Characterization of one- and two-photon excitation fluorescence resonance energy transfer microscopy

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Abstract

Advances in molecular biology provide various methods to define the structure and function of the individual proteins that form the component parts of subcellular structures. The ability to see the dynamic behavior of a specific protein inside the living cell became possible through the application of advanced fluorescence resonance energy transfer (FRET) microscope techniques. The fluorophore molecule used for FRET imaging has a characteristic absorption and emission spectrum that should be considered for characterizing the FRET signal. In this article we describe the system development for the image acquisition for one- and two-photon excitation FRET microscopy. We also describe the precision FRET (PFRET) data analysis algorithm that we developed to remove spectral bleed-through and variation in the fluorophore expression level (or concentration) for the donor and acceptor molecules. The acquired images have been processed using a PFRET algorithm to calculate the energy transfer efficiency and the distance between donor and acceptor molecules. We implemented the software correction to study the organization of the apical endosome in epithelial polarized MDCK cells and dimerization of the CAATT/enhancer binding protein α (C/EBPα). For these proteins, the results revealed that the extent of correction affects the conventionally calculated energy transfer efficiency (E) and the distance (r) between donor and acceptor molecules by 38 and 9%, respectively.

1. Introduction

Within the living cell, interacting proteins are assembled into molecular machines that function to control cellular homeostasis. These protein assemblies are traditionally studied using biophysical or biochemical methods such as affinity chromatography and coimmunoprecipitation. Recently, two-hybrid and phage-display methods have been used for detecting protein–protein interactions. These in vitro screening methods have the advantage of providing direct access to the genetic information encoding unknown protein partners [1]. These techniques do not allow direct access to interactions of these protein partners in their natural environment inside the living cell, but using the approach of fluorescence resonance energy transfer (FRET) microscopy, this information can be obtained from single living cells with nanometer resolution [2–9].

Advances in optics, digital computers, digitizers and image processing software, and low-light-level photodetectors have improved the application of FRET microscopy [10–12]. The development of mutant forms of green fluorescent proteins (GFP), which can be used to reveal the chemical and molecular dynamics of proteins in intact cells, has significantly broadened the usefulness of FRET microscopy, allowing localization and measurement of protein activity [13,14]. Visualizing fluorescently tagged proteins in living (or fixed) cells with conventional fluorescence microscopy is limited by the...
contribution of out-of-focus signal from above and below the focal plane [8]. The most widely used techniques to remedy these problems and produce more reliable three-dimensional data are digital deconvolution and confocal microscopy [15,16]. Laser scanning confocal microscopy (LSCM) uses a pinhole aperture to restrict the out-of-focus flare reaching a detector, the photomultiplier tube (PMT). Increased excitation intensity increases the signal, but also increases photobleaching and photodamage.

These limitations can be overcome with two-photon excitation FRET microscopy (2p-FRET). Two-photon excitation uses a diffraction-limited spot illumination of the specimen, which reduces photobleaching and photodamage outside the area of excitation [17–19]. Because excitation occurs only at the focal plane, a pinhole is not required and many more photons reach the PMT as compared with confocal microscopy. The use of infrared laser light excitation instead of ultraviolet laser light also reduces phototoxicity in the living cell. The extent of photobleaching with 2p excitation may be greater than one-photon (1p) excitation at the focal plane [20], but overall photobleaching of the sample is considerably reduced in 2p [18]. Further, it is possible to minimize the photobleaching in 2p-FRET microscopy by reducing the average power of the excitation IR laser at the specimen plane.

One of the important conditions for FRET to occur is the overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor [21–23]. As a result of spectral overlap, the FRET signal is always contaminated by donor emission into the acceptor channel and by the excitation of acceptor molecules by the donor excitation wavelength (see Fig. 1). Both of these signals are termed spectral bleed-through (SBT) signal into the acceptor channel. In principle, the SBT signal is the same for 1p- and 2p-FRET microscopy. In addition to SBT, the FRET signals in the acceptor channel also require correction for spectral sensitivity variations in donor and acceptor channels, autofluorescence, and detector and optical noise, which contaminate the FRET signal.

In this article, we describe the development of 1p- and 2p-FRET microscopy techniques and a methodology to characterize the systems for protein localization. We have described an algorithm for the FRET system that

Fig. 1. Absorption and emission spectra of fluorophore pairs. The absorption and emission spectra of different fluorophores used for labeling the proteins: (A) Alexa 488- and Cy3-labeled P1gA-R ligands and (B) BFP-GFP and (C) CFP-dsRED1 pairs were used to express the C/EBPα enhancer-binding proteins. The shaded regions are (A) the overlap integral and (A, B, C) the donor and acceptor spectral bleed-through (SBT). The fluorophore pair used for (A) 1p-FRET and (B, C) 2p-FRET imaging. $D_{AB}$, $A_{AB}$, donor and acceptor absorption spectra; $D_{EM}$, $A_{EM}$, donor and acceptor emission spectra.
corrects for donor and acceptor SBT and variation in signal intensity due to fluorophore expression (or concentration) levels in living or fixed cells. The energy transfer efficiency ($E$) and distance ($r$) between donor and acceptor molecules were calculated (or estimated) using the same cellular image used for FRET imaging.

2. Materials and methods

2.1. Cell preparation

2.1.1. Polarized epithelial cells

Polarized epithelial MDCK cells, stably transfected with polymeric IgA receptor (pIgA-R), were grown for 3 days in Transwell-Clear inserts, washed with phosphate-buffered saline (PBS), and followed by internalization of 160 µg/ml pIgA-R-IgG ligands ($[\text{Fab}]_2$ pseudo ligands) conjugated to Alexa 488 (www.probes.com) or Cy3 for 4 h at 17°C. The ligands were applied to the apical and basolateral plasma membrane (PM), respectively. At 17°C the pIgA-R-ligand complexes moved into the subapical region [24]. Then, the cells were washed with PBS to remove unbound ligands and immediately fixed with 4% paraformaldehyde/PBS. We internalized the Alexa 488- and Cy3-labeled ligands at a donor:acceptor ratio of 1:1. In all, three different samples were used: the double-labeled specimen, containing apically internalized Alexa 488-pIgA-R-ligand (donor) complexes and basolaterally internalized Cy3-pIgA-R-ligand complexes (acceptor), plus corresponding reference samples containing either Alexa 488 or Cy3 that were used to establish the spectral bleed-through levels (see below).

2.1.2. CAATT enhancer binding protein α (C/EBPα)

For the studies described here the sequence encoding the DNA binding and dimerization domain of the transcription factor C/EBPα [26] was fused in-frame to the commercially available CFP or dsRED1 color variants (www.clontech.com) to generate CFP-C/EBP Δ244 and dsRED1-C/EBP Δ244 [25]. Mouse pituitary GHFT1-5 cells [26] were harvested and transfected with the indicated plasmid DNA(s) by electroporation [27]. The total input DNA was kept constant using empty vector DNA. Cell extracts from transfected cells were analyzed by Western blot to verify the tagged proteins were of the appropriate size as described previously [27]. For imaging, the cells were inoculated dropwise onto a sterile coverglass in 35-mm culture dishes and allowed to attach prior to gently flooding the culture dish with medium. They were maintained for 18–36 h prior to imaging. The coverglass with attached cells was inserted into a chamber containing the appropriate medium and the chamber was then placed on the microscope stage.

2.2. Instrumentation and data acquisition

2.2.1. Confocal- or 1p-FRET microscopy

We configured the Nikon PCM2000 laser scanning confocal microscope for 1p-FRET microscopy. Plan-Fluor 60× (NA 1.2) water immersion (WI) objective lens was used for the image acquisition (1024 × 1024-pixel color). Simple PCI software (www.cimaging.net) was used to drive the hardware and for image acquisition. The fluorophore pair was selected depending on the availability of donor and acceptor excitation wavelengths and the filter configurations.

In this confocal FRET microscopy we have tested both live and fixed cells. Here we explain the polarized epithelial cells transfected with polymeric IgA receptor conjugated to Alexa 488 (donor) and Cy3 (acceptor). Double-labeled donor and acceptor specimens were positioned in a small chamber created by a coverslip between two metal rings and filled with a small amount of PBS. This whole assembly was placed on the microscope stage. An appropriate area of the specimen was chosen about 3.5 µm below the apical PM (cell height checked, 15–20 µm) using a green HeNe laser (acceptor excitation ($\lambda_A$, 543 nm). With the zoom settings at 2.3, and without any image processing, a single-scan image of the double-labeled specimen was taken with only acceptor excitation (see Fig. 2g). This was followed by a single-scan image with only the 488-nm ($\lambda_D$) donor excitation wavelength (see Figs. 2e and f). The single-labeled acceptor specimen was excited by $\lambda_D$ and $\lambda_A$ and the images were acquired in the acceptor channel (see Figs. 2c and d). The single-labeled donor was scanned at the donor excitation wavelength (Figs. 2a and b). Subjecting the donor to the acceptor excitation wavelength produces no signal. Seven images (see Table 1) of all three types of specimens were taken under the exact same conditions (optics, excitation laser power, and integration time of the signals) and stored in the hard disk for further processing (see Fig. 2).

2.2.2. 2p-FRET microscopy

The 2p-FRET system, which we integrated, consists of a Nikon TE300 inverted microscope equipped with a 100-W Hg arc lamp for epifluorescence illumination. Plan-Fluor 20× (NA 0.5) multimmersion (water and oil) and 60× (NA 1.2) water immersion objective lenses were used for the image acquisition. A built-in lens and the dichroic in the filter cube were used to couple the TE300 externally (no physical connection of the microscope and the confocal scan head) to the confocal scan head. A Coherent 5-W Verdi pumped, tunable (Model 900 Mira) mode locked ultrafast (76-MHz) pulsed ($<150$-fs) laser was coupled to the laser port of a Bio-Rad MRC600 laser scanning confocal scan head through beam steering optics (www.coherentinc.com).
This laser is equipped with X-wave optics for an easy tunable range of wavelength, 720–880 nm.

Below we describe the method followed to select the donor excitation wavelength for BFP-C/EBPα and GFP-C/EBPα pairs. Cells expressing the GFP (S65T)-tagged C/EBPα protein were identified at an excitation wavelength of 480/20 nm using an arc lamp light source coupled to the 2p-FRET microscope. We selected the appropriate filters and high-sensitivity PMTs to acquire donor (BFP–C/EBPα) and acceptor (GFP–C/EBPα) images. The emission filters for donor and acceptor were assembled in a filter cube with an appropriate dichroic mirror (see Table 3) (www.chroma.com). This filter cube was positioned and aligned in the Bio-Rad MRC600 scan head for maximum signal to the two respective PMTs or channels. In the excitation path a 700-nm LP dichroic mirror was positioned at the entrance port of the laser to the scan head. This dichroic mirror reflects the IR laser light toward the galvo mirrors (to the specimen plane) and transmits the visible spectrum to the PMTs through the emission filter configuration. For 2p excitation the titanium:sapphire laser wavelength was tuned from 720 to 880 nm (usable power in that range; 2–5 mW at the specimen plane) to detect the minimum or maximum signal for the BFP– and GFP–C/EBPα alone expressed proteins. We found that the minimum GFP signal and maximum BFP signal were seen at 735 nm. We also detected maximum GFP signal and minimum BFP signal at 790 nm. An excitation wavelength of 735 nm was used to acquire the donor and acceptor images from doubly expressed cells.

Table 1

<table>
<thead>
<tr>
<th>Wavelength excitation</th>
<th>Fluorophore</th>
<th>Emission donor images</th>
<th>Emission acceptor images</th>
</tr>
</thead>
<tbody>
<tr>
<td>λD</td>
<td>D</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D + A</td>
<td>e</td>
<td>f</td>
</tr>
<tr>
<td>λA</td>
<td>A</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D + A</td>
<td>g</td>
<td></td>
</tr>
</tbody>
</table>

*λD, donor excitation wavelength; *λA, acceptor excitation wavelength; a–g, images (see Fig. 2).

Fig. 2. Description of images acquired for 1p-PFRET (or 2p-PFRET) data analysis. Seven images including a contaminated FRET (f) image were acquired for PFRET data analysis. These images were acquired using Nikon PCM2000 laser scanning confocal FRET (1p-FRET) microscopy at a focal plane ~3.5 μm below the apical plasma membrane (see Tables 1 and 2). All the images (a–g) were acquired using the same optics, gain, excitation intensity, and integration time. As explained in the text and in Fig. 3, matrices were generated from the pixel gray level intensity values using the single- and double-labeled cells. Image f is the unprocessed FRET image, and image PF is processed using the PFRET algorithm.
(BFP–GFP–C/EBPα). The excitation power at the specimen plane was 3 mW for both donor (735 nm) and acceptor (790 nm) excitation wavelengths. As listed in Table 1 all seven images for data processing were acquired using slow scan mode. These images were further processed to obtain a 2p-FRET image as explained below. The above-described methodology for selection of donor and acceptor excitation wavelengths could be used for any possible 2p-FRET fluorophore pairs [28].

3. Algorithm

There are various methods to assess the SBT contamination in FRET image acquisition. Donor bleed-through can be calculated and corrected using the percentage of the spectral area of the donor emission spill over into the acceptor emission spectrum or FRET channel (see Fig. 1). In the case of acceptor bleed-through it is difficult to determine the fraction of excitation of the acceptor by the donor wavelength and its emission in the acceptor channel. Moreover, the available methods for FRET data analysis in the literature (see below) do not correct for any variation in the expression or concentration of the fluorophore labeled to the cells.

In the acceptor bleaching method, the difference between quenched donor (donor fluorescence in the presence of acceptor) and unquenched donor (donor fluorescence after the acceptor has been bleached) is used to calculate the energy transfer efficiency [29]. While this approach has the advantage of using a single double-labeled specimen, there are a number of limitations with this method. It cannot be used for live cell microscopy, it is not known whether the exposure of the specimen to extended laser energy has any ill effects, and a rise in fluorescence—postbleaching—has been reported, which could introduce artifacts into the data analysis. Depending on the level of sensitivity desired, other methods use two or three filter sets to normalize the FRET signal for the donor [3] and acceptor [30] bleed-through signal. There are other approaches that correct after image acquisition with single-label reference specimens [5]. These methods assume a linear relationship between single- and double-labeled specimens imaged under the same conditions and use that for the correction of donor and acceptor bleed-through signal. The relationship, however, is seldom linear, but for a certain level of sensitivity this approach may be adequate.

3.1. Precision FRET (PFRET) data analysis

Here, we describe a new algorithm or methodology that removes both the donor and acceptor SBT problems and corrects the variation in fluorophore expression level (FEL), associated with FRET imaging, to calculate the energy transfer efficiency (E%) and estimate the distance between donor and acceptor molecules using the same cell used for FRET imaging. Both the SBT and FEL correction are incorporated together into our mathematical calculations (see Appendix A.1 for mathematical equations).

Seven images were acquired with appropriate filters as shown in Tables 1–3 for PFRET data analysis (also see Section 2.2). Our approach works on the assumption that the double-labeled cells and single-labeled donor and acceptor cells, imaged under the same conditions, exhibit the same SBT dynamics. The hurdle we had to overcome was the fact that we had three (D, A, and D + A) different cells, where individual pixel locations cannot be compared. What could be compared, however, were pixels with matching fluorescence levels. Our algorithm follows fluorescence levels pixel-by-pixel to establish the level of SBT in the single-labeled cells, and then applies these values as a correction factor to the appropriate matching pixels of the double-labeled cell. PFRET then is

\[
P_{\text{FRET}} = \frac{U_{\text{FRET}}}{D_{\text{SBT}}} - A_{\text{SBT}},
\]

where \(U_{\text{FRET}}\) is uncorrected FRET, \(A_{\text{SBT}}\) is the acceptor spectral bleed-through signal, and \(D_{\text{SBT}}\) is the donor spectral bleed-through signal, established in the corresponding single-labeled cells described below.

### 3.1.1. Donor spectral bleed-through (DSBT) signals

To correct the DSBT, three images are required (one double-labeled and two single-labeled donor images), referenced in Tables 1–3 and Fig. 3. The gray level intensity values of the three images are assembled as

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Excitation wavelength (nm)</th>
<th>Emission filter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa 488</td>
<td>Argon 488</td>
<td>515/35</td>
</tr>
<tr>
<td>Cy3</td>
<td>Green HeNe 543</td>
<td>590 LP</td>
</tr>
<tr>
<td>CFP</td>
<td>Argon 457</td>
<td>480/30</td>
</tr>
<tr>
<td>dsRED1</td>
<td>HeNe 543</td>
<td>580/50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Excitation wavelength (nm)</th>
<th>Emission filter (nm)</th>
<th>Emission dichroic (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa 488</td>
<td>790</td>
<td>535/50</td>
<td>570</td>
</tr>
<tr>
<td>Cy3</td>
<td>735</td>
<td>610/60</td>
<td></td>
</tr>
<tr>
<td>CFP</td>
<td>820</td>
<td>480/30</td>
<td>520</td>
</tr>
<tr>
<td>dsRED1</td>
<td>940 (or 780)</td>
<td>610/60</td>
<td></td>
</tr>
<tr>
<td>BFP</td>
<td>735</td>
<td>460/40</td>
<td>505</td>
</tr>
<tr>
<td>GFP</td>
<td>790</td>
<td>535/50</td>
<td></td>
</tr>
</tbody>
</table>
matrix elements to implement the algorithm. Image $b$ is the bleed-through signal due to the donor molecules. Since images $b$ and $f$ are different cells it is not appropriate to subtract $b$ from $f$. Here we explain the algorithm to correct the DSBT signal. Fig. 3 shows a typical example of the matrix elements of part of an image size of $64 \times 64$ pixels (4096 matrix elements). A certain range of intensity values was chosen in the intensity matrix $e$ (e.g., 78–103 colored white see Fig. 3). The same range of selected pixel intensity values in $e$ (colored white) were marked in $a$ (colored white). Then the corresponding “coordinates” of marked intensity values in $a$ were selected in $b$ (colored white). The intensity values in $b$ are different compared with the intensity values of the same coordinates in $a$. We ratioed (R1) the sum of all the elements in $b$ to the sum of all the elements in $a$ as shown in the equation

$$R1 = \frac{\text{sum}(b)}{\text{sum}(a)}.$$  

(2)

Then, the R1 values were multiplied by the selected elements in $e$ (colored white). These corrections are applied only to those pixels, which satisfies the selected intensity range as explained above (see in Fig. 3). The resultant value (colored white, $[e \times (b/a)]$) is called the bleed-through factor (BTF) corresponding to the selected list of pixels in $e$ (see Fig. 3). Since there will be $n$ intensity ranges in a single image then one would have $n$ BTFs. This process was repeated for $n$ ranges of the

Fig. 3. Illustration of the precision FRET (PFRET) data analysis algorithm. The pixel intensity matrices shown here are from images of CFP–dsRED1–C/EBPα pair (not shown) acquired according to Table 1. Because of space limitations, we displayed a small matrix of pixel intensities ($10 \times 10$) of images $e$, $a$, and $b$ for donor SBT and variation in fluorophore expression level correction. The calculation of these numbers is explained in the text. $R$, Ranges of intensity; $X/Y$, number of pixel elements in $X$ and $Y$ directions.
Fig. 4. Demonstration of “considerable change” in energy transfer efficiency due to variation in fluorophore expression levels (conventional method). These images (A, B, C, D, E, CF) were acquired in the donor and acceptor channels using the donor excitation wavelength (488 nm) in single-labeled donor (A, B) and acceptor (C, D) cells and double-labeled (Alexa 488 + Cy3) cells (E, CF). All the images were acquired in 1p-FRET microscopy using the same optics, gain, excitation intensity, and integration time. Two equal-size ROIs were selected in (B) and (D) at bright (BR) and dim (DM) areas in the acceptor channel for single-labeled donor and acceptor cells. As explained in the text the data demonstrate that the variation in concentration of fluorophore changes the resultant FRET signal (BRF and DMF). But the PFRET data analysis for the same image shows only 6% error in $E$ compared with 51% for the conventional method (see text for more details).
pixel intensities until the whole of the image was covered. The donor bleed-through signal in the FRET channel for all the pixel elements of the whole image were determined using the equation

$$\text{DSBT signal} = \frac{\langle b \rangle}{\langle a \rangle} \times \langle c \rangle.$$  (3)

This kind of mathematical ratioing as shown in Eqs. (2) and (3) not only removes the SBT but also nullifies the effect of FEL variation within a cellular image (see Section 4).

### 3.1.2. Acceptor spectral bleed-through (ASBT) signal

The ASBT correction follows the same approach as DSBT using three images (one double-labeled and two single-labeled acceptor images) as outlined in the Tables 1–3 and Fig. 2. We ratioed (R2) the sum of all the elements in c to the sum of all the elements in d as shown in the equation

$$R_2 = \frac{\text{sum} \ (c)}{\text{sum} \ (d)}.$$  (4)

The acceptor bleed-through signal in the FRET channel for all the pixel elements of the whole image was determined using the equation

$$\text{ASBT signals} = \frac{\langle d \rangle}{\langle b \rangle} \times \langle g \rangle.$$  (5)

Eq. (5) not only removes the SBT but also nullifies the effect of the variation in FELs.

The results of the ASBT and DSBT corrections are applied to f (UFRET) as per Eq. (1) to obtain the precision FRET image pixel to pixel (Fig. 2PF). In this image both donor and acceptor SBT were removed and the expression level variation was also corrected. This image was used for further data analysis (such as calculation of distance between donor and acceptor molecules as described below) and the calculation of $E\%$. The sensitivity of PFRET data processing can be improved by increasing the number of BTFs and our algorithm provides an option to vary the number of BTFs. Moreover, the bandwidth of the emission filter is not an issue if one uses the PFRET algorithm for the FRET data analysis.

### 4. Results and discussions

#### 4.1. Comparison of conventional and PFRET methods in the determination of energy transfer efficiency due to variation in fluorophore expression levels (FELs)

It has been demonstrated in the literature that the concentration or uneven labeling of the fluorophore molecule is a common problem in biological signal measurement using fluorescence microscopy techniques [12]. As demonstrated in the literature, ratio imaging ($I_A/I_D$) would help to verify whether FRET occurred [8], but it does not provide any information about the energy transfer efficiency or the distance calculation between donor and acceptor molecules. The correction of the unevenness is important for quantitating the FRET signals in live or fixed cells. Here we demonstrate the change in energy transfer efficiency ($E$) due to the fluorophore concentration/expression level variation within a cellular image.

Images acquired using single- and double-labeled donor and acceptor cells with the donor excitation wavelength were used to determine the mean intensity at the selected region of interest (ROI) as shown in Fig. 4 (BR, DM). The mean data of any selected ROI (BR or DM) was used to remove the SBT from the acquired FRET image by pixel-to-pixel subtraction. The SBT contribution was greater in the brighter area of the uncorrected FRET image. However, the processed FRET image signal appeared to be much lower (Fig. 4, BRF) for the correction using BR ROI and higher (Fig. 4, DMF) for the DM ROI selected from the single-labeled donor and acceptor cells. This kind of processing would mislead the efficiency or distance calculation since the number varies depending on the variation in intensity or expression levels within the cellular images.

Therefore, we applied the PFRET data analysis in these images and found less variation in the statistical numbers, indicating that the algorithm does not change the resultant FRET image whether the intensity of the cell is bright or dim due to variation in intensity or expression levels. This is true for both 1p- and 2p-FRET microscopy. Our statistical data revealed that the pixel-to-pixel subtraction correction provides about 51% difference in energy transfer efficiency between the bright and dim areas, whereas the PFRET data analysis method provides only about 6% difference in efficiency. Similar percentages were obtained by selecting different single-labeled donor and acceptor live cellular images using CFP and dsRED1 expressed in C/EBPα proteins (see Fig. 5). The $t$ test clearly indicates that the standard error difference for two donor cells was about 1.733 and the upper and lower limits of mean difference lie between 3.67 and 3.38. We also tested for the two different single-labeled acceptor cells and the standard error difference was 1.72 and the lower and upper limits were −3.239 and 3.799, respectively. The described statistics clearly demonstrate that the bright and dim areas in the donor and acceptor images were due to the concentration variation in the cellular labeling, not the increase in energy transfer signal.

It is important to note that all the images (according to Table 1) should be acquired with good signal-to-noise ratio without saturation of the pixel intensity. This ensures a better statistical error in the determination of energy transfer efficiency and the distance between the donor and acceptor molecules.
4.2. Correction for one-photon FRET signals (1p-FRET)

We successfully implemented the PFRET analysis for the Alexa 488/Cy3 pair to study the clustered distribution of receptor–ligand complexes in apical endosomes in MDCK cells by colocalization of differently labeled pIgA-R ligand complexes. The acquired images were processed as explained in the Section 3 and the images are shown in Fig. 2. The PFRET analysis demonstrates that it is essential to implement the processing to correct the SBT and other variation in labeling conditions.

Fig. 5. Demonstration of “No” significant change in energy transfer efficiency due to variation in fluorophore expression level (PFRET method) for different control cells. PFRET data analysis was implemented for the CFP– and dsRED1–C/EBPα protein expressed in GHFT1-5 cells using 2p-FRET microscopy. We chose different donor control cells (a1, a2, a3) to correct the spectral bleed-through and found that there was no significant difference (4–8%) in the efficiencies in the resultant PFRET images as shown. Similar results were also observed for different acceptor control cells (not shown). This indicates that the developed PFRET software correction is independent of the expression levels of chosen control cells. As shown in the histogram, the cellular images have a good signal-to-noise ratio.
The UFRET image was acquired in the acceptor channel of the double-labeled cell under donor excitation. The statistical comparison of the UFRET signal with extent of correction, \(E\%\), and PFRET reveals many interesting observations as shown in Fig. 6. The extent of correction (the difference between UFRET and PFRET pixel intensity) in 1p-FRET is not a constant value and varied linearly with the UFRET (Fig. 6A). Also, the extent of correction was highly correlated (0.896) as shown in Fig. 6A. This demonstrates that a constant correction is not an ideal method when there is a variation in expression levels of the specimen. The decrease in \(E\%\) with increase in UFRET signal demonstrated that the behavior of donor molecules during the energy transfer processes was variable. The \(E\%\) variation was between 30 and 60% (Fig. 6B). Also, the PFRET signal increased with increase in UFRET and this pattern was more distinct in the case of 2p-FRET microscopy (Fig. 6C). The increase in PFRET signal could be accounted for by the advantages of 2p-FRET over 1p-FRET microscopy (see Section 1).

As we described in this article, the donor molecule plays an important role in determining the energy transfer process. In the presence of an acceptor, the donor molecule is quenched but this quenching mechanism depends on energy transfer processes. As shown in Fig. 7, \(E\%\) decreases rapidly as the donor fluorescence increases. Even though the spectral overlap determines the strength of the energy transfer efficiency, the transfer efficiency can still vary depending on the biological systems under investigation. In some situations, the decrease in \(E\%\) with rising donor level can be associated with clustered distribution of the Alexa 488 fluorophore-labeled receptor–ligand complexes, blocking others to transfer energy to an acceptor. The \(E\%\) decrease with increase in donor fluorescence gray level intensity showed the same general trend for 2p-FRET microscopy as shown in Fig. 7. We compared our new algorithm-based calculation of \(E\%\) with the acceptor bleaching method mentioned earlier. The efficiency of FRET using the acceptor bleaching method was 33%. For the same specimen, the efficiency calculated using our software correction method was about 39%, which is evidence that the two methods produce comparable results. Moreover, in the case of the donor, the correlation coefficient (CC) is 0.902, which means that the \(E\%\) is dependent on donor intensity (or concentration) increases (see Fig. 7). In the case of acceptor, the CC is 0.397, which suggests that the \(E\%\) is less dependent on the UFRET (see Fig. 6B).
4.3. Correction for two-photon FRET signals

Any combination of fluorophore pairs can be used for 2p-FRET if the microscopy system has a tunable Titanium:sapphire laser. Here we implemented the PFRET data analysis for 2p-FRET images with a correction for both DSBT and ASBT or only DSBT. There may be situations in 2p-FRET microscopy when it is not necessary to excite the acceptor with acceptor excitation wavelength. For example, in the case of the Alexa 488/Cy3 pair, zero emission signals was obtained in the FRET channel by exciting the single-labeled Cy3 MDCK cells with the donor excitation wavelength (790 nm). Consequently, this fluorophore pair required only donor SBT correction. In the case of a BFP-GFP fluorophore pair, there was an acceptor SBT signal in the FRET channel for GFP-C/EBPα proteins. So, the BFP-GFP-C/EBPα protein images required both ASBT and DSBT corrections. As shown in Fig. 8(i–iv) the processed signal for DSBT correction alone provides a higher gray level pixel intensity (Fig. 8(iii), $E = 68\%$) compared with the correction implemented for both ASBT and DSBT (Fig. 8(iv), $E = 61\%$). This is clearly indicated in the respective histograms as shown in Figs. 8(ii)h, (iii)h, (iv)h that the wrong estimation regarding the distance between protein molecules would have been obtained if the correction had not been implemented in the acquired FRET image. More pixels at higher gray level intensity in Figs. 8(iii)h and (iv)h were considerably reduced compared with Fig. 8(ii)h. A similar kind of change in number of pixels at higher and lower gray level intensity distribution was observed in the case of single (DSBT) versus both (ASBT and DSBT) corrections as indicated in the histograms in Figs. 8(iii)h and (iv)h. This demonstrates that the brighter signal (higher gray level intensity) was due to the SBT and variation in FEL.

It is also important to note that in some cases the acceptor channel appears to have sensitized signals, indicating the presence of protein–protein interactions as shown in Figs. 8(vi) and (vii)h, but most of the time those signals may be a combination of noise and SBT signals. As we have demonstrated in Fig. 8, the dimerization of C/EBPα proteins expressed with CFP and dsRED1 appears to have FRET signals (Figs. 8(vi) and (vii)h). But, the PFRET processing clearly demonstrates that there was no FRET signal or there was no dimerization of CFP–dsRED1–C/EBPα proteins (Figs. 8(vii) and (vii)h). Thus, we have demonstrated that PFRET corrects for the SBT whether FRET signals are present or not.

This kind of evaluation helps us to characterize intranuclear dimer formation for the transcription factor C/EBPα in living pituitary cells. We recently showed that GFP-tagged C/EBPα expressed in mouse pituitary GHFT1-5 cells was localized to subnuclear sites associated with pericentromeric heterochromatin [2,31], and this pattern was identical to that for the endogenous protein in differentiated mouse adipocytes [32]. Our studies indicated that the b-zip region of C/EBPα (AA 244–358) fused to GFP was sufficient for subnuclear targeting of the fusion protein in pituitary GHFT1-5 cells [2]. Since this region contains the dimerization domain, it is difficult to determine whether the expressed fusion proteins were associated as dimers in these subnuclear sites in the presence of SBT or due to FEL. The PFRET data analysis provided a higher confidence level in characterizing the intranuclear dimer formation.

4.4. Calculation of energy transfer efficiency ($E$) and distance ($r$) between donor and acceptor molecules

As shown in Eq. (6), the energy transfer efficiency is calculated by ratioing the donor image in the presence ($I_{DA}$) and absence ($I_{D}$) of acceptor. As we know, two cells under various conditions are involved in the efficiency calculation [see Eq. (6)]. But, it is appropriate to use the same cell used for FRET imaging to calculate the efficiency. We indirectly obtained the $I_{D}$ image by using the PFRET image. As described in the Section 3.1, the sensitized emission in the acceptor channel is due to the quenching of the donor or energy transferred signal from the donor molecule in the presence of acceptor. Therefore, if we add the PFRET to the intensity of the donor in the presence of acceptor we obtain the $I_{DA}$. This $I_{DA}$ is from the same cell used to obtain the $I_{D}$. Hence, the equation

$$E = 1 - (I_{DA}/I_{D})$$

(6)
will be modified to obtain the new transfer efficiency \( E_n \) from the same cell as shown in the equation

\[
E_n = 1 - \frac{I_{DA}}{I_{DA} + PFRET},
\]

where

\[
I_D = I_{DA} + PFRET.
\]

It is important to note that there a number of processes are involved in the excited state during energy transfer but here we accounted for some of the correction and added to the PFRET signal to obtain a new equation for energy transfer efficiency \( E_n \) (see Appendix B.1 for details); we added to the PFERT signal the detector spectral sensitivity for donor and acceptor channel images and the donor quantum yield:

\[
E_n = 1 - \left\{ \frac{I_{DA}}{I_{DA} + PFRET \times (\psi_{dd}/\psi_{aa}) \times Q_d} \right\},
\]

It is important to note that there a number of processes are involved in the excited state during energy transfer but here we accounted for some of the correction and added to the PFRET signal to obtain a new equation for energy transfer efficiency \( E_n \) (see Appendix B.1 for details); we added to the PFERT signal the detector spectral sensitivity for donor and acceptor channel images and the donor quantum yield:

\[
E_n = 1 - \left\{ \frac{I_{DA}}{I_{DA} + PFRET \times (\psi_{dd}/\psi_{aa}) \times Q_d} \right\},
\]
where
\[
\left( \psi_{dd}/\psi_{aa} \right) = \frac{\text{PMT gain of donor channel}}{\text{PMT gain of acceptor channel}} \times \frac{\text{spectral sensitivity of donor channel}}{\text{spectral sensitivity of acceptor channel}}
\]

and \( Q_d \) is donor quantum yield.

To estimate the distance between donor and acceptor, \( r \) has been changed to \( r_n \). Förster’s distance \( R_0 \) was calculated for various fluorophore pairs (see Table 4) as explained in Appendix C.1:

\[
r = R_0 \left\{ \left( 1/E \right) - 1 \right\}^{1/6},
\]

\[
r_n = R_0 \left\{ \left( 1/E_n \right) - 1 \right\}^{1/6}.
\]

The energy transfer efficiency was calculated and compared for the conventional method (two different cells, \( I_{DA}/I_D \)) and new method (same cell, \( I_{DA}/(I_{DA}+PFRET) \times (\psi_{dd}/\psi_{aa}) \times Q_d \)) in Table 5. The error in efficiency between the same cells versus different cells was about 43%. In the same way, we compared the distance between donor and acceptor molecules and the error was about 8%. This percentage calculation was for the ligand labeled Alexa 488 and Cy3 in 1p-FRET microscopy. The error varied depending on the energy transfer process for various proteins as demonstrated in Table 5. As shown in Table 5, the comparison of \( E \) and \( r \) for various methodologies of correction clearly demonstrates that the PFRET data analysis algorithm that we described in this article is a viable technique to study the spectroscopic nature of protein interactions for various protein molecules. The same cellular image approach was used for both 1p- and 2p-FRET microscopy. The results were significant when we compared the data for the distance calculation using the molecular size and other physical parameters.

### 5. Conclusion

In this article we have described the development of confocal (1p-FRET) and two-photon excitation (2p-FRET) FRET imaging microscopy systems. We analyzed the problems associated with FRET microscopy techniques in monitoring and quantifying FRET signals. Our approach to SBT correction is very effective and provides significant statistical data to substantiate the evidence for fixed (MDCK) and live (GHFT1-5) cells. The capability of this algorithm for precision FRET (PFRET) data analysis was clearly demonstrated by comparing the data with various correction methodologies. The algorithm also corrects for the expression level differences found between cells. This PFRET analysis provides a sensitive basis to estimate the distance between donor and acceptor molecules from the same cell used for FRET imaging. The spectral sensitivity correction provides significant data in estimating the distance between donor and acceptor molecules. The PFRET data analysis allowed us to characterize the 1p- and 2p-FRET microscopy system for intranuclear dimer formation of C/EBP\(\alpha\) proteins in the live GHFT1-5 cell nucleus and the clustered distribution of the Alexa 488–Cy3-labeled ligand molecules in MDCK cells.

### Appendix A

A.1. Algorithm described in the main text, is outlined here in the form of mathematical equations

The donor spectral bleed-through (DSBT) signal is the spillover of the donor signal into the acceptor

### Table 4

<table>
<thead>
<tr>
<th>Fluorophore pair</th>
<th>( R_0 ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFP and YFP</td>
<td>52.76</td>
</tr>
<tr>
<td>CFP and dsRED1</td>
<td>34.64</td>
</tr>
<tr>
<td>BFP and dsRED1</td>
<td>42.367</td>
</tr>
<tr>
<td>Alexa 488 and Cy3</td>
<td>67.49</td>
</tr>
<tr>
<td>BFP and GFP</td>
<td>41.40</td>
</tr>
</tbody>
</table>

*See Appendix C.1.*

### Table 5

<table>
<thead>
<tr>
<th>Microscopy technique</th>
<th>Conventional method</th>
<th>New method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Efficiency (%) ( E = 1 - (I_{DA}/I_D) )</td>
<td>( r ) (( \text{Å} ))</td>
</tr>
<tr>
<td>Confocal (CFP + dsRED1)</td>
<td>47.14</td>
<td>43.18</td>
</tr>
<tr>
<td>Confocal (Alexa 488 + Cy3)</td>
<td>49.30</td>
<td>67.80</td>
</tr>
<tr>
<td>Two-photon (CFP + dsRED1)</td>
<td>47.60</td>
<td>43.17</td>
</tr>
<tr>
<td>Two-photon (BFP + GFP)</td>
<td>53.67</td>
<td>40.41</td>
</tr>
</tbody>
</table>
channel. If both e and a are the same image, then the SBT is just b. However, since e and a are images from double- and single-labeled cells, the SBT can be estimated through these images only by applying a sequence of mathematical operations.

**A.1.1. Procedure for spectral bleed-through correction**

We divide the entire pixel intensity range 0–255 in the case of 8-bit images and 0–65535 in the case of 16-bit images into say \( k \) intensity ranges. We choose a set of pixels under a certain pixel intensity range in e and in a. Let \( n \) denote the number of pixels in e and m denote the number of pixels in a that satisfy the above condition. Find pixels in b that have the same coordinates as in a.

\[
\frac{\sum_{i=0}^{m} b_i}{\sum_{i=0}^{m} a_i} = r_j \quad \text{(for } j \text{th range of intensity } \forall j = 1 \ldots k\text{).}
\]

(A.1)

\( r_j \) is the ratio for one range of intensities.

\[
e_j \times r_j. \quad \text{(A.2)}
\]

This gives the SBT factor for all \( n \) pixels in image e. Repeat this process so that all the \( k \) ranges in image e are covered.

\[
e_j \times r_j \quad \forall j = 1 \ldots k. \quad \text{(A.3)}
\]

The result is the donor spectral SBT image e (DSBT).

Similarly in the case of acceptor SBT correction.

We choose a set of pixels under a certain pixel intensity range in g and in c. Let \( n \) denote the number of pixels in g and \( m \) denote the number of pixels in c that satisfy the above condition. Find pixels in c that are in the same coordinates as in d.

\[
\frac{\sum_{i=0}^{m} c_i}{\sum_{i=0}^{m} d_i} = r_j \quad \text{(for } j \text{th range of intensity } \forall j = 1 \ldots k\text{).}
\]

(A.4)

\( r_j \) is the ratio for one range of intensities.

\[
g_j \times r_j. \quad \text{(A.5)}
\]

This provides the SBT factor for all \( n \) pixels in image g. Repeat this process so that all the \( k \) ranges in image g are covered.

\[
g_j \times r_j \quad \forall j = 1 \ldots k. \quad \text{(A.6)}
\]

The resultant is the acceptor SBT image g (ASBT). Hence, precision FRET (PFRET) can be obtained by using the Eqs. (A.3) and (A.6) in the equation

\[
\text{PFRET} = \text{UFRET} – \text{DSBT} – \text{ASBT}.
\]

(A.7)

If \( p \) and \( q \) are the numbers of rows and column in the image, then

\[
\text{PFRET}_{ij} = \text{UFRET}_{ij} – \text{DSBT}_{ij} – \text{ASBT}_{ij} \quad \forall (i = 1 \ldots p, \ j = 1 \ldots q).
\]

(A.8)

**A.2. Correction for variation in fluorophore expression level**

In the algorithm described above we choose the same range of pixel intensities in the single-labeled cells and this need not correspond to any position as we do in double-labeled cells. If we could choose the single- and double-labeled cells, which appear to have similar intensities to the naked eye when visualizing with the microscope, then it is highly probable to match the double-labeled and single-labeled cells. Hence we overcome the difference in the expression level variation.

If we have the case that we do not find a pixel in the range from 20 to 35 gray levels in single-labeled cells, then we extend the range on either side to find a pixel from 10 to 45. This is done recursively in an incremental manner so that the intensity ranges are as small as possible and preserve the resolution for expression level variation.

\[
g_j \times \frac{\sum_{i=0}^{m} c_i}{\sum_{i=0}^{m} d_i}. \quad \text{(A.9)}
\]

For example, in Eq. (A.9), \( g_j \) and \( d_i \) are double- and single-labeled cells in a certain range of intensity. The operation is implemented for all intensity ranges and it is dependent on availability of the pixels in a certain range and not on the number of pixels in that range since the ratio of proportional variables would give the same result.

**Appendix B**

**B.1. Correction for calculation of energy transfer efficiency \((E)\) and estimation of distance \((r)\) between donor and acceptor molecules**

From the kinetics of energy transfer we know that the rate of energy transfer \((k_T)\) is dependent on the orientation of the dipoles of donor and acceptor molecules, the overlap of the emission spectra of donor with that of the excitation spectra of acceptor, and the distance between the donor and acceptor molecules. According to the equation

\[
E = 1 – (I_{DA}/I_D)
\]

(B.1)

the energy transfer efficiency \((E)\) was calculated using the intensity of the donor in the presence \((I_{DA})\) and the absence \((I_D)\) of the acceptor. Unfortunately, \(I_D\) and \(I_{DA}\) are two different cells with two different fluorophore contents and the value \(E\) will vary depending on the intensity of the cellular images. The accuracy of the value will be increased if the \(E\) calculation is carried out using the same cell used for FRET imaging.

The intensity \(I_{DA}\) is dependent on excitation intensity \(I_e D\), the number of molecules in the focal volume \(N_d\), the collection efficiency of the detector and other optics
of the donor emission in donor channel, quantum yield of donor when acceptor is present $Q_{da}$, quantum yield of donor alone $Q_d$, the absorption cross section of the donor at the donor excitation wavelength $\sigma_{dd}$, and collection efficiency in donor channel $\psi_{dd}$.

Hence

$$I_{DA} = I_{ex}D\sigma_{dd}N_d\psi_{dd}Q_{da},$$  \hspace{1cm} (B.2)

$$I_D = I_{ex}D\sigma_{dd}N_d\psi_{dd}Q_d.$$  \hspace{1cm} (B.3)

Therefore

$$E = 1 - \frac{I_{ex}D\sigma_{dd}N_d\psi_{dd}Q_{da}}{I_{ex}D\sigma_{dd}N_d\psi_{dd}Q_d} = 1 - \frac{Q_{da}}{Q_d},$$  \hspace{1cm} (B.4)

where $Q_d$ is known and standardized but $Q_{da}$ is unknown.

Quantum yield can be expressed in terms of the rate constants involved in the decay process of a fluorophore. If $k_d$ is the rate constant of radiative decay when only donor is present, $k_{di}$ is the rate constant for non-radiative decay, and $k_i$ is the rate constant for transfer of energy nonradiatively to the acceptor then

$$Q_d = k_d/k_d + k_{di},$$  \hspace{1cm} (B.5)

$$Q_{da} = k_d/k_d + k_{di} + k_i.$$  \hspace{1cm} (B.6)

We define

$$R_i = k_i/k_d + k_{di} + k_i.$$  \hspace{1cm} (B.7)

$R_i$ is the quantum yield corresponding to nonradiative transfer.

After algebraic simplifications using Eqs. (B.5)-(B.7) and from Eq. (B.5) $T$ is $Q_d$. Hence

$$Q_{da}/Q_d = Q_{da}/(Q_{da} + R_iQ_d).$$  \hspace{1cm} (B.8)

From Eqs. (B.4) and (B.8),

$$E = 1 - Q_{da}/Q_d,$$  \hspace{1cm} (B.9)

$$E = 1 - Q_{da}/(Q_{da} + R_iQ_d).$$  \hspace{1cm} (B.10)

From spectral bleed-through correction,

$$PFRET = UFRET - DSBT - ASBT,$$  \hspace{1cm} (B.11)

$$UFRET = I_{ex}D\sigma_{dd}N_d\psi_{aa}R_i + I_{ex}D\sigma_{dd}N_d\psi_{dd}Q_{da} + I_{ex}D\sigma_{dd}N_d\psi_{aa}Q_{da},$$  \hspace{1cm} (B.12)

$$DSBT = I_{ex}D\sigma_{dd}N_d\psi_{aa}Q_{da},$$  \hspace{1cm} (B.13)

$$ASBT = I_{ex}D\sigma_{dd}N_d\psi_{aa}Q_{da}.$$  \hspace{1cm} (B.14)

Hence, by using Eqs. (B.11)-(B.14),

$$PFRET = I_{ex}D\sigma_{dd}N_d\psi_{aa}R_i.$$  \hspace{1cm} (B.15)

From Eq. (B.2)

$$I_{DA} = \gamma\psi_{dd}Q_{da},$$  \hspace{1cm} (B.16)

where $\gamma = I_{ex}D\sigma_{dd}N_d$. Then,

$$PFRET = \gamma\psi_{aa}R_i.$$  \hspace{1cm} (B.17)

Multiplying numerator and denominator by $\gamma\psi_{dd}$ in Eq. (B.10) we obtain (B.19). Eq. (B.10) can be written as

$$E_n = 1 - \frac{\gamma\psi_{dd}Q_{da}}{(\gamma\psi_{dd}Q_{da} + \gamma\psi_{aa}R_i \times (\psi_{dd}/\psi_{aa}) \times Q_d)}.$$  \hspace{1cm} (B.18)

Then, using Eqs. (B.15)-(B.17) in (B.18)

$$E_n = 1 - I_{DA}/(I_{DA} + PFRET \times ((\psi_{dd}/\psi_{aa}) \times Q_d)).$$  \hspace{1cm} (B.19)

Eq. (B.19) is the final energy transfer efficiency equation.

For example, in the case of Alexa 488 and Cy3, the normalized spectral sensitivity of the donor channel is 1.023 and that of the acceptor channel is 1 (Nikon PCM 2000, PMT data sheets). Hence, the donor channel detects 1.023 times more photons than the acceptor channel. Similarly, we need to correct for the PMT gain settings of the donor channel and PFRET channel.

Therefore, for particular optics configuration the collection efficiency ratio between donor and acceptor channel is

$$\left(\psi_{da}/\psi_{aa}\right) = \left\{\left(PMT\ gain\ of\ donor\ channel\ /PMT\ gain\ of\ acceptor\ channel\right) \times \left(spectral\ sensitivity\ of\ donor\ channel\ /spectral\ sensitivity\ of\ acceptor\ channel\right)\right\}.$$  \hspace{1cm} (B.20)

In the case of Alexa 488 and Cy3, the PMT gain and the spectral sensitivity ratio were 2/3 and 1.023/1, respectively. The calculated efficiency with the correction $E = 35.6\%$ and without correction $E = 44.77\%$. This 25% difference in $E$ would influence the distance ($r$) estimation between donor and acceptor molecules by about 6%.

Appendix C

C.1. Förster distance ($R_0$) estimation

As explained in the literature [6,9,23], it is important to determine the $R_0$ value to estimate the distance between donor and acceptor molecules. As shown in the following equation these values depend on the quantum yield of the donor and the extinction coefficient of the acceptor:

$$R_0 = 0.211\left\{\kappa^2n^{-4}Q_0J(\lambda)\right\}^{1/6}.$$  \hspace{1cm} (C.1)

The value $\kappa^2 = 2/3$, the dipole orientation for the random movement of the molecule, has been widely used in the literature to calculate the distance [23]. Depending on the relative orientation of donor and acceptor, $\kappa^2$ can change from 0 to 4. Moreover, the sixth root of $\kappa^2$ is taken in calculating the distance (see Eq. (C.1)) between donor and acceptor. Many donors and acceptors display fundamental anisotropies of less than 0.4 owing to
overlapping electronic transitions. The range of \( k^2 \) values is more limited, and errors in distance are likely to be less than 10%, or error could be variable depending on the orientation values up to 35% [23]. So we assumed the orientation factor \( k^2 \) was 2/3 and the refractive index \( n \) was 1.4 to calculate the overlap integral, \( J(\zeta) \). \( J(\zeta) \) was calculated by numerical integration of \( f_D(\zeta) r_\lambda(\zeta) \zeta^4 d\zeta \) from 300 to 700 nm. We also assumed a fixed extinction coefficient for \( A \). The values for extinction coefficient and quantum yield were obtained from Clontech (www.clontech.com). Estimated \( R_0 \) values for various fluorophore pairs are listed in Table 4 (see the main text).

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