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TOPICAL REVIEW

Surfing on a new wave of single-molecule fluorescence methods

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Abstract
Single-molecule fluorescence microscopy is currently one of the most popular methods in the single-molecule toolbox. In this review, we discuss recent advances in fluorescence instrumentation and assays: these methods are characterized by a substantial increase in complexity of the instrumentation or biological samples involved. Specifically, we describe new multi-laser and multi-colour fluorescence spectroscopy and imaging techniques, super-resolution microscopy imaging and the development of instruments that combine fluorescence detection with other single-molecule methods such as force spectroscopy. We also highlight two pivotal developments in basic and applied biosciences: the new information available from detection of single molecules in single biological cells and exciting developments in fluorescence-based single-molecule DNA sequencing.

Introduction

Breakthroughs in understanding the natural world often rely on innovative instruments and analytical methods. Fluorescence, the process of light emission from molecules propelled to a light-induced excited state, has been contributing to the physical and biological sciences for decades (Lakowicz 2006). The past 20 years, however, have been marked by remarkable advances in fluorescence detection, especially as applied to the biosciences. During the 1990s this development was catalysed by the introduction of novel fluorescence probes, biological assays and instruments that helped the migration away from the use of radioactivity; such assays include fluorescence-based approaches for DNA sequencing, DNA arrays, real-time PCR methods and the increasing use of genetically encoded fluorescent proteins for cell-based assays.

In the early 1990s, adventurous physicists and physical chemists reached the ultimate detection limit by observing individual fluorescent molecules, heralding the era of single-molecule fluorescence detection. These measurements rapidly progressed from cryogenic to ambient temperatures, capturing the attention and imagination of many life scientists who saw enormous opportunities for capturing novel views of their favourite biological mechanisms. At that point, single-molecule fluorescence became one more sibling in the family of single-molecule biophysical methods, which already included single ion-channel recording, atomic force microscopy and optical tweezers (for reviews, see Walter *et al* (2008), Kapanidis and Strick (2009) and Deniz *et al* (2008)). These methods offered fresh ways of looking at existing materials and outstanding problems, often uncovering novel and exciting behaviours that had remained hidden due to the ensemble- and time-averaging inherent to conventional analysis. Single-molecule methods could study asynchronous reactions and full pathways in real time, uncover short-lived intermediates and combine optical and mechanical measurements on single molecules; these capabilities complemented high-resolution structural methods (since the latter provide extremely detailed but usually static snapshots of biomolecules), as well as conventional biochemical analysis.

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The single-molecule fluorescence methods included the detection of single molecules both in solution and on surfaces. Some methods used a point-detection format adapted from fluorescence correlation spectroscopy (Elson and Magde 1974, Schwille *et al* 1997, Haustein and Schwille 2007) (a 'small-ensemble' method that reports on diffusion and reaction kinetics from temporal fluctuations in fluorescence intensity). The point-detection approach (based on confocal optics) typically monitors only one single molecule at a time but with up to picosecond temporal resolution. Other methods employed a wide-field format, often involving evanescent waves, to excite immobilized molecules and highly parallel imaging of single molecules with millisecond temporal resolution (Zhuang *et al* 2000). These methods used a single excitation wavelength and a single emission wavelength to study singly-labelled molecules, reporting on properties such as fluorophore location on a surface, fluorescence lifetime, quantum yield and diffusion coefficients (Nie and Zare 1997; Weiss 1999). Steps towards higher complexity added a second detection channel, providing a handle on the rotational freedom of fluorophores or on spectral jumps (reviewed in Weiss (1999)) The subsequent use of two fluorophores on a single molecule led to interprobe distance measurements, either through high-resolution localization (Ha *et al* 1996a) or fluorescence resonance energy transfer (FRET; a.k.a. Förster resonance energy transfer (Förster 1948)), a dipole–dipole interaction occurring typically between two fluorophores. FRET has been used to study biomolecular structure and dynamics, since the steep distance dependence of FRET efficiency makes it useful as a nanoscale ruler (Schuler *et al* 2005, Stryer and Haugland 1967).

Not surprisingly, innovations in the field of single-molecule fluorescence continue to spawn new tools and instruments. Here we review some of the advanced single-molecule fluorescence methods, which we group under the umbrella of the new wave of modern fluorescence methods. These methods are characterized by increased instrumentation complexity and integration of measurement modalities and can address complex samples, such as living cells, or adhere to complex requirements, such as the ones dictated by the need for accurate genome sequencing. The new methods are also characterized by their ability to overcome some of the previously confounding issues of the first single-molecule fluorescence methods. We will discuss some of the most exciting advances and provide examples of applications to biological questions and biotechnological assays. Due to limited space, we will not be able to cover exciting advances in fields such as biomolecular structural analysis (Wozniak *et al* 2008, Muschielok *et al* 2008, Andrecka *et al* 2008), use of local-environment changes for single-molecule kinetics (Myong *et al* 2009, Sorokina *et al* 2009), single-molecule tracking (Kusumi *et al* 2005, Dahan *et al* 2003) and single-molecule FRET (Sako *et al* 2000), studies on the exterior of cells or novel methods of data analysis (Kalinin *et al* 2008, Nir *et al* 2006, McKinney *et al* 2006). For other excellent reviews of single-molecule fluorescence methods, we refer the reader to Walter *et al* (2008), Roy *et al* (2008), Brewer and Bianco (2008), Joo *et al* (2008) and Moerner (2007).

In this review, we focus mostly on instrumentation and we will not discuss molecular mechanisms in detail. We will discuss new multi-laser/colour techniques, super-resolution microscopy imaging and the development of instruments that combine fluorescence detection with other single-molecule methods. We also describe two pivotal developments in basic and applied bioscience, namely the ability to interrogate single molecules in single cells and the race for fluorescence-based single-molecule DNA sequencing.

The new wave: single-molecule fluorescence on the path of complexity

Recent advances in single-molecule fluorescence originate from necessity, availability of new tools such as instrumentation and fluorophores and specialized training. Many of the advances reflect the need to go beyond the initial, simple observations; the first reports of single-molecule detection were seminal proof-of-principle experiments, showing what can be achieved with simple samples such as fluorescent dyes and DNA fragments. These measurements, however, were fraught with problems due to fluorophore photobleaching, complex photophysical behaviour, inability to work at fluorophore concentrations higher than 200 pM, complications with multi-component biomolecules and surface-immobilization challenges, just to name a few. Over the years, several solutions have emerged to address these challenges and standardize the methods, gradually shifting the emphasis to answering biological questions and developing commercially viable biotechnological platforms. For example, encapsulation of proteins in vesicles (Boukobza *et al* 2001, Okumus *et al* 2004, Rhoades *et al* 2003) allowed monitoring over extended periods of time without potential perturbation due to direct surface immobilization. Moreover, our improved understanding of fluorophore photophysics (such as blinking, bleaching and triplet states) dramatically improved our ability to control photobleaching and blinking (Funatsu *et al* 1995, Rasnik *et al* 2006, Vogelsang *et al* 2008). Finally, the introduction of novel fluorophores, such as fluorescent quantum dots (Bruchez *et al* 1998, Resch-Genger *et al* 2008, Michalet *et al* 2005) and improved fluorescent proteins (Tsien 1998, Shaner *et al* 2007) provided two major alternatives to organic fluorophores. The serendipitous discovery of conditions that turn stable fluorophores into controllable photoswitches (Bates *et al* 2005, Heilemann *et al* 2005, 2009, Vogelsang *et al* 2009, Steinhauer *et al* 2008) was also central to the ever-expanding family of super-resolution methods (see section *Beyond the diffraction limit: super-resolution imaging*).

The contribution of technological innovation to the new wave cannot be overestimated. New and affordable components for custom-built fluorescence microscopes (robust lasers, low-fluorescence background microscope objectives, fluorescence filters with novel coatings, fast avalanche photodiodes, electronics for high resolution photon counting, electronics for set-up automation) reduced the overall cost, bulk and complexity of the experimental setups while adding new capabilities. In fact, commercial microscopes suitable

for single-molecule detection are currently available from several companies or microscopes can be home built for less than $100k, something very difficult 20 years ago. Micro- and nano-fabricated reaction chambers and microfluidics have also been useful (Brewer and Bianco 2008, Craighead 2006) for precise delivery of reagents, especially for commercial applications. A special mention should be given to CCD (charged-coupled device) cameras invented more than 40 years ago, the development of which captured the 2009 Nobel Prize in Physics. In particular, the electron-multiplying versions of CCDs (EMCCDs), can nowadays combine >90% quantum efficiency with high speed (30 ms per 512 × 512 pixel frame) to obtain diffraction-limited images of thousands of single molecules simultaneously either *in vitro* or in living cells (Goulian and Simon 2000, Xie *et al* 2008). This single piece of equipment has boosted the popularity of wide-field imaging methods such as total internal reflection fluorescence (TIRF) microscopy (Axelrod 2001, Moerner and Fromm 2003, Walter *et al* 2008).

Naturally, the methods can only advance if driven by young researchers with specialized training in the interdisciplinary field of single-molecule biophysics. This training has been provided mainly in the growing number of single-molecule biophysics laboratories, as well as through university courses, workshops and a range of books dedicated to single-molecule detection (Selvin and Ha 2008, Hinterdorfer and Oijen 2009, Bräuchle *et al* 2009, Zander *et al* 2002).

Single-molecule FRET using multi-parameter excitation and detection

In typical single-molecule FRET (smFRET) experiments, after excitation of the donor fluorophore via a light source, the energy can be transferred to a lower energy fluorophore (acceptor) in close proximity (<10 nm). Using two fluorophores covalently attached to DNA, RNA, protein or other biomolecules, distances in the range of 3–10 nm can be measured with up to single base-pair resolution (0.34 nm, see Christian *et al* (2009)). smFRET was first demonstrated on surface-immobilized molecules (Ha *et al* 1996b), followed by detection within diffusing molecules (Deniz *et al* 1999). This proof-of-principle work led to many ground-breaking experiments on (1) *dynamics*: DNA (Hohng *et al* 2004b), RNA (Ha *et al* 1999, Kim *et al* 2002) and proteins (Henzler-Wildman *et al* 2007); (2) *folding*: proteins (Deniz *et al* 2000, Schuler and Eaton 2008, Schuler *et al* 2002, Rhoades *et al* 2003) and RNA (Zhuang *et al* 2000, 2002, Solomatin *et al* 2010); (3) *interactions*: protein–DNA (Rothwell *et al* 2003, Ha *et al* 2002), protein–RNA (Abbondanzieri *et al* 2008) and protein–protein (Tan *et al* 2004) and (4) *molecular machines*: linear (Tomishige *et al* 2006, Mori *et al* 2007) and rotary (Borsch *et al* 2002, Diez *et al* 2004, Zimmermann *et al* 2006).

An important advance in the field of smFRET was the introduction of alternating-laser excitation (ALEX) spectroscopy, which allowed essentially simultaneous probing of FRET efficiency and relative probe stoichiometry within either diffusing or surface-immobilized single molecules (Kapanidis *et al* 2004, 2005, 2006, 2008, Lee *et al* 2005, Margeat *et al* 2006, Santoso *et al* 2008, 2010). This is achieved by using an excitation source that alternates between a wavelength that excites the FRET donor directly (and can thus probe the presence of FRET) and a wavelength that excites the acceptor directly (and can thus probe the donor–acceptor stoichiometry). The contribution of this family of FRET methods rested in its ability to identify and analyse molecules with the desired 1:1 donor:acceptor stoichiometry, which often represent only a small minority of the examined molecules due to incomplete labelling, photobleaching, blinking or incomplete formation of biomolecular complexes. Moreover, the ALEX approach can directly observe photophysical changes in the acceptor as well as formation of higher order complexes; both scenarios can complicate the analysis of single-laser excitation FRET measurements. The method has been used extensively for addressing mechanistic questions, especially in protein–DNA interactions (Kapanidis *et al* 2005, 2006, Margeat *et al* 2006, Santoso *et al* 2008, 2010).

The long list of successful projects using a single donor–acceptor pair is a testament to the usefulness of the approach; however, for complex conformational dynamics or multicomponent binding interactions, simultaneously measuring more than one donor–acceptor FRET pair would be insightful. Simply by adding a third fluorophore (a third 'colour') to an existing donor–acceptor pair, one could monitor multiple molecular interactions—each dye combination in total effectively yielding three two-colour experiments. Several studies have implemented three-colour FRET at the ensemble level (Ramirez-Carrozzi and Kerppola 2001, Haustein *et al* 2003, Galperin *et al* 2004, Klostermeier *et al* 2004). With the work of Hohng *et al*, three-colour FRET schemes were extended to the single-molecule level, in which a single donor and two alternative acceptors permitted observation of correlated conformational changes of different helical arms in individual surface-immobilized Holliday junctions (Hohng *et al* 2004a). Three-colour FRET was subsequently realized for freely diffusing molecules, where both binding stoichiometry and distance information were probed (Heinze *et al* 2004, Clamme and Deniz 2005).

While providing insight into the processes investigated, multi-colour methods, like their two-colour counterparts, lacked generality and were hindered for the following reasons: (1) *proximity limitations*: the methods introduced proximity constraints, relying heavily on substantial FRET between all probes to monitor interactions and distances, thus prohibiting quantitative analysis of molecular interactions, particularly when probes are separated by >7–10 nm; (2) *distance measurements*: accurate distance information cannot be easily obtained without detailed corrections and auxiliary experiments measuring FRET between each individual dye pair; (3) *special optics*: depending on interprobe distances and the characteristic Förster distance for different donor–acceptor FRET pairs, large spectral overlap between dye pairs (e.g., Cy5 and Cy5.5) requires careful selection of optics necessary to separate their signals, thus imposing instrumental constraints; (4) *biased detection*: species existing in interaction equilibrium but not appreciably

excited by the single-laser excitation scheme used in the studies cannot be detected; (5) *labelling efficiencies*: stoichiometric conditions other than 1:1:1 either cannot be sufficiently resolved or differentiated (e.g., 1:2:1 from 1:1:1); (6) *fluorescence pathways*: single-laser excitation schemes cannot readily distinguish between alternative FRET pathways and (7) *dye photophysics*: single-laser excitation schemes cannot adequately deconvolve individual dye photophysics from actual biological events (e.g., dye bright-dark state interconversion from conformational fluctuations). Most of these limitations arise from the clash between the need for spectral overlap between fluorophores with the need for spectral separation of donor and acceptor signals for detection.

To address these shortcomings, several methods have improved existing multi-colour techniques and maximized the information available from each measurement (figure 1). Along these lines, Lee *et al* extended two-colour alternating excitation schemes (Kapanidis *et al* 2004, Lee *et al* 2005) by introducing alternating laser excitation (ALEX) with three-colour FRET of diffusing molecules (Lee *et al* 2007); this development enabled monitoring of multiple interactions and distances without previous information about molecular structure. Specifically, modulated excitation of three lasers permitted virtual molecular sorting of singly-, doubly- and triply-labelled species independent of FRET efficiency (figure 2). Additionally, by simultaneously monitoring the three intermolecular distances of each dye pair, three-dimensional FRET histograms were used to resolve conformational heterogeneity more effectively than standard one-dimensional FRET histograms, with the additional benefit of being able to simultaneously monitor the translocation of protein from two different perspectives on a DNA track.

While solution-based measurements can uncover the presence of heterogeneity, recent development of multi-colour configurations designed to probe immobilized molecules opens avenues to study the sources of heterogeneity. Using confocal scanning microscopy and three-colour ALEX, Ross *et al* implemented programmable acousto-optical beamsplitters with user-defined wavelengths to achieve the same detection efficiency as single-excitation dual-colour setups. This setup allowed resolution of seven stoichiometric subpopulations, as well as several structural subpopulations, in the presence of competing FRET pathways, and permitted observation of correlated molecular movements (Ross *et al* 2007). Subsequent work in the Tinnefeld laboratory demonstrated the correlated movement and bending of three-way DNA junctions upon steroid binding, inferring conformational changes leading to adoption of a rigid DNA structure (Person *et al* 2009).

Three-colour methods have also been extended to TIRF microscopy. Friedman *et al* employed a novel microscope design in which the dichroic mirror traditionally used to spectrally separate excitation and emission paths was replaced with small broadband mirrors, allowing spatial segregation of excitation and emission paths and efficient collection over a large spectral range (Friedman *et al* 2006). Importantly, by placing two small mirrors on either side of the objective, the authors were further able to greatly reduce background contributions due to optics' autofluorescence. Subsequent work from the same laboratory used the above microscope to visualize splicing of single pre-mRNA molecules in whole cell extracts, using low excitation intensities to drastically reduce photobleaching while maintaining a high signal-to-noise ratio (Crawford *et al* 2008). Lastly, recent work from Roy *et al* utilized three-colour TIRF to monitor single-stranded binding (SSB) protein diffusion on single-stranded DNA (ssDNA). Using a donor-labelled SSB mutant (Alexa555) and two different acceptors (Cy5, Cy5.5) attached to opposite ends of the ssDNA, the authors observed rapid anti-correlated fluctuations in the FRET efficiency of the two acceptors, providing strong evidence that the SSB diffuses along the ssDNA (Roy *et al* 2009).

Despite significant advances, three-colour ALEX and three-colour TIRF struggle to deconvolve dye photophysics from biological dynamics. Thus, the development of multi-parameter detection schemes presents vast potential in truly delineating complex chemical, physical and biological behaviour. In independent papers published in 2005, two groups (Müller *et al* 2005, Laurence *et al* 2005) extended ALEX to the nanosecond regime, thereby adding sub-microsecond resolved fluorescence correlation spectroscopy and fluorescence cross-correlation spectroscopy to the other possibilities of ALEX. Specifically, Müller *et al* (2005) introduced pulsed interleaved excitation (PIE), wherein multiple excitation sources are interleaved such that the fluorescence lifetime is significantly shorter than the interval between adjacent excitation pulses. Critically, the absolute/relative arrival time and excitation source for each photon are recorded using time-correlated single-photon counting (TCSPC), and by using sub-nanosecond pulses, fluorescence lifetimes and FRET can be measured. Laurence *et al* used a similar approach (termed nanosecond ALEX) to study conformational fluctuations in DNA and proteins that occur at time scales faster than 100 μs; one of the results unearthed by this study was the presence of transient residual structure in the state of unfolded protein (Laurence *et al* 2005). Düser *et al* applied PIE to study the step size of proton-driven c-ring rotation in F_oF_1-ATP synthase (Duser *et al* 2009), obtaining absolute distance information and providing the first experimental determination of a 36° stepping mode.

Another sophisticated approach, multi-parameter fluorescence detection (MFD), is fully compatible with PIE schemes (Widengren *et al* 2006). Using an experimental configuration incorporating a polarizing beamsplitter and a dichroic beamsplitter, the fluorescence light is divided into four photon streams (red and green parallel and perpendicular polarized light, respectively). Implementing picosecond pulsed single-laser excitation, Rothwell *et al* exploited MFD to simultaneously measure fluorescence intensity, lifetime and polarization and reveal three populations, indicating heterogeneous behaviour of HIV-1 reverse transcriptase primer/template complexes (Rothwell *et al* 2003). By analysing the relation of polarization and FRET versus average fluorescence lifetimes and comparing the observed trends to theory, the authors excluded the lack of orientational mobility and local dye quenching as possible sources of the

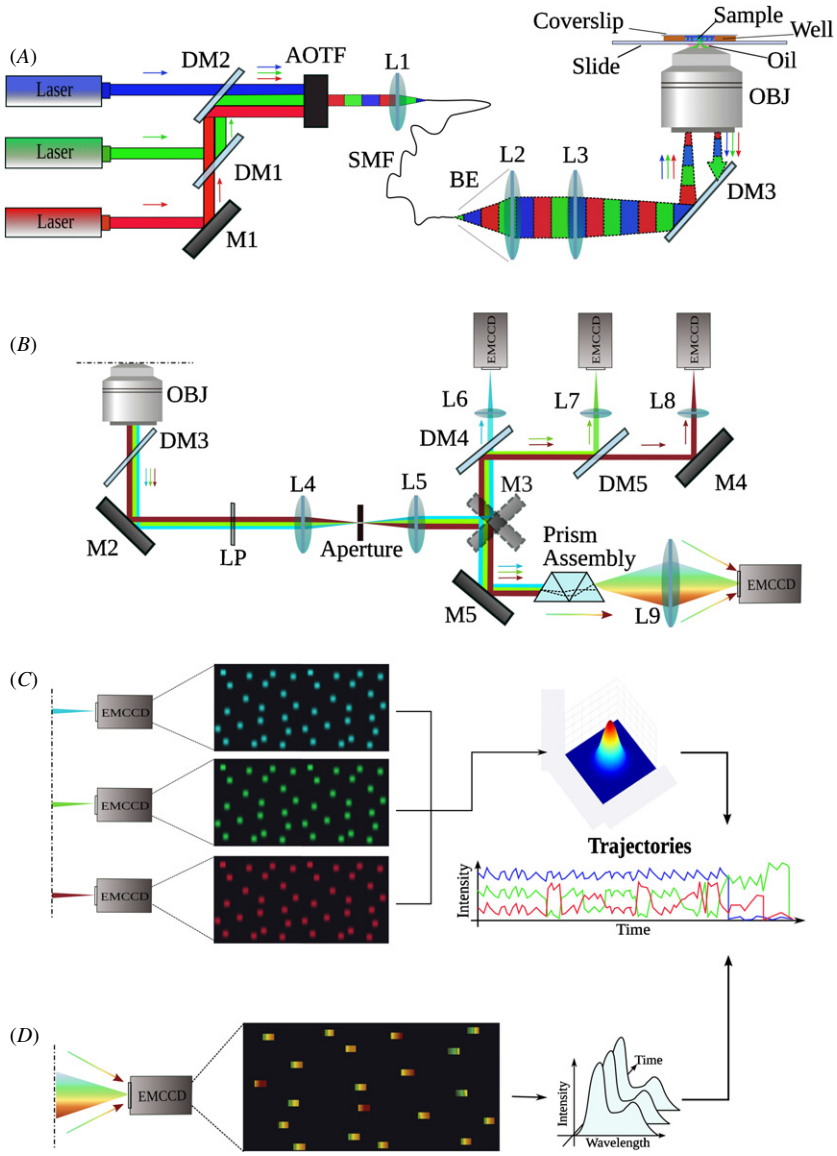

Figure 1. Schematic for TIRF (three-colour) ALEX. (*A*) *Objective-type total internal reflection (TIR) excitation*. Three frequency separated lasers are coupled into an acousto-optical tunable filter (AOTF), which modulates pulse wavelength and duration. The pulses are subsequently coupled into a single-mode optical fibre (SMF), yielding a point source which is expanded, collimated and focused at the periphery of the back focal plane of the microscope objective (OBJ). TIR occurs at the sample–glass coverslip interface, producing an evanescent electric field extending ∼100 nm into the sample, greatly reducing background signal contribution. (*B*) *Three-colour emission detection.* Fluorescence emission is collected through the objective and passed through an aperture to remove out of focus light. In the scheme provided, mirror M3 may reflect the light along one of the two paths. The upper path employs dichroic mirrors (DM) for frequency separation of emitted light, in which three frequency regimes are individually imaged on separate electron-multiplying charged coupled devices (EMCCD), thus maximizing the number of immobilized molecules which may be imaged at one time (*see panel C*). The lower path uses a prism assembly for frequency separation and images onto a single EMCCD (*see panel D*). (*C*) *Extracting single-molecule trajectories*. Individual particles are linked across EMCCDs, and individual point spread functions (PSF) are fit to Gaussian distributions and further subjected to methods commonly employed in crowded field analysis, yielding single-molecule trajectories. (*D*) *Extracting single-molecule spectra*. Importantly, instead of observing individual PSFs, the light constituting a PSF is dispersed over its spectrum on a single EMCCD. Individual molecule spectra are a convolution of each fluorophore emission spectrum, with relative contributions reflected in the intensity over a given wavelength range. Deconvolution may be achieved using multicomponent analysis, and individual fluorophore intensity trajectories along with the standard ALEX quantities of FRET and stoichiometry may thus be calculated directly from the spectra themselves, yielding similar results to *panel C*, but with the additional advantage of being able to directly monitor spectral shift (see, e.g., Chung *et al* 2009). AOTF: acousto-optical tunable filter; SMF: single-mode optical fibre; BE: beam expansion; OBJ: objective; L1–9: lenses; DM1–5: dichroic mirrors; M1–5: mirrors; LP: long-pass filter; EMCCD: electron-multiplying charged coupled device.

observed FRET changes. Moreover, the authors concluded that the observed heterogeneity could only be attributed to structural properties of the individual species. Recent work has further refined the statistical methodology (probability distribution analysis) associated with MFD, allowing for more robust identification and separation of events influenced by changes in dye brightness and other sources of heterogeneity (Kalinin *et al* 2008).

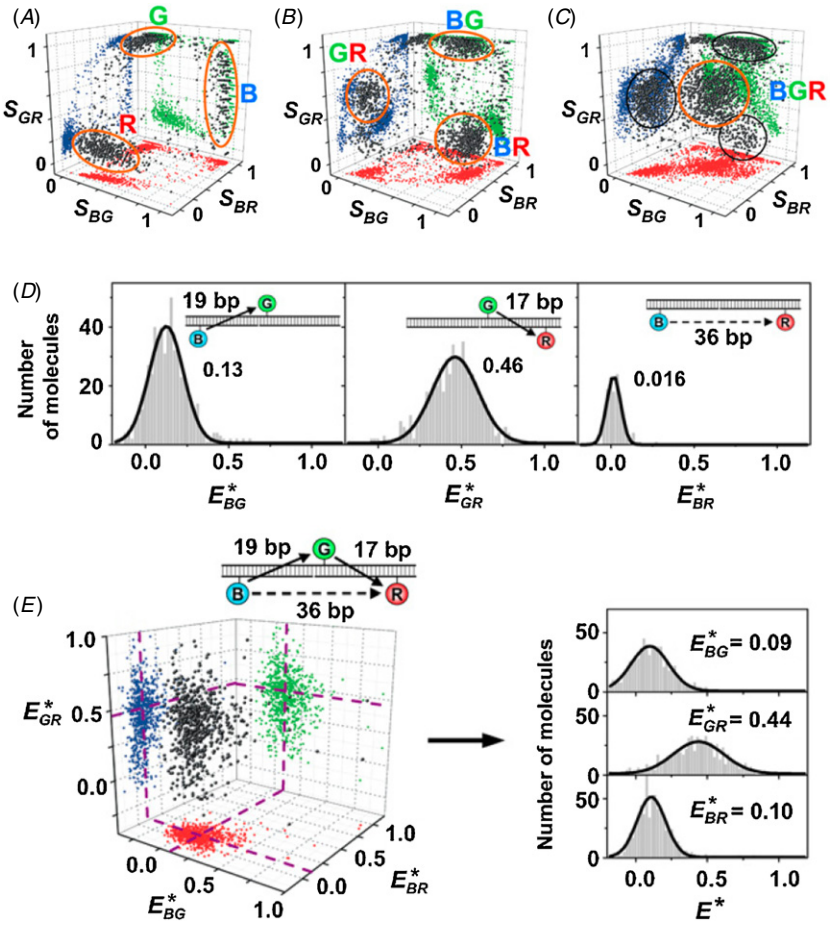

Figure 2. Molecule sorting for three-colour ALEX based on probe stoichiometry *S*. (*A*) Three-dimensional histogram for a 1:1:1 mixture of singly-labelled DNA species (B-only, G-only, R-only), where each species is localized by an orange ellipse and cluster projections are provided in each two-dimensional plane. (*B*) Three-dimensional histogram for a 1:1:1 mixture of doubly-labelled DNA species (B–G, G–R, and B–R), where the orange ellipse indicates species localization. (*C*) Three-dimensional histogram of triply-labelled DNA (B–G–R). The central cluster indicated by the orange ellipse is the species of interest, with the black ellipses corresponding to doubly-labelled species as may be confirmed in (*B*). (*D*) One-dimensional efficiency (E^*) histograms for each doubly-labelled species (B–G, G–R, B–R) identified in (*C*). The reported E^* values are the means of the fitted Gaussian distributions. (*E*) Projection of the triply-labelled species (B–G–R) identified in (*C*) onto each two-dimensional plane. Collapsing each two-dimensional projection into one-dimensional E^* histograms returns those at the right of the panel. As may be observed, the returned E^* values are in good agreement with those of the doubly-labelled species, illustrating the method's power in sorting molecular species of interest, as well as returning accurate distant information across multiple probes within the same sample and, more importantly, within the same molecule.

Chung *et al* extended the MFD approach by directly measuring entire emission spectra (whereas the original MFD approach simply separates spectral regimes), as well as polarization and absolute/relative photon arrival times using picosecond pulsed single-laser excitation (Chung *et al* 2009). The authors immobilized a 56-residue protein (GB1) on a glass surface, illuminated the sample and split the collected emission photons, sending half to an avalanche photo diode while dispersing the remaining with a spectrograph and imaging on a CCD (Jung *et al* 2007). Analysing the photon-by-photon trajectories, the authors deduced an upper bound of 200 μs for the transition path time for GB1 folding/unfolding kinetics. By acquiring single-molecule spectra, the authors were able to classify photons as being emitted from the donor or acceptor based on the emission wavelength, and to distinguish changes in FRET efficiency due to folding/unfolding transitions from those due to a previously unknown spectral shift of the donor (Alexa 488). Consequently, no trajectories were discarded because of perturbing photophysics, with 95% of the trajectories being understood in detail.

Subsequent unification of multi-parameter excitation and detection schemes and extension into multi-colour schemes will mark a significant step forward for single-molecule fluorescence applications. Combination of multi-colour excitation and spectra/polarization/lifetime detection permits multi-colour coincidence assays, allowing monitoring of complex multicomponent molecular interactions, where the distances fall outside the dynamic range of FRET (Rothwell *et al* 2003, Crawford *et al* 2008). Introduction of three or more 'colours' permits simultaneous measurement of multiple FRET pairs, enabling monitoring of concomitant conformational changes, and, used in conjunction with alternating laser excitation, of accurate FRET efficiencies (Lee *et al* 2007). Further, the vast improvement in immobilization (Selvin and Ha 2008), reduction of photobleaching (Donnert *et al* 2006, Funatsu *et al* 1995, Vogelsang *et al* 2008)

and the availability of TIRF microscopy (Friedman *et al* 2006) thus provide high-throughput monitoring of multiple trajectories, revealing insights into sources of heterogeneity. Lastly, maximizing the information obtained for each molecule reveals the tantalizing possibility of no longer having to rely on subjective criteria to delineate the 'good' from the 'bad', but, instead, we may understand almost every recorded trajectory in exquisite detail and isolate those species which are truly of biological relevance (Chung *et al* 2009).

Combination instruments

Single-molecule fluorescence methods can also be combined with other single-molecule methods such as atomic force microscopy (AFM), optical and magnetic tweezers and single ion-channel recordings (reviewed in Greenleaf *et al* (2007), Kapanidis and Strick (2009) and Neuman and Nagy (2008)). Such combinations provide exciting opportunities for probing complex reactions; for instance, such methods can report on local conformational changes (through smFRET) while they interrogate the energy landscape of a reaction and probe features such as reaction transition states (Koshland 1958). This intriguing prospect was clear to the pioneers of the single-molecule fluorescence field; as early as 1999, instruments combining single-molecule fluorescence with force-based or patch-clamp techniques were proposed (Weiss 1999). The development of such methods was relatively slow and required painstaking instrument development and overcoming of several technical challenges aside; recent reports, however, suggest a bright future for combination methods (Walter *et al* 2008). Here we review the combination of SMF with four other single-molecule techniques: atomic force microscopy (AFM), optical tweezers, magnetic tweezers (see figure 3 for visualization of those three combination methods) and single-channel ion recording.

In the case of combination of AFM and single-molecule fluorescence, one needs to mount a commercially available AFM setup on inverted single-molecule fluorescence microscopes (either objective-type TIRF or scanning confocal) (for a comprehensive review, see Shaw *et al* (2006)). The potential of AFM/TIRF was first demonstrated by probing the opto-mechanical cycle for single molecules (Hugel *et al* 2002): a chain of bistable photosensitive azobenzenes was attached between a cover glass and an AFM tip, and molecular conformational changes were measured using light-induced azobenzene photoswitching. A subsequent demonstration of an AFM/TIRF setup was presented by Sarkar *et al* in 2004, wherein a fluorescent AFM tip was used to measure the distance-dependent decay of an evanescent wave above a glass–water interface (Sarkar *et al* 2004). In that work, the high positioning accuracy of AFM resolved the intensity decay along the optical axis with nanometre accuracy. Using the calibrated evanescent wave, the authors exploited fluorescence and AFM to monitor the unfolding of the poly-ubiquitin protein.

AFM has also been used as a 'cut and paste' tool for single-molecule DNA assembly (Kufer *et al* 2008, 2009). Specifically, the AFM tip was used for transferring fluorescent DNA from a reservoir to a target site, where they were hybridized with immobilized non-labelled DNA to generate geometrical objects verified by fluorescence imaging (Kufer *et al* 2008). This technique has recently been combined with super-resolution imaging techniques and may allow studies of interactions with photonic nanostructures and between single fluorophores (Puchner *et al* 2008, Cordes *et al* 2010). Another recent development is the combination of polarized TIRF and AFM for studying phase separation of model membranes composed of saturated and unsaturated lipids and cholesterol (Oreopoulos and Yip 2009); this technique may enable studies of raft-mediated phenomena in phospholipid bilayers while interrogating them with an AFM tip.

Single-molecule fluorescence has also been combined with optical traps (a.k.a. optical tweezers), which use radiation pressure from a focused laser to control the position of dielectric objects, such as micron-size beads to which biomolecules can be attached. A pioneering instrument combining single-molecule fluorescence and optical trapping was introduced in 1998 (Ishijima *et al* 1998), allowing the simultaneous detection of mechanical events (force application) and ligand-binding events (ATP-binding) for single myosin molecules. The authors showed that, in contrast to prior literature, force generation did not always coincide with release of a bound nucleotide. A combination of optical traps with smFRET was introduced in 2003 (Lang *et al* 2003) and was extended by two groups (Hohng *et al* 2007, Tarsa *et al* 2007). Tarsa *et al* used optical traps to mechanically unzip DNA hairpins while simultaneously monitoring DNA conformational states using smFRET, where as Hohng *et al* used FRET to observe nanometre-scale motions within DNA Holliday junctions upon applying an external force.

A limitation in combining single-molecule fluorescence with optical traps is the rapid photobleaching of fluorophores due to the irradiation by the infrared laser beam used for trapping (Dijk *et al* 2004). The simplest solution for photobleaching is to separate the trapping and fluorescence excitations in space either by using one trapped bead and flowstretching the attached DNA (Hilario *et al* 2009) or by using a larger distance between two trapped beads and detecting fluorescence from the centre of the DNA strand (van Mameren *et al* 2009). It is also possible to separate trapping and fluorescence in time by using fast alternation between the trapping laser and fluorescence excitation beams (Brau *et al* 2006), a strategy that allowed fluorophore to survive for tens of seconds without compromising trap stiffness or by spacing. A second limitation is that, in contrast to wide-field single-molecule imaging, most force-based methods do not allow parallel detection and manipulation. In this respect, methods for generating four optical traps using acousto-optical devices after splitting two orthogonally polarized trapping beams or generating hundreds of traps using holographically engineered optical interference trapping patterns offer exciting prospects (Noom *et al* 2007, Grier and Roichman 2006).

Forces can also be applied using the technique of magnetic tweezers, which uses simple magnets to apply forces on magnetic beads attached to biomolecules. Compared with optical traps, magnetic tweezers have three distinct

advantages. First, whereas optical traps allow mainly translational movement of a bead with high precision, magnetic tweezers can also exert torque, making them the instruments of choice for studying DNA supercoiling and topoisomerases (Strick *et al* 2000, Greenleaf *et al* 2007, Charvin *et al* 2005). Moreover, since magnetic tweezers do not require a trapping beam, their use minimizes problems with fluorophore bleaching (Dijk *et al* 2004). Finally, magnetic tweezers have the simple instrumentation requirements of all force-based methods.

In one of the first combinations of magnetic tweezers and smFRET combination (Shroff *et al* 2005), it was shown that changing inter-dye distances between a smFRET pair could be used to tune a sensor's force response. The calibrated sensor was used to determine the accumulation of strain in the 0–20 pN range on a ssDNA molecule that can hybridize to form double-stranded DNA. A more recent report describes the use of electro-magnetic tweezers and TIRF illumination to study folding/unfolding kinetics of protein L (Liu *et al* 2009). Here, in contrast to AFM measurements, magnetic tweezers allow for a lower pulling force, dramatically extending the observation time for slow folding and unfolding reactions to over 30 min. By calibrating the intensity response of a fluorescent, paramagnetic bead in an evanescent wave and keeping the applied current of the electro-magnetic coil constant, changes in the bead-to-surface distance could be attributed to folding and unfolding in the protein L chain.

Combinations of magnetic tweezers and single-molecule fluorescence have been popular as means of studying DNA-processing proteins. An early example is the study of the viral genome packaging in the bacteriophage phi29 protein capsid (Hugel *et al* 2007). Using polarization-resolved TIRF microscopy, a single dye on the connector of the capsid reported on orientation of the connector during the process of DNA packaging, which was monitored by recording translocation of a magnetic bead attached to DNA. The connector did not rotate during packaging, addressing a long-standing mechanistic question regarding DNA packaging by viruses. Another application on DNA-processing proteins turned a labelled DNA strand into an 'optical encoder' for a DNA helicase (Wickersham *et al* 2010); translocation of a donor-labelled helicase DnaB on double-stranded DNA decorated periodically with multiple acceptors resulted in periodic FRET efficiency signals. As the distance between acceptors is known, the time between measuring high FRET events (during which a donor-labelled helicase bypasses an acceptor) directly correlates to the unwinding speed of helicase. This approach may be useful for monitoring the activity of DNA-processing machines along extended length scales.

Fluorescence detection can also be combined with the oldest single-molecule technique, which involved single ion-channel current recordings ('patch-clamp' technique (Neher and Sakmann 1976)). Early demonstrations of such a combination were used for studying Ca^{2+} signalling between single voltage-gated L-type Ca^{2+} channels and ryanodine receptors in heart cells (Wang *et al* 2001). In 2005, Demuro and Parker extended single ion-channel TIRF measurements to ligand-gated ion channels to study the Ca^{2+} flux through individual channels with single-digit millisecond temporal resolution and high parallelism by imaging hundreds of channels simultaneously (Demuro and Parker 2005). More challenging is the combination of smFRET with electrical recording as demonstrated in 2003 (Borisenko *et al* 2003, Harms *et al* 2003); such a combination will be vital for directly relating conformational changes within an ion channel (through FRET changes) with electrical recordings that report of the transport of ions through the channel. Towards this goal, two laboratories studied gramicidin channels formation in lipid bilayers. The first study showed a wide distribution of FRET efficiencies within single gramicidin molecules; this distribution was interpreted as the presence of multiple conformations (Harms *et al* 2003). The second study used FRET to detect homodimers and heterodimers of gramicidin and observed that the FRET appearance correlated with channel opening, as detected through the electrical measurements (Borisenko *et al* 2003). Current reports, however, suggest that these claims should be interpreted with care, since they involve imaging of an entire membrane (as opposed to a small area) to relate a fluorescent observable to an electrical one. This difficulty may be overcome by using water-in-droplet lipid bilayers that ensure the presence of only a single membrane protein per droplet (Heron *et al* 2009).

Overall, although combination methods add substantial complexity to single-molecule fluorescence experiments, they do provide unprecedented access to multidimensional landscapes of biological reactions and mechanisms, and, therefore, we anticipate significant developments in the application of combination methods in the near future. These developments will benefit from the standardization of individual single-molecule techniques, and the development of solutions that address the often conflicting requirements, length- and time scales of the different methods.

Single-molecule fluorescence for single-molecule DNA sequencing

One of the many remarkable facts about DNA is that only four slightly different bases encode the entire genome of any living organism, and thereby the blueprint of life in general. The three billion base pairs of the human genome comprise the genetic information for 20 000–25 000 protein-coding genes, non-coding RNA genes and regulatory sequences. Importantly, DNA contains hereditary genetic information, as well as genetic markers that may determine the likelihood of developing diseases; therefore, being able to read the genome accurately, quickly and inexpensively provides opportunities for learning more about our genetic background and future. As a result, a range of innovation in many areas of science have been fuelled by a precipitous decrease (by seven orders of magnitude) in sequencing costs during the past decade, whereas the first genome came at a cost of $3 billion, the company *Complete Genomics* announced the '$4400 genome' in late 2009 (Drmanac *et al* 2010). We believe that this remarkable improvement will dramatically transform biological and biomedical research

within the next decade, even though there is a lively discussion about the speed and final impact (Collins 2010). Here, after an overview of sequencing techniques (see also reviews in Shendure and Ji (2008), Kahvejian *et al* 2008, Gupta (2008), Pettersson *et al* (2009) and Bayley (2006)), we focus on single-molecule fluorescence-based sequencing (for non-fluorescence sequencing methods see Xu *et al* (2009).

First-generation sequencing relies on variations of the Sanger dideoxynucleotide termination method (Sanger and Coulson 1975), wherein fragments of amplified genomic DNA are copied by DNA polymerases in the presence of all four nucleotides. A fraction of each nucleotide is non-extensible (due to the absence of a 3′-OH group) and labelled with spectrally distinct fluorophores (e.g., a red dye coding for dATP), leading to stochastic chain-termination events that yield DNAs with different lengths. Since these DNAs can be separated with single base-pair resolution using gel electrophoresis, the sequence can then be easily determined by comparing fragment length and fluorescence wavelength. The Sanger method is still the most accurate sequencing technique, with an accuracy of 99.999% and read lengths of up to 1000 bases; however, this comes at a high cost and associated tedium (Shendure and Ji 2008, Xu *et al* 2009). The Sanger sequencing method also suffers from limited parallelization, although systems with 384 independent capillaries are commercially available.

Second-generation sequencing methods rely on the generation of clonal populations of DNA and achieve high parallelism, high speed and lower costs through massively parallel fluorescence (or luminescence) imaging (Shendure *et al* 2004, Margulies *et al* 2005). For example, the sequencing platform 454 (Roche) uses emulsion polymerase chain reaction (PCR) to create clonally amplified beads immobilized in picolitre wells. After adding a single nucleotide and polymerases, as well as luciferin and a modified adenosine, light generation in each well indicates the incorporation of a particular nucleotide. Read lengths of hundreds of bases are possible, but the accuracy is limited due to challenges with differentiating homopolymers. The Solexa/Illumina technology uses amplification of adaptor-flanked shotgun fragments on glass surfaces to achieve reads of up to 100 bases (Adessi *et al* 2000, Fedurco *et al* 2006). After annealing sequencing primers, each cycle of sequencing consists of adding all four nucleotides carrying reversible terminators and imaging the specific fluorophores of the four nucleotides in each clonal cluster; the fluorophores are cleaved, and the next single-base extension can occur. Finally, a method based on ligation of DNA fragments is used by the ABI/SOLiD platform (Life Technologies) (Shendure *et al* 2005, Macevicz 1998). Each sequencing cycle consists of flowing short DNAs to clonally amplified beads; incorporation and ligation is followed by four-channel imaging of the fluorophores attached to each short DNA and subsequent cleavage to reset the system. The system is high scalable accurate, but the read length is limited to ∼50 bases.

Third-generation sequencing is exclusively based on reading the base sequence on single DNA molecules. Here, no amplification of DNA is required, thus avoiding the costs

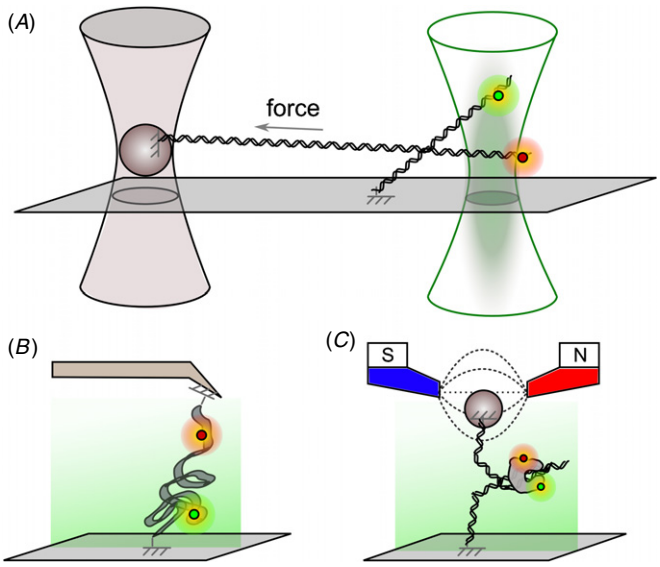

Figure 3. Combining single-molecule fluorescence with force-based techniques. Whereas single-molecule fluorescence primarily allows studies of local conformational changes of biomolecules, force-based methods, such as (*A*) optical traps, (*B*) atomic force microscopy and (*C*) magnetic tweezers, allow strain application or measurements of molecular motion. Here, (*A*) represents a force-based mechanical unzipping of DNA hairpins (see, for example, Tarsa *et al* 2007), (*B*) a suggested experiment of AFM-based unfolding of a protein and (*C*) a suggested experiment of supercoiling of DNA with magnetic tweezers and simultaneous monitoring of a fluorescently labelled topoisomerase. All force-based methods are combined with smFRET which allows measuring multi-dimensional energy landscapes with high spatial and temporal resolution. Panel (*A*) uses a confocal fluorescence excitation scheme, whereas panels (*B*) and (*C*) use a TIRF-based illumination scheme.

and errors associated with the use PCR-based amplification. Moreover, if the readout mechanism is non-destructive, a DNA template can be read several times, increasing the accuracy of the read. Such approaches can be highly parallelized, resulting in substantial reductions in the costs, time and tedium associated with genome sequencing. The appeal of single-molecule sequencing was apparent to the first practitioners of single-molecule fluorescence, such as Richard Keller and his group (Los Alamos National Laboratory), who worked on approaches that involved cleavage of labelled DNA combined with flow of the fluorescent base to a detection area for single-molecule detection (Werner *et al* 2003). Although this pioneering sequencing approach never reached maturity, it spawned several developments in the field of single-molecule fluorescence.

The first commercially available platform for single-molecule sequencing was presented by Helicos (Harris *et al* 2008). In this approach, the genomic DNA is fragmented and a poly-A strand is added to capture the DNA on a solid support (figure 4(*a*)). First, the positions of each immobilized fragment are determined by imaging a photocleavable dye. Then, species of single dye-labelled nucleotides and polymerases are added to the reaction chamber in a stepwise fashion. After sequence-dependent incorporation, the remaining non-incorporated nucleotides and polymerases are washed away

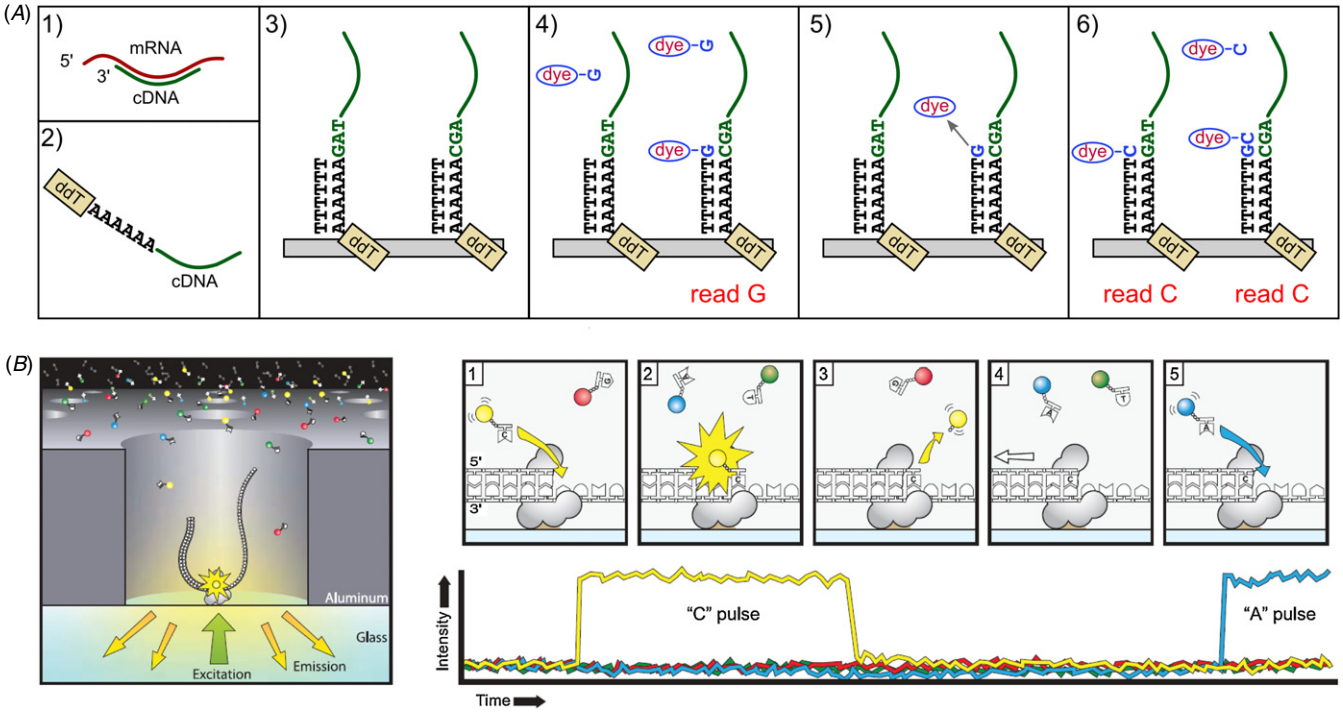

Figure 4. Two fluorescence-based platforms for single-molecule DNA sequencing. (*A*) Helicos uses a cyclic sequencing technique, here shown for the special case of starting with a RNA primer strand on which a DNA is synthesized. The DNA strand is flanked by a poly-dATP strand and dideoxyterminated with a ddTTP (2). This construct is hybridized to a surface-immobilized poly-dT strand (3). The position of each immobilized construct is initially determined with a cleavable dye (not shown). After flowing a particular dye-labelled nucleotide into the reaction chamber, the polymerase incorporates the nucleotide, the residual nucleotides are washed out and the incorporated dye-labelled nucleotide is imaged, determining one base of the particular sequence (4). The dye is photocleaved (5) and the next dye-labelled nucleotide is added (6). Adapted from Lipson *et al* (2009) with permission from Helicos Biosciences Corporation. Copyright 2009, Nature Publishing Group. (*B*) Real-time DNA sequencing utilizes polymerases immobilized in zero-mode waveguides (ZMW) as pursued by Pacific Biosciences. The polymerase captures a DNA molecule with a single-strand overhang. Excitation of single fluorescently labelled nucleotides is restricted to the bottom of the ZMW. If binding of a matching nucleotide to the polymerase occurs (1), the incorporation event (2) is far longer than a simple diffusion-only event (hundreds of milliseconds compared to a few microseconds) and results in a relatively constant level of intensity that is clearly distinguishable from the background. The formation of the phosphodiester bond leads to a cleavage of the dye–linker–pyrophosphate product, which then diffuses quickly out of the ZMW (3). The polymerase translocates one base on the template (4) and is ready for incorporating the next matching nucleotide (5). Reproduced with permission from Eid *et al* (2009). Copyright 2009, AAAS.

and the fluorescence intensity from every incorporated nucleotide is recorded and related to the initial position of each fragment. After cleavage of the dye of the incorporated nucleotide, the next labelled nucleotide is added into the chamber; the cycle is repeated, providing the positive signals that allow assembly of the DNA sequence. Although the method has been used for rapid sequencing of human genomes (Harris *et al* 2008) and a yeast transcriptome (Lipson *et al* 2009), it is hampered by short read lengths (30–35 bases), difficulties in dealing with base repeats and homopolymers, and high instrument costs.

The need for stepwise interrogation of each template base stems from a fundamental limit for any single-molecule fluorescence measurement: the need to keep the concentration of fluorescent molecules at pM–nM levels to allow detection of individual fluorescent molecules. Many biological interactions, however, have binding affinities in the micro- to millimolar range and cannot be studied at the single-molecule level if one of the fluorescent interacting partners is present at high concentrations. To enable real-time (rather than cyclic) sequencing, Levene *et al* introduced the concept of zero-mode waveguides for reducing the excitation volume by several orders of magnitude (Levene *et al* 2003) and allowed single-molecule detection even at micromolar concentrations. Based on this concept, the company Pacific Biosciences introduced an innovative approach for real-time single-molecule sequencing based on immobilized DNA polymerases (Eid *et al* 2009). Critically, the approach allows long read lengths (up to thousands of bases) and parallel sequencing of thousands of strands. Nanofabrication is used to create tiny holes (30–70 nm thick) within an aluminium layer (∼100 nm thick) on a glass slide; single DNA polymerases are stochastically immobilized within these cavities, which are smaller than the wavelength of light. Light focused through the slide cannot pass the aluminium layer but, instead, generates an evanescent wave within a small region at the glass/cavity interface. DNA is sequenced by polymerase-driven incorporation of fluorescently labelled nucleotides. During the actual event of incorporation, the nucleotide remains for a short time (∼100–1000 ms) at this position giving a constant fluorescence emission upon excitation. The cavities fulfil an important task by decreasing the local

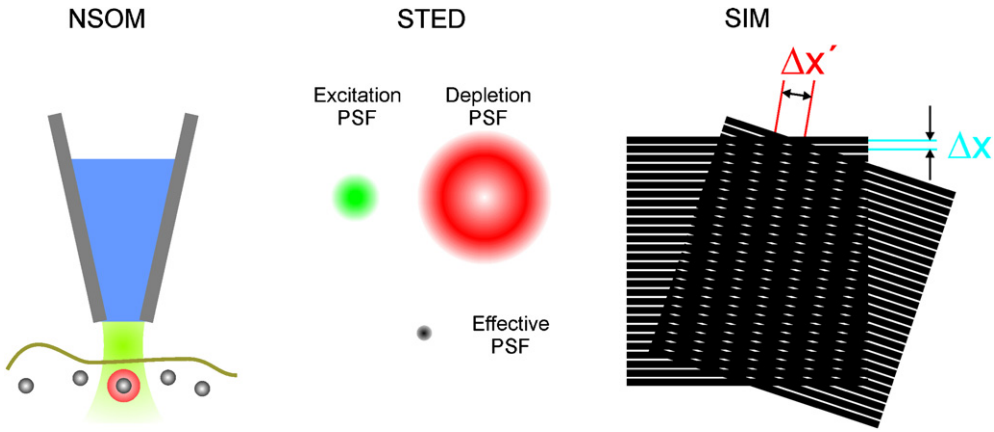

Figure 5. Super-resolution microscopy. Near-field scanning optical microscopy (NSOM) applies a scanning tip with light coupled into a sub-wavelength aperture that is moved along the surface of a sample. Stimulated-emission depletion (STED) and structured-illumination microscopy (SIM) are both far-field microscopic techniques that achieve subdiffraction resolution by controlling the light-intensity distribution. In the STED principle, this is achieved by overlaying two laser beams: a regular intensity profile of a first laser excites fluorophores, and a doughnut-shaped intensity distribution of a second laser depletes fluorophores everywhere apart from a local zero. For SIM, a sample is illuminated by a periodic light pattern which causes otherwise unresolved structures to become observable in the form of Moiré fringes.

concentration around each polymerase in a way that the bulk of detected fluorescence originates from the bound nucleotide (and not from nucleotides diffusing above the aluminium layer). Upon nucleotide incorporation, the polymerases remove the fluorophore attached to the terminal phosphate of the nucleotide (Korlach *et al* 2008). Detection of four different dyes (corresponding to the four bases) is achieved by dual-colour excitation and prism-based spectral separation of fluorescence. A limitation of this approach is colour separation, since it requires numerous photons to improve reading accuracy; this requirement limits reading speed to two to four bases per molecule per second, but improvements in fluorophores and instrumentation may increase the reading speed.

Another real-time sequencing approach was unveiled by Life Technologies during the 2010 Annual Biophysical Society Meeting (Previte *et al* 2010). The approach is partly based on technologies developed by the company Visigen and uses smFRET between a donor-labelled polymerase (labelled with a quantum dot with a high extinction coefficient) and acceptor-labelled nucleotides (with each nucleotide being labelled by a different acceptor). The FRET assay allows operation at high concentration of labelled nucleotides without nanocavities, since only fluorescent nucleotides transiently bound to the donor-labelled polymerase fluoresce (due to FRET-induced excitation); there is minimal excitation of any unbound nucleotides; another key element is the ability to exchange reagents (polymerases and nucleotides). The method is capable of long read lengths and high accuracy, and it may prove a competitive approach for single-molecule sequencing. Other approaches for working at higher concentrations for real-time DNA sequencing include the use of dark quenchers in conjunction with smFRET (JH, Ludovic Le Reste and ANK, in preparation). In that approach, a polymerase is labelled with fluorophores and nucleotides are labelled with dark quenchers (i.e. non-fluorescent FRET acceptors); base assignment relies on reading the quenching efficiency associated with binding of quencher-labelled nucleotides to immobilized polymerase molecules.

Although the field of third-generation sequencing is very active and includes many approaches not involving fluorescence (Xu *et al* 2009), we expect that fluorescence-based methods will be the main platforms for rapid and affordable genome sequencing for the next few years. We anticipate that advances in imaging will further boost the scalability and speed of the fluorescence-based methods, making genome sequencing affordable even for small research units. As combination instruments improve, it may also be the case that combinations of single-molecule methods may provide even more appealing solutions for affordable and accurate genomic sequencing, and eventually usher the long-awaited era of personal genomics.

Beyond the diffraction limit: super-resolution imaging

The resolution limit of light microscopy. A main advance within the third wave of fluorescence methods is the development of methods that overcome a fundamental physical limit in any kind of lens-based light microscopy, the diffraction limit. This phenomenon is a consequence of the wave nature of light and limits the attainable optical resolution in light microscopy to about 200–300 nm in the focal plane and >500 nm along the optical axis. A point-like object that emits light, e.g., an organic fluorophore with a size of 1–2 nm, will thus generate a blurred image of much larger size; this image is referred to as the point spread function (PSF). Two objects spaced closer than about 200–300 nm in the focal plane are, according to the Rayleigh criterion, defined to have a separation that is below the resolution limit and thus remain unresolved.

Biomedical research often encounters cellular or molecular structures at much shorter length scale than the diffraction limit, such that the latter obviates much insight into the underlying mechanisms, creating a clear motivation to develop techniques that break the resolution limit and

provide microscopic images with sub-diffraction or molecular resolution (Hell 2009, Huang *et al* 2009, Ji *et al* 2008). One of the first methods developed was near-field scanning optical microscopy (NSOM), where a scanning tip with light coupled into a sub-wavelength aperture is moved along the surface of a sample (Lewis *et al* 1984, Pohl *et al* 1984, Synge 1928) (figure 5). The resolution is related to the size of the aperture and reaches 30–100 nm (de Lange *et al* 2001). However, the application of NSOM is mainly limited to two-dimensional problems and to surfaces (as opposed to the cell interior).

Further research was directed towards the development of far-field microscopic methods that achieve sub-diffraction resolution to gain access to biological samples such as living cells. These methods can be divided into different groups based on the underlying principle, complexity, enhancement in resolution and degree of generality of the concept. Here, we distinguish between two different groups of methods that achieve super-resolution imaging: a group that combines a targeted read-out of fluorophores and a well-defined light-intensity distribution, and a group that combines a stochastic single-molecule read-out combined with fluorophore localization and image reconstruction.

Super-resolution applying a light-intensity distribution for targeted read-out. The first far-field microscopic method that applied a light-intensity distribution to achieve subdiffraction resolution was stimulated emission depletion (STED). STED locally depopulates the excited state of fluorophores by operating two laser beams simultaneously and generating a nanometric focus (Donnert *et al* 2006, Hell and Wichmann 1994, Klar *et al* 2000) (figure 5). A first laser is used to excite the fluorophores, and a second, red-shifted laser, with a doughnut-shaped beam profile, depletes the excited state everywhere except its zero-intensity centre. The depletion beam prevents the molecules from fluorescing anywhere except the zero-intensity centre in the nanometric focal region. The resolution enhancement can be adjusted with the irradiation intensity of the depletion beam, with a lateral resolution of down to ∼20 nm (Kasper *et al* 2010). STED has also been used to address many biological questions, such as the dynamics of dendritic spines in live neurons (Nagerl *et al* 2008) and the movement of synaptic vesicles in live neurons with video-rate acquisition (Westphal *et al* 2008). STED microscopy has become less complex and costly in recent years and readily available to non-expert users. However, compared to other super-resolution techniques that reach a near-molecular resolution, STED microscopy still requires the highest irradiation intensities of up to GW cm^{-2} and thus requires careful controls in particular in live-cell experiments.

A different concept is used in structured illumination microscopy (SIM) (Gustafsson 2000), where a sample is illuminated by a periodic light pattern which causes otherwise unresolved structures to become observable in the form of Moiré fringes. Linear SIM achieves a twofold increase in lateral resolution (figure 5), operates with low laser intensities and has no label restrictions. As a pure physical approach compatible with many types of fluorophores, SIM is ideal for studying dynamics in living cells (Hirvonen *et al* 2009, Kner *et al* 2009), with multi-colour SIM having been used to study the nuclear periphery of mammalian cells (Schermelleh *et al* 2008). The resolution enhancement of SIM can be extended if nonlinear optical effects are used (Gustafsson 2005).

Stochastic single-molecule read-out and localization. The PSF of a point-like single fluorophore emitting in the visible range of the electromagnetic spectrum has a full-width half-maximum of 200–300 nm in the focal plane and >500 nm in the axial direction. Precisely localizing a single emitting object is possible, however, by approximation of the PSF with a Gaussian function. The precision of this approximation depends mainly on the number of photons and the background (Thompson *et al* 2002). Provided that sufficient photons are collected, single fluorophores can be localized with nanometre precision—a fact used to study movements of motor proteins on filamentous proteins (Yildiz *et al* 2003, Yildiz and Selvin 2005).

A variety of super-resolution methods developed in the past few years are based on the common principle of combining high-precision single-molecule localization with stochastic read-out of single fluorophores (Henriques and Mhlanga 2009, Huang *et al* 2009, Ji *et al* 2008). Some prominent examples are photoactivated-localization microscopy (PALM) (Betzig *et al* 2006), fluorescence photoactivation localization microscopy (FPALM) (Hess *et al* 2006), stochastic optical reconstruction microscopy (STORM) (Rust *et al* 2006), direct STORM (dSTORM) (Heilemann *et al* 2008) and their variants (Flors *et al* 2007, Folling *et al* 2008, Heilemann *et al* 2009, Vogelsang *et al* 2009). The unifying theme of these methods is a temporal separation of the fluorescence emission of all fluorophores in the sample, so that single emitters are clearly identified (figure 6). In practice, this is achieved by starting off with all fluorophores in a non-fluorescent (dark) state. Next, a stochastic subset of molecules is activated, read out and switched off again with a second wavelength. Single fluorophores are localized with nanometre precision and the procedure is typically repeated for many thousands of cycles. A reconstructed microscopic image is generated from all single-molecule localizations, and a lateral resolution of ∼20 nm (Folling *et al* 2008, Heilemann *et al* 2008, 2009, Rust *et al* 2006) has been reported. Three-dimensional imaging has been realized using different concepts to improve the axial resolution, e.g. introducing astigmatism (Huang *et al* 2008), employing a helical PSF (Pavani *et al* 2009), recording two image planes simultaneously (Juette *et al* 2008) or using an interferometric arrangement (Shtengel *et al* 2009).

Localization-based super-resolution methods have also been extended using multi-colour implementations (Bates *et al* 2007, Shroff *et al* 2007, van de Linde *et al* 2009). Three-dimensional imaging has been realized using different concepts to improve the axial resolution, e.g. introducing astigmatism (Huang *et al* 2008), employing a helical PSF (Pavani *et al* 2009) or recording two image planes simultaneously (Juette *et al* 2008). First applications to biological questions included studies on the distribution of proteins in the plasma membrane of living cells using the

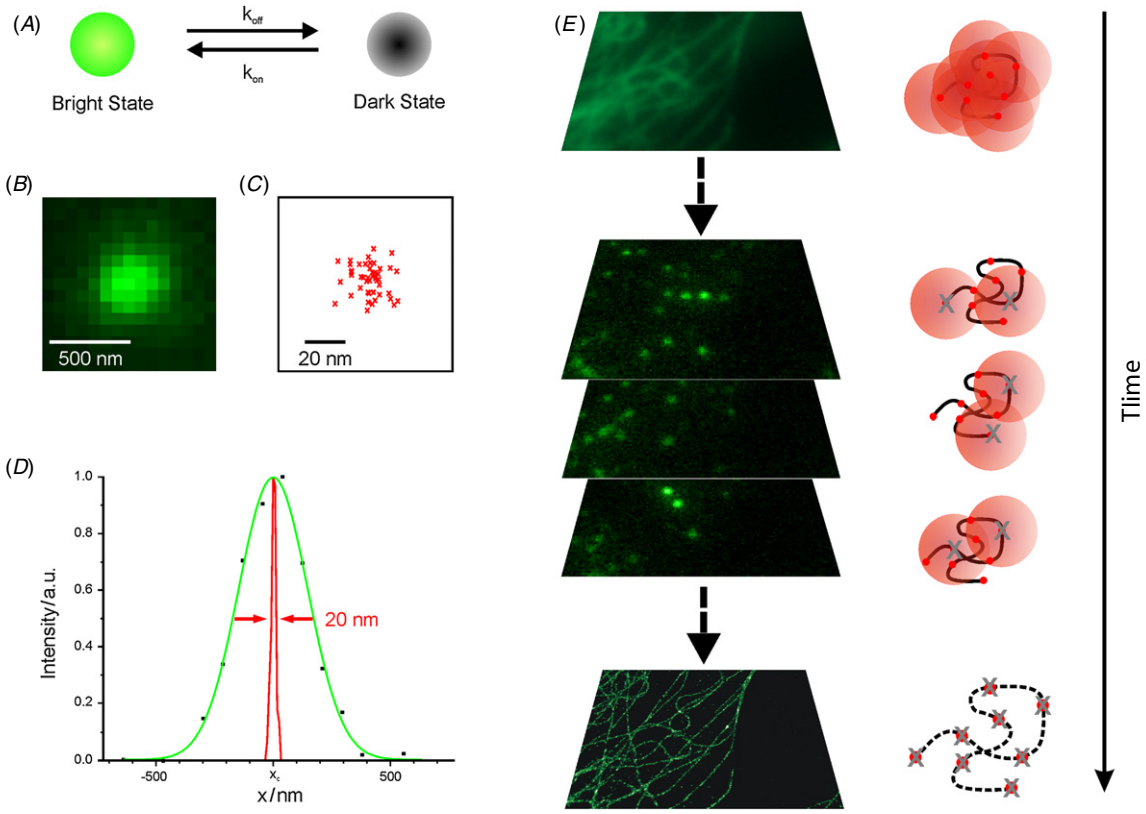

Figure 6. Super-resolution microscopy by stochastic photoswitching and single-fluorophore localization. Localization-based super-resolution techniques employ fluorescent probes that exist in at least two discernable states, e.g. a fluorescent (bright) state and a non-fluorescent (dark) state. (*A*) The transition between these states (photoswitching, photoactivation or photoconversion) is typically controlled via light and/or buffer conditions. (*B–D*) Emission profile of a single fluorophore in widefield microscopy. (*B*) Point spread function of a single fluorophore. (*C*) Both multiple single-molecule localizations with high precision can be approximated with a Gaussian function, demonstrating the increase in spatial resolution (*D*). (*E*) Subdiffraction-resolution images are obtained by temporal confinement of fluorescence emission of all fluorophores in a sample, combining stochastic photoswitching with high-precision single-molecule localization.

PALM approach (Hess *et al* 2007, Manley *et al* 2008). In a recent study, the clustering of chemotactic proteins in *Escherichia coli* was studied using PALM (Greenfield *et al* 2009). dSTORM has been used to study the spatial organization of proteins in the inner mitochondrial membrane (van de Linde *et al* 2008) and mRNA in living cells (Heilemann *et al* 2009). In contrast to SIM and STED, localization-based super-resolution methods have lower temporal resolution, as a stack of images was recorded to reconstruct a high-resolution image, limiting applications to slow dynamics of tens of seconds to minutes (Manley *et al* 2008). However, fast photoswitching and fast image acquisition allows super-resolution imaging with 1 Hz resolution, as shown in observations of fast dynamics of actin on a myosin-coated surface (Endesfelder *et al* 2010).

The key in localization-based super-resolution methods is exploiting fluorescent probes that exist in two discernable states, e.g., a fluorescent and a dark state (Heilemann *et al* 2009). Fluorescent proteins that can be photoactivated (such as paGFP) or photoconverted (such as mEos) can be genetically fused to a target protein. These fluorescent proteins, however, are relatively large, might affect the functionality of the target protein and have limited photon yield. Alternatively, a large number of much smaller organic fluorophores exhibit reversible photoswitching and high photostability (Heilemann *et al* 2009). Here, labelling a target molecule can either be achieved by direct chemical conjugation, by immunofluorescence or by employing a specific protein tag such as e.g. a SNAP-tag (Keppler *et al* 2003) or TMP-tag (Gallagher *et al* 2009).

Although the concepts for super-resolution fluorescence imaging have already been proposed almost 20 years ago, it was the development in the past few years together with the first impressive applications that led to a general interest in this research field (Method of the year 2008 (2009)). Simplified experimental schemes encouraged many research groups to build super-resolution microscopes themselves, and several techniques became available as commercial solutions. At the same time, biological and medical research groups that so far used conventional light microscopy became aware of the new opportunities that emerge with the use of super-resolution methods. We thus can anticipate that super-resolution methods in a few years will be implemented as standard tools in microscopy labs and imaging facilities and will be accessible to a large number of researchers.

Single-molecule fluorescence inside living cells

Our understanding of biomolecular structure, interactions and function arises mainly from decades of *in vitro* biochemical work using reconstituted processes from purified (and often modified) components. This reductionistic approach has been very successful: one has simply to consider the thousands of atomic-resolution structures in the Protein DataBank and the functional analysis of large complexes (e.g., eukaryotic transcription complexes) through site-directed mutagenesis. The precision and control one retains, however, in an *in vitro* setting comes at the expense of losing the all-important cellular context (e.g., thousands of different proteins, metabolites, macromolecular crowding, compartmentalization). Since most single-molecule measurements are performed *in vitro*, their ability to probe heterogeneity, real-time kinetics and stochasticity of a functional outcome carries the same limitations as any *in vitro* approach. Hence, to validate the biological significance of *in vitro* work, it is crucial to perform complementary *in vivo* single-molecule studies, thereby gaining access to protein machinery difficult or even impossible to reconstitute *in vitro*.

Many questions can be addressed by *in vivo* single-molecule studies. What are the kinetics and rate-limiting steps of biomolecular processes *in vivo*? How do *in vivo* observations compare to *in vitro* ones? How are rate-limiting steps influenced by DNA sequence or environmental factors? Are there distributions of behaviours (sub-cellular stochasticity)? How important is stochasticity for components with low abundance, such as genomic DNA or low-copy number RNAs and proteins? Our ability to address these difficult but vital questions will depend on novel methods that permit long, uninterrupted and sensitive fluorescence observations in living cells. These measurements will also be natural partners of systems biology approaches that perform predictive modelling of living cells.

Single-molecule fluorescence is arguably the best single-molecule technique for probing the cell interior, since other single-molecule methods (such as optical tweezers and AFM) are either too perturbative or require handles (such as micron-size beads) too large to introduce in many cells. Single-molecule fluorescence imaging is non-invasive and can offer sub-millisecond temporal resolution, nanometre spatial resolution and coding of biomolecules through use of genetically encoded fluorescent proteins. Here, we review examples of single-molecule fluorescence application in the interior of living cells, as opposed to studies focusing on molecules observed at the cell exterior. For an extensive discussion of single-molecule detection in living cells see Lord *et al* (2010), Xie *et al* (2008) and Yang (2010). We will also not cover the rich literature of single viral tracking in cells (Seisenberger *et al* 2001, Brandenburg and Zhuang 2007).

Until recently, mainly due to the experimental challenges entailed, few *in vivo* single-molecule fluorescence measurements had been reported. First, the background autofluorescence of the cell interferes with detecting fluorescence from labelled biomolecules. Second, due to size and labelling requirements, typical fluorophores used in living cells (organic fluorophores and fluorescent proteins) are of moderate brightness and photostability, leading to a low signal-to-noise ratio and fast photobleaching, which may lead to misinterpretation of molecular interactions and dynamics. Semiconductor quantum dots (Michalet *et al* 2005), novel probes displaying tuneable emission wavelengths, superior brightness and resistance to photobleaching may provide an alternative for *in vivo* labelling; however, quantum dot internalization and targeting to specific sites or proteins are not trivial. Third, fluorophore labelling methods in living cells can be complex and display poor specificity (Kapanidis and Weiss 2002). Despite the challenges, impressive progress has been made towards turning single living cells as the 'test tubes' of our times (Xie *et al* 2006).

An illustrative demonstration of the single-molecule approach to study processes in living cells involved studies of gene expression in *E. coli* (Yu *et al* 2006). These studies are based on remarkable experiments performed at the single-RNA level, which offered insight into the kinetics of RNA synthesis in single bacterial and eukaryotic cells (Golding *et al* 2005), as well as experiments that had demonstrated sensitive single-molecule detection in single cells (Ueda *et al* 2001). To monitor expression *in vivo*, Yu *et al* genetically modified the bacterial chromosome to insert a gene for a fast-maturing version of yellow fluorescent protein (Venus–YFP) fused to a membrane-localization protein fragment (Tsr) (figure 7(*a*)). The YFP–Tsr fusion product was placed under the control of a *lac* operator, a DNA sequence recognized by transcription factor *lac* repressor, which binds to its operator and blocks expression of YFP–Tsr. Stochastic dissociation of *lac* repressor led, however, to transcription and translation of the YFP–Tsr gene; after folding and membrane insertion, the newly synthesized protein was detected (as the protein fluorescence exceeded significantly the cellular autofluorescence) for a few frames before the fluorophore bleached in a single step (figure 7(*b*)). This method allowed direct counting of the proteins synthesized by a single cell, and reported on the timing of their appearance (which loosely correlated to the timing of synthesis). It was observed that proteins appeared in 'bursts' with a large variation in copy number (figure 7(*c*)), and each burst was attributed to the synthesis of a single RNA molecule that gave rise to a geometric distribution of protein copy numbers. Although its biological insight was significant, the study was arguably more important from the methodological standpoint, since it established that one could obtain quantitative information about fundamental processes in the cell and measure directly protein stoichiometries, mobility, subcellular localization and copy numbers, to name a few important observables.

This initial work was followed by an impressive sequel on the long-standing question on how transcription factors (and, in general, DNA-binding proteins) locate their targets in cells (Elf *et al* 2007). Transcription factors are responsible for controlling much of gene expression in cells, and some DNA-binding proteins (such as *lac* repressor) can locate a site on chromosomal DNA fragment with rates up to 100-fold faster than expected on the basis of pure 3D diffusion; theories that include a 1D search component on short stretches of DNA have been developed and experimentally tested *in vitro*

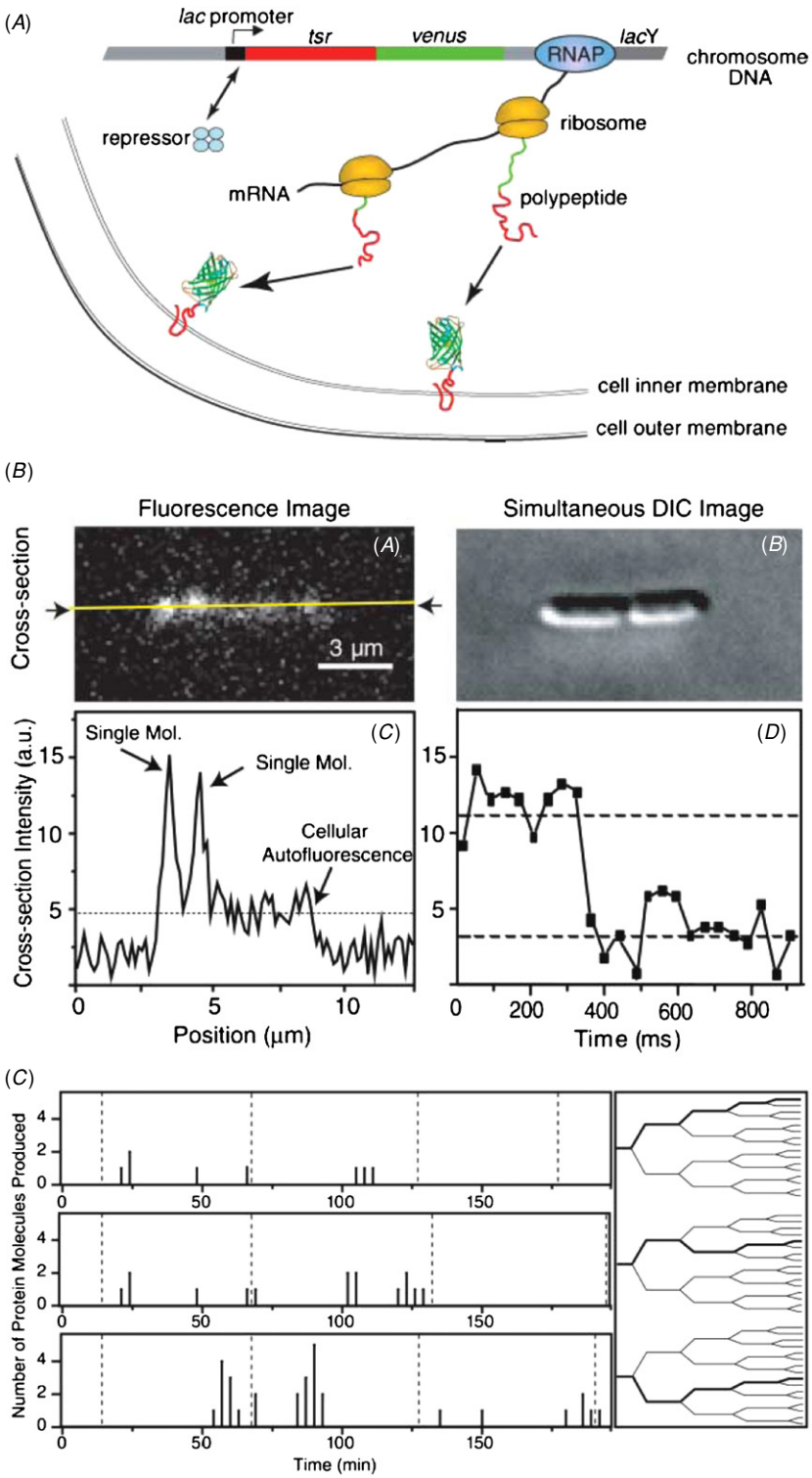

Figure 7. Single-molecule fluorescence detection inside living bacteria. (*A*) A genetic construct occasionally produces a rare protein fusion that localizes on the inner bacterial membrane and can be detected as a diffraction-limited spot. (*B*) DIC and fluorescence images of two bacterial cells, showing the presence of two fluorescence spots above the autofluorescence background; these spots correspond to single YFP molecules. (*C*) A time-series analysis of protein expression at the single-molecule level. Each protein expression event persists for a significant time, likely due to the rate-limiting steps of fluorescence development in the YFP fluorophore. Reproduced with permission from Yu *et al* (2006). Copyright 2006, AAAS.

to provide support for a hybrid mechanism that combines both 1D and 3D diffusion to account for the experimental observations (Wang *et al* 2006). However, no convincing data existed on whether these processes are relevant to the actual *in vivo* search process. This question was addressed using an inventive stroboscopic approach that illuminated YFP-labelled

lac repressor molecules for a short time interval during which minimal protein movement occurs due to diffusion, even when a protein freely diffuses in the cytoplasm. Equipped with this technique and performing mean-square displacement analysis, the group studied both the specific and non-specific interaction modes of *lac* repressor with DNA, characterizing the 3D diffusion of *lac* repressor in the cytoplasm and showing that the protein spends 90% of its time in 1D diffusion on DNA (while dissociating from DNA within 5 ms). These findings provided strong support for a combined 1D and 3D diffusion search mode for target search and paved the way for similar analysis on other DNA-binding proteins.

Further work from the same group (Choi *et al* 2008) demonstrated the importance of stochasticity in gene expression and phenotypic diversity seen in genetically identical cell populations; this was done by studying the mechanism for switching between a repressed phenotype, where only a few molecules of lacY (the permease of the *lac* operon) exist in the cell, and an 'activated' phenotype with high levels of lacY. It was found that a single molecule of lacY was insufficient for phenotype switching, in contrast to early proposals; instead, the lacY copy number threshold for switching was ∼400 molecules. Mutagenesis of the genetic control region showed that stochastic switching occurs due to a single dramatic event: the complete dissociation of a *lac* repressor molecule from the *lac* operon. In short, while partial dissociation of *lac* repressor (from one of the two *lac* operators on DNA, connected through a DNA loop) leads to short, frequent bursts of lacY insufficient for phenotype switching (since the number of synthesized lacY molecules is below the switching threshold), complete dissociation of lacY (along with the time delay for rebinding) leads to large bursts of synthesis that result in switching. This work provided another great demonstration of the potential of the single-molecule approach to address biological questions in the milieu of the cell.

Significant work, albeit with a different focus, has also been pursued in another bacterium, *Caulobacter crescentus.* Using single-molecule tracking to examine the *in vivo* diffusion of MreB (a bacterial actin homolog), Kim *et al* showed the presence of two distinct populations: one that diffuses freely (corresponding to single MreB-YFP molecules diffusing in the cytoplasm) and a second that performs a 'treadmilling', slow motion (Kim *et al* 2006). The latter motion was observed using time-lapse imaging (to minimize the effects of photobleaching) and was attributed to the addition of labelled MreB molecules to the growing end of the MreB filament (while depolymerization occurs at the opposite end), a behaviour that resembles that of actin filaments in eukaryotic cells. Using similar techniques, the same team characterized the diffusion properties of PopZ, a protein that link the chromosomal origins of replication to the cell poles (Bowman *et al* 2008).

Other important work in living bacteria involved the measurement of subunit stoichiometry and protein turnover in the bacterial flagella motor of *E. coli* using step-wise photobleaching as well as FRAP-based approaches (Leake *et al* 2006). Impressively, the work was performed in rotating cells, providing an instant functional assay about the performance of the molecular motor in operation. The same group also addressed similar questions about the bacterial Tat protein translocation system (Leake *et al* 2008). Similar techniques have been used to address questions regarding subunit stoichiometry in eukaryotic cells (Ulbrich and Isacoff 2007).

Progress has also been made in understanding the function of molecular motors *in vivo*. A number of reports described the use of very bright probes (either quantum dots or large vesicles with >100 GFP fluorophores) to achieve measurements of impressive spatial (∼1 nm) and temporal resolution (down to sub-millisecond). These techniques led to direct observation of the stepping behaviour of single kinesin and dynein molecules on microtubule tracks in living cells (Nan *et al* 2005, Toprak *et al* 2006), showing that the characteristic step size of 8 nm for kinesin seen *in vitro* is maintained *in vivo*; in addition, steps of 16- and 24 nm were also observed, raising the possibility that the cooperation of multiple kinesin molecules in carrying a single cargo may result in the larger steps for cargo movement. More recently, tracking of kinesin labelled with a smaller tag (a three-tandem monomeric Citrine tag) was also possible, showing agreement with *in vitro* work on the same motor. Similar work has also been done on single myosin V molecules labelled with quantum dots (Pierobon *et al* 2009). Finally, groundbreaking work on a large macromolecular machine, the nuclear pore complex, has reported on the dwell times and spatial trajectories for the movement of molecules through the pore (Yang *et al* 2004, Kubitscheck *et al* 2005).

From the examples above, it is obvious that the application of single-molecule fluorescence inside living cells has, and should continue to, unearth exciting new information that extends our understanding of biological mechanisms and the role of stochasticity in biology. With the recent addition of intramolecular smFRET observations (Sakon and Weninger 2010) to earlier observations of intermolecular smFRET in living cells (Murakoshi *et al* 2004), we expect to soon have access to molecular conformation and nanometre-scale conformational changes within cells, seeding another round of interesting studies, as well as insightful comparisons with *in vitro* observations.

A look into the future

Looking at our crystal ball, it seems likely that the main frontier will be the living cell, and possibly the living organism, since single-molecule measurements in live animals have already been reported (Schaaf *et al* 2009). Standardization of the new approaches through commercial superresolution instruments, as well as brighter and photostable probes (organic fluorophores, smaller quantum dots and nanodiamonds (Barnard 2009)), should aid research in this direction. The wide availability of single-molecule sequencing methods may also provide an unusual route for non-physicists to enter the world of single-molecule measurements, since the instruments used for the commercial platforms may also be used towards understanding basic processes, such as translation and reverse transcription.

Such instruments can also increase the throughput of single-molecule fluorescence measurements.

The next round of innovation will also benefit from the breakthrough developments in cameras; one can simply consider the introduction of scientific CMOS cameras which will offer an impressive 5 megapixel resolution at 100 frames per second. Another milestone in instrumentation should be the introduction of reliable and affordable white-light lasers (Kandidov *et al* 2003), which together with programmable filters and dichroic beam splitters (Ross *et al* 2007) could revolutionize the way the full spectrum of visible and near infrared light is used for detecting single fluorophores. Any innovation will also benefit from facile and robust site-specific labelling methods, techniques for gentle surface immobilization and new and efficient ways to internalize fluorescently labelled biomolecules in living cells.

Although we cannot guarantee that our predictions will turn out to be correct, we share the belief that single-molecule fluorescence will continue to advance and contribute to many areas of science in the next few years: the fluorescence future is definitely bright.

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