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## Assessing protein interactions in live-cells with FRET-sensitized emission

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**TITLE:**

# Assessing Protein Interactions in Live-Cells with FRET-Sensitized Emission

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**KEYWORDS:**

live-cell imaging, FRET, quantitative fluorescence microscopy, fluorophore, protein interaction, sensitized emission.

**SUMMARY:**

Förster Resonance Energy Transfer (FRET) between two fluorophore molecules can be used for studying protein interactions in the living cell. Here, a protocol is provided as to how to measure FRET in live cells by detecting sensitized emission of the acceptor and quenching of the donor molecule using confocal laser scanning microscopy.

**ABSTRACT:**

Förster Resonance Energy Transfer (FRET) is the radiationless transfer of energy from an excited donor to an acceptor molecule and depends upon the distance and orientation of the molecules as well as the extent of overlap between the donor emission and acceptor absorption spectra. FRET permits to study the interaction of proteins in the living cell over time and in different subcellular compartments. Different intensity-based algorithms to measure FRET using microscopy have been described in the literature. Here, a protocol and an algorithm are provided to quantify FRET efficiency based on measuring both the sensitized emission of the acceptor and quenching of the donor molecule. The quantification of ratiometric FRET in the living cell not only requires the determination of the crosstalk (spectral spill-over, or bleed-through) of the fluorescent proteins but also the detection efficiency of the microscopic setup. The protocol provided here details how to assess these critical parameters.

**INTRODUCTION:**

Microscopy-based analysis of Förster Resonance Energy Transfer (FRET) permits assessment of

interactions between proteins in live cells. It provides spatial and temporal information, including information on where in the cell and in which subcellular compartment the interaction takes place and if this interaction changes over time.

Theodor Förster laid the theoretical foundation of FRET in 1948[1]. FRET is a radiationless transfer of energy from an excited donor to an acceptor molecule and depends upon the distance of the molecules and the relative orientation of their transition dipoles as well as the overlap between the donor emission and acceptor absorption spectra. The rate of energy transfer is inversely proportional to the sixth power of the donor-acceptor distance. Thus, FRET can be used to measure molecular proximity in the range of 1-10 nm.

FRET competes with other de-excitation processes of the donor molecule and results in the so-called donor-quenching and sensitized emission of the acceptor. Donor-quenching is a reduction of the number of emitted donor photons, while sensitized emission is an increase in emitted acceptor photons. Many microscopic FRET analyses use fluorescence intensity measurements, including acceptor photobleaching[2], donor photobleaching[2], or FRET-sensitized photobleaching of the acceptor[3].

Here, a step-by-step experimental protocol and mathematical algorithm are presented to quantify FRET using donor quenching and acceptor sensitized emission[4,5], a method often referred to as ratiometric FRET. Many protocols on how to approximate sensitized emission have been published, few have quantified the absolute FRET efficiency[6-9]. The quantification of FRET efficiencies in the living cell requires determining (i) the crosstalk (spectral spill-over, or bleed-through) of the fluorescent proteins and, also (ii) the detection efficiency of the microscopic setup. While crosstalk can be assessed by imaging cells expressing only one of the fluorophores, the assessment of the relative detection efficiency of the donor and acceptor fluorescence is more complicated. It requires the knowledge of at least the ratio of the number of donor and acceptor molecules giving rise to the measured signals. The number of fluorophores expressed in live cells varies, however, from cell to cell and is unknown. The so-called $a$ factor characterizes the relative signal strengths from a single excited donor and acceptor molecule. Knowledge of the $a$ factor is a prerequisite for quantitative ratiometric FRET measurements in samples with variable acceptor-to-donor molecule ratios as encountered during live-cell imaging with fluorescent proteins. Using a 1-to-1 donor-acceptor fusion protein as a calibration probe permits the determination of the $a$ factor and also serves as a positive control. This genetically coupled probe is expressed by cells in unknown total amounts but in a fixed and known relative amount of one-to-one. The following protocol lays out how to construct the 1-to-1 probe and how to use it for quantification of FRET efficiency. A spreadsheet that includes all formulae can be found in the supplement and can be used by the readers to enter their own measurements in the respective columns as outlined below.

While the protocol uses the GFP-Cherry donor/ acceptor pair, the presented approach can be performed with any other FRET pair. The **Supplementary File 1** provides details on cyan-yellow pairs.

**PROTOCOL:**

## 1. Plasmid construction

1.1. For generating the eGFP-mCherry1 fusion probe, use an N1 mammalian cell expression vector (see **Table of Materials**) with mCherry1[10] inserted using the restriction sites *AgeI* and *BsrGI.*

1.2. Use the following oligonucleotides to amplify eGFP[11] without a stop codon as *SalI-BamHI* fragment: N-terminal primer 5'-AAT TAA CAG TCG ACG ATG GTG AGC AAG GGC GAG G 3' and C-terminal primer 5'-AAT ATA TGG ATC CCG CTT GTA CAG CTC GTC CAT GC 3'.

1.2.1. Insert this *SalI-BamHI* fragment into the multiple cloning site of the N1 vector to introduce RNPPV linker (five amino-acid) linker between the green and red fluorescent protein.

NOTE: This linker yields a mean FRET efficiency for the GFP-Cherry donor-acceptor pair of about 0.25 -0.3 (**Figure 1A**). The choice of stiff[12] and helical[13] linkers of varying lengths to scale measured FRET efficiencies has been discussed elsewhere but is not required for our purpose of the fusion protein. Going forward for simplicity we will call the fluorescent proteins 'GFP' and 'Cherry'.

## 2. Cell culture and transfection

2.1. Use any cell line, e.g., NRK cells, for FRET experiments in media, e.g., Dulbecco's modified Eagle's media (DMEM), *without* phenol red, to reduce background fluorescence. For the same reason, the usage of phenol red free trypsin is advised.

2.2. Once cells are 80% confluent, detach cells with 1 mL of 0.05% trypsin-EDTA, count the number of cells in suspension using a Neubauer chamber and seed about 10,000 cells per well of an 8-well chambered cover glass; alternatively, from a confluent cell culture grown in T25 flask, use 1 drop of cell suspension from a 2 mL pipette or 3 drops from a 5-mL cell suspension from a confluent culture grown in a T 12.5-cell culture flask.

2.3. Grow cells in 8-well chambers (0.8 $cm^2$/ well) with #1.0 cover glass for fluorescence live-cell microscopy at standard cell culture conditions (37˚C and 5% $CO_2$).

2.4. 24 h after plating transfect the cells using an appropriate commercially available transfection media (see **Table of Materials**), with GFP, Cherry, GFP/ Cherry mix (1:1 mix, i.e., 0.8 μg and 0.8 μg GFP and Cherry plasmid DNA), and the GFP—Cherry chimera.

2.4.1. For transfection, use 5 μL of the transfection reagent in 45 μL of DMEM and 1.6 μg of plasmid DNA. Stir by gently flicking the microcentrifuge tube.

2.4.2. After 15 min incubation of the mix at room temperature, add 1-2 μL of the transfection

reagent mixture to each well of the 8-well chamber slide. Return the chambered cover glass to the incubator.

2.5. Let 20 h after transfection elapse before live-cell imaging, to allow for proper fluorescent protein expression, folding and maturation, especially of the red fluorophore.

## 3. FRET Imaging

3.1. Image transfected cells in a humidified and heated environmental chamber at 37 ˚C. To buffer the cell media at physiological pH, use $CO_2$ gas set to 5% flow, or add 20 mM HEPES to render the cell media $CO_2$-independent.

3.2. Use a confocal laser scanning microscope. Set the excitation and emission as follows to optimize the signal and minimize cross-talk.

3.2.1. Use the 488-nm line of the argon ion laser to excite GFP and the 561-nm diode pumped solid state laser (or 543-nm Helium Neon laser, depending upon available laser lines) to excite Cherry.

3.2.2. Set the following in the software of a commercial confocal microscope. Set the **Dichroic mirror** to 488/ 561 by button click using the pull-down menu. Collect fluorescence using 488-nm laser light for excitation in channel 1 through an emission band of 505 - 530 nm (or 505 - 550 nm) and in channel 2 with a long pass filter >585 nm and use the 561-nm laser light for excitation in channel 3 with a long pass filter > 585 nm (type in wavelengths). Band pass filters e.g., 590 – 650 nm or similar can also be used which have the advantage of excluding Raman-scattering.

3.3. Excite with the two lasers sequentially and set the imaging mode to **Switch after each line** so that the excitation of the 512 x 512 pixels image alternates after each line (and not after each frame which would abrogate the ability to detect FRET due to diffusion of the labeled proteins while recording the images with different excitations; button click).

3.4. Set up a mini-time series of three images by button clicks to detect if significant photobleaching occurs, and potentially reduce the laser power. Photobleaching of less than 1% is optimal. High laser intensity can also lead to absorption saturation reducing the apparent FRET efficiency[14]. Laser power, up to 10-20 μW measured at the objective lens are safe to use.

3.5. First, image cells expressing the GFP—Cherry fusion construct. Set the parameters that define the time-integrated laser intensity per pixel in a confocal image, i.e., the pixel dwell time in microseconds, the acousto-optical tunable filter (AOTF) transmission in percent, and the zoom.

3.5.1. Image cells using a 63x oil objective and **Zoom** set to 3x. This provides sufficient magnification and resolution to image cells in its entirety. Aim for a pixel size of 70-80 nm.

3.5.2. Set **Pixel dwell time** to 2-4 μs and **AOTF transmission** for the 488-nm and 561-nm laser

such that images have a good signal-to-noise ratio without bleaching and no pixels showing fluorescence intensity saturation. It is advantageous to adjust the laser power of 488 and 561 such that signal levels in channel 1 and channel 3 are similar.

3.5.3. Set the **Photomultiplier (averaging mode) gain** to 600-800.

3.6. Image with these settings 15-20 cells expressing the GFP—Cherry fusion protein. 15-20 cells provide good statistics while keeping the total time of a FRET measurement session limited to a few hours to help ensure stability of the microscopic set-up.

3.7. Image with the same settings cells expressing GFP, Cherry, GFP and Cherry and non-transfected cells. Search for expressing cells in the green channel or red channel, respectively.

3.8. Then, image 15-20 cells co-expressing proteins of interest coupled to GFP and Cherry, respectively. Searching for expressing cells, avoid long exposure of the cells in order to not bleach the fluorescent proteins. Cherry has a lower photostability than GFP, and bleaching Cherry, the acceptor compromises FRET analysis.

NOTE: Absorption and emission spectra of GFP and Cherry is shown in **Supplementary Figure 1.** After measuring for 5-6 h, it is advisable to repeat imaging a few cells expressing the GFP-Cherry chimera at the end of the imaging session to document that the set-up remained stable and detected FRET efficiencies of the GFP—Cherry fusion protein did not significantly change during the course of an imaging session.

## 4. Image analysis for detecting absolute FRET efficiencies using donor quenching and sensitized emission

NOTE: Here, a practical step-by-step guide as to how to determine FRET efficiency with the use of the attached spreadsheet (**Supplementary File 2**) is provided. Theory and derivation of the presented equations can be found in detail in previous publications[4,15-17]. With the described settings, the following fluorescence intensities are collected.

4.1. Measure the donor signal $I_1$ in channel 1, the donor channel, with 488-nm excitation and an emission band of 505 – 530 nm.

$$I_1(488, 505-530) = I_D(1-E) + B_1,$$

where $I_D$ is the unquenched donor signal in channel 1 that would be measured in the absence of an acceptor, $E$ is the mean FRET efficiency, and $B_1$ the average background signal in channel 1.

4.2. Measure the acceptor signal $I_3$ in channel 3, the acceptor channel, with 561-nm excitation and emission at >585 nm.

$$I_3\,(561, > 585) = I_A + B_3\,,$$

where $I_A$ is the acceptor signal and $B_3$ the background in channel 3.

4.3. Measure the FRET signal $I_2$ in channel 2, the transfer channel, with 488-nm excitation and emission at >585 nm.

$$I_2\,(488, > 585) = I_D(1 - E)S_1 + I_A S_2 + I_D E\alpha + B_2\,,$$

Where, the signal in channel 2 is a sum of four different components: (i) $I_D(1 - E)S_1$ is the spectral spill over from the quenched donor signal into the >585 detection channel (with the cross-talk factor $S_1$), (ii) $I_A S_2$ is the acceptor signal from the direct excitation by 488-nm light (with the cross-talk factor $S_2$), (iii) $I_D E\alpha$ is the sensitized emission of the acceptor by FRET from the excited donor molecule ($\alpha$ will be detailed further in 4.8. - 4.10.), and (iv) $B_2$ is the background signal.

4.4. Measure average background intensities in channels 1, 2, 3 in non-transfected or mock-transfected cells; either is fine with negligible difference. For all cell measurements, use the free-hand tool to delineate regions of interests and avoid perinuclear vesicles with increased autofluorescence. It is important to avoid significant autofluorescence from these perinuclear vesicles.

4.4.1. Enter the measurements into the columns X, Y, and Z of the provided spreadsheet. Average background intensities in the 3 channels are entered into A2, B2, and C2 of the excel spreadsheet (**Supplementary File 2**).

4.5. Measure average intensities in channels 1, 2, 3 of cells expressing GFP or Cherry alone, and enter the measurements into columns C, D, E, and N, O, P. Respective background intensities are subtracted (in F, G, H, and Q, R, S).

4.6. In order to calculate $E$, the FRET efficiency, determine the cross-talk factors $S_1$ and $S_2$. The spectral cross-talk factor $S_1$ is calculated from cells expressing only GFP

$$S_1 = \frac{I_2 - B_2}{I_1 - B_1}$$

in column I. Enter the mean value for $S_1$ into cell D2 on the excel spreadsheet.

4.7. Calculate the spectral cross-talk factor $S_2$ from cells expressing only Cherry

$$S_2 = \frac{I_2 - B_2}{I_3 - B_3}$$

in column T. Enter the mean for $S_2$ into cell E2 on the excel spreadsheet.

4.8. Ensure that the $a$ factor relates the signal from any given number of excited GFP molecules in channel 1 to the signal of an equal number of excited Cherry molecules in channel 2, and is defined by

$$a = \frac{Q_A \eta_A}{Q_D \eta_D},$$

where $Q_A$ and $Q_D$ are the fluorescence quantum yields of Cherry and GFP; $\eta_A$ and $\eta_D$ the detection efficiencies of acceptor and donor fluorescence in channels 2 and 1, respectively.

NOTE: The $a$ factor could be determined from two samples expressing known absolute amounts of GFP and Cherry. It is, however, impossible to know the exact amount of GFP and Cherry expressed in a cell. Therefore, we calculated the $a$ factor by using cells that express the GFP—Cherry fusion protein. Here, while the absolute amount is still unknown, the ratio of donor and acceptor molecules is known to be one.

4.9. Measure average intensities in channels 1, 2, 3 of cells expressing the GFP—Cherry fusion protein, and enter the measurements into the columns AE, AF, AG. Background intensities are subtracted (in AH, AI, AJ).

4.10. Calculate the $a$ factor (AJ column) from the fluorescence intensities in channel 1, 2 and 3 of the GFP—Cherry fusion protein as follows:

$$a = \frac{(I_1 - B_1) \times S_1 + \left(1 + \frac{\varepsilon^D(488)}{\varepsilon^A(488)}\right) \times (I_3 - B_3) \times S_2 - (I_2 - B_2)}{I_1 - B_1}$$

4.11. Background-corrected intensities measured in channel 1 ($I_1 - B_1$), 2 ($I_2 - B_2$), and 3 ($I_3 - B_3$), respectively, are measured using the GFP—Cherry chimera. The spectral cross talk factor $S_1$ was determined using cells expressing GFP only (see 4.7.). $\varepsilon^D$ and $\varepsilon^A$ are the extinction coefficients of GFP, the donor, and Cherry, the acceptor, at 488 nm, and can be determined from the literature ($\varepsilon^{GFP}$ = 53,000 $M^{-1}cm^{-1}$)[18] and the absorption curve of Cherry ($\varepsilon^{Cherry} \approx 5560$ $M^{-1}cm^{-1}$). The ratio $\frac{\varepsilon^D(488)}{\varepsilon^A(488)}$ has been entered in cell G2 of the excel spreadsheet. Enter the mean value for the $a$ factor into J2.

4.12. Use the determined $a$ factor for the calculation of the FRET efficiency, $E$, as follows (column AK):

$$E = \frac{(I_2 - B_2) - (I_1 - B_1) \times S_1 - (I_3 - B_3) \times S_2}{\alpha \times (I_1 - B_1) + (I_2 - B_2) - (I_1 - B_1) \times S_1 - (I_3 - B_3) \times S_2},$$

4.13. Alternatively, determine FRET efficiency, $E$, for the negative controls, i.e., the co-expression of GFP and Cherry and the expression of GFP alone by adding the measurements of

channels 1, 2, and 3 in the excel sheet in column AD, AE, and AF under the GFP—Cherry fusion protein measurements. Determine FRET efficiencies between the GFP and Cherry labeled proteins of interest in the same way.

4.14. Determine the unquenched donor intensity, $I_D$ as $(I_1 - B_1)/(1 - E)$, and the acceptor intensity as $I_A = I_3 - B_3$; these values are proportional to the expression levels of the tagged proteins.

4.15. Determine the corrected acceptor-to-donor intensity ratio ($Q$) of the GFP—Cherry fusion protein as follows (column AL):

$$Q = \frac{(I_3 - B_3) \times (1 - E)}{I_1 - B_1}$$

4.16. For other co-transfected cells, calculate the acceptor-to-donor molecular ratio $N_A/N_D$ as follows:

$$\frac{N_A}{N_D} = \frac{(I_3 - B_3) \times (1 - E)}{(I_1 - B_1) \times Q} = \frac{I_A}{I_D \times Q}$$

NOTE: The rationale to determine the $N_A/N_D$ ratio and plot the mean cellular FRET efficiency $E$ versus $N_A/N_D$, is that one donor molecule can transfer energy to multiple acceptors while an acceptor molecule can only receive energy from one donor at a given time. Even if only one acceptor can interact with a donor because of the stoichiometry of the interaction, an increase of acceptor concentration is expected to increase the fraction of donors in complex with the acceptor because of the law of mass action. Thus, for a fixed (or narrow range of) donor expression, the FRET efficiency should rise with increasing $N_A/N_D$. When plotting the FRET efficiency $E$ versus $N_A/N_D$ for the co-expression of GFP and Cherry, i.e. the negative probe, however, an increase in $N_A/N_D$ should not result in an increase in FRET efficiency (at least at sufficiently low acceptor concentrations where random FRET due to the vicinity of acceptor dyes to donor dyes does not occur).

**REPRESENTATIVE RESULTS:**

**Figure 1** shows the images obtained in the donor channel, channel 1 (488, 505-530 nm), the transfer channel, channel 2 (488, >585 nm), and the acceptor channel, channel 3 (561, >585 nm), respectively. Representative images of cells expressing GFP only, Cherry only, co-expressing GFP and Cherry, and expressing the GFP—Cherry fusion protein. The mean cellular FRET efficiencies calculated in NRK cells expressing GFP—Cherry fusion protein (positive control, **Figure 2A**) and those co-expressing GFP—Cherry (negative control, **Figure 2B**) are plotted versus the acceptor-to-donor ratio intensity ratio ($Q$) or molecular ratio $N_A/N_D$ in each cell. **Figure 2C** illustrates an example on how to outline a region of interest and avoid perinuclear vesicles with high

autofluorescence.

The presented algorithm can be used to quantify FRET efficiency in any region of interest including the quantification in every pixel of the image in the transfer channel. **Figure 2D** shows normalized pixel-by-pixel FRET images of cells expressing the GFP—Cherry fusion protein, co-expressing GFP and Cherry as negative control, and expressing receptor subunits of the Ashwell-Morell receptor. The rat variant of this receptor, the rat hepatic lectin (RHL1 and RHL2), is a two-subunit receptor system that is known to hetero-oligomerize. All FRET efficiencies were normalized to that of the GFP—Cherry fusion protein. We labeled RHL1 and 2 with GFP and Cherry on the cytoplasmic side of the plasma membrane. The image shows distinct FRET values at the plasma membrane compared to intracellular vesicles. Deleting the stalk domain of RHL1 (GFP-RHL1$\Delta$stalk) which is thought to mediate the tight interaction between the two subunits decreases the detected FRET efficiencies. In **Figure 2E**, mean cellular FRET efficiencies of cells expressing GFP-RHL1 and Cherry-RHL2, and GFP-RHL1$\Delta$stalk and Cherry-RHL2 are plotted versus the acceptor-to-donor molecular *ratio* ($N_A/N_D$). For further FRET analyses of this receptor system the reader may refer to a previous publication[5].

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Representative images of cells expressing fluorescent proteins.** Cells expressing GFP only (**A**), Cherry only (**B**), GFP and Cherry co-expression (**C**), and GFP—Cherry fusion protein (**D**). Images in channel 1 (488, 505-530 nm), channel 2 (488, >585 nm), and channel 3 (561, >585 nm), respectively. Images were obtained with 63x oil objective and zoom set to 3x. Scale bar: 10 μm.

**Figure 2: Quantification of FRET images.** Mean cellular FRET efficiencies for cells expressing the GFP—Cherry fusion protein plotted versus the acceptor-to-donor *intensity ratio* ($Q$) (**A**) and versus the acceptor-to-donor *molecular ratio* ($N_A/N_D$) for cells co-expressing GFP and Cherry (**B**). Open circle thick line represents single cell expressing GFP—Cherry fusion protein. Open circle thin line represents a cell co-expressing GFP and Cherry. Note that at higher $N_A/N_D$ ratios the fraction of the useful signal, the sensitized emission ($I_D E\alpha$) in the transfer channel becomes smaller and smaller relative to the direct excitation of the acceptor ($I_A S_2$). This results in a larger error in the determination of $E$. Pixel-by-pixel FRET images calculated using the presented algorithm (**C**). This panel illustrates a cell co-expressing GFP and Cherry, the negative control, in the donor channel, the transfer channel, and the acceptor channel. It also shows a possible outline of a region of interest avoiding perinuclear vesicles with high autofluorescence which may negatively impact the precision of the FRET calculation. (**D**) All FRET efficiencies were normalized to that of the GFP—Cherry fusion protein. From left to right, GFP—Cherry fusion protein (positive control), GFP Cherry co-expression (negative control), GFP-RHL1 and Cherry-RHL2, and GFP-RHL1$\Delta$stalk and Cherry-RHL2. Scale bar: 10 μm. Color-coded scale bar: normalized mean FRET efficiency (normalized to the mean value of the positive control, the GFP—Cherry fusion protein). (**E**) This panel shows mean cellular FRET efficiencies for cells expressing the GFP-RHL1 and Cherry-RHL2 as well as GFP-RHL1$\Delta$stalk and Cherry-RHL2 plotted versus the acceptor-to-donor molecular ratio ($N_A/N_D$). Closed grey circle represents a single cell expressing GFP-RHL1 and Cherry-RHL2.

Closed black circle represents a cell expressing GFP-RHL1Δstalk and Cherry-RHL2.

**Supplementary File 1: Algorithm for other donor-acceptor pairs.** Algorithm for other donor-acceptor pairs such as different versions of cyan (ECFP, CyPet, mTFP1, Cerulean, mTurquoise2) and yellow (EYFP, Citrine, Venus, SYFP2, YPet) fluorescent proteins.

**Supplementary File 2: Spreadsheet with the presented FRET algorithm and use of the GFP—Cherry fusion protein to quantify FRET by sensitized emission of the acceptor and donor-quenching.** Cells A2, B2, C2: Mean background signals ($B_1$, $B_2$, $B_3$) in channels 1, 2, and 3, respectively. Cells D2 and E2: Mean values for cross-talk factors $S_1$, and $S_2$. Cell G2: Value for extinction coefficient ratio $\frac{\varepsilon^D(488)}{\varepsilon^A(488)}$. Cell I1, and I2: Extinction coefficients of GFP and Cherry at 488-nm laser light. Cell J2: Mean value for $a$ factor. Column C (C5 and up): measured fluorescence intensity of a cell expressing GFP in channel 1. Column D (D5 and up): measured fluorescence intensity of a cell expressing GFP in channel 2. Column E (E5 and up): measured fluorescence intensity of a cell expressing GFP in channel 3. Columns F, G, and H (F5, G5, H5 and up, respectively): measured fluorescence intensities subtracted by mean background intensities in all 3 channels. Column I (I5 and up): Calculated cross-talk factor $S_1$. Column M (M5 and up): measured fluorescence intensity of a cell expressing Cherry in channel 1. Column N (N5 and up): measured fluorescence intensity of a cell expressing Cherry in channel 2. Column O (O5 and up): measured fluorescence intensity of a cell expressing Cherry in channel 3. Columns P, Q, and R (P5, Q5, R5 and up, respectively): measured fluorescence intensities subtracted by mean background intensities in all 3 channels. Column S (S5 and up): Calculated cross-talk factor $S_2$. Column W (W5 and up): measured fluorescence intensity of a non- or mock-transfected cell in channel 1. Column X (X5 and up): measured fluorescence intensity of a non- or mock-transfected cell in channel 2. Column Y (Y5 and up): measured fluorescence intensity of a non- or mock-transfected cell in channel 3. Column AD (AD5 and up): measured fluorescence intensity of a cell expressing the GFP—Cherry fusion protein (or co-expressing GFP and Cherry, or any protein pair of interest) in channel 1. Column AE (AE5 and up): measured fluorescence intensity of a cell expressing the GFP—Cherry fusion protein (or co-expressing GFP and Cherry, or any protein pair of interest) in channel 2. Column AF (AF5 and up): measured fluorescence intensity of a cell expressing the GFP—Cherry fusion protein (or co-expressing GFP and Cherry, or any protein pair of interest) in channel 3. Columns AG, AH, and AI (AG5, AH5, AI5 and up, respectively): measured fluorescence intensities subtracted by mean background intensities in all 3 channels. Column AJ (AJ5 and up): Calculated $a$ factor. Column AK (AK 5 and up): Calculated mean FRET efficiency $E$. Column AL (AL5 and up): calculated corrected acceptor-to-donor intensity ratio ($Q$). Examples for calculated parameters from a FRET experiment as expressed as mean and standard deviation: $S_1$ = 0.2232 $\pm$ 0.0060. $S_2$ = 0.2039 $\pm$ 0.0074. $a$ = 1.9463 $\pm$ 0.1409. $E$ = 0.2713 $\pm$ 0.0220.

**Supplementary Figure 1: GFP and Cherry absorption and emission spectra.** Normalized absorption and fluorescence emission spectra of eGFP and mCherry. The excitation laser lines (488 and 543 nm) and filter transmissions used for the donor channel (ch1: 505-530 nm) and the transfer/acceptor channels (ch2 & 3: >585 nm) in the confocal microscope are marked by shading. For excitation of the acceptor, 561 or 590-nm laser lines can also be used. Source

fluorescent protein data base (fpbase.org).

**DISCUSSION:**

The presented protocol details the use of the genetically coupled one-to-one fluorescent protein calibration probe for quantifying FRET using the detection of sensitized emission of the acceptor and quenching of the donor molecule by confocal microscopy. This method can be applied to assess protein interactions in the physiological context of the living cell in different subcellular compartments. Spatial resolution can be further improved by applying the presented algorithm to calculate FRET efficiencies in each pixel of an image (pixel-by-pixel FRET). Intensity-based determination of absolute FRET efficiencies requires the determination of the cross-talk, quantified here with the $S$ factors, and the detection efficiency of donor and acceptor molecules by the given microscopic set-up, quantified by the $a$ factor. Here, a protocol is provided that permits quantification of both cross-talk and detection efficiency. Direct coupling of the genetic information of fluorescent protein guarantees equimolar expression in live cells and thereby makes the determination of the $a$ factor possible. Knowledge of the $a$ factor in turn is a prerequisite for the quantification of the FRET efficiency in live cells. Critical steps in the provided protocol are the proper cloning of the directly coupled fluorescent protein chimera, sufficient time after transfection to allow for fluorescent protein maturation, use of fluorescent proteins in a similar cellular microenvironment, e.g., fluorescent proteins in cytoplasm and on cytoplasmic side of plasma membrane, and a stable microscope set-up.

Experimental set-up and algorithm presented here are designed for the GFP-Cherry FRET pair. We provide in the **Supplementary File 1** the algorithm for different versions of cyan (ECFP, CyPet, mTFP1, Cerulean, mTurquoise2) and yellow fluorescent proteins (EYFP, Citrine, Venus, SYFP2, YPet). The advantage of these fluorescent protein pairs is a greater spectral overlap between the emission spectrum of the cyan and the absorption spectrum of the yellow protein (as compared to GFP—Cherry) resulting in somewhat higher $R_0$ values and larger FRET efficiencies. However, for the same reason, the number of non-negligible crosstalk factors and their magnitudes are also larger (see Supplementary Text).

Limitations of quantitative microscopic ratiometric FRET in live cells need to be considered and is discussed. Prerequisite of using FRET for detecting molecular interactions is the application of fluorescent tags. Our protocol uses genetically encoded fluorescent proteins. Since these fluorescent proteins may be comparable in size (27 kDa) to the tagged protein of interest, they may change localization and function of the protein of interest. Therefore, both localization and functionality of any tagged protein of interest should be tested and compared with those of the endogenous unlabeled protein. Another critical point to keep in mind is the endogenous unlabeled pool of the protein of interest. The interactions of the labeled with unlabeled endogenous proteins will decrease the FRET signal. Ideally, all proteins of interest are labeled. This can be achieved by using cells which do not have the protein of interest endogenously (such as in the example given in Figure 2C and 2E), using cells derived from knock-in mice, using CRISPR modified cells, etc. Even with the use of pixel-by-pixel FRET, the signal of donor and acceptor molecules will be averaged over a diffraction-limited spot, the resolution limit of a confocal microscope. Therefore, it is impossible to resolve different donor populations within a

diffraction-limited spot. There might be donors without an acceptor, or donors with multiple acceptors contributing to the average FRET signal. Dissecting different molecular subpopulations by FRET requires fluorescence lifetime imaging[22]. Another problem, especially at high expression levels is the so-called random FRET between fluorophores in close proximity without underlying interaction of the proteins of interest[23]. This random FRET can be significant in the plasma membrane given the 2-D confinement of the membrane proteins compared to freely diffusive cytoplasmic proteins. Therefore, control experiments should always be done such as deleting domains that mediate the presumed interaction between molecules and test for reduction in the detected FRET signal.

The uncertainty of exact stoichiometries of interacting proteins in live cells limits the use of FRET as a spectroscopic ruler to assess molecular distances between proteins in live cells. Even in the case of a strict one-to-one interaction, (i) the flexibility of the linker attaching the fluorescent protein to the protein of interest as well as (ii) the lack of knowledge of the relative orientation of the dipole moments of the dyes (determining the so-called $\kappa^2$ factor) may confound exact distance measurements. Studies on acceptor fusion constructs with stiff linkers of different lengths separating the two fluorophores and displaying different FRET efficiencies have been reported elsewhere[12]. Conversely, assessing stoichiometries with FRET measurements in live cells is complex, however possible using the presented ratiometric FRET quantification. The inclined reader may be referred to previous work detailing a feasible approach[5].

In summary, the quantitative FRET approach presented here permits the detection of (i) protein interactions in the physiological context of the living cell, (ii) changes in protein interactions over time and (iii) differences in interactions in different subcellular compartments down to the pixel-by-pixel level of a confocal image, and (iv) the dependence of the detected FRET signal upon the molecular acceptor-to-donor ratio expressed in a live cell.

**ACKNOWLEDGMENTS:**

We would like to thank the Neuroscience Imaging Service at Stanford University School of Medicine for providing equipment and space for this project. This research was supported by intramural funding of the Stanford Cancer Institute and the Gynecologic Oncology Division Stanford as well as GINOP-2.3.2-15-2016-00026, GINOP-2.3.3-15-2016-00030, NN129371, ANN135107 from the National Research, Development and Innovation Office, Hungary.

**DISCLOSURES:**

The authors have nothing to disclose.

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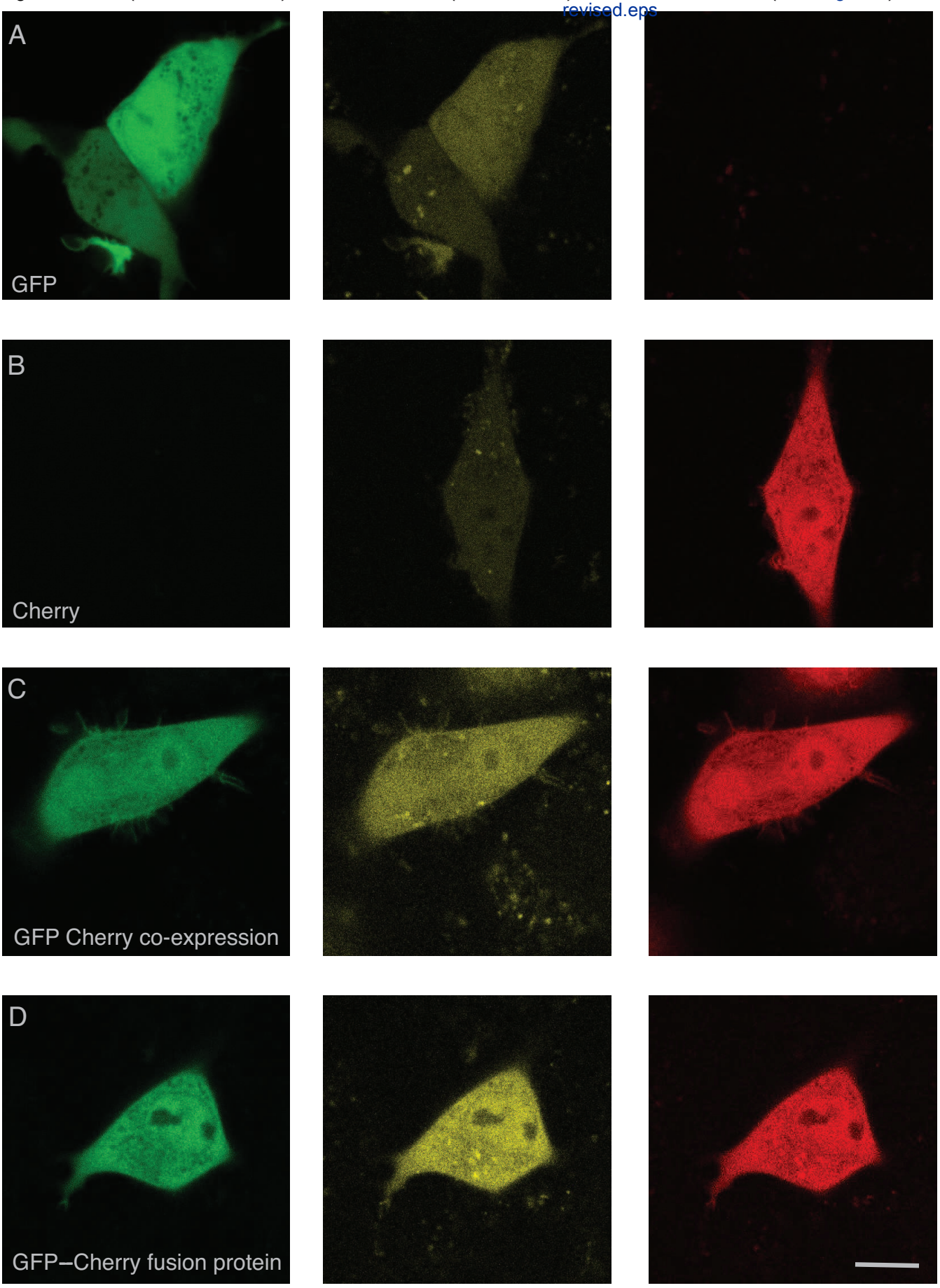


Vamosi et al. Figure 1

Figure



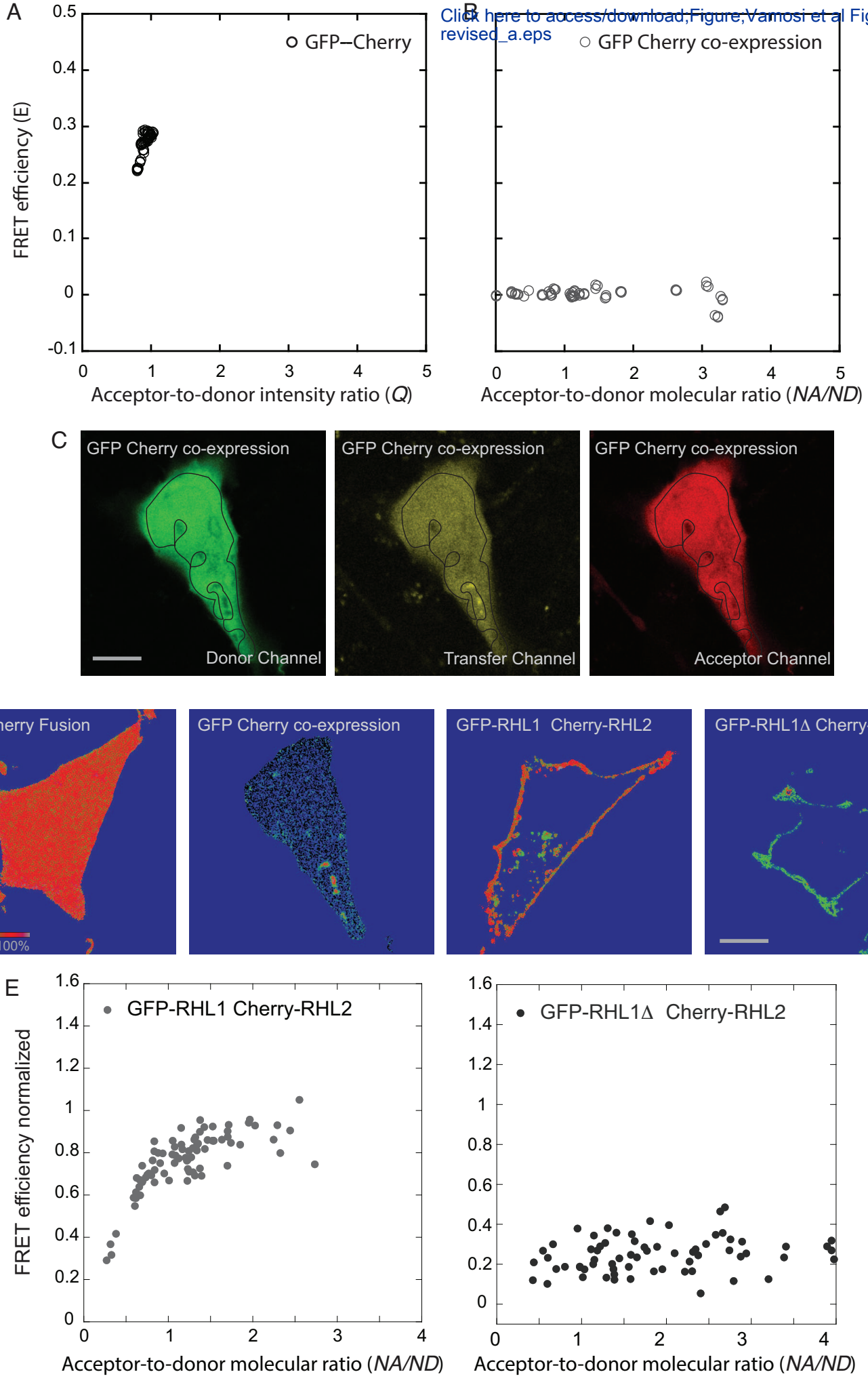

Table of Materials

Click here to access/download;Table of Materials;Vamosi, Renz_JoVE_Materials_revised.xls

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 0.5% Trypsin-EDTA without phenol red (10x) | Thermo Fisher Scientific | 15400054 | |
| Clontech mCherry N1 vector | Addgene | 3553 | |
| DMEM without phenol red | Thermo Fisher Scientific | 11054020 | |
| Fugene 6 | Promega | E2691 | |
| HEPES | Thermo Fisher Scientific | 15630080 | |
| LabTek 8-well chambers #1.0 | Thermo Fisher Scientific | 12565470 | |
| L-Glutamine (200 mM) | Thermo Fisher Scientific | 25030081 | |

Dear Editors,

We thank the reviewers for their detailed comments which helped improve the manuscript. Please see below the point-by-point answers to the reviewers. We uploaded the revised manuscript, the revised figure 2 and the new supplementary text, and the 3-page condensed protocol.

Thank you for your consideration of our manuscript.

Sincerely,
Malte

Malte Renz, MD, PhD
Gynecologic Oncology Division
Stanford University

**Editorial comments:**

1. The editor has formatted the manuscript to match the journal format. Please retain and use the attached file for revision.
2. Please address all the comments marked in the manuscript.

*Done*

3. Please address all reviewers' comments as well.

4. Please reword 52-54, 88-92 (primer sequence is ok), 105-109, 126, 150-157, 160-162, 165-168 as it matches with previously published literature.

*Done*

5. Once done please highlight up to 3 pages of protocol text including headings and spacings to be used for filming. Also, we cannot film calculations so please do not highlight step 4 of the protocol section.

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**Reviewers' comments:**

**Reviewer #1:**

Accept.

**Reviewer #2:**

Manuscript Summary:

The authors responded to most of my suggestions, but some might not be understood correctly.

Major Concerns:

>> The authors should explicitly explain importance of accurate quantification of FRET and what kind of information can be obtained by FRET and calculation proposed in this manuscript, for example, in the Introduction section.

> We thank the reviewer for bringing up this important aspect and expanded on it in the Introduction (page 2) and Discussion (page 11 and 12) section.

I would have liked to point out that what kind of information about protein interaction can be obtained by FRET measurement should be explained. For most of readers, the purpose is to investigate protein interaction. Measurement or quantification of FRET is just the means to achieve that. Protein interaction (the authors seem to assume binding interaction) has many aspects, including the structure of bound molecules, percentage of bound molecules, affinity of interaction, rates of association and dissociation, etc. Which of them or any other information can be obtained by the method the authors proposed? It should be explained why the readers have to use FRET measurement for investigating protein interaction.
The authors mentioned "interaction between proteins" and "spatial and temporal information" in the Introduction. If the purpose is only to detect binding interaction (occurrence of FRET), cancellation of spectral cross-talk by factor S1 may be sufficient. If relative difference or change in space or time should be detected, the apparent FRET efficiency Eapp = I2 / (I1 + I2) without any compensation may be sufficient to be compared. Why is more accurate calculation necessary?

*Thank you. We added another clarifying point to the end of the discussion. As detailed in introduction, protocol and discussion, only the assessment of the relative signal strength of an acceptor and a donor molecule allow the quantification of ratiometric FRET. The described quantification also allows the assessment of the detected FRET efficiency relative to the molecular acceptor-to-donor ratio in a live cell which otherwise would not be possible. Measuring different cells with different acceptor-to-donor ratios can give insight into the stoichiometry of the interacting proteins.*

Minor Concerns:

> We added the biological example of (i) the Ashwell Morell receptor, a two-subunit receptor system which is known to form hetero-oligomers, and the measurements of (ii) the same receptor subunits after deleting the stalk domain the domain mediating a tight interaction as 'biological control' (page 3: Results section and Figure 2C).

Why don't the authors make figures like Fig. 2A or B for new receptor samples. Fig.2 A and B can include data for all four samples shown in Fig. 2C for direct comparison.

*Added Figure.*

> We thank the reviewer for this comment and agree that a confocal laser scanning microscope is not a necessity to measure FRET. We decided to describe the protocol and actual steps for an actual microscope with the advantage that all steps can be shown in this video protocol.

It is still unclear how the "digital zoom" works. What is digital? I have never used microscopes equipped with "digital zoom".

*We recognize the issues with the use of 'digital' and 'optical' zoom and therefore talk in the current manuscript only about 'zoom'.*
*In commercial confocal laser scanning microscopes, the zoom which the user can apply through the software interface is often times a composite of optical and digital zoom. We recommend to use a zoom factor which fits a cell best in a picture and results in a pixel size of 60-80 nm as indicated in the manuscript.*
*(Optical zoom is achieved by reducing the angle of the scan mirrors (reducing the size of the scanned area). A smaller area is now represented by the same number of pixels. Thereby, the pixel size is reduced. This is limited by the optical resolution. Digital zoom is found in image processing software.)*

>> How large is variance of each parameter when calculated from experimental results? Examples of how those parameters calculated from 15-20 cells disperse should be shown. Practically, it is important how to treat those parameters when they have large variance. It should be explained.
> We thank the reviewer for this question. To show the variance of the results, the FRET plots have been implemented showing the measurements in a single cell and the variance from cell to cell (Figure 2).

I asked about variance of parameters (factors), such as S1, S2, a (α). They are not shown anywhere.
These factors are essential for calculation, but I guess they can largely vary cell to cell because intracellular environment may differ. The authors should show that calculated factors do not largely vary among cells or the guidance of how to treat those factors if obtained with large variance.

*Standard deviations for parameters S1, S2, $\alpha$, E added to the description of the supplementary material.*

>> Section 3.6., 3.7.: How many cells should be measured? 15-20 cells for each condition?
> Please see above. It is also advisable to repeat experiments three times independently.
It is still unclear. The authors claimed that three images are necessary to check photobleach as in 3.4. Imaging 15-20 cells is to calculate compensation factors in 3.6. So, in 3.7, because control samples are measured to decide other compensation factors, 15-20 cells should be imaged? In 3.8, where target proteins are measured, how many cell images are necessary to get conclusion on protein interaction?

*15-20 cells. Added to 3.8. In general, the more cells you measure, the better the statistics.*

>> Section 4.3.: Explain the definition of 'α' at first appearance. And 'α' and 'a' are used in the following part of the manuscript. If they are same, unify them. If different, explain.
> We expanded on the explanation of the importance of the factor, especially in Introduction (page 2) and Discussion section (pages 11 and 12).
I did not request such explanation. In the initial manuscript, 'α' was first introduced in 4.3 without any explanation. In the same section, other parameters were introduced with explanation, like " The FRET signal I2", "the cross-talk factor S2" and "B2 is the background signal".
Details of calculation should not be described in the Introduction.
Both 'a' and 'α'(alpha) are still used in the manuscript.

*As requested, we referred to the $\alpha$ factor in 4.3.*

**Reviewer #3:**
The revised version of the manuscript is improved, but I still have some recommendations.
1) Delete the adjective "flexible" in line 93. In fact, the inclusion of proline residues might increase the stiffness (Chen et al, 2013).

*Done.*

2) Add reference of the supplemental figure 1(emission and absorption spectra) to 3.2 section.

*Added source, fluorescent protein data base (fpbase.org).*

3) Add figure with example of regions of interest without perinuclear vesicles and reference to it in line 215-216.

*Added figure.*

4) Add your explanation of negative E in region NA/ND >3 to the manuscript.

*Done*

5) Change 2B to 2C in line 316 (typo).

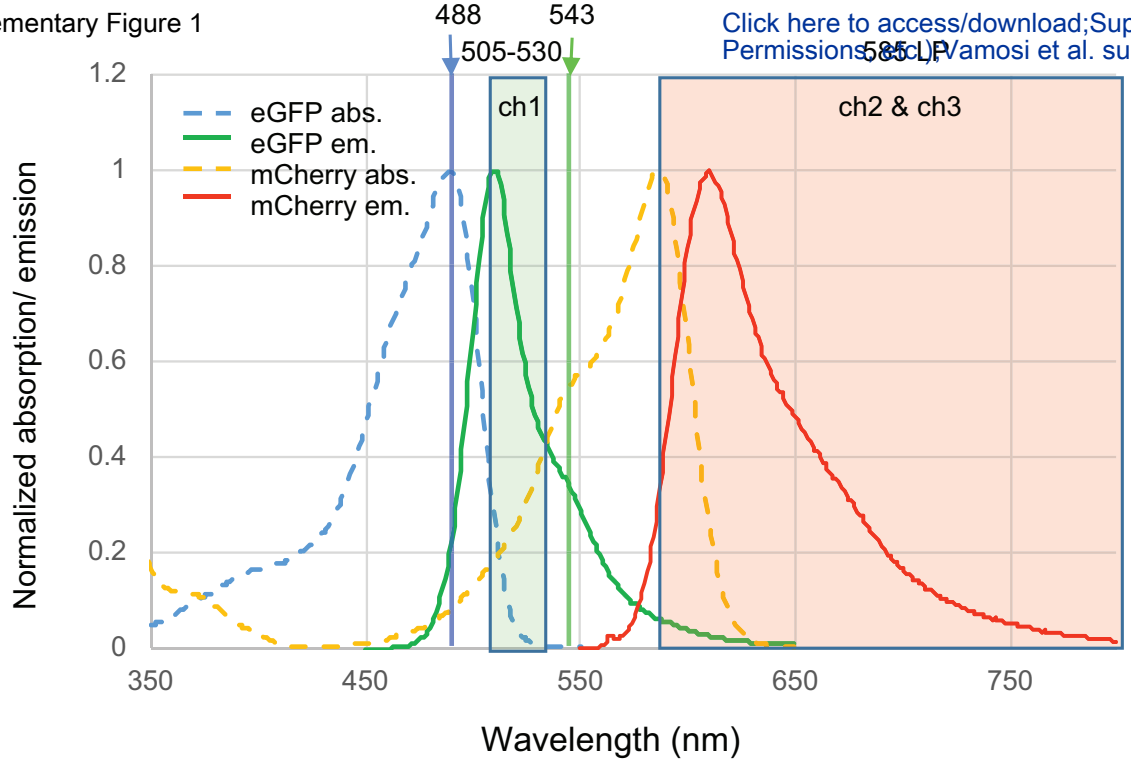
*Done*

6) Describe calculation of normalized mean FRET efficiency (Figure 2C). How was FRET normalized?

*We added the Figure 2D legend that the FRET values were normalized to the positive control GFP—Cherry fusion protein.*

Supplementary Figure 1





**Supplementary Text**

Other donor-acceptor pairs such as different versions of cyan (ECFP, CyPet, mTFP1, Cerulean, mTurquoise2) and yellow (EYFP, Citrine, Venus, SYFP2, YPet) fluorescent proteins are also suitable for FRET measurements[19-21]. The advantage of these pairs is a greater spectral overlap between the emission spectrum of the cyan and the absorption spectrum of the yellow protein (as compared to GFP-Cherry) resulting in somewhat higher $R_0$ values and larger FRET efficiencies. However, for the same reason, the number of non-negligible crosstalk factors and their magnitudes are also larger. For exciting the donor e.g. 405- or 458-nm, for the acceptor 488- or 514-nm laser lines are often used. The most general form of the FRET equations for cyan-yellow FRET pairs is given below[17].

$$I_1 = I_D(1-E) + I_A S_4 + I_D E\alpha \frac{S_4}{S_2}$$
$$I_2 = I_D(1-E)S_1 + I_A S_2 + I_D E\alpha$$
$$I_3 = I_D(1-E)S_3 + I_A + I_D E\alpha \frac{\varepsilon_4}{S_2}$$

For simplicity, all $I_x$ fluorescence intensities in the equations are corrected for autofluorescence ($B_x$ values based on non-labeled cells are subtracted). The spectral cross-talk factors $S_1$ and $S_3$ are calculated from cells expressing the cyan protein alone:

$$S_1 = \frac{I_2^D}{I_1^D} \qquad S_3 = \frac{I_3^D}{I_1^D}$$

$S_2$ and $S_4$ are measured using cells expressing the yellow protein alone:

$$S_2 = \frac{I_2^A}{I_3^A} \qquad S_4 = \frac{I_1^A}{I_3^A}$$

Depending on the actual excitation wavelengths and emission filters used, $S_3$ or $S_4$ may be negligible. This can be directly measured, or checked by comparing the absorption and emission spectra of the dyes with the laser wavelengths and transmission ranges of the emission filters. The terms $\varepsilon_2$ and $\varepsilon_4$ are ratios of the extinction coefficients of cyan and yellow proteins at the wavelengths used for exciting the donor ($\lambda_D$, e.g. 405 or 458 nm) and the acceptor ($\lambda_A$, e.g. 488 or 514 nm):

$$\varepsilon_2 = \frac{\varepsilon^{cyan}(\lambda_D)}{\varepsilon^{yellow}(\lambda_D)} \qquad \varepsilon_4 = \frac{\varepsilon^{cyan}(\lambda_A)\varepsilon^{yellow}(\lambda_D)}{\varepsilon^{cyan}(\lambda_D)\varepsilon^{yellow}(\lambda_A)}$$

Depending on the excitation wavelengths used, the value of $\varepsilon_4$ may be negligible (e.g., for $\lambda_A$=514 nm because cyan proteins do not absorb at this wavelength; however, they do at 488 nm).

The α factor can be determined using a fusion protein expressing the cyan and yellow proteins (e.g. ECFP-EYFP) at a 1:1 ratio as

$$\alpha = \frac{S_2\left(I_1\left(\left(1+\varepsilon_2\right)S_2S_3 - \left(1+\varepsilon_2\varepsilon_4\right)S_1\right) - \left(1+\varepsilon_2\right)I_3\left(S_2 - S_1S_4\right) + I_2\left(1 - S_3S_4 + \varepsilon_2\left(\varepsilon_4 - S_3S_4\right)\right)\right)}{\left(\varepsilon_4 - 1\right)\left(I_1S_2 - I_2S_4\right)}$$

The mean pixelwise FRET efficiencies are calculated as

$$E = \frac{S_2\left(I_2 - I_1S_1 - I_3S_2 + I_1S_2S_3 + I_3S_1S_4 - I_2S_3S_4\right)}{\alpha\left(1-\varepsilon_4\right)\left(I_1S_2 - I_2S_4\right) + S_2\left(I_2 - I_1S_1 - I_3S_2 + I_1S_2S_3 + I_3S_1S_4 - I_2S_3S_4\right)}$$

Note that the above formulas become simpler if any of the $S_3$, $S_4$ or $\varepsilon_4$ factors are negligible in the given microscope setup.

Supplemental File 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary File 2.xls

| B1 | B2 | B3 | S1 | S2 | | eGFP/mC | e(GFP,48 | 53000 | alfa | |
|---|---|---|---|---|---|---|---|---|---|---|
| 338.1713 | 356.8034 | 370.4393 | 0.22325 | 0.203902 | | 9.532374 | e(mCh1,4 | 5560 | | 1.71 |

| | Ch1 | Ch2 | Ch3 | D1-B1 | D2-B2 | D3-B3 | S1 |
|---|---|---|---|---|---|---|---|
| GFP 1 | 2449.99 | 817.28 | 367.76 | 2111.819 | 460.4766 | -2.6793 | 0.218047 |
| | 2511.7 | 833.01 | 368.48 | 2173.529 | 476.2066 | -1.9593 | 0.219094 |
| | 2395.02 | 807.92 | 366.93 | 2056.849 | 451.1166 | -3.5093 | 0.219324 |
| GFP 2 | | | | | | | |

| | Ch1 | Ch2 | Ch3 | A1-B1 | A2-B2 | A3-B3 | S2 |
|---|---|---|---|---|---|---|---|
| **Cherry 1** | 340.74 | 668.45 | 1903.88 | 2.5687 | 311.6466 | 1533.441 | 0.203234 |
| | 340.67 | 667.6 | 1899.63 | 2.4987 | 310.7966 | 1529.191 | 0.203243 |
| | 340.62 | 661.88 | 1881.42 | 2.4487 | 305.0766 | 1510.981 | 0.201906 |
| **Cherry 2** | | | | | | | |

| | Ch1 | Ch2 | Ch3 | |
|---|---|---|---|---|
| **Unlabeled 1** | 336.43 | 344.08 | 363.26 | **Fusion 1** |
| | 336.5 | 344.39 | 363.52 | |
| | 336.67 | 343.55 | 363.47 | |
| **Unlabeled 2** | | | | **Fusion 2** |

| Ch1 | Ch2 | Ch3 | D-B1 | T-B2 | A-B3 | alfa | E | Q |
|---|---|---|---|---|---|---|---|---|
| 938.04 | 1106.57 | 1141.21 | 599.8687 | 749.7666 | 770.7707 | 1.732779 | 0.308990688 | 0.887877 |
| 935.47 | 1103.72 | 1133.43 | 597.2987 | 746.9166 | 762.9907 | 1.716073 | 0.309586346 | 0.881936 |
| 935.69 | 1097.27 | 1134.67 | 597.5187 | 740.4666 | 764.2307 | 1.730774 | 0.306342552 | 0.887193 |

Förster Resonance Energy Transfer (FRET) is the radiationless transfer of energy from an excited donor to an acceptor molecule and permits to study the interaction of proteins in the living cell over time and in different subcellular compartments.
Here, a step-by-step experimental protocol and mathematical algorithm is provided to quantify FRET using donor quenching and acceptor sensitized emission, a method often referred to as ratiometric FRET. The quantification of FRET efficiencies in the living cell requires determining (i) the crosstalk of the fluorescent proteins and (ii) the detection efficiency of the fluorophores in the microscopic setup.
Crosstalk can be assessed by imaging cells expressing only one fluorophore. The assessment of the detection efficiency of donor and acceptor molecules requires the knowledge of the number of donor and acceptor molecules giving rise to the measured signal. The number of fluorophores expressed in live cells varies, however, from cell to cell and is unknown. The here introduced calibration probe, the 1-to-1 donor-acceptor fusion protein, permits the determination of the detection efficiency.

**1. Plasmid construction**

1.1. For generating the eGFP-mCherry1 fusion probe, use an N1 mammalian cell expression vector with mCherry1.

1.2. Design oligo-nucleotides to amplify eGFP[11] without a stop codon as a *SalI-BamHI* fragment. The five amino acid linker between the green and red fluorescent protein yields a mean FRET efficiency for the GFP Cherry donor-acceptor pair of 0.25 - 0.3.

**2. Cell culture and transfection**

2.1. Use any cell line but media, e.g. Dulbecco's modified Eagle media (DMEM), *without* phenol red, to reduce background fluorescence.

2.2. Once cells are 80% confluent, detach cells with 1 mL 0.05% trypsin-EDTA, seed about 10,000 cells per well of an 8-well by using 3 drops from a 5-mL cell suspension from a confluent culture grown in a T 12.5-cell culture flask.

2.4. 24 h after plating transfect the cells using an appropriate transfection media.

2.5. Let 20 h after transfection elapse before live-cell imaging, to allow for proper fluorescent protein expression, folding and maturation, especially of the red fluorophore.

**3. FRET Imaging**

3.1. Image cells in a humidified and heated environmental chamber at 37 degrees Celsius. To buffer the cell media at physiological pH, add 20 mM HEPES to render the cell media $CO_2$-independent.

3.2. Use a confocal laser scanning microscope.

3.2.1. Use the 488-nm line of the argon ion laser to excite GFP and the 561-nm diode laser (or 543-nm Helium Neon laser) to excite Cherry.

3.2.2. Set the dichroic mirror to 488/ 561. Collect fluorescence using 488-nm laser light for excitation in channel 1 through an emission band of 505 - 530 nm and in channel 2 with a long pass filter >585 nm, and using the 561-nm laser light for excitation in channel 3 with a long pass filter > 585 nm.

3.3. Excite with the two lasers sequentially, and set the imaging mode to 'switch after each line' so that the excitation of the 512 x 512 pixels image alternates after each line.

3.4. Set up a mini-time series of three images to detect if significant photobleaching occurs, and potentially reduce the laser power.

3.5. First, image cells expressing the GFP—Cherry fusion construct. Set the parameters that define time-integrated laser intensity per pixel in a confocal image.

3.5.1. Using a 63x oil objective and zoom set to 3x allows imaging of a cell in its entirety while providing sufficient magnification and resolution. Aim for a pixel size of 70-80 nanometer.

3.5.2. Set pixel dwell time to 2-4 μs and AOTF transmission for the 488-nm and 561-nm laser such that images show a good signal-to-noise ratio without any bleaching and no pixels indicating fluorescence intensity saturation. It is advantageous to adjust the laser power of 488 and 561 such that signal levels in channel 1 and channel 3 are similar.

3.5.3. Photomultiplier (averaging mode) gain should be set to 600-800.

3.6. Image with these settings, 15-20 cells expressing the GFP—Cherry fusion protein, cells expressing GFP, Cherry, GFP and Cherry and non-transfected cells.

3.8. Then, image cells co-expressing your proteins of interest coupled to GFP and Cherry, respectively.

**4. Image analysis for detecting absolute FRET efficiencies using donor quenching and sensitized emission**

For a detailed description on how to calculate FRET, we would like to refer to the online manuscript and the provided excel spread sheet. However, we would like to briefly describe the signals measured in the three different channels set up as described above

4.1. Measure the donor signal $I_1$ in channel 1, the donor channel, with 488-nm excitation and an emission band of 505 – 530 nm.

$$I_1(488, 505-530) = I_D(1-E) + B_1\,,$$

where $I_D$ is the unquenched donor signal in channel 1 that would be measured in the absence of an acceptor, $E$ is the mean FRET efficiency, and $B_1$ the average background signal in channel 1.

4.2. Measure the acceptor signal $I_3$ in channel 3, the acceptor channel, with 561-nm excitation and emission at >585 nm.

$$I_3\ (561, > 585) = I_A + B_3\ ,$$

where $I_A$ is the acceptor signal and $B_3$ the background in channel 3.

4.3. Measure the FRET signal $I_2$ in channel 2, the transfer channel, with 488-nm excitation and emission at >585 nm.

$$I_2\ (488, > 585) = I_D(1 - E)S_1 + I_A S_2 + I_D E\alpha + B_2\ ,$$

The signal in channel 2 is a sum of four different components: (i) $I_D(1 - E)S_1$ is the spectral spill over from the quenched donor signal into the >585 detection channel (with the cross-talk factor $S_1$), (ii) $I_A S_2$ is the acceptor signal from the direct excitation by 488-nm light (with the cross-talk factor $S_2$), (iii) $I_D E\alpha$ is the sensitized emission of the acceptor by FRET from the excited donor molecule, and (iv) $B_2$ is the background signal.

For a detailed workflow how to use the reader's data and the provided excel sheet to calculate FRET, please refer to the online manuscript.

The quantitative FRET approach presented here permits the detection of
(i) protein interactions in the physiological context of the living cell,
(ii) changes in protein interactions over time and
(iii) differences in interactions in different subcellular compartments down to the pixel-by-pixel level of a confocal image, and
(iv) the dependence of the detected FRET signal upon the molecular acceptor-to-donor ratio expressed in a live cell.