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FRET microscopy: from principle to routine technology in cell biology

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Summary

The phenomenon of resonance energy transfer first described by Theodor Förster presents the opportunity of retrieving information on molecular proximity, orientation and conformation on the nanometre scale from (living) samples with conventional fluorescence microscopes (or even macroscopic devices). During the past 10 years Förster (or fluorescence) resonance energy transfer (FRET) microscopy has been revolutionized by the vast progress in fluorescent protein and *in situ* fluorescent labelling technology as well as by the commercial availability of advanced quantitative microscopy instrumentation. FRET microscopy is now routinely used in modern cell biology research. This short review will guide the reader through the most established FRET microscopy techniques, their inherent strengths and limitations, potential pitfalls, and assist the reader in making an educated choice on the FRET microscopy method most suited for their specific application.

FRET basics

FRET is the physical phenomenon whereby energy is transferred from an excited fluorophore, called the donor (D), to a nearby chromophore, called the acceptor (A), by non-radiative dipole–dipole coupling (through space). FRET only occurs when D and A are in close proximity (nanometre range), when there is sufficient spectral overlap between donor emission and acceptor absorption and when the acceptor transition dipole moment is not perpendicular to the electric field of the dipole field of the donor. The amount of energy transfer, usually expressed as the FRET efficiency (E), is defined as the fraction of photons absorbed by donors whose energy is transferred to acceptors, and is highly dependent on the distance between donor and acceptor (r_{DA}). For a single DA pair this dependence is described in Eq. (1), where R_0 is the Förster radius at which 50% energy is transferred:

$$E = \frac{R_0^6}{R_0^6 + r_{DA}^6} \qquad (1).$$

The Förster radius R_0 depends on the fluorescence quantum yield of D (Q_D), the absorption coefficient of A (ε_A) (through the parameter J, the overlap integral), the refractive index (n) and on the relative angular dispositions of the donor emission and the acceptor absorption dipole moments (through the parameter: κ^2), see Eq. (2) in which C is a constant of 8.79×10^{-11} (units M cm nm^2):

$$R_0^6 = C\, n^{-4} Q_D \kappa^2 J \quad \text{(units nm}^6\text{)} \quad \text{and}$$
$$J = \int F_D(\lambda) \cdot \varepsilon_A(\lambda) \cdot \lambda^4 d\lambda \Big/ \int F_D(\lambda) d\lambda \qquad (2)$$

The overlap integral J (units M^{-1} cm^{-1} nm^4) depends on the donor fluorescence emission ($F_D(\lambda)$, arbitrary units), the acceptor absorption ($\varepsilon_A(\lambda)$, units M^{-1} cm^{-1}) and the wavelength (λ, in nanometre units).

Given the steep distance dependency of FRET (Eq. 1) and Förster radii of 3–6 nm for most DA pairs, FRET is only observed at DA separation of less than 10 nm, which is at the biomolecular scale. Therefore, FRET microscopy offers unique opportunities for studying static and dynamic molecular proximity (through r_{DA}) and conformation (through κ^2) with a resolution far below the diffraction limit of optical microscopy. Together with the revolution in fluorescent genetic encoded labelling [e.g. visible fluorescent proteins (FPs)] and the increasing availability of commercial quantitative fluorescence microscopy instrumentation, FRET microscopy has become a routine technology in modern cell biology with its most profound application being the study of

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cellular signalling phenomena. For more detailed description of Förster's theory, the reader is referred to the original literature and the many excellent reviews offer clear and helpful graphical visualizations (e.g. Vogel *et al.*, 2006; Clegg, 2009).

Intriguingly, the rate of energy transfer cannot be determined directly (because it is a dark process), and hence all FRET measurement techniques are indirect and monitor subtle changes in donor and/or acceptor photophysical properties. In order to appreciate the multitude of FRET microscopy techniques a thorough understanding of the changed properties of donor and acceptor molecules due to FRET is required. After absorption of a photon, the excited fluorescent donor molecule can relax back to the ground state through several different kinetically competing pathways. In case of FRET (donor in presence of acceptor, DA), the donor quantum yield is diminished ($Q_{DA} < Q_D$), resulting in reduced donor fluorescence intensity ($I_{DA} < I_D$). Because FRET offers an additional deactivation pathway from the donor-excited state, the donor fluorescence lifetime (τ_D), which is proportional to the average amount of time the fluorophore spends in the excited state, is shortened ($\tau_{DA} < \tau_D$). Other competing deactivation pathways from the singlet excited state (like intersystem crossing to triplet state and/or subsequent photobleaching) similarly become less probable in case of FRET. Therefore, FRET results in slower photobleaching kinetics (longer donor photobleaching time, $\tau_{bl,D}$) of the donor (i.e. $\tau_{bl,DA} > \tau_{bl,D}$). If the acceptor is a fluorophore, FRET will increase the acceptor fluorescence (I_A), called sensitized emission (SE), because the non-radiative energy transfer excites the acceptor in addition to direct excitation of the acceptor through absorbance of photons (hence $I_{AD} > I_A$). FRET is not dependent on the acceptor quantum yield (Q_A). FRET can also be observed by measuring the anisotropy of the fluorescence emission after exciting the donor with polarized light.

The efficiency of energy transfer (E) can be calculated from altered photophysical parameters, such as intensity, lifetime or bleaching kinetics (see Eq. 3). The subscripts denote donor in the presence (DA) or absence (D) of the acceptor and acceptor in the presence (AD) or absence (A) of donor.

$$E = 1 - \frac{Q_{DA}}{Q_D} = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{\tau_{DA}}{\tau_D} = 1 - \frac{\tau_{bl,D}}{\tau_{bl,DA}} = \frac{\varepsilon_A}{\varepsilon_D}\left(\frac{SE}{I_A}\right) = \frac{\varepsilon_A}{\varepsilon_D}\left(\frac{I_{AD}}{I_A} - 1\right) \quad (3)$$

In this review, we will restrict ourselves to the most established FRET microscopy techniques. However, for an overview of many more (exotic) FRET detection methods, some of which even not tested yet in practice, we refer to Jares-Erijman and Jovin (2003). Detailed considerations on the choice of fluorophores, either organic fluorescent dyes or visible FPs, although crucial for the success of a FRET experiment, are beyond the scope of this review, and can be found elsewhere. However, some specific characteristics of fluorophores will be highlighted if they are essential for execution of a particular technique.

Intensity-based FRET methods

Fluorescence intensity-based FRET methods determine the increased (i.e. sensitized) acceptor fluorescence (as compared to the donor and/or direct excited acceptor signal). For a comprehensive list of filter FRET techniques and original references, see Berney and Danuser (2003). An advantage of intensity-based FRET methods is that they can be implemented on conventional wide-field or confocal fluorescence microscopes. Filter FRET is the method of choice if intramolecular ratiometric FRET sensors are employed and fast dynamic measurements are required. In this case the DA labelling ratio is constant in the image and a simple ratio-image procedure can provide FRET contrast (because probe concentration and spatial variations of excitation light distribution and/or detection efficiency are largely divided out). The common ratio-imaging technique is emission ratioing, where excitation is done at a donor absorption wavelength and the fluorescence is recorded at the D and A emission wavelengths using bandpass filters. On confocal microscopes this is most easily implemented using simultaneous dual channel emission detection. On a wide-field microscope usually a filter wheel is required to change the emission bandpass filter to acquire two consecutive images with a charge-coupled device (CCD) camera, in which case the filter changes can limit the time resolution. The fastest and most sensitive FRET recording system can be achieved by using image splitters with dichroic mirrors projecting the D and A images side by side onto the same CCD. Ratiometric FRET can also be done using single emission wavelength recordings at the acceptor emission wavelength and performing dual excitation at the D and A absorption bands. For wide-field implementation this can be beneficial because image registration problems due to changing emission filters are avoided. The ratio values show FRET contrast and can be calibrated [e.g. by fluorescence lifetime imaging microscopy (FLIM) or acceptor photobleaching, see below] to provide quantitative images of E. However, for most ratiometric experiments such images are not required.

Although advantageous for ratiometric sensors, quantitative intensity-based FRET methods are highly problematic for situations, where D, A and E are distributed differentially in the specimen (as is the case for all intermolecular FRET studies). Here three-filter cube methods are necessary, requiring acquisition of three separate images with the settings allowing the detection of sensitized emission (S, using excitation at the donor absorption wavelength and detection at the acceptor emission wavelength), as well as donor (D, at donor excitation and emission wavelengths) and

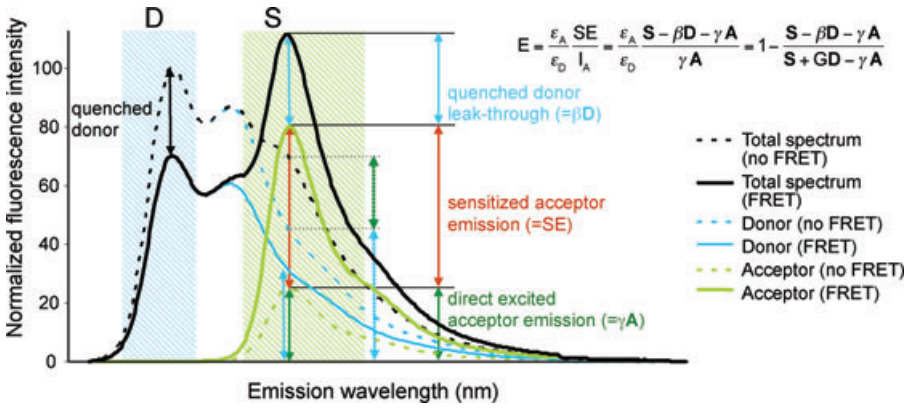

Fig. 1. Intensity-based and spectral FRET. Fluorescence emission spectra of donor (cyan line) and acceptor (green line) in absence (dashed line) and presence (solid line) of FRET are shown. Note the quenched donor and sensitized acceptor emission as a result of energy transfer. For intensity-based FRET small spectral regions (blue and green shaded areas) are selected for detection (e.g. by using bandpass emission filters). In the absence of FRET, the detected intensity in the sensitized emission image (S) contains direct acceptor excitation and donor emission contribution (bleed-through) (green and blue dashed arrows, respectively). In case of FRET, the S image contains direct acceptor excitation, donor bleed-through and sensitized emission (green, blue and red solid arrows, respectively). Here the donor emission filter to acquire the D image was chosen to avoid acceptor emission bleed-through. The calculation of net sensitized emission and E requires careful calibration with reference samples and determination of several correction factors (β, γ and G in the formula, see Jalink & van Rheenen, 2009).

acceptor (A, at acceptor excitation and emission wavelengths) images (Gordon *et al.*, 1998). In theory, from these three images and several calibration images and correction factors, E can be quantified (Jalink & van Rheenen, 2009) (see Fig. 1). In practice the method is beset with a number of problems, difficulties and error propagation in calculations. First, the S image, besides sensitized emission, contains inadvertent direct excitation of acceptor molecules. Second, the S image contains bleed-through of donor emission into the acceptor filter (see Fig. 1). Third, acceptor fluorescence (including sensitized emission) can leak into the D image, which can be avoided by choosing a restrictive donor emission filter. To calculate correction factors to cope with these problems and to obtain the fully corrected FRET image, a total of nine images are required. The accuracy of the FRET estimation will therefore be highly dependent on the reliability of the correction factors (please note that the correction factors are derived from measuring other samples than the FRET sample, and therefore might not yield exactly correct values, even if performed under exactly the same acquisition conditions). Detrimental for accurate calculation is the presence of different background intensities in the sample and calibration samples due to autofluorescence, scattered excitation light as well as possible inner filtering (absorption of excitation light or reabsorption of emission by highly concentrated absorbing molecules). Moreover, the method is very sensitive to slight photobleaching, image artefacts caused by the instrumental drift, excitation intensity fluctuations (both spatial and temporal), registration problems due to changing of filters, and dichroic beam splitters, dye photochromicity and chromatic aberrations (Jalink & van Rheenen, 2009). Also detector gain and laser intensities (in case of confocal filter FRET) need to be constant for the acquisition of all nine images, and each experiment needs its own calibration, precluding easy day-to-day comparisons. In practice, because of the large amounts of corrections required, obtaining quantitative results with filter FRET methods is very complicated, and requires a thorough knowledge of the setup being used and excellent microscopy and image processing skills from the researcher. For a full description of many challenges present in filter FRET techniques and considerations of its quantitative possibilities (aiming also at the unification of the correction factors nomenclature) the reader is referred to Jalink and van Rheenen (2009). Although quantitative filter-FRET menus and wizards are advertised by confocal microscope manufacturers, and may seem simple as a push-button computer application, we strongly suggest trying more robust techniques (like acceptor bleaching or FLIM, see below) unless acquisition speed and low fluorescent signals become a limitation, and the aforementioned issues can be addressed.

Spectral FRET

FRET can also be calculated from spectral images (spectral FRET) in which each pixel encodes the composite spectrum from all different fluorescent species present at the corresponding location in the specimen. The spectra from the different fluorescent species are extracted with linear unmixing algorithms and used for the calculation of E (Zimmerman *et al.*, 2002; Thaler *et al.*, 2005). Spectral FRET microscopy requires the availability of specialized instrumentation, but in return offers several advantages over three-filter cube FRET method: (1) the possibility to use D and A fluorophores with highly overlapping emission spectra

(generally characterized by higher R_0 values and hence enhanced FRET); (2) background fluorescence including autofluorescence can be attributed to distinct spectral components and adequately corrected for by unmixing and (3) the entire emission spectrum is collected whereas in filter FRET methods large portions of the emission are discarded by selective bandpass filters (see Fig. 1). However, because some spectral detectors display relatively low quantum efficiencies (especially in confocal imaging), and emission photons are inherently distributed into several parallel detector channels to acquire the spectrum, the method requires relatively long integration times and/or high fluorescence levels in the specimen. Quantitative spectral FRET with linear unmixing requires careful acquisition of reference spectra under the same excitation conditions as the FRET sample (needs frequent calibration), and similar restrictions apply as compared to filter FRET. Spectral FRET is sensitive to photobleaching, photochromicity (see below) and possible inner filtering.

Intensity-based and spectral FRET methods require relatively photostable donor and acceptor molecules, and both techniques benefit from acceptors with high quantum yield, which increases sensitized emission, but has no effect on E. Furthermore, to perform meaningful FRET quantification, the donor and acceptor signals should be within the same order of magnitude, especially for unmixing in spectral FRET.

Acceptor depletion FRET methods

Acceptor depletion methods aim at quantifying the reduced donor quantum yield or intensity due to FRET. In practice, the donor emission (usually in terms of intensity or spectrum but also τ_D can be determined) is measured before (providing I_{DA}) and after (providing I_D) selective photodestruction of the acceptor. The relative increase in donor emission – or lifetime – after complete acceptor photobleaching is proportional to E (see Fig. 1) (Bastiaens *et al.*, 1996; Bastiaens & Jovin, 1998). The advantages of acceptor depletion methods are: the ease of implementation and compatibility with standard (confocal) microscopes; the ease of FRET estimation and insensitivity to inner filtering artefacts. In addition, the method, unlike FLIM, is very effective when (part of) the donor molecules display very high transfer efficiencies causing their 'disappearance'. After acceptor depletion these molecules contribute most to the detected FRET signal. However, this technique can be relatively slow and therefore is most often applied to fixed samples. In addition, it is inherently destructive precluding dynamic measurements, and it can induce severe phototoxic effects when applied to live samples. Fast photobleaching requires a relatively low photostable acceptor and a strong and selective excitation source that does not affect donor photostability. Inadvertent bleaching of the donor during the acquisition of I_{DA} and I_D images, results in erroneous (under-)estimation of E. Moreover, measurements are affected by scattering, autofluorescence and by movement in the sample between the acquisition of the pre- and post-bleach images. The quantification of E using acceptor photobleaching strictly relies on complete photobleaching of the acceptor (Berney & Danuser, 2003). However, continuous monitoring of donor and acceptor intensities during acceptor photobleaching and subsequent curve fitting of donor and acceptor bleaching kinetics eliminates the need of performing additional correction measurements. This technique improves the determination of FRET efficiency and allows measurements with partially bleached acceptors (van Munster *et al.*, 2005). For acceptor depletion methods it is assumed that photobleaching of the acceptor also destroys its absorption. Some acceptor fluorophores however can be converted to (reversible) absorbing dark states with low quantum yield. In this case acceptor fluorescence is lost (temporarily), but FRET remains, leading to an underestimation of E. Photochromic acceptor behaviour can also be used for FRET microscopy if the acceptor absorption can be switched (cycled) repeatedly between FRET competent 'on' and FRET incompetent 'off' states using specific excitation wavelengths (photochromic FRET, pcFRET). In the past a few organic probes have been used as reversible switching FRET acceptors (Jares-Erijman *et al.*, 1997). The recent development of a reversible photoswitchable red fluorescent protein rsTagRFP enabled excellent intra- and intermolecular pcFRET using YFP as donor (Subach *et al.*, 2010) and holds great prospects for dynamic pcFRET microscopy of live cells in the future.

Donor photobleaching FRET method

Another technique employing photodestruction is based on altered donor photobleaching kinetics in the presence of FRET (photobleaching FRET, pbFRET; Jovin & Arndt-Jovin, 1989). pbFRET can be applied if photodestruction of the donor fluorophore involves and depends on the population of the donor singlet excited state. In this case the reduced donor fluorescence lifetime due to FRET decreases the probability/rate of donor bleaching. In practice, the technique requires recording of the donor fluorescence intensity as a function of time, and the kinetics of donor bleaching is compared in presence and absence of FRET (Young *et al.*, 1994). Because bleaching occurs generally in the seconds to minutes range, the kinetic measurements can be performed using conventional fluorescence microscopes. Another advantage of the method is that high excitation power and long integration times can be used which gives rise to high signal to noise data. Disadvantages of this technique are the requirement of fixed samples, the need for a photostable acceptor and the influence of factors other than FRET influencing the photobleaching rate like presence of oxygen, radical scavengers and (spatial dependent) excitation light intensity in the sample (e.g. introduced by inner filtering). Even if all these can be controlled photobleaching can be a

rather complex process and the accuracy of FRET estimation relies on the correctly fitted decay of donor intensity. Final caveats of methods employing photobleaching strategies are the induction of photodamage and/or photochromicity, and their inherent destructiveness, which is not compatible with dynamic (live cell) FRET measurements.

FLIM–FRET method

FLIM is a technique used to resolve the spatial distribution of nanosecond (ns) excited state lifetimes within microscopic images (Gadella & Jovin, 1995; van Munster & Gadella, 2005). The two most common implementations are the frequency- and time-domain FLIM. Measurements in the time-domain employ ultra short excitation pulses and recording of the (ns) time-resolved donor fluorescence emission decay from which the donor fluorescence lifetime (τ_D) can be determined. In the frequency domain, (sinusoidally) intensity-modulated excitation light and a gain-modulated detector are used, and τ is derived from the phase shift and demodulation of the fluorescence emission as compared to the excitation light. Because of the frequency-domain technique is most easily implemented on wide-field microscopes (using a gain-modulated intensified-CCD detector) and the time-domain technique is usually implemented on a confocal scanning microscope (using time-correlated single photon counting), they are often described separately. However, both methods can be implemented on either confocal scanning or wide-field microscopes and the wide-field FLIM implementations are compatible with Nipkow disc multi-beam techniques.

Although FLIM–FRET requires specialized instrumentation, the independence of τ from fluorescence intensity makes the technique very robust because variations in excitation intensity, inner filtering, moderate donor photobleaching and detector sensitivity do not influence τ. Furthermore, by contrast to the bleaching methods, the method is non-destructive, not particularly phototoxic and hence can be applied to living cells and used for monitoring dynamic FRET changes. Factors possibly affecting the measurements are scattered excitation light and autofluorescence. Although the latter is a general problem for any form of quantitative fluorescence microscopy, the former problem can be reduced by usage of a proper emission filter or accounted for by FLIM analysis because scattering is an ultrafast process ($\tau = 0$). A disadvantage of FLIM analysis is that it requires the detection of relatively large numbers of photons in order to get accurate results, especially for (time-correlated single photon counting) confocal imaging. This can result in long acquisition times (generally >30 s/frame). Wide-field FLIM, despite its usage of low quantum yield (gated or frequency modulated) intensified detectors, is usually one to two orders of magnitude faster than confocal FLIM because all time-resolved measurements in the image are done in parallel: even video-rate FLIM systems have been described. So depending on the type of instrumentation FRET–FLIM can be compatible with monitoring dynamic processes.

There are two concerns related to the process of photodestruction for FLIM measurements. First, (partial) acceptor bleaching leaves the donor unquenched, leading to underestimation of E. Second, photoconversion of the donor fluorophore can alter its fluorescent lifetime. Therefore the ideal FRET pair for FLIM consists of two relatively photostable fluorophores with well-separated emission spectra (because FLIM requires the donor-only part of the spectrum). For FRET–FLIM one has to rule out the possibility of lifetime changes due to altered pH, ion concentration or viscosity in the sample. Small organic fluorophores are especially affected whereas most recent monomeric FPs are far less sensitive to such artefacts. Furthermore, donor fluorophores with long lifetimes, increasing the dynamic range of the lifetime measurement, and monoexponential decay, for easier fitting and quantification, are preferred (Goedhart *et al.*, 2010). Assumptions involved in curve fitting, such as the appropriateness of specific decay models for a particular sample, or even the specific method used for fitting must be considered to attain reasonable results. Although multispecies fitting procedures in FLIM such as global analysis (Verveer & Bastiaens, 2002) or phasor/polar plot analysis (Clayton *et al.*, 2004) can be complicated, they enable the identification of fractions of molecules involved in FRET, which is impossible for methods like filter FRET, spectral FRET, pbFRET, pcFRET or acceptor bleaching.

Anisotropy FRET method

Another powerful and still underappreciated parameter reporting on energy transfer is the anisotropy of the fluorescence emission. Upon excitation of the sample with polarized light, only molecules that, by chance, have their excitation dipole moment oriented favourably (i.e. parallel) to the excitation light polarization direction are excited (a process called photoselection). If rotational movement of the molecule within its fluorescence lifetime is limited or negligible (as is the case for visible FPs due to their size), the emitted light will also be polarized, the extent of which depends on the angle between excitation and emission dipole moments. In case of FRET to a fluorescent acceptor, the ensuing sensitized emission is partially depolarized because it results from acceptor molecules whose emission dipoles can be differently orientated with respect to the excitation polarization direction (see Fig. 2). Both steady-state emission anisotropy and (life)time-resolved anisotropy decay measurements are possible. In practice, the sample is excited using linearly polarized light and the fluorescence intensity is measured in both parallel and perpendicular polarization directions (Clayton *et al.*, 2002). Anisotropy measurements can be performed using scanning or wide-field microscopes. Complicating

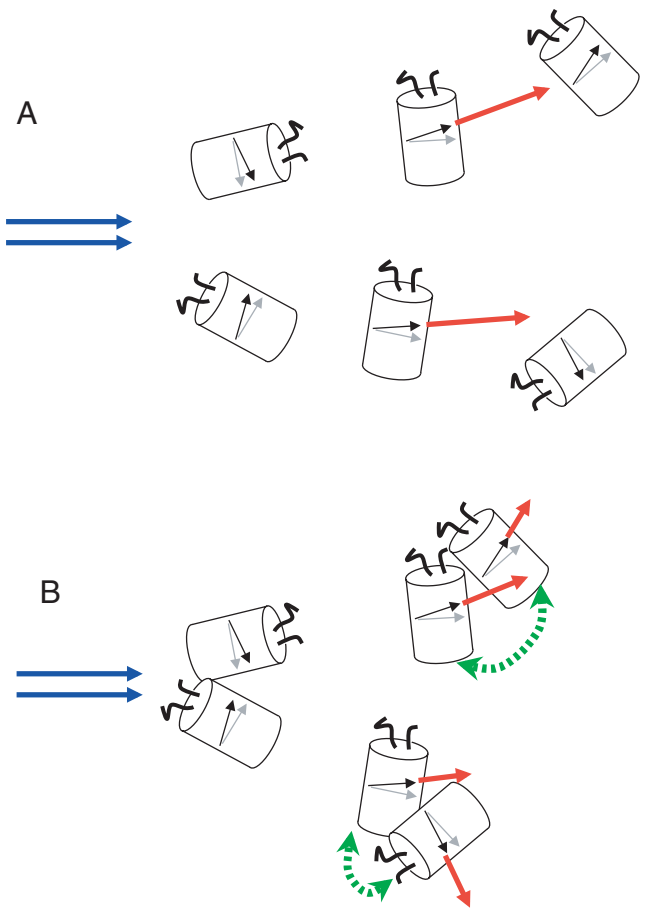

Fig. 2. Homo-FRET by fluorescence anisotropy measurement. A population of randomly oriented FPs is excited with linearly polarized light (the polarization direction is indicated by the leftmost blue arrows). In situation A, no FRET occurs because of extended distance between the FPs (here, photoselection only excites two molecules). Due to a small intrinsic angle between excitation (grey) and emission (black) dipole moments, the total resulting fluorescence emission (red arrows) will have nearly the same polarization direction as the excitation light. In case of homo-FRET (in situation B, note the close proximity of FPs), the resulting fluorescence emission consists of remaining highly polarized emission from the initial photoselected FPs and a much less polarized sensitized emission from their interacting partner FPs. This mixed polarization results in reduction of the detected anisotropy. Note that FRET (green arrow) can be bidirectional and that the total emission (summed lengths of the red arrows) is identical for situation A and B, i.e. no overall quenching or fluorescence lifetime reduction will occur.

instrumental factors are (de)polarizing effects of filters, mirrors and lenses (especially lenses with high numerical aperture), leading to a G factor (= relative detection efficiency of parallel and perpendicular directions) substantially deviating from 1. As a result, microscopy-based anisotropy measurements are restricted to lenses with numerical aperture ≤ 1, usually limiting the possible magnification for the measurement. Furthermore, contrast techniques such as phase contrast and Nomarski differential image contrast that utilize polarization and interference to enhance contrast, perturb with the anisotropy measurement. Hence, the microscopy setup should be free of these functional elements in order to ensure correct anisotropy calculations. Besides these hardware considerations, the anisotropy measurement is sensitive to background autofluorescence and scattered excitation light. In hetero-FRET applications (different spectral properties of D and A), both direct excitation of the acceptor and bleed-through of the donor emission will introduce highly polarized components. Hence, three-filter cube-like corrections are required for isolation of the partially depolarized sensitized emission in hetero-FRET anisotropy.

Importantly, anisotropy measurement holds a unique advantage over all other FRET methods in that it is the only technique that can detect homo-FRET: the energy migration between spectrally identical fluorophores (see Fig. 2). A requirement for homo-FRET (also called energy migration) is a fluorophore with a small Stokes shift and hence overlapping excitation and emission spectra. Homo-FRET is particularly useful for the investigation of oligomerization (Varma & Mayor, 1998). Homo-FRET (employing 1 type of fluorophore) provides a higher sensitivity than hetero-FRET (employing two different types of fluorophores) for studying homo-oligomerization. In hetero FLIM–FRET pairing of two acceptor-tagged molecules will not be detected and worse, pairing of two donor-tagged molecules will dilute the FRET signal from donor-acceptor paired molecules. Other advantages of homo-FRET are that it relieves the researcher from dual labelling, from controlling relative expression levels of differently labelled but otherwise identical proteins and from using narrow emission bandpass filters, because the entire donor spectrum can be used.

Choosing the best FRET microscopy method

Considering the many different methods and principles for detecting FRET in a microscope, choosing the best microscopy technique for a FRET measurement can be a daunting task. There is no overall best method, because each has its own pros and cons. Furthermore, the choice will be determined by the microscopy instrumentation available, including (excitation/emission) filters and excitation options (laser lines), the specimen and probes used. To assist the interested reader in selecting the best FRET microscopy technique we included Fig. 3 in which answering a few questions will guide you to the most straightforward and applicable techniques. The figure is presented without pretensions: by no means other choices, solutions or new options considering new hardware developments are excluded, it should be regarded as a well-meant advice after 20 years of FRET experimentation in practice.

Some general considerations to keep in mind are: regard the use of a simple technique in case only a qualitative answer is needed (a FRET change over time) or in case

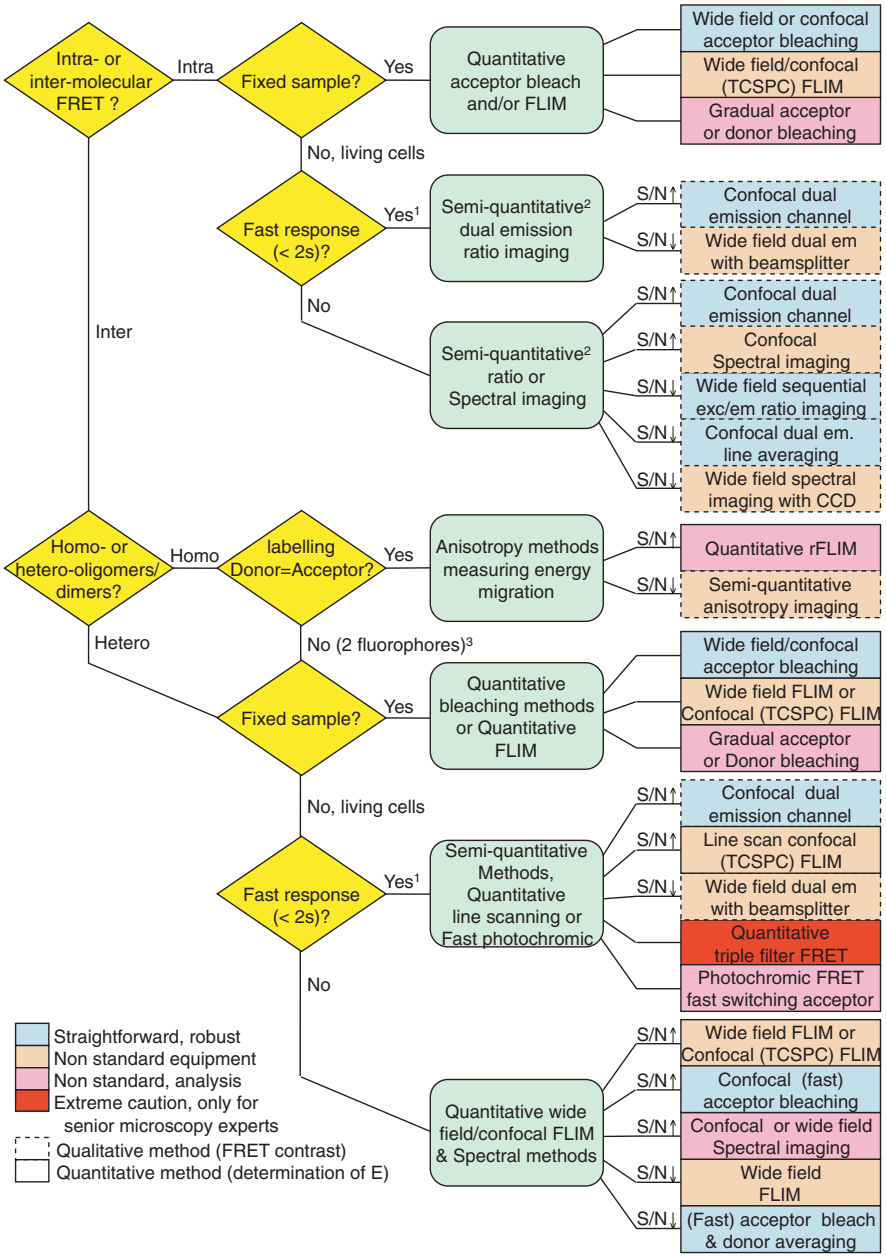

Fig. 3. FRET-microscopy method selector. [1]Or slow response with fast moving cells or subcellular structures. [2]Can be made quantitative after calibration using ratiometric constructs with known E. [3]In this case donor-only and acceptor-only labelled oligomers will not contribute to the FRET signal. S/N↑ means the method of choice in case of high fluorescence levels and S/N↓means the method of choice in case of low fluorescence levels (noisy images).

where a ratiometric intramolecular FRET sensor (with fixed donor to acceptor labelling ratio) is used. Another advice is to pay particular attention to setting up the proper set of controls – also perform measurements of non-labelled, donor-only, acceptor-only and dual-labelled non-FRET and FRET samples, even if they do not seem necessary (like acceptor bleaching of a donor-only labelled specimen). Such controls will report on autofluorescence, scattering, bleed-through, photochromicity, registration problems, etc. Upon awareness of these problems, measures can be taken to reduce or eradicate them by changing the experimental conditions, like filters, laser intensity, fixation method, medium, immersion oil, etc. Finally, if possible, try to confirm results with two different techniques, i.e. one based on donor quenching and one on sensitized emission. In case of intermolecular FRET, methods monitoring only the donor (like acceptor bleaching and FLIM) are less prone to artefacts because acquisition with only one excitation wavelength circumvents registration problems, chromatic aberrations and allows usage of so-called dirty acceptors (e.g. labelled antibodies recognizing multiple

epitopes) or even dark non-fluorescing acceptors. On the other hand, methods monitoring both donor and acceptor populations provide an invaluable control for the behaviour of both interacting species. This can be of great importance in a situation where the distribution of molecules changes during the measurement.

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