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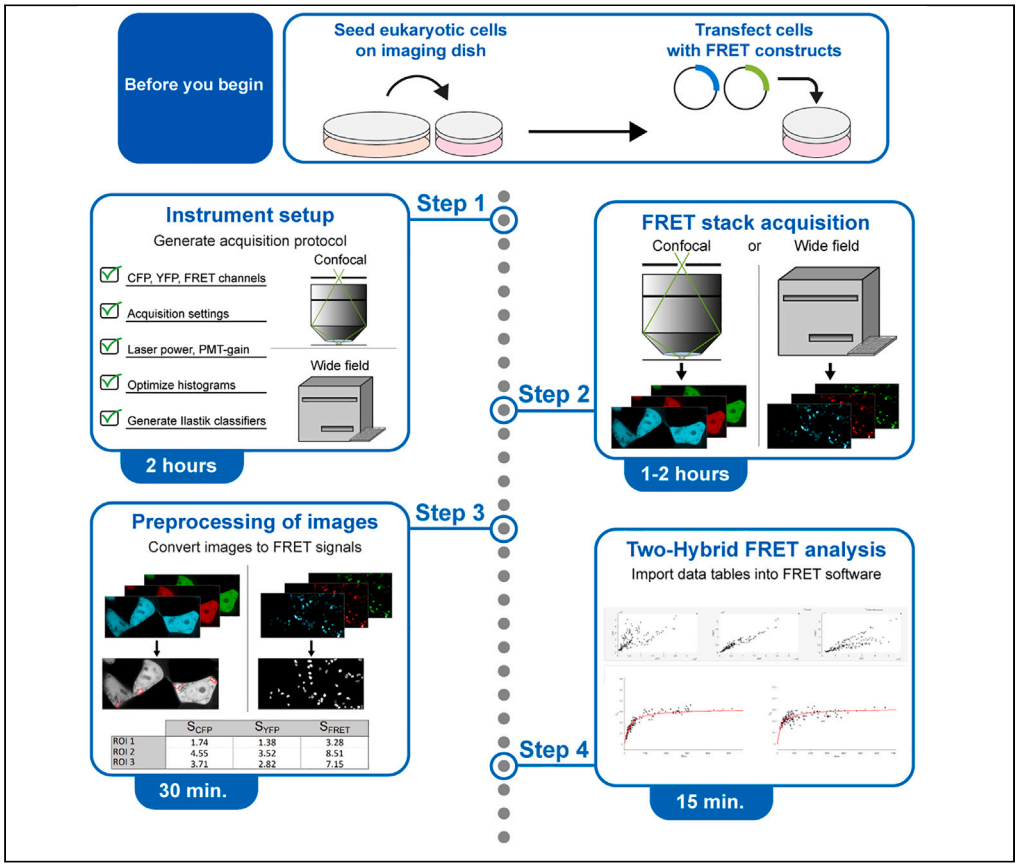
Protocol

Protocol for deriving proximity, affinity, and stoichiometry of protein interactions using image-based quantitative two-hybrid FRET



Two-hybrid Förster resonance energy transfer (FRET) provides proximity, affinity, and stoichiometry information in binding interactions. We present an image-based approach that surpasses traditional two-hybrid FRET assays in precision and robustness. We outline instrument setup and image acquisition and further describe steps for image preprocessing and two-hybrid FRET analysis using provided software to simplify the workflow. This protocol is compatible with confocal microscopes for high-precision and imaging plate readers for high-throughput applications. A plasmid-based reference system supports fast establishment of the protocol.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

FRET imaging derived proximity, affinity, and stoichiometry of protein interactions

Graphical analysis software for rapid and intuitive data evaluation

Included reference system for quick establishment of the FRET assay

Adaptable to a variety of confocal or wide-field instruments

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Protocol for deriving proximity, affinity, and stoichiometry of protein interactions using image-based quantitative two-hybrid FRET

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SUMMARY

Two-hybrid Förster resonance energy transfer (FRET) provides proximity, affinity, and stoichiometry information in binding interactions. We present an image-based approach that surpasses traditional two-hybrid FRET assays in precision and robustness. We outline instrument setup and image acquisition and further describe steps for image preprocessing and two-hybrid FRET analysis using provided software to simplify the workflow. This protocol is compatible with confocal microscopes for high-precision and imaging plate readers for high-throughput applications. A plasmid-based reference system supports fast establishment of the protocol.

For complete details on the use and execution of this protocol, please refer to Rivas et al.[1]

BEFORE YOU BEGIN

Förster resonance energy transfer (FRET) is a non-radiative process where energy is transferred between a donor and an acceptor located in close proximity to each other (1–10 nm). In case donor and acceptor molecules are close enough for FRET to occur, the transfer of energy from the donor to the acceptor results in reduced donor emission (donor quenching) and increased acceptor emission (sensitized emission) (Figure 1A). The Two-Hybrid FRET assay quantifies these FRET related changes in emission[1,2] using three distinct spectral channels (CFP, FRET, YFP) to measure fluorescence intensities as shown in Figure 1B. These channels are labeled for consistency according to previous publications.[2] It is important to note that in this protocol we employ mTurquoise2 and mVenus as FRET donor and acceptor fluorophores due to their improved photo-physical properties in comparison to CFP and YFP.[3] The FRET assay presented here allows for absolute quantification of donor- and acceptor-related FRET efficiencies (E_D and E_A),[4] which are comparable between different instruments. Two distinct binding curves are generated for each protein-protein interaction, correlating E_D and E_A with the concentration of the corresponding FRET partner (Figure 1I). These binding curves provide additional information about protein binding stoichiometry[5] and relative protein binding affinity ($K_{D,EFF}$).[6]

This protocol describes an image-based approach to determine Two-Hybrid FRET binding curves from fluorescence images (i_{CFP}, i_{FRET}, i_{YFP}) acquired with a confocal microscope or wide-field plate reader system in the corresponding channels. We also provide a software package that automates the workflow: It

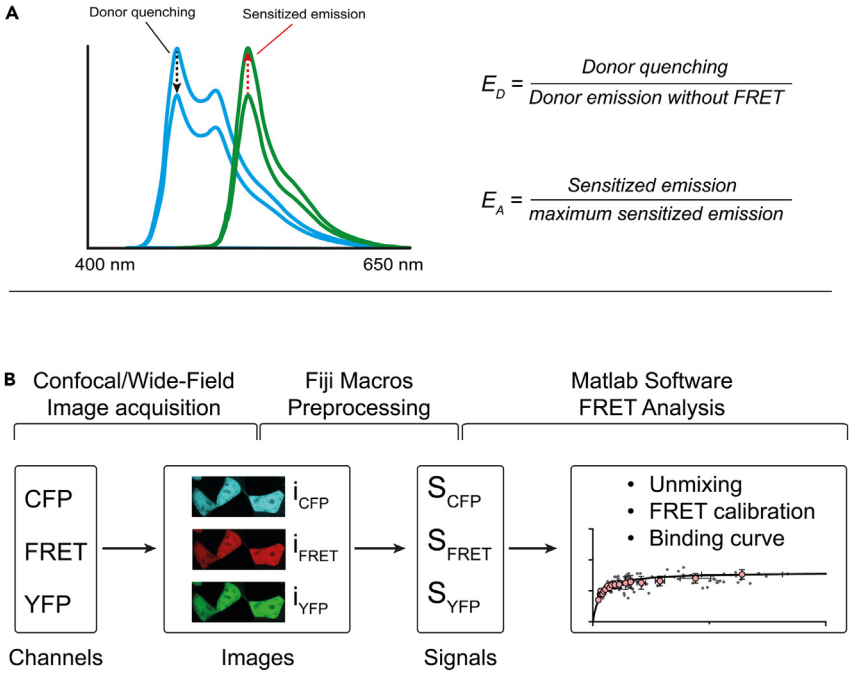


Figure 1. Principles of Two-Hybrid FRET

(A) The fluorescence emission spectra for mTq2 (blue) and mVenus (green) are shown in the absence and presence of FRET. FRET is observed as decreased fluorescence emission of the donor fluorophore (donor quenching, black dotted arrow) and increased emission of the acceptor (sensitized emission, red dotted arrow). Quantifying donor quenching and sensitized emission yields two distinct FRET efficiencies: E_D for the donor and E_A for the acceptor.

(B) Conceptual framework of image-based Two-Hybrid FRET. Images are acquired via three distinct acquisition channels. ROI data from these images are converted into fluorescence data tables. Data tables are imported into the FRET analysis software for analysis.

includes Fiji macros that convert hand-selected ROIs or automatically detected cells from images to fluorescence signals (S_{CFP}, S_{FRET}, S_{YFP}). A custom Matlab script can then be used to calculate the Two-Hybrid FRET binding curves from these signals for an immediate protein binding analysis (Figure 1B).

The protocol details the steps for an LSM980 (Zeiss™) confocal microscope and alternatively a Cytation5 (Biotek™) wide-field imaging plate reader. The protocol can be adapted to other confocal or wide-field instruments that can capture fluorescence images in the required CFP, FRET, and YFP spectral channels.

We also provide a set of reference constructs, comprising essential calibration constructs and two test protein interaction partners, which are known to interact with each other and which have been validated by the FRET Two-Hybrid method (Table 1). These constructs can be used in order to establish the technique.

Table 1. Required samples for Two-Hybrid FRET assays using the set of reference constructs

Determined parameters	Constructs transfected	File name (*czi, *csv)	Availability
Spectral correction R_{A1}, R_{D1}, R_{D2}	mTq2 (donor only) mVen (acceptor only)	mTq2 mVen	AddGene#198196 AddGene#198192
Calibration constants G_{ratio}, F_{ratio}	Dimer D3 Dimer D42 Dimer DT2A	D3 D42 DT2A	AddGene#198197 AddGene#198198 AddGene#198199
Test interactions	mTq2-CaM + mVen-IQ-WT mTq2-CaM + mVen-IQ-6A	myo_cam-WT or myo_cam-6A	AddGene#198200 AddGene#198201 AddGene#198202
Scramble DNA	empty vector backbone	bg	Thermo Fisher Scientific (CAT:V79020)

Cell culture

Timing: 3 days

This section describes the steps for culturing HEK293T cells. Other adherent cell lines can also be used for the experiments described in the protocol. We have successfully used HEK293T, Hela and CHO cells.

1. Seed 1 × 10^5 HEK293T cells in 6 Well Plates (Thermo Fisher Scientific, Cat#140675).
2. On the following day, seed transfected cells on optical-grade glass bottom imaging dishes (Ibidi, Cat#81158) or well plates (Cellvis, Cat#P06-1.5H-N) that contain a glass bottom with a thickness of 1.5H (170 μm) or lower.
3. Transfect FRET constructs on the next day, using Lipofectamine 2000 (Thermo Fisher Scientific, Cat#11668030). Follow the manufacturer's instructions for preparing transfection solutions (https://www.thermofisher.com/de/de/home/references/protocols/cell-culture/transfection-protocol/lipofectamine-2000.html).
   a. Use 1 μg DNA for each construct specified in Table 1, except the test interaction.
   b. Transfect multiple stoichiometries of the test interaction at varying ratios (up to 2 μg:0.5 μg DNA for Donor:Acceptor and vice versa).
4. Prior to imaging, replace growth medium with Tyrode's solution. This decreases background fluorescence and increases overall image quality.

Note: Imaging dishes with similar specifications can be used, for instance Nunc™ glass bottom imaging dishes (Thermo Fisher Scientific, Cat#150682).

Note: Other lipophilic transfection reagents can be used instead. An alternative is Turbofect (Thermo Fisher Scientific, Cat#R0533)

This set includes two constructs for determining spectral correction factors. These are expression plasmids for mVenus (mVen) or mTurquoise2 (mTq2) alone. mTq2 and mVen are significantly less pH sensitive, more resistant to photobleaching and have a larger Förster radius than CFP and YFP fluorophores. Furthermore, three dimer constructs are used to determine FRET calibration coefficients. These dimers contain mTq2 and mVen separated by a linker composed of 3 (Dimer D3), 42 (Dimer D42) amino acid residues or by a cleavable T2A sequence (Dimer DT2A). The T2A sequence is cleaved immediately after translation, resulting in a 1:1 stoichiometry of mTq2 and mVenus in the cell without being covalently bound to each other. In addition, three constructs are included, by which two different FRET binding curves can be determined and by which the method can be established. The first construct is designed by fusing the fluorescent protein mTurquoise2 to the N-terminus of the Ca^{2+} sensor protein calmodulin (CaM). It is termed mTq2-CaM. For the second construct (mVen-IQ-WT) the fluorescent protein mVenus is fused to the N-terminus of a canonical CaM binding IQ-domain, in this particular case, to the IQ-domain 6 of MyosinVa (Figure 2). The third construct, termed mVen-IQ-6A, is similar to mVen-IQ-WT, but the arginine residue in position 6 of the IQ motif is mutated to alanine. This mutation lowers the affinity of the IQ-domain to CaM. The interaction between mTq2-CaM and mVen-IQ-WT or mVen-IQ-6A are robust, have been validated in FRET experiments (Figure 11) and will be used as test pairs to determine binding curves. Once the whole FRET assay is established, any pair of proteins of interest can be used instead. For the background sample, use an empty expression vector for transfection. The second rightmost column displays the file names for the provided sample data, while the rightmost column indicates the corresponding Addgene ID.

Download and install software

Timing: 15 min

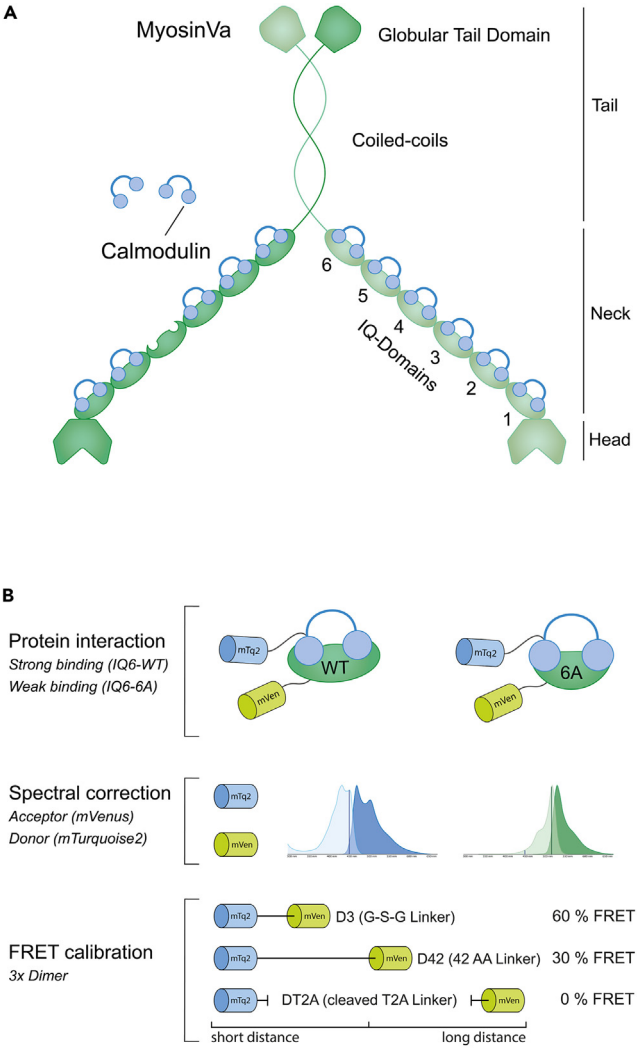


Figure 2. Two Hybrid FRET: Set of reference constructs

(A) Cartoon of myosinVa with bound calmodulin (CaM) proteins. A dimer of two myosinVa monomeric proteins is shown. The head, neck and tail region of MyosinVa monomers are indicated. The neck region contains 6 canonical IQ domains, each of which bind calmodulin. The constructs mVen-IQ-WT and mVen-IQ-6A are derived from the IQ domain 6 of myosinVa.

(B) Set of reference constructs. Protein interaction: Co-transfection of mTq2-CaM with either IQ6-WT or IQ6-6A results in two protein interactions exhibiting distinct binding affinities. Spectral correction: Spectral correction factors are determined by measuring the fluorescence of the expressed donor-only (mTurqoise2) and acceptor-only (mVenus) constructs. FRET calibration: Three constructs coding for linked donor-acceptor dimers with distinct linker lengths that result in defined FRET efficiencies are provided as indicated. The linker region of the DT2A dimer is cleaved after translation.

This section lists the software that is necessary for the protocol.

5. Required third party software:
   a. Download and install Matlab: https://www.mathworks.com/help/install/install-products.html.
   b. Install the Matlab Toolboxes "Curve Fitting", "Signal Processing" and "Statistics and Machine Learning".
   c. Download and install Fiji: https://imagej.net/software/fiji/downloads.
   d. Download and install Ilastik: https://www.ilastik.org/.
   e. Install the Ilastik Fiji-plugin: https://www.ilastik.org/documentation/fiji_export/plugin.

6. Download custom made software written for this protocol: [https://doi.org/10.5281/zenodo.7625519].

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
HEPES	Merck	Cat#7365-45-9
KCl	Merck	Cat#7447-40-7
$CaCl_2$	Sigma-Aldrich	Cat#10043-52-4
$MgCl_2$	Sigma-Aldrich	Cat#7786-30-3
D-Glucose	Sigma-Aldrich	Cat#50-99-7
NaCl	Sigma-Aldrich	Cat#7647-14-5
Cycloheximide solution	Sigma-Aldrich	Cat#66-81-9
Critical commercial assays		
Lipofectamine 2000 Transfection Reagent	Thermo Fisher Scientific	Cat#11668019
Turbofect Transfection Reagent	Thermo Fisher Scientific	Cat#R0533
Deposited data		
Plate Reader – exemplary images (.tif export)	This paper	https://zenodo.org/record/7670415
Plate Reader - ILASTIK trained cell-and object identifier (exemplary data)	This paper	https://zenodo.org/record/7670415
Plate Reader - CSV Processed data tables for MatLab import (exemplary data)	This paper	https://zenodo.org/record/7670415
Confocal microscope - exemplary images (.czi export)	This paper	https://zenodo.org/record/7670415
Confocal microscope - ROI-maps for the images to import in FIJI (exemplary data)	This paper	https://zenodo.org/record/7670415
Confocal microscope - XLS data tables for MatLab import (exemplary data)	This paper	https://zenodo.org/record/7670415
Flow Cytometer – exemplary data (.fcs2.0 export)	This paper	https://zenodo.org/record/7670415
Flow Cytometer – gated .ffa files for MatLab import (exemplary data)	This paper	https://zenodo.org/record/7670415
Wide-Field Photometry –.xls files for MatLab import (exemplary data)	This paper	https://zenodo.org/record/7670415
Experimental models: Cell lines		
HEK293t	DSMZ	Cat# ACC-635; RRID:CVCL_0063
Recombinant DNA		
Plasmid: Dimer D3: mTurquoise2-mVenus with a 3 amino acid linker	This paper	Addgene: 198197
Plasmid: Dimer D42: mTurquoise2-mVenus with 42 amino acid linker (randomized sequence)	This paper	Addgene: 198198
Plasmid: Dimer D2A: mTurquoise2-mVenus with T2A linker (cleaved linker) The original plasmid "mNeonGreen-2A-mTurquoise2" was a gift from Dorus Gadella (Mastop et al., 2017).	This paper	Addgene: 198199 Original Plasmid: Addgene: 98885
Plasmid: mTurquoise2 expression	This paper	Addgene: 198196
Plasmid: mVenus expression	This paper	Addgene: 198192
mVenus tagged MyosinVa IQ6-Domain (NM_010864.2, pos. 3080–3187)	This paper	Addgene: 198201
mVenus tagged MyosinVa IQ6-Domain [6A] mutant	This paper	Addgene: 198202
mTurquoise2 tagged Calmodulin (NM_017326.3)	This paper	Addgene: 198200
Empty vector backbone	Thermo Fisher Scientific	CAT: V79020
Software and algorithms		
Ilastik	Ilastik	RRID:SCR_015246
Fiji	ImageJ	RRID:SCR_002285
Fiji "Cell_Detect" macro	This paper	https://zenodo.org/record/7670415
Fiji "FRET_ROI" Two-Hybrid FRET Pre-processing macro	This paper	https://zenodo.org/record/7670415
Fiji "FRET_PIX" Two-Hybrid FRET Pre-processing macro	This paper	https://zenodo.org/record/7670415
MatLab (R2021a)	MathWorks	RRID:SCR_001622

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
MatLab "FRET2Hybrid" analysis script	This paper	https://zenodo.org/record/7670415
MatLab FACS gating script	This paper	https://zenodo.org/record/7670415
ZEN Blue 3.4 Software for confocal imaging	Zeiss	RRID:SCR_013672
Gen5 software for Cytation 5 imaging	BioTek	RRID:SCR_017317
Other		
µ-Dish 35 mm, high (microscope imaging dish), glass coverslip	ibidi	Cat#81158
Nunc™ Cell-Culture Treated Multidishes	Thermo Fisher Scientific	Cat#140675
Nunc™ glass bottom imaging dishes	Thermo Fisher Scientific	Cat#150682
Glass bottom 12 well plate	Cellvis	Cat#NC0799106
Cytation5	BioTek	RRID:SCR_019732
20X Phase-contrast Objective for the Cytation 5	BioTek	Part#1320517
'CFP FRET' Filterblock for the Cytation 5 (CFP-channel)	BioTek	Part#1225107
'CFP YFP FRET V2' Filterblock for the Cytation 5 (FRET-channel)	BioTek	Part#1225110
'YFP' Filterblock for the Cytation 5 (YFP-channel)	BioTek	Part#1225104
Zeiss LSM-980	Zeiss	https://www.zeiss.com/microscopy/en/products/light-microscopes/confocal-microscopes/lsm-980-with-airyscan-2.html

MATERIALS AND EQUIPMENT

Tyrode Imaging-solution	
Reagent	Final concentration
NaCl	140 mM
KCl	5 mM
$CaCl_2$	2 mM
$MgCl_2$	1 mM
HEPES	10 mM
D-Glucose)	10 mM
ddH_2O	N/A
Adjust pH to 7.4 with NaOH. Store Tyrode at 4°C for up to 12 months.	

STEP-BY-STEP METHOD DETAILS

This section describes the step-by-step procedure for two different instruments. The basic workflow is modular. It consists of four main sections: instrument setup, image acquisition, data preprocessing and FRET analysis. The first three sections are instrument-specific, while the section for FRET analysis is common for both workflows and described at the end of the protocol.

In the "instrument setup" section, an acquisition protocol containing three spectrally distinct channels (CFP, FRET, YFP) is set up to obtain the corresponding images i_{CFP}, i_{FRET}, i_{YFP}. In the "data preprocessing" section, the mean fluorescence values from ROIs in the images (i_{CFP}, i_{FRET}, i_{YFP}) are determined using Fiji macros and stored as fluorescence data tables containing intensities of all channels (S_{CFP}, S_{FRET}, S_{YFP}). The "FRET analysis" section uses the custom Matlab software to calculate Two-Hybrid FRET binding curves from these fluorescence data tables (Figure 1B).

Confocal microscope protocol

Instrument setup

Timing: 2 h

This section describes how to create an acquisition protocol on the microscope to capture the three images required for the Two-Hybrid assay (Figure 1B).

1. Generate an acquisition protocol to capture image stacks in the form (i_{CFP}, i_{FRET}, i_{YFP}). This order is important for data processing.
   a. Create a confocal track with 445 nm laser excitation (Figure 3B).
   b. Within this track, define two channels, termed CFP and FRET with the respective spectral properties shown in the blue box and the snapshot in Figure 3B.
   c. Create a second confocal track with 514 nm laser excitation (Figure 3C).
   d. Define the YFP channel with the respective spectral properties shown in the yellow box and the snapshot in Figure 3C.
2. Select an objective with a numerical aperture ≥ 1 (oil or water immersion).
   a. Adjust the correction collar to correct the objective to the bottom thickness of the imaging dish.
   b. Maximize image brightness by slightly adjusting the correction collar further.
3. Define acquisition properties.
   a. Prepare the dish transfected with the shortest dimer (D3) for imaging.
   b. Analyze emission in all three channels using histograms (Figure 3E).
   c. Laser power, PMT voltage gain and pixel dwell time affect image brightness. Adjust these parameters to cover 25%–75% of the histogram for all channels (Figure 3E).
   d. Refer to the CRITICAL note below to optimize the acquisition further. Exemplary settings are shown in Figure 3D.

   Note: Adjusting the correction collar improves focus alignment in multi-wavelength image acquisition and minimizes aberration.

   Note: The D3 construct is characterized by the highest FRET efficiency and will yield the highest fluorescence signals. Using this construct to adjust acquisition properties helps to avoid oversaturation of pixels in subsequent measurements that include bright samples with high FRET efficiencies.

   Note: High quality imaging is essential for Two-Hybrid FRET. The acquisition settings must be carefully configured to accommodate all samples (protein interaction and calibration samples). Once set, they must not be altered between measurements. The laser power and pixel dwell time influence the number of photons collected. Collecting a higher number of photons improves the signal-to-noise ratio (SNR). However, increasing these excitation parameters can lead to photo-bleaching. Averaging in the form of scan repeats can increase SNR but also photo-bleaching. Averaging also extends the acquisition time. The PMT gain can enhance data quality by optimizing histogram utilization. Live cell imaging is susceptible to sample movement during the acquisition, particularly when investigating organelles. Track switching should be set to line mode to reduce motion artefacts. Lowering the image resolution reduces the acquisition time. However, this is limited by the required resolution to see the biological system of interest clear enough to draw ROIs. Optimizing the parameters discussed above require finding a compromise. In contrast, the following adjustments should always be implemented: Avoid digital amplification of signals. Do not use a zoom factor lower than 1 to minimize image inhomogeneities. Always use a 16-bit signal depth. Acquisition settings shown in Figures 3B–3D were suitable for the reference system.

Image acquisition (confocal microscope)

Timing: 1–2 h

This section describes the acquisition of image stacks (i_{CFP}, i_{FRET}, i_{YFP}) on the confocal microscope.

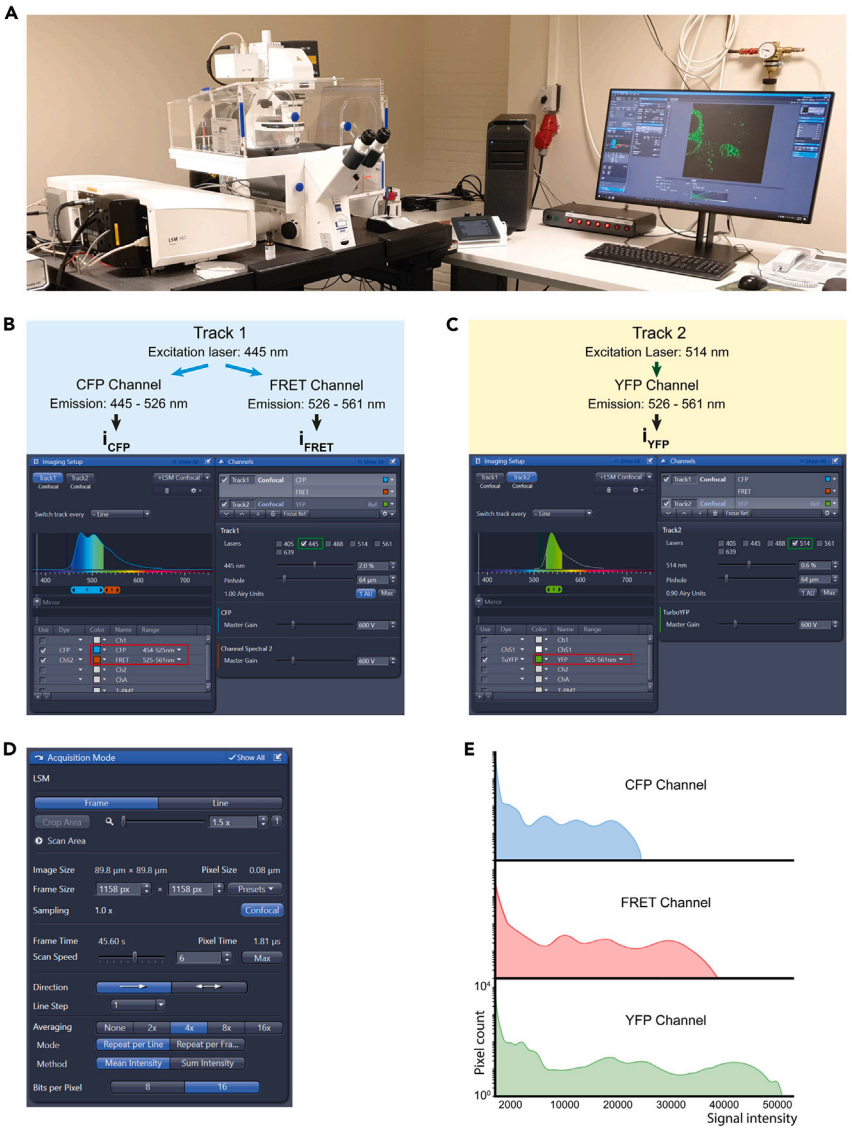


Figure 3. Setup of FRET acquisition channels (confocal microscope)

(A) The Zeiss LSM980 confocal microscope used for image acquisition.

(B) The top part of the panel highlighted in blue provides an overview of the Track 1 organization: The CFP channel and the FRET channel are incorporated in Track 1, since both use the 445 nm excitation laser. The wavelength interval in nm used for spectral detection in both channels are set up individually (red square). Both channels are scanned simultaneously in Track 1.

(C) The top part of the panel highlighted in yellow provides an overview of Track 2. The YFP channel is scanned separately in Track 2 with the 514 nm excitation laser (green square). The wavelength interval in nm for the YFP channel is defined as shown.

(D) Exemplary image settings for image acquisition.

(E) Histograms from the Dimer D3 sample acquired with the settings shown above.

⚠ CRITICAL: Do not change acquisition settings during the subsequent data collection process.

Note: Quantitative FRET methods are sensitive to photo-bleaching of fluorophores. Minimize illumination of cells when selecting a field of view for imaging.

4. Measurement process.
   a. Acquire a minimum of five fluorescent cells per calibration sample and at least 20 fluorescent cells per protein interaction sample.
   b. Save images in a lossless image format supported by Fiji's Bio-Formats importer (*.czi for Zeiss instruments).

Data preprocessing (confocal microscope)

Timing: 30 min

The following steps describe how the image stacks acquired in the previous section are converted into fluorescence data tables for FRET evaluation.

5. Create fluorescence data tables (S_{CFP}, S_{FRET}, S_{YFP}) from image stacks (i_{CFP}, i_{FRET}, i_{YFP}).
   a. Load an image stack from step 4 b into Fiji by dragging the image on the Fiji user interface (Figure 4, step 1).
   b. Optional: Apply a weak Gaussian blur to the image stack (r = 1 px). Click on the drop down menu Process->Filters->Gaussian Blur and set the radius to 1.
   c. Load the "FRET_ROI" macro (refer to section "download and install software") by dragging the macro file on the Fiji main window.
      i. Start the macro by pressing "Run" (Figure 4, step 2).
   d. Draw ROIs in the image stack and press "t" to add them to the ROI manager, (Figure 4, step 3).
      i. Use at least 10 ROIs for each calibration sample and at least 30 ROIs for protein interaction samples.
      ii. Press "OK" to generate data tables.
   e. Save data tables as comma separated values (*.csv).
      i. Repeat this step for all samples from Table 1. We suggest to use the naming convention from Table 1.
   f. To increase data abundancy, repeat step 5 for multiple images. Data tables can later be merged in the FRET analysis software.
   g. Continue with step 15 in the section "FRET analysis".

Note: Applying a Gaussian blur reduces stochastic noise and can improve data processed from small ROIs or when using the "FRET_PIX" macro.

Note: The FRET_ROI macro generates a mean fluorescence value from all pixels within a ROI for all three channels (i_{CFP}, i_{FRET}, i_{YFP}) and generates .csv data tables containing those fluorescence values (S_{CFP}, S_{FRET}, S_{YFP}) for each row as shown in Figure 4, step 4. An alternative "FRET_PIX" macro computes one data triplet for each pixel within a ROI, thus generating more data points than the "FRET_ROI" macro. The workflow, layout and subsequent calibration results are identical for both macros. We recommend to use the "FRET_PIX" macro for calibration samples instead of the "FRET_ROI" macro to increase data visibility in diagrams.

Note: Draw ROIs depending on your proteins of interest: For cytosolic proteins of interest and all calibration samples, draw ROIs in cytosolic regions exclusively.

Imaging plate reader (wide-field) protocol

Instrument setup

Timing: 1.5 h

This section describes how to set up an acquisition protocol on the imaging plate reader to capture the three images (i_{CFP}, i_{FRET}, i_{YFP}) required for the Two-Hybrid assay (Figure 1B).

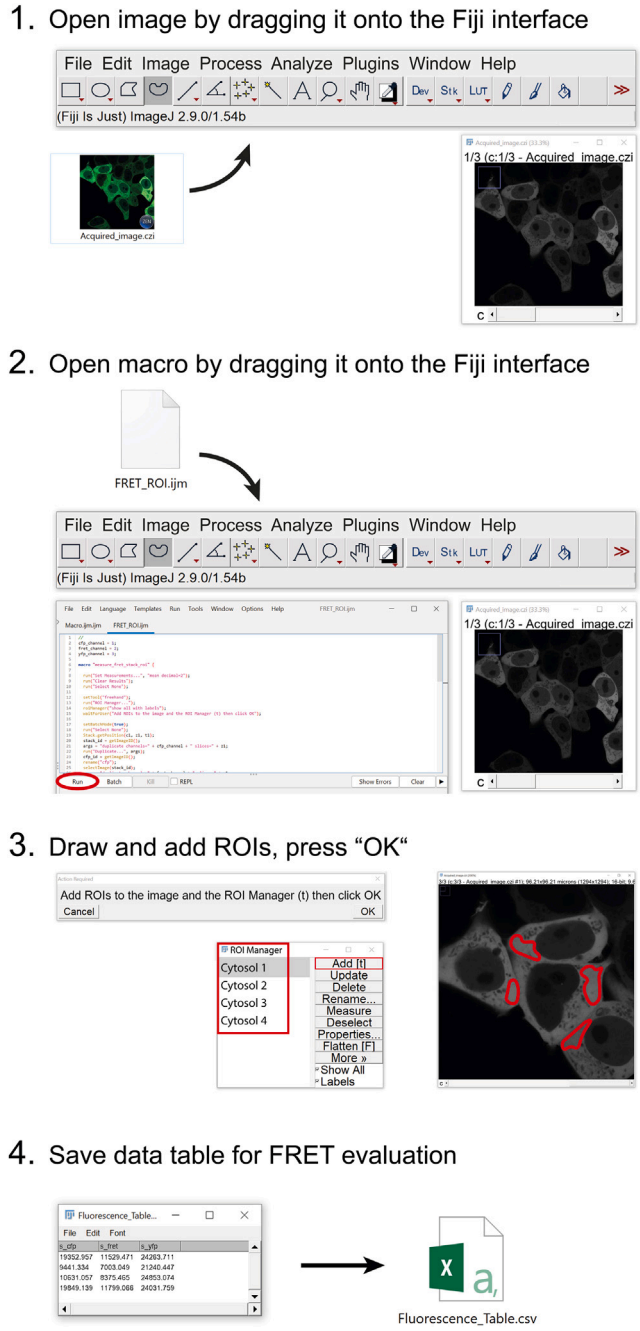


Figure 4. Workflow of data preprocessing (confocal microscope)

(1.) The confocal image stack (i_{CFP}, i_{FRET}, i_{YFP}) is opened in Fiji by dragging the image file onto the Fiji user interface.

(2.) The "FRET_ROI" macro (or alternatively the "FRET_Pix" macro, see Note) is opened similarly by dragging the file onto the Fiji user interface. Both the image stack and the macro are now open in the Fiji application. The "Run" button starts the macro (red ellipse).

(3.) After pressing the "Run" button in step 2, two pop-up windows appear as displayed in step 3. ROIs are drawn in the image and are added to the ROI manager by pressing (t). In this figure, four ROIs were added to the ROI manager (Cytosol 1–4). After adding all ROIs, this step is completed by pressing the "OK" button.

(4.) The fluorescence data table is generated and can be saved as shown in step 4.

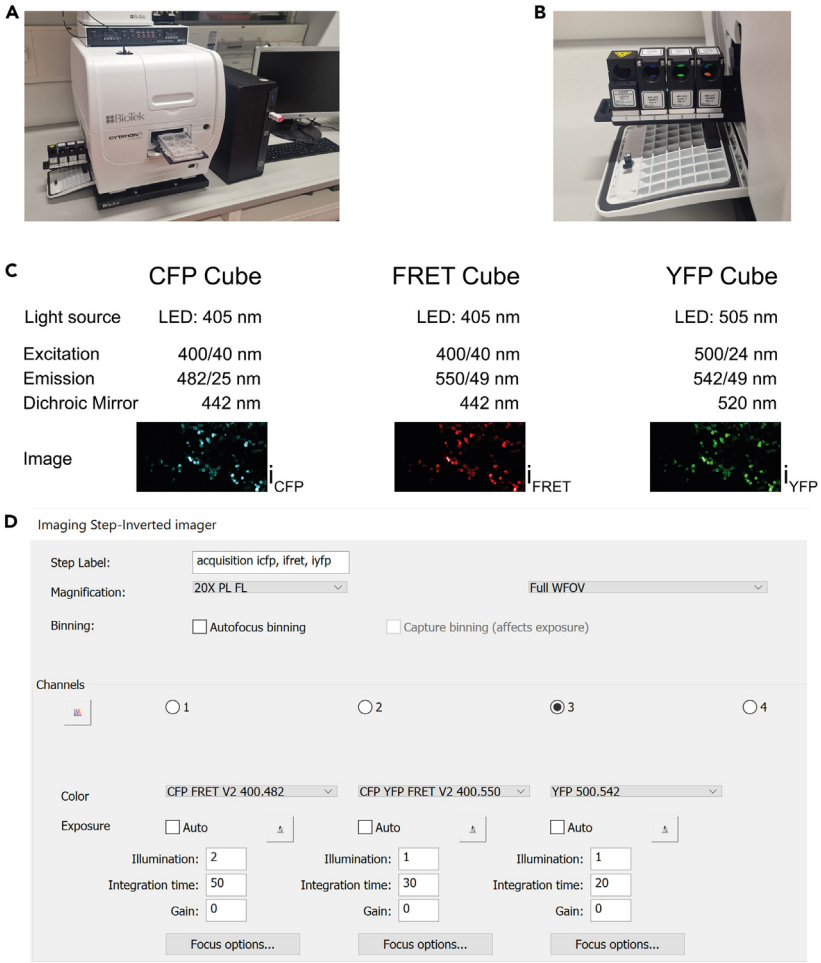


Figure 5. Setup of FRET acquisition channels (Plate reader/wide-field microscope)

(A) Image of the Cytation5 wide-field imaging plate reader used for this protocol.

(B) Installation of the filter cubes to acquire images in all three channels.

(C) Filter cube properties.

(D) Screenshot of the acquisition panel of Gen5 software (Biotek™): Setup of Two-Hybrid FRET acquisition channels with exemplary illumination settings.

6. Install Filter cubes and LEDs corresponding to Figures 5B and 5C on your device according to the manufacturer's instructions.
7. Create an acquisition protocol that includes the three required channels with the corresponding filter cubes shown in Figure 5D. This protocol generates the images i_{CFP}, i_{FRET}, i_{YFP}.
8. Select the 20× objective and adjust the correction collar to the bottom thickness of the well plate as described in step 2.
9. Adjust instrument specific parameters and take a representative image.
   a. Define LED power, illumination time and gain on your device (exemplary settings are displayed in Figure 5D).
   b. If available, use the laser autofocus option.
   c. We recommend using image stitching to capture a larger number of cells per image. A 3 × 3 field allows capturing several hundred cells per acquisition.
   d. Start imaging the shortest dimer sample (D3). Using the shortest dimer will ensure well balanced acquisition settings for all following measurements.

e. Analyze emission in all three channels and adjust Illumination time and LED power parameters to cover 25%–75% of the histogram in all channels (a representative histogram is displayed in Figure 3E and applies to wide-field instruments as well).
f. Adjust integration time (acquisition time) and illumination power from the presented acquisition settings in Figure 5D. Leave the Gain at 0.
g. Save the acquisition protocol and acquire a representative image in all three channels.
h. Create a folder on your processing computer called "Training".
i. Save or export one representative image (any channel) into the "Training" folder and label it "TrainingImage" (used for training of classifiers in step 10 and 11).

Setup of automatic cell detection

Timing: 30 min

The following steps explain how to train Ilastik classifiers for automatic cell detection.[7] The pixel classifier segments an image in cells and background. The object classifier identifies and distinguishes healthy cells from debris and artefacts, acting as a quality control measure.

We recommend reading the Ilastik documentation for pixel classification and object classification for background information (https://www.ilastik.org/documentation/).

10. Generate a pixel classifier.
    a. Start Ilastik, select "Pixel classification".
    b. Open the training image in the "Training" folder from step 9 i.
    c. Label cells and background according to Figure 6A. Ordering of these categories is important, with "cell" being the first category and "background" the second.
    d. Press "Live Update" and test the cell/background prediction. The image should look similar to Figure 6A with faint segmentation of cells (yellow) and background (blue).
    e. If the prediction of cells and background is acceptable, save the pixel classifier in the "Training" folder.
    f. Export the Prediction.h5 file from the pixel classifier project into the "Training" folder.
11. Generate an object classifier.
    a. Start Ilastik, select "Object classification".
    b. Load the training image and the Prediction.h5 file from the "Training" folder.
    c. Annotate objects according to Figure 6B: Single, healthy cells should be classified as "good". Cell clusters, dead cells and artefacts should be marked as "bad". Ordering of the two categories is important, with "good" being the first category and "bad" the second.
    d. Press "Live Update" to analyze the object prediction. The image should now look similar to Figure 6B, classifying cells in light green and artefacts in light red.
    e. If the prediction is acceptable, save the object classifier in the "Training" folder.
    f. Verify that the "Training" folder contains the training image, the pixel classifier, the pixel prediction map (.h5) and the object classifier.

⚠ CRITICAL: The TrainingImage, trained PIXEL_classifier, Prediction.h5 file and the trained OBJECT_classifier need to be saved in the same folder to allow processing by the Fiji macro. File and directory names must not include whitespace.

Note: Due to the non-linearity of the FRET calculation, confluent cells and cell clusters should be classified as "bad".

Image acquisition (plate reader/wide-field microscope)

Timing: 1–2 h

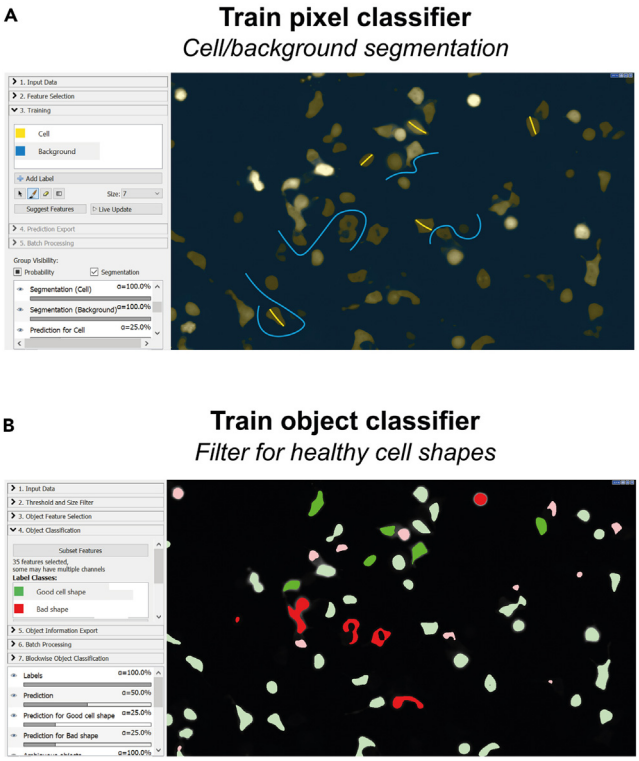


Figure 6. Training of Ilastik classifiers for cell detection

(A) Pixel classification window: Blue and yellow lines represent user annotations for cells (yellow) and background (blue). Cell prediction confidence is displayed in light yellow shade, background in dark blue.

(B) Object classification workflow: Dark green objects are user annotated "good" cell shapes, dark red objects are annotated objects that should be excluded ("bad"). Predicted objects are shown in light green and light red. The object classifier builds upon the pixel classification and serves as a quality control to exclude unwanted objects from evaluation.

This section describes the acquisition of image stacks (i_{CFP}, i_{FRET}, i_{YFP}) on the confocal microscope.

⚠ CRITICAL: Do not change acquisition settings during the subsequent data collection process.

12. Measurement process.
    a. Run the acquisition protocol for each sample (Table 1).
13. Data storage.
    a. Copy image data from the Gen5 image folder to your processing computer. We recommend labeling each image according to table 1 and the acquisition channel. Do not use whitespace in file names.

Data preprocessing (plate reader/wide-field microscope)

Timing: 30 min

The following steps describe how the image stacks acquired in the previous section are converted into fluorescence data tables for FRET evaluation.

14. Generate fluorescence data triplets (S_{CFP}, S_{FRET}, S_{YFP}) from images i_{CFP}, i_{FRET}, i_{YFP}.
    a. Load and start the "Cell_Detect" macro by dragging the macro file onto Fiji's main window and pressing "Run" (Figure 7, step 1).

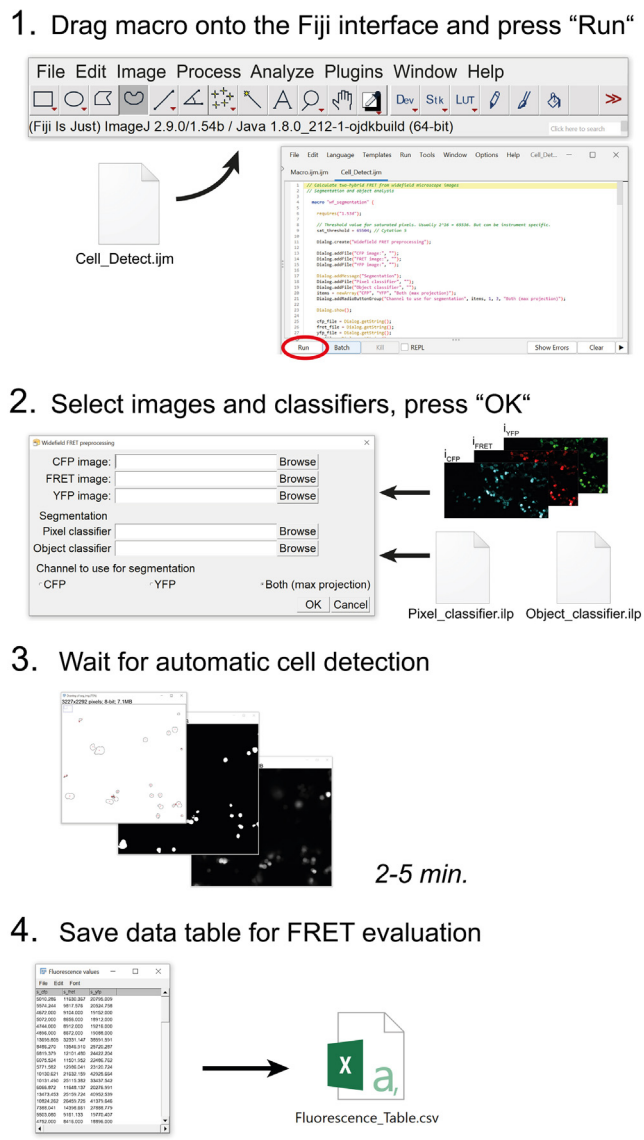


Figure 7. Workflow of data preprocessing (Plate Reader/wide-field microscope)

(1.) After dragging the Cell_Detect macro into Fiji, the macro window is opening. Press “OK” to start the macro (red ellipse).

(2.) Click on “Browse” beside the corresponding image dialogs and select images of the corresponding channels from one sample. Load the pixel- and object-classifier from step 10 and 11 into the corresponding fields. The images are converted to a hyperstack image. Leave the “channel to use for segmentation”-setting on the bottom on “Both”. This will create an additional maximum projection from the CFP and YFP channel from the hyperstack image, which is used for the automatic cell detection.

(3.) The macro segments the image using the pixel classifier and then filters the detected objects according to the object classifier. The remaining objects are converted into ROIs. Mean fluorescence intensities are quantified from these ROIs and listed as tabulated values (S_{CFP}, S_{FRET}, S_{YFP}). Do not click on the images while the macro is computing.

(4.) The macro will generate a fluorescence data table as shown. Save this data table for each construct and continue with the Two-Hybrid FRET analysis.

b. Specify the files to load in the dialog box (Figure 7, step 2).
   i. Load the three images i_{CFP}, i_{FRET}, i_{YFP} from a sample (exclude the background sample) acquired in step 12.

ii. Load the classifiers generated during step 10 and 11 in section "setup of automatic cell detection".

c. Press "OK" to start the automatic cell detection process. This will apply the specified classifiers to the images and compute FRET data triplets (S_{CFP}, S_{FRET}, S_{YFP}), (Figure 7, step 3).

d. Save the three-column data table (.csv) using the naming convention from Table 1 (Figure 7, step 4).

e. Repeat step 14 for all samples from Table 1 except the scramble DNA (background) sample.

f. Background values from the scramble DNA sample are determined manually.

i. Run the "background" Fiji macro.

ii. Specify the background image files to load in the dialog box and press "OK".

iii. Draw a large ROI and add it to the ROI manager by pressing "t", press "OK".

iv. Save the data table as .csv file.

g. Continue in the next section "FRET analysis".

Note: If the automatic ROI generation via Ilastik is not desired, an alternative macro for manual ROI generation as shown for confocal microscopy is provided for wide-field instruments as well ("WF_manual-preprocessing").

FRET analysis

Timing: 15 min

This section describes the FRET analysis to create Two-Hybrid FRET binding curves. This part applies to data obtained with either of the two instruments.

The mathematical background to all calibration constants and binding curve parameters obtained here is described in detail in the "quantification and statistical analysis" section.

15. Start the Matlab analysis software FRET2Hybrid.m. The user interface is shown in Figure 8, the subpanels are displayed in Figure 9 in detail, corresponding to the workflow.

16. Import the three-column data tables (S_{CFP}, S_{FRET}, S_{YFP}) (Figure 9, step 1).

a. Select the directory in which all data tables are contained (Background, Acceptor, Donor, Dimers and Proteins of interest) by clicking the "Pick Directory" button (Figure 9, step 1).

b. Import background and spectral correction data tables by clicking the black "+" button (Figure 9, step 1 "*Data table import button*") in the background, acceptor and donor import window, respectively. File names appear in this window after importing.

i. Examine the fluorescence intensities in each channel (S_{CFP}, S_{FRET}, S_{YFP}), which are plotted in the three raw fluorescence diagrams (Figure 8 "*Raw Fluorescence Diagrams 1–3*") for each data table that is uploaded.

ii. Optionally, remove files from the import windows by clicking the black "-" button beside the data upload ("+") button.

c. Import all three dimer data tables (D3, D42, DT2A) into the "Dimer" import window.

i. Click on the "Plot All" box on top of the "*Raw Fluorescence Diagram 3*" (Figure 8).

ii. Examine the fluorescence intensities which are plotted in the "*Raw Fluorescence Diagrams 1–3*" for each imported dimer.

d. Import data tables of the protein interaction in the "Constructs" import window.

i. Merge multiple data tables of a protein interaction by clicking on the "Merge Constructs" button (Figure 9, step 1) after importing all datasets of the interaction.

17. Calculate calibration constants (Figure 9, step 2).

a. Click on the "Background" button (Figure 9, step 2). The resulting background values are displayed below the calibration button (BG.CFP, BG.FRET, BG.YFP, Figure 9, step 2).

b. Click on the button "Setup RA". The numeric value for R_{A1} appears in the box below the button (Figure 9, step 2).

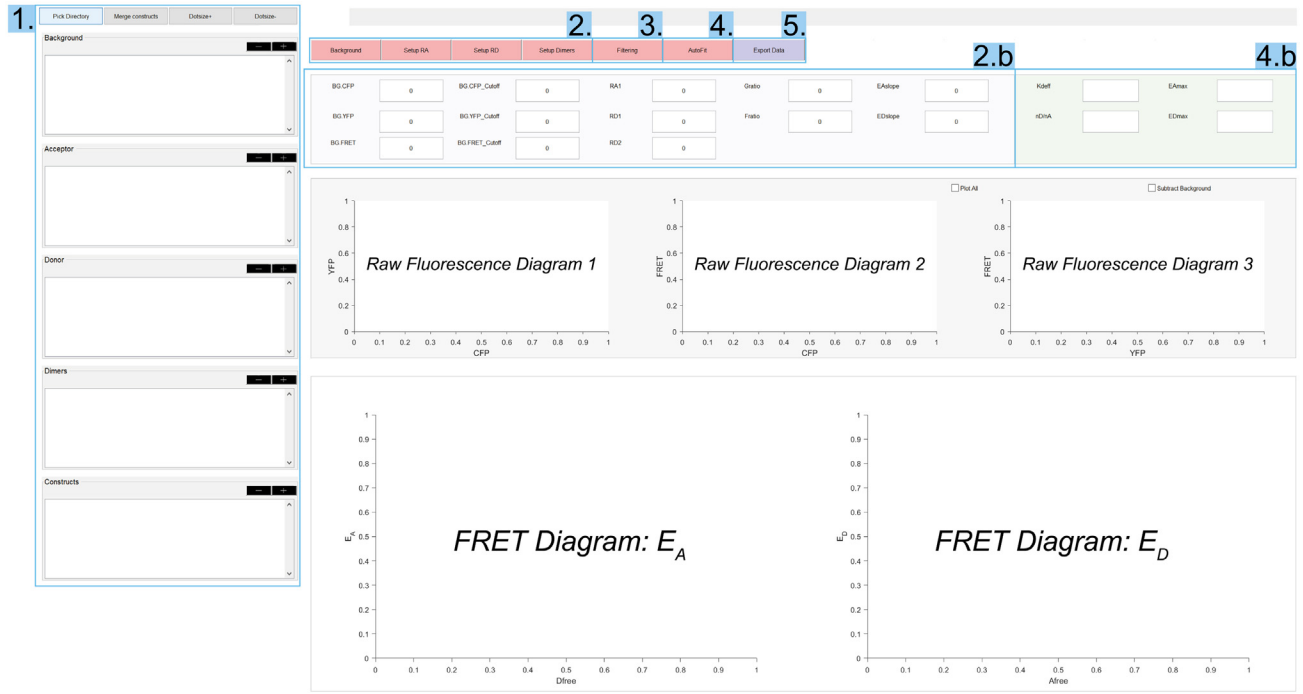


Figure 8. Main panel of the FRET analysis software

Sections (1.-4.) are numbered according to the workflow (Figure 9) and described below.

(1.) Top buttons from left to right: "Pick Directory", "Merge constructs", "Dotsize+" and "Dotsize-" (Figure 9, step 1). "Pick Directory" selects the root folder for data files. The five panels for the data upload from top to bottom are: "Background", "Acceptor", "Donor", "Dimers" and "Constructs". By clicking the "+" data files can be added to the boxes from the directory selected before (Figure 9, step 1) Multiple protein interaction data tables can be merged by clicking the "Merge constructs" button. "Dotsize" buttons alter the size of data points in the diagrams.

(2.) Calibration buttons: "Background", "Setup RA", "Setup RD" and "Setup Dimers". Clicking one of these buttons after upload of the respective data table calculates the background ("Background" button), spectral correction ("Setup RA" and "Setup RD" buttons) and FRET calibration ("Setup Dimers" button) (Figure 9, step 2). Calibration constants are displayed as numerical values below the calibration buttons (panel 2.b).

(3.) The "Filtering" button opens a window to adjust minimum and maximum signal values, as well as the maximum donor-acceptor imbalance for binding curve evaluation (Figure 9, step 3).

(4.) The "AutoFit" button offers two fitting options for protein interaction data, as displayed in Figure 9, step 4. Binding curve parameter are displayed as numerical values in panel 4.b after selecting a fitting procedure.

(5.) The blue "Export Data" button is used to copy binding curve data into the clipboard as displayed in step 5 of Figure 9.

"*Raw Fluorescence Diagrams 1–3*": These diagrams display raw fluorescence values of the three channels in relation to each other. They are used for the spectral calibration (Figure 10A). After clicking on "Setup RA" and "Setup RD" (Figure 9, step 2), these diagrams show the linear relationship between channels to unmix emission signals as described in the expected outcomes section (Figure 10A).

"*FRET Diagrams*": Calculated FRET efficiency diagrams. Dimer FRET efficiencies for FRET calibration (Figure 10C) and protein interaction binding curves (Figures 11A and 11B) are displayed here.

i. Examine the fluorescence data in the "*Raw Fluorescence Diagram 3*" corresponding to the spectral correction factor R_{A1} (Figure 10A).

c. Click on the button "Setup RD".

i. Analyze the spectral correction data for R_{D1} and R_{D2} which appear in the "*Raw Fluorescence Diagram 1 and 2*", respectively (Figure 10A).

d. To compute FRET calibration, click on the "Setup Dimer" calibration button.

i. Examine the pop-up window, displaying a diagram containing all three dimer data (Figure 9, step 2), similar to Figure 10B.

ii. Close the pop-up window. The values for F_{ratio} and G_{ratio} are displayed in the respective boxes (Figure 9, step 2).

iii. Analyze the calculated FRET efficiencies of all dimers which are displayed in the "*FRET Diagram (E_A and E_D)*" (Figure 8).

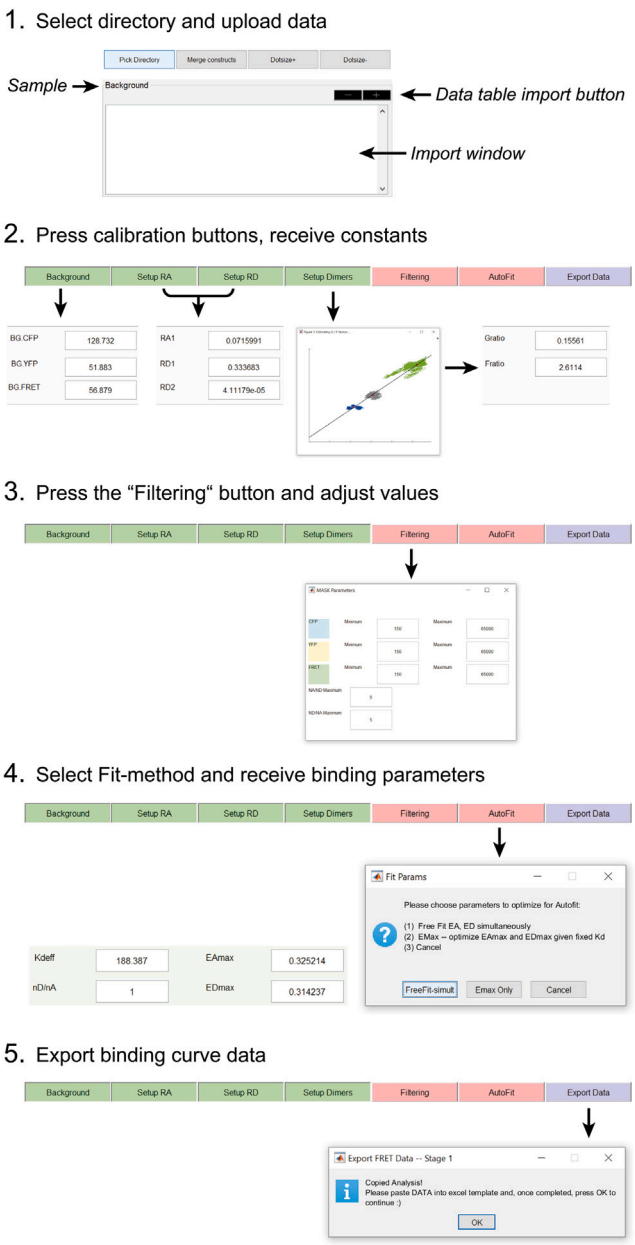


Figure 9. Workflow of the FRET analysis software

Steps correspond to the subpanels in the main panel of the FRET analysis software (Figure 8, 1.-4.).

(1.) Data upload: "Pick Directory": Selection of root directory for data import. "Merge Constructs": Merge multiple construct data tables. "Dotsize +/-": Change data point size. Import Window: Displays imported files. Data import button "+": Import data from selected root folder. Data import button "-": Remove imported file.

(2.) Calibration steps: "Background", "Setup RA", "Setup RD" and "Setup Dimers". These buttons initiate the calibration procedures described in step 17. After calibration, the buttons turn green. The calculated numerical values of all constants are displayed below. After pressing the "Setup Dimers" button, a pop up window will appear that correlates sensitized emission with donor quenching. Close this window to finish calibration. All calibration fluorescence data are displayed in the "*Raw Fluorescence Diagrams 1–3*".

(3.) Fluorescence data filtering: After pressing the "Filtering" button, a new dialog box appears. Fluorescence data can be filtered regarding minimum and maximum signal values and the upper limit for the donor:acceptor ratio taken into account for evaluation.

(4.) Binding curve generation: After pressing the "AutoFit" button, a pop-up window opens, offering two binding curve fit options. "The "Emax only" option fits the data with a binding model with coefficients $E_{A,MAX}$ and $E_{D,MAX}$ and a fixed $K_{D,EFF}$. FreeFit-simult" option fits the data with a binding model with coefficients $E_{A,MAX}$, $E_{D,MAX}$ and $K_{D,EFF}$.

Figure 9. *Continued*
(5.) Export: Binding curve data points can be exported by clicking the blue "Export Data" button. All data of the binding curve, binding curve parameter and filtering options are copied to the clipboard and can be pasted into a spreadsheet software, such as excel.

18. Define filtering options (Figure 9, step 3).
    a. Click on the "Filtering" button. A new window will appear as shown in Figure 9, step 3.
    b. Adjust the maximum N_D/N_A and N_A/N_D ratios in the corresponding dialogs to 5.
19. Plot Two-Hybrid FRET binding curves (Figure 9, step 4).
    a. Click on the "AutoFit" button. A window pops up with two different fit options: "FRET-Max" and "FreeFit-simult".
    b. Select a "FRET-Max" fit first to evaluate a binding curve with respect to FRET-Efficiencies only.
    c. Select "FreeFit-simult" to fit binding curve with respect to both FRET-Efficiencies and $K_{D,EFF}$.
20. Export binding curve data (Figure 9, step 5).
    a. Click on "Export Data" to copy data from binding curves to the clipboard. Paste the clipboard into Excel or any other spreadsheet software.

Note: The N_D/N_A values in the filtering options (step 18) determine the maximum ratio between the number of donors and the number of acceptors considered in the evaluation. Large values can improve the coverage of the binding curve, but can lead to increased scattering of the curve.

EXPECTED OUTCOMES

The FRET analysis of the reference protein interaction between CaM and IQ-WT or IQ-6A should result in accurate E_A- and E_D-binding curves similar to those presented in Figures 11A and 11B. The maximum FRET efficiencies of WT and mutant should be similar to values given in Figure 11C. The maximum FRET efficiencies between the two binding curves (E_A and E_D) should be similar, indicating the 1:1 stoichiometry of the interaction (Figure 11C). Binding of calmodulin to the mutated IQ6 domain (IQ-6A) is expected to be weaker, thus having a higher $K_{D,EFF}$ (Figure 11D).

Once the protocol is established and validated using the reference interaction, unknown protein-protein interactions can be evaluated in terms of their relative binding affinity,[6] absolute FRET efficiency[2] and binding stoichiometry.[5]

QUANTIFICATION AND STATISTICAL ANALYSIS

Determination of Two-Hybrid FRET binding curves involves multiple calculation steps categorized into three sections: spectral correction, FRET calibration, and binding curve generation. This section presents the formulas underlying the calculation for the Two-hybrid FRET analysis.

Spectral correction factors (R_{A1}, R_{D1}, R_{D2})

In a sample containing interacting proteins A and B, labeled by donor and acceptor, respectively, three fundamental components are present in the emission signal of the FRET channel: 1.) Emission due to direct excitation of the donor (donor bleed through; CFP_{direct}), 2.) emission due to direct excitation of the acceptor (cross excitation of the acceptor at the donor excitation wave length; YFP_{direct}) and 3.) sensitized emission (YFP_{FRET}). These quantities have to be obtained from the raw fluorescence signals (S_{CFP}, S_{FRET}, S_{YFP}) to calculate FRET efficiencies. Three spectral correction factors are required to calculate CFP_{direct}, YFP_{direct} and YFP_{FRET} from the raw fluorescence signals and subtract them from total fluorescence in the FRET channel in order to quantify sensitized emission:

R_{A1}

A fraction of YFP is cross-excited in the FRET channel from donor excitation light. In a sample containing acceptor fluorophores only, R_{A1} is defined as the ratio of excitation in the FRET channel relative to the YFP channel (1).

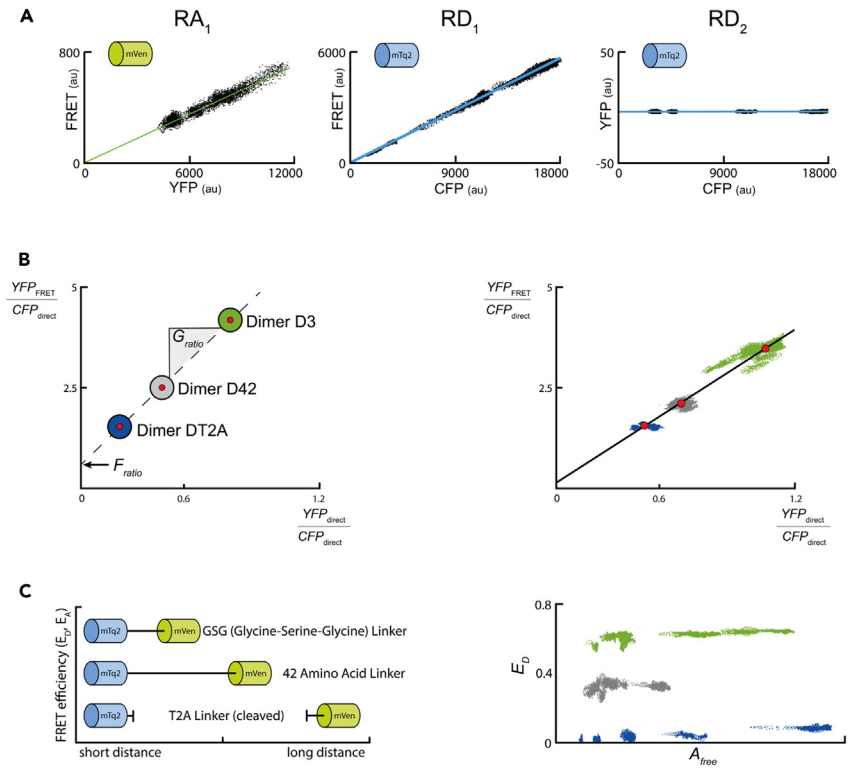


Figure 10. Expected outcomes: Spectral correction factors and FRET calibration

(A) Determination of spectral correction factors: R_{A1}: Plot of fluorescence in the FRET channel (y-axis) vs. fluorescence in the YFP channel (x-axis). R_{A1} describes cross excitation of YFP by CFP excitation (FRET/YFP). R_{D1}: Plot of fluorescence in the FRET channel (y-axis) vs. fluorescence in the CFP channel (x-axis). R_{D1} describes the bleed-through of CFP emission into the FRET emission detection range (FRET/CFP). R_{D2}: Plot of fluorescence in the YFP channel vs. the fluorescence in the CFP channel. R_{D2} describes cross excitation of CFP from the YFP excitation (YFP/CFP). These spectral correction factors are determined by linear regressions between the specified channels.

(B) Correlating sensitized emission versus donor quenching displays an affine relationship between these quantities when using dimer constructs. The G_{ratio} corresponds to the slope of a linear regression line, while the F_{ratio} is calculated from the y-axis offset.[1]

(C) Left: cartoon of the dimer constructs. Right: Plot of FRET efficiencies determined from donor quenching (E_D) vs. A_{free} for the shown dimers. FRET calibration results in stable FRET efficiencies independent of the fluorophore concentration (A_{free} or D_{free}). The plot for sensitized emission (E_A) should look similar. All calibration images were processed with the "FRET_PIX" macro instead of the "FRET_ROI" macro.

$$R_{A1} = S_{FRET}/S_{YFP} \quad \text{(Equation 1)}$$

R_{D1}

The emission spectra of CFP and YFP overlap, therefore a fraction of CFP emission will be detected in the FRET channel. In a sample containing donor fluorophores only, R_{D1} is defined as the ratio of CFP emission in the FRET channel relative to the CFP channel (2).

$$R_{D1} = S_{FRET}/S_{CFP} \quad \text{(Equation 2)}$$

R_{D2}

A small fraction of CFP is cross-excited in the YFP channel from acceptor excitation light. In a sample containing donor fluorophores only, R_{D2} is defined as the ratio of excitation in the YFP channel relative to the CFP channel (3).

$$R_{D2} = S_{YFP}/S_{CFP} \quad \text{(Equation 3)}$$

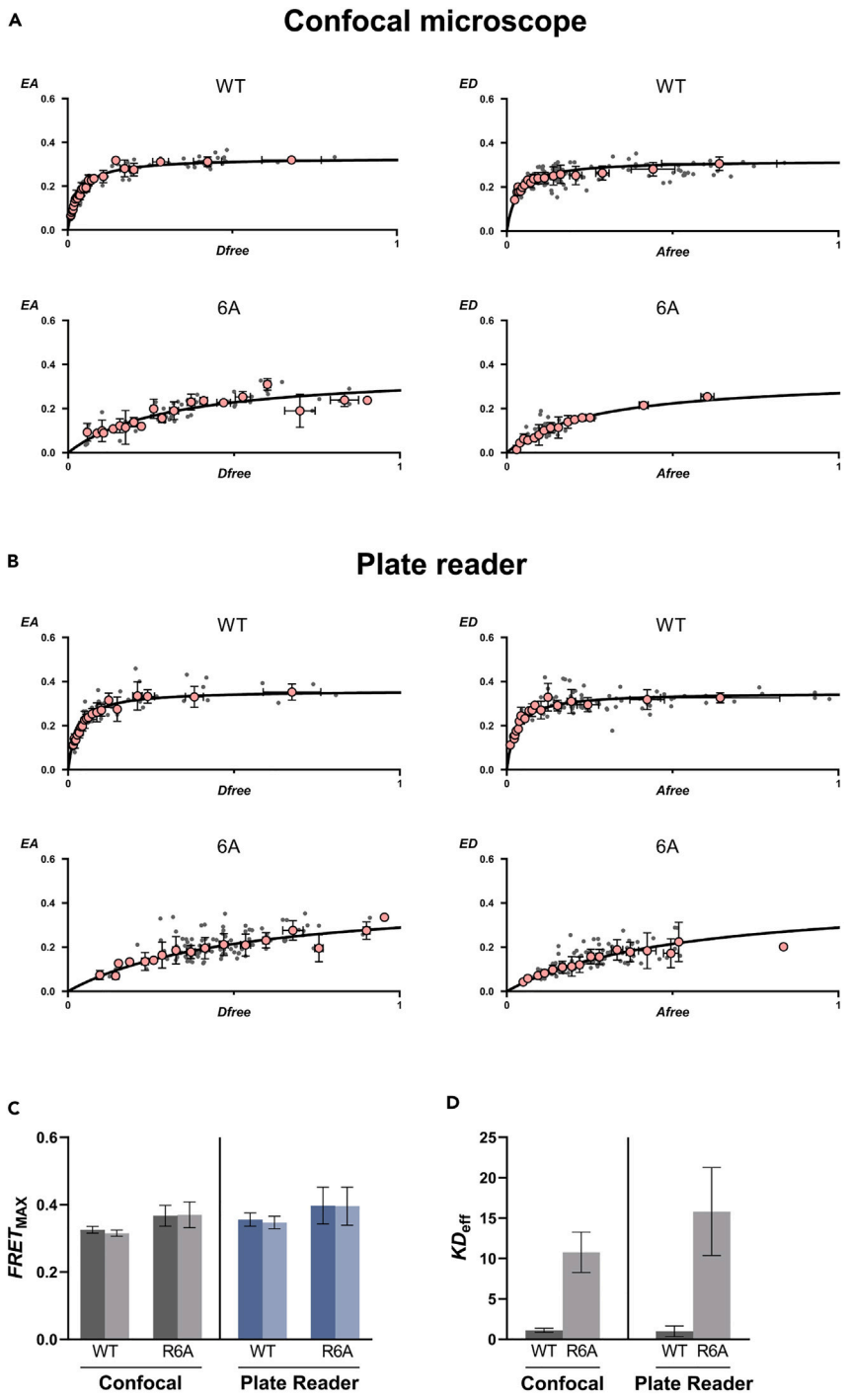


Figure 11. Expected outcomes: Image based FRET binding curves

Binding curves of the interactions between mTq2-CaM (FRET donor) and mVenus-IQ6-WT or mVenus-IQ6-6A (FRET acceptors). Each gray dot represents data from a single ROI, evaluated with the "FRET_ROI" macro. Red dots represent XY-binned data.

(A) Measurements performed with a confocal microscope.

(B) Binding curves were generated using an imaging plate reader. Fitting includes a small spurious FRET correction as described in the "troubleshooting" section (problem 8).

Figure 11. *Continued*

(C) Comparison of the maximum FRET efficiencies of the E_A binding curves (darker) and the E_D binding curves (lighter) for each graph shown in A and B. Similar $E_{A,max}$ and $E_{D,max}$ efficiencies indicate a 1:1 interaction stoichiometry. Values obtained from fitting the binding model. Error bars represent confidence intervals (95%).

(D) Relative dissociation constants of the wild type and mutated protein interaction, shown for both instruments. Values normalized, wild type $K_{D,EFF}$ set to 1. Error bars represent confidence intervals (95%) from the binding model fit.

Using the spectral correction factors, the raw fluorescence data (S_{CFP}, S_{FRET} and S_{YFP}) can be unmixed into the three emission quantities in the FRET channel:

$$CFP_{direct} = R_{D1} * S_{CFP} \quad \text{(Equation 4)}$$

$$YFP_{direct} = R_{A1} * (S_{YFP} - R_{D2} * S_{CFP}) \quad \text{(Equation 5)}$$

$$YFP_{FRET} = S_{FRET} - R_{A1} * (S_{YFP} - R_{D2} * S_{CFP}) - R_{D1} * S_{CFP} \quad \text{(Equation 6)}$$

FRET calibration (G_{ratio} and F_{ratio})

For absolute quantification of FRET, two ratios are determined: The G_{ratio} ($\frac{g_A}{g_D}$) corresponds to the relative absorption ratio of the acceptor to that of the donor. The F_{ratio} ($\frac{f_A}{f_D}$) corresponds to the relative emission ratio of the acceptor to that of the donor.

In terms of microscopic and instrument specific parameters the three fundamental emission quantities obtained after spectral correction can be written as:

$$CFP_{direct} = N_D * g_D * f_D * (1 - E_D) \quad \text{(Equation 7)}$$

$$YFP_{FRET} = N_D * g_D * f_A * E_A \quad \text{(Equation 8)}$$

$$YFP_{direct} = N_D * g_A * f_A \quad \text{(Equation 9)}$$

N_D and N_A describe the number of donor- and acceptor molecules, respectively. Further, g_D and g_A describe excitation dependent aspects for both donor and acceptor. Additionally, f_D and f_A describe the instrument- and fluorophore-dependent aspects of emission for the donor and acceptor.

Calculating absolute FRET efficiencies E_A and E_D requires knowledge of the calibration constants $\frac{g_A}{g_D}$ (G_{ratio}) and $\frac{f_A}{f_D}$ (F_{ratio}), which are determined in the following steps:

Equations 8 and 9 yield the absolute FRET efficiency from sensitized emission, by solving for E_A:

$$E_A = \frac{g_A}{g_D} * \frac{YFP_{FRET}}{YFP_{direct}} \quad \text{(Equation 10)}$$

Equations 8 and 7 yield the absolute FRET efficiency resulting from donor quenching by solving for E_D, with using the identity $N_A\ E_A = N_D\ E_D$:

$$E_D = \frac{YFP_{FRET}}{YFP_{FRET} + \frac{f_A}{f_D} * CFP_{direct}} \quad \text{(Equation 11)}$$

For Cer-Ven dimers with a 1:1 stoichiometry, $E_A = E_D$. Thus, the Equations 10 and 11 can be rearranged into a linear function, with the G_{ratio} ($\frac{g_A}{g_D}$) as the slope and F_{ratio} ($\frac{f_A}{f_D}$) as the y-intercept.

$$\frac{YFP_{FRET}}{CFP_{direct}} = \frac{g_D}{g_A} * \frac{YFP_{direct}}{CFP_{direct}} - \frac{f_A}{f_D} \quad \text{(Equation 12)}$$

Equation 12 can now be solved for G_{ratio} and F_{ratio} by using the dimer constructs (Table 1): Both ratios are determined as outlined in Figure 10B by plotting the dimer data according to the diagram.

Two-Hybrid FRET binding curve generation

With the three unmixed emission signals from the spectral correction and the F_{ratio} and G_{ratio}, it is possible to approximate the relative amount of donor and acceptor molecules from the three emission signals (S_{CFP}, S_{FRET}, S_{YFP}). Additionally, the relative bound and unbound fraction of FRET partners can be determined, resulting in the ability to impose a binding model to generate the displayed Two-Hybrid FRET binding curves.

Imposing a binding model requires the knowledge of the abundance of donor- and acceptor molecules.

The relative amount of donor and acceptor molecules are determined as follows. This is possible by assuming $f_D * g_D = 1$, as only the relative values for g_A versus g_D and f_A versus f_D (G_{ratio} and F_{ratio}) can be acquired from the previous steps.

$$N_D = \frac{CFP_{direct}}{1 - E_D} \quad \text{(Equation 13)}$$

$$N_A = \frac{YFP_{direct}}{G_{ratio} * F_{ratio}} \quad \text{(Equation 14)}$$

A given protein interaction sample consist of a mixture of bound and unbound FRET partners. The bound fractions (D_B and A_B) are calculated as follows:

$$D_b = \frac{N_D+N_A+K_D - \sqrt{(N_D+N_A+K_D)^2 - 4N_DN_A}}{2N_D} \quad \text{(Equation 15)}$$

$$A_b = \frac{N_D+N_A+K_D - \sqrt{(N_D+N_A+K_D)^2 - 4N_AN_D}}{2N_A} \quad \text{(Equation 16)}$$

D_{free} and A_{free} are then determined as:

$$D_{free} = N_D * (1 - D_b) \quad \text{(Equation 17)}$$

$$A_{free} = N_A * (1 - A_b) \quad \text{(Equation 18)}$$

The fraction of bound molecules (D_b and A_b) determine the apparent FRET-efficiency as shown:

$$E_A = A_bE_{max} \quad \text{(Equation 19)}$$

$$E_D = D_bE_{max} \quad \text{(Equation 20)}$$

This leads to the following equations for E_A and E_D: Both apparent FRET efficiencies depend on the approximated abundancy of the opposite FRET partner (D_{free} and A_{free}). $E_{A,MAX}$, $E_{D,MAX}$ and $K_{D,EFF}$ can be evaluated by using multiple measurements and fitting a binding curve plotting E_A or E_D on the y-axis and D_{free} or A_{free} on the x-axis, respectively.

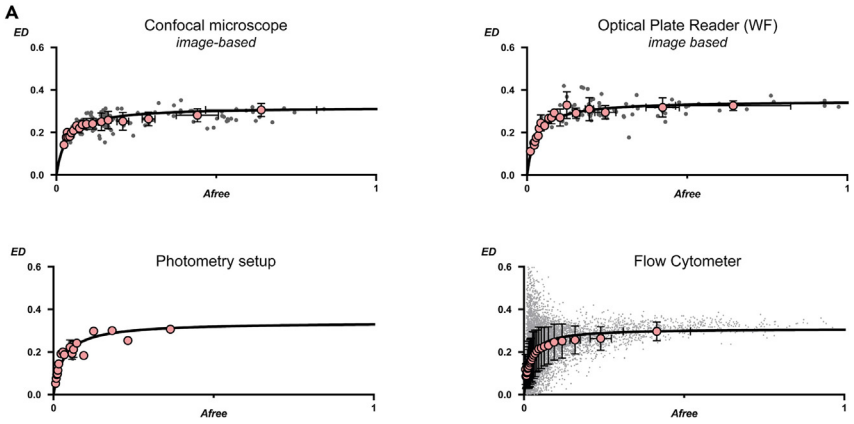
$$E_A = E_{A,MAX} * D_{free}/(D_{free}+K_{D,EFF}) \quad \text{(Equation 21)}$$

$$E_D = E_{D,MAX} * A_{free}/(A_{free}+K_{D,EFF}) \quad \text{(Equation 22)}$$

Equations 21 and 22 are fitted to the experimental data using a nonlinear, iterative least-square fitting algorithm (Matlab function: nlinfit), resulting in Two-Hybrid FRET binding curve data as shown in Figure 11. The $K_{D,EFF}$ is the half-maximum of the binding curve, $E_{D,MAX}$ and $E_{A,MAX}$ are the asymptotic maxima.

LIMITATIONS

While the FRET efficiencies and stoichiometries are absolute and apply to all devices, the calculated binding affinity is a relative value. As a consequence, binding affinities for different protein-protein interactions can only be compared if measured using the same device.



B

	CONFOCAL MICROSCOPE	OPTICAL PLATE READER	FLOW CYTOMETRY	PHOTOMETRY SETUP
SPEED	Medium	High	Very high	Low
PRECISION	Very high	High	Low	Very high
VERSATILITY	Subcellular	Only cytosolic	Only cytosolic	Only Cytosolic

Figure 12. Comparison of binding curves obtained by image-based and non-image based instruments

(A) Non-image based Two-Hybrid FRET assays using a photometry setup[2] (bottom left) or a flow cytometer[1] (bottom right) are compared to image based FRET setups such as a confocal microscope (top left) or optical plate reader (top right). The set of reference constructs was used for all binding curves presented. E_D binding curves for the interaction between CaM and IQ-WT are shown. Each gray dot represents one whole-cell readout. For confocal measurements, each gray dot represents one subcellular ROI. Red dots: XY-Binned data values.

(B) Summary of characteristics from instruments used for Two-Hybrid FRET assays.

Overall, quantitative FRET experiments outlined here can be reliably performed with high precision and robustness using most confocal or wide-field imaging systems, compared to traditional approaches that are not image-based (Figure 12A and 12B). One important requirement for these assays is an optimal image quality in a short acquisition time frame and a precise alignment of all three images (i_{CFP}, i_{FRET}, i_{YFP}). Those requirements become stricter when applying the assay to subcellular regions and organelles, as organelles can move between images. Another limitation is that FRET techniques can only indirectly serve as a molecular ruler, as tagged fluorophores may be positioned in unfavorable conformation relative to the protein of interests, resulting in reduced FRET signals. Another challenge is to obtain a balanced and controlled expression of the two proteins of interest, which can be difficult to achieve in some cases, especially when subcellular expressed proteins are under investigation. To overcome fluorophore-dependent limitations, we recommend using pH-stable and bright fluorophores with strong monomeric properties.

TROUBLESHOOTING

Problem 1

Unbalanced expression resulting in fluorescence imbalance between channels (related to step 4 or step 12).

Potential solution

Adjust the amount of transfected DNA. Design the plasmids in such way that the brighter fluorophore (mVenus) is tagged to the weaker expressed protein of interest.

Problem 2

The coverage of the binding curve is insufficient. This leads to either poor fits, or the inability to perform a $K_{D,EFF}$-dependent fit (related to step 19).

Possible solution

Insufficient coverage of the binding curves indicates that the apparent donor-acceptor ratios of the protein interaction partners are not different enough in one or both directions. It is recommended to expand the number of protein interaction samples with additional transfection stoichiometries (e.g., transfect twice the amount of the donor compared to the acceptor FRET partner, if the E_A binding curve is insufficiently covered): This results in more data points along the binding curve and allows for robust fitting.

Problem 3

Poor signal-to-noise ratio in any channel (related to step 3 or step 9).

Potential solution

Increase pixel dwell time in confocal microscopy, or increase acquisition time for wide-field microscopy. Increase the laser power instead of PMT gain if the emission signal is still insufficient. Optimize correction collars. As a rule of thumb, sufficient fluorescence emission should be detectable with the human eye via the eyepiece for both confocal and wide-field microscopy measurements. Digital signal amplification does not increase the signal-to-noise ratio.

Problem 4

High scattering of binding curves, usually accompanied by the inability to perform a fit (related to step 19).

Potential solution

To ensure fluorescent signals are sufficient for evaluation, observe cells using the eyepiece in epi-fluorescence mode. Fluorescent cells should be detectable with the eye when moderately excited in the correct excitation range. Compare fluorescence values of untransfected cells with fluorescent cells: Fluorescent cells should have at least 20 times higher fluorescence intensities than non-fluorescent cells. Use the "Filtering" button in the MatLab software to reduce maximum imbalance between donor and acceptor fluorophores to 3:1 or 1:3 (see step 18). Filter out measurements with very low fluorescence intensity as well as very bright outliers. Avoid photo-bleaching: Refer to Problem 6. Proteins of interest that co-localize only partially have to be analyzed on a confocal microscope to limit evaluation to regions that co-localize. Scattering around a FRET value of 0% with the inability to perform a fit in the Matlab software indicate that proteins are not associated with each other. Ensure that background signal intensities are stable throughout all images. Static background values are subtracted within the software. Fluctuating background values degrade data quality.

Problem 5

Spectral correction factors (R_{A1}, R_{D1} and R_{D2}) are unstable between measurements (related to step 17).

Potential solution

The spectral correction factors provide a reference for instrument stability and should not change over multiple days. Fluctuations of spectral correction factors are usually caused by the excitation or emission system of the instrument. The problem is located at the hardware site of the setup, including light source, beam pathway, wavelength filtering and light detection. Check the mechanical switching between the CFP, FRET and YFP cubes in your wide-field instrument and analyze if overlay images are shifted. Scan in unidirectional instead of bidirectional scan mode. Check if the sample dish is properly fixed on the mount. If using a system with a xenon-arc lamp or argon laser, turn on the light source at least 20 min before starting measurements to ensure stable illumination.

Problem 6

Spectral factors are stable, but dimer constructs result in varying FRET efficiencies (related to step 17).

Potential solution

One possible cause is the photo-bleaching of the fluorophores during the acquisition, which leads to different or unbalanced FRET efficiencies of the dimer constructs. Measure a dimer construct in one area five times sequentially and compare the total emission values in all three channels. Fluorescence values should not change significantly between these images. Minimize illumination of cells if photo-bleaching effects are detected. Use cycloheximide to your transfected cells 4 h prior measurement to stop translation of fluorophores. This will minimize maturing fluorophore proteins and likely results in improved stability.

Problem 7

The fitting process results in a very high estimated $E_{A,max}$ or $E_{D,max}$ value (Figure 9, step 4, green panel) that is higher than the experimental data indicate (related to step 19).

Potential solution

This problem can occur if the data points are not covering the binding curves sufficiently to estimate the maximum FRET efficiencies precisely. This problem is much more likely to occur for low binding affinity interactions, where the saturated region of the binding curve is hard to cover experimentally, combined with a concentration dependent FRET efficiency that corrupts the binding curve fit. Analyze the D3 Dimer: Increasing FRET values at higher concentrations in the "*FRET diagram:* E_A" and "*FRET diagram:* E_D" indicate concentration dependent FRET efficiencies and can corrupt the binding curve fit. Adjust this issue in the Matlab software by using the $E_{A,slope}$ and $E_{D,slope}$ dialogs (Figure 8, panel 2 b). Adjust both values manually: Start with very low values (e.g., 1×10^{-5}), until the dimer sample displays a constant FRET efficiency independent of concentration (A_{free} or D_{free}) in both diagrams ("*FRET diagram:* E_A" and "*FRET diagram:* E_D").

Problem 8

The Fiji macro "Cell_Detection" which uses the Ilastik plugin for automatic ROI detection does not complete the segmentation and automatic ROI generation process (related to step 14).

Potential solution

This macro requires increasing amount of RAM for larger images. If large or stitched images need to be analyzed, increase the amount of RAM used for the Fiji-Plugin in the settings of the software to at least 8 GB (Follow command: Edit -> Options -> Threads & Memory).

RESOURCE AVAILABILITY

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Materials availability

All plasmids related to the reference system are available on AddGene under the following reference ID's:

#198196.

#198192.

#198197.

#198198.

#198199.

#198200.

#198201.

#198202.

Data and code availability

The accession number for the software developed for this protocol and sample data reported in this paper is Zenodo: https://doi.org/10.5281/zenodo.7670415

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AUTHOR CONTRIBUTIONS

Conceptualization, C.F., M.S., M.B.-J.; methodology, C.F., M.S., M.B.-J., C.W.-S.; software, M.S., M.B.-J.; validation, C.F., M.S.; formal analysis, C.F., M.S., M.B.-J.; investigation, C.F.; data curation, C.F.; writing – original draft, C.F.; writing – review & editing, M.S., M.B.-J., C.W.-S.; visualization, C.F.; supervision, C.W.-S.; project administration, C.W.-S.; funding acquisition, C.W.-S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Rivas, S., Hanif, K., Chakouri, N., and Ben-Johny, M. (2021). Probing ion channel macromolecular interactions using fluorescence resonance energy transfer. Methods Enzymol. *653*, 319–347. https://doi.org/10.1016/bs.mie.2021.01.047.

2. Butz, E.S., Ben-Johny, M., Shen, M., Yang, P.S., Sang, L., Biel, M., Yue, D.T., and Wahl-Schott, C. (2016). Quantifying macromolecular interactions in living cells using FRET two-hybrid assays. Nat. Protoc. *11*, 2470–2498. https://doi.org/10.1038/nprot.2016.128.

3. Cranfill, P.J., Sell, B.R., Baird, M.A., Allen, J.R., Lavagnino, Z., de Gruiter, H.M., Kremers, G.J., Davidson, M.W., Ustione, A., and Piston, D.W. (2016). Quantitative assessment of fluorescent proteins. Nat. Methods *13*, 557–562. https://doi.org/10.1038/nmeth.3891.

4. Ben Johny, M., Yang, P.S., Bazzazi, H., and Yue, D.T. (2013). Dynamic switching of calmodulin interactions underlies Ca2+ regulation of CaV1.3 channels. Nat. Commun. *4*, 1717. https://doi.org/10.1038/ncomms2727.

5. Ben-Johny, M., Yue, D.N., and Yue, D.T. (2016). Detecting stoichiometry of macromolecular complexes in live cells using FRET. Nat. Commun. *7*, 13709. https://doi.org/10.1038/ncomms13709.

6. Liu, G., Papa, A., Katchman, A.N., Zakharov, S.I., Roybal, D., Hennessey, J.A., Kushner, J., Yang, L., Chen, B.X., Kushnir, A., et al. (2020). Mechanism of adrenergic Ca(V)1.2 stimulation revealed by proximity proteomics. Nature *577*, 695–700. https://doi.org/10.1038/s41586-020-1947-z.

7. Berg, S., Kutra, D., Kroeger, T., Straehle, C.N., Kausler, B.X., Haubold, C., Schiegg, M., Ales, J., Beier, T., Rudy, M., et al. (2019). ilastik: interactive machine learning for (bio)image analysis. Nat. Methods *16*, 1226–1232. https://doi.org/10.1038/s41592-019-0582-9.