for quantitative AB-FRET analysis. A sample size of at least 15 cells (experiments) was mostly appropriate for our purposes^{20,23,24}. When planning FRET experiments, it is important to note that AB-FRET efficiency is not a simple measurement of protein binding affinities, which may be determined by absolute distances between FRET pairs; rather, it represents an aggregate measure of the number of complexes that undergo energy transfer, as well as their respective distances²³. Thus, AB-FRET experiments in cell-based systems with multiple donors

and acceptors are not particularly suitable and should not be used to determine absolute distances between proteins (spectroscopic ruler)³², in particular when the proteins are detected by antibodies⁵. This is not to say that it is impossible to determine absolute distances between FRET pairs using AB-FRET³³, but when studying protein interactions in multi-protein complexes, it is more useful to determine relative molecular distances. The quantification of relative changes in proximity for the same proteins under different experimental conditions is possible and works very well.^{5,23,24}

Discrete endpoints that can be measured during experiments must also be identified. For example, determining FRET and thus the relative proximity of proteins between treated and untreated samples can be done in fixed cells, and quantified readings can be readily compared when the same targets are investigated^{20,23,24}. In addition, appropriate controls should be tested. Proteins that co-localize at the same subcellular compartment or that even reside within the same multi-protein complex but that do not directly interact with one of the investigated partners may be good negative controls for FRET experiments^{20,23}.

AB-FRET experiments are particularly sensitive to the choice of donors, and this decision can profoundly affect FRET values. Unbound donors not within energy transfer distance to their respective FRET acceptors negatively influence the overall detectable FRET signal, whereas unbound acceptors have little effect on FRET efficiency due to acceptor bleaching. Thus, in an experiment, donors should be the proteins with the lower stoichiometry in order to minimize the percentage of unpaired partners⁵. However, due to these inherent assay properties, differences in FRET values upon the exchange of donors and acceptors may also provide information about the stoichiometry of interaction partners⁵.

AB-FRET experiments can be performed with ectopically expressed, fluorescence-tagged proteins or with one or both partners labeled by antibodies^{4,5,17-20,23,24,28,34}. Thus, AB-FRET is also suitable for investigating native proteins^{5,20,23}. When performing experiments with overexpressed FRET partners, expression levels and possible localization artifacts should be taken into consideration^{5,20,23}. For the detection of FRET signals with native proteins labeled by antibodies, additional points should be considered. When labeling native proteins in large protein complexes with antibodies, it initially seems unlikely that FRET is possible, since additional distance is added to the “detection array” by the respective antibodies. Such variables complicate the relationship between FRET signals and the distance of proteins to the bound donor and acceptor antibodies^{9,35}. Still, labeling native proteins in large complexes with antibodies also provides advantages by introducing additional degrees of freedom and flexibility. The conjugation of multiple dyes to the antibodies further enhances the probability of a detectable FRET signal⁵. Previous studies performed by our group indicate that these assays work very well and can produce reproducible measurements of relative changes in protein proximity for the study of the interactions within large multi-protein complexes at different subcellular compartments^{19,20,23,24}. However, particularly for experiments with native proteins, FRET should not be used as a spectroscopic ruler for absolute distances⁵.

Here, we present a concise protocol for performing AB-FRET experiments, both with overexpressed fluorescence-tagged proteins and with native antibody-labeled FRET pairs. Using the E-cadherin adhesion complex, we demonstrate the application of AB-FRET to measure the relative proximity of known binding partners, as well as suitable approaches to control specificity. E-cadherin is a transmembrane protein that mediates cell-cell adhesion in conjunction with intracellular binding partners³⁶. E-cadherin trans-dimerizes between adjacent cells in a calcium-dependent manner³⁶⁻³⁸. On the cytoplasmic side, E-cadherin directly binds to beta-catenin³⁷, which helps to mediate a connection to the underlying actin cytoskeleton via additional factors, such as alpha-catenin^{39,40}. Alpha-catenin is known to directly bind to beta-catenin, but not to E-cadherin^{40,41}. Calcium depletion (e.g., by EGTA) breaks up E-cadherin-mediated adherens junctions, resulting in the loss of beta-catenin binding and the internalization of E-cadherin by endocytosis⁴². We have therefore used this system of published molecular interactions to characterize and validate our protocol for quantitative AB-FRET analysis in control cells as well as upon the dissolution of adhesion complexes by EGTA.

PROTOCOL:

1. General considerations before performing the experiment

1.1. Choose donors and acceptors according to the protein stoichiometry.

1.2. For the detection of native proteins, choose immunofluorescence-tested primary antibodies that are best directed against a defined protein epitope.

1.3. Choose fluorescent dyes with good overlap of donor emission and acceptor excitation spectra.

Note: The emission should be readily separable using a confocal microscope, and the acceptor dye should be accessible to efficient bleaching. Good FRET pairs include Cy3-Cy5, CFP-YFP, GFP-mCherry (monomeric), GFP-mRuby (monomeric), GFP-Alexa-568, and Alexa-488-Alexa-568.

1.4. Consider the sub-cellular localization and the dimensions of the structures when investigating protein interactions with AB-FRET using confocal microscopes.

Note: For example, when detecting protein interactions in cell-cell adhesion junctions using green and red dyes, structures of interests easily fit in one confocal plane, and pinholes can be set to airy to increase the spatial resolution in the Z-axis. If acquiring structures that require several overlapping confocal planes for full acquisition, the pinhole may be opened, an easy solution to accommodate the full structure dimensions with less Z-resolution.

1.5. Choose proper control experiments.

1.5.1. For a negative-control FRET pair, use proteins that reside within the same compartment or are part of the same protein complex but that do not interact directly.

1.5.2. Perform “donor-only” controls by analyzing the FRET in samples where only the donor, and not the acceptor, is stained; in the absence of an acceptor, no FRET should be detected.

1.5.3. When using an overexpressed acceptor, perform an acceptor titration curve to determine the dependence of the FRET signal on the acceptor concentrations.

Note: “Acceptor-only” and “donor-only” samples will help to determine optimal bleaching conditions during the experimental setup. For the newer confocal laser scanning systems, especially those equipped with HyD hybrid detectors, instrumental errors are negligible. To determine the “aggregated instrumental error,” use the FRET experimental settings, as well as the same detector used for the donor acquisition. Turn off the excitation to record images that can be used to determine the instrumental error after the extraction of the intensity data.

1.6. Fix samples with 3.5% formaldehyde in order to avoid the excessive crosslinking of proteins, which may produce artifacts.

1.7. Mount cells with a mounting medium that does not strongly stabilize fluorescence dyes in order to enable the bleaching of the acceptors.

1.8. Stain samples and controls within the same batch on the same day to reduce the variance. Perform at least three independent experiments per condition.

2. Representative experiments

2.1. Choice of donors and acceptors

2.1.1. For FRET donors (low stoichiometry), use E-cadherin-GFP or E-cadherin (native).

2.1.2. For FRET acceptors, use beta-catenin (native) or alpha-catenin (native).

2.2. Choice of FRET pairs

2.2.1. For donor dyes, use E-cadherin-GFP or E-cadherin (mAb)-anti-mouse-Alexa-488.

2.2.2. For acceptor dyes, use beta-catenin (rb)-anti-rabbit-Alexa-568 or alpha-catenin (rb)-anti-rabbit-Alexa-568.

Note: Protein interactions are detected at adherens junctions and can be visualized in a single confocal plane to increase the Z-resolution.

2.3. FRET negative controls

2.3.1. Use E-cadherin (mAb)-anti-mouse-Alexa-488 and alpha-catenin (rb)-anti-rabbit-Alexa-568.

2.4. Donor-only controls

2.4.1. Use E-cadherin-GFP-E-cadherin (mAb)-Alexa-488 incubated with acceptor dye secondary antibody only (anti-rabbit-Alexa-568).

2.5. Experimental setup

2.5.1. Use AB-FRET E-cadherin-GFP and beta-catenin-Alexa-568 w/wo EGTA.

2.5.2. Use AB-FRET E-cadherin-Alexa-488 and beta-catenin-Alexa-568 w/wo EGTA.

2.5.3. For a negative control, use E-cadherin-Alexa-488 and alpha-catenin-Alexa-568.

2.5.4. For a donor-only control, use E-cadherin-GFP and anti-rabbit-Alexa-568.

2.5.5. For aggregated instrumental error, perform an acquisition of E-cadherin-GFP (donor signal) in AB-FRET samples without excitation.

3. Immunofluorescent staining of cells for AB-FRET analysis

3.1 Place 15 cm x 15 cm coverslips in 12-well plates and coat them with 1 µg/mL collagen I for 1 h.

3.2 Seed 150,000 Caco-2 cells on the coated coverslips.

3.3 On the next day, transfect the Caco-2 cells with the indicated constructs using commercial transfection solution according to the manufacturer's protocol. Use 2.5 µg of DNA, 4 µL of transfection solution, and 5 µL of P3000 reagent.

3.4 After 48 h, treat the confluent Caco-2 cells with 2 mM EGTA for 1 min to dissolve the E-cadherin-mediated adherens junctions.

3.5 Fix the cells with 3.5% formaldehyde solution for 20 min at room temperature (RT).

3.6 Wash the samples three times for 5 min each with phosphate-buffered saline (PBS).

3.7 Quench the remaining formaldehyde by incubating it with 0.1 M glycine in PBS for 5 min.

3.8 Wash the samples twice with PBS for 5 min each.

3.9 Permeabilize the cells with 0.1% Triton X-100 for 3 min.

3.10 Wash the samples three times with PBS for 5 min each.

3.11 Block the samples using PBS with 5% FCS and 0.05% Tween-20 (blocking buffer) for 20 min.

3.12 Incubate the samples with primary antibodies in 300 μ L of blocking solution: anti-beta-catenin 1:100 (H-102), anti-E-cadherin 1:100, and alpha-catenin 1:100 for 2 h.

3.13 Wash samples five times with PBS for 5 min each.

3.14 Incubate the samples with fluorescent secondary antibodies in 300 μ L of blocking solution: goat anti-mouse IgG-Alexa-488 1:400 and goat anti-rabbit IgG-Alexa-568 1:400 for 2 h.

3.15 Wash the samples five times in PBS for 5 min each.

3.16 Mount the samples on coverslips using mounting solution.

3.17 Let the samples dry at RT for 48 h and store them at 4 °C in the dark.

4 AB-FRET microscopy using a confocal laser scanning microscope

4.1 Pre-warm samples to RT for several hours before use; RT for AB-FRET microscopy should be well-controlled to avoid artifacts due to thermal drift during acquisition.

4.2 Set up a confocal laser scanning microscope for AB-FRET imaging.

4.2.1 Acquire an automated time-bleach image series of the donor and acceptor (8-bit): take one image (pre-bleach), bleach the acceptor to the background, and take one more image (post-bleach). Here, use the dedicated “FRET AB Wizard” drop-down menu tab to gain access to the imaging and bleach settings.

4.2.2 If available, use a highly sensitive HyD detector with a superior signal-to-noise ratio, single-photon sensitivity, and a high dynamic linear range to acquire the donor signals.

4.2.3 In order to set a baseline threshold for the linear detection of specific FRET signals, acquire 8-bit images with 256 gray-levels to produce relevant and reproducible FRET values.

4.2.4 In the “Acquisition” tab in the FRET AB Wizard, set the image size to 1024 x 1024 pixels and the pinhole to airy. Set the scan speed to 200-400 Hz during acquisition. Use appropriate zoom settings for the respective objectives.

4.2.5 In the “Workflow” tab, press the “Donor” button to set up the donor imaging conditions. Turn on the argon laser, excite the donor with the 488-nm argon laser, and set the acousto-optical tunable filter (AOTF) laser power to 0.1-0.35% in order to avoid bleaching during acquisition.

4.2.5.1 Detect the donor signals by activating a PMT or HyD detector. Set the

detection range (GFP, Alexa-488) to 505-535 nm using the sliders for the acousto-optical beam splitter (AOBS) in the spectrum display.

4.2.5.2 For HyD detectors (representative results), set the detection intensities by modulating the laser power settings (photon-counting HyD detectors only have a virtual, digital gain that should be set to 100%).

4.2.5.3 For PMTs, do not exceed a detector gain of 930 V in order to reduce the image noise. Set the virtual or PMT detector gain using the USB Control Panel “Smart Gain” turn wheel.

4.2.6 For the empirical determination of the donor settings, test for bleaching artifacts during scanning by acquiring 10 images, followed by detecting the fluorescence intensity in a region of interest (ROI).

Note: The signal intensity should not drop more than 10%. If there are bleaching artifacts, reduce the laser power.

4.2.7 In the “Workflow” tab, press the “Acceptor” button to set up the acceptor imaging conditions. Turn on the 561-nm, diode-pumped, solid-state laser (DPSS laser) and set the ATOF laser power to 0.1-0.4%.

4.2.7.1 Detect the acceptor signals by activating a PMT detector. Set the detection range (Alexa-568) to 571-620 nm using the sliders in the spectrum display. Do not exceed a detector gain of 930 V to reduce the image noise. Set the PMT detector gain using the USB Control Panel “Smart Gain” turn wheel.

4.2.8 Set the donor and acceptor channels by adjusting the laser power and/or gain settings to within the linear range of detectors, with no saturated pixels in the designated bleach-ROI. For image acquisition, use a scan speed of 200 Hz.

4.2.9 In the “Bleach” tab in the “Wizard,” draw an ROI in the setup image that will represent the bleach and detection region for the AB-FRET experiment.

4.2.9.1 Set the laser power for the bleaching to 100% (ATOF) and enter the number of bleach cycles.

Note: For Alexa-568 dye, 2-5 bleach cycles (representative experiment: 4 cycles, 100% laser power) were sufficient to bleach the acceptor to background intensity levels. The bleaching will be processed with the same scan speed used during image acquisition (200 Hz, 1.2- μ s pixel dwell time). Here, donor-only and acceptor-only controls will help to determine the optimal bleach settings.

4.2.10 Do not “overbleach” the sample; if samples are bleached too excessively, the donor intensity will also be reduced (bleach artifact).

4.2.10.1 In case of “overbleaching,” reduce the laser power and/or the number of bleach cycles until the acceptors are effectively bleached without affecting the donors.

4.2.11 Once the setup is complete and the bleach-ROIs are defined, press the “Run experiment” button; the microscope will run the automated time-bleach series.

4.2.12 To determine the readings for the “aggregated instrumental error,” acquire images with settings similar to those of the donor, but without laser excitation.

Note: Upon completion, the “FRET AB Wizard” will automatically display the results in the “Evaluation” tab. The acquired images—DonAcc_PreBleach, Bleach (images series), and DonAcc_PostBleach—can be selected in the “Project” sidebar. The macro will also display donor/acceptor pre- and post-bleach intensity values (8-bit) and FRET efficiency (% FRET).

4.3 Save the data after every experiment by exporting it to an XML file using the “Report” function. Right-click into one of the images and click “SAVE ROIs” to permanently keep the bleach-ROI after every experiment.

4.4 Export (*.lif) image raw data to tiff files by right-clicking on the respective experiments in the results tab of the “FRET AB Wizard.”

4.5 For the quantitative analysis, acquire at least 15 cells from three independent experiments for all conditions, as well as the respective controls using identical laser power, gain, and bleach settings. Randomly select cells with co-localized donors and acceptors. When analyzing overexpressed proteins, do not select cells with massive overexpression, as this may generate unwanted artifacts.

5 Quantitative analysis of AB-FRET experiments using ImageJ

5.1 Download and install the MBF “Image J for Microscopy” collection with ~200 plugins, collated and organized by Tony Collins at the McMaster Biophotonics Facility (<https://imagej.nih.gov/ij/plugins/mbf/index.html>).

5.1 To normalize the quantitative analysis between different AB-FRET experiments, measure the intensity changes in the donor pre- and post-bleach images within the bleached region and use a number of equally-sized sub-ROIs (representative experiment: six equally-sized sub-ROIs randomly placed at adherens junctions within the bleach-ROI).

5.2 Open the donor/acceptor pre- and post-bleach images.

5.3 Open the “Multi measure” plug-in (Plugin/ROI) and generate a “reference ROI” for the sub-analysis, to be used during the entire quantitative analysis procedure.

5.3.1 Save the “reference ROI” for future use.

5.3.2 Set the measurement parameters in the “Set Measurements” drop-down menu. Select the area, mean gray value, and standard deviation. Select at least 3 decimal

places.

5.4 Place the reference ROI on the structure of interest in the pre-bleach donor image and press the “Add” button to save the position. Repeat as necessary and verify the placement within the bleached region by uploading the saved ROIs in the post-bleach acceptor image. Use the same procedure for all experiments.

5.5 Select the ROIs and press the “Multi” measure button. The results will be displayed in a new text window.

5.5.1 Draw the position of the sub-ROIs in the images by pressing the “Draw” button. Save the modified image.

5.5.2 Save the positions of all sub-ROIs by pressing the “Save” button.

5.6 Repeat step 5.5 for every open image to quantify the intensity, in sub-ROIs, of the donor and acceptor pre- and post-bleach images.

5.7 Collect the donor/acceptor pre- and post-bleach intensities of all ROIs in a spreadsheet.

5.8 Calculate the FRET efficiency (% FRET) for each sub-ROI using the following equation:

$$\% \text{ FRET} = [(Intensity_{\text{DonorPost}} - Intensity_{\text{DonorPre}}) / Intensity_{\text{DonorPost}}] * 100$$

for DonorPost > DonorPre

5.8.1 If the exported microscopy FRET report (XML file, see step 4.3) indicates no FRET within the bleach-ROI ($DonorPost \leq DonorPre$), set the “0” values to be equivalent to the number of sub-ROIs for the respective experiment.

5.8.2 If the sub-ROI analysis indicates “no FRET” within a sub-ROI, set the respective values to “0”.

5.8.3 Calculate the mean % FRET across all sub-ROIs within the same experiment.

5.9 To control a quantitative FRET analysis with the same proteins and specific endpoints, also calculate the mean intensities for the donor/acceptor pre- and post-bleach sub-ROIs.

REPRESENTATIVE RESULTS:

Here, we present a quantitative AB-FRET analysis to evaluate the relative proximity/interaction of E-cadherin and beta-catenin in controls as well as after the dissolution of adhesion complexes by EGTA. We also demonstrate that alpha-catenin can serve as a suitable negative control for experiments.

E-cadherin and beta-catenin co-localize

As a prerequisite for FRET, the proteins need to be physically present at the same structure. **Figure 1A** demonstrates the co-localization of ectopically expressed E-cadherin with native beta-catenin in control cells and after treatment with EGTA. Here, cell-cell adhesion is visibly disturbed, as indicated by the disrupted cell-cell connections at multi-cell junctions. **Figure 1B** demonstrates the co-localization of native E-cadherin and beta-catenin under the same experimental conditions.

Quantitative AB-FRET analysis of E-cadherin and beta-catenin in controls and EGTA-treated samples

A quantitative AB-FRET analysis was performed to compare the extent of protein interactions for E-cadherin-GFP and endogenous beta-catenin in control cells and after the dissolution of adherens junctions by calcium depletion (**Figure 2A**). **Figure 2B** displays the same experimental setup for native E-cadherin and beta-catenin. Indeed, EGTA treatment significantly decreased the % FRET values, as shown in the statistical analysis of the AB-FRET experiments, which is in line with published data^{38,42} (**Figure 2C** and **2D**).

Dedicated AB-FRET analysis controls

To further control AB-FRET experiments, donor and acceptor pre-bleach intensities were evaluated and compared. **Figure 3A** shows an equal distribution of donor pre-bleach intensities for ectopically expressed E-cadherin-GFP, demonstrating an unbiased selection of cells during the experiments. Acceptor intensities were also not significantly different. Donor and acceptor pre-bleach values were also determined for native E-cadherin and beta-catenin. Here, intensity values may provide information that could help to conclude whether the changes in the % FRET values are caused by an altered number of complexes that undergo energy transfer or by altered proximity. In line with the published data on the internalization of E-cadherin after the dissolution of cell-cell contacts⁴², we show significantly reduced E-cadherin levels at adherens junctions upon EGTA treatment, whereas the acceptor was not significantly affected (**Figure 3B**).

AB-FRET negative controls

The specificity of our AB-FRET experiments with the antibody-based detection of native proteins was further controlled by performing AB-FRET studies with alpha-catenin. Alpha-catenin is a member of the cytoplasmic adhesion complex that does not directly bind to E-cadherin, but it was shown to interact with beta-catenin (negative control). **Figure 3C** demonstrates the co-localization of both proteins but no detectable FRET between E-cadherin and alpha-catenin. Negative-control FRET experiments were statistically analyzed and demonstrated highly significant differences when compared to the E-cadherin-beta-catenin interaction (**Figure 3D**). To include a measure for the accuracy of the FRET acquisition performed with the microscope system and the HyD detector, we determined the “aggregated instrumental error” during the acquisition of the donor images (**Figure 3E**). “Instrumental error” images were acquired with settings similar to those of the FRET studies, but without laser excitation. The intensity in 8-bit images recorded for the instrumental error amounted to 0.01002 ± 0.001953 (mean and

SEM, $n = 15$ experiments, 6 sub-ROIs), which is negligible compared to the more than 100-times greater intensities of the donor signals, displayed in **Figure 3A** and **B**. To determine the “experimental errors,” we also performed “donor-only” controls, where fluorescent donor samples were incubated solely with acceptor secondary antibodies. **Figure 4A** shows the example of such a control, with E-cadherin-GFP as a donor fluorophore. Quantitative analyses of these samples indicated no detectable FRET due to the absence of a dedicated acceptor (**Figure 4B**).

Thus, using AB-FRET at the E-cadherin adhesion complex, we have analyzed changes in the organization/interaction of known binding partners under different conditions, verifying our experimental protocol.

Figure 1: Co-localization of E-cadherin with beta-catenin in Caco-2 cells.

A) Upper panel: Confluent Caco-2 cells expressing E-cadherin-GFP (E-cad-GFP) co-localize with native beta-catenin. Lower panel: Confluent Caco-2 cells expressing E-cadherin-GFP were incubated with 2 mM EGTA for 1 min to break down the adherens junctions. Fixed immunofluorescence samples were stained with anti-beta-catenin primary and Alexa-568 secondary antibodies. B) Upper panel: Native E-cadherin and beta-catenin co-localized in confluent Caco-2 cells. Lower panel: Co-localization of native E-cadherin and beta-catenin in Caco-2 cells treated with 2 mM EGTA for 1 min. Fixed immunofluorescence samples were stained with anti-E-cadherin and anti-beta-catenin primary, as well as with Alexa-488 or Alexa-568 secondary antibodies, respectively. Samples were documented using a confocal microscope with similar settings for all conditions. White arrows mark the breakdown of adherens junctions after EGTA treatment. The images depict single confocal sections. Scale: 10 μm .

Figure 2: Quantitative AB-FRET analysis of E-cadherin and beta-catenin in controls and EGTA-treated samples.

A) Quantitative AB-FRET analysis of the interaction of E-cadherin-GFP with native beta-catenin labeled with Alexa-568 antibodies in control and 2 mM EGTA-treated Caco-2 cells. Exemplary images depict donor and acceptor pre-bleach as well as post-bleach states for the interaction of E-cadherin-GFP with beta-catenin. For the control cells in this example, we determined a mean % FRET of 19.64, and for the EGTA-treated sample, a mean % FRET of 8.86. B) Quantitative AB-FRET analysis for the interaction of native E-cadherin-Alexa-488 with native beta-catenin-Alexa-568 in control and 2 mM EGTA-treated Caco-2 cells. Exemplary images depict donor and acceptor pre-bleach as well as post-bleach states for the interaction of E-cadherin with beta-catenin. For the control cells in this example, we determined a mean % FRET of 5.30, and for the EGTA-treated sample, a mean % FRET of 3.51. White arrows highlight the position of bleach-ROIs, and sub-ROIs have been indicated by white circles. Images depict single confocal sections. Scale bar: 10 μm . C) Quantitative AB-FRET analysis for the interaction of E-cadherin-GFP with native beta-catenin in control and EGTA-treated Caco-2 cells. The graph shows the mean \pm SEM of % FRET ($n = 15$ cells, three independent experiments). D) Quantitative AB-FRET analysis for the interaction of native E-cadherin with native beta-catenin in control and EGTA-treated Caco-2 cells. The graph shows the mean \pm SEM of % FRET ($n = 15$ cells, three independent

experiments). * $p < 0.05$. Statistical significance test: two-tailed unpaired Student's t-test.

Figure 3: Distribution of donor/acceptor intensities and negative controls.

A) Distribution of donor and acceptor pre-bleach intensities for all cells evaluated in Figure 2C. Graph: mean intensities and SEM; $n = 15$ cells; 8-bit images. B) Distribution of donor and acceptor pre-bleach intensities for all cells evaluated in Figure 2D. Graph: mean intensities and SEM; $n = 15$ cells; 8-bit images. Statistical significance tests for A and B: two-tailed unpaired Student's t-test. * $p < 0.05$; ns, not significant. C) Quantitative AB-FRET analysis for the interaction of native E-cadherin-Alexa-488 with native alpha-catenin-Alexa-568 (negative control). Exemplary images depict donor and acceptor pre-bleach as well as post-bleach states for E-cadherin and alpha-catenin. The white arrow highlights the position of bleach-ROIs, and sub-ROIs have been indicated by white circles. The images depict single confocal sections. Scale bar: 10 μm . D) Quantitative AB-FRET analysis for the interaction of native E-cadherin with native beta-catenin (results from Figure 2D, control) in comparison to the FRET analysis of native E-cadherin with native alpha-catenin. The graph shows the mean \pm SEM of % FRET ($n = 15$ cells, three independent experiments). **** $p < 0.0001$. Statistical significance test: two-tailed unpaired Student's t-test. E) Determination of the "aggregated instrumental error." Images of the donor without laser excitation (E-cadherin-GFP, Figure 2A), recorded using the HyD detector with acquisition settings also employed during the AB-FRET experiments performed in Figure 2A. "Instrumental error" images were processed in a manner similar to the FRET experiments in A in order to extract intensities from sub-ROIs. Box blot: median intensity with whiskers (5th-95th percentile) for $n = 15$ cells; 8-bit images. The numbers indicate the intensity for the mean instrumental error with SEM (8-bit images).

Figure 4: AB-FRET "donor-only" experimental controls.

A) Exemplary AB-FRET analysis for E-cadherin-GFP with "non-specific" anti-rb-Alexa-568 secondary antibodies. The images depict donor and acceptor pre-and post-bleach states for E-cadherin-GFP and anti-rb-Alexa-568. The bleach-ROI is marked in green. The images depict single confocal sections. Scale bar: 10 μm . B) Quantitative AB-FRET analysis for the interaction of E-cadherin-GFP with native beta-catenin (results from Figure 2C, control) compared to the FRET analysis of E-cadherin-GFP with anti-rb-Alexa-568. The graph displays the mean \pm SEM of % FRET ($n = 15$ cells, three independent experiments). **** $p < 0.0001$. Statistical significance test: two-tailed unpaired Student's t-test.

DISCUSSION:

Advantages and caveats of AB-FRET

AB-FRET is a robust and versatile tool to study the proximity interaction of proteins in multi-protein complexes. Compared to other methods, AB-FRET has several advantages. Experiments can be performed with standard confocal laser scanning microscopes by acquiring a time-bleach image series of the donor and acceptor. Software macros are available on microscopes to guide the setup of the imaging procedures. In contrast to sensitized-emission FRET, AB-FRET is insensitive to donor fluorescence bleed-through, since FRET is only calculated from donor intensities.

Moreover, a quantitative FRET analysis can be performed by calculating the % FRET values from a simple equation^{5,20,23}. However, a methodological disadvantage is the destruction of the acceptor dye, which does only allow for one measurement in a region of interest. AB-FRET is also sensitive to “overbleaching” that may inadvertently result in donor fluorescence loss. Thus, parameters during the acquisition of experiments must be carefully controlled. There are also additional caveats, such as false FRET signals due to the photoconversion of an acceptor Cy5 dye into a fluorescein-like fluorophore^{5,22} or false signals that stem from moving samples due to thermal drift during image acquisition. Furthermore, acceptor dyes that are too stable for sufficient bleaching to background levels may produce artifacts. However, if these factors are accounted for by carefully controlling experimental settings, quantitative studies can be performed in a robust and reproducible manner.

Antibody-based AB-FRET experiments with native proteins

AB-FRET may be used to investigate the relative proximity/interaction of ectopically expressed, fluorescence-tagged or native proteins detected by antibodies. Previous studies performed by our group indicated that AB-FRET experiments with native protein are very effective in investigating the organization of large protein complexes under different conditions. In the above-presented experimental setup, we were able to show a significant reduction in the % FRET values between endogenous E-cadherin and beta-catenin upon the dissolution of the adhesion complexes by calcium depletion. Although in close proximity within the adhesion complex, the mean % FRET for E-cadherin and alpha-catenin was drastically lower. Our observations^{20,23,24} also indicate that, due to the larger, antibody-based “detection arrays,” assays are also very sensitive to even the smallest changes in protein proximity, which may translate into larger distances, ultimately exceeding FRET distances. The antibody-based assay format is particularly efficient when unbound interaction partners leave protein complexes. Thus, in our experiments, we faithfully detected AB-FRET for directly interacting proteins (**Figure 2**), whereas for adjacent factors that do not directly bind, FRET was barely detectable (**Figure 3C and D**). Provided the negative controls work reliably, antibody-based AB-FRET may be used to access relative differences in proximity/interaction by direct comparison^{20,23,24}. These kinds of experiments do not only work in cultured cells: we have previously published a quantitative analysis for the interaction of native E-cadherin with beta-catenin utilizing immunohistochemistry in mouse intestine tissue sections²³.

Limitations and the necessity of proper controls

AB-FRET can provide a valuable tool to assess and compare the relative proximity/interaction of proteins in multi-protein complexes at discrete end points, such as non-stimulated or stimulated cells^{20,23}. For example, we have used AB-FRET to study the interactions of proteins following the phosphorylation of a binding partner²³. Actin-GFP monomer incorporation into actin filaments stained by Rhodamine-Phalloidin was analyzed to detect the relative localized actin polymerization²³, whereas Vinculin-GFP-F-actin-binding properties were investigated by quantitative AB-FRET analysis with Rhodamine-Phalloidin at the adherens junctions²³. Thus, AB-FRET has numerous applications, but proper controls need to be provided to allow for the concise evaluation

of the results. In order to control the experiments, FRET studies should be performed with ectopically expressed proteins and native factors. For ectopic expression of donors/acceptors (E-cadherin-GFP, **Figure 3A**), a measure for the equal distribution of pre-bleach signals between the different experimental conditions should be provided to ensure the unbiased selection of cells during the experiments. If experiments are performed with native proteins (E-cadherin-Alexa-488 and beta-catenin-Alexa-568, **Figure 3B**), the distribution of donor and acceptor pre-bleach signals may also show whether relative changes between end points are the result of changes in the number of complexes that exhibit FRET or of altered relative proximity²³. In our representative results, we were able to show a significant reduction in E-cadherin complexes upon EGTA treatment for native proteins, but not for exogenously expressed E-cadherin-GFP (**Figures 3A, 3B**). In line with the literature⁴², the reduction of the native E-cadherin signal at the adherens junctions after EGTA treatment may be the result of internalization due to endocytosis. Furthermore, FRET negative controls that at least co-localize at similar structures are required⁵. We also suggest that donor-only controls and acceptor titration curves may be provided to demonstrate the specificity. Although, AB-FRET is very effective in probing and quantifying relative changes in protein proximity and interactions, results should always be supported and verified by additional biochemical studies, such as co-immunoprecipitation experiments or pulldown assays^{20,23}. Antibody-based AB-FRET with native proteins should not be used as a stand-alone tool to analyze novel protein interactions without further independent verification by biochemical methods. In summary, we have provided a hands-on protocol for the performance of quantitative AB-FRET studies with a standard confocal laser scanning microscope, described the analysis of the experimental data, and designated the appropriate controls for the experiments.

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The authors declare no competing financial interests.

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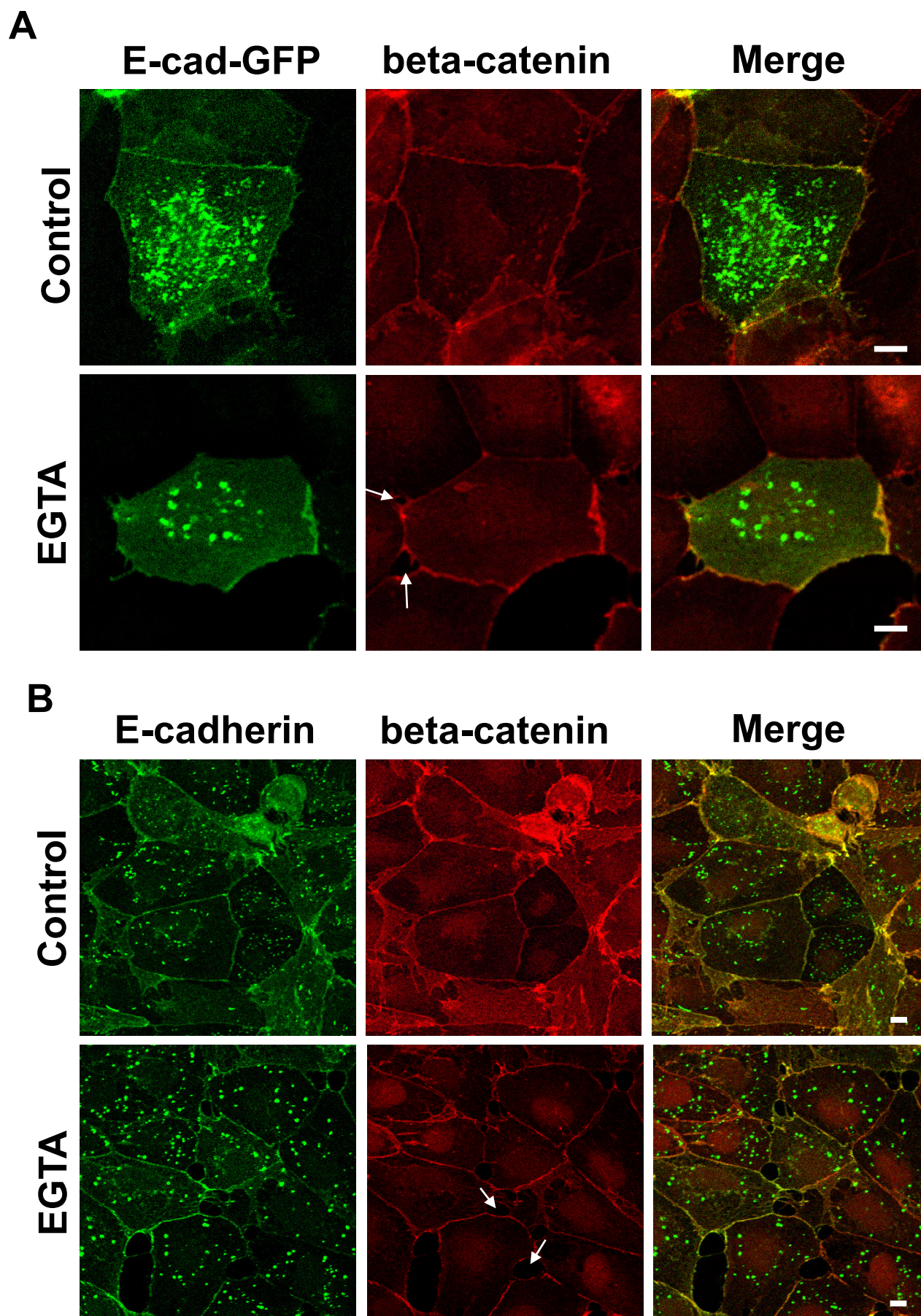


Figure 2

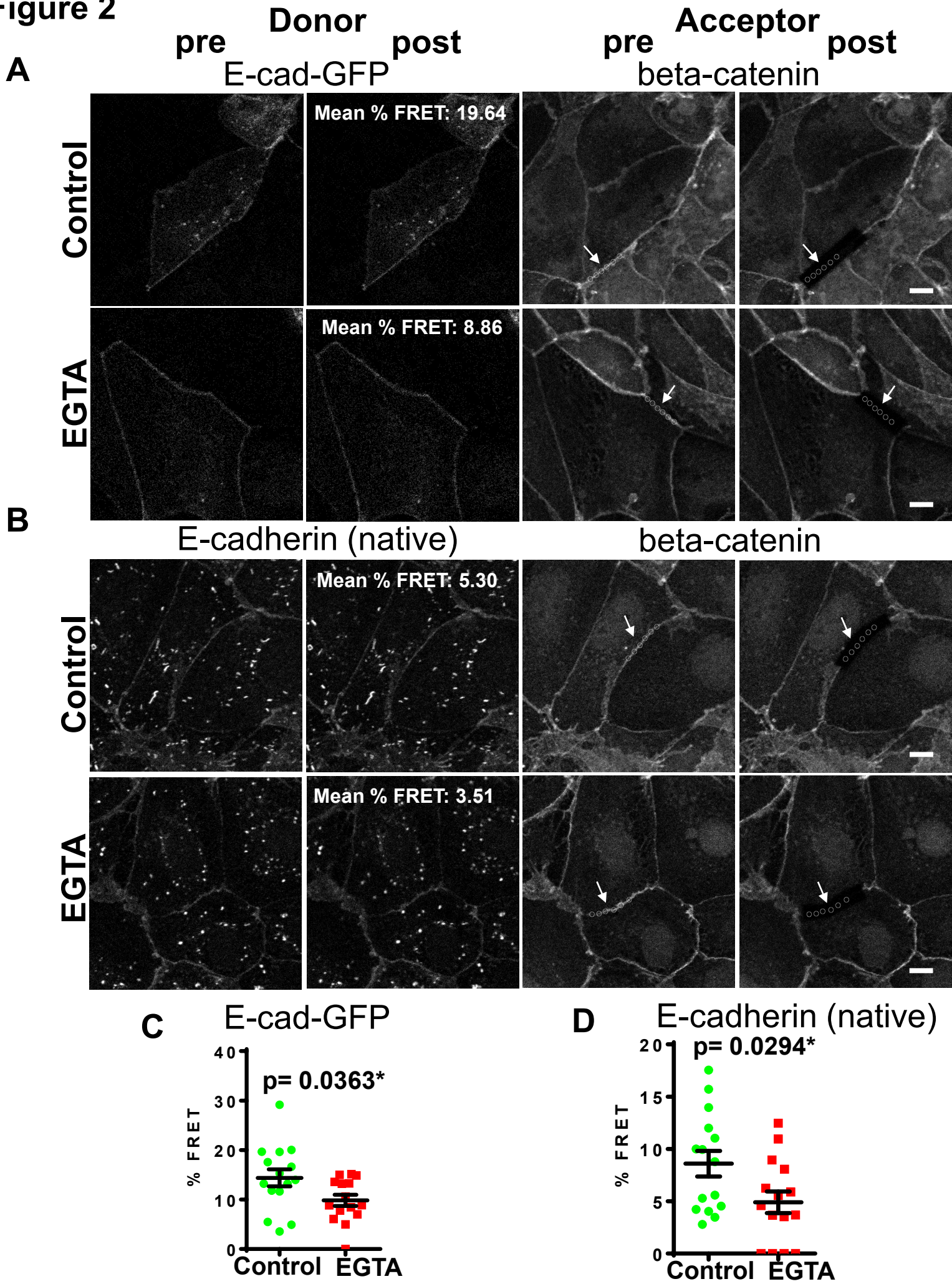
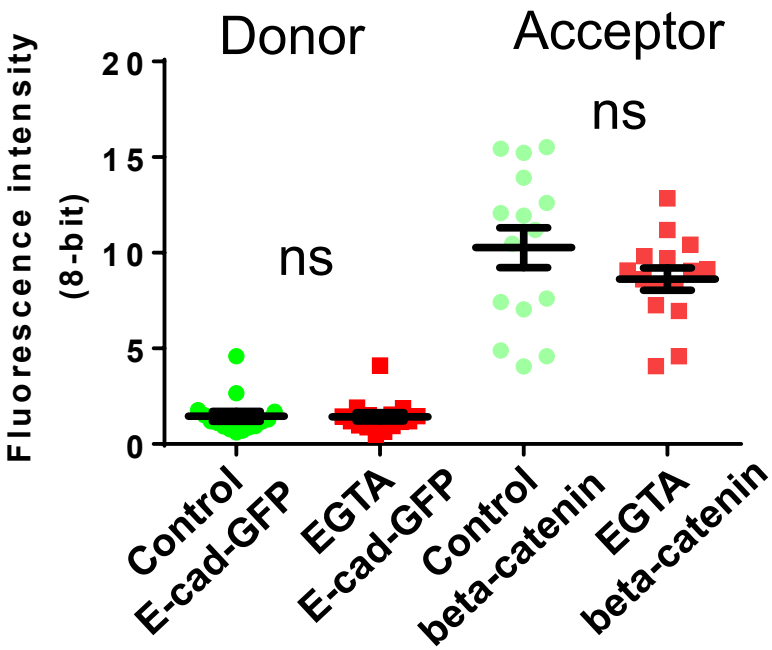
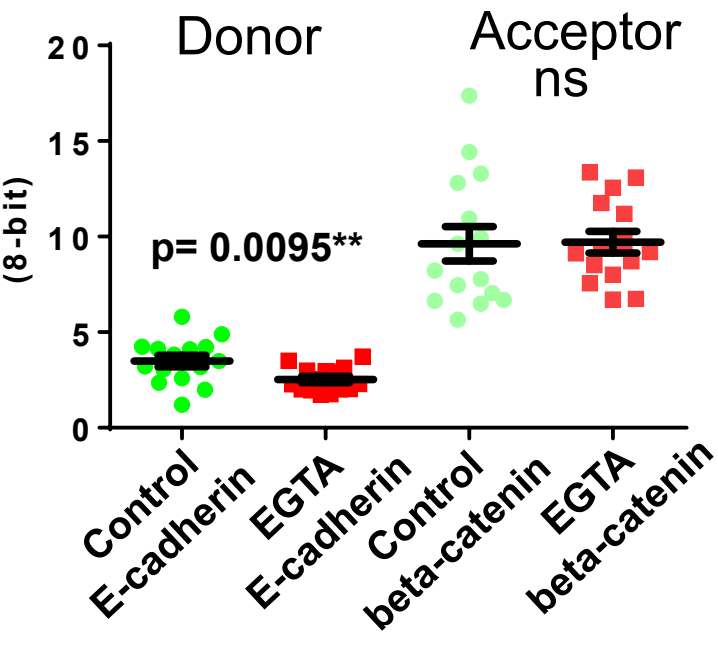


Figure 3

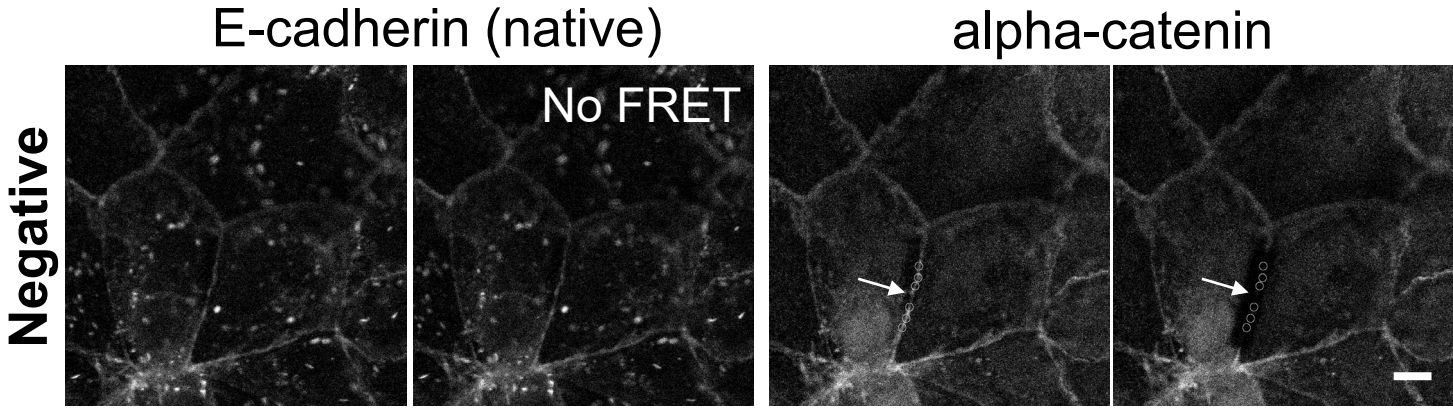
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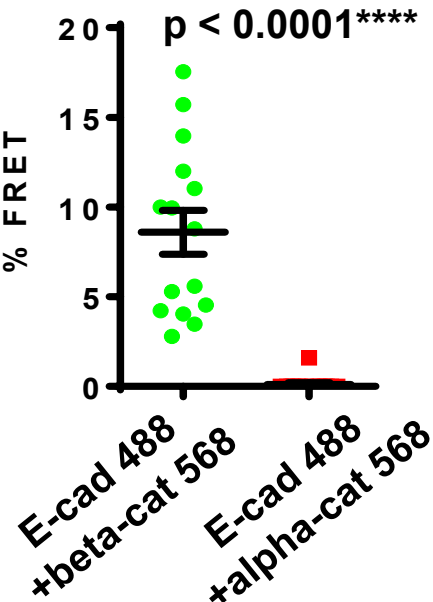
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C



D



E

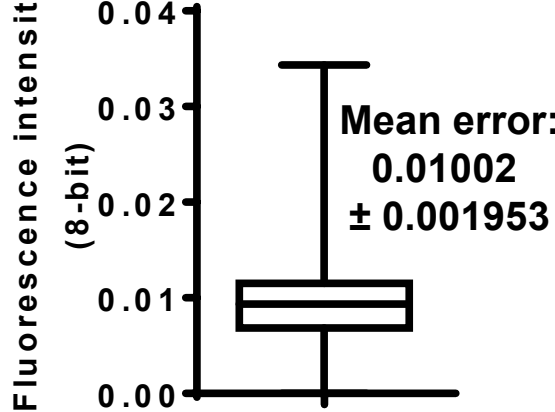
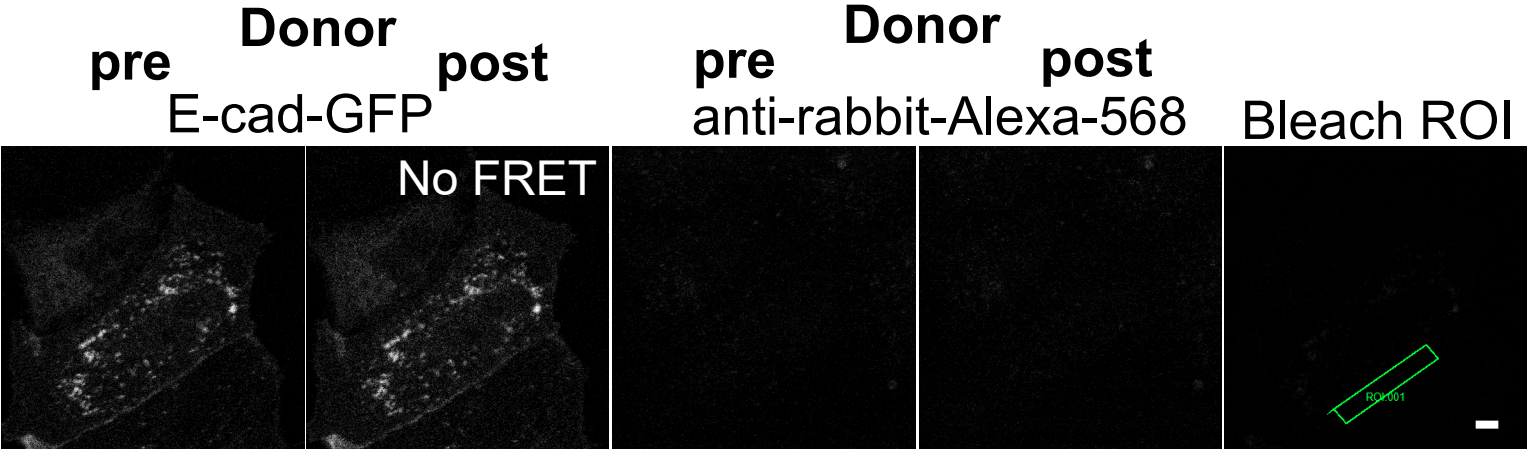
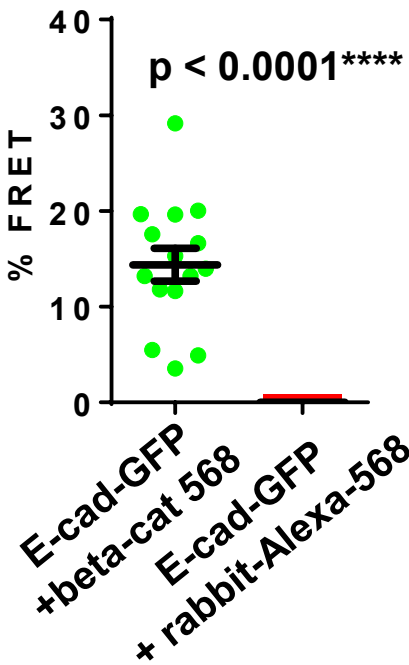


Figure 4

A



B



Name of Reagent/ Equipment	Company	Catalog Number
Caco-2 cells	DSMZ (German collection of microorganisms and cell cultures)	ACC169
		665180
12-well cell culture plate	Greiner bio-one	
3.5% formaldehyde solution	Fischar Otto	PZN2652965
anti-alpha-catenin (EP1793Y) (rb)	Abcam	ab51032
anti-mouse-Alexa-488		A11031
	ThermoFisher Scientific	
anti-rabbit-Alexa-568		A11011
	ThermoFisher Scientific	
anti-beta-catenin (H-102) (rb)	Santa Cruz Biotechnology	sc-7199
Collagen I, Rat Tail (5 mg/ml)	Invitrogen	A10483-01
Cover slips 15 x 15 mm	Menzel-Gläser/ VWR	631-0710
E-cadherin (clone 36) (mAB)	BD Bioscience	610182
E-cadherin-GFP	Addgene	28009
EGTA	Carl Roth	3054.3
FCS	Biochrome	S 0115
Fluoromount G	Southern Biotech	0100-01
Glycine	Sigma-Aldrich	33226-1KG
	ThermoFisher Scientific	L3000001
Lipofectamine 3000 Transfection Reagent		
MBF "Image J for Microscopy" collection		
MEM, GlutaMAX Supplement	Gibco	41090028
Microscope slides	Carl Roth	1879
PBS (1x) Dulbecco's Phosphate Buffered Saline	Gibco	14190-094
Penicillin-Streptomycin (10,000 U/ml)	ThermoFisher Scientific	15140122
Prism software version 6.00	GraphPad Software	
TCS SP8-HCS system	Leica	
Triton X-100	Sigma-Aldrich	T8787-250ML
Tween 20	Sigma-Aldrich	P7949-500ML

Comments/Description

cultivated in MEM medium (GlutaMAX, with 20% FCS, 1x non-essential amino acids, 1x penicilli

Dilution 1:100

Dilution 1:400

Dilution 1:400

Dilution 1:100

Dilute 1:5000 in 1x PBS for coating coverslips

Dilution 1:100

generate 0.5 M EGTA stocksolution in ddH₂O and adjust a pH 7.5; dilute 0.5 M EGTA to 2 mM E

Use 0.1 M Glycine in PBS for quenching remaining formaldehyde in microscope probes
according to the optimized protocol provided by the manufacturer

<https://imagej.nih.gov/ij/plugins/mbf/index.html>

Warm in 37 °C water bath before use

dilute for 500 ml medium 1:100

1% (v/v) TritonX-100 dissolved in PBS

0.05% (v/v) Tween + 5% (v/v) FCS solved in PBS (blocking buffer)

n-streptomycin)

EGTA solution for experiment



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Signature:		Date: 08/17/2016

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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 widefield 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 510¹, 710^{2,3} or even more recent systems from Zeiss as well as the SP5⁴ to SP8³ 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 manuscript¹⁻⁵. However, HyD hybrid detectors are even better suited to detect signals with a good linear response and a high dynamic range. HyDs also possess 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 PMTs⁶.*

* 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.

- *We 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 / β -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 cells¹⁰⁻¹².*

Citation 5 is heavily referenced, often in cases where 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-FRET¹³, we also suggest in line with Snapp et al. 2006¹⁴ 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 than 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: 1,2 μ s). 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 stacks². 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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