As a result of heightened interest in elucidating protein–protein interactions, techniques for mapping the protein interactome are frequently used by biologists. In an era when a multitude of molecular biological and superresolved microscopy techniques are available for this purpose, Förster resonance energy transfer (FRET) preserved its status as a fashionable, accurate and relatively easy-to-use approach (1). FRET is a nonradiative energy transfer process from an excited fluorophore (donor) to an acceptor by dipole–dipole coupling. Its sensitivity to molecular interactions and conformations stems from the fact that its efficiency steeply decreases with the donor–acceptor separation in the 2–10 nm range. Although it is already valuable as a standalone approach, its usefulness can be further increased by combining with other modalities, like confocal microscopy (2). Its flexibility and versatility are enhanced by the generation of sensors, that is, donor–acceptor fluorescence protein constructs connected by a linker, whose conformation responds to certain properties of the environment. The FRET efficiency of such sensors can be used as a readout parameter for a multitude of biological processes (3,4). Although a large number of approaches are available for the determination of FRET, intensity-based (ratiometric) measurements remain the cornerstone in its biological applications since these experiments are the easiest to implement and they usually provide the answer to the questions asked about protein clustering by ordinary biologists (1). The method typically involves the measurement of fluorescence intensities in three channels corresponding to donor fluorescence, directly excited and FRET-sensitized acceptor emissions. In order to solve the equation set, two different kinds of correction parameters are required:

i. None of these channels is pure in the sense that they are contaminated by contribution from the other molecular species. In order to correct for these spectral overspills, donor-only and acceptor-only samples are measured to obtain the fractional contribution of these fluorophores to the other fluorescence channels (5). Different approaches are available for calculating these overspill parameters. They can be obtained as the mean of individual overspill factors (mean of ratios) calculated for single pixels (in microscopy) or single cells (in flow cytometry), as the ratio of mean intensities or as the slope in different kinds of regression approaches (6,7). The approach based on calculating the mean of ratios is a biased estimator, while the ratio of the means approach is an asymptotically unbiased estimator of the overspill parameter (6). The overspill factor has been reported to show apparent intensity dependence as a result of poor photon statistics (7). If, for any reason, the overspill parameters exhibit real intensity dependence, a method has been developed to calculate these bleedthrough parameters for different intensity ranges (8).
ii. As a result of FRET, an excited donor disappears and an excited acceptor is generated. The consequent increase in acceptor emission and the decrease in donor fluorescence (quenching) are related to each other. This relationship is expressed by the parameter, variably termed $\alpha$, $G$ or $\gamma$, which is required for calibration of intensity-based FRET measurements (9-11). Basically, this parameter characterizes how efficiently an excited acceptor can be detected in the FRET channel vs. an excited donor in the donor channel. Since it is only determined by the quantum yields and detection efficiencies of the donor and the acceptor, it is constant for a particular fluorophore pair and for a given experimental setup (Fig. 1).

Although the definition of the calibration factor, introduced in point (ii) above, is simple, its experimental determination is more challenging. While quantum efficiencies can be determined relatively easily, detection efficiencies are much more difficult to measure in practice. Therefore, the experimental determination of this calibration parameter is based on a different principle. Although several methods have been devised for this purpose, all of them are based on the concept, introduced by Trön et al. (9), of comparing the intensities of an equal number of excited acceptors and excited donors.

The original approach was developed for measuring FRET between fluorescent antibodies. In such a system, a sample is labeled with only donor-conjugated antibodies, and another sample is labeled with only acceptor-conjugated antibodies against the same epitope. These two samples contain an equal number of donor-tagged and acceptor-tagged antibodies if a large enough number of cells are averaged. So that the ratio of the intensities of the acceptor-only sample, measured in the FRET channel ($M_A$, and the donor-only sample, measured in the donor channel ($M_D$), correspond to an equal number of donors and acceptors, the intensities are to be corrected with the degrees of labeling (DOL) of the donor-tagged ($L_D$) and acceptor-tagged antibodies ($L_A$). If the requirement of an equal number of excited donors and acceptors is to be met, the intensity ratio is to be further corrected with the molar absorption coefficients of the donor ($\varepsilon_D$) and the acceptor ($\varepsilon_A$) at the donor excitation wavelength:

$$G = \frac{M_A L_D \varepsilon_D}{M_D L_A \varepsilon_A}$$

Since the DOLs ($L_D$, $L_A$) are determined for the antibody stock solutions, the correctness of the formula hinges upon the assumption that the DOL of the stock is identical to the mean DOL of the cell-bound antibody fraction. It has recently been explicitly shown that the average DOL of the cell-bound fraction is often lower than the mean DOL of the stock due to diminished affinity of fluorescently labeled antibody species. Therefore, correction of the intensities with the DOL of the stock leads to a misestimation (12). It has also been pointed out that a slightly modified formula is required for the calculation of parameter $\alpha$ if fluorophore saturation takes place, that is, when most fluorophores are in the excited state (13). This situation is common when using confocal microscopes equipped with high numerical aperture objectives.

Since it is fairly easy to generate a 1:1 donor–acceptor ratio using fluorescent protein-based FRET constructs, a plethora of approaches have been devised for such systems. In one of these methods, the loss in sensitized acceptor emission, due to partial acceptor photobleaching, is compared to consequent donor dequenching in order to obtain parameter $G$ (14). Several approaches based on a series of donor–acceptor fluorescent protein constructs exhibiting different FRET values have been developed. Although the formalisms are somewhat different, the donor intensity and the sensitized acceptor intensities are compared in all of them. Initially, a regression approach utilizing an arbitrary number (at least two) of donor–acceptor constructs have been published (15) followed by another paper using two such constructs (16). Practically, the same principle is used in single-molecule experiments, although the formula is modified (17) to correct for the dequenching of the donor.

**Figure 1.** Interpretation of the parameter variably termed $G, \alpha$ or $\gamma$. In the absence of an acceptor, a certain fraction of the excited donors (designated by $D^*$ in the star symbol) emit a photon. The fraction of donors emitting fluorescence is determined by the fluorescence quantum efficiency (or yield) of the donor ($Q_D$), assumed to be 0.5 in the figure. The rest of the excited donors return to the ground state by thermal relaxation (concentric, dotted circles). Besides the fluorescence quantum yield, the donor fluorescence intensity is proportional to the number of excited donors ($N_D$) and the detection efficiency of donor photons in the donor channel ($\eta_D$). All donors are assumed to be complexed with an acceptor. If the FRET efficiency ($E$) is 50%, half of the excited donors transfer their energy to an acceptor. Consequently, the donor fluorescence quantum yield is reduced by a factor of (1-$E$). In the example shown in the figure the donor quantum yield in the presence of the acceptor is $Q_D(1-E)=0.5 \times 0.5=0.25$. As a result, the donor fluorescence intensity is reduced (quenched) by $\Delta I_D \propto N_D Q_D \eta_D E$. Due to FRET, an extra number of acceptors are excited (ground state) and excited acceptors are designated by A and A* (respectively). The number of FRET-excited acceptors is proportional to $N_D E$. A fraction of these acceptors, equal to the fluorescence quantum yield of acceptors ($Q_A$, assumed to be 0.5 in the figure), will fluoresce, and the corresponding FRET-sensitized acceptor emission ($\Delta I_A$) is also influenced by the detection efficiency of acceptor photons in the FRET channel ($\eta_{A2}$): $\Delta I_A \propto N_A Q_A \eta_{A2}$. The factor, variably called $G$, $\alpha$ or $\gamma$, is the ratio of how much intensity is gained on the acceptor side to how much intensity is lost on the donor side. As shown by the bottom equation in the figure, this factor is determined by the ratio of the fluorescence quantum yields of the acceptor and the donor and the ratio of their detection efficiencies.
FRET measurements, in which the series of different donor-acceptor fluorescent protein constructs is replaced by different conformations of a single construct. The calibration factor, designated by $G$ or $\alpha$ in other FRET approaches, is termed $\gamma$ in single-molecule FRET methods, and it is determined by regression (10). In two consecutive publications, an iterative and a closed-form approach has been developed for determining $G$ (or $\alpha$, or $\gamma$) for a single donor–acceptor fluorescent protein construct (17,18).

Recently, different measures of central tendency were evaluated for the determination of parameter $G$ using a previously published method based on two donor–acceptor fluorescent protein constructs (16). Although the mean, the median, and the mode provided statistically insignificantly different estimates for $G$, the precision (reproducibility) of the mode was the best (19). Menaesse et al. (in this issue, page XXX) went one step further in simplifying the determination of $\gamma$ by using a single donor–acceptor fluorescent protein construct with known FRET efficiency. Using the calibrated FRET efficiency of the construct $G$ was determined by regressing the sensitized emission on the quenched donor intensity. Besides describing the calibration method, the authors also compare this new approach to a previous one based on two FRET constructs with unknown energy transfer efficiencies (16,19). The authors concluded that a single construct with known FRET efficiency provides a more precise estimation with fewer images compared to the previously used method. It was pointed out that the confidence interval of the estimation is broadened if a FRET construct with low FRET efficiency is used. An admitted drawback of the proposed approach is the requirement for a FRET construct with known energy transfer efficiency. Inaccuracy or uncertainty of the FRET efficiency of the calibration construct (e.g. because of the presence of unpaired donors due to incomplete maturation of the acceptor fluorescent protein) could obviously undermine the reliability of the proposed method. Since FRET calibration constructs with known energy transfer efficiency and rapidly maturing fluorescent proteins suitable for FRET are increasingly available, this approach is a viable and simple alternative for the determination of parameter $G$. Since the real value of FRET measurements compared to other, less quantitative approaches is the predictability and modelability of the readout parameter, the energy transfer efficiency, any improvement increasing the reproducibility and simplicity of the approach will definitely contribute to its successful implementation in biological research.

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AUTHOR CONTRIBUTIONS
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