FRETcalc plugin for calculation of FRET in non-continuous intracellular compartments

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Abstract

FRET has emerged as an important tool for studying intracellular processes and interactions between biomolecules. Intracellular donor and acceptor molecules are distributed in individual organelles that usually have complex non-continuous shape. Consequently, background pixels arising from fluorophore-free regions of the cell are proximal to FRET-positive pixels, leading to systemic errors in the estimated FRET values. This study introduces a new FRET algorithm for FRET estimation by acceptor photobleaching that separates the FRET-positive pixels from the background by applying user-defined thresholds for pixel selection. The FRET algorithm was validated by analysis of interactions between fluorescently tagged proteins in the endoplasmic reticulum using acquired and simulated images. The novel algorithm showed superior performance to the regular FRET calculation algorithm in acquired images and in most simulations. The developed algorithm was incorporated into the FRETcalc plugin for ImageJ program that enables user-defined choices of thresholds for calculation of FRET by acceptor photobleaching.

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Fluorescence resonance energy transfer (FRET) is an important tool in the modern biomedical research that allows analysis of inter- and intra-molecule interactions and intracellular processes [1,2]. Cells expressing fluorescently tagged biomolecules can be easily generated and studied using fluorescent and confocal microscopes that are accessible to most researchers. Accessibility of the above mentioned techniques and equipment has resulted in a steady increase in the number of publications that report FRET data. However, the majority of the studies do not apply standards for FRET analysis, and do not report validation of the applied FRET estimation approaches [3]. As a result, the robustness of FRET estimation and the reliability of the reported FRET data are difficult to ascertain.

FRET estimation is complicated due to technical limitations, such as the resolution of the microscope and sensitivity of the fluorescence detection systems [4], and complexity of biological samples. In the cells, both donor and acceptor are compartmentalized to individual organelles as a function of their physicochemical and biochemical properties and intracellular trafficking. Organelles possess different shapes and forms, and fluorescently tagged molecules that are located within the organelle are usually highly mobile and homogeneously distributed [5]. In some cases, such as nucleus, the dimensions of the organelle are much larger than the resolution limit of the microscope. Hence, donor and acceptor fluorescence arising from the nucleus in confocal images are not intermixed with the contaminating signals from other compartments. In this case, FRET estimation using the regular techniques and algorithms is expected to be robust and reliable, with diffraction and detection noise being the major factors that introduce errors in the calculated FRET values.

Other organelles have more complex non-continuous shapes and the size of one or more of their dimensions can be comparable to the resolution limit of the micro-
scope. One example is the endoplasmic reticulum (ER), the site of intracellular metabolism and folding, that comprises a mesh of interconnected tubuli with a diameter in the range of 60–100 nm that are spread through most of the cytosol [6,7]. Even when there is a uniform distribution of donor or acceptor molecules in the ER, fluorescence images include pixels with ER-originating signal that are surrounded by background pixels. The calculated FRET values in this case are lower than the real FRET values, and separation of the signal-containing ER pixels from the background is essential for reliable FRET calculation.

The objective of this study was to analyze the precision and reliability of FRET estimation for the donor and acceptor molecules distributed in intracellular compartments with non-continuous shape, and to identify the factors that affect them. The study applied fluorescently tagged HLA-A2, TAP1, and tapasin proteins that interact in the ER and form MHC class I peptide loading complex [8]. A new FRETTH algorithm for FRET estimation by acceptor photobleaching is introduced that is based on selection of pixels that match a set of user-defined thresholds. Effects of diffraction, detection noise, and image smoothing on FRET estimation were studied by analysis of the confocal images of the cells expressing the studied proteins, or of the simulated ‘real’ or ‘ideal’ ER images with known FRET efficiencies. FRETTH was superior to the regular FRET calculation approach in most conditions. The algorithm was incorporated into the FRETcalc plugin for ImageJ software that enables user-defined threshold choices for FRET calculation and visualization of the FRET analysis results. The outcomes of the study, the FRETTH algorithm, and the FRETcalc plugin, are applicable for FRET estimation in other organelles with non-continuous and continuous shapes, as well as for non-protein fluorophores.

Materials and methods

Constructs. The studied transmembrane ER proteins [8,9] were tagged with CFP or/and YFP groups in their C-terminal cytosolic regions. The tagged HLA-A2 vectors were kindly provided by Dr. M. Edidin [10]. The C-terminal tagged TAP1 construct was created by PCR-deletion of the stop codon of TAP1 and ligation into HLA-A2-CFP, replacing HLA-A2. The tagged tapasin construct was created by PCR-mutation of the sequence upstream of the ER retention signal to introduce a Sall restriction site that was used to insert CFP-encoding sequence. The coding sequences of the obtained constructs were transferred into pLPCX or pLNCX2 vectors (Clontech).

Cells and retroviral transduction. HeLa M, a IFNγ-sensitive clone of HeLa human cervical cell carcinoma, was used in this study because of cellular dimensions (approximately twice as big as HeLa cells) and spreading upon attachment, that are favorable for imaging of intracellular structures. The cells were cultured in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 5% FBS. Retroviral production and infection of the cells were performed according to the manufacturer’s protocol (Clontech), and cell lines expressing the tagged proteins were generated by FACS-sorting for YFP and/or CFP-positive cells and selection with antibiotics.

Sample preparation and image acquisition. Cells were seeded on sterile alcan blue-pretreated coverslips, allowed to adhere overnight, fixed with 2.5% formaldehyde, washed, and the coverslips were mounted on slides using 10% Mowiol 4-88 solution (Calbiochem). FRET between CFP and YFP groups was determined using Leica TCS SP2 Confocal Microscope and FRET by acceptor photobleaching module of the Leica confocal software. The microscope was equipped with a 63× 1.4 oil immersion objective (NA = 1.4), and 458 and 514 nm laser lines were used to excite the CFP and YFP fluorophores, respectively. 8-bit 512×512 pixel images were acquired with 4× digital zoom, resulting in 116.2×116.2 nm pixel size. After acquisition of prebleach images, YFP was bleached by at least 85% using repetitive scanning with a full intensity of 514 nm laser, and the postbleach images were acquired. The background fluorescence in the cell-free regions of the YFP and CFP channels, was ~8 ± 6 (mean ± SD).

Simulated ‘real’ and ‘ideal’ ER images. Simulated ER image sets (four slices for donor and acceptor pre- and postbleaching fluorescence) were prepared using custom-developed plugins for ImageJ software. Real’ ER images with 0% FRET were produced by duplicating the donor prebleaching image of cells expressing tapasin-CFP and HLA-A2-YFP. For ‘ideal’ ER images, image with uniform background (I = 10) served as a template, and 1-pixel wide horizontal and vertical lines of higher intensity (I = 100) were created using ImageJ. The width and distance between the lines were chosen to resemble the ER morphology in the electron microscope images of the studied cell lines that showed average ER branch thickness and interbranch distance of ~60 and ~300 nm, respectively (data not shown).

Simulations of FRET, point spread function (PSF), detection noise, and image smoothing. Effects of individual experimental factors of FRET calculation were studied in ‘ideal’ and ‘real’ ER images, and sequential changes were applied to the studied images in the following order: simulation of FRET, PSF (optional), and detection noise, and image smoothing (optional).

FRET arising from ER in the ‘ideal’ and ‘real’ ER images was simulated by multiplying the intensity of the pixels in the donor channel above a selected threshold (I = 40) by a factor of 0.5–1.0, reducing the pixel intensity as corresponding for 0–50% FRET.

For PSF determination, Z-stacks of Fluoresbrite YG carboxylate beads (1.0 μm diameter, Polysciences Inc.) were acquired using the same parameters as used for the experimental samples. PSF was estimated using the PSF module of the Leica confocal microscope, and PSF convolution of the simulated image sets was performed using the Real Convolver plugin for ImageJ [11].

As an approximation of Poisson noise, every pixel’s intensity in the simulated image sets was increased/decreased by a uniformly distributed random number within (I – I/4, I + I/4) range, subject to the constraint of non-negativity. Image smoothing was performed by 3×3 averaging filter.

FRETcalc plugin and FRET calculation. FRET calculation used the FRETTH and FRETALL algorithms implemented in the custom-developed FRETcalc plugin [12]. For the acquired and simulated ‘real’ ER images rectangular regions of interest (ROI) were selected covering part of the ER in the individual cell, and for the ‘ideal’ ER images ROI covered the entire image. FRET analysis was based on all the pixels in the selected ROI (FRETALL) or on the pixels with donor and acceptor fluorescence higher than the threshold value (I = 50, FRETTH). Additional thresholds that could be specified in FRETcalc plugin (% acceptor bleaching, % FRET) were not applied. For both algorithms, FRET was calculated from the sum of the individual pixel’s fluorescence intensities (I) as:

\[
\% \text{ FRET} = \left( \frac{I_{\text{Donor PostBleaching}} - I_{\text{Donor PreBleaching}}}{I_{\text{Donor PostBleaching}}} \right) \times 100\%
\]

Results

The studied proteins are part of the MHC class I peptide loading complex that loads the MHC class I heavy chain \(\beta\)-m dimers with peptides in the ER. TAP1 and tapasin are ER-resident proteins, while HLA-A2 molecules after loading with peptides traffic from the ER to Golgi, cell surface, and the endosomes. Thus, only a small fraction of the HLA-A2 molecules is in the ER at any given time, with sig-
significant amounts distributed in other compartments. Fluorescent images confirm our expectations regarding the localization of the fluorescently tagged proteins; tapasin-CFP and TAP1-CFP showed the expected reticular ER pattern, while the tagged HLA-A2 proteins showed a combined pattern with predominantly ER and endosomal components (Fig. 1). Tapasin-CFP and TAP1-CFP colocalized with the markers of the ER, while tagged HLA-A2 proteins colocalized with the markers of ER and endosomes (data not shown). Analysis of fluorescence intensities using histograms showed that the background noise can reach intensities of up to $I = 40–50$, while majority of the intracellular pixels had higher intensities (Fig. 1E).

**Analysis of acquired ER images**

Interaction between the different pairs of the interacting proteins was studied using FRET by acceptor photobleaching, in comparison to the positive (tandem YFP–CFP tag, with estimated FRET efficiency of ~20% [10]) and negative (CFP alone) controls (Fig. 2). Two algorithms were used for the analysis: FRET$_{\text{ALL}}$ that is based on all the pixels in the selected ROIs, and FRET$_{\text{TH}}$ that did not include background pixels ($I_{\text{DONOR}} < 50$ and $I_{\text{ACCEPTOR}} < 50$). Elimination of the background pixels from the analysis using the FRET$_{\text{TH}}$ algorithm resulted in higher FRET values, especially for the samples where ER and endosomally-distributed HLA-A2-YFP molecules were serving as acceptor (Fig. 2A). Tapasin interacts directly with HLA-A2 and with TAP1, while HLA-A2 is in close proximity to TAP1 in the MHC class I peptide loading complex [8], and all the protein interactions presented in Fig. 2 were expected to exhibit significant FRET. Thus, results obtained with FRET$_{\text{TH}}$ algorithm are consistent with expectations, while analysis using FRET$_{\text{ALL}}$ algorithm was not able to reveal FRET efficiencies, potentially leading to the false conclusion that the proteins do not interact. Smoothing of the images resulted in lower FRET values calculated with the FRET$_{\text{TH}}$ algorithm, apparently due to reduced separation between the FRET-containing pixels and the background (Fig. 2B). Since smoothing does not change the total fluorescence intensities in the selected ROIs, it did not affect the calculation by FRET$_{\text{ALL}}$ algorithm, as expected.

**Generation of simulated ER images**

To elucidate the robustness of FRET$_{\text{TH}}$ algorithm and the factors that limit its application, I applied a simulation approach. Simulated images with known FRET efficiencies were generated based on either the acquired images of ER fluorescence (‘real’ ER, Fig. 3A) or the ‘ideal’ ER with complete separation between the ER branches from the background (Fig. 3B). PSF convolution, detection noise, and smoothing were applied to the simulated images, and their effect on the estimation of FRET values was studied.

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![Fig. 1](image-url)  
*Fig. 1. Fluorescence images of HeLa M cells stably expressing tapasin-CFP (donor) and HLA-A2-YFP (acceptor). Tapasin-CFP is confined to the ER, while HLA-A2-YFP is localized predominantly in the ER and endosomal compartments. (A–D) Donor and acceptor pre- and postbleaching images. (E) Histogram of donor fluorescence intensity for the individual pixels in the representative extracellular or intracellular ROIs.*
Representative example of detection noise and PSF images are shown in Fig. 3 C and D, respectively, along with their effect on the simulated 'ideal' ER images (see Fig. 3 E–H). Performance of FRET TH and FRET ALL algorithms was studied for a wide range of FRET efficiencies and detection noise intensities (Fig. 4).

Analysis of simulated ER images

Analysis of the simulated ‘real’ ER images is shown in Fig. 4. The FRET TH algorithm had a tendency to overestimate the FRET values by approximately 1%, while FRET ALL underestimated FRET efficiency, and the error increased with increase in the FRET values (Fig. 4A). Smoothing introduced a trend of underestimation for FRET TH without affecting FRET ALL (Fig. 4B). Thresholding algorithm FRET TH was more sensitive to noise than FRET ALL, but this sensitivity was cancelled by image smoothing (Fig. 4C and D). Thus, in ‘real’ ER images FRET TH algorithm was superior to FRET ALL in most cases, with exception of the samples with low efficiency of FRET (<10%) and high noise. It should be noted that these simulations most probably overestimate the effect of noise on the performance of FRET TH, since the ‘real’ ER images originate from the acquired images that are not noise-free.

Analysis of ‘real’ ER images is influenced by the noise, diffraction, and incomplete resolution between the ER and the background that stem from the nature of the specific sample and the characteristics of the confocal microscope that was used for the acquisition. To study the individual influence of each one of these factors on the FRET estimation algorithms, I generated and analyzed ‘ideal’ ER images (Fig. 3). In absence of diffraction, FRET TH was very robust over a wide range of FRET efficiencies and noise levels, while FRET ALL had a tendency for FRET underestimation that was augmented with increase in FRET (Fig. 4E and G). Upon image smoothing,
FRET_TH was still superior to FRET_ALL, although its robustness was compromised (Fig. 4F and H). PSF convolution of the images reduced the separation between the ER-positive and background pixels, and increased the error for FRET_TH. Consequently, FRET_TH was marginally superior to FRET_ALL in the whole range of studied FRET efficiencies (Fig. 4I), while at high noise levels (\(I > 15\)) it reduced the error in FRET estimation, apparently due to higher sensitivity of FRET_TH algorithm to detection noise that increases the calculated FRET values (Fig. 4K). Image smoothing did not affect the trends of FRET estimation errors in PSF-convolved images (Fig. 4J and L). Thus, although diffraction and image smoothing reduce efficiency of separation between ER-positive and background pixels, FRET_TH consistently reduces error in FRET estimation as compared to FRET_ALL in simulated ‘ideal’ ER images.

Discussion

Precise and robust determination of FRET values is important for studying intracellular processes and analyzing intracellular interactions between fluorescently tagged molecules. In this study I analyzed the estimation of FRET values for tagged molecules distributed and interacting in intracellular compartments with non-continuous shape, with specific focus on the endoplasmic reticulum, and introduced a new FRET_TH algorithm that is based on threshold-based selection of pixels for data analysis. Analysis of original experimental data using this algorithm revealed significant interactions between the tagged proteins in the ER, consistent with the biochemical data [8,9], while threshold-less analysis based on all the pixels in the selected ROI (FRET_ALL algorithm) failed to reveal some of the interactions. Robustness of the FRET_TH algorithm was assessed further in simulated ‘real’ and ‘ideal’ ER image sets, and effects of the individual experimental (FRET efficiency), acquisition-related (diffraction, detection noise), and image processing (image smoothing) factors on the error in the estimation of FRET values were determined (see Fig. 4 and Results).

From the results of FRET analysis, it is evident that error in FRET estimation is dependent on efficiency of separation between the ER and background pixels in the analyzed images, which in turn is dependent on the shape of studied organelle, intensity of the background and specific fluorescence, resolution, and noise of the acquisition system, and user choices of image processing (e.g., smoothing). Consequently, results of FRET estimation were different in the ‘real’ as compared to the ‘ideal’ ER images. For ‘real’ ER, only partial separation between the ER and
background pixels could be achieved, limiting the robustness of FRET_{TH} algorithm, and the resolution is improved for the samples characterized by high FRET efficiency and low detection noise. Error in estimation of FRET can be reduced in some cases by combining the FRET_{TH} algorithm with image smoothing that partially balances the overestimation and underestimation trends in the FRET calculation (see Fig. 4B and D). For ‘ideal’ ER, the ER-rich and ‘empty’ pixels were efficiently separated using thresholds, and FRET_{TH} was superior to FRET_ALL, for a wide range of FRET efficiencies and intensities of detection noise, while image smoothing reduced the separation between the ER and background pixels and induced systemic underestimation of FRET values by FRET_{TH}. Better separation between the ER and background pixels and higher robustness of FRET_{TH} algorithm could be potentially achieved by deconvolution of the experimental images. However, deconvolution is computation-intensive, prone to errors in fluorescence reassignment, and usually requires acquisition of Z-stacks of the experimental sample, a procedure that can substantially increase the error in FRET calculation due to photobleaching.

FRET_{TH} algorithm is based on pixel selection and pixel-to-pixel analysis of the experimental images. Several pixel-based algorithms have been proposed previously for FRET calculation, and they can be classified as (a) algorithms for spectral bleed-through correction, (b) FRET normalization algorithms, and (c) colocalization algorithms. The majority of these algorithms, including PixFRET plugin [13] and PFRET algorithm [14] that correct the spectral bleed-through, and FRETN and N_{FRET} normalization algorithms [15,16], are not suitable or do not offer advantage for FRET analysis when donor and acceptor molecules are distributed in organelles with non-continuous shapes. A colocalization algorithm, such as ‘FRET and Colocalization Analyzer’ [17], can potentially be applied to select the donor and acceptor-positive pixels, and to exclude background pixels from the analysis. However, ‘FRET and Colocalization Analyzer’ requires manual selection of the colocalized pixels in each analyzed image that is potentially prone to bias, and is restricted to analysis of FRET by sensitized emission.

It appears thus that the FRET_{TH} algorithm developed in this study offers substantial benefits over the previously described pixel-based algorithms in analysis of FRET in intracellular organelles with non-continuous shape. The algorithm was assessed for the analysis of protein interactions in the ER, but it is applicable to other organelles, and to non-protein fluorophores. The precision and robustness of FRET estimation using FRET_{TH} is dependent on the shape of the organelle under study, the resolution of the microscope, the noise of the detection systems, and image manipulations, such as smoothing. Selection of the threshold values can affect the efficiency of the FRET_{TH} algorithm. Efficiency of separation between the specific and the background signals will be reduced if the thresholds are too low, while too high threshold values can skew the calculation results by eliminating part of the specific pixels from the analysis. Additional source of error in FRET estimation for organelles with non-continuous shape can be misalignment of the analyzed images due to shift in the imaged area during the acquisition. Image misalignment should be monitored, and misaligned images should be re-aligned or discarded from the analysis.

While the FRET_{TH} algorithm reduces the error in FRET estimation for compartments with non-continuous shape by separating the signal-containing pixels from the background, it can not separate the organelle-derived and background signals that are intermixed within the same pixel. Therefore, for organelles with non-continuous shape and fine morphology, if dimensions of the individual structures are close to the resolution limit, the estimated FRET values will be lower than the true FRET efficiency. The true FRET values can be generally revealed by normalizing the FRET signal in the individual pixel (voxel) by the relative volume of the ER vs. background. However, this normalization requires standards with known ER vs. background volumes, and is expected to be highly sensitive to detection noise and image misalignment.

In summary, the FRET_{TH} algorithm is useful for estimation of FRET arising from complex compartments with non-continuous shape that result in non-homogeneous fluorescence of the fluorophores in the analyzed images. The FRET_{TH} algorithm was incorporated in the FRETcalc plugin for ImageJ program that enables platform-independent (PC and Mac computers-compatible) analysis of FRET by acceptor photobleaching. In addition to user-defined thresholds of donor and acceptor intensities, pixels for the data analysis can be restricted based on the extent of bleaching, and FRET efficiency. Quality and robustness of FRET analysis can be assessed using the output options of FRETcalc plugin that include histograms and plots of the donor, acceptor, and FRET intensities in the analyzed ROIs.

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