University of Groningen
Faculty of Mathematics and Natural Sciences

Fluorophore stabilization and applications in the life-sciences

Mustapha Tisan Abdu-Aguye
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Supervisor
Dr. T. M. Cordes

Molecular Microscopy Research Group
Single Molecule Biophysics
Zernike Institute for Advanced Materials

Abstract

Fluorescence has become an important scientific tool since its discovery in the 1800s. Since then fluorescent molecules or fluorophores have become important materials for several industrial and technological applications, particularly in the life sciences, where they enable non-invasive studies on systems of biological importance. This essay provides an overview of the latest applications of fluorophores in single molecule studies and the problems associated with them. It starts with an introduction to classes of common luminescent materials, and discusses their advantages and disadvantages; furthermore, it gives an introduction to single molecule studies using fluorescence microscopy and discusses state-of-the-art techniques to overcome identified challenges to the use of fluorophores in the biological sciences based on their photophysical properties. Finally, it highlights the applications of stabilized fluorophores for single molecule based resonance energy transfer (FRET) and super-resolution microscopy giving perspectives on future possibilities.

Keywords: fluorescence, synthetic fluorophores, fluorescence microscopy, single-molecule spectroscopy, photophysics, Forster resonance energy transfer (FRET), super-resolution imaging

Contents

1. Introduction to fluorescence and fluorophores

Luminescence is the emission of light from any substance, and occurs from electronically excited states. Depending on the nature of the excited state [1], Luminescence can be either termed fluorescence or phosphorescence. Fluorescence occurs from excited singlet states, an electron in the excited orbital is paired by opposite spin to a second electron in the ground-state orbital. Consequently, return to the ground state is spin allowed and occurs rapidly by emission of a photon on timescales of approximately ten nanoseconds. On the other hand, phosphorescence is emission of light from triplet excited states, in which the electron in the excited orbital has the same spin orientation as the ground-state electron. Transitions to the ground state are spin-"forbidden", thus emission rates are relatively low compared to fluorescence implying that phosphorescence occurs on a timescale of at least milliseconds, and can often be as long as seconds. Since fluorescence was first observed by Sir John William Herschel in the mid-1800s, several applications have been found for fluorescence in fields ranging from lighting (as in fluorescent tubes), engineering to scientific research (forensics, spectroscopy, laser physics etc.) [17]

A fluorophore is an emissive molecule that can re-emit light upon excitation by light. Several applications of fluorophores are based on their property of often being strongly coloured – this has to do with their ability to absorb certain wavelengths of light better than others, thus several dyes, paints, optical brighteners etc. contain some kind of fluorophore; to be exact, the part of a molecule responsible for absorption of light is called the chromophore. More importantly however, they are important technological materials commonly used in light emitting diodes and solar cells; thus they are of interest in materials science, nanotechnology, and also in the life sciences; where they are a valuable method of tagging molecules of interest to track them and analyse their behaviour in a non-invasive manner.

A good illustration of the processes that can occur between excitation and emission of light by a fluorophore are given by a Jablonski diagram (see figure below); when a fluorophore is excited by radiation of sufficient energy (purple or blue arrows) from the electronic ground state S_0 to an excited state (such as S_1); it usually undergoes fast vibrational relaxation (internal conversion) to the lowest vibrational level of the excited state before fluorescence (green arrows) is observed, because of this relaxation, the energy of fluorescence photons is less than the absorbed photon by an amount called the "Stoke's shift". Additionally, it is possible for intersystem crossing to occur where the molecule switches to the spin-forbidden triplet excited state (T_1) which is characterised by delayed emission termed Phosphorescence [1]

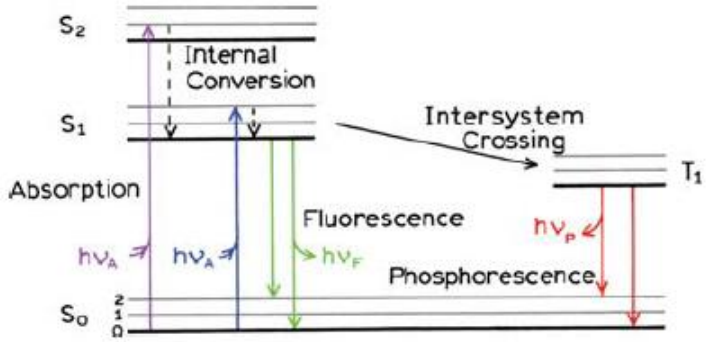

Figure 1: Jablonski diagram showing elementary photophysical processes that can occur after excitation of a fluorophore, figure adapted from [1]

2. Types of common fluorophores

(a) Organic Fluorophores

The ultimate performance of any fluorescence application depends heavily on the fluorescent molecule used to emit light (=fluorophore); in general, there are three common families of emissive materials. The first most common are (small) synthetic fluorophores or "dyes"; which consist of a pi-conjugated section (i.e. C=C bonds) which contain delocalized pi-electrons, and several other functional parts for solubilisation etc. Several families of organic dyes exist which cover the entire visible spectrum from blue to red – their ubiquity mainly stems from the fact they have been very well studied over the past century, with applications in diverse fields such as laser physics and textile manufacturing (hence the name "dye" coming from their colour). Their main advantages lie in their good wavelength selection, small size (1-2nm), commercial availability and low cost. Limitations to their use however are posed by the fact that it is necessary to use sometimes complex strategies to "tag" them to the feature to be observed, and that they are foreign inorganic objects which when inserted into biological samples; which could alter biological function and complicate analysis of results [7].

The figure below shows some families of common organic fluorophores; the shaded regions in the molecular structures represent the π-conjugated systems responsible for their colour and fluorescent properties.

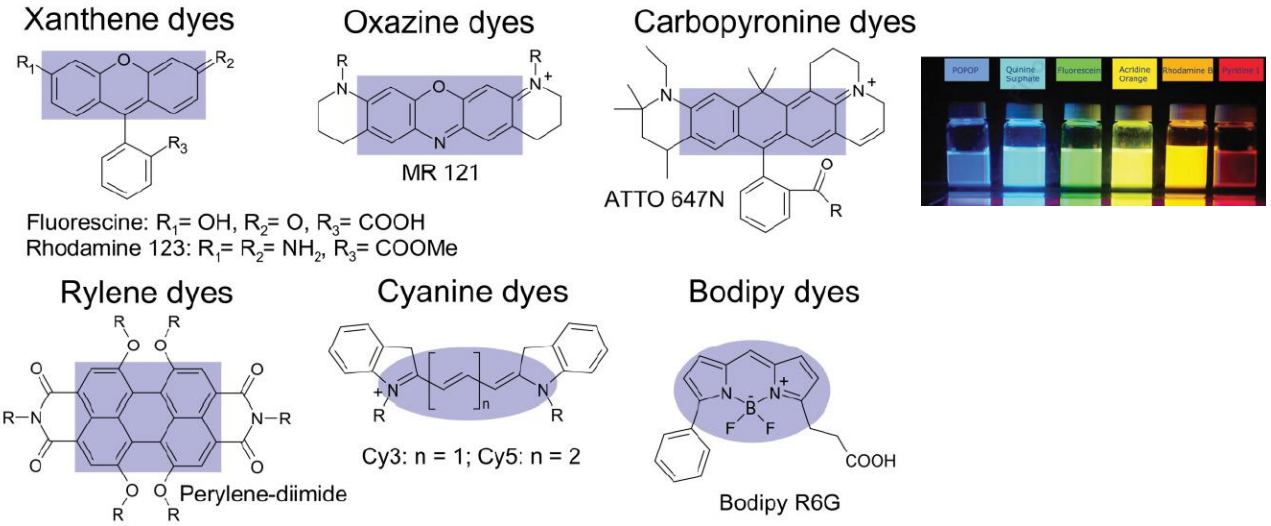

Figure 2: Common families of organic fluorophores, shaded region represent the pi-conjugated system responsible for fluorescence. **Right inset**: dyes ranging from blue to red; figures obtained from [7]

(b) Fluorescent Proteins

A second family of fluorophores are fluorescent proteins, which were first discovered in 1961 by Shimomura and Johnson [1]; who were later awarded the Nobel Prize in Chemistry 47 years later. They successfully extracted wild-type Green Fluorescent Protein (wt-GFP) from the bioluminescent *Aequorea Victoria* specie of jellyfish. Structurally speaking, fluorescent proteins consist of a small organic chromophore covalently linked to and surrounded by a barrel of 11 β-

sheet proteins and 6 α-helices [7] as shown in figure 3; this barrel contains a covalently bound chromophore (one part of the barrel is cut away to show the chromophore as a ball-and-stick model for clarity). Since the initial discovery of wt-GFP several more advanced fluorescent proteins (FPs) have been successfully engineered by inducing mutations in original wt-FP which affect folding efficiency and colour; resulting in drastically improved fluorescence, brightness and photostability. Currently there are several GFP mutants and other FPs which emit from blue to red [1]; their main advantage lies in the fact that the chromophore forms spontaneously upon protein folding without the need for any additional enzymatic synthesis – this means that it is possible to express the GFP gene into cells and even entire organisms [3].

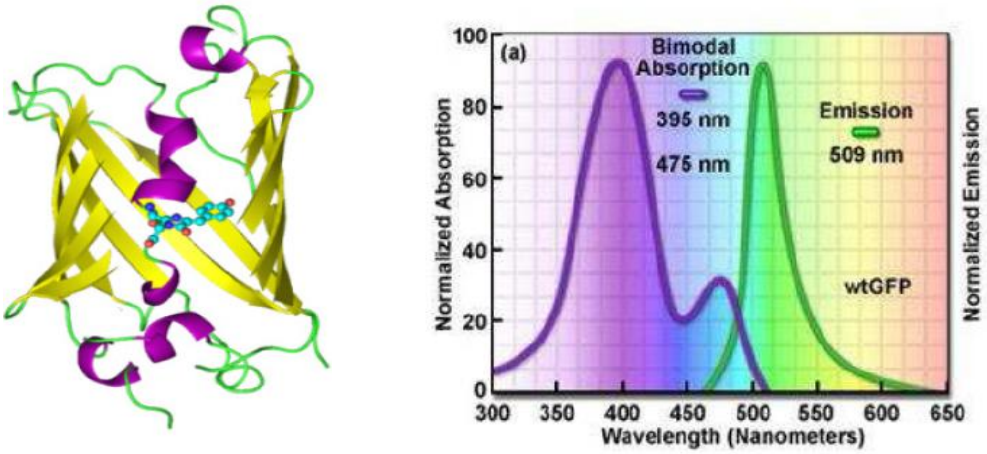

Figure 3: Structure and spectral characteristics of wt-GFP, (**left**) cartoon showing the chromophore housed inside the barrel formed by the proteins (www.wikipedia.org) (**right**) spectral characteristics of wt-GFP obtained from [7]

(c) Quantum Dots

Lastly, the third family of fluorescent probes are small crystalline aggregates of purified semiconductors consisting of hundred to a few thousand atoms called quantum dots. Quantum dots used in fluorescence microscopy generally have a core-shell anatomy where a core of one material (say CdSe) is over-coated with a shell on wider bandgap material such as ZnS to improve the optical properties by surface passivation. The shell is then conjugated to biologically active peptides to enable tagging to the structure of interest. Quantum dots are fundamentally different from dyes or FPs in the nature of photoexcitation and emission which is by correlated electron-hole pairs rather than a $\pi \rightarrow \pi^*$ transition [18]. The main advantages of quantum dots are their size tunable absorption and emission characteristics, exceptional wavelength selection, brightness and stability. However, toxicity due to heavy metals such as Pb and Cd remains a drawback for many applications [2].

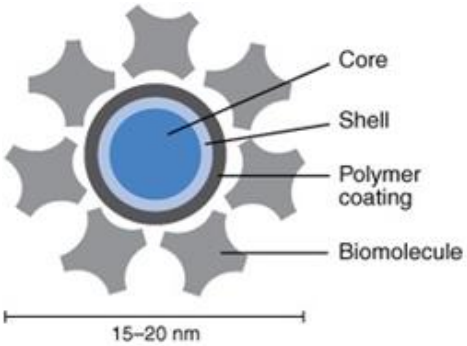

Figure 4: Schematic diagram of a fluorescent quantum dot probe, average size after bioconjugation is 15-20nm, significantly larger than dyes or FPs (www.lifetechnologies.com)

3. Single Molecule Studies using Fluorescence Microscopy

Microscopy refers to the technical field of viewing objects smaller than the resolution of the human eye under a microscope; traditional optical microscopy has several limitations including: (i) resolution, dictated by the wavelength of the light used – typically around 250 nm for UV light, a situation called the "Rayleigh criterion" [19] which limits the maximum magnification obtainable when viewing objects under a microscope. The Rayleigh criterion is given by:

$$\Theta = 1.22\frac{\lambda}{D}$$

Where Θ, λ and D are respectively the angular resolution in radians, the wavelength of light and the diameter of the lens aperture. The airy diffraction patterns arising from light passing through a circular aperture such as a lens are circular discs if we assume that the wave-front passing the aperture is a plane wave. When light from two objects beside each other satisfy the Rayleigh criterion: they are clearly resolvable, points closer to each other than that are not clearly resolvable. (ii) Aberration which arises from optical imperfections in the lens, which causes light rays at different distances from the optical axis to be focused to different points. Other limitations include insufficient contrast (i.e. the ability to distinguish between objects of different colour and brightness in the same field of view) to image several samples precisely, particularly in samples with transparent features.

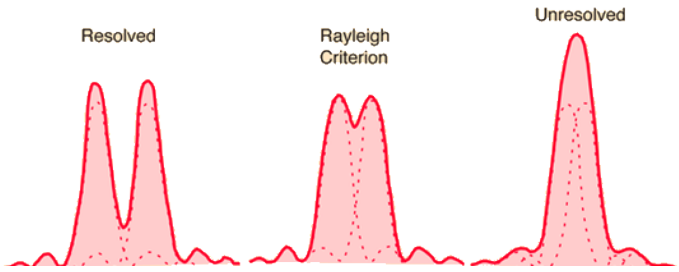

Figure 5: Airy disks from two nearby objects (positions shown by dotted lines), when there is sufficient distance they can be clearly resolved (left, at the Rayleigh criterion they can just be resolved (middle), and beyond that they are indistinguishable (right). Image from Hyperphysics (http://hyperphysics.phy-astr.gsu.edu/)

Since fluorescence was first reported in the mid-1800s by Sir John William Herschel followed by the subsequent observation of auto-fluorescence in plant and animal tissues by Heimstädt & Lehmann in the 1900s [7]; microscopy using fluorescence as contrast (= fluorescence microscopy) has led to a revolution in the biological sciences with far reaching implications in other diverse fields such as materials science where sub diffraction limited resolution is a huge advantage. A fluorescence microscope has the basic function of irradiating a sample with a desired and (often very) specific band of wavelengths, followed by collection of the emitted light; which is generally much less intense than the excitation light [1]. A basic fluorescence (epifluorescence) microscope consists of a light source such as a laser, mercury lamp or Xenon lamp; which is focused on a sample by an objective lens via filters and a dichroic mirror. The

emitted fluorescence from the sample is focused on the detector (or ocular lens) by the same objective lens; which ideally should have a large numerical aperture for the best sensitivity.

Over the past two decades, several more advanced fluorescence microscopes have been developed such as total internal reflection (TIRF) and confocal microscopes.

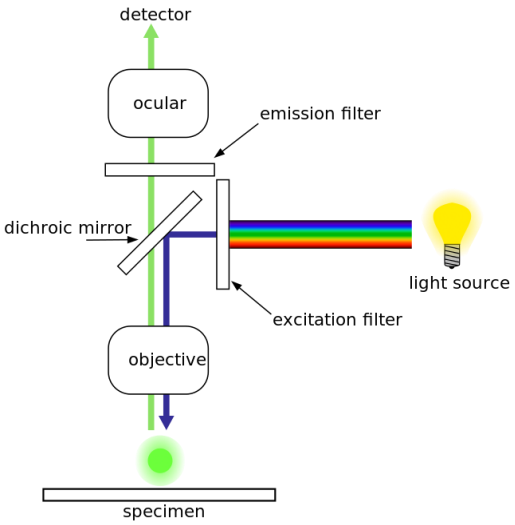

Figure 6: Simple design of a fluorescence microscope (epimicroscope) showing the basic parts: light source, objective and ocular lenses, dichroic mirror and filters for excitation and emission as well as the detector

The development of these advanced microscopes, fluorescent probes and imaging techniques have made it possible to monitor elementary biophysical processes with a high degree of clarity and precision [4]; allowing scientists to answer fundamental questions about enzyme-mediated reactions, membrane processes and even the mechanism of disease infection on a single molecule level [3]. The main power in single molecule studies lies in the ability to characterize distribution of individual molecular properties and their fluctuations, avoiding ensemble averaging which then allows the observation of heterogeneity and the detection of rare events. In addition, it is possible to measure time-dependent phenomena without the need for synchronisation [7].

In addition, new methodologies have been demonstrated using a combination of spatial and temporal localization of fluorescence, intrinsic photophysical properties of fluorophores and advanced signal detection and processing techniques which yield good contrast and resolution below the diffraction limit. These techniques are summarily referred to as super-resolution techniques.

The requirements for the fluorescent probes for these reasons are quite demanding – and since all fluorophores do not behave ideally (as easily linkable, stable, bright, long lasting light sources) but are rather affected by their environment, and their own intrinsic photophysical properties; it is appropriate to quote Tinnefield and Ha [3], who stated that every fluorophore has a somewhat unique “personality”. Vast amounts of information can be obtained from singe molecule studies using fluorescent probes, information regarding position, population, distance between

molecules of interest, rotational dynamics, diffusional movement and the local environment can be gleaned from fluorescence intensity, polarization and lifetime measurements [1].

The requirements placed on fluorophores for application in single molecule studies primarily require good photostability, brightness and minimal intensity fluctuations. Therefore some of the major challenges to the use of fluorophores have focused on these.

4. Limitations to use of fluorophores: photobleaching and blinking

Photobleaching is an irreversible pathway through which a fluorophore permanently enters a non-emitting dark state and is thereafter considered inactive. Fluorescence quenching by photobleaching can occur either when the fluorophore enters the reactive triplet state in the presence of molecular oxygen, which then interacts with the fluorophore by the formation of radical ions in solution. Strategies to minimize photobleaching in organic fluorophores use enzymatic oxygen scavenging systems such as glucose oxidase catalase (GOC) to reduce oxygen in solution from mM to lower μM concentrations [4]; there has also been a report where a system consisting of protocatechuic acid and protocatechuate-3,4-dioxygenase (PCD) was used to allow experiments at even lower oxygen concentrations, although it is reported to act slower than the GOC-based route [3].

Efficient removal of oxygen sometimes leads to a lower total intensity, and also a phenomenon called blinking; which refers to reversible intensity fluctuations between emitting (on) and dark (off) states. Blinking becomes apparent in fluorescence traces of organic fluorophores (see figure 7 below) as a consequence of the increase in the spin forbidden triplet excited state lifetime which occurs when oxygen is removed, since oxygen is an efficient triplet quencher [3, 4]. Note also that the triplet state is not the only source of intensity fluctuations in organic fluorophores; it can also be a consequence of redox reactions which occur when alternative redox agents are added to fluorophore solutions to quench the triplet state such as Trolox and β-mercaptoethanol. This is because the likelihood of photo-induced electron transfer leading to the formation of radical ions is higher for longer lived excited states; resulting from their lower oxidation potentials and increased electron affinity. Finally, reversible transformation induced by light between chemical species can also result in blinking fluorescence traces, such behaviour has been observed in cy5 with thiols in solution and some fluorescent proteins.

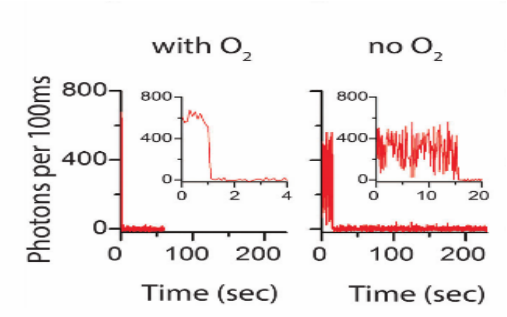

Figure **7**: Representative fluorescent trace for cy5 in the presence (**left**) and absence (**right**) of oxygen; removal of oxygen leads to intensity fluctuations called *blinking*, figure from [4]

5. Photophysics of fluorescent probes

For organic fluorophores, when a molecule is excited (EX, see figure 8) by radiation of suitable energy from the electronic ground state S_0 to an excited state S_1 (or S_n), the efficiency of which is dependent on the fluorophore's extinction coefficient (that is a measure of how well it absorbs light). It can return to S_0 via non-radiative internal conversion (IC) or by the emission of a photon (fluorescence F). Due to the fast vibrational relaxation, the energy of a photon emitted from S_1 is less than the excitation energy (by an amount called the Stoke's shift). Experimentally, it is sometimes observed that a fluorophore in S_1 undergoes intersystem crossing (ISC) to the spin-forbidden, non-fluorescent triplet excited state (T_1); which despite being a rather rare event (occurring once every 1000 or so excitation cycles), has a relatively long lifetime compared to S_1 and decreases the fluorophore's emission rate resulting in blinking [1]

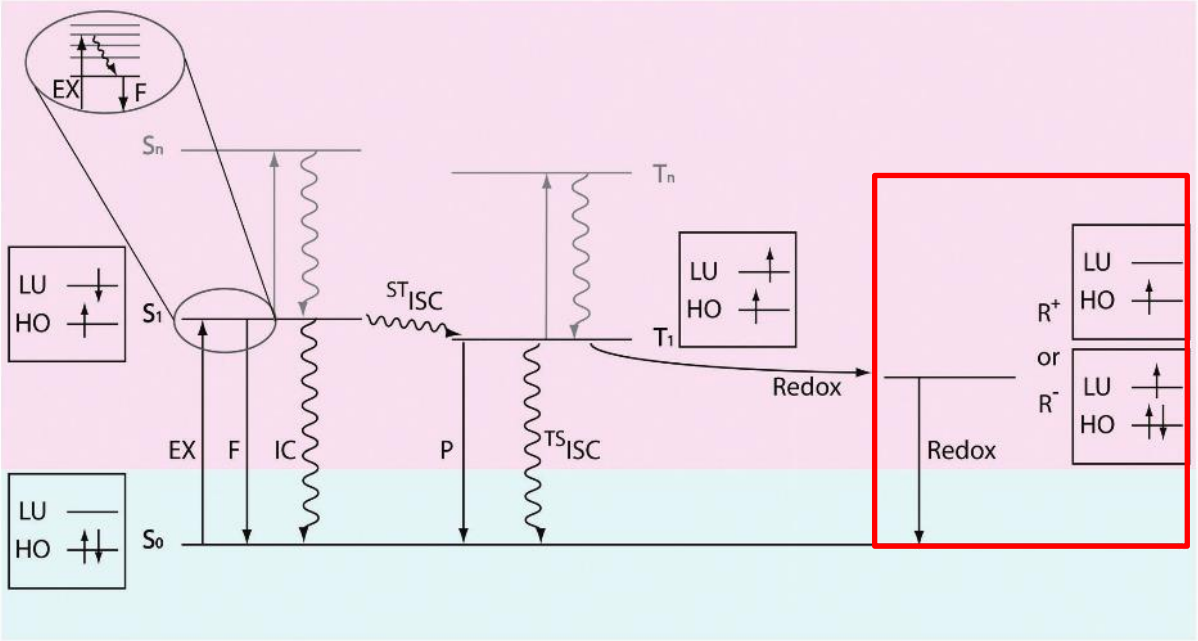

Figure 8: Extended Jablonski diagram showing photophysical process and reactions that can occur when a fluorophore is excited, adapted from [4]; note the new pathways indicated for redox reactions which depopulate the triplet state (red box)

Additionally, the triplet state by virtue of its lower oxidation potential and higher electron affinity [20] opens pathways that can lead to irreversible damage (photobleaching); such as the formation of non-fluorescent radical cation or anion states (R^+ and R^- in the red box in figure 8). T_1 is particularly susceptible to electron transfer (redox) reactions which can be mediated by solvent impurities, molecular oxygen present in solution, another fluorophore or even components of the biomolecule to which the fluorophore is attached.

The photophysics of fluorescent proteins is also rather complicated; in addition to blinking arising from triplet and radical states, the conformation of both the chromophore (cis/trans) and dynamics of the β-barrel scaffold can result in blinking. It is also possible that proton transfer is a further factor in the observed flickering; however, in general, it is believed that the chromophore is fluorescent provided it is kept in a planar configuration by the protein scaffold. Interestingly,

the isolated chromophore is non-fluorescent, thus the protein barrel must be important to the photophysical properties of FPs and their derivatives. Limitations to the widespread use of FPs in single molecule studies compared to organic fluorophores are their order-of-magnitude lower photostability and higher intensity fluctuations [3].

The exceptional size-tunable optical properties of QDs can be improved by growing a shell of a wider band-gap semiconductor around them to improve surface passivation, and also the attachment of long insulating ligands [18]. Quantum dots emit based on an excitonic transition, and have also been shown to display fluorescence intermittency [5] even under continuous excitation [21]. These fluctuations occur as a result of the formation of charged nanocrystals, which then lead to the formation of so-called trions, consisting of a coulomb correlated electron-hole pair and the extra charge; this increases the efficiency of non radiative processes such as Auger recombination, with fluorescence recovering after the particle loses the extra charge. It has however been demonstrated [6] that it is possible to synthesize non-blinking QDs by using ternary CdZnSe, with a ZnSe shell or epitaxially grown InGaAs on GaAs, explained by a soft-confinement potential enabling radiative recombination of trion states. Though in both cases, solubility in water was not achieved. Despite these advances and their higher brightness and photostability relative to organic fluorophores and FPs, metal-induced toxicity still remains a huge issue for their use in in-vivo studies.

Below is a summary of the advantages and disadvantages of organic fluorophores, FPs and QDs obtained from [7], this usually informs the choice of probe for particular applications

Property	**Dyes**	**Fluorescent Proteins**	**Nanocrystals**
Wavelength Selection	good	good	good
Brightness	good	moderate	excellent
Stability	good	low	excellent
Toxicity	organic material	none	inorganic material
Size	small (1-2nm)	moderate (4-5nm)	big (> 15nm)
Linkage via	tagging	expression	tagging

6. Stabilization Techniques

As mentioned earlier, the ideal fluorescent probe for single molecule studies is bright, extremely photostable, easy to link to intended targets - causing minimal interference with ordinary function, does not display any intensity fluctuations and is readily available commercially. It is

therefore no surprise that organic fluorophores have been the workhorse for this purpose. To improve their utility for single molecule studies (in terms of signal/noise ratio and observation times) several strategies have been tested and found to yield good results.

(a) Solution based additives

Unlike many molecules, oxygen (O_2) is more stable in the triplet form and plays a huge role in dye stability. Triplet O_2 is an efficient dye triplet quencher – this, however results in the formation of the less stable, higher energy singlet oxygen which rapidly reacts with exposed groups on dye molecules and leads to photobleaching. Oxygen scavenging systems consisting of glucose oxidase and glucose catalase (GOX) and protocatechuic acid-protocatechuate-3,4-dioxygenase (PCD) have been used successfully for oxygen removal from dye solutions; in the GOX system, glucose oxidase oxidizes glucose into glucolactone, converting oxygen into hydrogen peroxide in the process. Since hydrogen peroxide could harm the fluorophores, glucose catalase converts the hydrogen peroxide into water and oxygen. The concentration of oxygen in solution is around (0.3 – 0.5) mM, this reduces the oxygen concentration to around 3 μM [3, 4].

Additives for depletion of the triplet state rely on either intermolecular collisions with redox additives via photo-induced electron transfer, or other well-known triplet quenchers via a Dexter electron transfer process as used in dye-lasers [6]. Frequently, combinations of these have been used with good results [4, 8, 20]. This intermolecular collision based strategy employs phenomenologically tested redox reagents (also referred to as protective agents) and has resulted in a shortlist of preferred additives (shown below).

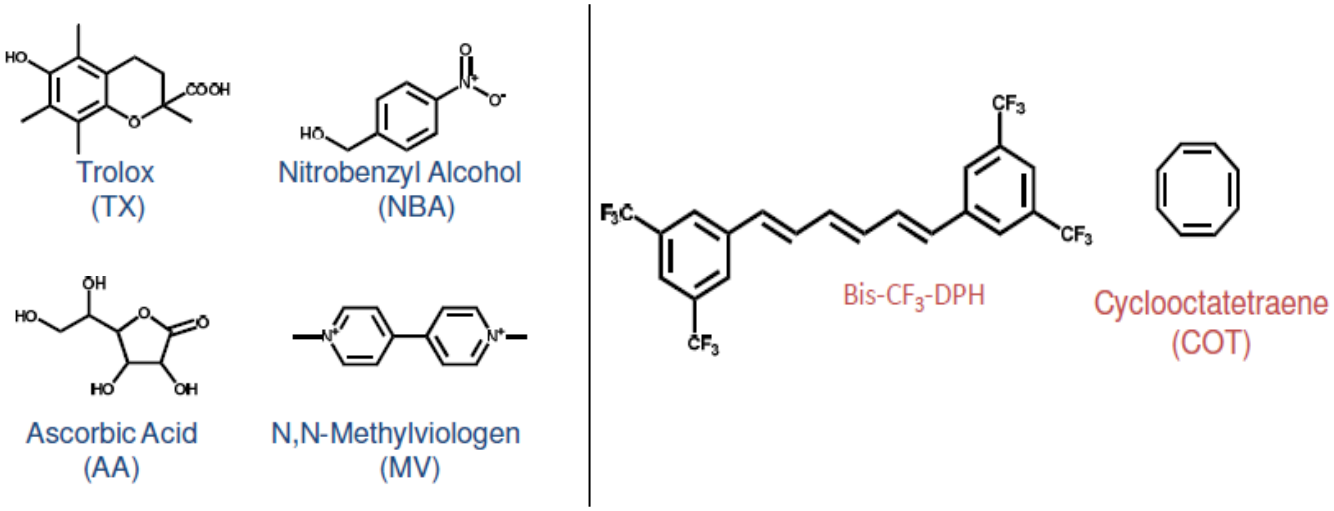

Figure 9: (**left**) Common molecules groups used either as redox active photoprotective groups (labelled in **blue**) or (**left**) triplet state quenchers (labelled in **red**); frequently, combinations of both are used [4, 6]

Though the exact mechanisms for their action are still a matter of debate; the improvement in fluorophore stability, decrease in intensity fluctuations, blinking frequency, and increase in mean fluorescence intensity is generally common for all of them; also consistent with the assertion that reactive oxygen species are detrimental to fluorophore performance [3, 4, 8]. Note however, that

it is currently not clear which dyes tolerate the presence of oxygen and that large variability in results on solution based additives depending on dye are observed.

The first demonstration [9] of this anti-fading cocktail of COT, NBA and Trolox under oxygen deficient conditions yielded a dramatic improvement in fluorophore lifetime, and mean intensity via a mechanism now believed to be diffusion based intermolecular collisions [10]. Currently, there are already several commercial anti-fading agents based on the ROXS system which effectively supress dark states in common fluorophores [4]. This mostly occurs through triplet quenching via the formation of radical fluorophore cations or anions, which are then rapidly depleted via the reagents themselves, repopulating the ground state.

(b) Covalent-linkage based techniques

Though there have been unquestionably many advances in solution-based additives for fluorophore stabilization, challenges remain from the fact that protective agents are nonetheless additional exogenous entities to the systems to be studied, and probably alter ordinary function in living cells. It has already been established the COT, Trolox and NBA can affect the function of membrane proteins [11]. Another consideration is that non-specific inhibition may arise from additives since they operate via collision mechanisms and therefore must be present in solution in high enough concentrations [4, 12] for frequent collisions to occur (mM in general), which incidentally is close to the solubility limit for several of them, this complicates analysis of results as their effect on every experiment must be carefully considered.

A slightly different strategy of fluorophore stabilization was demonstrated by Blanchard and co-workers in 2012 using methods already developed for dye-laser research and improving the aqueous solubility of dyes [22, 23]. Their method involved the covalent linkage (via a flexible 12 Carbon linker) of triplet state quenchers (TSQs)/protective agents to Cy5, resulting in enhanced photostability in both *in-vitro* and *near-in-vivo* (on the surface of living cells) contexts without any apparent change in its intrinsic spectral characteristics [8]; this study revealed that (a) linking a protective agent proximally to a fluorophore increases its effective local concentration, and can perform better than the same additive at near saturating conditions and (b) wider applicability could be gained since improvements were also noticed in oxygenated buffers where additives had little or no effect. The mechanism of *self-healing* was suggested by Cordes and Tinnefeld as a double electron transfer between the fluorophore and the protective agent [13]. Further studies on ds-DNA [12] and synthetic developments such as increased proximity between the fluorophore and protective agent [4] also revealed enhancement of photostability without the need for any additional solution based additives.

This self-healing concept also suggests the possibility of high temporal resolution imaging using these stabilized fluorophores studies by Blanchard and co-workers on cy5 and the self-healing cy5-COT3 (linked to COT via a 3 C linker) in a Potassium chloride (KCl) and β-mercaptoethnol (BME) buffer with TIRF under oxygen depleted conditions revealed a linear increase in photon emission rates over a wide range of excitation intensities (see next section).

Figure 10 below, shows a schematic diagram of the difference between solution based stabilization and the intra-molecular stabilization route.

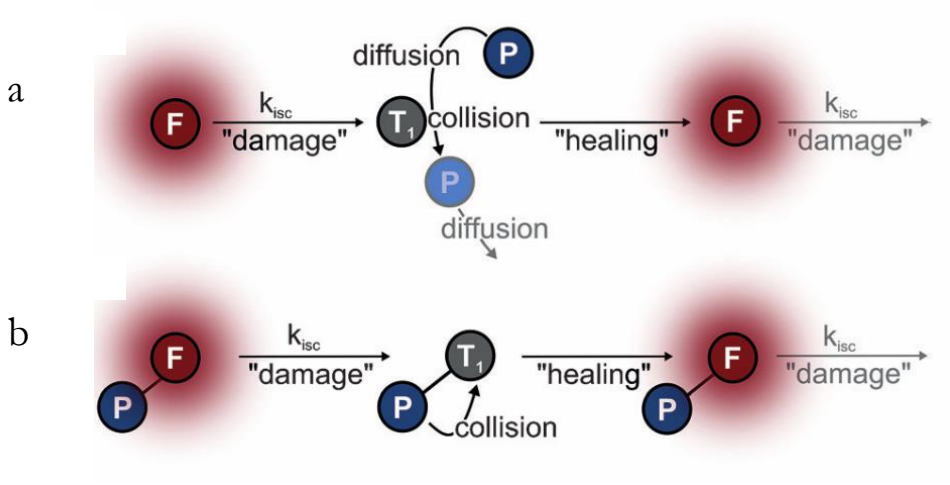

Figure 10: Photostabilzation techniques using (a) solution based techniques, collisions between the triplet state (T_1) of the fluorophore (F) and additives in the imaging buffer; (b) a covalently linked photoprotecting group (P) creates a high local concentration resulting in collisions with the fluorophore triplet. Image adapted from [12]

7. Applications of stabilized fluorophores

Recent advanced applications of stabilized fluorophores have certain requirements in common: (a) high signal-noise ratio and prolonged observation times which should be a consequence of robust, photostable fluorophores and (b) a reasonable amount of control on the emission of a single emitter (for example: minimal blinking for Forster resonance energy transfer (FRET), and switchable blinking for super resolution (SR) imaging). The successes with both additives-based fluorescence stabilization and the self-healing concept have enabled new experiments with developments in imaging technology such as high speed sCMOS cameras and feedback driven instruments to answer fundamental questions regarding gene expression, protein translation and even membrane dynamics [4, 8, 11].

(a) Forster Resonance Energy Transfer (FRET)

FRET is a mechanism describing energy transfer between two nearby chromophores via a radiationless dipole-dipole interaction from an excited donor (D) and an acceptor (A). Since FRET efficiency falls off as the sixth power of distance R between the donor and acceptor – monitoring FRET efficiency is a powerful tool in the biological sciences as an ad-hoc "nano-ruler" for distances between 1-10 nm (figure 11)

For most applications, fluorophores for FRET are required to have comparable emission quantum yield i.e. both donor and acceptor; and, large spectral separation between donor and acceptor to reduce "bleed-through" of donor emission into acceptor emission, and also to minimize direct excitation of the acceptor by the laser source.

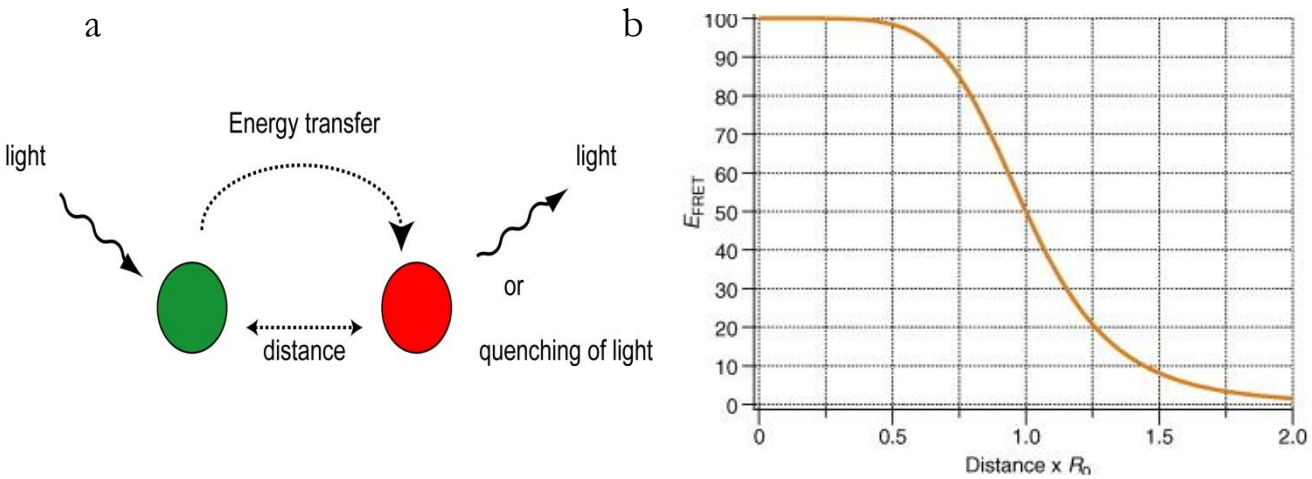

Figure 11: (a) schematic representation of FRET the green molecule (donor) undergoes a radiationless energy transfer to the red molecule (acceptor) which then emits, the presence of an acceptor is deduced by quenching of fluorescence from the donor; (b) FRET efficiency falls of as the sixth power of distances between donor and acceptor, the Forster radius R_0 is when FRET efficiency is 50%

Depending on the particular application, there have been several dyes used as donors such as cy3 and TMR, and acceptors such as cy5 and ATTO647N, each with their own attendant peculiarities. In FRET it is necessary to understand the underlying photophysical and/or biophysical processes during experimental data interpretation. For instance, a "low FRET" state can be mimicked by a transient photophysical OFF state in an acceptor or donor molecule, hence, the requirement for ultra-stable fluorophores where undesirable photophysics are kept to a minimum [3].

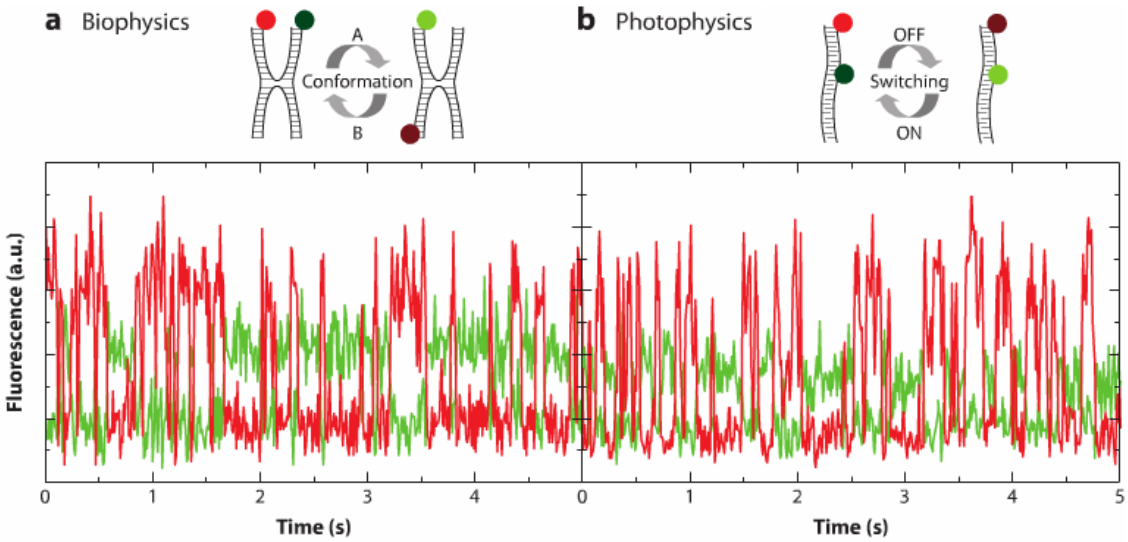

Figure 12: (a) a conformational change in a holliday junction labelled with the cy3/cy5 FRET pair, (b) ds-DNA labelled with ATTO647N/ATTO680; a transient photophysical OFF state in the acceptor can mimic a low FRET state giving similar fluorescence traces [3]

Generally, single molecule fluorescence studies are limited by temporal resolution (in the order of 10ms at least) due to the constraint on number of detectable photons per time step (> 20 is acceptable) over periods meaningful in a biological context. Self-healing fluorophores have been demonstrated to enable millisecond timescale single molecule FRET imaging, Blanchard and co-workers used cy3-COT3/cy5-COT3 (that is, cy3/cy5 linked covalently to COT via a 3 atom linker) versus the traditional FRET pair cy3/cy5, to label a ribosome complex where

spontaneous conformational changes facilitate inter sub-unit rotation [4, 12]; their results showed that the self-healing fluorophores recorded reliable FRET recordings with good photon emission rates/frame over an extended period (1s) with little intensity fluctuations, whereas under similar conditions the cy3/cy5 pair was photobleached in less than a frame (1ms). However, they never benchmarked their self-healing dyes against solution additives in the same experiment to compare the utility of intra- vs. inter-molecular stabilization.

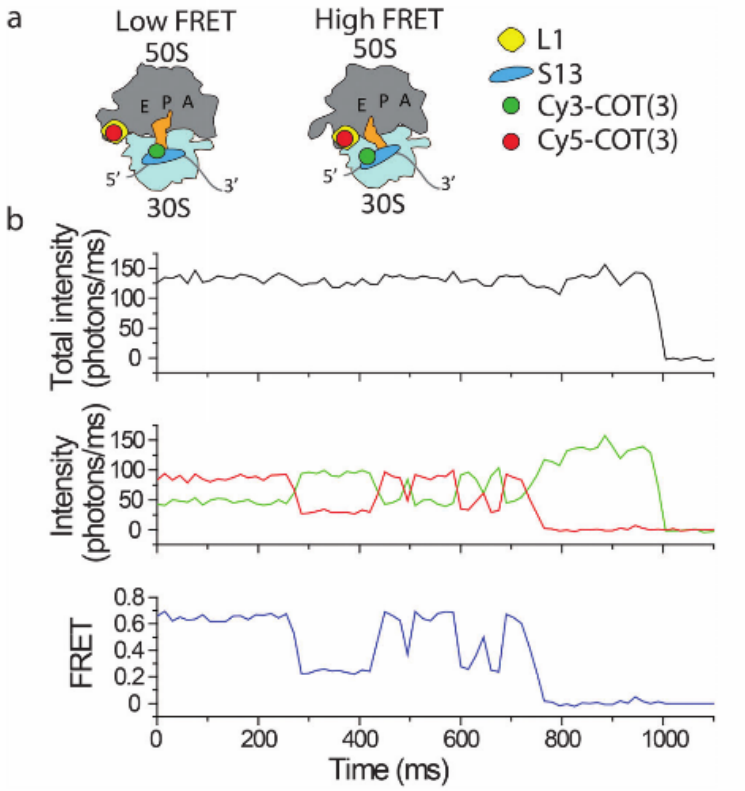

Figure 13: Results from [12] showing (a) Low and High FRET conformations in the bacterial ribosome; (b) Self-healing fluorophores show robust emission rates compatible with time scales for imaging of around 1ms. Data measured by TIRF at a frame rate of 66Hz.

(b) Super-resolution Imaging

Optical super-resolution (SR) is a term used to refer to techniques of fluorescence microscopy where the "*Abbe limit*": $d = \lambda/2NA$, where d, λ *and* NA respectively represent the resolution, wavelength of light and numerical aperture of the lens; imposed on the maximum achievable resolution arising from the wave nature of light (based on diffraction) is surpassed. SR can broadly be classified into (i) techniques that use information contained in evanescent waves such as total internal reflection (TIRF) microscopy; or (ii) those that use experimental techniques and known limitations on the matter being imaged to gain additional information beyond the diffraction limit such as confocal microscopy. Over the past two decades, SR imaging has seen a gamut of techniques such as patterned illumination, reversible saturable optical fluorescence transitions (RESOLFT) and several stochastic type methods, each of which has its own variations, advantages and drawbacks. A full review of SR imaging is beyond the scope of this

essay; moreover, the author notes that there are already several excellent reviews covering SR microscopy for those interested [4, 15].

For the so-called "functional" SR i.e. those utilizing experimental techniques to surpass the diffraction limit, two main classes exist: deterministic type; achieved by the spatial suppression of fluorescence from probes to a desired region (fluorescence confinement) and stochastic type; achieved by the temporal modulation of emitters between an ON and OFF state for each time frame (i.e. fluorescence localization); both followed by a subsequent determination of a "most probable" position via a 2-D Gaussian point spread function.

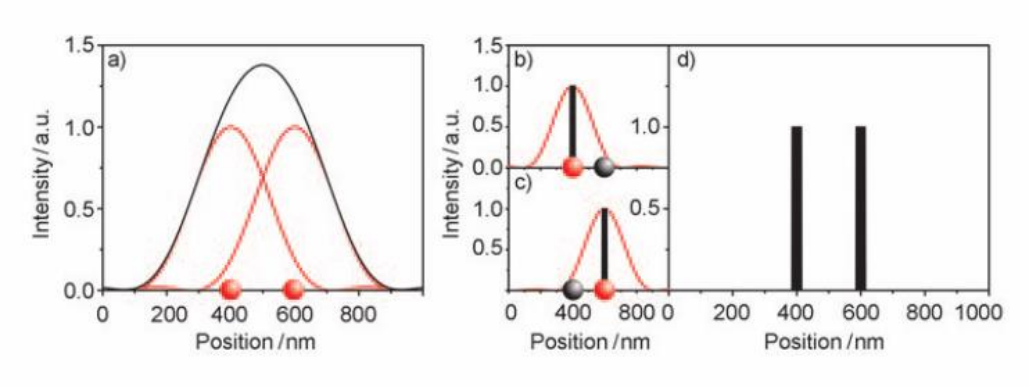

Figure 14: (a) when two fluorescent molecules are closer than ~ 250nm their exact positions cannot be determined; (b, c) in SR imaging, they are switched ON/OFF sequentially and their positions are precisely determined; (d) a histogram is made for reconstruction of the final image [15]

Regardless of the technique used for SR imaging, the requirements on the fluorophores are that they be both photostable and also reversibly switchable between distinct ON and OFF states, though the exact switching mechanism may differ. Generally, for fluorescence confinement techniques, only one photon is required to measure the position of a molecule, neglecting noise and background effects (this does not hold for certain variants utilizing inverse fluorescence confinement); however, for localization based techniques the main demand is to be able to control the switching of emitters since only one out of a population of (often) identical emitters is allowed to be active in a diffraction limited area within a certain time interval. In addition the resolution d achievable by localization based SR is related to the full width at half maximum (FWHM) of the point spread function by $\mathrm{d} \approx \mathrm{FWHM}/\sqrt{\mathrm{N}}$; where N represents the number of photons detected during the ON state [7].

Both these requirements are afforded by fluorophores with stabilization via oxygen depletion (for added photostability) and ROXS. While on first thought ROXS reduces blinking which is beneficial for stochastic type SR imaging, we have learnt from the ROXS system that redox blinking can be intentionally induced by additives in solution with OFF lifetimes that are somewhat controllable by for example, adjusting the concentration(s) of additives. Moreover, redox blinking occurs on a slower timescale than triplet blinking and is accompanied by the formation of radical ions; thus, exploiting radical ion states for localization based SR is achievable [15].

Fluorescent confinement based stimulated emission depletion (STED) microscopy where fluorescence from emitters is supressed via an additional beam which induces stimulated

emission from excited species, shelving them into a dark state until the additional STED beam intensity is turned off in their vicinity (see figure 15(a) below); has been reported by Sauer and co-workers [16] where they used ATTO647N-labelled 60-base pair oligonucleotides immobilized under aqueous buffer conditions with oxygen removal and the MV + Trolox ROXS system. They show that under STED beam excitation intensities as high as 100mW on an area of ≈ 10^{-9} approximately 50% of fluorophores are still active whereas in the absence of ROXS, more than 90% of molecules are photobleached. They further demonstrated a resolution of around 35nm on the same labelled oligonucleotide.

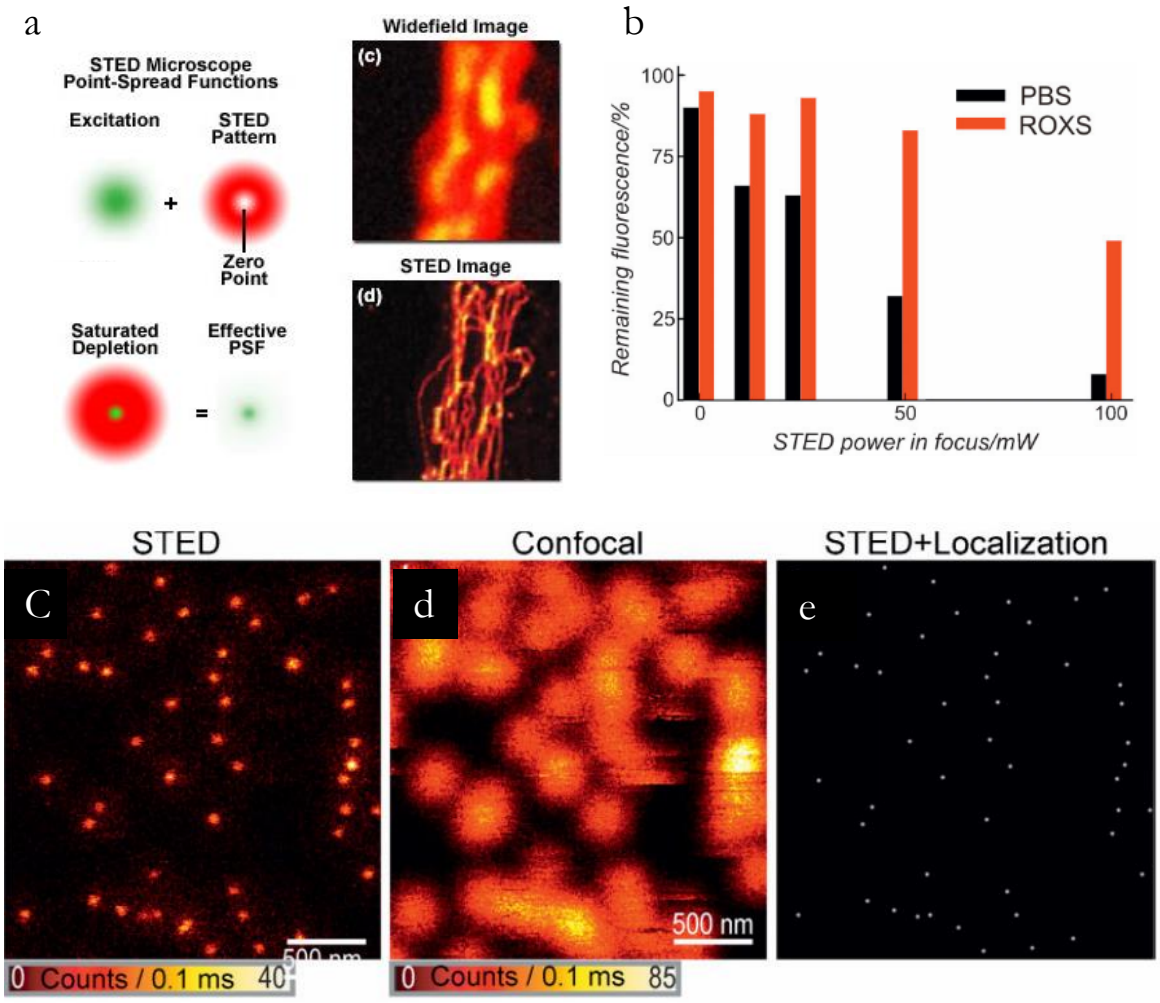

Figure 15: (a) General STED scheme showing the excitation and depletion beams and the effective PSF which enables SR imaging (image credit: www.zeiss-campus.magnet.fsu.edu) (b) results from [16] showing the improvement in fluorescence with ROXS under high STED beam intensities; (c-e) STED and confocal images of the ATTO647N-labelled 60-bp oligonucleotide, localization provided a resolution of about 35nm from [16]

In addition, a study by Cordes and Co-workers [12] on ds-DNA covalently linked to cyanine fluorophore via a 6-carbon linker suggests the general applicability of the self-healing concept to stochastic STORM-type imaging; thus despite the order-of-magnitude better stabilization afforded by solution based ROXS buffers, self-healing is also a viable option.

8. Conclusion and Outlook

The ultimate utility of fluorescence microscopy is heavily dependent on the fluorescent probes vis-à-vis their stability and brightness. Over the past two decades, advances in our understanding of the photophysical properties of several fluorophores have led to improved strategies of "photo-protection" – oxygen removal and using protective groups either as additives in solution or the more recent self-healing concept. However, we must keep in mind that the redox properties of fluorophores are dependent on the particular one in use, biological context and buffer condition; thus it is likely that there is no *one-size-fits-all* solution to enhancing photostability but rather we have to look on a case-by-case basis for guidelines that apply to specific dye families.

Solution additives are useful in improving photostability and brightness for some fluorophores but often fall short in biological settings; where their redox properties and hydrophobicity inhibit ordinary biological activity or is toxic to living cells. Consequently, self-healing fluorophores will be an important improvement for single molecule studies such as FRET and SR microscopy. Going forward, this will require concerted effort in non-natural amino-acid engineering, enzymatic peptide tagging, expressed protein ligation and other means of making organic fluorophores attach site specifically to biomolecules of interest.

It was the aim of this essay to give a short but vigorous overview of fluorophores for single molecule studies, highlighting the main challenges and the strategies that have been employed to circumvent them. In addition, we give examples of new experiments that have been enabled by the latest developments and possibilities for future experiments.

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