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Current Opinion in Chemical Biology

The bright future of single-molecule fluorescence imaging

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Single-molecule Förster resonance energy transfer (smFRET) is an essential and maturing tool to probe biomolecular interactions and conformational dynamics *in vitro* and, increasingly, in living cells. Multi-color smFRET enables the correlation of multiple such events and the precise dissection of their order and timing. However, the requirements for good spectral separation, high time resolution, and extended observation times place extraordinary demands on the fluorescent labels used in such experiments. Together with advanced experimental designs and data analysis, the development of long-lasting, non-fluctuating fluorophores is therefore proving key to progress in the field. Recently developed strategies for obtaining ultra-stable organic fluorophores spanning the visible spectrum are underway that will enable multi-color smFRET studies to deliver on their promise of previously unachievable biological insights.

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Current Opinion in Chemical Biology 2014, **20**:103–111

This review comes from a themed issue on **Molecular imaging**

Edited by **Christian Eggeling** and **Mike Heilemann**

For a complete overview see the Issue and the Editorial

Available online 21st june 2014

http://dx.doi.org/10.1016/j.cbpa.2014.05.010

Introduction

The 20th century saw the identification and characterization of the macromolecular constituents of life. A plethora of powerful techniques were developed to study these molecules at the ensemble level to understand their behavior and function and, perhaps most importantly, their ''malfunction'' due to disease. When the optical detection and spectroscopy of individual molecules in condensed matter became a reality in the late 1980s [1], it was soon recognized that this breakthrough would help researchers gain an entirely new perspective on the inner workings of biological systems [2,3].

While typical biochemical bulk experiments provide ensemble-averaged measurements of molecular properties, single-molecule approaches not only reveal the full probability distribution functions and their time dependence, but also enable the identification of sub-populations and transient intermediates. The resulting insights into heterogeneities and time-dependent fluctuations are fundamental for an accurate mechanistic description of bio-molecular function [3].

Among the possible far-field, optical readout modes for single molecules [1], fluorescence is notable for its simplicity of implementation, molecular specificity, contrast, and compatibility with multi-color and live-cell imaging [2,4]. Within the past two decades, single-molecule fluorescence techniques have proven their potential and are now routinely used in many biological investigations [5]. A key limitation that has been noted, however, is the need to broaden the range of imaging time scales that can be achieved to gain deeper insights into both rapid and slow time scale processes [6,7••,19•].

Another key challenge of contemporary single-molecule fluorescence imaging relates to the growing need to correlate multiple events in space and time. The function of many — if not most — complex biological systems entails both time-dependent changes in conformation and composition. If the goal is to dissect the macromolecular machinery in all of its complexity, the observation of only one molecular species or the interaction of just one pair of species at a time, providing only a partial view, is vastly insufficient. Fortunately, fluorescence techniques readily lend themselves to the simultaneous observation of multiple processes through the use of spectrally distinct fluorophores. Owing to instabilities of the available fluorophores, however, their selection is often a performance-limiting factor [8•,7••,34].

In this review, we focus on single-molecule Förster resonance energy transfer (smFRET) using small-molecule organic fluorophores, a technique that is widely used to probe macromolecular binding and conformational dynamics [5]. While multi-color smFRET for the correlation of multiple events was introduced nearly a decade ago [9], it has only recently gained traction as a tool to solve important biological problems (reviewed in [10••,11]).

The mainstream use of multi-color smFRET has been substantially held back by the paucity of bright and long-lasting complementary fluorescent probes, which are required to enable the imaging of complex systems at

biologically relevant timescales [8•]. In this review, we therefore highlight recent innovations in the design of organic fluorophores that have the potential to expand the palette of bright and stable fluorescent probes spanning the visible spectrum. Particular emphasis is placed on ''self-healing'' dyes developed in our lab, where undesirable dark states are quenched intrinsically through an incorporated protective moiety [12••,13]. We further consider how the combination of ultra-stable dyes with other emerging technologies, including faster detectors and high-throughput imaging platforms, will expand the scope of smFRET experiments to new physical and kinetic regimes currently beyond reach.

The power of multi-color smFRET

FRET (Förster resonance energy transfer) is a powerful tool to investigate the dynamics of macromolecular machines by detecting nanoscale conformational changes as well as binding events. FRET is based on an interaction occurring between two fluorophores in close proximity (10–90 Å) [14] (Figure 1a). Excited-state energy from a donor fluorophore is partially transferred to the nearby acceptor through non-radiative dipole–dipole coupling, leading to fluorescence emission of the acceptor accompanied by (partial) quenching of the donor. The transfer efficiency is strongly distance-dependent (following an inverse sixth-power law), thus allowing the experimentalist to infer changes in the relative position of the two fluorophores from the ratio of donor and acceptor fluorescence emissions [15] (Figure 1a). For excellent practical introductions to smFRET we refer the reader to [15] and [16].

Since the first observation of a single donor–acceptor pair [17], the use of smFRET has grown rapidly [5]. It has enabled important insights into biological systems [18], including protein folding and binding [19•,66], RNA folding and catalysis [20], transcription [21], translation [6,22], and membrane transporters [23–25].

The observation of a single FRET pair, however, yields only a single distance vector. Thus, multiple FRET measurements from distinct structural perspectives are typically required to deduce the origin of the observed motion [26–28]. This limitation leaves open the possibility that separate measurements may be difficult to compare due to experimental variability. Multicolor-FRET imaging has the potential to circumnavigate such challenges to reveal whether conformational events are correlated, uncorrelated or partially coordinated in nature. Figure 1b illustrates how multi-color smFRET may help differentiate between alternative biological models. In our example, multiple conformational events are required to achieve an ''unlocked'' (*i.e.* activated transient intermediate) configuration of the ribosome on path to translocation of the mRNA and tRNAs [27]. Probing the interplay of three fluorophores enables discrimination of whether these events proceed in a coordinated or partially uncoupled fashion.

The first implementation of multi-color smFRET was demonstrated using a combination of one donor (Cy3) with two acceptors (Cy5 and Cy5.5) [9]. A key shortcoming of these early experiments related to the considerable spectral overlap of the Cy5 and Cy5.5 acceptor fluorophores. This was significantly improved by the introduction of the dye combination Cy3/Cy5/Cy7 [29] (Figure 1c/d), which exploits the previously unoccupied near-infrared spectral region and remains in common use for three-color smFRET imaging [30••,31]. The Cy7 fluorophore is, however, particularly labile under the intense illumination that is required for single-molecule imaging [12••].

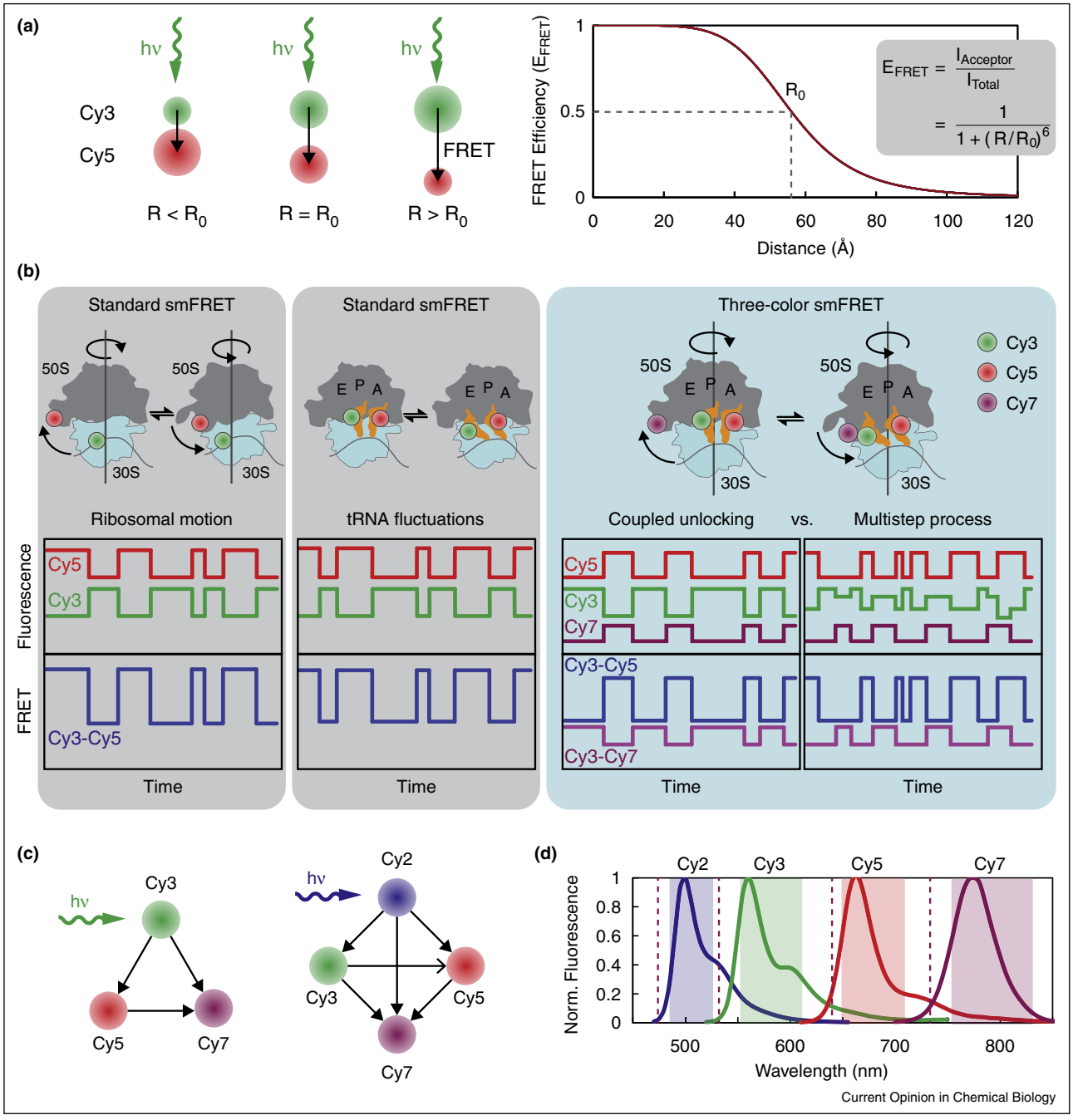
Three-color smFRET, while expanding the scope of measurable phenomena, is not capable of probing two fully independent molecular interactions. This capability was recently introduced with the advent of four-color smFRET [32••,33], where Cy2 was used as the additional donor fluorophore (Figure 1c/d). While four-color smFRET is capable of probing complex biological systems by exploiting the multitude of pairwise interactions that can occur be (Figure 1c), the increased demands associated with optically separating several fluorescence signals and high-intensity illumination at multiple wavelengths tends to cause rapid photobleaching and severely limit the signal-to-noise ratio and imaging time — and thus the interpretation — of such experiments.

Advances in organic fluorophores for single-molecule imaging

Detecting single molecules requires bright, long-lasting fluorophores. This requirement is amplified in multicolor techniques, where several such fluorophores must be observed simultaneously before photobleaching. Like most fluorophores, organic dyes are compromised by the prevalence of non-emissive ''dark states'' that limit the brightness and duration of fluorescence emission [7••,8,34]. Hence, the choice of suitable fluorophores, as well as specifically optimized buffer conditions to maximize photon emission, are indispensable for a successful experimental outcome.

Phenomenologically, fluorophore dark states can be divided into intermittent transitions (blinking) and permanent damage to the fluorophore, rendering it non-emissive (photobleaching). Photophysically, such transitions disrupt the excitation-induced cycling of a fluorophore between its electronic ground state S_0 and the first excited singlet state, S_1. Figure 2a illustrates some of the most important processes, where orange arrows indicate the desired S_0-S_1 cycling. Intersystem crossing (ISC) of a fluorophore from S_1 to a non-fluorescent triplet state, T_1, has been identified as a key transition determining fluorophore performance [7••]. While ISC is a rare event for typical fluorophores (quantum yield <0.01), triplet lifetimes are orders of magnitude longer (10^{-6}–10^{-4} s) compared to those of singlet

Figure 1

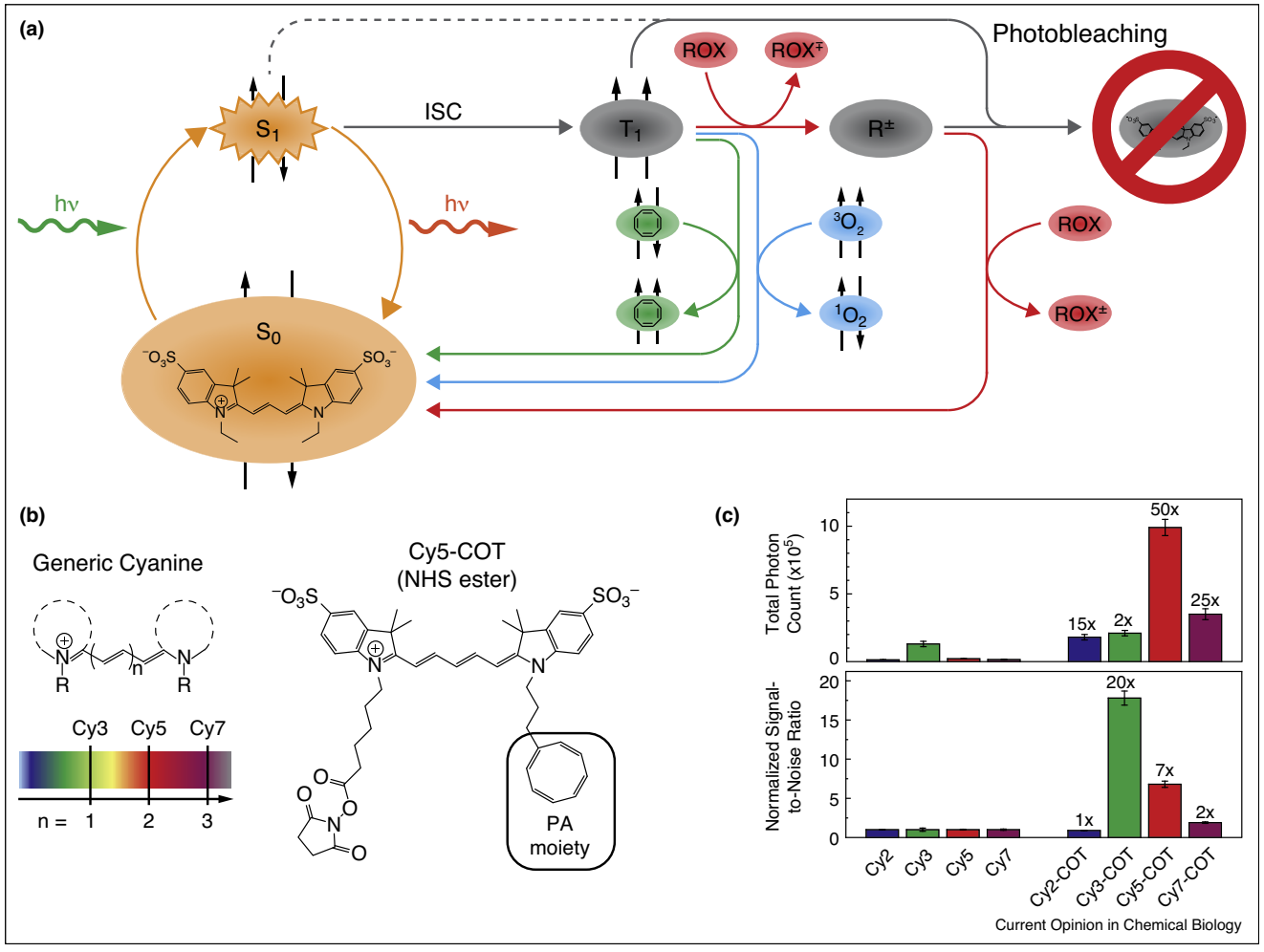


Principles of multi-color smFRET. **(a)** Left: illustration of energy transfer from a donor fluorophore (here: Cy3) to an acceptor fluorophore (here: Cy5). Right: distance dependence of energy transfer. **(b)** Illustration of the correlation of conformational events by multi-color smFRET. Grey panels: two independent structural perspectives monitored by standard two-color smFRET experiments. Blue panel: three-color smFRET experiments distinguishing between fully coupled (left) and partially uncoupled (right) modes of ribosomal motions. **(c)** Possible FRET interactions between three and four fluorophores. **(d)** Emission spectra of common cyanine dyes frequently used in smFRET imaging, along with typical excitation laser wavelengths (dashed lines) and detection windows (shaded areas).

excited states (10^{-10}–10^{-9} s). In addition to directly causing blinking, triplet excursions also mediate downstream photobleaching due to the highly reactive nature of the triplet state.

Although molecular oxygen is a strong triplet state quencher that tends to suppress triplet-induced blinking events (blue arrow in Figure 2a), the oxidation of the fluorophore by molecular oxygen is particularly detrimental to

Figure 2



Fluorophores for single-molecule imaging. **(a)** Schematic view of key photophysical processes related to fluorophore instabilities and their mitigation (S_0 – ground state; S_1 – first excited singlet state; T_1 – triplet state; $R^{\pm}$ – radical ion; ISC – intersystem crossing; ROX –reduction/oxidation). **(b)** Left: generic structure of cyanine fluorophores, characterized by the central polymethine chain, whose length is a key determinant of spectral properties. Right: NHS ester of a protective agent (PA)-conjugated self-healing Cy5 molecule. **(c)** Comparison of total photon emission and brightness (as represented by the signal-to-noise ratio) of conventional and COT-conjugated cyanine dyes across the visible spectrum.

single-molecule imaging [35,36], as it generates a highly reactive superoxide radical (O_2^-) along with a cationic radical form of the fluorophore that are susceptible to photobleaching. The common practice of removing molecular oxygen from the imaging buffer through enzymatic oxygen scavenging systems [37–40], therefore, has the ambivalent result of improving overall fluorophore lifetimes before photobleaching at the expense of increased triplet-induced blinking.

To further improve the emission rate and total photon emission of organic fluorophores, a variety of soluble small-molecule protective agents [41] have been used in many single-molecule fluorescence studies (green arrow in Figure 2a). For an in-depth discussion of these, mostly redox-active compounds, we refer the reader to [7••]. An especially elegant and successful strategy to control the blinking behavior of fluorophores *via* solution additives is the use of a combined, carefully balanced reducing and oxidizing system (ROXS, red arrows in Figure 2a) [42,43,74]. This has proven to be of particular importance in localization-based super-resolution microscopy, where finely tuned control of both bright and dark states is desirable [8•,75].

While solution-based protective agents have afforded remarkable improvements in fluorophore performance, several factors limit their effective use in biological studies. The millimolar concentrations required to provide sufficient collision frequencies with dye molecules are often near the solubility limit of these substances. In addition, their use may interfere with the integrity of biological systems [46]. Finally, the effect of a given protective agent on different fluorophores is not universal [42,43]: what benefits one fluorophore may be detrimental to another. This is

especially problematic for multicolor approaches, which require combinations of fluorophores that all perform well under the same environmental conditions.

In an effort to generate intrinsically stabilized fluorophores, we have recently synthesized derivatives of commonly used cyanine fluorophores [45•] with protective agents covalently attached to the fluorogenic center (Figure 2b) [12••] [13]. The goal of this strategy was to achieve the maximum ''local concentration'' of the single, specific protective agents, 1,3,5,6-cyclooctatetraene (COT), 4-nitrobenzylalcohol (NBA), and Trolox. These engineered fluorophores were shown to exhibit markedly reduced blinking and lower photobleaching rates than the parent compounds.

Recent studies [35,46,47] have provided further insights into the mechanisms of photostabilization of these so-called ''self-healing dyes'', showing that COT operates through a triplet-triplet energy transfer mechanism while NBA and Trolox appear to function through a less controllable charge transfer mechanism. Focus was therefore given to the generation of COT-linked cyanine fluorophore derivatives where substantial benefits were observed in fluorophore performance across the visible spectrum (Figure 2c). It was recently shown that COT-linked derivatives of Cy5 exhibit negligible saturation of fluorescence excitation under increasing illumination intensities — a finding compatible with single-molecule FRET imaging at millisecond time resolution [7••]. By extension, such efforts may also apply to multicolor techniques. To ultimately achieve such a goal, synergistic strategies are likely to be required to reduce propensities of cyanine fluorophores to undergo cis–trans isomerization of the polymethine chain [48]. Considerations related to solvent shell effects may also need to be included as water itself can be a quenching determinant [49,50].

While cyanine dyes remain the most commonly used fluorophores in single-molecule studies [8•,45•], a variety of alternative dye combinations have been used for multicolor smFRET, including dyes from the Alexa [51–53] and ATTO [54] families of commercially available dyes. It should be noted, however, that certain dyes can suffer from spectral shifts that can be problematic in multi-color imaging [32,8•]. Although it remains to be determined whether analogous self-healing strategies can be applied to rhodamine, oxazine, and carbopyronine dyes, such results are anticipated given the universally negative impact of triplet states on fluorophore performance [7••]. Achievements in this area are expected to broadly impact diverse imaging modalities.

Expanding the scope of smFRET measurements

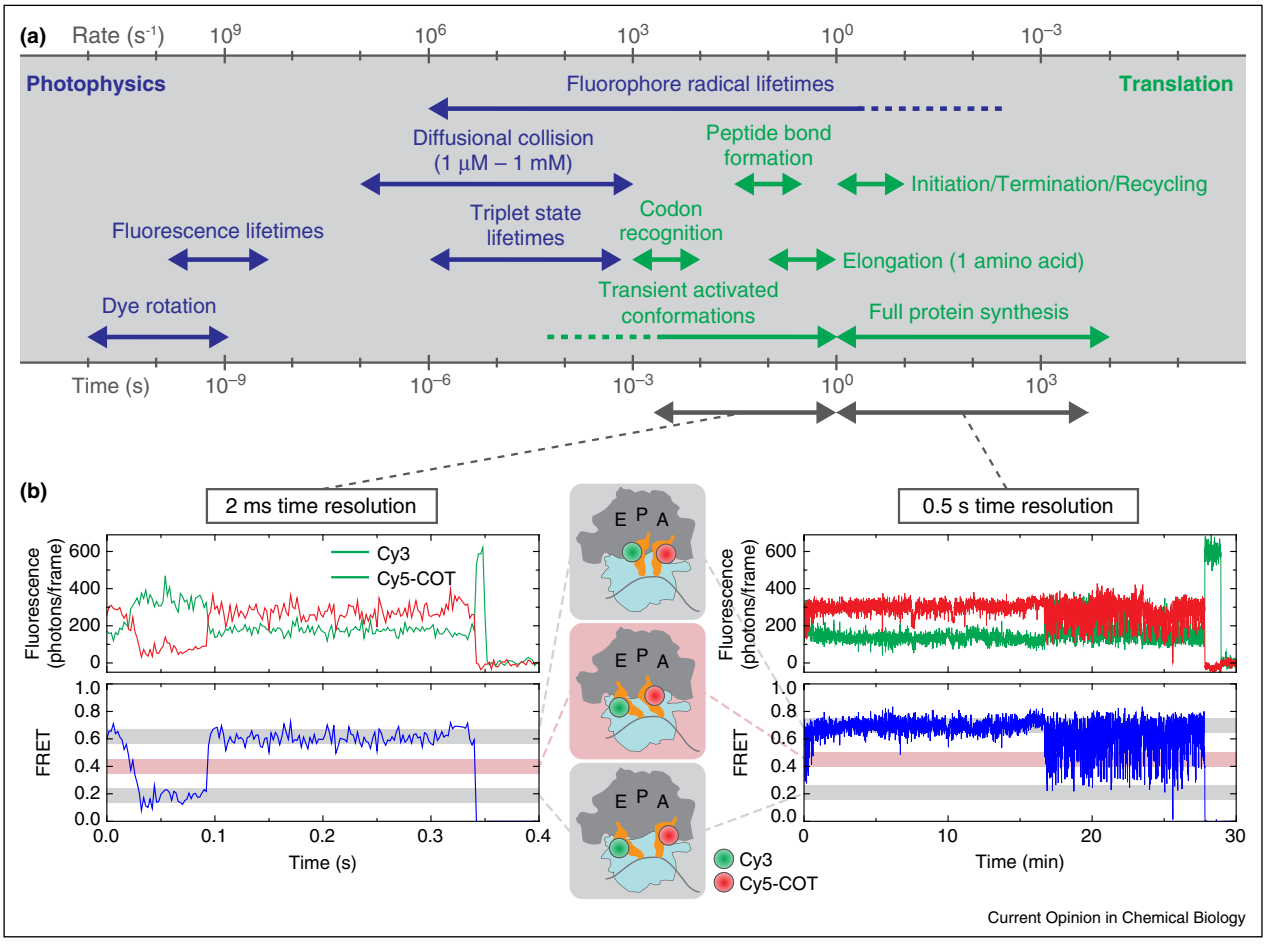
While fluorophore development is only one aspect of the multifaceted challenge to increase the scope and depth of information accessible by smFRET imaging [10••,11], the spectral versatility, long lifetimes before photobleaching, and high photon emission rates exhibited by the emerging new generation of fluorophores have the potential to enable single-molecule investigations of unprecedented spatiotemporal resolution. Important biological events occur in nature at timescales that typically exceed the rates of photon emission that can be achieved with commercially available dyes due to saturation of triplet states or photobleaching under intense illumination. Observing fast timescales of milliseconds to microseconds through fluorescence has mostly been limited to the analysis of short emission bursts, *e.g.* in fluorescence correlation spectroscopy (FCS) [55] or smFRET implemented on confocal microscopes [56].

Wide-field approaches that facilitate the generation of robust statistics over short time periods and that enable pulse-chase type, pre-steady state measurements [15] are typically limited in temporal resolution by the readout speed of electron-multiplying charge-coupled device (EM-CCD) cameras. Recently developed, scientific complementary metal-oxide semiconductor (sCMOS) cameras with much faster readout and larger field of view have, however, been shown to have sufficient performance to increasingly replace EM-CCDs for single-molecule imaging at fast time resolutions (milliseconds and below) [57,58•]. Combined with sophisticated microfluidic systems for millisecond scale mixing of reagents [59•], these novel technologies will enable fast pre-steady state kinetic measurements *in vitro*. For live-cell studies, combining smFRET with fast, active single-particle tracking [60] and tracking FCS [61] technologies may enable the investigation of conformational processes in an undisturbed functional context.

The ability to perform experiments over a vast range of timescales is essential for investigations of an increasing number of molecular machines, whose distinct functions dictate dynamics in entirely different temporal regimes. Even processes within the same machine may be orders of magnitude apart in timescale, as different functional units perform individual steps of a complex process. In the case of the translating ribosome, the selection of aminoacyl-tRNA occurs on the sub-millisecond to millisecond timescale [28], while the synthesis of an entire protein may take many minutes (Figure 3a). The development of ultra-stable fluorophores will therefore be paramount to gaining direct insights into the mechanistic features of processive reactions spanning all timescales relevant to function.

Self-healing cyanine fluorophores, in combination with current detector technology, already enable investigations covering a wide range of timescales. To illustrate this, we imaged dynamic processes within ribosomal pre-translocation complexes at both 2 ms and 0.5 s temporal resolution using sCMOS technology (Hamamatsu Orca-Flash 4.0 and W-View Gemini image splitting optics) (Figure 3b). While the fast measurements highlight the occurrence of non-accumulating, transient species related

Figure 3



Temporal regimes accessible in smFRET imaging. **(a)** Overview of relevant timescales for the photophysics of organic fluorophores (blue) and for translation (green). **(b)** Fluctuations in ribosomal pre-translocation complexes observed using site-specifically labeled tRNA molecules as previously described [62](see cartoon) imaged at 2 ms and 0.5 stime resolution (1.4 W and 8 mW laser excitation at 532 nm, respectively). Data were collected in Tris–polymix buffer using ribosomal complexes and a wide-field TIRF illumination system as previously described [62].

to activated reaction intermediates [26,62,63] (red shaded area), the longer timescale measurements demonstrate that the same system exhibits signatures of dynamic disorder that may be of unknown importance to ribosome function.

These data are meant to emphasize the importance of both fine-grained and coarse-grained observations to obtain a comprehensive picture of biological function. While advanced fluorophores and imaging technologies are enabling ever higher time resolution, they do not yet enable the ultimate goal of millisecond-resolution imaging with hour-long observation times.

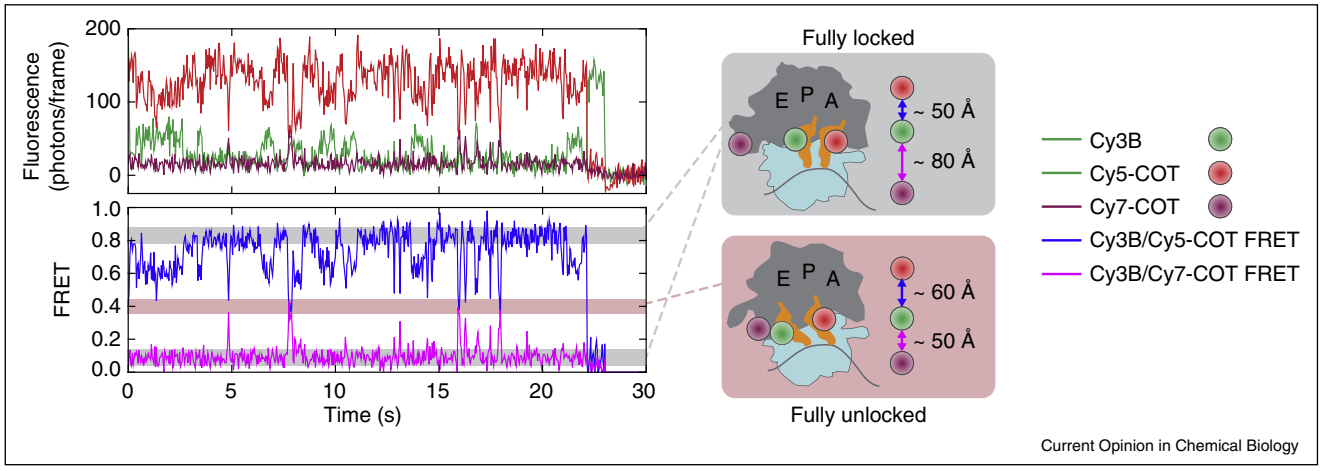
As dye performances tend to be broadly distributed, the study of complex, processive reactions of benefit from large numbers of single-molecule observations. High-throughput, potentially automated instrumentation has the potential to enable highly parallelized observations. An example of such an instrument has been achieved with the recent adaptation of a commercially available DNA sequencing machine into a widely applicable high-throughput platform for up to four-color single-molecule experiments [64••].

Biological impact of multi-color smFRET

As exemplified by the photophysical shortcomings discussed above, smFRET imaging with multiple fluorophores remains remarkably challenging. Proof-of-principle and other early studies have often focused on oligonucleotides [9,54,65], which are photophysically favorable [34] but enable only a limited scope of investigations.

Owing to the advent of optimized dyes and imaging conditions, three-color smFRET has successfully been applied to a variety of biological systems and made an impact on the study of ribosomal dynamics and assembly [27,30••] as well as protein folding and binding (reviewed in [66]). Recent studies have investigated elastic deformations

Figure 4



Multi-color smFRET with self-healing fluorophores. Ribosomal pre-translocation complexes were labeled as shown (cartoon) on tRNA molecules and ribosomal protein L1 and imaged as described previously [27]. Fluorescence (top) and FRET traces (bottom, defined here as acceptor fluorescence divided by total fluorescence) of labeled pre-translocation complex imaged at 40 ms time resolution during continuous laser excitation (120 mW, 532 nm wavelength).

of the rotary motor of a bacterial ATP synthase [67], the quarternary structure of RecR oligomers [68], the activity and folding of 10–23 deoxyribozyme [52], conformational dynamics and ligand binding of the maltose-binding protein [31] and dynamics of intrinsically disordered proteins [53]. Four-color smFRET, by contrast, has yet to prove its potential outside of oligonucleotide systems [32••,33,69].

While the scarcity of suitable combinations of fluorophores, as well as the challenges associated with the potential need for orthogonal biochemical labeling strategies are serious impediments, the demand for fluorophore performance in these kinds of experiments is an especially limiting aspect of three-color and four-color single-molecule fluorescence imaging. We therefore expect developments in the area of highly stabilized fluorophores that span the visible spectrum will have a major impact in this field by improving signal-to-noise ratios and extended observation times.

To illustrate this point, we have used the dye conjugates Cy5-COT and Cy7-COT, in combination with Cy3B [48], to perform three-color smFRET imaging of intrinsic dynamic processes in the *E. coli* ribosome with high photon counts (Figure 4). As we have highlighted in our initial discussion (Figure 1b), measurements of this kind are able to differentiate between correlated and uncorrelated motion, in contrast to standard smFRET experiments, where individual structural perspectives are observed in isolation. In our multicolor example, ribosomal domain motions and tRNA fluctuations appear as two partially uncoupled processes that contribute to the formation of an activated reaction intermediate, consistent with previous observations [27].

Conclusions

More than a decade into the 21st century, the significance of single-molecule biology is no longer a matter of contention (compare [2,5]). However, despite their ubiquity, single-molecule fluorescence studies have mostly focused on certain ''sweet spots'' on the map of spatiotemporal regimes relevant to biology [70].

As we have outlined in this review, some of the key obstacles to be overcome in cartographing new terrain include the photon emission rate and longevity of fluorescent dyes [7••], the concentration limit imposed by extended observation volumes [71], the challenge to combine sufficient experimental throughput with high time resolution [19•,64••], the development of bio-orthogonal labeling strategies [72], and the application of single-molecule techniques to living cells [73•]. Important inroads have been achieved to all of these aspects. While no technology can transcend its basic physical limitations, it is important to approach further development from the perspective of what is required to enable practical investigations.

Multi-color smFRET has just begun to find its stride as a viable tool for insightful biological investigations. A synthesis of efforts in dye and detector development, labeling chemistry, data analysis, and advanced instrumentation design will enable multi-color smFRET imaging to leverage its unique potential to elucidate correlated conformational events *in vitro* and in living cells.

Acknowledgements

Fluorophore and ribosome research in the Blanchard lab are supported by the US National Institutes of Health (GM098859 and GM079238, respectively to S.C.B.). M.R.W. is supported by US National Institutes of Health Research Service Award fellowship 5F31DC012026. M.F.J. is supported by a postdoctoral fellowship from the German Academic Exchange Service (DAAD).

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