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Single-molecule spectroscopic methods

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Being praised for the mere fact of enabling the detection of individual fluorophores a dozen years ago, single-molecule techniques nowadays represent standard methods for the elucidation of the structural rearrangements of biologically relevant macromolecules. Single-molecule-sensitive techniques, such as fluorescence correlation spectroscopy, allow real-time access to a multitude of molecular parameters (e.g. diffusion coefficients, concentration and molecular interactions). As a result of various recent advances, this technique shows promise even for intracellular applications. Fluorescence imaging can reveal the spatial localization of fluorophores on nanometer length scales, whereas fluorescence resonance energy transfer supports a wide range of different applications, including real-time monitoring of conformational rearrangements (as in protein folding). Still in their infancy, single-molecule spectroscopic methods thus provide unprecedented insights into basic molecular mechanisms.

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Abbreviations

FCCS	fluorescence cross-correlation spectroscopy
FCS	fluorescence correlation spectroscopy
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
MPE	multiphoton excitation
TCSPC	time-correlated single photon counting
TIR	total internal reflection

Introduction

Biological macromolecules are inherently heterogeneous, exhibiting different folded states, distinct configurations or varying stages during an enzymatic process. Single-molecule techniques require no synchronization of underlying molecular kinetics. Observing a single member of the ensemble guarantees that only one configuration can be assumed at any given time. From the temporal evolution of the single-molecule signal, the conformational changes underlying elementary processes such as hydrolysis, binding or even enzymatic activity may be determined. With a sufficiently high temporal and spatial resolution, even rare intermediates or subpopulations can be directly probed, which would otherwise be hidden beneath more populous species in the ensemble regime.

Detection of the single fluorescent molecule of interest is hampered by the presence of billions, maybe trillions, of solvent molecules and further impeded by additional noise from the setup. Therefore, highly diluted fluorescently labeled sample solutions, devoid of any fluorescent impurities, must be combined with small probe volumes defined by focusing, spatially confined samples or special excitation techniques. A good signal-to-noise ratio is crucial, which depends largely on the quantum yield and photostability of the selected chromophore in combination with the filter set used to suppress residual excitation light and Raman scattering. Originally limited to 'seeing' immobilized fluorophores at liquid-helium temperatures [1,2], nowadays a vast variety of minimally invasive fluorescence techniques provide useful tools to gather information about the structure and function of biological macromolecules.

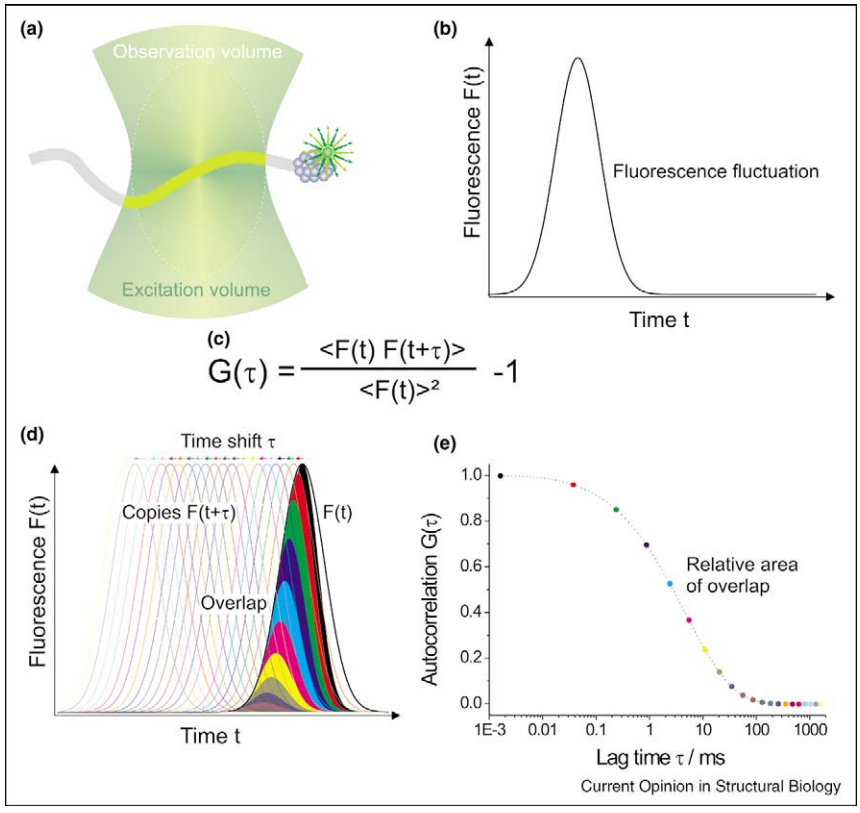
Fluorescence correlation spectroscopy — a single-molecule-sensitive technique with high variability

The biological function of molecules depends not only on their structure, but also on their mobility and dynamic properties, which are strongly influenced by the environment. The single-molecule-based technique fluorescence correlation spectroscopy (FCS) allows inherent averaging over a large number of single-molecule passages through the measurement volume (c.f. Figure 1) and thus is ideally suited to assess molecular movements. The excitation volume is defined by laser light focused by a high numerical aperture objective, as schematically depicted in Figure 2a. At thermal equilibrium, fluorescent molecules diffusing through the illuminated region give rise to minute signal fluctuations [3–7] (Figure 1), which are analyzed by FCS. In principle, the autocorrelation function, $G(\tau)$, is a measure of the self-similarity of the signal after a lag time (τ). It resembles the conditional probability of finding a molecule in the focal volume at a later time, τ, given it was there at $\tau = 0$. This function provides real-time access to a multitude of system parameters, for example, particle concentration or diffusion coefficients [8,9] (Figure 3).

Diffusion studies

An ideal target for FCS is therefore molecular binding and aggregation. The individual diffusion characteristics

Figure 1



Molecules diffusing through the optically defined observation volume **(a)** give rise to tiny fluctuations in the detected signal F(t), as schematically depicted in **(b)**. These can be analyzed by FCS, which, in principle, determines the self-similarity of the signal after a lag time τ. The corresponding mathematical expression is given in **(c)**. Graphically, this is demonstrated in **(d)**; a copy of the observed fluctuation $F(t + \tau)$ is shifted by a lag time τ relative to the original signal F(t). Both are multiplied and the area under the resulting curve (i.e. the overlap integral) gives the value of the autocorrelation for this lag time **(e)**. For short time delays, the (normalized) overlap is large and decreases gradually for larger time shifts. The average residence time of the particle in the focal volume can be determined as the time at which the amplitude has decayed to 50% of its original value.

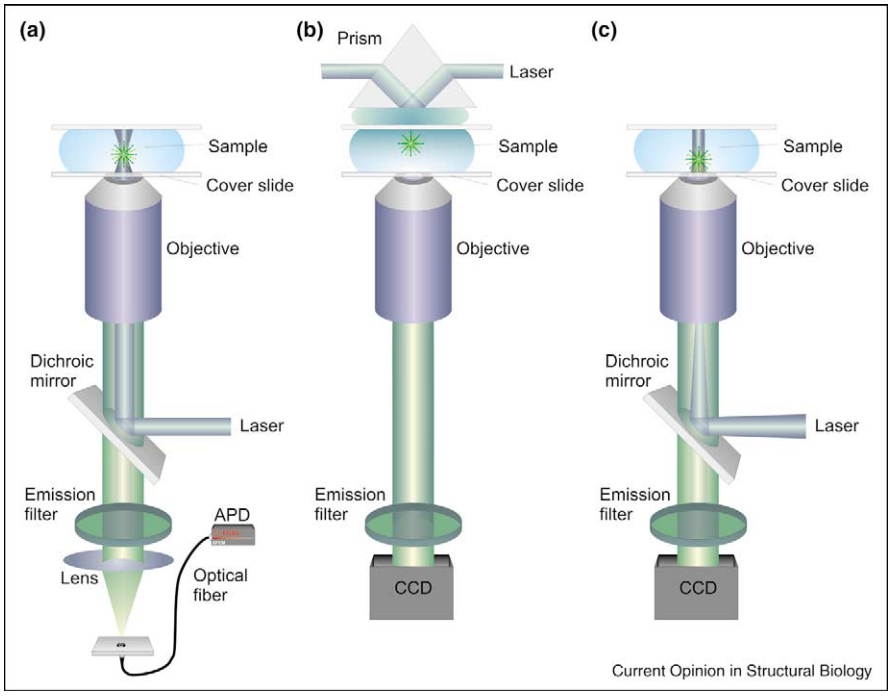
allowed Diez *et al.* [10] to determine the dissociation constant for the F_1b_2 complex of *Escherichia coli* ATP synthase and hence the Gibbs free energy of binding. Even conformational changes can be monitored, provided the diffusion coefficient is sufficiently altered by structural rearrangements, such as the 15% increase seen for the F_1 part of the H^+-ATPase upon nucleotide binding [11]. The polymer-like dynamics and structural flexibility of large double-stranded (ds) and single-stranded (ss) DNA can also be assessed and characterized in this manner [12].

When investigating the aggregation of fluorescently labeled molecules, the resulting clusters are significantly both larger and brighter than the individual molecule; this has been exploited, for example, to investigate ionotropic 5HT3 receptor clustering [13]. However, the sensitivity of autocorrelation analysis is restricted, because the diffusion coefficient depends on the molecule's hydrodynamic radius and thus on the cubic root of the molecular mass for spherical particles.

Cross-correlation

To overcome these limitations for reaction partners of comparable weight (e.g. homodimers), dual-color fluorescence cross-correlation spectroscopy (FCCS) was established. Two differently colored labels are excited by independent lasers and the light emitted in the common measurement volume is split onto two separate detectors by a wavelength-sensitive dichroic mirror [14]. In this case, it is the coincidence of signal fluctuations in each of the channels, indicating concomitant movement of two distinct labels, that is of interest, as depicted in Figure 4 [14,15]. First proclaimed by Eigen and Rigler [16], this concept was experimentally demonstrated by monitoring the annealing kinetics of two differently labeled ssDNA strands [15]. Among the multitude of successful applications *in vitro* are enzymatic cleavage [17] and protein aggregation [18]. Lately, several promising *in vivo* measurements have been reported, including endocytosis of cholera toxin, for which separation of the toxin subunits was confirmed to occur in the Golgi [19•]. However,

Figure 2



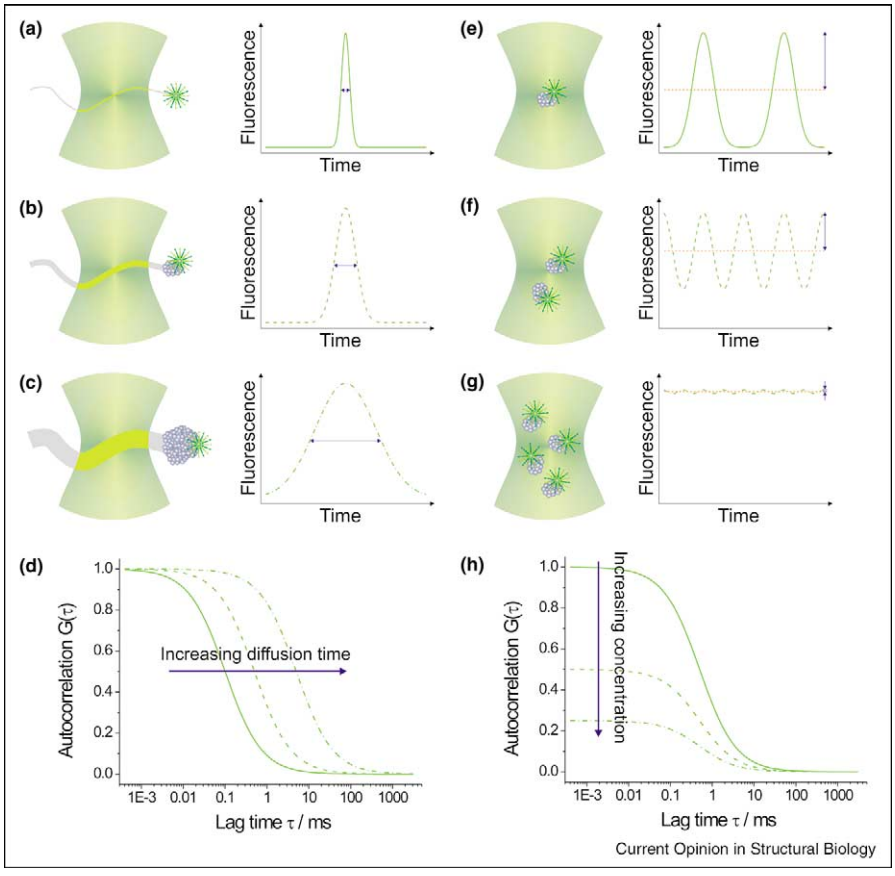
Different illumination techniques used for imaging. **(a)** Confocal scanning microscopy. Except for the scanning unit, this setup is, in principle, the same as that used for FCS measurements. A parallel laser beam is deflected by a dichroic mirror and focused by a high-numerical aperture objective onto a diffraction-limited spot. The fluorescence signal then passes through the dichroic mirror, is focused onto a pinhole (here, the fiber entrance aperture) and finally reaches the (point) detector (i.e. a photomultiplier or an avalanche photodiode [APD]). The excitation volume is confined in the *x–y* plane by focusing; the *z*-resolution in the detection pathway is provided by the aperture, which blocks light emanating from regions not in the immediate vicinity of the focal volume. Sequential scanning in two or even three dimensions is controlled by a computer, which also calculates the final image. **(b)** TIR microscopy using a prism. When light propagating within a dense medium (e.g. the quartz prism) reaches an interface with a less dense medium (e.g. aqueous solution), Snell's law describes how light is refracted or reflected at the interface as a function of the incident angle, depending on the refractive index differences between the glass and water phases. At a specific critical angle, however, the light is totally reflected from the glass–water interface and an evanescent field is generated that extends into the aqueous solution. The penetration depth is typically in the range of half the wavelength of the incident light, thus ensuring high surface selectivity. Only fluorophores located within this thin layer will be imaged by the area detector (i.e. camera). Molecules in bulk solution cannot be excited and therefore do not contribute to the background noise. As the excitation light is totally reflected away from the detector, one can discriminate even weakly fluorescent particles and obtain very high signal-to-noise ratios. **(c)** Epifluorescence microscopy. This is a form of conventional wide-field microscopy. Collimated laser light is directed via a dichroic mirror into the microscope objective, which acts as condenser. As the sample is illuminated by parallel light and there is no additional confinement in *z*, this technique only works for thin samples. The emitted fluorescence passes the dichroic mirror and is then collected either by the eye-piece or by the connected camera, as in transmitted wide-field microscopy.

cellular measurements are prone to suffering from extremely low molecular mobility, causing bleaching artifacts and impeding fluctuation analysis. Scanning dual-color FCCS [20] may help by utilizing temporal and spatial fluorescence fluctuations simultaneously.

Two-photon excitation

Based on the quasi-simultaneous absorption of several long-wavelength photons, multiphoton excitation (MPE) provides intrinsic spatial sectioning due to the higher-order dependence of the fluorescence on the excitation intensity. Fluorescence is no longer observed along the double cone of the focused incident light, but is emitted solely from a small ellipsoidal region in the center, where the intensity is sufficient for efficient excitation. As scattering is reduced for longer wavelengths, it also offers larger penetration depths in biological tissue. When combined with FCS, MPE offers advantages especially beneficial to intracellular measurements [21], such as reduced autofluorescence and less photobleaching in off-focus areas. It also allows excitation of intrinsic tryptophan fluorescence in the visible spectral range. This was used to determine oxygen binding curves for single tarantula hemocyanin molecules and to discriminate among different models describing cooperativity (i.e. distributions of molecular conformations characterized by different oxygen affinities [22,23]). Moreover, for cross-correlation studies, two or more spectrally distinct dyes may be excited by a single laser line in automatically overlapping excitation volumes, thereby significantly

Figure 3



Various parameters can be extracted from the autocorrelation curves. The most prominent are the diffusion coefficient **(a–d)** (and hence the approximate size) and the concentration **(e–h)** of fluorescent molecules. The half-value decay time provides a good estimate of the mean diffusion time (d), from which the diffusion coefficient can be determined. With increasing mass, the residence time of the molecules in the focal volume is increasing (a–c) and thus the corresponding autocorrelation curves shift to longer diffusion times (d). The amplitude, G(0), is proportional to the inverse particle number and thus to the concentration of the fluorescent particles (e–h). An increase in particle number leads to an increase in average fluorescence (e–g), but the signal change caused by one specific molecule entering or leaving the observation volume becomes less pronounced. This is illustrated in (e,f) by the maximal deviation (arrows) of the idealized fluorescence signal from the mean value (straight line). The autocorrelation amplitude, however, is a measure of fluctuation strength and thus decreases for higher concentrations (h).

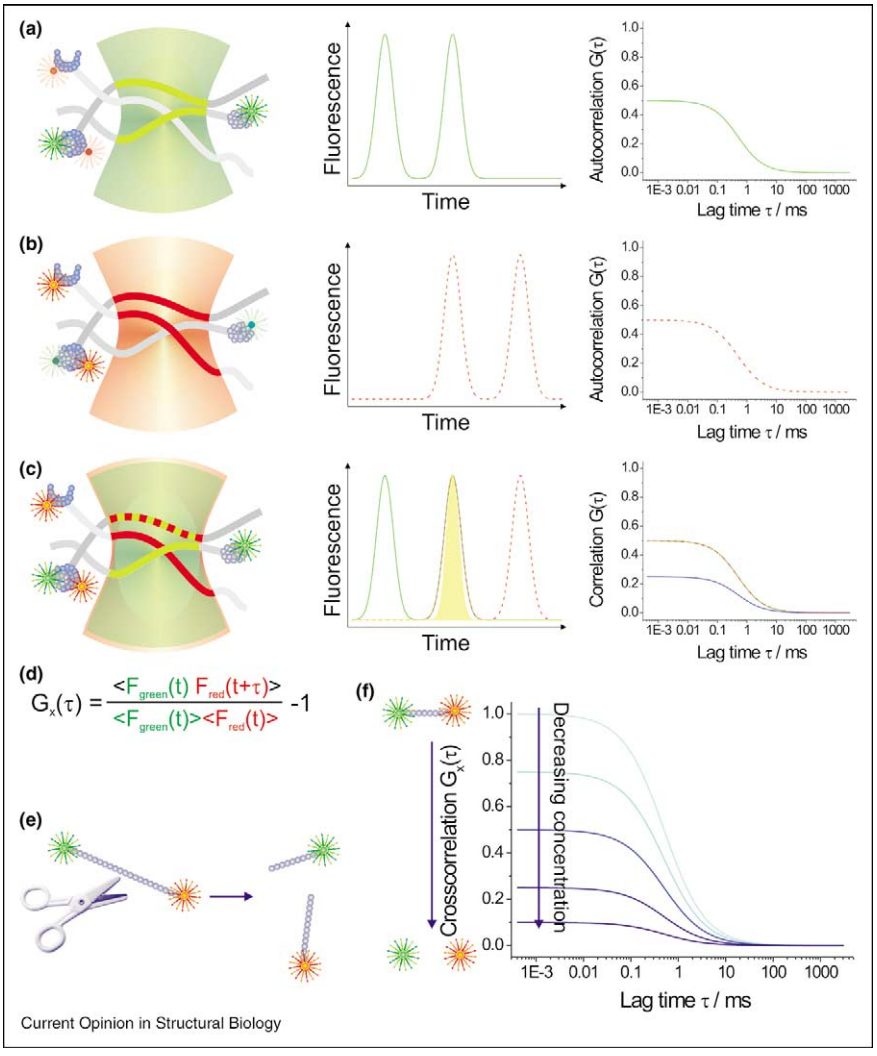
simplifying optical alignment [24]. This versatile technique can be extended also to autofluorescent proteins, as shown for the proteolytic cleavage of a fully cloneable GFP–peptide–DsRed construct (DsRed is a red fluorescent protein from *Discosoma* coral) [25]. *In vivo*, the advantages mentioned above were demonstrated for the binding of calmodulin to Ca^{2+}/CaM-dependent protein kinase II [26] and the oligomerization of G-protein-coupled receptors in CHO cells [27].

Further developments

To enhance temporal resolution in two-color applications, reduce sampling times and thus render the technique suitable for high-throughput screening, coincidence analysis can be employed. By discarding all diffusion-related information, the cross-correlation amplitudes can be calculated much faster [28,29]. This allows real-time monitoring of enzymatic digestion or even cofactor binding in signaling cascades. Only recently, this concept has been extended to the simultaneous excitation of three spectrally separable chromophores by two-photon excitation [30•]. This allows the pairwise interactions of the three reaction partners and the ternary complex to be monitored, and might finally lead to direct measurement of Michaelis–Menten kinetics in real time.

Traditional FCS, however, is rather insensitive to the brightness of the molecules under investigation. A promising extension, termed PAID (photon-arrival-time interval distribution), has just been presented [31•] that measures brightness, coincidence, concentration and diffusion time simultaneously. With this technique, the

Figure 4



Principles of dual-color cross-correlation. The fluctuations in two spectral regions [e.g. green (a) and red (b)] are recorded in separate channels. Taken separately, they contain only the information about **(a)** the green or **(b)** the red labeled species. Combining both single-channel setups in a two-channel apparatus, the two partially independent signals can be compared, looking for common features **(c,d)**. As before, both autocorrelations can be determined and contain information about the chromophores detected in the individual channels, irrespective of a second label. The cross-correlation, however, depends only on the particles carrying both labels. The yellow area in (c) indicates the overlap of the distinct channels for $\tau = 0$; only a double-labeled particle gives rise to simultaneous fluctuations in both channels. The mathematical formalism (d) is extended by using both the green $F_{green}(t)$ and the red $F_{red}(t)$ signal either for the original F(t) or for the shifted copy F(t+τ). For stationary samples, both forward ($G_{green–red}$) and backward ($G_{red–green}$) cross-correlation give the same result. As the cross-correlation amplitude is directly proportional to the number of double-labeled molecules **(f)**, it is ideally suited to monitoring association and dissociation reactions **(e)**.

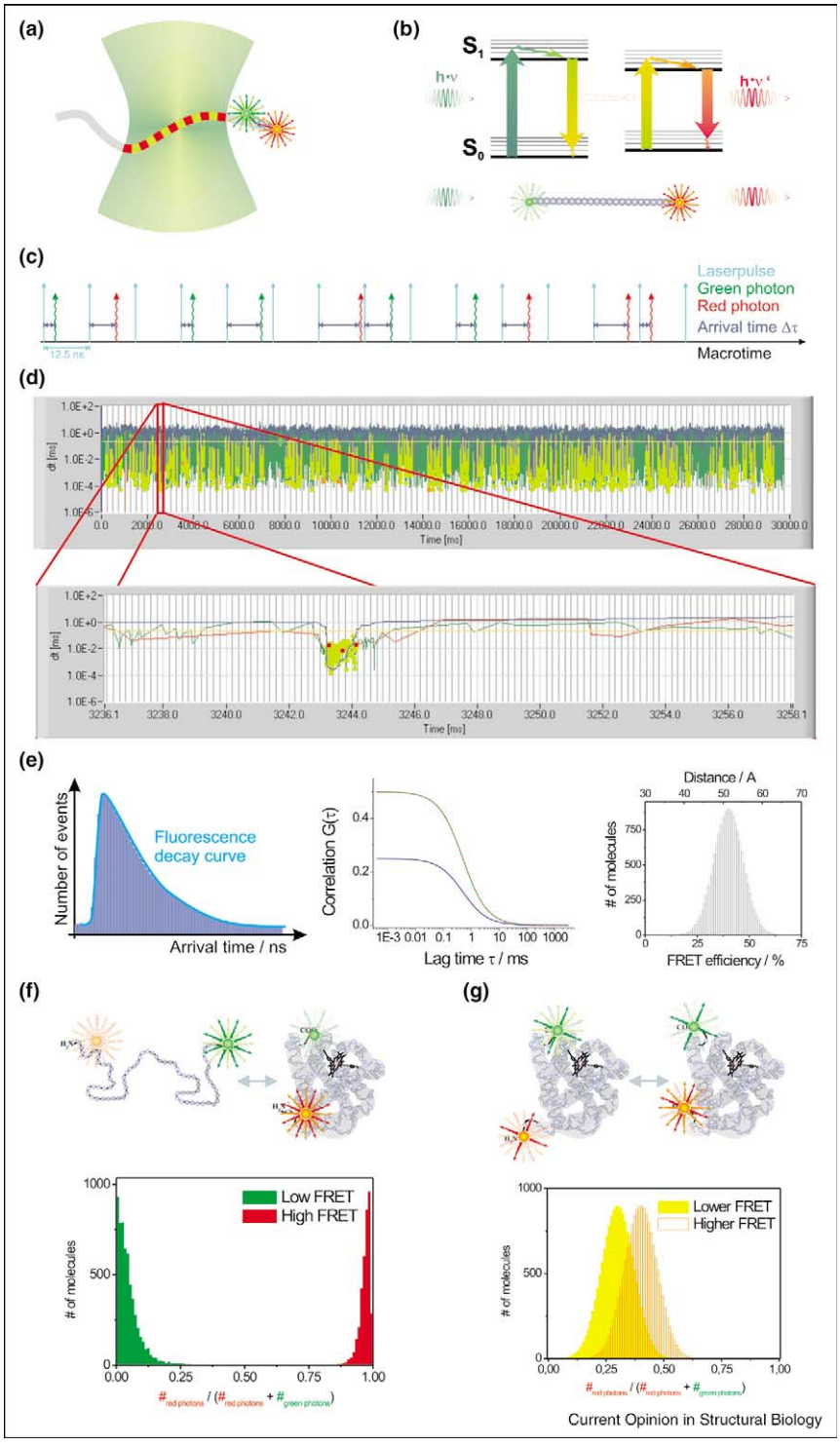
authors successfully distinguished DNA carrying one, two identical or two different labels [31•].

Single-molecule imaging

The fluctuation techniques mentioned above are completely at a loss if precise spatial localization is required. Moreover, single-molecule imaging techniques also allow each molecule to be monitored for as long as possible before photobleaching. Confocal laser scanning methods give three-dimensional images, although with a low temporal resolution (Figure 2a). For single-molecule imaging, the wide-field methods of epifluorescence (Figure 2c) and total internal reflection (TIR) microscopy (Figure 2b) are therefore generally preferred.

Using total internal epifluorescence microscopy (TIRF), Yildiz *et al.* managed to locate a single fluorescently tagged molecule to within 1.5 nm. Monitoring the step size during the translocation of the molecular motors myosin V and kinesin, they favored a hand-over-hand model of motility compared to an inchworm model [32••,33] for both molecules. For myosin V, this is

Figure 5



For FRET measurements, the donor molecule is excited with a single laser line **(a)**. If the chromophores are sufficiently close, the energy is transferred non-radiatively to the acceptor, which then emits a red fluorescence photon. The corresponding schema is depicted in **(b)**. TCSPC uses the full information from every photon. In addition to the arrival time of the photon, the time relative to the last laser pulse and the detection channel are also stored **(c)**. In **(d)**, a real data trace can be seen, with single-molecule passages highlighted in blue. The arrival times for the individual photons detected during these 'bursts' are marked by bright green (green channel) or red (red channel) dots. Single-molecule passages through the focal volume can be determined via the sudden increase in intensity (i.e. the number of photons counted per unit time interval, not shown) or via differences in photon-arrival times between successive photons (dt) (d). Photons arrive at much closer intervals at the detector if an emitter resides in the focal volume. These single-molecule bursts are isolated from the trace data via an upper limit for the arrival time

corroborated by fluorescence polarization studies monitoring the tilting motion of the myosin light chain during force generation [34•].

The observation of single molecules in living cells is a special challenge, mainly because of the cellular autofluorescence originating from natural cellular fluorophores such as flavins, NADH and so on, which are present at high concentrations in the cell. Thus, great care is mandatory in selecting proper (long-wavelength) fluorophores and appropriate filters, assisted by growing the cells in fluorophore-free media. Nevertheless, the limited number of photons detected per molecule is compensated by the enormous advantage of probing biologically relevant macromolecules *in situ* with minimal perturbation. A summary of current achievements is given in [35,36]. Using epifluorescence, the signaling from H-Ras to cRaf-1 was visualized [37] and, by monitoring H-Ras only, domains in the cytoplasmic leaflet of the cell membrane were discovered [38].

Fluorescence resonance energy transfer for single molecules

Of all dual-color applications, fluorescence resonance energy transfer (FRET) has elevated fluorescence colocalization experiments to a new level of specificity, providing a direct measure of proximity on molecular length scales.

In a typical FRET experiment, a biological macromolecule is labeled with a donor and an acceptor fluorophore at two different positions. Upon excitation of the donor, energy is transferred non-radiatively via induced dipole–dipole interaction [39] to the acceptor; most of the detected photons originate from the acceptor (Figure 5a,b). Ligand binding or internal motions can cause distance changes, which are related to fluctuations in relative acceptor intensity. As the FRET efficiency is inversely proportional to the sixth power of the distance between the two chromophores, this serves as a spectroscopic ruler for measuring molecular distances [40] between 10 and 100 Å. Hence, single-pair FRET (spFRET) can report on binding events and conformational changes of biological molecules in real time that are often difficult to observe in conventional ensemble studies [41].

There are two basic approaches to record single-molecule FRET. Whereas immobilized molecules may be observed for longer times, enabling the study of multiple reaction cycles, their proximity to a surface may induce artifacts. In solution, a molecule is only visible while it is diffusing through the focal volume. Without adhesion artifacts, both techniques should give the same result for an ergodic system, in which the ensemble average over a physical quantity at a given time yields the same information as the time average over the same quantity for one particular particle.

Folding

Large changes in the FRET efficiency are observed in binding or folding studies, where, in principle, the system under investigation is changing from a no-FRET state to a high-FRET state (Figure 5f). Nevertheless, protein folding is inherently a heterogeneous process because of the multitude of unfolded conformations leading to the same native structure. Even for a 255 base pair ribozyme, different folding intermediates displaying partially non-ergodic behavior emerge with increasing Mg^{2+} concentration [42]. Using small cold-shock proteins (CspTm) and a chemical denaturant, the height of the free-energy barrier to folding was estimated [43•,44].

Chaperonins facilitate the (re-)folding of newly translated or stress-damaged proteins. With its central nano-cavity capped by GroES, GroEL provides a safe folding cage for the non-native protein. In contrast to the current doctrine, the active cycle was found, using single-molecule imaging and FRET, to consist of two major steps: binding of the free cofactors and the actual folding event, immediately followed by product release [45•].

However, when working with surface-bound molecules, great care must be taken to avoid adhesion artifacts. The folding of RNase H, for example, depends strongly on the coating of the glass substrate it is tethered to [46].

Conformational changes

In the absence of diffusion, the main fluorescence fluctuations arise from conformation-dependent FRET. To determine these transition rates, such as those of the Mg^{2+}-facilitated conformational change of an RNA three-helix junction [47] or dsDNA looping by NgoMIV restriction endonuclease [48], correlation functions can also be employed. In particular, the structural dynamics of all kinds of oligonucleotides, such as Holliday and four-way RNA junctions [49], and ssDNA flexibility [50] have been studied extensively.

Unraveling functionally relevant conformational changes in proteins is highly complex, mainly because of the

(Figure 5 Legend Continued) (yellow horizontal line in d). From these raw data, various physical parameters can be determined; **(e)** for example, by histogramming the arrival times you get a fluorescence decay curve and hence the fluorescence life-time of the molecule (e, left). It is still possible to correlate the intensities calculated from the raw data (e, center). Moreover, for each single-molecule passage, the relative intensity or the relative number of detected photons in the individual channels can be determined. Hence, finally the FRET efficiency and thus the distances between the donor and acceptor chromophore can be calculated (e, right). In folding-unfolding transitions, a large difference in the FRET efficiencies can be expected for the two distinct states **(f)**, whereas conformational rearrangements give rise to only small changes **(g)**.

rather small changes in relative distance involved (Figure 5g). Besides careful selection of a chromophore pair, this also necessitates exactly defined environmental conditions to avoid artifacts induced by slight changes in temperature, buffer conditions, illumination power and so on. Therefore, only recently have studies involving protein conformations been reported, for example, determining the orientation of an *E. coli* Rep helicase monomer bound to a labeled 3′-(ss/ds) DNA junction in solution [51]. In the absence of crystal structures, FRET between different locations on nanometer-scale complexes can assist in roughly defining three-dimensional structures, as impressively performed for the bacterial RNA polymerase holoenzyme and the bacterial RNA polymerase–promotor open complex [52].

One of the major caveats is dye-induced artifacts. Changes in their relative orientation and mobility can influence the FRET efficiency and are easily misinterpreted as distance changes. To circumvent these pitfalls of single-molecule FRET, fluorescence life-times of the donor and acceptor dyes, and their steady-state and time-resolved anisotropies can be simultaneously determined by time-correlated single photon counting (TCSPC) for each molecule diffusing through the focal volume (Figure 5). With sophisticated analysis schemes that utilize all available information, distance distributions can be deduced quantitatively and discriminated against potential dye artifacts, such as quenching or spectral jumps. Thus, three structurally distinct HIV-1 reverse transcriptase–DNA complexes were revealed that are yet to be observed by X-ray crystallography [53]. In the same manner, the authors found that free syntaxin switches between two distinct configurations [54••] and monitored the rotation of the γ-subunit of the F_0F_1-ATP synthase [55••]. With a total of 14 independent measurement parameters per single fluorophore, Prummer *et al.* [56] recently extended this scheme even to nanosecond spectral dynamics.

Concluding remarks

After the first proof-of-principle measurements more than a decade ago, single-molecule methods have taken the life sciences by storm and meanwhile form a universally applicable class of standard technique. Based on considerable achievements in physics and (bio-)chemistry, the detection and analysis of single molecules have been extended to ultrasensitive FRET measurements *in vitro* and even *in vivo*. We can now catch glimpses of the inner life of cells, and visualize and characterize protein dynamics even on the single-molecule level. Thereby, the application of FCS to the intracellular milieu may still hold enormous potential in facilitating observations due to the intrinsic averaging.

Acknowledgements

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- •• of outstanding interest

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