rFRET: a comprehensive, Matlab-based program for analyzing intensity-based ratiometric microscopic FRET experiments

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Abstract

Fluorescence or Förster resonance energy transfer remains one of the most widely used methods for assessing protein clustering and conformation. Although it is a method with solid physical foundations, many applications of FRET fall short of providing quantitative results due to inappropriate calibration and controls. This shortcoming is especially valid for microscopy where currently available tools have limited or no capability at all to display parameter distributions or to perform gating. Since users of multiparameter flow cytometry usually apply these tools, the absence of these features in applications developed for microscopic FRET analysis is a significant limitation. Therefore, we developed a graphical user interface-controlled Matlab application for the evaluation of ratiometric, intensity-based microscopic FRET measurements. The program can calculate all the necessary overspill and spectroscopic correction factors and the FRET efficiency and it displays the results on histograms and dot plots. Gating on plots and mask images can be used to limit the calculation to certain parts of the image. It is an important feature of the program that the calculated parameters can be determined by regression methods, maximum likelihood estimation and from summed intensities in addition to pixel-by-pixel evaluation. The confidence interval of calculated parameters can be estimated using parameter simulations if the approximate average number of detected photons is known. The program is not only user-friendly, but it provides rich output, it gives the user freedom to choose from different calculation modes and it gives insight into the reliability and distribution of the calculated parameters.

Keywords: FRET, image analysis, microscopy, shot noise

INTRODUCTION

Fluorescence or Förster resonance energy transfer (FRET) is widely used by biologists to study the clustering and conformation of fluorescently-labeled molecules (1-4). In the FRET process an excited donor fluorophore, instead of emitting a photon, transfers energy to an acceptor if (i) they are sufficiently close to each other (2-10 nm); (ii) the emission spectrum of the donor overlaps substantially with the absorption spectrum of the acceptor; and (iii) the donor and acceptor are suitably oriented. Although the many manifestations of FRET allow the process to be measured in a multitude of ways, probably the most straightforward and widespread method is to record the directly-excited donor and acceptor fluorescence intensities alongside with the sensitized emission of the acceptor. This approach is variably referred to as ratiometric or intensity-based FRET (1-3,5). Although it is not our intention to give a comprehensive review of this method, a brief summary is required so that the evaluation tool to be described is understandable. A list of the parameters required for the FRET calculation is provided in Table 1.

In an intensity-based, ratiometric FRET measurement the fluorescence intensity of a donor-acceptor double-labeled sample is recorded in three fluorescence channels: (i) the donor channel (I_1) excited and detected at the absorption and emission wavelength range, respectively, of the donor; (ii) the FRET channel (I_2) excited at the donor absorption wavelength but detected at the acceptor emission wavelength range characterizing the sensitized emission of the acceptor; and (iii) the acceptor channel (I_3) whose absorption and emission wavelengths match the corresponding spectral ranges of the acceptor and this channel characterizes the direct emission of the acceptor. The measured intensities ($I_{1\text{-}3}$) are described by the following set of equations (2,5,6):

$$\begin{aligned} I_1 &= I_D\left(1-E\right)+I_A S_4 + I_D\ E\ \alpha\frac{S_4}{S_2} \\ I_2 &= I_D\left(1-E\right)S_1 + I_A S_2 + I_D\ E\ \alpha \\ I_3 &= I_D\left(1-E\right)S_3 + I_A + I_D\ E\ \alpha\frac{S_3}{S_1} \end{aligned} \qquad (1)$$

Intensities I_1-I_3 are assumed to be background-corrected in the equation set above. The simplicity of the method described in the paragraph preceding equation 1 is in striking contrast to the many terms in equation set 1 arising from the fact that none of the measured intensities is pure, but they are contaminated with overspill intensities. S_1 and S_3

characterize the fractional overspill of the donor fluorescence from the donor channel to the FRET and acceptor channels, respectively, and they are to be determined using samples labeled with the donor only. S_2 and S_4 are to be determined from the fluorescence intensities of samples containing pure acceptor only and they describe the fractional overspill of the acceptor fluorescence from the acceptor channel to the FRET and donor channels, respectively. I_D, I_A and E stand for the unquenched intensity of the donor, the directly excited emission of the acceptor and the FRET efficiency, respectively. α relates the intensity of an excited acceptor molecule detected in the FRET channel to that of an excited donor molecule detected in the donor channel according to the following equation:

$$\alpha = \frac{Q_A}{Q_D}\frac{\eta_{A,2}}{\eta_{D,1}} \tag{2}$$

where Q_A and Q_D are the fluorescence quantum efficiencies of the acceptor and donor, respectively, and $\eta_{A,2}$ and $\eta_{D,1}$ characterize the detection efficiency of an acceptor photon in the FRET channel and that of a donor photon in the donor channel, respectively.

Equation set 1 is only valid if channels I_2 and I_3 are recorded by the same detector adjusted identically. In many cases the direct and sensitized emission of the acceptor are measured by different detectors requiring a slightly modified equation for I_3:

$$I_3 = I_D\left(1-E\right)S_3 + I_A + I_D\ E\ \frac{\varepsilon_{R2}}{S_2},\ \varepsilon_{R2} = \frac{\varepsilon_D^A\ \varepsilon_A^D}{\varepsilon_D^D\ \varepsilon_A^A} \tag{3}$$

where ε stands for the molar absorption coefficient of the donor or acceptor indicated in the subscript measured at the emission range of the species labeled in the superscript. In most practical cases the two different forms of the equation for I_3 are equivalent since the terms in which they differ are zero ($S_3 \approx 0$, $\varepsilon_D^A \approx 0$, $\varepsilon_A^D \approx 0$). Solving the above equation set for the FRET efficiency (E) yields different results depending on which overspill constants can be neglected and on the form of the equation describing I_3. The solutions have been published elsewhere (2,5-8).

Although user-friendly programs are inevitable for biologists lacking computer skills to analyze intensity-based FRET measurements in microscopy due to the complexity of the equations to be solved, only a limited number of such software tools are available. While some of them are aimed at high-throughput evaluation without allowing the user much control over the calculations (7), others require more user-input to fine-tune the evaluation (6,9). The software tool presented in the current manuscript belongs to the second group

performing scientifically rigorous and fully overspill-corrected calculations with many user-adjustable options. Many different FRET-related parameters (FRETN (10), NFRET (11) and the FRET index used in the FRET Analyzer plugin of ImageJ) have been introduced to replace FRET efficiency as a measure of interaction between the donor and the acceptor. However, the quantitative correlation of these FRET-related parameters with protein clustering and the underlying physical processes is questionable (3,12). Therefore, only the FRET efficiency itself is reported by our program. Development of the tool has been initiated by the limitations in the capabilities of software tools performing fully overspill-corrected, scientifically rigorous FRET calculations (e.g. PixFRET (9) and RiFRET (6) in ImageJ):

- 1D and 2D histograms of calculated parameters are not displayed and therefore the distribution of overspill parameters and the FRET efficiency is unknown to the user. The distribution of parameters is important for interpreting and choosing an appropriate central value (mean, trimmed mean, median) to be used in the calculations. The spread of the calculated parameters may be related to biological variation or measurement error. It is reasonable to assume that the only source of variability of overspill parameters is measurement error, while the FRET efficiency may show variability due to biological reasons as well. If the signal-to-noise ratio is low, the spread of calculated parameters as a result of measurement error related to the statistical nature of photon detection may be significant. In these cases the parameter distribution is skewed, and consequently the mean is an unreliable estimate for the central tendency (13). Therefore, it is important to look at the distribution of parameters to appreciate these factors.
- Gating on histograms, which is widely used in flow cytometric analysis, is either not possible or very limited. Consequently, back-mapping of selected pixels on the original image is not available.
- It is not possible to define a mask specifying which pixels are to be included in the evaluation. Although simple histogram-based thresholding is available in other applications, this approach has severe limitations if the signal is weak relative to the background.
- Only pixelwise calculation of overspill factors and the FRET efficiency is usually available, although it has been shown that regression-based and maximum likelihood

estimation of these parameters as well as the use of summed fluorescence intensities is superior to pixelwise calculation if the signal-to-background ratio is low (13,14).

- The capability of the available programs to calculate parameter α is limited although many such methods have been published (7,8,15-17).

The software tool, rFRET, presented in the current manuscript is capable of performing rigorous analysis of intensity-based FRET experiments in microscopy and remedies the shortcomings of available tools listed above. The major novelty of our tool is (i) the display of the distribution of parameters in histograms and dot plots; (ii) flexible selection of pixels to be included in the calculation using mask images and gating on histograms or dot plots; and (iii) the availability of several methods for the calculating overspill parameters and the FRET efficiency. rFRET has been written in Matlab (Mathworks Inc., Natick, MA). Handling of images is made easier in Matlab by the free DipImage toolbox (Delft University of Technology, Delft, The Netherlands; http://www.diplib.org/). The rFRET application can handle both Matlab and DipImage variable types. Installation of DipImage is advised, but not required for running rFRET. rFRET has been tested in R2015a of Matlab and version 2.7 of DipImage, and it is freely available for download at http://peternagy.webs.com/fret. The source code of the most important functions is available at http://peternagy.webs.com/Matlab/rfret-main-functions.m. We have chosen Matlab since application development is relatively straightforward and users can use the functionality of Matlab and DipImage to process images before and after performing the analysis with our program. While our tool addresses several issues of previous programs, its limitations include that it requires Matlab and a continuous user intervention for analyzing images (i.e. it is not a high-throughput application) and that it is not currently capable of analyzing spectral FRET experiments, which are valuable alternatives for single wavelength measurements.

RESULTS AND DISCUSSION

Evaluation of a ratiometric, intensity-based FRET experiment is divided into four parts which is mirrored in the structure of the main panel of the application (Fig. 1A). First, the overspill parameters of the donor and the acceptor must be determined using samples labeled with the donor or the acceptor, respectively, followed by calculation of parameter α and the FRET efficiency itself. A detailed documentation of the most important features of

the program is presented in the Supplementary Material. In addition, a set of sample images is also provided with a brief description of their analysis and the results.

Determination of donor and acceptor overspill parameters

The rFRET application can be started by typing 'rfret' at the Matlab command prompt. In order to determine the donor and acceptor overspill parameters, samples labeled with donor or acceptor only must be imaged in the donor, FRET and acceptor channels and the images must be loaded into Matlab variables. The name of the Matlab variables can be arbitrary, and the rFRET application imports these images from the Matlab workspace by specifying the names of these variables in the graphical user interface of the application (Fig. 1). In addition to the three variables holding the images recorded in the donor, FRET and acceptor channels a fourth variable containing a mask image can be defined. The concept of masking is a significant feature of the program which can be used to exclude pixels with low signal to noise ratio or those not of interest to the user from the analysis, or it is possible to select certain cells or subcellular compartments to measure and compare FRET in distinct locations. It is important to exclude background pixels from the calculations since they result in nonsense values significantly distorting the estimation by any of the available methods. The mask is a binary image in which ones correspond to the pixels to be analyzed. The mask can be generated by a segmentation algorithm. Manually-seeded watershed segmentation is powerful for segmenting images of cells with membrane labeling (Fig.2) (18). An implementation of the manually-seeded watershed algorithm is available at http://peternagy.webs.com/matlab.htm. After defining the input variables background subtraction must be performed. The background, which will be subtracted pixel-by-pixel from the intensities, can be determined by drawing a region of interest in a label-free area in the image. This approach does not take the autofluorescence of cells into account. If cellular autofluorescence is high, the intensity of an unlabeled sample must be determined and its mean fluorescence intensity can be entered manually as the background. The donor and acceptor overspill parameters ($S_{1\text{-}4}$) can be determined by one of the following methods: (a) pixel-by-pixel calculation; (b) Deming regression; (c) maximum likelihood estimation (MLE); and (d) calculation from summed intensities. Deming regression is an alternative for ordinary least-squares linear regression. When fluorescence intensity is regressed on another fluorescence intensity, the measurement error of both variables is comparable which

violates the requirements of ordinary-least squares linear regression. Deming regression has been shown to be useful in such cases (7,19,20). MLE, taking the Poissonian nature of photon detection into consideration explicitly, has been shown to be a powerful approach for estimating both overspill parameters and the FRET efficiency (13). In most other applications for the evaluation of ratiometric microscopic FRET experiments (e.g. RiFRET (6), pixFRET (9)) only the pixel-by-pixel calculation approach is implemented. As demonstrated previously (13,14) the pixelwise calculation method is the most sensitive for distortions introduced by outlier pixels. This drawback is the most prominent at low signal-to-noise ratios. In such cases careful observation of the histogram showing the distribution of the calculated parameters and comparison of this histogram to that of simulated parameter distributions are advisable (Fig. 2). While the overspill parameter is calculated by taking the mean of the parameters calculated for each pixel in the pixelwise method ("mean of ratios"), the intensities are first summed followed by taking their ratio in the method using summed intensities ("ratio of sums"). In each calculation method the evaluation can be restricted to pixels gated on the displayed histograms or dot plots (Fig. 2). Once the determination of the overspill parameters is finished, the calculated values are exported back to the main panel.

Determination of parameter α

Parameter α, introduced by equation 2, is probably the most difficult to determine from among all the calibration constants required in a FRET experiment. Six different methods have been implemented in the rFRET application:

(1) Comparison of intensities of a sample labeled with acceptor-tagged antibodies measured in the FRET channel to the intensity of a sample labeled with donor-tagged antibodies against the same epitope measured in the donor channel (15): The efficiency of detection of an acceptor photon in the FRET channel can be compared to the detection efficiency of a donor photon in the donor channel by labeling a sample with donor-conjugated antibodies against a certain epitope and another sample with acceptor-tagged antibodies against the same epitope. Taking the molar absorption coefficients and the degree of labeling (i.e. number of fluorophores/antibody) into consideration α can be determined according to the following equation:

$$\alpha = \frac{M_A}{M_D}\frac{\varepsilon_D^D}{\varepsilon_A^D}\frac{L_D}{L_A} \quad (4)$$

where M_A is the mean intensity of the acceptor-labeled sample in the FRET channel and M_D is the mean intensity of the donor-labeled sample in the donor channel, ε_D^D and ε_A^D are the molar absorption coefficients of the donor and the acceptor, respectively, at the donor excitation wavelength and L_D and L_A are the degrees of labeling of the donor-conjugated and acceptor-conjugated antibodies, respectively. Although the method is straightforward, reliable determination of the mean intensities (M_D and M_A) requires the measurement of a large enough number of cells. This requirement can be met by analyzing several pairs of images and averaging the M_D and M_A intensities. The molar absorption coefficients can be determined by spectrophotometry or according to the method of Tolar et al. (21). Although the degree of labeling of an antibody solution is easy to determine by spectrophotometry, the number of fluorophores/antibody is likely to be different for the bound fraction if the degree of labeling is high (>3-4). This observation undermines the reliability of the calculation, since overlabeled antibodies do not bind efficiently to the antigen (22).

(2) Iterative method for a tandem donor-acceptor fluorescent protein construct (8): In a tandem donor-acceptor fluorescent protein conjugate equation 4 cannot be used directly for the determination of α, since the unquenched donor intensity (M_D) and the directly excited acceptor intensity in the FRET channel (M_A) cannot be determined in one step. However, a successive approximation method can be used in which equation set 1 is solved for I_D and I_A assuming E=0 followed by determining α according to the following equation:

$$\alpha = \frac{I_A S_2}{I_D}\frac{\varepsilon_D^D}{\varepsilon_A^D} \quad (5)$$

This α is then used to solve equation set 1 for E. The resulting FRET efficiency is used again to determine α according to equation 5. The algorithm usually converges within 5-10 steps.

(3) Closed form solution for a tandem donor-acceptor fluorescent protein construct (7): If the donor and acceptor are expressed as a tandem conjugate, then the same

number of donor and acceptor fluorophores are present, and the ratio of their fluorescence intensities is related according to equation 5. Inclusion of this equation in equation set 1 makes the simultaneous determination of α and the FRET efficiency possible. Methods 2 and 3 provide results identical within experimental error. In addition to the calculation from summed intensities suggested in the original publication, maximum likelihood estimation-based determination of α has also been implemented in the program (Fig. 3).

(4) Fitting approach for a series of donor-acceptor fluorescent protein constructs (16): If there are several conjugates of donor and acceptor fluorescent proteins in which the two fluorophores are separated by linkers of different lengths, one can determine parameter α and the $\varepsilon_D/\varepsilon_A$ absorption ratio simultaneously. Since the parameters are estimated by regression, the algorithm provides confidence intervals for the parameters. A simpler implementation of the same idea using two donor-acceptor tandem conjugates has also been published (23).

(5) Acceptor bleaching-induced dequenching of the donor (17): Incomplete bleaching of the acceptor in cells expressing a tandem donor-acceptor conjugate results in dequenching of the donor. Comparison of the decrease in the directly-excited emission of the acceptor (I_A in equation 1) detected in the FRET channel (numerator in the following equation) to the increase in the donor emission (I_D in equation 1) detected in the donor channel (denominator in the following equation) yields α provided S_3 and S_4 are negligible:

$$\alpha = \frac{\left(I_2 - S_1 I_1 - S_2 I_3\right) - \left(I_{2,\,post} - S_1 I_{1,\,post} - S_2 I_{3,\,post}\right)}{I_{1,\,post} - I_1} \tag{6}$$

where "*post*" designates intensities measured after partial acceptor photobleaching.

(6) Spectral method: The most direct way of determining α is according to equation 2 actually defining the parameter. In order to make the equation experimentally applicable it has to be extended:

$$\alpha = \frac{Q_A}{Q_D} \frac{\int f_A(\lambda)\, T_2(\lambda)\, DQ_2(\lambda)\, d\lambda}{\int f_D(\lambda)\, T_1(\lambda)\, DQ_1(\lambda)\, d\lambda} \tag{7}$$

where Q_A and Q_D stand for the fluorescence quantum efficiency of the acceptor and the donor, respectively. The detection efficiencies can be obtained by integrating the

product of the normalized fluorescence emission spectrum (*f*) of the fluorophore, the transmission of the optics (*T*) and the quantum efficiency of the detector (*DQ*) in the fluorescence channel detecting the fluorescence. Subscripts A and D designate the acceptor and the donor, respectively, whereas subscripts 1 and 2 stand for the first (donor) and second (FRET) detection channels, respectively. It must be noted that equation 7 can only be used if the detectors are run in photon-counting mode. Although this approach would be the most straightforward way to determine α, the fact that the transmission profile of the detection system and the quantum yield of the detector are typically unknown makes this method difficult to apply in practice.

Determination of the FRET efficiency

Once the overspill parameters and α have been determined, the user can choose from three different methods to calculate the FRET efficiency: (a) pixel-by-pixel; (b) FRET calculated from summed intensities; and (c) maximum likelihood estimation (13). After defining the input images recorded in the donor, FRET and acceptor channels the mask image must be chosen and background correction must be performed. Solutions for the FRET equation set with the emission wavelength range identical (equation set 1) and different (equation 3) for the FRET and acceptor channels have been implemented in the program and the user has to choose from the two options. The three calculation methods available in the program typically provide identical results with images having a high signal-to-noise ratio. However, if an image is noisy, the FRET values calculated according to the pixelwise method lead to the largest deviation from values known from previous measurements and the variability of FRET values between different segmentation methods is also the most significant for this calculation approach (Fig. 4, Table 2). This conclusion is in accordance with our previous simulation results (13) and it is further demonstrated in the Supplementary Material in which images of the same sample recorded with either high or low intensity excitation are analyzed. Both maximum likelihood estimation and the calculation from summed intensities provide a single estimate for the FRET efficiency thereby making the detection of biological heterogeneity impossible. However, in the case of noisy images widening of the FRET histogram due to the statistical nature of photon detection is significant enough to obscure any biological heterogeneity. In such cases a reliable single estimate for the FRET efficiency is more valuable than a questionable FRET

histogram dominated by noise-related artifacts. At the same time, if the signal-to-noise ratio is high, FRET values calculated on a pixel-by-pixel basis can reveal biological heterogeneity. Such pixelwise distributions ("FRET images") can be generated by the program and the image can be exported to the Matlab workspace.

Simulation of parameter distributions

The number of detected photons follows a Poisson distribution whose coefficient of variation is inversely proportional to the square root of the mean number of photons. Since the error in photon detection propagates to any parameter derived from intensities, the FRET efficiency and the overspill parameters are also characterized by a certain distribution whose width is determined by the underlying statistics of the intensity measurement. Since this effect is significant if intensities are low, knowledge of the width of the distributions due to error propagation is important in such cases. Given the mean number of detected donor and acceptor photons the rFRET application can simulate the distribution of the FRET efficiency due to the Poissonian nature of photon detection assuming all pixels are characterized by the same, user-defined FRET value (Fig. 5). The same kind of simulation can be carried out for the overspill parameters. If the width of the simulated distribution is the same as that of the experimentally determined one, there is no evidence for any biological heterogeneity, i.e. the width of the experimentally determined distribution is solely due to detection noise.

Conclusions

rFRET is a versatile tool for evaluating ratiometric, intensity-based FRET experiments. Users can select pixels to be included in calculations using a mask image or gating on histograms and dot plots. Several methods have been implemented for the determination of all the required correction parameters and the FRET efficiency. The effect of the statistical nature of photon detection can be observed using parameter simulations and it can be eliminated using maximum likelihood estimation. Usage of the program is facilitated by the graphical user interface and extensive help available from the application.

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Table 1 Parameters used during the analysis of intensity-based FRET measurements

Parameter	Description
S_1	overspill of donor emission to the FRET channel
S_2	overspill of acceptor emission to the FRET channel
S_3	overspill of donor emission to the acceptor channel
S_4	overspill of acceptor emission to the donor channel
α	ratio of intensity of an excited acceptor measured in the FRET channel to that of an excited donor measured in the donor channel
ε_D^D	molar absorption coefficient of the donor at the excitation wavelength of the donor
ε_A^D	molar absorption coefficient of the acceptor at the excitation wavelength of the donor
ε_D^A	molar absorption coefficient of the donor at the excitation wavelength of the acceptor
ε_A^A	molar absorption coefficient of the acceptor at the excitation wavelength of the acceptor
I_1	intensity in the donor channel
I_2	intensity in the FRET channel
I_3	intensity in the acceptor channel
I_D	unquenched donor intensity (intensity of the donor in the absence of the acceptor, i.e. when E=0)
I_A	directly excited acceptor intensity
E	FRET efficiency

Table 2 Evaluation of FRET efficiency using different mask images

segmentation method	pixelwise	MLE	summed intensity
manual	0.04	0.06	0.07
intermeans	0.13	0.08	0.1
watershed	0.13	0.07	0.1

Cells were labeled with donor- and acceptor-conjugated trastuzumab antibodies against the cell surface protein ErbB2 to measure its homoassociation. In parallel flow cytometric experiments the FRET efficiency was calculated to be 0.08±0.01 (mean±SD). In the microscopic measurements the FRET efficiency was calculated using masks generated using histogram-based thresholding (manual, intermeans) and manually-seeded watershed segmentation. Only pixels inside the masks were included in the calculations. The masks are shown in Fig. 4. The FRET efficiency was calculated using pixelwise calculation, maximum likelihood estimation (MLE) and from summed intensities. The FRET efficiencies for the pixelwise method represent the 5% trimmed mean of the histograms shown in Fig. 4E.

FIGURE LEGENDS

Figure 1. Panels of the rFRET program. (A) Main panel from which panels performing calculation of overspill parameters and the FRET efficiency can be opened. Users can save and load the application environment, upgrade the program and access extensive help using the push buttons in the bottom row. (B) Panel for calculating donor overspill parameters. There is a similar panel for performing acceptor overspill parameters. (C) Panel performing calculation of parameter α. (D) Panel for calculating the FRET efficiency.

Figure 2. Representative calculation of donor overspill factors using the pixel-by-pixel method. A sample labeled with a donor-conjugated antibody against ErbB2, a receptor tyrosine kinase expressed in the cell membrane, was measured in the donor, FRET and acceptor channels and the image recorded in the donor channel is shown in A. Since a membrane protein was labeled, manually-seeded watershed transformation was used to segment the image. The membrane pixels identified by the watershed algorithm are shown in B and the overlay of the segmented and original images in C. The graphs generated when pixelwise calculation of the donor overspill parameter is performed are shown in D. Histogram of S_1 and S_3 and dot plots are generated. A polygon gate was placed on the dot plot showing the intensity in the FRET and donor channels. The orange dots and curves correspond to the gated pixels. The scale bar corresponds to 20 μm.

Figure 3. The graph panel generated during the maximum likelihood estimation of parameter α. Maximum likelihood estimation of the FRET efficiency and α was performed with pixels gated in the I_3-I_1 dot plot (7). Gated pixels are shown in orange. The estimated values for the FRET efficiency and α are shown in the blue text boxes on the top. The surface plot at the bottom displays the confidence plot of the determined FRET and α values. The log-likelihood distribution of pixels at the estimated FRET and α values is shown in the lower right corner. A more detailed description of all the adjustable parameters is provided in the Supplementary Material.

Figure 4. Representative calculation showing the determination of FRET efficiency. A sample was labeled with both donor and acceptor (donor-tagged and acceptor-tagged trastuzumab antibodies against the cell surface receptor ErbB2 to measure its homoassociation). The

image recorded in the acceptor channel is shown in A. The image was segmented by manual thresholding aimed at identifying most of the membrane pixels (B), using the intermeans algorithm (C) and manually-seeded watershed transformation (D). The corresponding distributions of pixelwise FRET efficiencies are shown in E and the calculated trimmed means in Table 2. The scale bar corresponds to 20 μm.

Figure 5. Simulation of the distribution of the FRET efficiency at different number of detected photons. The number of detected photons from the unquenched donor (I_D) and from the directly excited acceptor (I_A) were assumed to be 20, 100 and 200, and the distribution of the calculated FRET efficiency of 100,000 pixels was simulated assuming all of them have a FRET value of 0.35.