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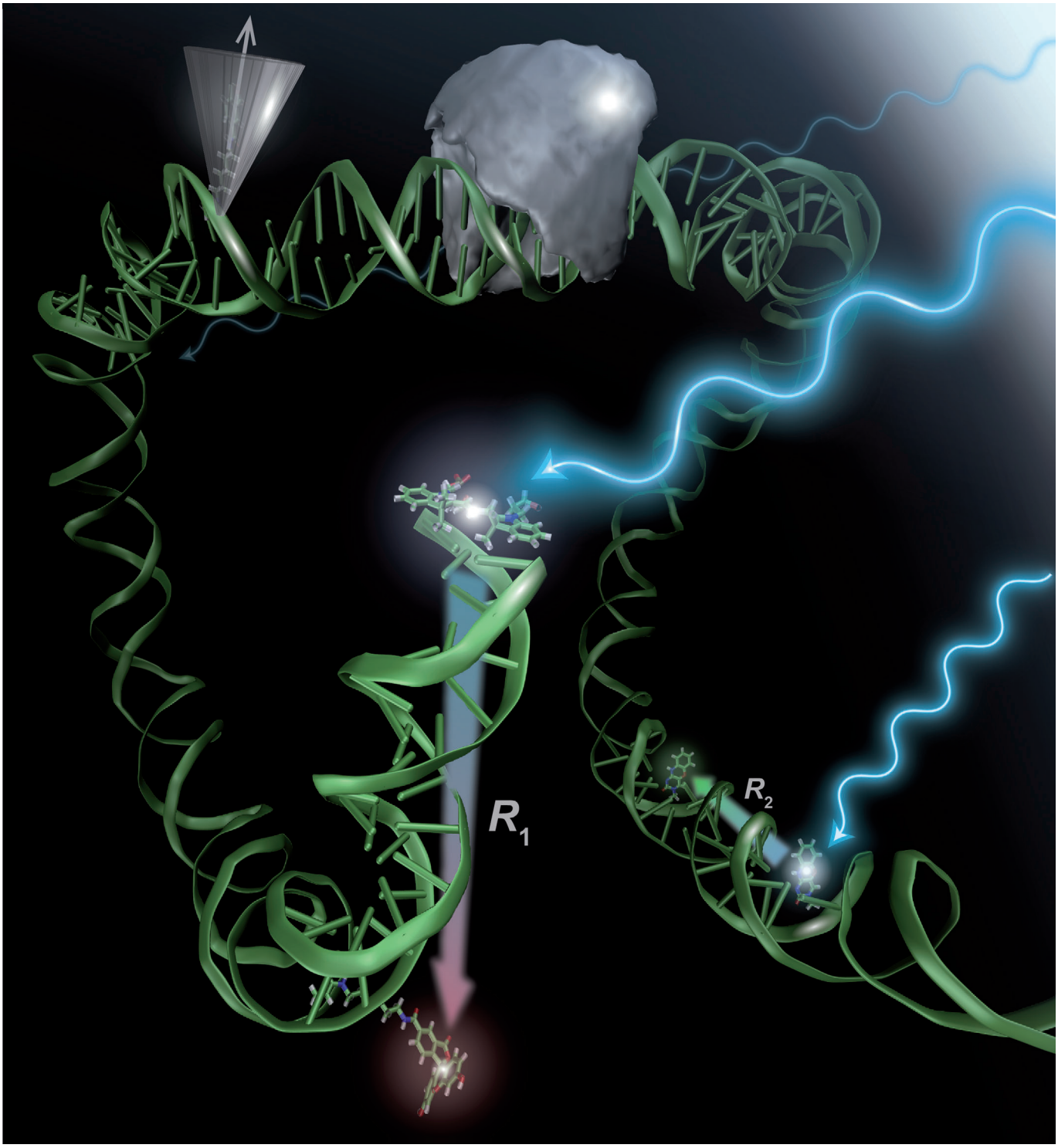
Advances in Quantitative FRET-Based Methods for Studying Nucleic Acids

Søren Preus[b] and L. Marcus Wilhelmsson*[a]



Förster resonance energy transfer (FRET) is a powerful tool for monitoring molecular distances and interactions at the nanoscale level. The strong dependence of transfer efficiency on probe separation makes FRET perfectly suited for "on/off" experiments. To use FRET to obtain quantitative distances and three-dimensional structures, however, is more challenging. This review summarises recent studies and technological advances that have improved FRET as a quantitative molecular ruler in nucleic acid systems, both at the ensemble and at the single-molecule levels.

1. Introduction

The three-dimensional structures and conformational dynamics of nucleic acids are directly related to their functions in living organisms. Traditionally, structural information on biomolecules is obtained at atomic resolution by X-ray crystallography[1] and NMR spectroscopy.[2] Although the importance of both methods is indisputable, the main drawback of crystallography is the need for molecular crystals, whereas NMR primarily suffers from complexity, the need for large amounts of sample and an upper molecular weight limit. Lower-resolution techniques include small-angle scattering,[3] cryo-electron microscopy[4] and "molecular rulers" such as FRET. Of these, FRET possesses the prominent advantage of being cheap and easy, and provides rapid measurements with just a few nmol of sample even in complex media or large molecular complexes. In addition, FRET is routinely performed at the single-molecule level.[5] The main disadvantages of FRET as a quantitative ruler, however, are its dependence on fluorophore photophysical properties, which depend on the interaction between the probe and its microenvironment, and the uncertainty in probe position and orientation relative to the biomolecule. These problems are often associated with the linker connecting the probe to the biomolecule. In single-molecule experiments, FRET suffers from low, fluctuating signals and bleaching. In addition, FRET is limited to distances of < 10 nm.

The limitations of FRET have motivated the development of alternative molecular rulers. Site-directed spin labelling offers measures of relative distances and orientations.[6] Despite its high potential, spin labelling is not widely used for structural studies of nucleic acids, probably due to limitations in the number of commercially available spin labels.[7] In the past six or seven years the interesting electronic properties of metal nanoparticles (NPs) have been exploited in molecular rulers based on NP–NP coupling ("plasmon rulers")[8] and small-angle X-ray scattering interference (SAXSI),[9] whereas dye–NP coupling has been exploited in rulers based on nanometal surface energy transfer (NSET)[10] and surface-enhanced Raman scattering (SERS).[11] The main advantages of these techniques are high and robust signals and probe separations exceeding 10 nm. However, each of these methods still requires refinement and more thorough characterization before it can have broad applicability in quantitative structural studies. Most notably, the heterogeneity of colloidal NPs in preparation limits the interpretation of quantitative measurements because the sizes and shapes of NPs are directly related to their optical properties.

As a result, none of these rulers is yet as ubiquitous in the biosciences as FRET. Whereas the theory and applications of FRET have been reviewed previously,[12] this review seeks to provide a short, but comprehensive, overview of recent studies and techniques that have addressed the above issues of FRET and advanced the use of quantitative FRET in nucleic acid studies. We define "quantitative FRET" as when a distance, a distribution of distances, or dynamical parameters are derived from the measured FRET signal. The properties of common FRET probes in nucleic acid environments are reviewed first, followed by an overview of alternative fluorophores. After focusing on the probes we summarise methods developed to measure and to analyse FRET quantitatively, both at the ensemble and at the single-molecule levels, including methods developed to model probe fluctuations. Finally we describe new techniques based on FRET and provide a list of advanced FRET simulation and analysis software. Throughout the article, particular emphasis is given to the advantages and limitations of each strategy.

2. Dye Properties in Nucleic Acids

Almost all quantitative FRET experiments are performed by tethering the dyes externally to nucleic acids through linkers (Figure 1 A). This highly versatile approach allows practically any probe to be implemented site-specifically by several different methods. The inherent drawback, however, is that quantitative interpretation of experiments becomes complicated by dipole diffusion and reorientation, influencing the donor–acceptor distance (R) and the orientation factor (κ^2), respectively. Only in ideal cases is the almost universal assumption of freely rotating fluorophores—that is, $\kappa^2 = 2/3$—valid (κ^2 can take values between 0 and 4). In addition, interactions between the dye and the nucleic acid, such as electrostatic interactions with the negatively charged backbone, intercalation and/or end-stacking, influence not only the position and orientation of the probe but also its photophysical properties, thus directly affecting the critical Förster distance (R_0; readers interested in the equations describing this relationship are referred to Lakowicz[12c]). It is a rule rather than an exception that fluorophore quantum yields and lifetimes depend on dye position and the surrounding DNA sequence.

[a] *Assoc. Prof. L. M. Wilhelmsson*
Department of Chemical and Biological Engineering
Chalmers University of Technology
41296 Göteborg (Sweden)
E-mail: marcus.wilhelmsson@chalmers.se

[b] *S. Preus*
Department of Chemistry, University of Copenhagen
Universitetsparken 5, 2100 Copenhagen (Denmark)

Because the energy transfer is dependent on the above parameters, a large number of studies addressing the properties of FRET dyes attached to DNA have been performed (summarised in Table 1). Most of the early studies employing quantitative FRET in nucleic acids combined fluorescein (FAM) as the donor and rhodamine or tetramethylrhodamine (TMR) as the acceptor (Figure 1 B).[14]

Fluorescein, being negatively charged under biological conditions, is repelled by the DNA backbone, resulting in an approximately random dynamic orientation as observed by the fluorescence anisotropy of the dye coupled to DNA.[15] The drawbacks of fluorescein, however, are its relatively poor photostability, pH dependency and a marked quenching effect by guanine.[16]

In contrast to fluorescein, the positively charged rhodamine and its derivatives interact strongly with DNA.[15a–c, 16e, 17] Neubauer et al. showed that end-labelled rhodamine 6G populates several distinct, end-stacked conformations and suggested that different quenching kinetics, resulting from electron transfer from a nearby guanine, govern the different states.[17h] The popular carboxytetramethylrhodamine (TAMRA) also interacts with DNA[15c] and displays complex decay kinetics in nucleic acid environments[16c, 17f] resulting from quenching by guanine[16e, 17i,j] and the presence of Mn^{2+}.[17k]

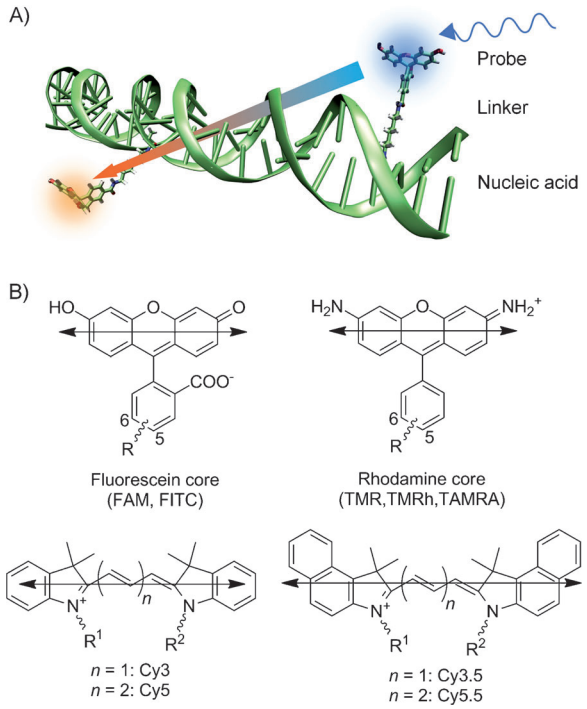


Figure 1. A) Illustration of tethered FRET probes. B) Examples of common FRET probes. Arrows denote *emission* transition dipole moments (note that most rhodamines have an additional slightly blue-shifted transition with a transition moment perpendicular to the lowest in energy[13]).

Marcus Wilhelmsson received his PhD, mainly focusing on interactions between small ligands and DNA, from Chalmers University of Technology (2003). He has also been a guest researcher at Stanford University in Prof. Richard Zare's lab (2000). After his PhD he switched to DNA nanotechnology and was a supervisor in an EU STREP project involving 25 researchers in four countries. Since 2008 he has had his own group at Chalmers. His main focus is on the design, development and use of fluorescent base analogues and on methodologies to facilitate quantitative FRET. He is also founder of the conference FB^3.

Søren Preus is currently finishing his PhD in the optical spectroscopy laboratory of Assoc. Prof. Kristine Kilså at Københavns Universitet. His research frequently brings him to the laboratories of Prof. Bo Albinsson and L.M.W. at Chalmers University of Technology, and he was a visiting scholar in Prof. Daniel Herschlag's laboratory at Stanford University. His current research is focused on the application of fluorescent dyes, especially nucleobase analogues to study biological systems at the nanoscale level.

Texas Red, a zwitterionic derivative of fluorescein and rhodamine, has been suggested to bind significantly into the minor groove of DNA.[15c]

For the last ten years, the most popular FRET probes in nucleic acid studies have been the Cy dyes, primarily owing to their high brightness in single-molecule experiments (Figure 1 B). Due to their popularity, the literature on the properties of Cy dyes in nucleic acid environments is comprehensive[10d, 17a, e, 18] (summarised in a recent review by Levitus and Ranjit[18a]). Levitus and co-workers showed that the photophysical properties of Cy3 in DNA differ substantially from the properties of the free dye and display a strong dependency on dye position,[18d] whereas Sabanayagam et al. showed that the fluorescence quantum yield of Cy3 even varies from molecule to molecule.[18i] This property is a consequence of a *cis–trans* photoisomerisation decay pathway competing with fluorescence from the first excited singlet state of Cy3 and related polymethine dyes.[19] When the microenvironment of the probe hinders rotation of one end of the molecule with respect to the other the fluorescence quantum yield increases. Interestingly, in Cy3B the isomerisation is prevented by rigidification of the trimethine chain, which increases the fluorescence quantum yield and excited state lifetime of the probe,[18d, 20] but at the expense of lower photostability.[20c]

Lilley and co-workers have shown that end-labelled Cy3 and Cy5 tend to stack on top of the ending base-pair of the helix, resulting in a partially constrained dipole orientation (Figure 2).[18b, l] The stacking ability is independent of linker length[18e] but the precise orientation of the stacked probe is

Table 1. Selected references for commercially available FRET probes characterized in nucleic acid environments.

Dye	Property[a] and references	Suppliers[b]
Alexa 488	lifetime/QY[15d,18k] position/orientation[15d,18k]	[c]
ATTO 610/647/680	lifetime/QY[23c,d] position/orientation[23c]	[d]
Cy3	lifetime/QY[17e,18d–j] position/orientation[17e,18b–f]	[e, f, g]
Cy3B	lifetime/QY[18d,20]	[e]
Cy5	lifetime/QY[10d,17e,18j,k] position/orientation[10d,17a,e,18k,l]	[e, f, g]
fluorescein/FAM	lifetime/QY[10d,15b,d,16c,d,24] position/orientation[10d,15,16d,17b–d,24b]	[c, e, f, g, h, i, j]
rhodamine 6G	lifetime/QY[17e,h] position/orientation[17e,h,25]	[i]
rhodamine/TMR/TAMRA	lifetime/QY[15b,16c,17d–g,18g,24c] position/orientation[15a–c,17a–g]	[c, f, g, h, i, j]
Texas Red	lifetime/QY[16c] position/orientation[15c]	[c]
tC/tC^{O}/tC$_{nitro}$	lifetime/QY[26] position/orientation[26a,27]	[f]

[a] QY: Fluorescence quantum yield; "position/orientation" covers studies of the structure and dynamics of the dye in DNA as studied by fluorescence anisotropy, NMR, MD simulations etc. [b] Suppliers of monomeric dyes (phosphoramidites or other reactive forms): [c] Life Technologies, [d] ATTO-TEC, [e] GE Healthcare, [f] Glen Research, [g] Lumiprobe, [h] Sigma–Aldrich, [i] AAT Bioquest, [j] Azco Biotech, Inc. For their commercial incorporation into nucleic acids see reference list.[28]

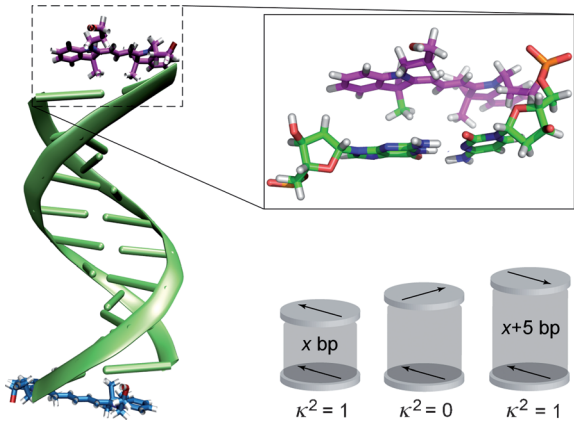


Figure 2. Orientations of end-labelled Cy3 (purple) and Cy5 (blue) in B-DNA. Coordinate file courteously provided by Prof. David Lilley. Bottom right: schematic depiction of the effect of duplex length on the transition dipole moment orientations for three extreme cases of κ^2 (dyes represented as discs). Adapted with permission from ref. [18e], copyright 2011, Biophysical Society.

linker-dependent, as shown by NMR.[18c] Molecular dynamics (MD) simulations, partly supported by lifetime measurements,[18e] suggested that the stacking interaction depends on the identity of the ending base pair.[18f] The effect of end-stacking on the energy transfer efficiency between terminally attached Cy dyes was demonstrated by Iqbal et al.[21] As a result of the constrained transition moments, the FRET efficiency between Cy3 and Cy5 units terminally attached to duplexes of varying lengths (Figure 2, insert) resulted in a fine-structured FRET efficiency as a function of duplex length with a periodicity in phase with the helical periodicities of the B-form DNA and A-form DNA/RNA helices being probed. Upon internal labelling, Cy dyes are believed to bind into and along the minor or major groove.[10d,17a,22]

Recently, the ATTO dyes (trademark of ATTO-TEC, Siegen, Germany) have found increased use in single-molecule FRET experiments, due to their high brightnesses and photostabilities. To date, only a few studies have been directed at characterizing this dye series in nucleic acids.[23] The oxazine ATTO 655 was shown to interact strongly with double-stranded DNA and is transformed into a reduced, quenched state by guanine.[23a,b] Kupstat et al. found that ATTO 610 and ATTO 680 interacted with DNA, however, with the assumption of freely rotating probes giving reasonable agreement between expected and measured FRET efficiencies.[23c]

Under the high irradiation conditions of single-molecule setups, most organic dyes undergo blinking or long-time on/off photoswitching.[29] Although photoswitchable dyes are useful in, for example, super-resolution imaging for determining dye localizations one probe at a time,[30] in FRET experiments blinking can be a serious source of error if interpreted as a FRET state.[29a] Blinking can be greatly reduced by use of additives such as a reducing and oxidising system (ROXS)[29i] or a triplet-state quencher such as Trolox, either added to the solution[29c] or conjugated directly to the dye.[29d] In fact, the mechanism of Trolox as an anti-blinking agent was shown by Tinnefeld and co-workers to be similar to that of a ROXS.[29e] Trolox can also be used in aerobic solution in combination with an oxygen radical scavenger such as cysteamine.[29f] Interestingly, the identities of some long-lived dark states were recently determined for red cyanine dyes[29g] and ATTO 655.[29h]

3. Alternative Probes

Many alternative FRET probes have been developed for various purposes (for a review see Sapsford et al.[31]). FRET between genetically encoded fluorescent proteins is extremely useful for studying protein interactions in vivo,[32] and fluorescent proteins have been coupled covalently to DNA in the pursuit of light-addressable photonic nanodevices.[33] Fluorescent proteins, though, will introduce more unnecessary complexity into quantitative FRET studies than small organic dyes in many in vitro applications. Quantum dots (QDs, semiconductor nanoparticles) offer extreme intensities and stabilities relative to organic fluorophores, and for analytical and imaging purposes the potential of QDs is tremendous (for reviews see, for example, ref. [34]). The main disadvantages of QDs in quantitative FRET are their large sizes (30–40 nm in diameter), the difficulty in coupling only one biomolecule per QD, and limitations in donor–acceptor distances due to the thickness of the QD coating.

Background signals resulting from scattering, direct excitation of acceptor and/or autofluorescence can be greatly reduced by use of long-lifetime donors such as lanthanides and transition metal complexes (see ref. [35] and references there-

in). Besides providing the ability to filter out background fluorescence through time-gated detection of acceptor emission, lanthanide emission is unpolarized, resulting in less uncertainty associated with the orientation factor of the energy transfer process (reviewed by Selvin[35a]). However, reported studies using long-lifetime probes for quantitative nucleic acid studies have been sparse in relation to those involving conventional labels. This is likely a result of limitations in available probes and the difficulty in introducing lanthanide and transition metal complexes site-specifically and noninvasively into nucleic acids.

Recent efforts have been put into the development of dyes that exhibit less uncertainty in position and orientation relative to the nucleic acid.[18k,36] Diffusional mobility is reduced by using short or rigid linkers (Figure 3 A),[18k,36a–e] but this can

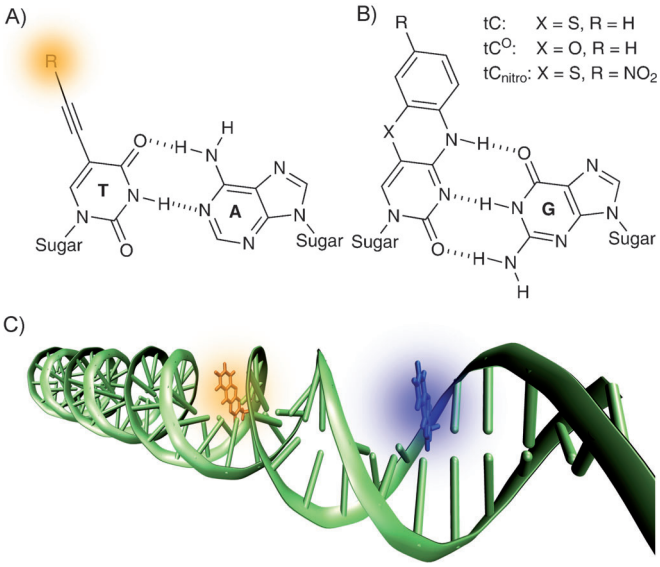


Figure 3. FRET probes designed to display limited diffusional and rotational mobility. A) Fluorophore tethered through a rigid ethynyl linker. B) The tricyclic cytosine base analogue FRET probes. C) The base probes positioned in B-DNA.

be at the expense of limited rotational freedom of the probe.[18k,36a] In some cases, the orientation of the probe can be controlled in a predictable manner.[36f,g] Lewis and co-workers demonstrated control of dye orientation in a series of "end-capped" DNA duplexes in which the donor was rigidly attached to both strands of the duplex while the acceptor was allowed to stack on the ending base-pair.[36f] Ranjit et al. tested probes consisting of Cy3 and Cy5 rigidly attached to the backbone of DNA and found unusually high FRET efficiencies at large donor–acceptor distances, explained by favourable transition dipole moment orientations.[36g]

Our group has developed nucleobase analogue FRET pairs with very high degrees of position and orientation control of both donor and acceptor transition dipole moments (Figure 3 B, C).[26d,27c] To date, the combination of two donors (tC and tC^O) and one acceptor (tC$_{nitro}$) has been reported (base–base FRET), but fluorescent base analogues can also be used in combination with other labels.[37] By mimicking the Watson–Crick hydrogen bonding and base-stacking of the natural bases these probes position themselves inside the nucleic acid structure as a replacement for one of the canonical bases, adapting the position and orientation of the substituted base, usually without perturbing the overall nucleic acid structure (reviewed previously[38]). The main advantage of this approach is the ability to position the reporters inside the site of interest, where it is impossible to use external fluorophores. In addition, the very high degree of control of both position and orientation of the probes allows more detailed structural information to be retrieved from FRET experiments without complications associated with linker flexibility. Furthermore, the photophysical properties of the tC bases are relatively environment-insensitive, allowing better control of quantum yields and spectral overlaps than with most external dye pairs. Despite their great promise, the base probes cannot compete with external labels on overall brightness and large R_0 distances, and still no fluorescent base analogues sufficiently bright and photostable for single-molecule experiments have been reported. In addition, as a result of their highly constrained orientations, which as mentioned above are unique to these FRET pairs, the value of κ^2 cannot be assumed constant (e.g., $\kappa^2 = 2/3$) but must be included in the analysis. Moreover, theoretical calculations have suggested that the ideal dipole approximation, on which the FRET theory is based, in some cases does not fully describe closely separated, constrained donors and acceptors.[39]

4. Measuring FRET Accurately

Aided by the increasing versatility in available probes and technological advances in single-molecule fluorescence spectroscopy (recently reviewed by Hohlbein et al.[40]) the FRET toolbox is continuously expanding, allowing FRET to be used quantitatively for more and more complex biological systems. Single-molecule fluorescence detection is generally performed either with TIRF (total internal reflection fluorescence) microscopy on surface-immobilized dyes or with confocal microscopy on freely diffusing dyes (see Selvin and Ha for a practical description of both methods[5c]). In TIRF smFRET (single-molecule FRET) microscopy, movies of up to hundreds of individual donor–acceptor pairs are recorded simultaneously, and individual intensity time traces are deduced and analysed from these. In diffusion-based smFRET, intensity time traces are recorded from a small confined excitation volume, detecting the short "bursts" of photons emitted from dyes diffusing into the confocal spot. In both strategies the difficulty in measuring quantitative FRET mainly comes from the requirement for corrections of spectral cross-talk, detection efficiencies and relative donor and acceptor fluorescence quantum yields. These parameters are usually obtained by alternating laser excitation (ALEX) for molecules in solution[41] (reviewed by Kapanidis et al.[41a]) or by acceptor photobleaching for surface-immobilized fluorophores.[42] In ALEX, the donor and acceptor are sequentially excited in a rapid, alternating fashion (Figure 4). A two-dimensional histogram of FRET efficiencies and relative donor–acceptor stoichiometries can be made, enabling molecules to be

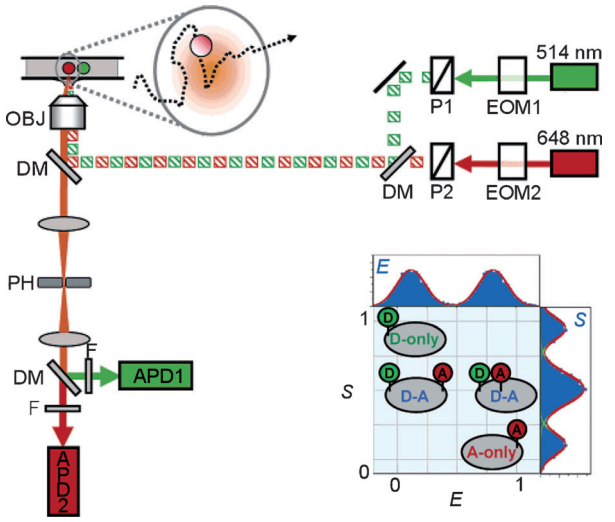


Figure 4. Schematic illustration of setup used in ALEX. Bottom right: resulting two-dimensional histogram of FRET efficiencies (*E*) and relative donor–acceptor stoichiometries (*S*) used to sort molecules. Adapted with permission from ref. [41a]; copyright 2005, American Chemical Society.

sorted into subpopulations of different physical states (Figure 4, insert). In acceptor photobleaching the corrections are obtained by comparing fluorescence intensities before and after destroying the acceptor by photobleaching.[42]

The ability to detect multiple fluorescence parameters at the single-molecule level experimentally offers vast potential in future quantitative FRET.[43] ALEX with nanosecond alternation (nsALEX)[43a]/pulsed interleaved excitation (PIE)[43b] can be used to measure lifetimes, time-resolved fluorescence anisotropies and high-temporal-resolution fluorescence correlation spectroscopy (FCS) at the single-molecule level. Seidel and co-workers have developed single-molecule multiparameter fluorescence detection (MFD)[43c–h] in which all possible experimentally ascertainable fluorescence information (intensity, lifetime and anisotropy) is collected simultaneously by time-resolved, polarized detection in both donor and acceptor emission channels (reviewed by Sisamakis et al.[43c]). In time-resolved measurements, FRET efficiencies can be calculated directly from the donor decay rates without the need for many of the correction factors required in intensity-based measurements.[43g] Recently, MFD was combined with the alternating excitation scheme of PIE, paving the way for accurate smFRET of freely diffusing molecules in a single experiment.[43h] Although single-molecule multiparameter techniques are highly promising, the main disadvantage is their complexity, requiring equipment not available in most microscopy facilities.

The determination of FRET efficiencies from ensemble measurements has been described[44] well both for ensemble spectroscopy[44a,b] and for FRET microscopy (refs. [44c–e] and references therein). Small signal changes are particularly difficult to measure accurately through conventional intensity or lifetime-based measurements, thus decreasing the accuracy when measuring long donor–acceptor distances. Widengren and co-workers recently showed that by monitoring the acceptor triplet state even very low FRET efficiencies (<5%) can be measured accurately, potentially allowing longer distances to be probed with conventional microscopy setups.[45] At very short donor–acceptor separations, on the other hand, measured FRET efficiencies are often systematically lower than expected.[41b,46] Di Fiori and Meller showed that at short separations direct dye–dye interactions can result in a mixture of quenched and unquenched dye states, leading to lower apparent measured FRET efficiencies.[46a] In single-molecule experiments, direct dye–dye interactions can be distinguished from FRET states, leading to more accurate measurements.

For quantitative measurements, a "calibration ruler" with known structure and donor–acceptor separations is valuable as a reference for testing of new FRET-based methods or for studying the influence of photophysical, structural and instrumental factors.[47] The standards employed are usually polyprolines[47a,b] or oligonucleotides[41b,46a,47d] (to reference but a few), both possessing the advantages of being simple and cheap to fabricate and allowing donor–acceptor separations to be varied in a systematic manner. The main disadvantage of polyprolines as model systems, however, is their deviation from ideal rigidity,[47a,b] whereas the quantitative analysis of FRET in oligonucleotides can be complicated by the helicity of dsDNA. Recently, a rigid DNA origami block was demonstrated as a quantitative FRET ruler (Figure 5).[47c] This model system is more complex in some aspects and increases the expense of fabrication relative to regular dsDNA, but it provides an intriguing versatility in structure design and the ability to position the dyes on the same side of the DNA surface.

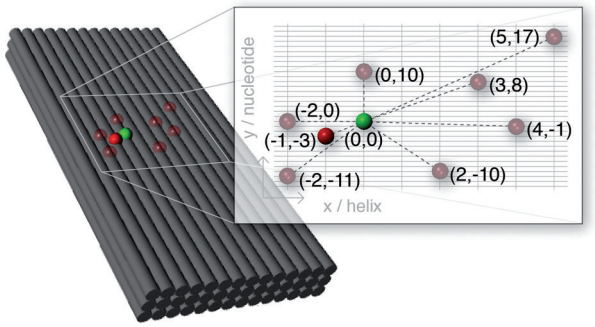


Figure 5. DNA origami calibration ruler. Reprinted with permission from ref. [47c]; copyright 2011, Wiley-VCH.

5. Quantitative Analysis of smFRET

The quantitative analysis of FRET measurements is particularly challenging in single-molecule experiments as a result of low signal-to-noise ratios. The theory and methods used to analyse smFRET data are therefore under continuous development, in studies both of immobilized[48] and of freely diffusing dyes.[48i,49]

The main advantage of using immobilized dyes in smFRET is long observation times of each individual molecule (up to minutes), allowing kinetic rates between discrete states to be extracted from histograms of dwell times in the various states. However, the interpretation of intensity traces into quantitative

distances is difficult, due to shot-noise and the low numbers of photons detected in each time-bin. Addressing this issue, Watkins et al. used smFRET to derive distance distributions by combining a maximum information analysis of the photon traces and a model-free maximum-entropy-based deconvolution of the raw probability distribution histograms.[48a] Landes and co-workers have reported a denoising algorithm for smFRET trajectories of immobilized dyes based on wavelet shrinkage.[48b,c] Backović et al. recently reported a distribution-based method for generating smFRET efficiency histograms from intensity trajectories by use of Bayesian updating.[48d]

Usually, smFRET efficiency histograms are analysed by fitting a sum of weighted Gaussians, from which the average FRET efficiency is extracted. In order also to extract quantitative information, such as structural heterogeneities and molecular dynamics, from the widths and shapes of smFRET histograms, the contribution from shot-noise must be accounted for. Here one must distinguish between the real FRET efficiency and the often used proximity ratio (PR), a convenient but uncorrected measure of FRET calculated as the ratio of the acceptor intensity (I_A) to the total intensity ($I_A + I_D$) upon donor excitation:

$$PR = \frac{I_A}{I_A + I_D} \quad (1)$$

Although the PR provides a relative measure of the degree of FRET it cannot be translated directly into quantitative distances. In diffusion-based measurements, the shot-noise limited PR histogram (PRH)—that is, the histogram broadened only by shot-noise—can be calculated by using the measured burst size distribution and Poisson statistics. Any additional broadening of the PRH beyond shot-noise is then assigned to static and/or dynamic heterogeneities of the system under investigation (Figure 6). This strategy is used in PRH analysis, described by Nir et al.,[49a] and probability distribution analysis (PDA), developed by Seidel and co-workers.[49b–d] PDA has been used to model both multiple static states[49d,e] and dynamics between interconverting states[49c,f] (for a review covering PDA see Sisamakis et al.[43c]), although the model in a given study must be chosen on the basis of prior assumptions. Kapanidis and co-workers showed that dynamics can be distinguished from multiple static states by burst variance analysis (BVA), in which the standard deviation of FRET in each burst is calculated and compared to that expected from shot-noise only.[49g] Nir and co-workers also reported a statistical method based on photon arrival times, in which the internal photon distribution is analysed and used to classify bursts according to various properties of interests, thus acting as a filter to identify subpopulations in FRET and ALEX measurements.[49h] The derivation of distance distributions from diffusion-based smFRET measurements by the method of classic maximum entropy, having the advantage of analysing the data in a model-free manner, has also been demonstrated.[49i]

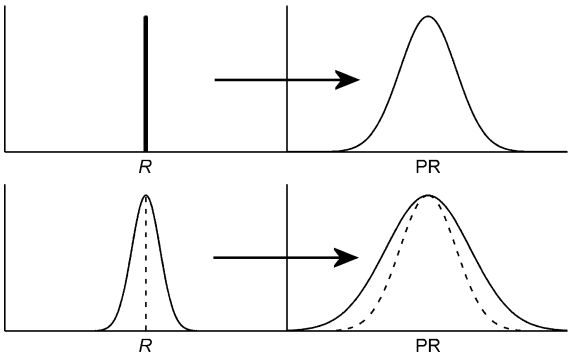


Figure 6. Illustration of the proximity ratio histogram resulting from shot-noise only (top) and shot-noise including additional broadening due to a distribution of D–A distances (bottom).

6. Modelling Probe Dynamics

In order to interpret measured FRET efficiencies into quantitative structural information about the nucleic acid, both the positions and the orientations (including diffusion and reorientation) of the tethered probes relative to the biomolecule must be known or somehow included in the analysis. Although R and κ^2 might in some situations be directly correlated,[50] dye diffusion and reorientation are usually modelled separately for purposes of simplicity. The simplest way of modelling probe diffusion in ensemble measurements is by fitting the donor intensity decay with use of a Gaussian distribution of donor–acceptor distances (referred to as time-resolved FRET).[17d,51] In this case, the width and centre of the fitted distribution provide quantitative measures of dye diffusion and average donor–acceptor distance, respectively. Because the broadening of the distribution results both from tether flexibility and from global nucleic acid dynamics, time-resolved FRET can be used to derive information on the flexibility of structural elements in nucleic acids, in particular if the contribution from the linker is known (a detailed review is provided by Klostermeier and Millar[51a]). The often overlooked power of time-resolved FRET is its ability to reveal trends in the donor decay reflecting molecular dynamics and to extract more than one quantitative molecular parameter from experiments, facilitated by the large number of data points analysed in such experiments.[46b]

Although a distance distribution does not itself give information on rotational dynamics, orientation factor distributions can be implemented in time-resolved FRET in a similar manner for the description of dye reorientation (for a useful discussion see Parkhurst et al.[51b]). Traditionally, the distribution of dye orientations during the energy transfer is described by a simple wobbling-in-cone model, with semi-angles obtained from fluorescence anisotropy measurements (or directly from microscope images[52]), from which upper and lower limits of $\langle\kappa^2\rangle$ can be estimated (Figure 7 A).[51b,53] This model was recently explored for quantitative interpretation of FRET efficiency histograms in single-molecule FRET.[54] In the cone model, the transition moment of the dye is assumed to be equally distributed within a cone. Rindermann et al. recently modelled dye orientation and reorientation by using the Mises–Fisher directional probability distribution (Figure 7 B).[46b] This model describes the direction of the transition moment vector by an axial Gaussian probability density function weighted most heavily at the

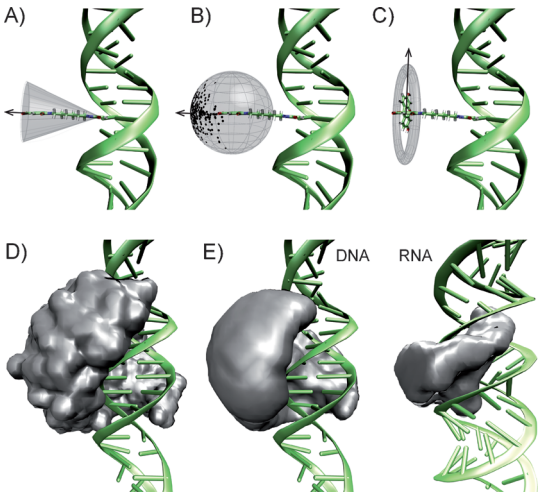


Figure 7. Modelling probe dynamics. A–C) Representing dye reorientation by: A) a wobbling-in-cone model, B) the Mises–Fisher distribution, and C) a distribution-in-disc model. D, E) Illustration of tethered dye diffusion modelled by: D) MD simulation, and E) an accessible volume model. Coordinate files used in (D) and (E) were courteously provided by Prof. Claus A. M. Seidel and Simon Sindbert.[18k]

mean orientation of the dye. Through the use of the direction and distribution width of the transition dipole vectors as fitting parameters, this strategy provided estimates of the three-dimensional positions, orientations and rotational dynamics of the dyes in DNA.[46b] For dyes with transition moments perpendicular to the linker axis—FAM and TAMRA, for example—a distribution-in-disc model might be more physically accurate (Figure 7C).[18k]

The downside of using theoretical distance or orientation distribution models in describing probe diffusion and reorientation is that the inherent assumptions imposed by such models—that the distributions are isotropic and uncorrelated, for example—are nearly impossible to validate in practice. If the local structure of the nucleic acid around the tether is known, the exact positions and orientations of the probes can be predicted by use of MD simulations (Figure 7D).[17a, 18k, 50, 55] MD simulations provide the most detailed insight into probe position, orientation and dynamics by offering an atomic-level view of distance and κ^2 distributions, as well as any correlation between the two. MD simulations, however, are still rather cumbersome and time-consuming for everyday use. Recently, very simple and fast geometric search algorithms have been implemented for the prediction of dye[18k] and spin label[6b, 56] positions in DNA. The accessible volume (AV) of the dye can be determined simply by calculating the volume within the extended linker–dye radius and the point of tether, excluding all positions resulting in a steric clash between the tethered dye and the nucleic acid surface (Figure 7E).[18k] Assuming that all dye positions within the AV are equally populated provides a reasonable representation of the dye, as shown by Sindbert et al. (this recent paper presents a very thorough investigation of fluorophore–linker fluctuations in DNA and RNA[18k]). Alternatively, more customized Metropolis-Monte-Carlo simulations have also been used to model tether flexibility in DNA.[57]

7. Emerging FRET-Based Techniques

An interesting development in quantitative FRET is the use of multiple FRET-based distance measurements to obtain three-dimensional structural information.[55a, 58] By exploitation of partially known geometrical elements of the biomolecular structure, the three-dimensional position of an unknown structural element can be determined accurately by means of triangulation/trilateration.[55a, 58b–j] Triangulation-based FRET techniques exploit the concept of three or more "satellite dyes", positioned in the known part of the structure, in determining the position of one or more "antenna dyes" (Figure 8). Using FRET

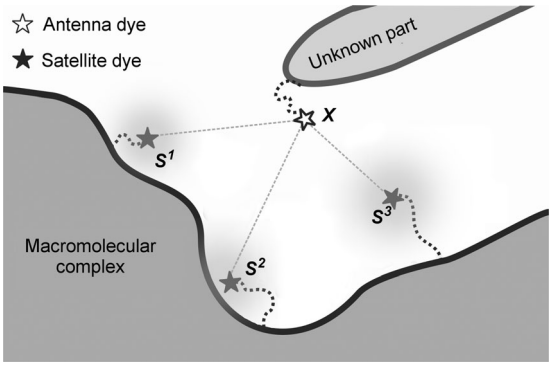


Figure 8. Schematic illustration of antenna and satellite dyes used in FRET triangulation. Adapted with permission from ref. [58b]; copyright 2008, Macmillan Publishers Ltd.

triangulation, Wozniak et al. determined the three-dimensional geometries of adenine bulges of various sizes by combining several donor and acceptor positions across the B-DNA helical arms.[55a] Muschielok et al. have developed a "nano-positioning system" (NPS) for the analysis of data from FRET triangulation measurements with the aid of Bayesian parameter estimation.[58b, c] By accounting for many of the uncertainties in FRET experiments, the NPS provides an informative picture of the information obtainable from the experiment by producing probability distributions of fluorophore positions. Using FRET triangulation and the NPS, Andrecka et al. determined the position of nascent RNA exiting RNA polymerase II,[58d] as well as the positions of the non-template and upstream DNA in the same complex.[58e] More recently, Balci et al. used FRET triangulation and the NPS to probe the position of the ss/dsDNA junction in a helicase–DNA complex,[58f] and Treutlein et al. determined the three-dimensional architecture of an RNA polymerase II open promoter complex.[58j] Using a slightly different approach, Sabir et al. combined several smFRET measurements with MD simulations in determining the global structure of a four-stranded DNA fork[58k] and, more recently, a three-way DNA junction.[58l]

The simultaneous combination of more than one donor–acceptor pair can be exploited for multistep and multicolour FRET (put in a general theoretical framework by Watrob et al.[59]). Through the introduction of mediator dyes in between the donor and acceptor, energy has been transferred

over distances longer than 20 nm by multistep FRET (Figure 9A).[60] The use of such systems as photonic wires,[60a–c] photonic switches[60d–f] or as a trick for long-range FRET measurements[60g] has been suggested. Labelling with more than one donor–acceptor pair (multicolour FRET) can be used to monitor multiple distances simultaneously (Figure 9B).[61] Multicolour FRET can be used in ensemble measurements to limit the number of samples needed[61a–c] but is particularly powerful in single-molecule experiments for the simultaneous monitoring of multiple structural elements in real time without hiding molecular heterogeneity and dynamics behind an ensemble average.[61d,f–i]

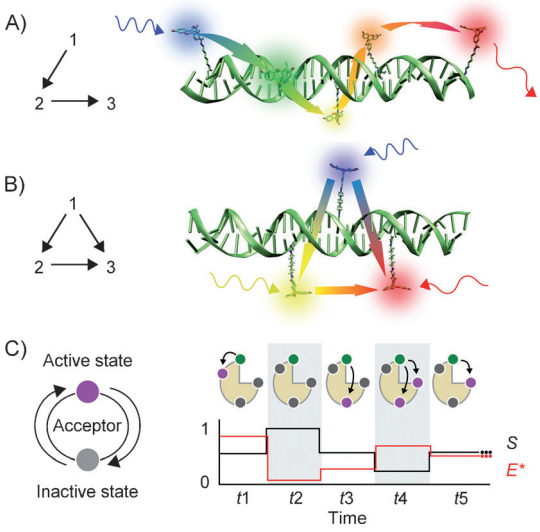


Figure 9. Illustration of: A) multistep FRET, B) multicolor FRET (represented by three-color ALEX[61f]), and C) switchable FRET. *S* is the donor/acceptor stoichiometry and *E** is the apparent FRET efficiency. Part (C) is adapted with permission from ref. [62a]; copyright 2010, Macmillan Publishers Ltd.

The limitation of multicolour FRET is its complexity. After excitation a cascade of events occurs, complicating quantitative data analysis. In addition, multicolour FRET requires several fluorophores that are spectrally separated but have sufficiently large spectral overlaps for FRET. To address these issues, Kapanidis and co-workers reported switchable FRET in which photoswitchable dyes are used to confine individual donor–acceptor pairs temporarily within a multilabelled system.[62] In switchable FRET, single FRET pairs are sequentially activated and deactivated by stochastically switching the acceptor dyes between active and inactive states (Figure 9C). Monitoring the stoichiometry between active donor and acceptor dyes in each biomolecule by ALEX allows individual FRET states to be resolved in real time.[62a]

8. FRET Simulation and Analysis Software

The customized software developed in individual laboratories for the simulation and analysis of FRET is occasionally made publicly available.[63] Table 2 lists a selection of such software.

Table 2. Selected software packages for the simulation and analysis of FRET data.

Name	Capabilities	Free	Link
TwoTone	automated ALEX and TIRF-FRET image analysis[63a]	MATLAB-based	[63b]
CPLC package	construction of smFRET traces and histograms from TIRF-FRET measurements[5d]	MATLAB-based	[63c]
vbFRET	identification of states and kinetic rates from sm-FRET trajectories by HMM and ME[48e]	MATLAB-based	[63f]
HaMMy	determination of kinetic rates from smFRET trajectories by HMM and ML[48f]	yes	[63d]
QuB	determination of kinetic rates from smFRET trajectories by HMM and ML	yes	[63e]
CSM	identification of states from smFRET trajectories by causal state modelling[48g]	MATLAB-based	[63i]
FRETshrink	denoise smFRET trajectories by wavelet shrinkage[48b]	MATLAB-based	[63j]
FretTrace	reconstruction of distance trajectories from smFRET burst trajectories by ML[49k]	yes	[63k]
Tatiana	analysis of smFRET histograms by PDA; part of software for MFD analysis[49b]	no	[63l]
exiFRET	simulation of FRET in complex geometries[63m]	yes	[63n]
FRETmatrix	simulation and analysis of FRET in three-dimensional nucleic acid geometries	MATLAB-based	[63o]
FRETlab	simulation of time-resolved FRET by use of the Mises–Fisher distribution[46b]	IGOR Pro-based	[63p]
FRETsg	translation of multiple FRET distances into three-dimensional positions[58g]	yes	[63q]
FRETnps	simulation and analysis of FRET by the NPS triangulation method[58b]	MATLAB runtime	[63r]

Some freeware packages offer smFRET trajectories and histograms from raw TIRF image data, such as TwoTone from the Kapanidis lab[63b] and the data acquisition and analysis software package from the Ha lab.[5d,63c] The analysis of smFRET trajectories into kinetic rates between different conformational states can be accomplished by hidden Markov modelling (HMM) with a maximum likelihood (ML) optimisation as in HaMMy[48f,63d] and QuB[63e] or a maximum evidence (ME) optimisation as in vbFRET[48e,63f] (smFRET analysis by HMM is reviewed by Blanco and Walter[64]). Software packages for analysing bursts of diffusing molecules are available from a number of commercial suppliers: SymPhoTime from PicoQuant[63g] and Burst Analyzer from Becker & Hickl, for example.[63h] Analysis of MFD data, including the analysis of smFRET histograms by PDA,[49b] can be performed with a package from the Seidel lab.[63l] Corry and co-workers have developed exiFRET, a web-based platform for simulating FRET between probes distributed in various complex geometries.[63m,n] Our own software package, FRETmatrix, provides a general framework for the simulation and analysis of FRET in nucleic acids.[63o] FRETmatrix directly correlates three-dimensional nucleic acid structures with FRET efficiencies and can be used to analyse both nucleic acid structure and dynamics from multiple time-resolved FRET measurements. FRETmatrix is particularly powerful when the positions and orientations of the probes are highly or partly constrained, such as in base-base FRET.

9. Summary and Outlook

We have summarised recent methodological advances in the use of FRET as a quantitative molecular ruler, with particular focus on studies of nucleic acid structure and dynamics. New knowledge on the relative positions, orientations and dynamics of several commercially available dyes attached to DNA should aid the design and interpretation of FRET experiments (Section 2). Alternative FRET probes designed with well-defined positions and orientations within DNA now offer interesting alternatives for more specialized studies such as retrieval of orientation information and site-specific probing of nucleobases (Section 3). FRET can now be measured accurately at the single-molecule level even for diffusing molecules in solution, and this field is pushed further by the development of multiparameter fluorescence techniques capable of monitoring several dye properties simultaneously (Section 4). The quantitative analysis of smFRET signals into donor–acceptor distance distributions has the potential to provide quantitative insight into structural and dynamic heterogeneities of DNA and RNA systems (Section 5), further aided by new methods optimised to model local dipole fluctuations (Section 6). The systematic probing of multiple FRET pair positions in DNA/RNA structures can be used to triangulate the three-dimensional positions of unknown structural elements, whereas multicolour FRET can now be used to monitor two or more distances simultaneously at the single-molecule level (Section 7).

We believe that continuing progress in quantitative FRET-based methods will be greatly catalysed by sharing of the knowledge and specialized software developed in individual laboratories (Section 8). As such, it is our hope that the overview provided here will inspire further development and use of quantitative FRET as a tool complementing more complex, higher-resolution techniques in the study of nucleic acids.

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