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Photophysics of Fluorescence Probes for Single Molecule Biophysics and Super-Resolution Imaging

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Abstract

Single-molecule fluorescence spectroscopy and super-resolution microscopy are important elements of the ongoing technical revolution to reveal biochemical and cellular processes in unprecedented clarity and precision. Demands placed on the photophysical properties of the fluorophores are stringent and drive the choice of appropriate probes. Such fluorophores are not simple light bulbs of certain color and brightness but instead have their own ‘personalities’ regarding spectroscopic parameters, redox properties, size and water solubility, photostability and several more. Here, we review the photophysics of fluorescent probes, both organic fluorophores and fluorescent proteins, used in applications such as particle tracking, single molecule FRET, stoichiometry determination, and super-resolution imaging. Of particular interest is the thiol-induced blinking of Cy5, a curse for single molecule biophysical studies which was later overcome using Trolox through reducing/oxidizing system, but a boon for super-resolution imaging due to the controllable photoswitching. Understanding photophysics is critical in design and interpreting single molecule experiments.

Keywords

FRET; single particle tracking; single molecule stoichiometry; triplet state; redox blinking; photochromic blinking

INTRODUCTION

The ability to manipulate and monitor single biological molecules in vitro and in the cell is revolutionizing modern biological inquiry, allowing us to look at elementary biochemical reactions with unprecedented precision and clarity and to obtain vivid cellular images at resolutions that are an order of magnitude better than what is possible using conventional light microscopy(1). Started as a novel pursuit of physical scientists, detection of single fluorophores rapidly moved into the main stream applications in biological sciences(2), ranging from studies of central dogma enzymes(3–5) and membrane processes (6) and to the various processes involved in infectious diseases and cancer. High quality and information-rich data can be obtained in vitro using purified and well-defined molecular components and novel insights are emerging from observing single molecule processes in living cells. Most recently, single molecule pull-down approaches are making it possible to analyze the

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composition of cellular protein complexes(7) and to perform single molecule biochemical analysis of the pull-down complexes *in situ*(7, 8), bridging the gap between *in vitro* and *in vivo* contexts. It is realistic to expect that in a few years, most biological publications may include a figure or a panel of a figure that is based on single-molecule data.

Because native biological molecules generally are not optically detectable at the single molecule level, a probe needed to be attached to the biomolecule for fluorescent detection. These probes ideally need to be bright and long-lasting (i.e. slow photobleaching) in addition to being specifically linkable to a biomolecule of interest. For single molecule biophysics applications, the probe also needs to emit light steadily because fluorescence intermittence can hinder continuous observation of biological processes. However, fluorescence intermittence is a very useful feature for single molecule-based super-resolution imaging(9). In this review, we focus on photophysical properties of organic fluorophores (10) and fluorescent proteins (11) that are widely used for single-molecule biophysics and super-resolution imaging.

Single molecule biophysics using fluorescence

What can a fluorophore tell us about the biomolecule to which it is attached? First, it tells us that the biomolecule exists and also where it is. This alone can be very useful because binding and dissociation of an enzyme to and from a substrate, for example, can be directly observed if the enzyme is labeled fluorescently. As a corollary, the copy number of molecules in a complex can be determined by following the photobleaching steps or by comparing the total intensity of a single complex to the intensity of a single fluorophore. Also, diffusional movements as well as directional movements powered by molecular motors can be followed with nanometer precision by localizing the fluorophore in the laboratory frame. Multicolor versions of this reporter function can be used to follow colocalization of different proteins in the same complex and to follow the consequence of an encounter between two proteins, for example on DNA.

Second, dipole-dipole interaction between two adjacent fluorophores, typically of different colors called donor and acceptor, results in energy transfer from the donor to the acceptor in a process called fluorescence resonance energy transfer (FRET) (12, 13). Because FRET is a very sensitive function of distance, FRET at the single molecule level (smFRET) can be used to report on the intramolecular conformational changes of a biomolecule and intermolecular interactions between two or more molecules(14).

Third, the polarized excitation and emission of a single fluorophore can be used to follow its rotational dynamics which can report on the local environment changes and on the orientational changes of the host molecule to which the fluorophore is attached(15).

Fourth, the fluorescence lifetime in the nanosecond range and fluorescence intensity can also report on the local environment, for example protein proximity, which can cause enhancement or reduction in fluorescence lifetime and intensity.

Super-resolution imaging using switchable probes

The principle of single-molecule based super-resolution fluorescence imaging is very simple (Figure 1). All it requires is that only a small fraction of fluorophores in the sample are fluorescently active at a given time. For example, if there are many fluorophores, N of them, in a diffraction-limited imaging volume, their internal organization cannot be deduced based on conventional imaging alone because of limited resolution, about half the wavelength of the light used. However, if only one of the N fluorophores is active, its pixelated image on a camera can be fit using a two-dimensional Gaussian function to determine the center position with a precision that roughly scales with the wavelength divided by the square root

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of the number of photons detected (16). In the extreme cases, the center position could be determined to 1.5 nm accuracy(17) and most recently to a sub-nm level(18). By iteratively repeating the process through other molecules in the same excitation volume, either waiting for them to turn on spontaneously or through photo-activation, their positions can be precisely determined down to about 10–20 nm (19–21)(Figure 1).

In one scheme, all fluorophores are initially active and exciting them with intense laser light can turn them all off. And subsequent lower intensity excitation with a laser of the same or different wavelength can activate a small subset only (Figure 1a) for localization. In some cases, thermal, spontaneous activation can replace photo-activation. In another scheme, all fluorophores start in the inactive form and are then converted in small numbers to the active form via photo-excitation (Figure 1b). It is also possible to control the fraction of active fluorophores using chemical compounds in solution in combination with photo-excitation (Figure 1c). In all of the scenarios above, the ultimate resolution is determined by the probe density (22) and the lowest active fraction possible. For example, if there are only 25 molecules spread over $(0.3\ \mu m)^2$ area, one cannot achieve resolution better than 60 nm $(=\mathrm{sqrt}((0.3\ \mu m)^2/25))$. Even if there are 2,500 molecules in the same area, potentially enabling 6 nm resolution, if more than 1 % of the molecules are active at a given time, one can rarely have only one photoactive molecule in the same area, making it very difficult to determine the center positions of individual molecules.

Length scales of single molecule fluorescence methods

Single molecule fluorescence detection can provide information on length scales ranging from an Å all the way to the size of the cell, which can be ~100 μm. (1). Direct contact between fluorophores can cause electron transfer, for example in H-aggregates and in rhodamine dimers(23). (2). Local viscosity change due to proximity to proteins can show fluorescence changes in 1 to 3 nm range(24). (3). FRET reports on distances between 3 nm and 8 nm, (4). Particle tracking can go from sub-nm to hundreds of μm and (5). Super-resolution imaging tools can image cellular objects down to 10 nm spatial resolution. In all cases, the precision with which the distance or position information is obtained is determined by photon flux, i.e. how many photons are detected per second, which in turn determines the time resolution. Therefore, photophysical properties such as how quickly a single fluorophore can be recycled between the ground state and the excited state, and how high the fluorescence quantum yield is are the important considerations in evaluating single molecule fluorescence probes.

PHOTOPHYSICS OF ORGANIC FLUOROPHORE

Overview

For many biophysical applications where single fluorophore imaging is used to detect the presence and whereabouts of a biomolecule, an ideal fluorophore should be a robust “light bulb” that emits steadily independent of its environment. In other applications, specific properties of fluorophores allow them to report on the local environment such as pH, binding, or flexibility. Another approach is FRET where the colocalization of two differently labeled molecules is indicated by the occurrence of FRET and the efficiency of FRET reports on the distance between a donor and an acceptor fluorophore(14).

The necessity to be mindful of the photophysics of single organic fluorophores is illustrated in smFRET data in Figure 2. High intensity of the acceptor (red) indicates a high-FRET state whereas high donor intensity (green) indicates a low FRET state. Both sets of donor and acceptor intensity time trajectories show anti-correlated fluctuations, indicating FRET fluctuations on the time scale of hundreds of milliseconds. Interestingly, the origin of these two fluctuations is fundamentally different. In Figure 2a, the FRET fluctuations indicate

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conformational transitions of a DNA Holliday junction that is labeled by Cy3 and Cy5 on different branches (25). Figure 2b, however, shows FRET fluctuations of a simple double-stranded DNA labeled with ATTO647N as donor and ATTO680 as acceptor. In this case, the low FRET state is induced by transient one-electron reduction of the acceptor yielding a radical anion that does not absorb in the visible range. Therefore, the acceptor is in a photophysical off-state, giving rise to the low FRET state which can be mistakenly attributed to biomolecular activity.

In this section we describe the photophysical properties of organic fluorophores and discuss the sources of intensity fluctuations. In later sections we extend our photophysical discussion including mechanisms that additionally influence applications such as stoichiometry determination, smFRET, and super-resolution microscopy.

Organic fluorophores exhibit a conjugated π-electron system for absorption in the visible or near-visible. Due to photobleaching and background fluorescence issues, almost all single molecule studies have used fluorophores that absorb above 450 nm. These fluorophores can be classified into groups such as rhodamines, cyanines, oxazine, bodipy and perylenes many of which were originally developed for applications in photography or dye lasers. To make them compatible with biomolecular applications they were further developed by adding functional groups for labeling (NHS, maleimide, hydrazide), increasing water solubility through charged substituents such as sulfonic or carboxylic acids and increasing photostability by blocking reactive positions and sterically reducing chromophore reactivity by the introduction of bulky groups. Most recently, the palette of fluorophores was further expanded especially in the longer wavelengths region, i.e. the near-infrared, which is advantageous for many applications because of much reduced light scattering and fluorescent impurities.

Two main problems for single-molecule biophysical studies using fluorescence have been photobleaching and undesired intensity fluctuations. The two problems are related in some cases, e.g. when a long-lived dark state such as the triplet state is an intermediate toward photobleaching reaction (26, 27). In fluorescence microscopy, there has been a long quest to solve the photobleaching problem and a multitude of anti-fading agents have been tested (see e.g. (28–31). Since the pioneering work of Yanagida (31), β-mercaptoethanol has been the most popular choice as triplet state quencher and it has the added benefit of functioning as radical scavenger. The empirical search continued and only in recent years, a converging and comprehensive picture of the underlying mechanisms has emerged along with the methods to dramatically improve the quality of single-molecule fluorescence data.

A fluorophore is said to 'blink' when its intensity repeatedly drops to zero and then comes back to normal. In contrast, photobleaching is an irreversible switching-off event. Blinking appears universal for all fluorophores exposed to the elements, the only exceptions being emitters that are well-shielded from fluctuations in the local environment such as nitrogen vacancy centers in diamond and specially engineered quantum dots(32). Common causes of blinking include triplet blinking, redox blinking, and photochromic blinking as are described below. We also discuss widely used methods to slow photobleaching in the following.

Photobleaching and oxygen removal

Molecular oxygen is primarily responsible for photobleaching, either through direct interaction with a fluorophore in one of the long-lived excited states or indirectly by producing free radicals in solution. Therefore, it is essential to remove molecular oxygen for achieving long observation times. The concentration of oxygen in buffers at ambient conditions is ~0.5 mM. Because it is difficult to efficiently remove oxygen by physical means, enzymatic oxygen scavenging systems are most widely used. For example, a

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combination of glucose, glucose oxidase, and catalase (33) can reduce the oxygen concentration to a few micromolar, lengthening the photobleaching time by several orders of magnitude for some fluorophores. An alternative system consisting of protocatechuic acid and protocatechuate-3,4-dioxygenase can reduce the oxygen concentration further (34) and does not induce pH drop over time in contrast to the glucose oxidase-based system (35), but in our hands, it tends to act more slowly.

Triplet blinking

Fluorescence photons are emitted as a fluorophore cycles between the single excited state and the ground state. Occasionally, about once every thousand excitations, the molecule visits the triplet state. Because the lifetime of the triplet state is at least 1,000 times longer, the molecule stops emitting until it returns to the ground state. This would result in on-off switching in single-molecule fluorescence intensity but because the lifetime of the triplet state is typically a few microseconds in aqueous solution due to efficient quenching by molecular oxygen, such on-off transitions are invisible (Figure 3a). If molecular oxygen is removed from solution to increase the photobleaching lifetimes, the triplet state lifetime increases to milliseconds (36–38) so that distinct on and off transitions can be seen in single molecule time trajectories (Figure 3b). Because a blinking light source is undesirable for most applications, one therefore needs an alternative way to quench the triplet state, which is not molecular oxygen. β-mercaptoethanol has long been used for this purpose but the triplet state is not as effectively quenched so that the time trajectories still show significant triplet-state blinking, especially at high time resolution (Figure 3c). We empirically found that Trolox, a vitamin E analog, is much better in quenching the triplet state (Figure 3d), and combination of oxygen scavenger system and Trolox have been powerful in extending the observation time without introducing any side effect (39). Another problem of a lengthened triplet state lifetime upon oxygen removal is that the emission intensity saturates to a much lower level compared to the case of short triplet state lifetime, and Trolox helps mitigate this problem (Figure 3e). We will discuss the detailed mechanism on how Trolox and other additives function below.

Redox blinking

Photo-induced electron transfer is one mechanism for blinking because the resulting radical ions are generally non-fluorescent. For molecules in excited states both oxidation and reduction are possible. First, the electron in the singly-occupied molecular orbital is removed more easily so that the triplet state has a lower oxidation potential than the ground state. Second, the highest occupied molecular orbital is singly-occupied so that also the electron affinity is increased (higher reduction potential than ground state). Consequently, collisions of a fluorophore in an excited state with electron donors or acceptors can easily lead to reduction or oxidation, yielding radical ions. Due to the longer lifetime of the triplet state photo-induced electron transfer is much more likely to originate from the triplet state than from the first excited singlet state. Dependent on the concentration of the oxidant or reductant such electron transfer processes can depopulate the triplet state so fast that the triplet state is efficiently quenched and cannot be detected. Observed dark states can then be ascribed to reduced or oxidized states (-> redox blinking).

Meanwhile, it is accepted that most off-states longer than several milliseconds are not related to triplet states but have other origins such as specific photochemistry(40, 41) or redox blinking (27, 37, 42, 43). Interestingly, for single molecules in solution, redox blinking can easily be induced by the presence of oxidants such as oxygen or methylviologen or by reductants such as ascorbic acid or ferrocene derivatives. Depending on the conditions and the redox potentials of the fluorophores, radical ions can have almost infinite lifetimes (44).

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Photochromic Blinking and Photochemistry

Photochromism refers to the reversible photo-induced transformation between two chemical species and appears as blinking in single-molecule trajectories. Such switching was observed for single fluorescent proteins already in the nineties (45). In the context of material science, other single-molecule switches were developed that consisted of the combination of a fluorophore with a photochromic chromophore (46) where the two communicate via FRET. The first ordinary organic fluorophore that could be switched at the single molecule level was Cy5. Upon photo-excitation, Cy5 reacts with thiol in solution and turns dark (39, 41, 47). The dark state has a very long lifetime, at least a minute long, and can return to the emitting state spontaneously. This recovery can be enhanced by excitation below <540 nm and can be dramatically enhanced if an excited Cy3 molecule is nearby (20, 41). The distance required for such an effect is smaller than typical FRET distance (20). In recent years, switching and induced blinking of Cy5 and other photo-switchable or photo-activatable organic fluorophores such as rhodamine derivatives and azide functionalized fluorogens (48–50) have become key ingredients for super-resolution imaging.

Stable fluorescence by the reducing and oxidizing system

Photo-stabilization has always been concerned with the quenching of triplet states and the scavenging of radicals and photo-oxidized states (formed e.g. through multiple excitations). Therefore, a large number of potentially useful reagents have been phenomenologically screened to reduce blinking and improve photostability. Among the typical reagents were thiols such as β-mercaptoethanol, β-mercaptoehylamine as well as l-glutathione, propyl galate, ascorbic acid, and diazabicyclo[2,2,2]octane (27, 51, 52).

A unifying model on how reducing and oxidizing agents can help recycle the fluorophore rapidly from the triplet state was recently presented (27). As discussed above, triplet states can be depopulated using single-molecule electron transfer reactions yielding redox blinking. The idea to achieve stable fluorophores is to also rapidly depopulate the resulting radical ions using the complementary redox reaction (see Figure 4). If, for example, a triplet state reacts with a reductant such as Trolox, a radical anion is formed. The lifetime of this radical anion strongly depends on the availability for a second, reverse electron transfer reaction to return to the ground state. The rate of this process can be massively enhanced by adding an oxidizing agent. The working mechanism of this stabilizing scheme is depicted in Figure 4. Upon removal of oxygen, blinking due to long-lived triplet states is observed. In the presence of only a reductant, blinking is observed with a lifetime of 27 ms for ATTO647N due to the formation of radical anions. In the presence of an oxidant only, blinking is observed due to the formation of radical cations. All blinking beyond shot-noise is removed when both the reductant and the oxidant are simultaneously present because each species quickly reacts with either the oxidant or the reductant until the ground state of the fluorophores is reached. Typically, upper micromolar to lower millimolar concentrations of the redox agents are required and we generally avoid higher concentrations that may quench the singlet states or may influence the biological processes being observed.

Among many choices of redox agents (53), we avoid too strong reductants and oxidants which may react directly with each other. The combination of ascorbic acid and methyl viologen works well with ease of handling. However, methyl viologen is toxic and positively charged and might thus interfere with biological function. Interestingly, Trolox is similarly efficient in blinking and photobleaching suppression even in the absence of separately added oxidant (27, 39). It turns out that (54) Trolox partly degrades upon dissolving in buffer forming a quinone-derivative(54). Therefore, Trolox itself acts as a reductant while the Trolox-quinone fulfills the role of the oxidant. Trolox-quinone formation

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can be controlled e.g. by UV-light and a simple absorption measurement helps to determine the fraction of quinone formed (54).

Because this photo-stabilization scheme relies on diffusion of small additives in solution, processes that occur on faster time scales cannot be efficiently controlled. When photobleaching occurs directly through multi-photon processes or through subsequent absorption of photons from the first excited singlet state leading e.g. to rearrangements, the reducing and oxidizing scheme cannot intervene. Evidence that such direct photobleaching pathways are also significant comes from experiments in which red fluorophores such as Cy5 were additionally excited with blue light. Simultaneous pulsed excitation, lead to increased photobleaching whereas alternating laser excitation had negligible effect indicating successive absorption of two photons in the singlet manifold leading to photobleaching (55–57). The reducing and oxidizing system works more efficiently for fluorophores with lower excitation energy, with some exceptions(58), probably because the higher energy photons of blue light are more capable of inducing photobleaching through higher excited states.

Protein-induced fluorescence enhancement

Cyanine fluorophores have an elongated structure with a polymethylene chain connecting two aromatic groups. Due to the twisting around this bond (called cis-trans isomerization) which results in non-radiative decay, the quantum yield of Cy3 by itself is generally low, below 10%. However, if the viscosity of the solution is increased (59), the twisting reaction is hindered, deactivating the non-radiative decay pathway, resulting in increased emission (and quantum yield) and an extended fluorescence lifetime. Likewise, when these fluorophores are conjugated to nucleic acids or proteins, the quantum yield increases to above 20%, likely due to an increase in local viscosity. Now if the fluorophore is attached to a strand of DNA and a protein binds nearby, an additional increase in viscosity may occur leading to fluorescence increase. This protein-induced fluorescence enhancement was first used for ensemble biophysical studies where a helicase moving directionally on single stranded DNA was detected via Cy3 enhancement (60). More recently, several single-molecule studies exploited this effect to study the dynamics of proteins that function on DNA or RNA (59, 61–63). Distance calibration using a DNA ladder and restriction enzymes showed that the effect falls off rapidly (less than 3 nm), demonstrating that it may probe events that are not accessible to FRET (24). In addition, there is no need to label the protein in this scheme, greatly simplifying sample preparation. A related approach is to use the fluorescence quenching induced by iron sulfur cluster of an XPD helicase (64).

PHOTOPHYSICS OF FLUORESCENT PROTEINS AND QUANTUM DOTS

Fluorescent proteins

Fluorescent proteins are small 25–30 kDa proteins that can be attached to essentially any protein of interest as genetically encoded fluorescence marker. The prototypical green fluorescent protein (GFP) consist of 238 amino-acid that folds into a rigid 11-sheet beta barrel with a helix in the center. The fluorescent chromophore 4-(p-hydroxybenzyliden)-5-imidazolinone forms autocatalytically from a tripeptide (Ser65-Tyr66-Gly67 for GFP) (11). The advantages of these labels have rapidly led to applications in single-molecule biophysics and fluorescent proteins have gained further popularity with the development of single-molecule based super-resolution schemes (19) (21).

Just a few years after the wide dissemination of GFP, the Moerner group demonstrated single-molecule fluorescence detection of GFP and YFP (yellow fluorescent protein) (45) and found that these molecules blink, showing intermittence in emission intensity . A number of sources of blinking (or flickering) has been identified for fluorescent proteins on

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essentially all time scales from nanoseconds to minutes. Besides common mechanisms such as triplet states and radical states (65–67), many fluorescent protein derivatives exhibit further fluctuations due to proton transfer and conformational dynamics involving different states such as cis-trans isomerization of the chromophore (68–70). Interestingly, the chromophore can be fluorescent in both the cis and the trans conformations as long as the protein scaffold keeps the chromophore in a planar conformation. In contrast, the isolated chromophore is non-fluorescent. Hence, the rigidity of chromophore fixation in the protein pocket is a measure of the complexity of photochemical states of a specific FP derivative. For some derivatives it could be shown that fluctuations can be significantly frozen when the fluorescent protein (here eqFP611) is immobilized on bare glass, indicating the role of the protein rigidity for the chromophore's photophysics (71). In general, the intensity fluctuations and the limited photostability (typically one tenth of the photostability of organic fluorophores) of fluorescent proteins hamper their applicability in protein tracking and stoichiometry determination. Attempts to use additives have not yet yielded positive effects although it was shown that redding is related to redox processes (67). But, useful for many other applications is the fact that some proteins can be switched reversibly ON and OFF and some others can be converted between two spectrally distinct states. Both types of switching are mechanistically related to cis-trans isomerization and the structures of the isomers, that is their planarity is the decisive factor whether an isomer is fluorescent or not. The Moerner group also discovered a long-lived dark state of YFP and showed that excitation at ~400 nm can bring the molecule back to the emitting state (45). Such photoswitching of YFP was subsequently used for super-resolution imaging of bacterial proteins in living cells (72).

The CFP/YFP pair has long served as the most effective cellular FRET reporter and the first single molecule application on calcium dependent reporter demonstrated the expected conformational changes with calcium. However, it was difficult to follow the conformational changes reliably due to the blinking of the probes and extremely poor photostability of CFP (73). We also found CFP to be a poor single-molecule probe in part due to its 'blueness' where excitation at ~400 nm generates high fluorescence background from solution and sample surface.

Quantum dots

Quantum dots, also known as nanocrystals, are made of semiconducting materials, and brighter and longer-lasting than organic fluorophores(74). They also have emission spectra that are narrower than those of organic fluorophores, allowing easier multiplexing. However, quantum dots are large, at least as big as fluorescent proteins but in general much larger once they are made soluble and bio-conjugatable through surface modifications and coating. They are therefore in general unsuitable for FRET applications (75), but are ideal for particle tracking for example on the cell surface (76) or on DNA (77). Blinking of quantum dot has been noted for many years and could be effectively suppressed with thiols in solution (78). Recently, a method was developed to synthesize a quantum dot that does not blink at all (32) but a water-soluble version has not been reported yet.

EFFECTS OF PHOTOPHYSICS ON SINGLE MOLECULE APPLICATIONS

Ideal fluorophores for single molecule fluorescence studies have to be 1) photostable, 2) bright (high extinction coefficient and quantum yield of emission), 3) showing little intensity fluctuation, at least in the time scale of interesting biological events under study, 4) excitable and emitting in the visible wavelength, 4) relatively small so that they introduce minimum perturbation to the host molecule and 5) commercially available in a form that can be conjugated to biomolecules. FRET poses additional photophysical demands on the probes.

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FRET

An ideal pair of fluorophores for smFRET studies would have 1) large spectral separation to minimize the donor emission leakage into the spectral range of acceptor emission and to reduce the amount of direct excitation of the acceptor by the laser and 2) comparable emission quantum yield for donor and acceptor. The latter is useful because it guarantees clearly anti-correlated intensity changes of donor and acceptor when FRET changes occur. In addition, fluorophores that can undergo substantial spectral shifts by themselves are not suitable because such a change can cause fluctuations in the apparent FRET efficiency even in the absence of any conformational changes of the host molecule. For example, ATTO647N and Alexa488 display substantial spectral shifts, complicating FRET analysis, an effect largely absent for Cy3 and Cy5.

Cy3/Cy5 has arguably been the most popular donor and acceptor pair for smFRET because 1) their spectral separation is large (~100nm), 2) they are both photostable in oxygen-free environment, 3) the quantum yields (~0.2) are comparable, and 4) they are commercially available in amino-, thiol- and carbonyl-reactive forms. When Cy5 is used as the acceptor, even when other assays indicate that the donor is very close to Cy5 ($R \ll R_0$) with 1:1 stoichiometry, there is a fraction of donor/acceptor complex that shows only donor emission. This is presumably due to an inactive Cy5, likely due to pre-bleaching, and accounts for 15% – 55% of total population depending on the batch of Cy5 used. Repeated freezing and thawing of the sample also increases this so-called “donor-only” fraction. The issue can be overcome altogether by using alternating excitations of the donor and the acceptor so that only the molecules or time periods with the active acceptor are analyzed (79). In addition to the inactive fraction of Cy5 (80), the Cy3/Cy5 pair has other potential issues: (80)(i) both fluorophores have relatively low quantum yields, (iii) Cy3 shows strongly environment-dependent fluorescence (10, 63, 81), (iv) both fluorophores exhibit cis-trans isomerization complicating autocorrelation analysis (41, 82) and (v) Cy5 exhibits the above-mentioned photochemistry with thiols (20, 41, 47). An alternative FRET pair of Alexa488/Cy5 has a greater spectral separation with minimal crosstalk and essentially no direct acceptor excitation at 488 nm (83, 84). In protein folding the Alexa488/Alexa594 pair has been frequently used (85–88) despite problems with spectrally varying donors (89). A very bright FRET-pair (possibly the brightest available) is Cy3B/ATTO647N that has been used in several smFRET experiments (90–94). This specific pair can be problematic if a narrow FRET distribution is desired for quantitative distance measurements because ATTO647N shows several distinct emissive states with different emission spectra (27)(unpublished results).

The question arises why the Cy3/Cy5 pair is still widely and successfully used and not completely replaced by other brighter fluorophore pairs. It will often depend on the specific application and an important parameter for the selection is whether the fluorophores come in physical contact in the course of the experiment as described in the next section.

A frequently encountered problem with many FRET pairs occurs when the donor and acceptor come in close proximity. Fluorophores tend to form complexes and non-fluorescent aggregates when orbital interactions occur. Recently, Di Fiori et al studied the interaction of FRET pairs along double-stranded DNA for different FRET pairs including Cy3 and TMR as donors and ATTO647N and Cy5 as acceptors (95).

Below a separation of 8 basepairs corresponding to ~3 nm, the fluorophores can physically interact resulting in simultaneous quenching of the donor and acceptor fluorescence signals to various intermediate levels. A characteristic set of time trajectories for the TMR/ATTO647N pair at 6 bp distance is depicted in Figure 5. Interestingly, such a behavior with unexpected FRET values and quenching is rather the typical case than an exception. For the

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permutations of the four fluorophores used in this study, only the Cy3/Cy5 pair exhibited <10% molecules showing such FRET fluctuations while for the other FRET pairs the fraction with intensity fluctuations was >75% (see Figure 5). This tendency might be related to the better water solubility of the Cy3/Cy5 pair (95) and show the advantage of Cy3/Cy5 pair in giving reliable signals even at short distances. It is interesting to note that the direct contact between Cy3 and Cy5 was even necessary for the FRET pair to work well in thiol-containing buffer. The thiol induced blinking described above had long plagued smFRET applications using Cy5 as the acceptor because Cy5 blinking mimics a zero FRET state. The short range re-activation of Cy5 by an excited Cy3 explains why many smFRET studies were still possible using β-mercaptoethanol as additive. In conclusion, the Cy3/Cy5 pair is the FRET pair of choice when physical contact between donor and acceptor might occur.

Quenching between interacting fluorophores can also be advantageously used. Cordes et al. recently sensed DNA opening in transcription by the occurrence of a FRET population that was quenched due to the physical contact of Cy3B and ATTO647N before the transcription bubble was formed (93). Complex formation between fluorophores can involve significant binding energy, for example about 19 kJ mol^{-1} for two ATTO655 molecules (96), which may alter the energy landscape of the biomolecule.

FRET can provide distance constraints that can yield detailed structural information when used in combination with complementary information from other techniques or by measuring multiple FRET-pairs in different positions (97–101). smFRET measurements can be considerably more accurate than ensemble measurements because populations can be sorted and separately be analyzed. The sorting ability is important because complex biomolecules are difficult to produce or maintain without considerable sample heterogeneity either of the biomolecules themselves or due to imperfect labeling or inactive fluorophore populations. Besides the average FRET-value reporting on the average distance between the donor and acceptor, the width of the FRET-distribution reports on possible biomolecular dynamics. Interestingly, such FRET histograms are commonly broader than expected from shot-noise considerations even for simple stiff model systems such as double-stranded DNA (86). Photophysics plays a key role in broadening, including variations in spectrum or quantum yield (83, 90) (84).

Blinking can also induce broadening of smFRET histograms. Consider an acceptor in the triplet state. It is sometimes assumed that during this period the donor emits as in the absence of an acceptor. This is, however, often not the case because the triplet state of the acceptor is also able to absorb and in many cases the absorption spectrum of the triplet state displays considerable spectral overlap with donor emission. Hence, the acceptor in the triplet manifold can still act as acceptor. Since the spin of the acceptor is not changing upon absorption, energy transfer to triplet states (as well as to excited singlet states) is Förster-allowed and is referred to as singlet-triplet annihilation (and as singlet-singlet annihilation, respectively). For perylene imide, the Förster radii of the three possible energy transfer processes have been determined (102). The Förster radius for singlet-triplet annihilation was largest and that for homo energy transfer was smallest with the radius for singlet-singlet annihilation in between. In single-molecule time trajectories, FRET to the triplet states appears as “collective blinking”, that is both fluorophores are quenched because the acceptor in the triplet state thermalizes the energy after acting as FRET acceptor (42, 102). Similarly, Cy5 in the triplet state acts as FRET acceptor similar to Cy5 in the ground state as was shown for the TMR/Cy5 pair (103). The situation is different for other dark states such as radical states. For the radical anion of ATTO655 or ATTO680, there is no detectable spectral overlap with donors such as Cy3B or ATTO647N (104). Accordingly, the fluorescence of the donor resumes completely upon photo-reduction of ATTO680 (see “photophysics” fluctuations in Figure 2). This has recently been exploited for the design of

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an "exciton blockade" that exhibits a superlinear fluorescence dependence of the excitation intensity allowing an increase in resolution of confocal microscopes (104).

For Cy5 another form of intensity fluctuations has to be considered, that is cis-trans isomerization. The cis state has a similar absorption spectrum as the trans state and isomerization is photo-induced in both directions. Therefore, a photodynamic equilibrium between the states is reached already at moderate excitation intensities so that the fraction of Cy5 in the cis-state remains quite constant (in contrast to the transition rate) (82); (105). Since cis-Cy5 also is FRET acceptor similar to trans-Cy5, the measured FRET is not strongly affected and the effect is partly accounted for by a reduced quantum yield.

Single molecule stoichiometry

A hallmark of single fluorophore detection, although not without some exceptions, is a digital, single step decrease in fluorescence signal to the background level upon photobleaching. An ensemble of fluorophores would instead show a gradual decrease in fluorescence signal. One can therefore use the number of photobleaching steps to deduce the number of fluorophores in one fluorescent spot. This phenomenon can be used as a way to determine the stoichiometry of a protein complex if the protein is labeled with a known stoichiometry. For example, a trimer would show three photobleaching steps (Figure 6). In reality, it is difficult to obtain 100% labeling efficiency, and a simple calculation shows that only 1.5% of hexameric protein complexes would show six photobleaching steps if the labeling efficiency is 50%. In such cases, binomial distribution is used to deduce the protein stoichiometry in vitro (106, 107), in vivo (108), and from protein complexes directly pulled down from cell extracts (7). Also if the fluorophore has multiple levels of brightness as in the case of TMR attached to DNA (109), or if the labeling is non-specific, the task becomes more challenging. Although GFP and YFP do blink they generally do not show multiple levels of fluorescence. In contrast, the red fluorescent proteins are often found to have inferior photophysical properties when compared to GFP variants (69, 110, 111), making it difficult to perform stoichimometric analysis of two different proteins in the same complex.

Super-resolution imaging

For organic photo-switchable probes, schemes a and b in Figure 1 are most widely used for super-resolution imaging. Photo-activation can be achieved by directly exciting the inactivated fluorophore using the laser or via collision with a nearby fluorophore of a different color that is excited with a separate laser (112, 113). For Cy5 and Alexa647, fluorophore inactivation requires a photo-induced reaction with a thiol-containing compound (41, 47). Cy5.5 also displays similar photo-switching but its spontaneous reactivation is too frequent for high density cellular imaging(114).

Currently, there are three ways of using the blinking of organic fluorophores for super-resolution imaging. The first report used Cy3 and Cy5 in close proximity. After Cy5 was switched off in the presence of thiols it could be recovered with minimal green light because the activation was enhanced by an excited Cy3 nearby (112). Second, Cy3 could be omitted since Cy5 can also be activated using higher intensity of blue/green light (113), bypassing the need to label antibodies with Cy3/Cy5 in close proximity, and became the method of choice for single-color imaging. The combination of different activator fluorophores and reporter fluorophores for the activator/reporter pair system, however, enables straightforward multicolor imaging (114). Thousands of photons detected per on state allow high localization accuracy compared to FP-based imaging but the necessity of oxygen removal to avoid rapid Cy5 photobleaching can be problematic for certain live cell imaging applications.

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Third, super-resolution imaging has also been accomplished with ordinary organic fluorophores exploiting long-lived generic dark states (scheme c in Figure 1) (38, 115, 116). In most cases the dark states can be ascribed to radical ion states that can have essentially infinite lifetime dependent on the redox properties of the fluorophore and the buffer (44, 117). In vitro, the conditions can be adapted so that a large number of fluorophores can be used for this simple kind of super-resolution microscopy that only requires a single excitation wavelength. It has also been demonstrated how tuning of the on- and off-times by changing the environmental conditions determines the achievable resolution(118). TMR and ATTO655 that have been incorporated by recently developed labeling protocols fulfill the requirements for super-resolution imaging in living nuclei (119, 120).

The second main class of fluorophores for single-molecule based super-resolution imaging are fluorescent proteins (FPs) exploiting the photochromic mechanisms presented above. In the original work, irreversible activation of photoactivatable GFP derivatives, which we call photo-conversion, was employed (19). By irradiation with 400 nm light the backbone is cleaved leading to an additional double bond that extends the π-conjugation and shifts the emission to the red. Recently presented versions of FPs show more switching cycles, brighter fluorescence at longer wavelengths and different combinations of wavelengths that switch on, off and that are used for readout (70, 121, 122). FPs that are switched between two fluorescent variants are often advantageous since they allow visualizing the structures of interest before starting the super-resolution imaging by activating the other conformation (121). In addition, a variant termed mIrisFP that can be both photo-converted and photo-switched has been presented, allowing a combination of pulse-chase imaging with super-resolution (123).

The movements of molecules often aggravate super-resolution imaging in living cells. In many instances it is more meaningful to activate subpopulations and to track molecules in cells to visualize the activity, function and interactions. For such applications high photostability and brightness as well as red-shifted emission are of particular importance. Since the localization of single activated molecules is accurate and activation can be repeated this represents an elegant combination of super-resolution and single-particle tracking (22, 124, 125).

FINAL THOUGHTS

Now that we have methods to photo-stabilize organic fluorophores by eliminating various non-emitting states, what might be the highest photon flux one can achieve? Assuming that a triplet state is visited once in one thousand excitations and that the shortest it takes to bring the fluorophore to the ground state is 100 ns, the emission will almost achieve the saturating rate limited by cycling the fluorophore between the singlet ground and excited states, which is a rate of 2 to $5*10^8$ s^{-1}. If we further assume that the emission quantum yield is 0.5 and 10% of emitted photons are detected, the photon flux could exceed 10^7 detected photons per second. Since about 100 photons are needed for medium accuracy FRET determination, this would mean that the ultimate time resolution for smFRET analysis is about 10 μs. This has not be realized experimentally yet, but it is interesting to note that this is the time scale that is becoming accessible to all atom molecular dynamic simulations(126). In the future, merging of time scales accessible to single molecule fluorescence measurements and computational simulations may allow direct comparison.

A major strength of single molecule methods is that multiple observables may be followed and correlated with each other in a single molecule, which can be most easily achievable in multi-color fluorescence. Whereas this has been achieved in smFRET (up to four colors) (127, 128), particle tracking(129) and super-resolution imaging(114), we are not aware of

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reliable way of determining absolute stoichiometry of protein complexes in multiple colors due to the inferior photophysical properties of available red fluorescence proteins. For the future, more stable FP with such functionalities are required in order to improve the imaging quality, to extend to longer wavelength for background reduction and multiplexing, and to better combine imaging with tracking (22, 130). In addition to an anticipated push for better FPs, we may also imagine that stoichiometric labeling of minimally modified cellular proteins with organic fluorophores may become possible(131). Another method that currently lacks the 'second color' is protein-induced fluorescence enhancement.

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Acronyms

FRET	Fluorescence (Förster) resonance energy transfer
FP	Fluorescent protein
GFP	Green fluorescent protein
smFRET	FRET at the single molecule level

Key Terms

Fluorophore	A molecule that emits fluorescence photons after being excited
Fluorescence Lifetime	An average period of time a fluorophore spends in an excited state before undergoing relaxation
Oxygen Scavenger System	Generally composed of glucose oxidase and catalase which converts oxygen and glucose molecules into gluconic acids

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SUMMARY POINTS

1. There have been conceptual advances in our understanding of photophysics of organic fluorophores relevant to single molecule applications.
2. Photobleaching can be dramatically reduced by removing molecular oxygen enzymatically.
3. Oxygen removal causes triplet state blinking which can be overcome with reducing/oxidizing system.
4. Thiol-induced Cy5 switching-off could be overcome by using alternative quenching of the triplet state, for exampling using Trolox.
5. Thiol-induced Cy5 switching-off and its re-activation underlies the organic fluorophore based super-resolution imaging.
6. Photo-conversion and photo-switching of fluorescent proteins mainly involve changes in the planarity of the chromophore.
7. Developments of fluorescent proteins for the ‘second color’ in stoichiometric determination are needed.
8. Understanding photophysics of your probes is essential in proper design and interpretation of experiments.

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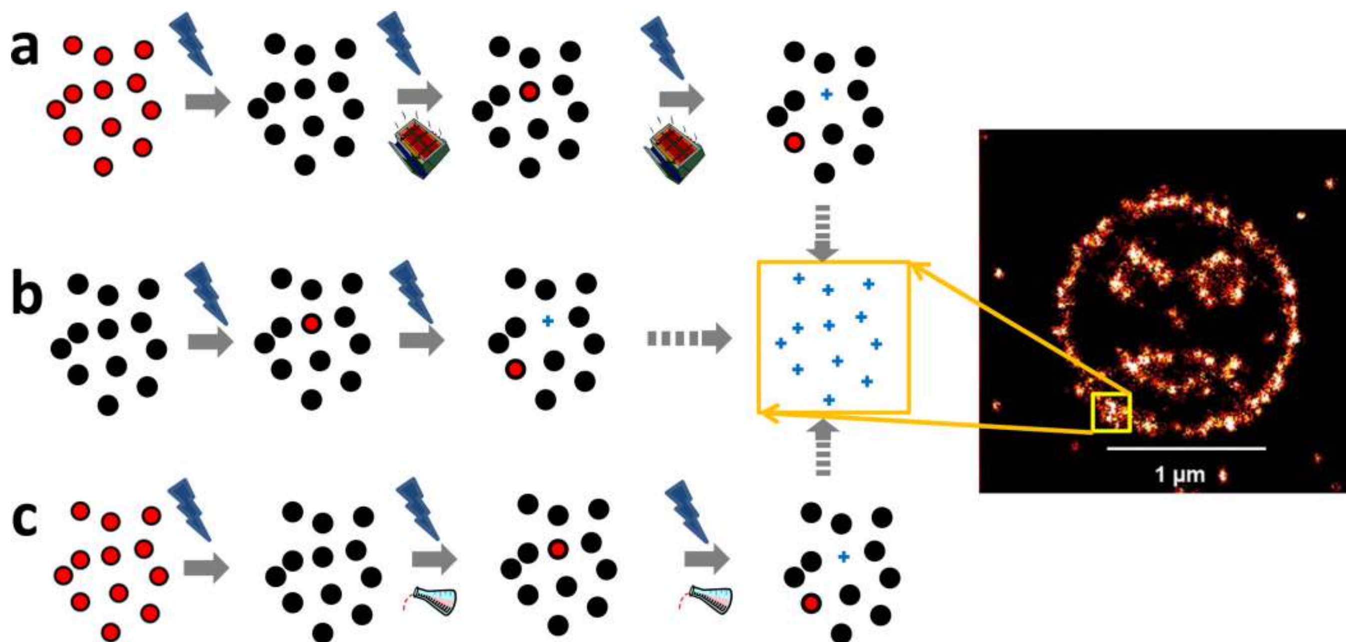

Figure 1.

Three schemes of single-molecule based super-resolution imaging. (a) Probes are active initially and then are turned off. A small fraction is activated thermally or by light for localization. This process is repeated many times to result in an image. The smiley face is an actual image, adapted from ref. (118). (b) Probes are initially dark and then activated in small numbers. (c) Probes are active initially and turned off, and a small fraction becomes active by controlling additives in solution.

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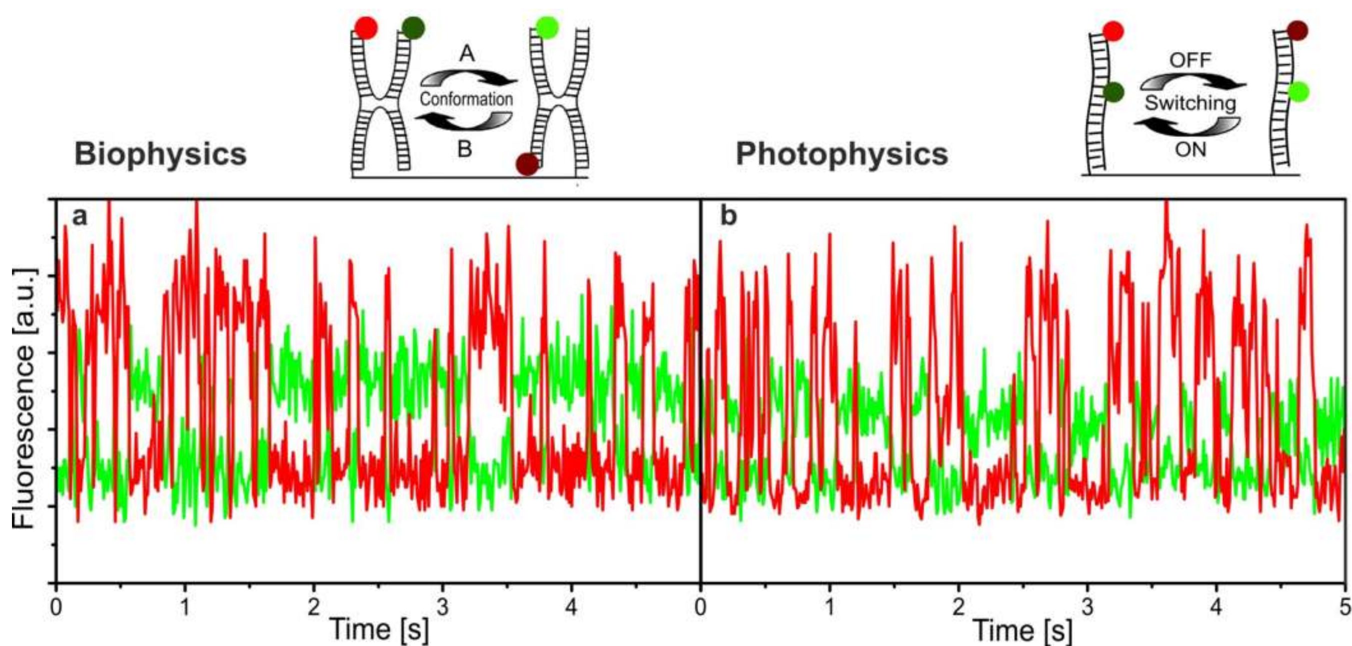

Figure 2.

SmFRET time trajectories indicating the potential similarity of biomolecular dynamics and photophyscially induced FRET fluctuations. (a) FRET fluctuations representing conformational transitions between two conformers of a Holliday junction labeled with Cy3/Cy5. B: FRET fluctuations originating from transient off-states of the acceptor in double-stranded DNA labeled with ATTO647N and ATTO680.

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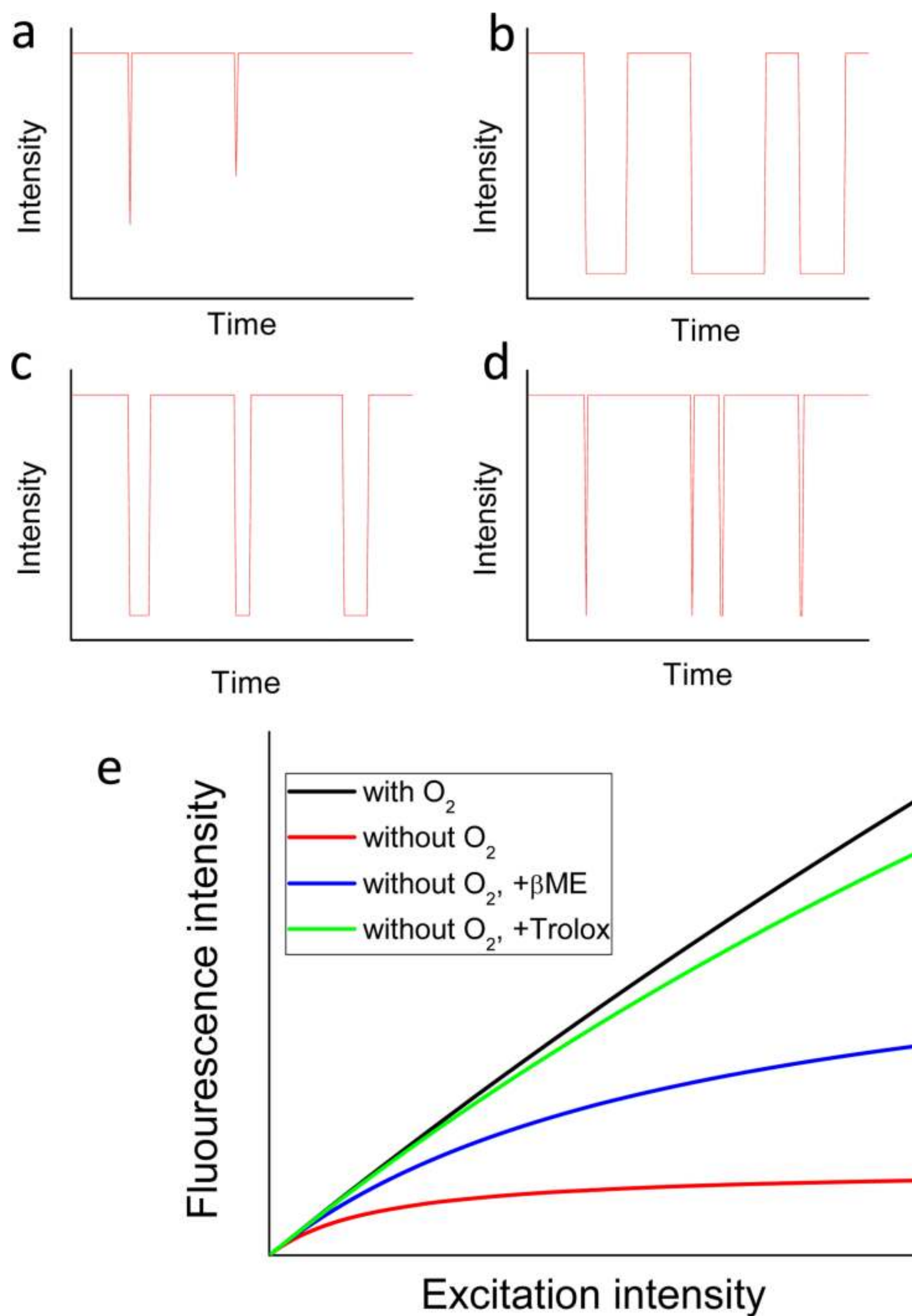

Figure 3.
Illustration of triplet-state blinking and its control. (a) Without oxygen removal, a single molecule spends only a small fraction of time in the off state (triplet state). (b) When oxygen is removed, the triplet state lifetime increases and the molecule spends long periods in the off state. (c) β-mercaptoethanol shortens the triplet state lifetime but not very well. (d) Trolox is highly efficient in shortening the triplet state lifetime. (e) Long triplet state lifetimes can cause early saturation of emission at high excitation rates under de-oxygenation condition, a problem that can be mitigated by using Trolox.

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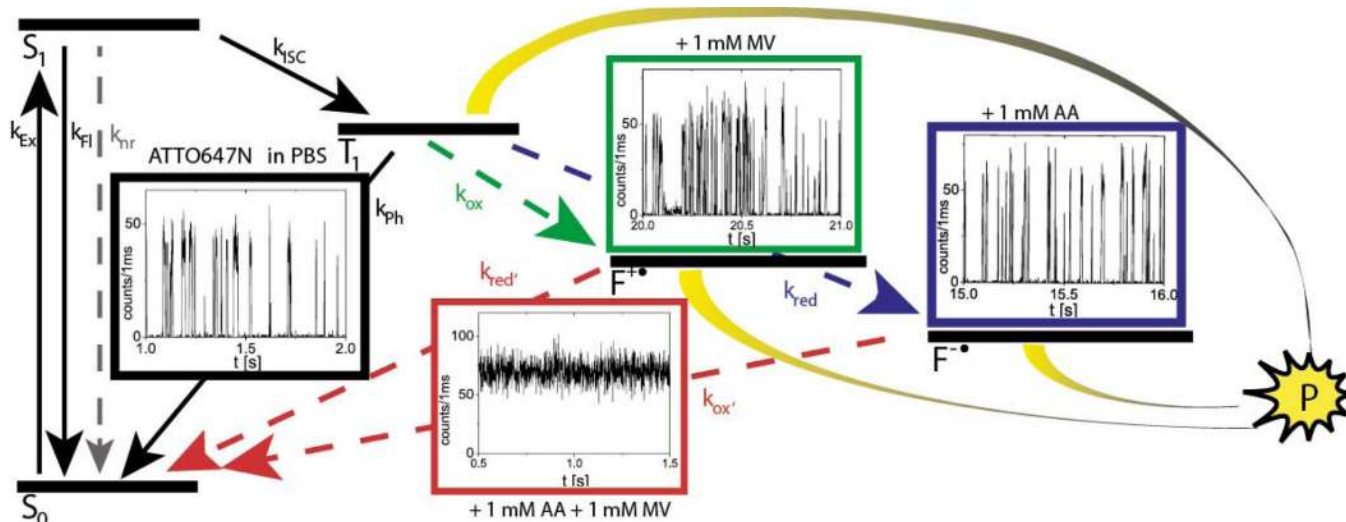

Figure 4.
Simplified Jablonski diagram with accessible states under different conditions. The fluorophore is excited from its ground state (S_0) to the first excited state (S_1) with an excitation rate k_{ex} and fluoresces with the rate k_{fl} after a few nanoseconds. Competing processes are non-radiative decays to S_0 with rate constant k_{nr} and intersystem crossing to the triplet state k_{isc} with a lifetime in the millisecond range. If an oxidant or a reductant is added, the triplet state is depopulated quickly and radical cations ($F^{+\partial}$) or anions ($F^{-\partial}$) are formed with rate constants k_{ox} or k_{red}, respectively. Depending on their redox potential these dark states are comparatively stable but can be recovered to S_0 by the complementary process in a buffer containing a reducing and an oxidizing system. The resulting short lifetimes of the triplet and radical ion states additionally improve photostability, as photobleaching (P) usually occurs from excited states. Time trajectories were measured with enzymatic oxygen scavenging. Reductant: Ascorbic Acid (AA), Oxidant: Methylviologen (MV).

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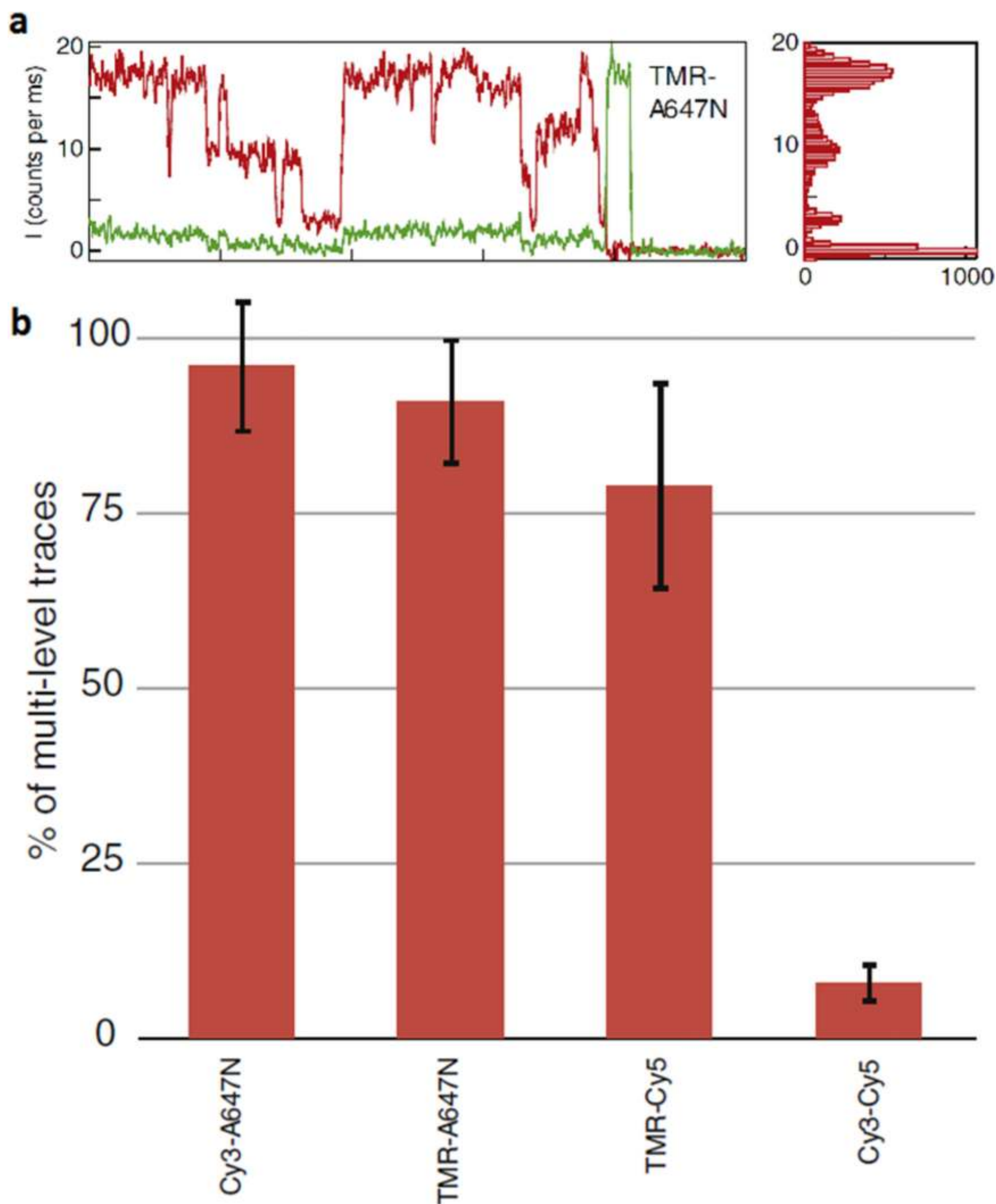

Figure 5.

(a) Fluorescent transient of a TMR-ATTO647N pair attached to double stranded DNA at a distance of 6 base pairs. Intensity fluctuations are caused by direct donor-acceptor contact. (b) Only the FRET-pair Cy3/Cy5 shows comparably stable FRET even at short distances. Adapted with permission from ref. (95).

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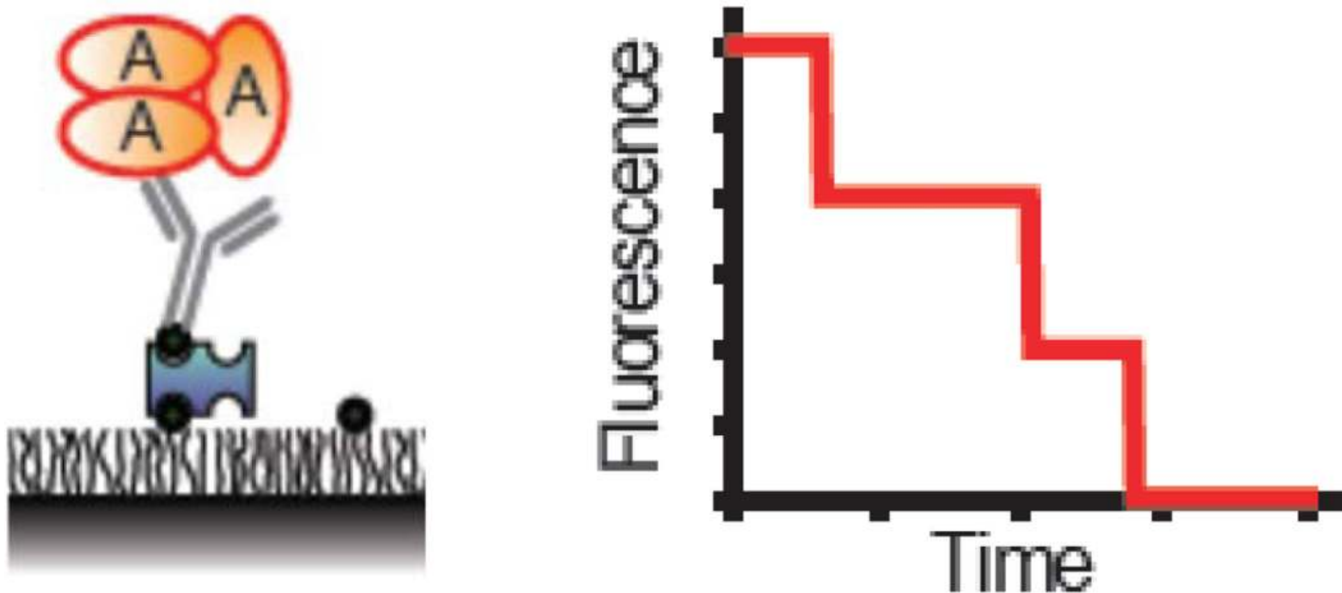

Figure 6.
Illustration of how a single molecule fluorescence intensity trajectory can reveal the number of proteins (A) in a complex by showing three steps during photobleaching. Adapted with permission from ref. (7).