FRET microscopy in the living cell: Different approaches, strengths and weaknesses

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New imaging methodologies in quantitative fluorescence microscopy, such as Förster resonance energy transfer (FRET), have been developed in the last few years and are beginning to be extensively applied to biological problems. FRET is employed for the detection and quantification of protein interactions, and of biochemical activities. Herein, we review the different methods to measure FRET in microscopy, and more importantly, their strengths and weaknesses. In our opinion, fluorescence lifetime imaging microscopy (FLIM) is advantageous for detecting inter-molecular interactions quantitatively, the intensity ratio approach representing a valid and straightforward option for detecting intra-molecular FRET. Promising approaches in single molecule techniques and data analysis for quantitative and fast spatio-temporal protein-protein interaction studies open new avenues for FRET in biological research.

Keywords:
biochemical activities; FLIM; fluorescence microscopy; protein-protein interactions

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Abbreviations:
FLIM, fluorescence lifetime imaging microscopy; **FP,** fluorescent protein; **FRET,** Förster resonance energy transfer; **TCSPC,** time-correlated single photon counting.

Introduction

Fluorescence-based methods encompass different disciplines and a vast number of technical approaches. Among the different fluorescence-based applied methodologies, Förster resonance energy transfer (FRET) [1] stands alone as a technique that allows the investigation of molecular processes with nanometer resolution [2]. During the last 40 years FRET has been used to understand a great variety of molecular interactions, both in vitro and in vivo. Advances in different photonic imaging techniques and the development of fluorescent probes, particularly fluorescent proteins (FP) [3, 4], made FRET microscopy a breakthrough, going from the biophysics to the biology lab. When applied to live cells, FRET occurs between two labeled proteins at separation distances of a few nanometers. Such distances are often interpreted as a protein-protein interaction. This is the case for the two proteins shown diffusing independently in Fig. 1A, one is tagged with a green FP (donor) and the other with a red FP (acceptor). When the two proteins interact, the two tags are close and inter-molecular FRET occurs. Similarly, by tagging the same protein with donor and acceptor fluorophores, FRET is used to monitor protein conformational changes and, in the case of well-adapted biosensors, is interpreted as a biochemical activity modulation. In Fig. 1B, biochemical activity (such as phosphorylation) modifies the FRET biosensor leading to its structural change monitored by differential intra-molecular FRET measurement. In summary, protein-protein interactions and biochemical activities in living cells can be directly monitored using FRET (Fig. 1A and B). This aspect is critical to improve our understanding of different processes occurring in vivo such as biochemical protein cascades and when performed quantitatively, to build or improve biological mathematical models [5]. FRET is a unique method to monitor the spatio-temporal changes of macromolecular interactions, biochemical activities, conformations, and particularly transient interactions between macromolecules in real time. Here, we present a review of the different methodological approaches used in microscopy to measure FRET in live cells with their

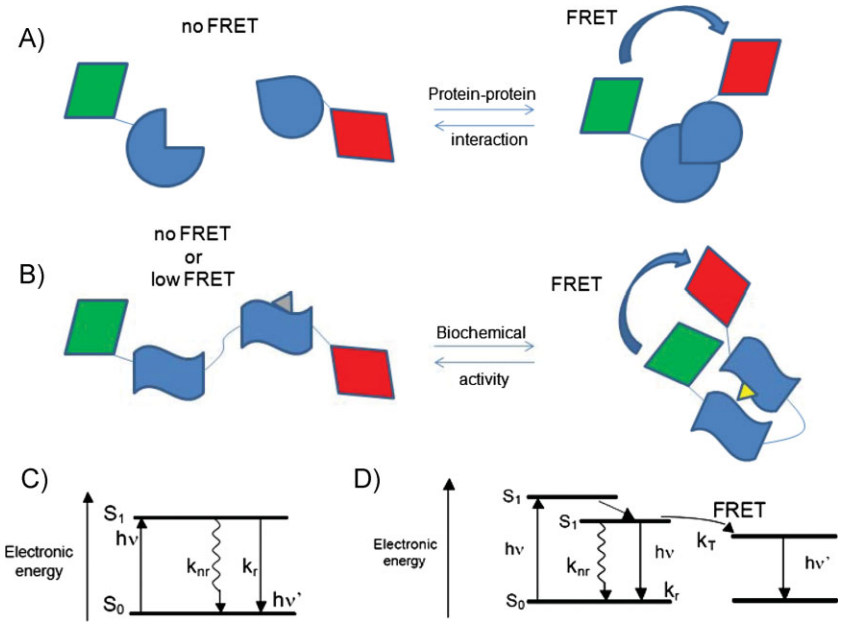


Figure 1. Inter-molecular and Intra-molecular energy transfer. **A:** The cartoon describes two proteins diffusing independently, one tagged with a green FP (donor) and the other tagged with a red FP (acceptor). When interaction occurs the distance and orientation of the tagged proteins allow FRET to occur. **B:** A protein with two subunits is tagged with a green FP (donor) and a red FP (acceptor). When a conformational change occurs both donor and acceptor are close enough for FRET to happen. **C:** Modified Perrin-Jablonski diagrams showing photonic processes: after initial excitation ($\sim 10^{15}$ s^{-1}), fluorescence occurs as a result of a photon being emitted during the decay of an excited electron from S1 to the ground level (S0) with a particular radiative kinetic constant ($k_r \sim 10^9$ s^{-1}). Note that other non-radiative phenomena for relaxation are also possible ($k_{nr} \sim 10^9$ s^{-1}). **D:** The process of FRET is described in this diagram; the process of energy transfer is indicated with a horizontal arrow (kT). In this case there is competition between three pathways of relaxation: energy transfer ($kT \sim 10^9$ s^{-1}), fluorescence ($k_r \sim 10^9$ s^{-1}) and non-radiative phenomena ($k_{nr} \sim 10^9$ s^{-1}).

strengths and weaknesses in terms of technology and quantification.

Theory of FRET

Förster resonance energy transfer is a non-radiative process which occurs in the excited state of fluorophores when the energy is transferred from a donor fluorophore to an acceptor fluorophore with a rate constant that depends on: (i) the distance between the two fluorophores, (ii) the extent of the overlap between the donor emission and acceptor excitation spectra, (iii) the quantum yield of the donor, (iv) the fluorescence lifetime of the donor, and (v) the relative orientation of the donor and acceptor. In practice, it is the FRET efficiency, E, which is determined. E is the quantum yield of the transfer process and depends on R_0, the Förster distance (which corresponds to the distance between the donor and the acceptor at which 50% of FRET efficiency occurs) and on R, the actual distance between the donor and the acceptor (Box 1). R_0 can be calculated from the spectroscopic and mutual dipole orientation parameters of the donor and acceptor and generally ranges between 2 and 8 nm. Due to this very short working range, specific interactions between two proteins can be determined only if the fluorescent tags are close enough to each other; in practice, at a distance around R_0.

It must be noted that FRET does not detect the interaction of the two tagged macromolecules by itself but the distance between the two fluorescent tags. The absence of FRET does not necessarily mean that the tagged macromolecules are not in interaction and therefore this relatively short FRET working distance constitutes a limitation for studying multi-protein complexes or interactions between big proteins.

FRET by steady-state intensity

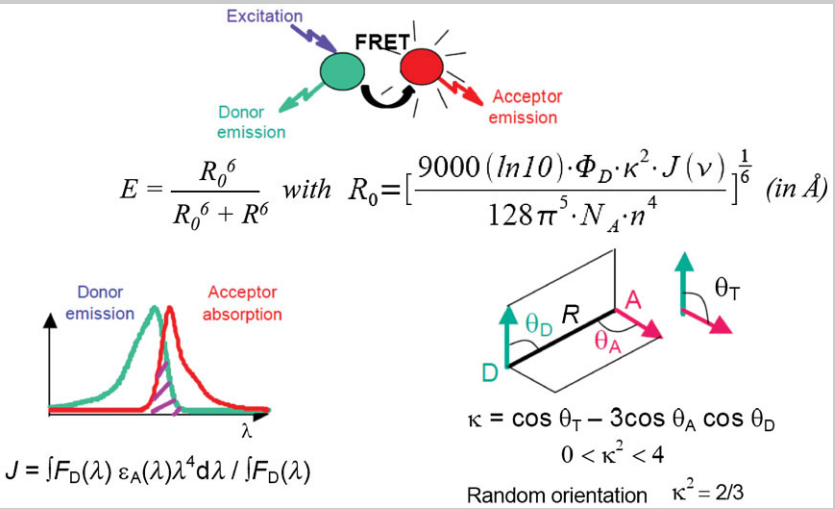
When FRET occurs between donor and acceptor fluorophores, the fluorescence quantum yield of the donor decreases and the acceptor is excited by non-radiative energy transfer. This gives rise to a diminution of the donor fluorescence intensity concomitantly to the appearance of acceptor fluorescence intensity. When measuring intramolecular FRET to monitor biochemical activities (Fig. 1B), direct ratiometric measurement can be applied (Box 2). Since steady state intensity FRET measurements depend upon the bleed-through between donor and acceptor channels, the ratio is not sufficient when donor and acceptor concentrations are not known beforehand (i.e. FP expressed in live cells). Many methods based on the same principle (see Box 3) have been developed during the last decade to overcome this problem associated with the measurement of FRET: (i) normalized FRET ($FRET_N$) [6], (ii) mean normalized FRET (N_{FRET}) [7], (iii) precision FRET (PFRET) [8], and its refinement [9], (iv) time resolved FRET (TR FRET) [10], (v) FRET efficiency calculated relative to donor fluorescence (E_D) [11], (vi) apparent FRET efficiency (E_{app}) [12], (vii) intensity ratio (I_F/I_A) [13], and (viii) E from three filter cube [14]. The overlap of fluorophore spectra due to their wide emission range is a common problem when performing multi-channel/color fluorescence microscopy, especially employing FPs (due to the long emission tails toward the red part of the spectrum of all FPs). The combination of sensitized emission with linear unmixing to measure FRET [13] or the subtraction approach [15] have been proposed as solutions to separate overlapping donor and acceptor emissions. It is worth mentioning that the acceptor photobleaching method is also quite popular to measure FRET but normally it is applied on fixed cells or highly immobile environments and hence is out of the scope of this review (more focused on live cell applications).

Quantitative FRET can be performed by taking advantage of steady-state intensity measurements but it only allows

Box 1

The basic principles of FRET

When FRET occurs, after excitation of the donor, its deexcitation is a competition between conventional fluorescent process (with donor fluorescence emission) and resonant energy transfer from donor to acceptor (long range dipole-dipole interaction). The FRET efficiency, E, corresponds to the transfer quantum yield and represents the probability that the excited donor will return to its ground state by transfer. E decrease in $1/R^6$ and is equal to 0.5 at the Förster distance, R_0, which depends on Φ_D, the fluorescence quantum yield of the donor, n, the refractive index of the medium, N_A, the Avogadro's number, J, the quantitative overlap of the donor emission spectrum with the acceptor absorption spectrum (presented on the figure), and κ, the orientation factor. κ depends on θ_T the angle between donor emission dipole and acceptor excitation dipole, θ_D the angle between the donor emission dipole and the vector joining the donor and the acceptor and θ_A the angle between the acceptor excitation dipole and the vector joining the donor and the acceptor. Note that in addition to distance, the other essential geometrical requirements for effective transfer are the orientations of the transition dipole of donor and acceptor which is in practice not known (the orientation factor, which lies somewhere between 0 and 4, depending on the donor and acceptor mutual orientation).



determination of the apparent FRET efficiency (E_{app}, defined as the product of the true FRET efficiency (E) and the fraction of interacting donor, f_D), the relative concentration of donor, [D], and the concentration of acceptor, [A] [16]. In all cases, attention must be paid to the excitation and acquisition conditions while collecting data (since the final E_{app} calculation depends on the concentration of the fluorophore, the optical pathway and the intensity of the laser; the settings of the microscope must not change throughout the whole experiment; this includes the acquisition of the different spectra and negative controls such as the donor alone, the acceptor alone and the donor in the presence of the acceptor). Finally we would like to stress once more that results obtained using these methodologies are relative and not absolute, therefore inter-molecular FRET (i.e. protein-protein interactions) should be interpreted with caution.

It must be added that in the last few years, steady state approaches have been successfully applied to intramolecular or unimolecular genetically encoded FRET biosensors to visualize and measure biological activity. The detection and quantification of small GTPase activity [17] is a good example of this. Thanks to the development of Raichu probes [18] important insights in the regulation of the small family of GTPases (Rho, Rac, and Cdc42), which play a fundamental role in morphology, motility and gene expression, and more specifically in endocytosis and the regulation of the actin cytoskeleton [19], have been revealed.

FRET by fluorescence lifetime measurements

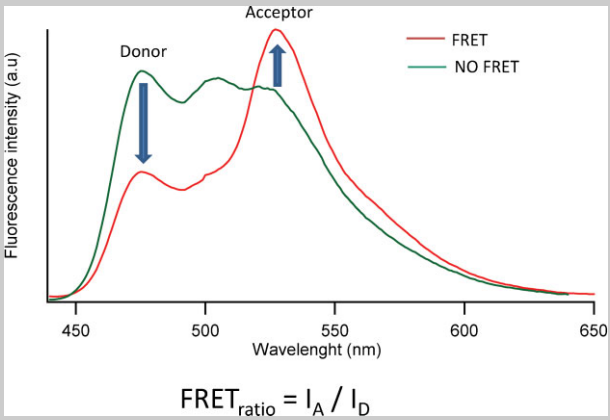
Fluorescence is a radiative process (rate constant kr), which takes place when molecules excited by light absorption revert to their original state by light emission. Fluorescence is a transient phenomenon associated with a very short time (from picosecond to nanosecond range) in which the molecules stay in the excited state S1 (Fig. 1C). The kinetics of the fluorescence decay (rate constant $k = k_{nr} + k_r$) depends on the relative proportion of the various pathways to return to ground state, radiative (k_r) and non-radiative (k_{nr}), thus giving information about the environment of the fluorophore in the excited state. FRET takes place in the excited state of the donor through a non-radiative process. The energy transfer rate is thus added to the total rate constant of deactivation of the excited donor. As a consequence, the fluorescence lifetime of the donor decreases relative to the one of the donor measured in the absence of FRET (Fig. 1D).

When studying protein-protein interactions by FRET, the fraction of interacting protein is a crucial biological parameter to be followed. In contrast to methods based on steady state intensity measurements, fluorescence lifetime imaging microscopy (FLIM) applied to live cells to quantify FRET is particularly well suited to detecting protein-protein interactions in live cells because it allows determining independently the FRET efficiency (E) and the fraction of interacting donor (f_D) (Box 4). f_D quantification is particularly important, since it determines the percentage of the interacting protein

Box 2

Ratiometric FRET

By monitoring the global fluorescence spectral properties after donor excitation during a FRET experiment (here the well-known CFP/YFP couple), one can see that when FRET increases, the donor fluorescence is decreased and the acceptor fluorescence is increased (see arrows). It is thus natural to follow the FRET process by ratiometric measurement of acceptor intensity divided by donor intensity. But this approach suffers from multiple spectral cross-talks: direct acceptor excitation when exciting the donor and spectral bleed through of the donor in the acceptor spectral emission channel. If the relative concentration of donor and acceptor is not controlled, the ratio measurement is not efficient and it can be used only for intramolecular FRET (Fig. 1B) where donor and acceptors exist in a molar ratio of 1:1.



population, and makes it possible to follow the spatio-temporal evolution of the interaction under investigation.

Different methodologies are used to measure fluorescence lifetime under the microscope. To date, different microscopy apparatus are available to measure the lifetime in an imaging mode (FLIM systems), such as the time-correlated single photon counting (TCSPC) method under confocal microscope (Becker and Hickl, Picoquant), single photon counting in wide field (Europhoton), single photon on a confocal microscope (LIMO from Nikon), streak camera (Hamamatsu), fast gated intensifier CCD camera (Kentech instrument, Lavision) and a modulated intensifier in frequency domain (LIFA Lambert Instrument). From the literature (for a detailed review consult [20]) and from our own expertise using TCSPC [21–24] and time-gated intensifiers [25–27], all these approaches have been successfully applied in biology for FRET experiments with their own advantages and limitations. TCSPC methods have a very good temporal resolution and signal to noise ratio, which allows precise quantitative analysis by using fitting methods. But the main limitation is the acquisition, which does not allow time-lapse to be much faster than one FLIM image per minute. This limitation comes from both: (i) confocal mode and eventual photobleaching and/or photodynamic effects on live sample expressing FP-tagged proteins, and (ii) the photon counting rate limitation of the TCSPC method by itself. Coming from our previous expertise, FLIM for biology has to be as fast as possible in terms of acquisition rate to increase the spatio-temporal resolution of the FRET signal. In that respect, methodologies using wide field detection are of high interest. For time-domain, fast gated intensifiers are mounted onto wide field [28] or confocal pseudo-wide field microscopes [24, 29].

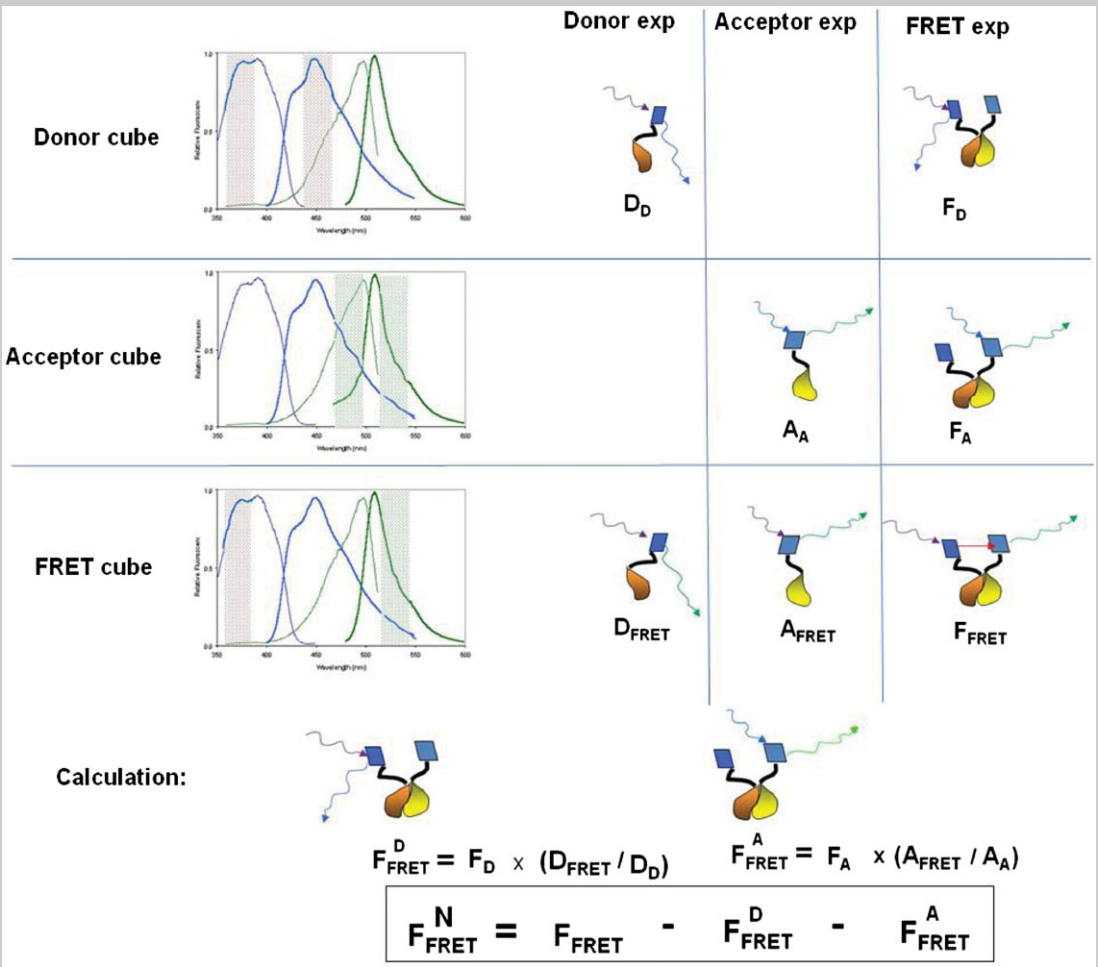
Usually, analyses based on the least-squares method to fit fluorescence data coming from single photon detection systems are employed to recover fluorescence kinetics parameters (i.e. least squares utilizing an exponential model or the maximum entropy method). In FRET-FLIM experiments, f_D is related to the fluorescence decay behavior of the donor and its quantification is typically carried out by fit procedures based on nonlinear least squares methods. This approach becomes difficult (i) when donors' decays are better fitted with a multi-exponential function, e.g. employing the cyan fluorescent protein (CFP) [30]; (ii) when the number of acquired photons is too low and so a compromise between the acquisition times and the high accuracy of quantitative analysis is needed. In that respect we would like to stress the importance of novel analyses based on "non-fitting" approaches which allow the recovery of direct quantitative data employing fast acquisition times (at the second time scale, even using multi-lifetime donors) like the minimal fraction of interacting donor method (mf_D) [26, 27] or the phasor plot approach [31–35]. A high number of events (at least thousands of photons per pixel) is needed to get statistically reasonable values to recover the mechanistic parameters of the system (i.e. lifetimes, pre-exponential factors, FRET efficiency) to apply fitting procedures; hence the time required for such measurements is too long: in the order of minutes. Under these circumstances, the FRET signal is averaged out (in a biological system, the kinetics of the interaction should not be faster than the acquisition time; within minutes this situation is quite likely to occur). This "FLIM resolution" is not related to the optical resolution but to the impact of long acquisition times in the final lifetime image. On the contrary, since non-fitting approaches allow quantitative analysis of signals with considerably less events compared to the referred fitting techniques [36], they are more convenient when working in vivo. The minimal fraction of interaction approach and the phasor plot approach, permit one to obtain a quantitative image with low numbers of photons per pixel (e.g. 100) by hypothesizing a two population system (FRET and no-FRET), a situation that in biology is quite likely to occur.

In recent years, FRET by FLIM has become a classic technique to probe and quantify protein-protein interactions in live cells. A couple of examples of the technique can be mentioned here. Multiphoton FLIM allowed the detection of the interaction between chemokine receptor CXCR4 and protein kinase C (PKC) α in carcinoma cells [37]. The authors found that the complex between CXCR4-eGFP:PKCα could be detected in intracellular vesicles. Moreover, the use of the eGFP/mRFP1 pair in FRET allowed precise quantification thanks to the single exponential decay coming from eGFP. Other examples of inter-molecular protein interactions employing this FRET pair with FLIM are the interaction of

Box 3

3-cube FRET

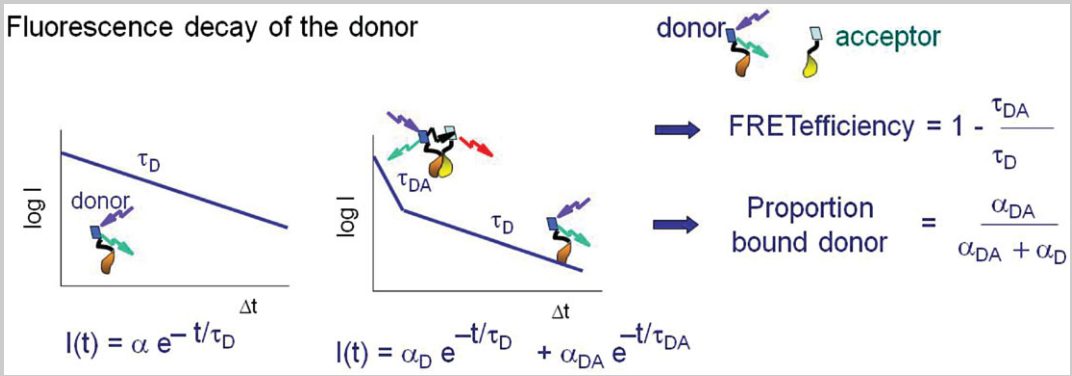
The 3-cube FRET is used to measure the FRET signal (excitation of the donor and emission of the acceptor) corrected from the donor spectral bleed-trough into the acceptor channel and the direct acceptor excitation. The principle consists of measuring the fluorescence images following three spectral configurations: the donor cube (donor excitation and donor emission, D in subscript), the acceptor cube (acceptor excitation and acceptor emission, A in subscript) and the FRET cube (donor excitation and acceptor emission, FRET in subscript). The measurements pertaining to the three cubes are carried out for the FRET experiment (F) and two controls having donor only (D) and acceptor only (A). The donor spectral bleed-through into acceptor channel during FRET experiment (F^{D}_{FRET}) is determined by calculating the product of the intensity of FRET experiment in the D-cube (F_D) by the correction ratio of the intensity of the same donor image in the FRET-cube (D_{FRET}) and in the D-cube (D_D). The acceptor direct excitation using FRET-cube during FRET experiment (F^{A}_{FRET}) is determined by calculating the product of the intensity of FRET experiment in the A-cube (F_A) by the correction ratio of the intensity of the same acceptor image in the FRET-cube (A_{FRET}) and in the A-cube (A_A). These two bleed-through corrections are then subtracted from the measured intensity in the FRET-cube during FRET experiment (F_{FRET}) to recover the real FRET intensity of the experiment (F^{N}_{FRET}). This value corresponds to the corrected signal coming from the FRET process received in the acceptor channel after direct excitation of the donor. Its quantity depends directly on the concentration of donor and/or acceptor and is usually normalized by the donor intensity for the FRET experiment (F_D), the acceptor intensity for the FRET experiment (F_A) or the square root of the product of both. It is essential to be able to compare the FRET measurements obtained from different samples and locations.



Box 4

FRET by FLIM

When FRET occurs, the fluorescence lifetime of the donor is decreased. Since the fluorescence decay profile is independent from the concentration, FRET can be characterized by comparing the fluorescence decay of the donor alone and the fluorescence decay of the donor during FRET experiment from different live samples. The donor is usually chosen to exhibit mono-exponential decay (such as mTFP1 or EGFP, but it's not true for ECFP). Using the mono-exponential fluorescence decay of the donor, the donor lifetime is determined (τ_D). During FRET experiment for protein-protein interaction study, the situation of the donor-fused protein is not homogeneous: some are effectively in interaction with acceptor-fused protein but some others are alone. This gives rise to a bi-exponential fluorescence decay with two different lifetimes, the lifetime of the donor in the presence of the acceptor (τ_{DA}, the FRET lifetime) and the lifetime of the donor alone (τ_D). Through data analysis, it is possible to determine these two lifetimes with their respective amplitude and to recover not only the FRET efficiency but also the proportion of donor in interaction. This last parameter is of high interest in biology and is not accessible by using steady state intensity methods.



a translocation reporter (CD3ζ-eGFP, mCherry-tSH2ZAP-70) [38], as well as examples from our own work [25–27].

Single molecule FRET

Single molecule FRET (smFRET) has been mostly applied to immobilized molecules [39] and is a technique that, in spite of its success in some biological studies, is still the domain of biophysicists (for an extensive review about smFRET see [40]). During the last decade, however, smFRET has been also applied to elucidate mechanisms related to membrane proteins in living cells: activaton of GTPases [41], EGFR signaling [42], study of Ca^{2+} release activated channels [43], and in general, the study of cell-surface receptor interactions [44, 45]. Therefore, it has great potential and the relatively simple instrumentation needed to implement it in any laboratory (Total Internal Reflection excitation combined with single FRET pair emission detection collected with one or two EM-CDD cameras) makes it an attractive approach to quantify protein interactions related to the plasma membrane. It is also worth mentioning that problems related to bleedthrough, photobleaching, correction of the emission and excitation spectra inherent of ratiometric FRET must also be addressed in smFRET. Moreover, the image analysis of data is even more important here, since normally high signal to noise (S/N) background precludes straightforward spatio-temporal analysis of the FRET signal for single molecules. In that respect, the use of filters to subtract or minimize the background fluorescence (i.e. Gaussian filters), or the mathematical determination of the center of mass for each fluorescent punctum by using specific algorithms [46] are mandatory here. Therefore, despite the simple instrumentation required to implement smFRET, one of the reasons why it is not extensively employed in biology is the combination of difficulties arising from classical ratiometric FRET techniques (Box 1) combined with the inherent problems of data analysis for single molecule detection.

Conclusions and outlook

In this article we have reviewed different approaches to measuring FRET in living cells. Table 1 summarizes the different FRET approaches with their spatio-temporal resolution, their accuracy and their best-dedicated applications. Both intensity based methods and spectroscopic approaches based on lifetime calculation present a number of advantages and drawbacks. The strengths of FRET detection based on intensity approaches are: (i) they are easy and cheap to implement in any confocal or wide-field microscope, and (ii) they are well optimized for intra-molecular FRET (it must be said that during the last 15 years a number of FRET based probes have proven to be very useful to measure, for example pH [47], Cl^- [48], or Ca^{2+} [49, 50] concentrations or small GTPase activity [51, 52] in living cells). The weakness of intensity FRET are: (i) difficulties in correctly calibrating the system coming from the bleedthrough of the donor into the acceptor channel, photobleaching, and/or photoconversion of the donor, and (ii) the limitation of obtaining only relative parameters such as the apparent FRET efficiency (E_{app}). This is particularly

Table 1. Summary of the different FRET approaches

Approaches	Material	Acquisition procedures	Acquisition time	Data analysis	Quantitative results	Applications
Ratiometric FRET	Wide field, confocal or spinning disk microscope	Simultaneous acquisition	Seconds to tens of ms	Ratio imaging	Only for known donor/acceptor stoichiometry	FRET biosensors
3-cube FRET	Wide field, confocal or spinning disk microscope	Sequential acquisition and bleed through controls	Seconds	Image calculation	Semi quantitative Apparent FRET efficiency	Quantitative FRET biosensors Qualitative prot-prot interaction
FLIM by single photon counting	Confocal microscope with TCSPC module	Single photon by single photon	Few minutes	Pixel by pixel fit	FRET efficiency and fraction of donor in interaction	Quantitative FRET biosensors Quantitative prot-prot interaction
FLIM by time gated or frequency domain	Time-gated or fast-modulated intensifier on wide field or spinning disk microscope	Sequential stack of time-gated or phase-shift images	Minute to seconds	Fit or direct analysis	FRET efficiency and fraction of donor in interaction	Quantitative FRET biosensors Quantitative prot-prot interaction

relevant in the detection of protein-protein interactions (inter-molecular FRET) since the quantification of E_{app} is not only hampered by spectral overlap or the excitation pathway but also the concentration of donors and acceptors, which depending on the transfection/infection conditions changes a lot from cell to cell. Strengths and weaknesses of FRET detection based on lifetime methods are also apparent. The strengths are: (i) One can straightforwardly obtain the true FRET efficiency and the fraction of interacting donor independently, (ii) lifetime measurements are independent from fluorophore concentration and the light pathway or the instrument employed. The weaknesses are: (i) expensive instrumentation, (ii) high levels of expertise required to analyze and interpret results. In some reviews it is argued that the long acquisition times necessary to obtain enough photons to fit the fluorescence decay represent another weakness of FRET by FLIM; this problem, however, has been overcome by the development of non-fitting approaches (for a detailed description see [53]) that reduce the acquisition time down to a few seconds. In conclusion, we recommend the use of FLIM to detect inter-molecular interactions quantitatively, as the intensity ratio approach is a valid and straightforward option for detecting intra-molecular FRET.

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