Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Chemical Biology

The bright future of single-molecule fluorescence imaging

Manuel F Juette[1], Daniel S Terry[1], Michael R Wasserman[1], Zhou Zhou[1], Roger B Altman[1], Qinsi Zheng[1,2] and Scott C Blanchard[1,2]

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.

Addresses
[1] Department of Physiology and Biophysics, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10065, United States
[2] Tri-Institutional Training Program in Chemical Biology, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10065, United States

Corresponding author: Blanchard, Scott C (scb2005@med.cornell.edu)

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**

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