Imaging molecular interactions in living cells by FRET microscopy

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Förster resonance energy transfer (FRET) is applied extensively in all fields of biological research and technology, generally as a 'nanoruler' with a dynamic range corresponding to the intramolecular and intermolecular distances characterizing the molecular structures that regulate cellular function. The complex underlying network of interactions reflects elementary reactions operating under strict spatio-temporal control: binding, conformational transition, covalent modification and transport. FRET imaging provides information about all these molecular processes with high specificity and sensitivity via probes expressed by or introduced from the external medium into the cell, tissue or organism. Current approaches and developments in the field are discussed with emphasis on formalism, probes and technical implementation.

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Introduction

Förster resonance energy transfer (FRET) is a photophysical phenomenon in which energy is transferred from the first excited electronic state (S_1) of a fluorophore (the donor D) to another nearby absorbing (but not necessarily emitting) molecule (the acceptor A). Thus, there is a concerted quenching of D and activation of A fluorescence (Figure 1). For this reason, the acronym FRET is often, albeit incorrectly, used to designate 'fluorescence' resonance energy transfer. The process involves the resonant coupling of emission and absorption dipoles and is thus non-radiative. That is, it competes with other radiative (fluorescence) and non-radiative pathways for deactivation. The underlying formalism first elaborated by Theodor Förster establishes a parametric proportionality between the rate of transfer (k_t) and the radiative rate constant (k_f) and it is this relationship, operative over region of ~1–20 nm, that forms the basis for the extensive application of FRET in virtually every field of biology, chemistry, physics and engineering.

This survey of FRET in imaging applications extends our last review of the subject in 2003 [1]. The format does not permit a comprehensive coverage of the vast literature but is rather intended as a selective guide to the present status and projected development of the field. Most specific citations are from 2004 to the present. Because of space limitations, we do not systematically consider single-molecule and fluorescence correlation spectroscopy, nor the use of molecular beacons, aptamers and other bioengineered FRET biosensors of ions, second messengers, and covalent modification (for a recent review see [2••]).

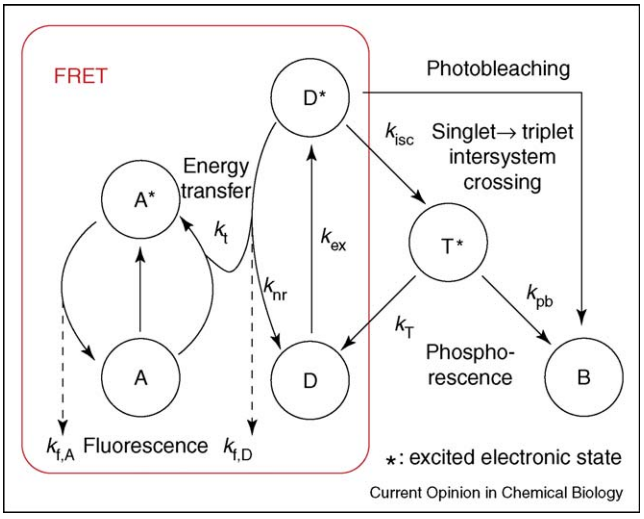
FRET formalism

FRET can be employed for probing or for systematically altering states of matter; the former use by far outweighs the latter in the reported literature. Its rational application necessarily involves (i) understanding the fundamental basis and parametric dependencies of the phenomenon; (ii) selecting D–A pairs and the means for their introduction into systems of interest; (iii) performing measurements and/or perturbing the system; and (iv) analyzing the results so as to confirm or reveal a molecular model. The master Förster equation applicable for a given D–A pair and in universal use is:

$$E \equiv k_t \tau_{DA} = \left[1 + (r/R_o)^6\right]^{-1}; R_o^6 = c_o J n^{-4} \kappa^2 Q_o$$
$$c_o = 8.8 \cdot 10^{-28} \text{for } R_o \text{ in nm,}$$
$$\text{and } J = 10^{17} \int q_{D,\lambda} \varepsilon_{A,\lambda} \lambda^4 d\lambda \text{ in nm}^6 \cdot \text{mol}^{-1} \quad (1)$$
$$q_{D,\lambda}, \text{normalized donor emission spectrum}$$
$$\varepsilon_{A,\lambda}, \text{acceptor extinction coefficent}$$
$$Q_o = k_f \cdot \tau_D; \tau_D^{-1} = k_f + k_{nr} + k_{isc} + k_{pb}; \tau_{DA}^{-1} = \tau_D^{-1} + k_t$$

where the energy transfer efficiency (E), defined as the product of k_t (Figure 1) and the fluorescence lifetime (τ_{DA}) of the donor subjected to quenching by the acceptor, depends on the sixth power of the ratio of the D–A separation distance (r) to the Förster 'constant' (R_o), a value generally in the range of 2–8 nm for most D–A pairs in general use. R_o is the indicated function of (i) the overlap of the donor emission and acceptor absorption spectra (parameter: overlap integral J); (ii) the refractive index (parameter: n^{-4}, range ≈ 1/3–1/5); (iii) the relative orientation of the donor absorption and acceptor

Figure 1



Trafficking in the excited state subjected to FRET. A donor D is excited by absorption of light (k_{ex}) to its excited state (S_1 = D*) and deactivates via fluorescence emission ($k_{f,D}$), non-radiatively (k_{nr}), intersystem crossing to the triplet state (k_{isc}), photobleaching (k_{pb}), and resonance energy transfer (k_t) to a nearby acceptor (A). The latter, in turn, undergoes deactivation, including by emission ($k_{f,a}$) in the event it is fluorescent. See [1] for a 'photophysical primer'. Energy dissipation in multichromophoric systems is much more complex [3].

transition moments (parameter: κ^2, range 0–4); and (iv) the unperturbed (by FRET) donor quantum yield (Q_o), given by the product of k_f and the donor fluorescence lifetime (τ_D). We refer to [1] and citations therein, and to a recent multi-authored compendium on FRET [4••] for extensive discussions of this classical FRET formalism and its application in imaging, particularly of living cells, tissues and organisms (for excellent reviews see [2••,3,4••,5–7,8••,9–13] as well as a previous survey in this series [14] and the PhD thesis of Alessandro Esposito, University of Utrecht, 2006).

Methods for the determination of $E_{x,y,z,time}$, based on the evaluation of *donor* emission alone, require a comparison of a donor property (δ_i) in the presence (subscript DA) and absence (subscript D; the reference condition) of the acceptor (Equation 2). Examples are steady-state emission (I), lifetime (τ), and steady-state emission anisotropy ($\bar{r}$). (The initial and limiting anisotropies, r_0 and r_∞, respectively, in Equation 2 correspond to the simple model generally applicable to most molecules of an apparent spherical rotator undergoing anisotropic, i.e. hindered, rotational relaxation: $r(t) = (r_0 - r_\infty)e^{-t/\phi} + r_\infty$. We assume in Equation 2 that τ, and possibly the rotational correlation time (ϕ), change upon approximation of A to D.) Unfortunately, the comparison is often difficult, if not impossible to achieve in practice, particularly with living cells. One reason is that the reference condition may vary from one position to another, imposing the necessity of performing its determination locally instead of globally with a separate sample. Some of the existing FRET methods (e.g. those of the *acceptor depletion* category ([1]; Table 2 Ie) circumvent the latter problem, but most other measurement techniques do not.

$$E \equiv \frac{k_t}{k_t + \tau_D^{-1}} = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{\tau_{DA}}{\tau_D} = 1 - \left(\frac{r_o - \bar{r}_{DA}}{\bar{r}_{DA} - r_\infty}\right)\left(\frac{\bar{r}_D - r_\infty}{r_o - \bar{r}_D}\right)\frac{\phi_{DA}}{\phi_D} = 1 - \delta_i \ldots \quad (2)$$

An alternative formulation of the Förster relationship, more fundamental than that represented by E (Equation 1), is given by Equation 3, in which we adopt and extend the nomenclature of [1]. Equation 3 features a reduced Förster constant (Γ_o), better suited to measurements based on *acceptor* properties (see below), in that it (i) avoids the arbitrary introduction of the unperturbed donor lifetime into the definition of R_o; and (ii) obviates the requirement for an estimation of E (Equations 1,2), thereby eliminating the need for explicit knowledge of the unperturbed donor parameters.

$$\frac{k_t}{k_f} = \left(\frac{\Gamma_o}{r}\right)^6; \Gamma_o^6 = c_o \kappa^2 J n^{-4}; \left(\frac{\Gamma_o}{r}\right)^6 \rightarrow \rho_i \Omega_i \quad (3)$$

The focus in most FRET determinations is on the D–A separation r as an index of molecular proximity and association [15]. However, FRET is sensitive to *all* of the indicated parameters and thus can be employed in a given situation for systematically exploiting or evaluating any of them, alone or in combination. In addition, in images of structures with components exhibiting FRET, every pixel (2D) or voxel (3D) may exhibit arbitrary degrees of heterogeneity with respect to composition and/or molecular environment. It follows that the assumption in FRET imaging of the invariance of not only Q_o (Equation 1) but also of J, n^{-4}, and/or κ^2 implied by a fixed R_o (Equation 1), may often, perhaps even generally, be unjustified and therefore misleading. Thus, one may wish to consider in any given experiment the suitability of factoring $(\Gamma_0/r)^6$ into a particular targeted parameter of interest (ρ_i) and an associated 'FRET constant' Ω_i (Equation 3). Concrete examples are the assessment of (i) changes in conformation or orientation ($\rho = \kappa^2$), (ii) spectral perturbations as measures of microenvironment and/or binding ($\rho = J$), or (iii) temperature ($\rho = r^{-6}, \kappa^2, J, n^{-4}$). The ease with which the FRET process can be detected is maximal for measurements centered about $\rho_i\ \Omega_i = 1$.

The new treatment (Equation 3) has little purpose unless it can lead to concrete and useful results. We stated above that Equation 3 is more suitable than Equation 2 in FRET determinations based on acceptor (± donor) properties (e.g. sensitized emission) [16]. In such cases, k_t can be expressed directly and conveniently in terms of

measured experimental parameters. We provide here one such example (Equation 4), valid for the generally utilized regime of low excitation intensity (negligible donor and acceptor saturation). We assume an experimental design based on alternating excitations at/near the donor and acceptor absorption peaks ([17,18,19•]; intensities $I_{D,ex}$ and $I_{A,ex}$, respectively), and the acquisition of fluorescence signals ($f_{exc,em}$) in the two corresponding emission bands (D,A). Under such conditions, the ratio (k_t/k_f) at every image position is given by:

$$\frac{k_t}{k_f} = Q_A^{-1}\left[\frac{d_{D,D}}{d_{A,A}}\right]F;$$
$$F \equiv \left(\frac{f_{D,A}}{f_{D,D}}\right) - \left[\frac{\varepsilon_{A,D}}{\varepsilon_{A,A}}\right]\left(\frac{f_{A,A}}{f_{D,D}}\right)\frac{I_{D,ex}}{I_{A,ex}} - \left[\frac{d_{D,A}}{d_{D,D}}\right] \quad (4)$$

in which the fixed (constant) ratios in square brackets relate to (i) extinction coefficients $\varepsilon_{A,\lambda}$ (or, equivalently, absorption cross-sections $\sigma_{A,\lambda}$) of the acceptor at the two excitation wavelengths, or (ii) relative detection efficiencies ($d_{A\text{ or }D,\ A\text{ or }D\text{ emission band}}$) reflecting both $q_{D,\lambda}$ or $q_{A,\lambda}$ and the wavelength-dependent instrument response function. The second and third terms in Equation 4 represent direct acceptor excitation and spillover of donor emission in the acceptor emission band, respectively; inverse $A \rightarrow D$ overlap can also be incorporated into Equation 4. It is interesting and fortunate that the presumably/generally invariant acceptor quantum yield (Q_A) appears as a factor in Equation 4 instead of the varying donor quantum yield (Q_D). The generation of a 'k_t k_f' image provides a direct measure of $(\Gamma_0/r)^6$ or of its factored form ρ_i Ω_i (Equation 3). Useful and reliable images of relative values can also be based on the function F alone (Equation 4), assuming constancy of the proportionality constants.

Equation 4, as in the case of most FRET imaging formalisms, represents the donor in each particular position *j* as a virtual species with an apparent $k_t(j)$; it applies for arbitrary absolute and relative local concentrations of donor and acceptor. Other expressions related to Equation 4 incorporate alternative or additional experimental parameters such as donor and acceptor emission anisotropies and lifetimes, or exploit non-linear phenomena such as ground state depletion of the donor and/or acceptor in the high-intensity excitation regime ([1,20,21]; M Beutler, R Vermeij, TM Jovin, R Heintzmann, unpublished). Such a multiparameter approach facilitates interpretations of image data in terms of population distributions [22] and binding fractions although doing so in a necessarily model-dependent manner.

FRET probes

The identification of optimized D–A pairs is a perennial quest (see Update) and much value can be derived from perusal of the flow cytometric literature on this subject (see, for example [23]). Ideal fluorophores serving as donors have large extinction coefficients for single and/or multiphoton absorption, high emission quantum yields (or more precisely, large radiative rate constants k_f; Figure 1; Equation 1), large Stokes shifts (separation between donor and acceptor emission bands), high photostability, a 'reasonable' lifetime ('very short' is hard to measure in FLIM and 'very long' yields low rates of fluorescence [1]), reasonably small size, relative insensitivity to microenvironment (polarity, pH, ionic strength) — unless this property is the one under investigation — and a facile, stable means for chemical conjugation to a target of interest. Organic fluorophores meeting many if not all of these objectives are under constant development [24] and the reader is directed to the websites of Amersham, AnaSpec, Atto-Tec, Denovo Biolabels, Dyomics, Few Chemicals, Marker Gene Technologies, Molecular Probes (Invitrogen), and other (http://www.fluorescence-resource.com) firms supplying such reagents.

Up to this point, we have considered FRET between a D and an A with distinctive properties; this process is termed heteroFRET. A donor with a large Stokes shift facilitates discrimination between the D and A emissions. Interesting new compounds with this characteristic are Pacific Orange™ (Invitrogen; λ_{exc} 400 nm, λ_{exc} 551 nm), the voltage-sensitive 'Pittsburgh dyes' [25•], and the visible fluorescent protein (VFP) *Keima* [26]. FRET can also occur between identical fluorophores, but this case requires that the Stokes shift be *small* enough to result in a finite value of the overlap integral J (Equation 1). In such homoFRET or energy migration FRET (emFRET; [1,14,27,28]), the donor ensemble population exhibits no quenching (reduction of intensity and lifetime) but rather a depolarization (reduction of emission anisotropy) because of the virtual loss of correlation between photoselective excitation and emission orientation upon transfer. HomoFRET has the virtue of requiring only a single expression probe in studies of homo-association in cellular systems.

Ideal acceptors for FRET share some of the above characteristics, above all a large absorption cross-section (larger J, Equation 1). Photostability is generally, but not always, desirable, as for example in acceptor photobleaching FRET ([1], Table 2) that obviously requires a photobleacheable acceptor. The lifetime should be matched appropriately to that of the donor (e.g. in luminescence RET (LRET) [29•]; in which the sensitized emission is characterized by two lifetimes (τ_D, τ_A) with opposite amplitudes representing the build-up and decay phases). In the case of lanthanide donors, $\tau_A \ll \tau_D$, such that the long decay phase is free of 'contamination' by directly excited acceptor emission (the direct donor emission is isolated spectrally; see Equation 3) and thus provides a sensitive measure of FRET. A 'dark' (non-fluorescent)

acceptor can, under some circumstances, be preferable [30], for example in strategies based on multi-probe multi-transfer FRET. In other instances, an acceptor with modulatable absorption properties is highly desirable. Such a property potentiates deterministic '*J* engineering' (Equation 1) or indirect reporting of the state of a dark acceptor via the fluorescent donor (Equation 2). Other examples of molecular photoswitches [31] are photoconvertible VFPs [32,33••,34–39], caged acceptors [40], photochromic diheteroarylethenes devised for photochromic FRET [1,41–43], and photochromic spiro-naphthoxazines/benzopyrans devised as modulators of molecular structure and function [44]. Such molecules can be exploited for achieving super-resolution by non-linear optical imaging (STED; [45]).

Another category of FRET probes includes nanoparticles serving either as donors or acceptors. Quantum dots (QDs), in particular, exhibit almost all of the properties listed at the outset of this section, particularly efficient absorption and extreme photostability. Their use as probes of living cells is rapidly increasing [46,47•,48,49]. One can define a measure of probe brightness as the integrated emission over an observation time T. Under non-saturating illumination conditions, this quantity is given by the product of excitation efficiency ($\sigma\ \Psi$;Ψ = illumination photon flux), emission efficiency ($k_f\tau = Q$), photon turnover rate ($k_f = Q\tau^{-1}$), and T, resulting in $\sigma Q\tau^{-1}\cdot\Psi T$. QDs exhibit large values of σ and Q but characteristically longer (>10 ns) lifetimes than organic dyes. Thus, they are 'bright' but not exceptionally so under conditions of short, weak illumination. However, in the high intensity (saturation) illumination regime, the corresponding brightness parameter is $\left[\left(T^{-1}+k_{pb}\right)\tau\right]^{-1}$. For QDs, k_{pb} is generally very small and thus $\ll T^{-1}$, whereas the opposite inequality holds for most organic dyes. For this reason, QDs are easily detected and identified visually and by electronic imaging, providing single molecule sensitivity, positional super-resolution [50], and confirmation of molecular identity (e.g. of substances to which they are conjugated), as a consequence of their blinking behavior and distinctive narrow emission spectra [43]. As FRET donors, QDs also benefit from their large absorption cross-section that increases continuously from their narrow emission bands to the UV. Thus, despite their relatively large size [1], requiring close proximity of the acceptor(s) to the nanoparticular surface, QDs function efficiently as FRET donors in many bioanalytical and imaging applications [41,43,51,52•]. One can anticipate the development of QDs with smaller stabilization-conjugation coats for enabling FRET probing at locations removed from the particle surface. Small fluorescent noble-metal clusters [49] and silicon-based dye-encapsulated nanoparticles [53] constitute other potential sources of FRET probes with unique characteristics, whereas larger gold nanoparticles can *enhance* the fluorescence of nearby weak (low Q_o) fluorophores in a distance-dependent manner [54] reminiscent of FRET.

Expression probes are generally required for FRET measurements in living cells, tissues, embryos and organisms. The VFPs serve very well in this capacity [2••,5–7,8••,9–14,55–58] but their large mass (27 kDa) often leads to alteration or abrogation of biological function. Fortunately, numerous other expression systems are emerging (Table 1; see also [2••,5,59] and Update), which in spite of the general requirement for an exogenous probe offer distinct advantages in that they (i) require much smaller peptide segments as specific fusion tags; (ii) are flexible in the choice of fluorophores for targeting; and (iii) can be used in a multiplexed spatio-temporal sequence tailored

Table 1

Expression tags based on protein fusions.

	Added kDa	Target	References
Visible fluorescent proteins (VFPs)	27	–	See text
Spectral variants			
'Cameleon' dual-VFP constructs for sensing ions, pH, covalent modification, Photoactivatable, photoconvertible, photochromic VFPs			
Bimolecular complementation (half VFP molecules: BiFC)	2 × 27/2		
Mutant dehalogenase (HaloTag™; probe-substituted haloalkanes) [probe-substituted haloalkanes]	33	Asp	Promega
DNA alkyltransferase (AGT, SNAP-tag™) [*para*-substituted benzylguanines]	20	Cys	[64]; Covalys
Dihydrofolate reductase (DHFR) [methotrexate-linked probe — LigandLink™]	18	–	[59]; Active motif
Acyl and peptidyl carrier protein (ACP, PCP) [CoA-linked probe]	9	Ser	[65]
Biotin-mimetic peptides (nano-tags) [avidin, streptavidin, anti-biotin linked probe]	1–1.7	–	[66]
Tetracysteine (-CCxxCC-) motifs [bisarsenical probe derivatives]	0.7–1.3	Cys_4	See text
Biotin ligase acceptor protein (AP) [ketone analog of biotin]	0.9	Lys	[67]
Transglutaminase recognition sequence (Q-tag) [probe-linked primary amine]	0.7	Gln	[68,69]
Oligohistidine [trifunctional: NTA-linked photoreactive probe]	0.7	$His_{4–6}$	[70]
N-terminal cysteine (from protein cleavage) [thioester-linked probes]	–	Cys	[71]

Square brackets denote fluorophore or QD probes.

to the system. The bisarsenicals specific for tetracysteine tags [2••,60•] are good examples in which two probes (e.g. FlAsH and ReAsH, MJ Roberti, CW Bertoncini, R Klement, EA Jares-Erijman, TM Jovin, unpublished; FlAsH and VFPs [61]; and the recently introduced, much more photostable and efficient FlAsH fluoroderivatives, CC Spagnuolo, RJ Vermeij, EA Jares-Erijman, unpublished; see Update) constitute useful D–A pairs. In addition, the resorufin-based ReAsH permits correlative optical and electron microscopic imaging [2••]. A current challenge is to develop D–A pairs based on systematic combinations of the various expression probes (Table 1) for multicomponent FRET imaging of living cells.

To follow a complex cascade of signals simultaneously, one would ideally wish to have at disposal a family of probes featuring the parallel use of 'orthogonally directed, compatible labels', particularly for use with imaging systems. This aim requires the development of novel probes and strategies for monitoring several signals concurrently. Given the multiplexing capabilities [43,46,47•,62] and photostability of QDs, a wide variety of independent methods can be conceived for the simultaneous assessment of multiple cellular functions. These probes exhibit an additional feature of great utility: the number of groups bound to their surface can be varied from 1 to >10 in a controlled manner. That is, one can add single or several small molecules to provide particular binding specificities and/other functional properties. Methods of specific delivery have been combined with small fluorophores and lately with QDs [63] for the targeting of cell surface proteins. Further efforts devoted to the facile introduction of diverse organic and nanoparticle probes into cells will serve to exploit established as well as new fluorophores in an optimal manner, particularly for FRET.

FRET imaging technology

In previous papers [1,72], we provided a classification scheme for FRET imaging techniques based on the measurement parameters and modes of signal acquisition. We employ the same system in Table 2, in which we limit the entries to developments posterior to those cited in the previous reviews; other relevant citations are given in the text of this review. Much of the FRET literature originates or is associated with topics such as strategies for optical sectioning (confocal microscopy), superresolution, and the determination of diffusion properties of ensemble and single-molecule populations. Space limitations preclude a detailed comparative assessment of the current repertoire of FRET imaging techniques and the reader is urged to consult the cited literature.

Future directions

In our estimation, major new FRET developments will lie in the area of the acceptor depletion techniques (adFRET, Table 2 Ie), particularly those based on non-linear phenomena such as ground state-depletion (excited state saturation), and multiparametric

Table 2

Methods for determining FRET in fluorescence microscopy[a].

No.	Method	Comments	References
I. Donor quenching and/or acceptor sensitization			
	Ia Combined donor (D) and acceptor (A) emission signals		
Ia1	2,3 Signals; spectra	Multispectral; spectral unmixing	[10,18,73–76]
Ia3	Bioluminescence RET (BRET)	New reagents and detection methods	[77]
	Ib Fluorescence-detected excited state lifetime(s) (FLIM, FLI)		
Ib1	**D** lifetime	Time and frequency domain	[72,74,78,79,80•, 81,82,83••,84–88]
Ib2	Luminescence RET (LRET)	New reagents and procedures	[29•]
Ib4	Spectral FLIM (sFLIM)	Calibration, spectral and temporal resolution	[76,89]
	Id donor depletion		
Id1	**D** pb kinetics (pbFRET)	Improved procedures and analysis techniques	[22,90]
	Ie acceptor depletion FRET (adFRET)		
Ie1	Direct **A** pb (irreversible)	Method in widespread use	[16,75,90]
Ie2	Photochromic **A** (pcFRET)	Reversible adFRET: organic and QD reagents	[41,42,91]
Ie3	**A** saturation (frustrated FRET)	Reversible adFRET: acceptor saturation (new)	
II. anisotropy			
	IIa steady-state anisotropy		
IIa1	**D** anisotropy $\bar{r}$	Molecular association → ↑ (lower τ)	[17,27,92,93]
IIa2	**A** anisotropy $\bar{r}$	Sensitized emission is depolarized	[92,94]
	IIb homotransfer, energy migration FRET (homoFRET, emFRET)		
IIb1	Steady-state anisotropy $\bar{r}$	Homo-association; concentration depolarization	[27,28,95]

[a]See [1,72] for previous versions of the table and citations therein. [b]A Esposito, PhD thesis, University of Utrecht, 2006. [c]M Beutler, R Vermeij, TM Jovin, R Heintzmann, unpublished.

approaches. In this connection it is appropriate to stress the fundamental importance of fluorescence lifetime determinations, either in the time or frequency domain. FLIM (or FLI, fluorescence lifetime-resolved imaging) can be combined with multispectral, polarization-sensitive, and optical-sectioning modalities and as such offers the prospect of a 'do-it-all' form of fluorescence microscopy (Table 2 Ib). We also anticipate the emergence of numerous new techniques based on parameter modulation and perturbation, permitting the reliable phase-sensitive detection of extremely low level FRET signals. Significant driving technology will lie in the areas of illumination sources (LEDs, diode lasers), advanced optical imaging techniques, and improvements in the theoretical framework dictating modes of data (image) acquisition and analysis.

Update

A comprehensive survey has appeared of probes suitable as FRET donors and acceptors [96•]. A new expression probe has been reported consisting of a complex of an oligo-Asp tag with Zn(II) [97].

The work referred in the text as (CC Spagnuolo, R Vermeij, EA Jares-Erijman, unpublished) is now in press [98•].

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