JOURNAL OF THE ROYAL SOCIETY Interface

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J. R. Soc. Interface 2008 **5**, 15-45
doi: 10.1098/rsif.2007.1021

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doi:10.1098/rsif.2007.1021
Published online 22 May 2007

REVIEW

Single-molecule biophysics: at the interface of biology, physics and chemistry

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Single-molecule methods have matured into powerful and popular tools to probe the complex behaviour of biological molecules, due to their unique abilities to probe molecular structure, dynamics and function, unhindered by the averaging inherent in ensemble experiments. This review presents an overview of the burgeoning field of single-molecule biophysics, discussing key highlights and selected examples from its genesis to our projections for its future. Following brief introductions to a few popular single-molecule fluorescence and manipulation methods, we discuss novel insights gained from single-molecule studies in key biological areas ranging from biological folding to experiments performed *in vivo*.

Keywords: single-molecule fluorescence; force; FRET; tracking; AFM; optical tweezers

1. INTRODUCTION

During the last two decades, the scientific community has witnessed the breakneck pace of growth of the field of single-molecule research, propelled by technological advances and the promise of revolutionary abilities to uncover usually hidden information. In conventional ensemble experiments, large numbers of molecules are interrogated simultaneously, and primarily averaged-out properties are observed. In contrast, single-molecule experiments provide access to an incredible wealth of molecular information, often seemingly limited only by the practitioner's creativity and resourcefulness. These powerful capabilities are now allowing the scientific community to ask and address previously 'unanswerable' questions in the physical, chemical and biological sciences. The broad impact of these advances is clear, with applications in fields as diverse as quantum optics, polymer- and photophysics, chemical bonding theory, and the *in vitro* and *in vivo* folding, assembly, dynamics and function of proteins and other cellular machines. The field of single-molecule science can be roughly divided into two general areas, as depicted in figure 1, one aimed at improving and developing single-molecule and supporting methodologies, and the other at using these methodologies to address important scientific questions. Owing to the diverse expertise critical to making key advances in the field, single-molecule research over the past decade has fostered much excellent collaboration, sparking a rich array of truly interdisciplinary research involving elements from biology, chemistry and physics.

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Several reasons make single-molecule methods especially attractive and powerful for the study of complex biological and chemical systems and processes. First, molecular properties are measured one molecule at a time in a single-molecule experiment. Hence, in contrast with conventional ensemble experiments, distributions in molecular properties are more directly measured. For example, multiple or distributions of folding states can be directly observed in a single-molecule experiment, but must generally be inferred indirectly in ensemble experiments. Rare species or states can also be detected in single-molecule experiments, which would be averaged in an ensemble. Second, dynamics of systems are routinely measured under equilibrium conditions for single molecules (or small ensembles). This is important not only for studies of equilibrium dynamics, but also for those of dynamics in systems that cannot be easily synchronized (as is generally required for ensemble measurements of kinetics). For example, the motions of DNA enzymes or molecular motors along their tracks can be complex and stochastic, making them virtually impossible to synchronize for significant distances. Single-molecule methods have found enormous popularity for studying such systems. The connectivity and kinetic rate constants between different states of a system with multiple discrete states can also be most directly measured using single-molecule methods. For example, the different folding and enzymatic steps during the reactions of ribozymes have been elegantly quantified using single-molecule fluorescence (Nahas *et al.* 2004). Additionally, single-molecule manipulation allows the direct measurement of molecular forces, as well as the molecular structural and functional responses to mechanical manipulation and control. Finally, the excellent

Received 23 February 2007
Accepted 23 April 2007

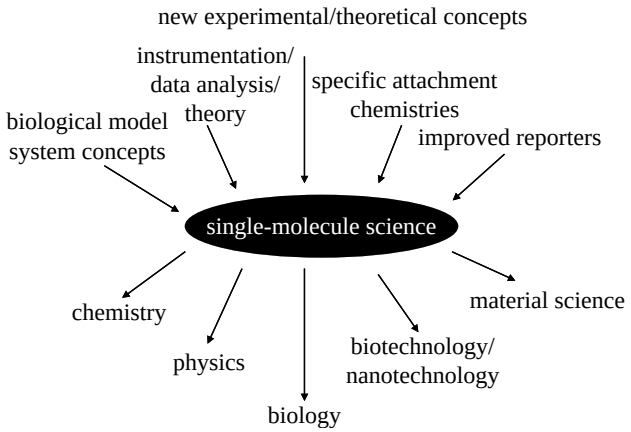

Figure 1. Components and scope of single-molecule science.

sensitivity of single-molecule methods also has direct benefits. Some biological molecules are prone to aggregation at ensemble concentrations; single-molecule methods operating at low concentrations or with immobilized molecules allow the properties of the monomeric species to be monitored at equilibrium. Additionally, many components in the cell are present in only a few (or even one) copies (Guptasarma 1995; Ghaemmaghami *et al.* 2003; Bon *et al.* 2006). Single-molecule methods allow these to be studied at 'native' concentrations in living cells.

Early steps towards optical single-molecule detection are apparent in the work of Hirschfeld in the 1970s, with detection of multiply labelled single antibody molecules (Hirschfeld 1976). The 1970s also saw the pioneering detection of currents from single ion channels in membranes (Neher & Sakmann 1976). Several exciting developments in modern optical and mechanical single-molecule detection were made starting in the late 1980s, including optical detection and spectroscopy of single chromophores in solids at low temperatures (Moerner & Kador 1989; Orrit & Bernard 1990); detection of single fluorophores under biologically relevant conditions (Shera *et al.* 1990); the use of the newly developed scanning tunnelling microscopy (STM; Binnig & Rohrer 1982) and atomic force microscopy (AFM; Binnig *et al.* 1986) to image individual DNA molecules in water (Lindsay *et al.* 1988, 1989; Engel 1991); and gliding filament and optical tweezers measurements of individual kinesin movement along microtubules (Howard *et al.* 1989; Block *et al.* 1990). The early years of the field resulted in several exciting developments and improvements in a variety of fluorescence and manipulation methods, and these methods were generally applied to probe simpler chemical and biological systems. In the context of single-molecule fluorescence, imaging was translated from low to room temperature (Betzig & Chichester 1993), a significant advance for studying chemical and biological systems under 'normal' conditions, and the popularization of a confocal far-field format made these techniques more accessible (Eigen & Rigler 1994). Towards the mid-1990s, more complex biological systems began to be explored using single-molecule fluorescence and manipulation techniques. For example, DNA stretching studies using glass fibres and optical tweezers led to the discovery of an overstretching transition (Cluzel *et al.* 1996; Smith *et al.* 1996); fluorescence imaging allowed the direct observation of individual ATP turnovers during myosin movement (Funatsu *et al.* 1995); epifluorescence imaging of F_1-ATPase provided a direct and dramatic visualization of its rotary motion (Noji *et al.* 1997); and mechanical protein unfolding began to provide insights into protein unfolding landscapes (Kellermayer *et al.* 1997; Rief *et al.* 1997; Tskhovrebova *et al.* 1997). Very promising and exciting results, increased funding opportunities, more convenient formats for experiments and commercial availability of several components and systems, and a large influx of talent and expertise into the field have since accelerated the number and quality of problems addressed by these techniques. Biological applications have included the discovery of static and dynamic heterogeneity during the activity of single enzymes; direct observation of multiple folding states and pathways of proteins and RNA; dissection of the folding and activity of a ribozyme; and strong evidence for a hand-over-hand mechanism for the movement of myosin and kinesin, to name just a handful. During the last few years, these observations have even been extended to studies of membrane structure, the initial events of signal transduction and the dynamics of proteins and RNA on and in living cells.

This review aims to briefly describe some of the most important capabilities of single-molecule optical and manipulation methods and to highlight how they can be used to answer key biological and chemical questions. The remainder of the review is organized in three main sections. Section 2 deals with a primer on single-molecule methods, with a focus on a few commonly used techniques including intensity and fluorescence resonance energy transfer (FRET)-based fluorescence microscopies, optical tweezers and AFM. In §3, we discuss a few key applications of single-molecule methods in biology. This section is organized by problem, and different approaches and the insights they provided are discussed about a given problem. In §4, we discuss our aspirations and projections for the field during the coming years. For readers interested mainly in applications, we envision that they can read through §§3 and 4, while referring to the methods primer (§2) as necessary for clarification. Owing to the size of the field, we have chosen to focus on a few illustrative examples and common techniques, resulting in a limited and perhaps somewhat idiosyncratic overview of the field. We refer the reader to other excellent reviews of the field for fuller and complementary viewpoints (Basche *et al.* 1996; Mehta *et al.* 1999; Moerner & Orrit 1999; Weiss 1999, 2000; Tamarat *et al.* 2000; Leuba & Zlatanova 2001; Moerner 2002; Rigler *et al.* 2002; Michalet *et al.* 2006; Muller *et al.* 2006), and hope that this review will encourage the general reader to explore the wealth of incredibly fascinating and diverse single-molecule literature not covered here.

2. SINGLE-MOLECULE METHODS PRIMER

Although desirable, no individual ensemble or single-molecule technique allows us to simultaneously study all the features of complex biological systems. Hence, multiple single-molecule techniques have often been

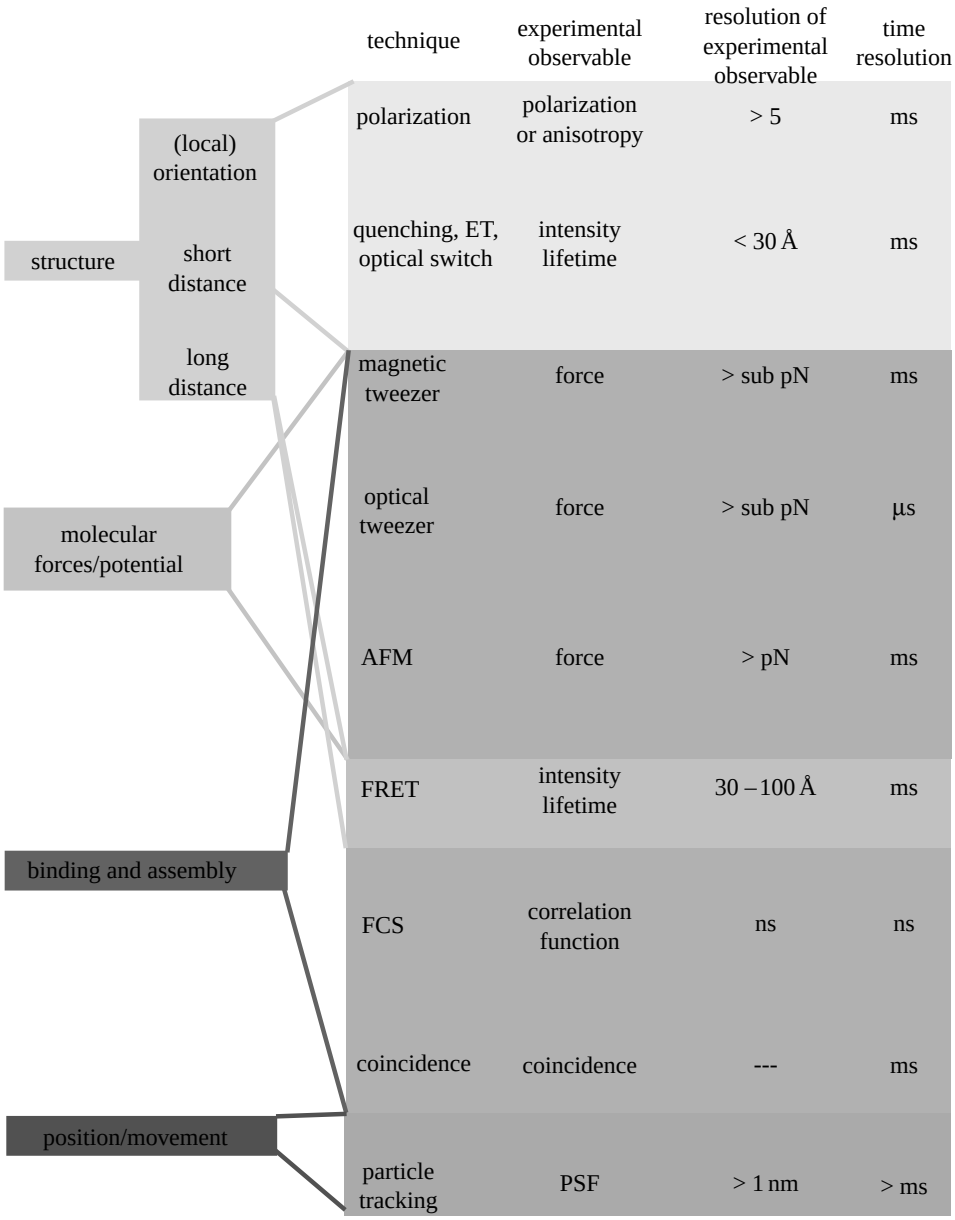

Figure 2. Overview of single-molecule methods and their main applications and strengths for measuring molecular properties.

applied to study different aspects of many important biological machines and processes, in many cases, complementing and building upon a host of other ensemble studies on the same or similar systems. While single-molecule techniques are not able to provide atomic resolution pictures of large molecules such as proteins, their strengths lie in probing detailed structural distributions, as well as real-time and stochastic dynamics. In this section, we provide a basic overview of a few commonly used single-molecule techniques. This overview of methods is not meant to be comprehensive, but rather to provide a basic understanding of the capabilities of and rationales behind the most commonly used techniques, while highlighting aspects of particular techniques that would be most useful for accessing or controlling a molecular property of interest, such as structure (distance or orientation), binding, conformational dynamics (time-scales), energy landscapes, force or nanoscale motions (see also figure 2).

2.1. Single-molecule experimental strategies and instrumentation

2.1.1. Fluorescence methods. Molecular fluorescence occurs due to radiative relaxation of molecular excited singlet states, and has been used extensively as a readout of molecular properties such as local environment (quenching), distance (FRET) and orientation (polarization). Single-molecule fluorescence detection is achieved by repeatedly exciting the fluorophore of interest, followed by detection and analysis of the emitted fluorescence photons. Since the rate and number of photons emitted by a single fluorophore is limited, fluorescence detection at single-molecule resolution has several key requirements, including the use of high-efficiency and low-background photon detection, and bright fluorophores (usually extrinsic dyes). Efficient photon collection is achieved by using high-efficiency optics, i.e. objective, filters and lenses in the detection pathway of the instrument. In particular, high numerical aperture objectives (numerical

aperture, NA; 1.2 water or higher oil objectives) are used, so as to collect a significant fraction of the emitted photons. To maximize detection efficiency, single-molecule instruments also have high-sensitivity detection units, usually either point detectors with high time resolution (e.g. avalanche photodiodes (APDs)) or CCD cameras (area detectors with lower millisecond time resolution). Background photons can originate from various sources such as scattering and fluorescence from buffers and impurities. Since the number of background photons is a function of the detection volume (because a larger volume of buffer will have more solvent and impurity molecules), a key element in minimizing this background is to minimize the detection volume using one of the several optical techniques. Based on these considerations, two popular geometries for single-molecule fluorescence detection, confocal and total internal reflection (TIR), are used most often for single-molecule fluorescence studies. Figure 3*a–f* shows the basic outlines of the methods discussed in the next few paragraphs.

Confocal microscopy (figure 3*a*). Confocal single-molecule detection is usually carried out using continuous wave (cw) or pulsed laser light to excite single fluorescent molecules. The emitted fluorescence is separated from excitation light using a dichroic mirror. Confocal detection is achieved by introducing a small aperture (pinhole) at an image focal point in the detection pathway, such that out of focus light is rejected by the pinhole. Such a configuration typically produces a very small, femtolitre (fl) detection volume (Pawley 1989). The resulting light is then passed through additional optics (e.g. high-performance filters and dichroic mirrors) to further reject background, to isolate fluorescence emission and, if needed (e.g. in FRET or polarization experiments), to split the light into separate spectral or polarization components. Detection is generally via APD point detectors. We note that similar small detection volumes can also be created by using two-photon microscopy, without the use of a pinhole (Denk *et al.* 1990). Two-photon microscopy has the advantage that illumination and detection volumes overlay, avoiding out of focus photobleaching, an important concern when studying cells or tissue. However, the nonlinear excitation has been reported in some cases to cause faster photobleaching in the focal volume when compared with cw excitation (Patterson & Piston 2000). Measurements can be carried out on diffusing molecules, or scanning the sample allows acquisition of images or time trajectories on immobilized single molecules.

Total internal reflection fluorescence microscopy (*TIRFM*, figure 3*b*,*c*). When an incident light beam undergoes TIR at the interface between a high and a low refractive index material, a very thin (approx. 100–200 nm thick) evanescent field penetrates the low refractive index material (for a review, see Axelrod 2001), providing the physical basis for TIRFM. This thin evanescent field can be used to selectively excite samples that are very close to the interface, while avoiding fluorescent background from the rest of the sample. Two general configurations of TIRFM are commonly used: prism-type and through-objective TIRFM (figure 3*b*,*c*, respectively). In the prism-type configuration, TIR occurs at the interface between the surface of a quartz microscope slide (optically coupled through glycerol or other fluid to a quartz prism) and the sample. The emitted fluorescence light is then collected on the opposite side by an objective. In this configuration, the illumination and detection optics are completely separated from each other and thus can be more easily modified; for example, variable angle TIR can be used to measure surface to object distances (Olveczky *et al.* 1997). In the through-objective mode, a very high numerical aperture objective (oil NA 1.4 or higher) is used to couple in excitation light at a super-critical angle, and the evanescent field is generated in the sample at the cover-slip–water (sample) interface. Key advantages of this latter approach are that the sample is more easily accessible from the open side (useful, for example, in live cell studies) and the higher NA objectives can allow for increased lateral resolution. Detection is generally carried out using a CCD camera to acquire real-time series of images of immobilized or slowly diffusing species (e.g. in live cells).

Freely diffusing and immobilized molecules. An important consideration for molecular studies is whether the system is immobilized or freely diffusing in solution (for reviews, Deniz *et al.* 2001; Rasnik *et al.* 2005). Most studies are carried out on surface-immobilized molecules, using either confocal or TIR geometries, because this permits the acquisition of long fluorescence signal time traces for individual molecules. An analysis of the signal fluctuations provides information about different states and lifetimes. Although successful in many cases, a significant drawback of this method for smaller and dynamic molecular systems is that there is a significant danger of surface-induced perturbations in the molecular properties. Additionally, a method of non-covalent or covalent immobilization must be used. Immobilization of RNA and DNA molecules is well worked out, but protein immobilization methods are still evolving (Rhoades *et al.* 2003; Kuzmenkina *et al.* 2005; Rasnik *et al.* 2005). Additionally, at least some of the dynamics of such polymers may be modified due to confinement close to a surface or in small enclosures (Friedel *et al.* 2006). A complementary method that is increasingly used is to observe freely diffusing molecules in solution using confocal detection at low (approx. 100 pM) concentrations, thereby minimizing surface-induced problems (Deniz *et al.* 2001). An analysis of the bursts of photons emitted by molecules as they traverse the focal volume provides information about molecular properties. It should be noted, however, that the latter method is not useful for studying longer dynamics of individual molecules, since the observation time is limited by the diffusion time of the molecules through the observation volume, generally to a few milliseconds or less. Rather, this method is very useful to quickly provide information on distributions of molecular properties (such as distance) as well as sub-millisecond fluctuations for a statistically significant number of single molecules. Methods where the molecules of interest are 'pseudo-immobilized' in vesicles or gels, so that molecules are still diffusing, but confined to sub-diffraction volume, can provide some of the advantages

of both the above modes, and are being used in a few applications (Dickson *et al.* 1996; Rhoades *et al.* 2003; Rasnik *et al.* 2005). A novel method of trapping water droplets confining individual molecules is very promising, because reactions can be initiated by fusion of two droplets containing different species (Reiner *et al.* 2006). Another novel method has been recently described that uses an electrokinetic trap to suppress the Brownian motion of individual small particles in solution, and could be very useful for extended studies of single molecules without physical immobilization (Cohen & Moerner 2006).

Multiparameter fluorescence detection. Although most single-molecule fluorescence instruments rely on intensity measurements, multiparameter data acquisition is a powerful tool. An efficient scheme has been described to collect information about arrival time, corresponding excited-state lifetime, colour and polarization, for each photon. Using this information, it is possible to calculate several parameters of interest, such as intensity time trajectories, fluorescence lifetimes, anisotropy and correlation functions (see below; Rothwell *et al.* 2003; Widengren *et al.* 2006).

2.1.2. Manipulation methods. Several manipulation methods have been used successfully to study molecular structure, potential energy landscapes and function at the single-molecule level. These methods provide ways to exert force or torque on molecular species and observe their responses to these applied forces. Important basic considerations for using these techniques are (i) how to attach the molecule of interest to one or two surfaces, (ii) how to manipulate and monitor the distance or rotational state between the two attachment points, and (iii) how to measure and/or control forces or torques exerted on the molecule of interest. Attachment to surfaces is generally achieved by using biotin–streptavidin, antibody, histidine tag or even non-specific binding strategies. Forces can be exerted by a variety of physical means, including mechanical (AFM, microneedles, etc.), optical (optical tweezers), magnetic (magnetic tweezers), fluid flow or electrical forces. Measurement of forces is often carried out by measuring the displacement in a potential, but can also be achieved by more direct means (Smith *et al.* 1996; Strick *et al.* 1996). Position sensing is achieved using position sensitive or other detection schemes (Engel & Muller 2000; Gosse & Croquette 2002; Neuman & Block 2004). Among the most popular single-molecule manipulation techniques are AFM, and optical and magnetic tweezers, which we discuss in more detail below.

Atomic force microscopy (*AFM*, figure 3*d*). AFM (Binnig *et al.* 1986) is part of the family of scanning probe microscopies, the first of which was scanning tunnelling microscopy (Binnig & Rohrer 1982). During the last two decades, AFM has increasingly become a powerful tool for studying molecular processes at single-molecule resolution (Bustamante *et al.* 2000; Clausen-Schaumann *et al.* 2000; Engel & Muller 2000; Fisher *et al.* 2000; Zlatanova *et al.* 2000; Kienberger *et al.* 2006). Two kinds of measurements are commonly made with such an instrument. In the first (mode 1), a scanning mode is used where structural features of the molecule of interest are probed, and changes as a function of time or reaction can also be monitored. The second (mode 2) is a pulling mode, where unfolding or unbinding forces and dynamics can be measured, providing detailed information about the folding or binding energy landscapes of biomolecules and complexes. The AFM instrument generally contains a moveable (x, y, z) piezoelectric sample stage, a small and highly flexible cantilever which ends in a narrow tip and a quadrant photodiode detector, as shown in figure 3*d*. The sample is deposited on a mica (or other) surface on the sample stage (mode 1) or connected between the surface and the tip using various chemistries (mode 2). A laser beam is reflected off the back surface of the cantilever and its reflection is detected with high precision using the quadrant detector. Since a change in the deflection of the cantilever will change the reflection angle of the laser beam, the detector signal provides a high-resolution measurement of the cantilever deflection. The tip can be raster scanned over the surface (mode 1, usually while tapping the tip on the surface to avoid scratching or scraping soft biological samples), and the recorded cantilever deflection as a function of position provides a topographic map of the surface-immobilized molecules, with a resolution limited by the size and shape of the tip. In addition to topographic information, such images can provide information about forces that arise from interactions of the tip (functionalized or not) with the surface, even in the absence of covalent linkages between the two. In mode 2, using the same arrangement, the molecule of interest is instead tethered between the tip and the surface, and the surface is withdrawn from the tip using the piezoelectric stage. In this case, the deflection of the cantilever (within approximately a harmonic potential) provides a readout of the force being exerted on the molecule, while the distance between the surface and the tip is simultaneously controlled. This can provide a force–extension curve for the molecule. Additionally, in mode 2, feedback-based force clamps can be used to study system transitions under quasi-equilibrium conditions, where time trajectories of molecular distances can be obtained under constant force conditions.

Optical tweezers (figure 3*e*). Optical or laser tweezers (also called optical traps) use light forces to manipulate molecules and measure the associated forces (Perkins *et al.* 1994; Ashkin 1997; Bustamante *et al.* 2000; Clausen-Schaumann *et al.* 2000; Grier 2003; Neuman & Block 2004) originating in the seminal experiments of Art Ashkin in the 1980s (Ashkin *et al.* 1986). As in the case of the pulling mode of the AFM, the molecule of interest is tethered between two surfaces, one being of a bead manipulated using an optical tweezer and the second of a cover-slip or a second bead held in place by suction using a micropipette or another optical tweezer. The optical tweezer is achieved by strongly focusing a laser beam into the bead. Light gradient forces then act to centre the bead at the focal point of the laser beam (while scattering, thermal forces and forces due to intra- or inter-molecular attractive forces will tend to pull the bead out of this position). By changing the position of the laser beam focus, it is possible to manipulate the position of the bead and the force acting on the molecular system. Since the deflection of the bead relative to the laser focus is a

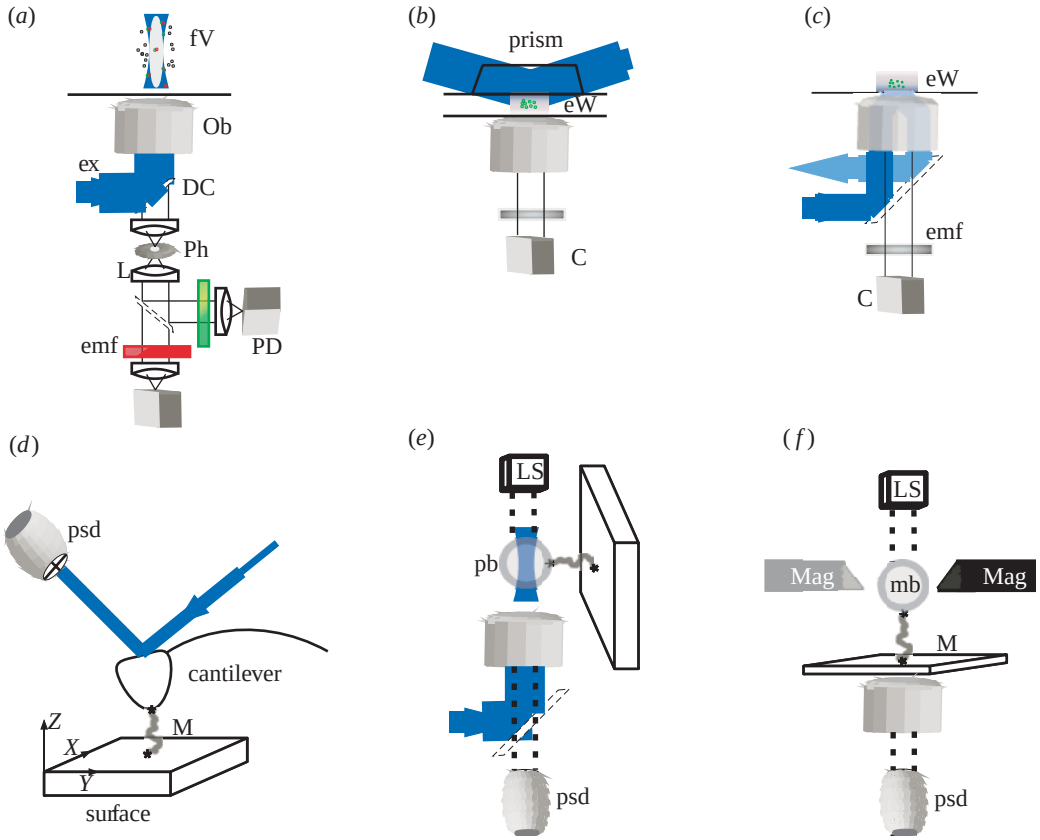

Figure 3. Overview of single-molecule instrumentation. Six examples for typical single-molecule instrumentation. Components that occur in more than one type of instrumentation are often labelled only in the panel where they appeared first for better clarity of the picture. (*a*) Confocal geometry and dual-colour detection; focal volume (fV), objective (Ob), excitation with a laser (ex), dichroic mirror (DC), pinhole (Ph), point detector (PD), emission filter (emf). (*b*) Prism-type TIRFM. (*c*) Through-objective TIRFM; evanescent wave (eW), area detector/camera (C). (*d*) AFM; position-sensitive detector (psd), molecule (M). (*e*) Optical tweezer; light source (LS), polysterene bead (pb). (*f*) Magnetic tweezer; magnet (Mag), magnetic bead (mb).

function of the force exerted on the molecule, force–extension curves can also be measured in this case. Since the beads are typically micron sized, this technique was generally used for studying larger systems such as those involving large duplex DNA, protein polymers or motor proteins, although improved methods are also allowing the study of smaller systems such as individual proteins and RNA molecules.

Magnetic tweezers (figure 3*f*). Magnetic tweezers use magnetic fields to apply forces on molecules (Smith *et al.* 1992). The technique is especially useful because, in addition to application of linear forces, it also allows torque to be easily applied to individual molecules (Strick *et al.* 1996; Gosse & Croquette 2002). Magnetic tweezers have been successfully used to study the action of DNA-processing proteins on DNA twisting, e.g. the effects of topoisomerase II on DNA supercoiling (Strick *et al.* 2000). In this case, one end of the DNA is attached to a surface and the other end is attached to a micron-sized magnetic bead by multiple attachments to prevent free rotation. By using magnets close to the surface, a force can be applied to the molecule, and it can be stretched. More interestingly, because the bead is superparamagnetic, it can be rotated by using the external magnet assembly. Since the DNA (or other) molecule is multiply attached to the bead, this causes a torque to be applied on the tethered molecule. This allows elegant studies of the effects of torque and linear forces on the extension and biology of the molecule to be performed. The method has even been used to reverse the chemistry of the ATPase enzyme using the exerted torque to cause the rotary motor to reverse its spontaneous direction of rotation (Itoh *et al.* 2004).

2.2. Measuring molecular properties at single-molecule resolution

2.2.1. Structural information. Distance. A significant effort in the single-molecule field has gone into developing methods to measure intra- and inter-molecular distances in single molecules and complexes. Two kinds of methods are mainly used in fluorescence measurements, based on (i) distance-dependent alteration of dye emission properties or (ii) localization of point spread functions (PSFs). The latter one is especially useful to follow the displacement of a particle. The first method will be discussed here, and the latter in §2.2.2. Additionally, size estimates for single molecules can also be obtained using fluorescence correlation spectroscopy (FCS) or polarization measurements, as described in later sections.

A popular and powerful tool for measuring single-molecule distances is FRET (Ha *et al.* 1996; Deniz *et al.* 1999; Weiss 1999). Site-specific attachment of a fluorescent donor (D) and acceptor (A) to a biological macromolecule allows measurement of the FRET

efficiency (E) between the D–A pair. E, which is the radiationless transfer of energy from D to A, has a steep dependence on distance, given by $E=1/(1+(R/R_0)^6)$. Here, R is the distance between the D–A pair, and R_0 is the Förster radius (distance for $E=0.5$), and is a characteristic for each dye pair in a given environment. Choice of the dye pair permits sensitive distance measurements for molecular distances of interest, with the maximum sensitivity to distance changes being around R_0. In practice, however, the distances that can be measured with reasonable accuracy range between approximately 3 and 10 nm, because E plateaus towards 1 and 0, respectively, outside these values for common single-molecule dye pairs. Extension of this method to additional dyes is emerging as a powerful method to analyse the correlation between conformational changes in molecules or complexes. For example, three-colour smFRET measurements can provide information about up to three different distances in a molecule or complex (Hohng *et al.* 2004; Clamme & Deniz 2005; Lee *et al.* 2007). Correspondingly, an n-dye system could report on up to $n(n-1)/2$ distances, although the associated distance measurement errors and demands on dyes and instrumentation will increase significantly.

E is generally measured using either ratiometric intensity measurements or lifetime measurements. Ratiometric intensity measurements are somewhat less instrumentation-demanding to perform, requiring only cw laser illumination of the donor, followed by spectral separation of the donor and acceptor emission signals and subsequent data analysis. E is then calculated as a ratio of the corrected emission signals, as $E=I_A/(\gamma I_D+I_A)$, where I_D and I_A are the donor and acceptor signals, respectively, and γ is a correction factor for differential quantum yields and detection efficiencies on the two channels. For the purpose of monitoring subpopulations, changes in distance and fluctuations, an observed E or proximity ratio is often reported with γ set to 1. For more accurately estimating molecular distances, γ can be estimated using control measurements with dyes of known properties (Schuler *et al.* 2002), photobleaching (Kitamura *et al.* 1999) or alternating laser excitation (ALEX; Lee *et al.* 2005*b*). The latter method also provides a means to identify and quantify low-FRET peaks (below $0.1E$), which usually overlap with a peak (zero peak) due to non-fluorescent acceptor (e.g. due to incomplete labelling or photobleaching) in measurements on diffusing molecules (Kapanidis *et al.* 2004). Fluorescence lifetime measurements need more sophisticated time-correlated single photon counting set-ups with picosecond pulsed laser excitation. Here, the arrival time and delay time between the excitation pulse and the photon detection event are recorded for each photon, allowing lifetimes to be estimated for individual molecules. Such experiments allow direct measurement of distances and do not require separate γ measurements (Rothwell *et al.* 2003).

For distances shorter than approximately 3 nm, quenching methods are showing significant promise. For example, quenching due to electron transfer from a fluorescent flavin to an adjacent tyrosine allowed enzyme fluctuations to be monitored during the activity of the enzyme flavin reductase (Yang *et al.* 2003). Another method that uses site-specifically attached dye and TEMPO quencher moieties, similar to FRET, has been shown to be useful for measurement of distances below 3 nm (Zhu *et al.* 2005). An interesting alternative is a dye pair that has been described to function as an optical switch in a distance-dependent manner, also allowing short distances to be measured (Bates *et al.* 2005).

Single-molecule distances longer than 10 nm have not been readily accessible by spectroscopic means. A recently described method that shows promise uses plasmon resonance between gold nanoparticles, which can be used to measure distances up to tens of nanometres (Sonnichsen *et al.* 2005). Other methods for measuring longer distances involve localization of PSFs or beads, and work for immobilized or slowly moving molecules (discussed in §2.2.2). One relatively simple yet useful method for measuring such longer distances is tethered particle motion, which uses the Brownian motion of particles tethered to a surface to learn about the length of the tether (Schafer *et al.* 1991; Yin *et al.* 1994).

In addition to manipulation of individual molecules, AFM and optical tweezers also allow separation distances to be measured for the molecular system under consideration (see also §2.2.3).

Binding and assembly: FRET, coincidence, correlations and pulling. Binding and assembly of molecular complexes can be detected in several ways. When the complexes are small or their structures are known, smFRET can be used as discussed above, by labelling each interacting partner with a donor or acceptor dye. Binding can also be detected when the dyes are not within FRET range, such as for a complex of unknown structure, by using the spatial or temporal coincidence of fluorescence signals on two spectral channels (Li *et al.* 2003; see also cross-correlation analysis (CCS) and ALEX measurements). For example, two interacting components can each be labelled with one of the two dyes with well-separated spectra, and experiments carried out using two excitation lasers. At low concentrations, where overlap (in time for bursts from diffusing molecules or in an image for immobilized molecules) of signals for two separate molecules occurs with low probability, overlap provides a convenient measure of binding. Binding can also be assessed at somewhat higher concentrations using the related fluorescence auto- or cross-correlation methods (Eigen & Rigler 1994; Schwille *et al.* 1997).

These methods can be extended to provide a powerful means to study binding and assembly of multi-component complexes, often key components of cellular machinery. For example, recently described three-colour coincidence can provide binding information for three interacting components, while three-colour smFRET or ALEX measurements can provide simultaneous distance and binding information (Heinze *et al.* 2004; Hohng *et al.* 2004; Clamme & Deniz 2005; Lee *et al.* 2007).

One key problem has to be kept in mind when designing experiments on complexes with multiple labelled components. Since the concentration of labelled components in single-molecule experiments is often in the

sub-nanomolar regime, these complexes should have high affinities (nanomolar range), or a slow dissociation constant. Alternatively, methods reducing the size of the observation volume can also allow for studies at higher concentrations. Various approaches may be used employing nanotechnology (Levene *et al.* 2003, and for a review, Craighead 2000), near field techniques (Betzig & Chichester 1993) and methods that allow observation of a very small volume (e.g. smaller than a confocal volume), such as TIR microscopy (Thompson & Axelrod 1983) or other far-field methods with high spatial resolution (Hell *et al.* 2004).

Pulling experiments via various manipulation methods also provide a means to mechanically measure binding, unbinding forces and binding energy landscapes, and have been used to measure a variety of non-covalent and covalent binding affinities.

Molecular orientation: fluorescence polarization. If a single fluorophore is excited with polarized light and the emitted light is split into light with parallel and perpendicular polarizations, the fluorescence anisotropy can be calculated in a ratiometric manner (Schaffer *et al.* 1999). The value of this parameter is inversely related to the rotational mobility and becomes zero in the limit where the molecule tumbles fast compared with the fluorescence lifetime (typically a few nanoseconds). Since rotational mobility is dependent on the size of the molecule, this parameter provides a measure of molecular size. For immobilized molecules, if information is available about the orientation of the fluorophore relative to the molecule of interest it is attached to, even the absolute orientation in space can be determined (for a review, see Rosenberg *et al.* 2005), with a resolution as good as 5° angle precision and down to greater than 100 µs time resolution.

Since the energy transfer from a donor to an acceptor is dependent on the orientational factor κ^2, the rotational mobility of dyes is important for more accurately determining distances in FRET measurements. This factor is often set to 2/3 in single-molecule measurements. However, a lack of orientational averaging during the donor lifetime (signalled by high anisotropy values) is probable to result in values of κ^2 different from 2/3 and thus need to be taken into account when calculating absolute distances (Lakowicz 1999).

2.2.2. Following movement of molecules: tracking techniques. The basic idea behind fluorescence single-molecule tracking (SMT) and single particle tracking (SPT) is as follows. Using illumination from a microscope, the image of each fluorescent molecule or small particle, no matter how small, appears with an intensity distribution that is specific for every microscope and called a PSF. When imaged on a two-dimensional detector, this distribution can often be approximated by a two-dimensional Gaussian function. Many particle tracking algorithms are available to accurately determine the position of this distribution, most commonly using centre of mass or direct Gaussian fitting for sub-resolution particle tracking (Cheezum *et al.* 2001; Thompson *et al.* 2002). More complex tracking algorithms might be required for large particles (Cheezum *et al.* 2001; Thompson *et al.* 2002; Gennerich & Schild 2005). A precise fitting routine is crucial for a good position estimate. In addition to using low-noise detectors, bright fluorophores and high NA objectives, using a small pixel width with respect to the width of the PSF is essential to avoid precision loss from undersampling. Thus, choosing pixels that are too large might cause neighbouring points to fall within one pixel. In general, collecting more photons (thus decreasing the acquisition rate) increases the signal to noise ratio and thus the precision of the tracking algorithm. By collecting a large number of photons, a precision down to a few nanometres has been achieved (Yildiz *et al.* 2003; Nan *et al.* 2005). There is always a trade-off between high sampling rates, precision and duration of the study, mainly limited by photobleaching of the fluorophore. Although SMT and SPT are similar, blinking in the single-molecule case can cause the fluorophore to disappear and appear in an image, which increases the problems with tracking one individual fluorophore over time. Research in the area is continuously adding new mechanistic understanding and tools to reduce such blinking and photobleaching of single-molecule dyes (Eggeling *et al.* 2006; Rasnik *et al.* 2006). PSF localization analysis combined with information from photobleaching has also been used to measure nanometre distances between single fluorophores (Gordon *et al.* 2004). Many SPT studies also make use of quantum dots, which are superior to most dyes in terms of photobleaching (Michalet *et al.* 2005). Particle tracking inside living cells is also possible with non-fluorescent labels, such as gold particles (Tkachenko *et al.* 2005) or even cellular structures that have a sufficient contrast in the light microscope. Particle tracking algorithms are also often applied in optical or magnetic tweezer configurations to determine the position of the bead. Fluorescence-based particle tracking methods can be extended to the dual- or multi-colour case, if the dyes are sufficiently spectrally separated. Objects can also be tracked along the optical axes, for example, optical slices are recorded by stepping the objective in the axial direction. Since the precision can be readily increased by having a sharper intensity distribution, techniques enhancing axial resolution, such as confocal or 4Pi microscopy, can be especially advantageous (Egner & Hell 2005).

2.2.3. Molecular forces and potentials. A number of processes in biology exert or rely on mechanical forces, ranging from muscle proteins, unfoldases, and motor and transport proteins to the possible role of strain in enzyme catalysis (Bustamante *et al.* 2004). Single-molecule manipulation methods allow the direct measurements of forces generated during these processes. Additionally, the application of forces on molecular systems allows studies of their structure and function following controlled changes to their potential energy landscapes. Force resolution depends on the stiffness of the potential used for their measurement. Forces that can be exerted/measured range from down to femtonewton up to approximately 10 pN and 1 nN by magnetic and optical tweezers, respectively,

due to the low stiffness of the trap potentials. For optical tweezers, the higher-end forces are accessible using dual laser traps, with counter-propagating laser beams, which stabilize the trap by cancelling reflected light forces. The corresponding distance resolution or stability in optical tweezers measurements is usually limited to the 2–3 nm range, and a similar range applies to magnetic traps. However, near-angstrom resolution has been recently achieved using an ultrastable dual optical trap (Abbondanzieri *et al.* 2005; Moffitt *et al.* 2006). Another approach using microfabricated fiduciary reference marks has also resulted in extreme stabilization of tweezers set-ups to the 1 Å level in all three dimensions (Carter *et al.* 2007). The AFM can make measurements in the piconewton to 100 nN force range, depending on the stiffness of the cantilever used, with angstrom distance resolution being possible in the axial dimension (Bustamante *et al.* 2000; Samori *et al.* 2005). The AFM lateral distance resolution during single-molecule imaging is determined by the stability of the set-up, the reproducibility of the piezoelectric movement and the shape of the tip. For feedback-stabilized set-ups, the resolution is usually limited by the radius of curvature of the tip, typically approximately 10 nm with regular silicon tips, but can be in the low nanometre range. Variance analysis has even allowed a sub-angstrom conformational change to be detected using AFM experiments in a recent study (Walther *et al.* 2006). Carbon nanotubes, with tip resolution of 2–6 nm, also allow tall or deep structures to be imaged (Wong *et al.* 1998; Martinez *et al.* 2005) with less danger of tip breakage. Additionally, sub-nanometre resolution has been reported for imaging of two-dimensional arrays of single molecules (Muller *et al.* 2006). Finally, an important advance in using optical tweezers for studying molecular potentials has been recently made by implementing a passive force clamp. This method avoids the limited response time of feedback-based optical traps, by cleverly using the region of the trapping potential where force varies minimally with position in the trap (Greenleaf *et al.* 2005).

2.2.4. Molecular dynamics: time resolution and observation time. Within the limits determined by photon emission rates of single dye molecules, fluorescence studies of very fast processes are ultimately limited by the detection quantum efficiency and effective acquisition rate. Thus, time trajectories of molecular distances, orientations or other properties can be plotted with a time resolution of few milliseconds (imaging) or less than a millisecond (point detection). A more detailed analysis of histogram peak shapes can also provide information about fluctuations somewhat faster than this resolution (down to approx. tens of microseconds; Pljevaljcic *et al.* 2004; Gopich & Szabo 2005). SmFRET or polarization histogram analysis requires at least a few minutes of data collection. However, this dead time has been decreased to approximately 100 ms by using a continuous-flow mixer (Lipman *et al.* 2003). Recently, in the context of rotary motors, a time resolution of a few microseconds was achieved for studying the rotation of individual ATPase molecules, by using light scattering from an attached gold nanorod (Spetzler *et al.* 2006).

Even sub-microsecond processes can be studied by correlation analyses of fluorescence fluctuations recorded using the kilohertz to megahertz sampling rates of APDs in a small detection volume. Although possible at single-molecule concentrations, such experiments are typically carried out at somewhat higher concentrations (nM) of freely diffusing molecules, with a few molecules present on average in the focal volume. Under such conditions, while information is not obtained about each individual molecule, several properties of the molecular system can be extracted by an analysis of fluctuations in such a small ensemble of molecules over time. Along these lines, FCS (Magde *et al.* 1972, 1974; Thompson 1989; Rigler *et al.* 1993) is perhaps the best known, using the autocorrelation of fluorescence fluctuations to extract parameters such as concentration, diffusion coefficients and reaction kinetics. In the context of molecular dynamics, such an analysis has been used to study microsecond and even nanosecond conformational fluctuations in biological molecules (see §3). The molecular brightness of an individual species is also accessible by this method, while for a mixture of more than one species, complementary methods can be used. Conveniently, these techniques (such as photon counting histogram, fluorescence intensity distribution analysis and photon arrival time-interval distribution) do not require major new instrumentation, but in principle, can be used to extract molecular brightness by an analysis of the same photon emission streams as FCS, and thus provide a means for quantifying binding or aggregation (Qian & Elson 1990; Chen *et al.* 1999; Kask *et al.* 1999; Palo *et al.* 2000; Laurence *et al.* 2004). If two fluorescent particles are studied, whose emission spectra can be well separated, additional parameters like interaction of those particles with each other can be obtained by a CCS of the two measured fluorescence fluctuation time traces (Schwille *et al.* 1997). Properties of the detector, such as dead time and after pulsing, limit the time resolution in the sub-microsecond time range. These problems can be overcome by splitting the light onto two detectors, increasing the time resolution down to the sub-nanosecond regime (Hanbury Brown & Twiss 1956). Finally, nanosecond time-scale information about the existence of molecular subpopulations can be obtained from multiple decays in single-molecule fluorescence lifetime data (Deniz *et al.* 2001; Laurence *et al.* 2005). For the study of slow processes, the acquisition time can be quite long, being limited by dye photobleaching and instrument stability to several seconds or longer.

3. APPLICATIONS IN BIOLOGY

In §§3.1–3.6, we describe a handful of select examples, showing the power of different single-molecule experiments to tease out structural and mechanistic details at the molecular level that would be hard or impossible to obtain from an ensemble. We touch on six general areas, with examples classified into protein folding, DNA and RNA structural studies, biological assembly and function, single-molecule enzymology, molecular motors and studies in living cells. We note

that the classification of systems or problems into the different subsections is not completely unique; rather, it is merely provided as a rough framework. Here again, the presentation is far from comprehensive, but rather is meant to provide a flavour for the wide variety of problems tackled and insights obtainable by such studies. A few select figures are also presented to give the reader a feel for a range of data from different methods.

3.1. Protein folding

The question of how an unfolded polypeptide chain is converted into a natively folded and functional protein continues to fascinate biologists, chemists and physicists. Conceptual breakthroughs and technological developments have flourished in this area during the past four decades. Early views of protein folding relied on theoretical descriptions developed for small-molecule reactions, involving one or a small number of specific folding pathways and intermediates (Kim & Baldwin 1982). In the 1980s, the concept of protein folding based on a statistical mechanical theory of spin glasses was developed (Bryngelson & Wolynes 1987; Bryngelson *et al.* 1995; Dill & Chan 1997). In this 'New View', the energy landscape of protein folding was described in terms of a folding funnel (figure 4*a*). Intermediates were proposed to occur as a result of ruggedness in this funnel. Such intermediates could slow down the folding process and sometimes result in a kinetically trapped and misfolded conformer of a protein. Folding reactions on such an energy landscape could be complex, exhibiting distributions of conformational states and folding trajectories (Kuzmenkina *et al.* 2005). Much of this interesting behaviour is obscured by the averaging inherent in ensemble experiments. In contrast, observing the folding of single proteins promises to directly resolve the heterogeneity of states and folding pathways, and to disclose intermediate states even if they are only transiently and incoherently populated.

FRET has evolved into a very powerful probe of folding at single-molecule resolution. The first single-molecule FRET experiment to probe folding was reported by Jia *et al.* (1999) for a GCN4 coiled-coil peptide non-specifically immobilized on a glass surface. This work enabled the observation of anti-correlated changes in donor and acceptor fluorescence intensities, due to conformational fluctuations. Talaga *et al.* (2000) reported single-molecule FRET measurements on the folding and unfolding conformational equilibrium distributions and dynamics of a disulfide cross-linked version of the two-stranded coiled coil from GCN4. In a correlation analysis of their data, they observed folding dynamics on a time-scale in agreement with ensemble experiments, but also showed slow conformational fluctuations for the unfolded peptide, probably because of interactions with the surface.

Deniz *et al.* (2000) introduced the use of ratiometric smFRET to study protein folding at single-molecule resolution under freely diffusing conditions. Using this methodology, which minimizes any extraneous factors related to protein–surface interactions, the two-state conformational behaviour of chymotrypsin inhibitor 2 (CI2) was directly monitored as a function of denaturant concentration (figure 4*b*). Properties of folded and unfolded subpopulations were analysed to extract a single-molecule denaturation curve, as well as a possible small expansion in the unfolded state as a function of increasing denaturant concentration. In a subsequent study, the cold-shock protein Csp*Tm* also showed bimodal distributions of FRET efficiencies, similar to CI2, at moderate denaturant concentrations where both the native and the unfolded protein coexist (Schuler *et al.* 2002). The FRET peak corresponding to the population of the unfolded form clearly showed progressive movement, indicating a decrease in the average end-to-end distance due to a collapse to more compact denatured conformations at low denaturant concentration. Also, by showing that the width of the FRET efficiency distribution of denatured Csp*Tm* was similar to that of a rigid polyproline type II helix, a 25 µs upper limit for the reconfiguration time in the unfolded state was estimated. Using Kramer's theory of chemical kinetics and ensemble microsecond-stopped-flow kinetic experiments, it was possible to estimate the limits on the height of the folding free-energy barrier (Δ): 11 $k_{\mathrm{B}}T > \Delta > 4k_{\mathrm{B}}T$ (where k_{B} is the Boltzmann constant and T is the temperature). The unfolded ensemble of Csp*Tm* was further investigated using smFRET in two recent studies. In one study, distance distributions for the unfolded state obtained using FRET pairs attached at different positions on the protein chain agreed well with a Gaussian chain model, both under low and high denaturant concentration (Hoffmann *et al.* 2007). In the other study, differences in average donor fluorescence lifetimes from photons corresponding to high- and low-efficiency parts of the unfolded peak in the FRET histograms were taken to potentially indicate slow ($\gg$1 ms) polypeptide chain dynamics in the unfolded state (Merchant *et al.* 2007).

Two-state folding behaviour has also been observed for ribonuclease H (RNase H) immobilized on star-polymer surfaces, using single-molecule FRET (Kuzmenkina *et al.* 2005). FRET time trajectories from these immobilized proteins demonstrated that fluctuations between folded and denatured states could be directly observed. Folding free energies and cooperativity for such immobilized molecules were similar to the values for diffusing molecules, showing the promise of the method of immobilization for single-molecule folding studies.

Insights have also been gained into protein unfolding and folding using mechanical unfolding experiments (Mehta *et al.* 1999; Clausen-Schaumann *et al.* 2000; Bustamante *et al.* 2004; Samori *et al.* 2005). Early work in this area focused on pulling on multiple protein repeats, mainly on the muscle protein titin using optical tweezers or AFM (Kellermayer *et al.* 1997; Rief *et al.* 1997; Tskhovrebova *et al.* 1997). In these studies, a sawtooth-like pattern was observed due to the sequential unfolding of multiple protein units. An interesting insight that has emerged from such experiments on other proteins is that protein mechanical stability is dependent on the pulling locations on the protein (Brockwell *et al.* 2003;

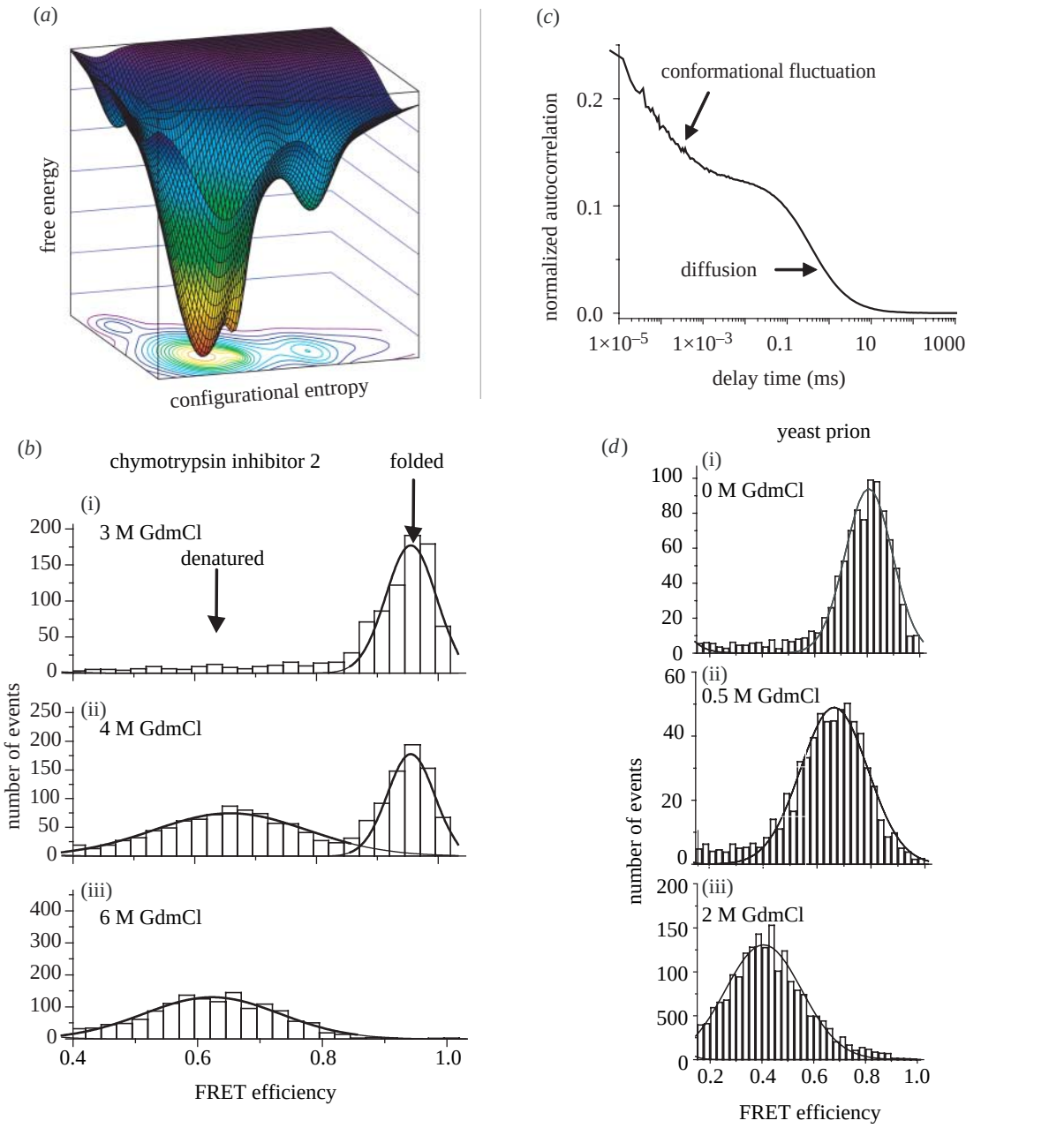

Figure 4. Protein folding studies. (*a*) Schematic energy landscape for protein folding. (*b*) FRET efficiency histograms showing folded and denatured subpopulations for CI2. (i) 3 M GdmCl, (ii) 4 M GdmCl and (iii) 6 M GdmCl. Adapted from Deniz *et al.* (2000). (*c*) FCS correlation function for Alexa488-labelled prion domain of Sup 35 yeast prion protein, displaying nanosecond time-scale conformational fluctuations in addition to a slower diffusion decay component. Adapted from Mukhopadhyay *et al.* (2007). (*d*) Progressive shift of FRET efficiency during the unfolding of monomeric prion domain. (i) 0 M GdmCl, (ii) 0.5 M GdmCl and (iii) 2 M GdmCl. Figure 4*b* and *d* clearly show the difference between a natively folded globular protein (CI2) and a natively unfolded yeast prion. Adapted from Mukhopadhyay *et al.* (2007).

Carrion-Vazquez *et al.* 2003), potentially important in cellular proteolytic or other unfolding machinery. Recently, three-state behaviour has been directly observed for refolding of RNase H by Cecconi *et al.* (2005) using optical tweezers. This study is notable because the protein studied was monomeric, and attachment to beads was achieved using specific tethering via long duplex DNA to avoid non-specific interactions between the protein and the beads. Using these experiments, it was possible to determine that the folding intermediate is an on-pathway intermediate. While the mechanical unfolding behaviour of most proteins studied so far has been nonlinear, surprising linear force–extension behaviour has been observed recently for ankyrin repeats (Lee *et al.* 2006*a*). In this case, inter-repeat protein–protein interactions appear to make the initial unfolding behaviour of this system behave as a Hookean spring, which may play an important role in the protein's biological function.

More complex folding behaviour has been uncovered by both fluorescence and AFM experiments. For example, the folding of adenylate kinase has been studied by encapsulating it in lipid vesicles immobilized onto a bilayer surface in order to minimize the surface-induced perturbation of the protein molecule (Rhoades

et al. 2003). Time-dependent FRET fluctuations in the single-molecule folding trajectories at denaturant concentrations around the midpoint of transition were indicative of a complex and rugged energy landscape. Similar trajectories for a two-state folder, Csp*Tm*, were less complicated and showed two-state fluctuations between high- and low-FRET states (Rhoades *et al.* 2004), demonstrating that the complexity observed for adenylate kinase was probably due to the protein system and not an artefact of the observation method. AFM experiments on ubiquitin using a force clamp uncovered continuous refolding behaviour consisting of multiple phases and large end-to-end distance fluctuations (Fernandez & Li 2004). In recent work on the same protein, Brujić *et al.* observed a surprisingly broad distribution of unfolding rates, taken to be evidence for glassy nature of the unfolding energy landscape of this protein (Brujic *et al.* 2006). In the context of membrane proteins, multiple features have been detected in the mechanical unfolding force curves, which have been ascribed to the unfolding of individual structural elements of these proteins (Oesterhelt *et al.* 2000; Sapra *et al.* 2006).

Fast conformational fluctuations in an unfolded protein have also been measured at a small ensemble level by fluorescence quenching using FCS (Chattopadhyay *et al.* 2002, 2005; Frieden *et al.* 2002). The method takes the advantage of fluorescence intensity fluctuations on the microsecond time-scale (faster than the diffusion time of protein molecule through the confocal volume) due to the rapid conformational fluctuations of the protein chain. Using this method, Chattopadhyay *et al.* (2005) have directly measured the rate of conformational fluctuations of unfolded intestinal fatty acid-binding protein. The observed microsecond time-scale fluctuations provided evidence to support the existence of transient structure in the unfolded state. In a recent paper, single-molecule photon statistics showed that the global reconfiguration of unfolded Csp*Tm* protein molecules occurs approximately on the 50 ns time-scale, and slows down as a function of chain collapse, indicative of a diffusive collapse process (Nettels *et al.* 2007).

As we have discussed, single-molecule studies have clarified and uncovered several aspects of the protein folding reaction. These methods will continue to be used to more deeply probe the physics and biologically relevant aspects of this complex and interesting process. For example, single-molecule techniques have been applied recently to study a natively unfolded and amyloidogenic yeast prion protein (Mukhopadhyay *et al.* 2007), with a key advantage being that the protein remains monomeric at the low concentrations used in these studies. FRET, dual-colour coincidence and FCS results (figure 4*c*,*d*) indicated that the native monomeric protein is composed of an ensemble of structures, having a collapsed and rapidly fluctuating N-terminal region juxtaposed with a more extended middle region. This combined single-molecule approach holds considerable promise to understand the energy landscape of the aggregation process during amyloid assembly, which is of significant biological and health interest.

3.2. DNA and RNA structural studies

DNA and RNA are other key cellular players, whose complex structural biology has been probed using a variety of single-molecule methods. Indeed, these molecules have a longer and more prominent history in the single-molecule field, in part due to their generally simpler synthesis, labelling and immobilization. For example, DNA is a key information molecule in the cell; hence, its structural and dynamical properties have been the targets of several single-molecule studies. For the simplest case of a duplex DNA molecule, mechanical stretching studies have provided many insights (Bustamante *et al.* 2004). Low-force (few piconewtons or less) stretching studies using magnetic fields and flows, as well as later studies using optical tweezers, showed that DNA can be modelled as a worm-like chain (Smith *et al.* 1992, 1996; Marko & Siggia 1995). At low stretching forces (few piconewtons or less), it displays an interesting phenomenon known as entropic elasticity, a manifestation of entropic forces that make a polymer chain prefer a more compact form over stretched-out conformations (which would have less degrees of freedom). At higher stretching forces (and below approximately 65 pN), DNA shows an approximately linear elasticity characterized by a stretch modulus ('enthalpic elasticity'). An interesting observation was made upon increasing the force on the DNA even further—it underwent a stretching transition at approximately 65 pN (Smith *et al.* 1996). In more recent measurements, Bloomfield and colleagues have found that this transition represents an equilibrium form of DNA melting (Williams *et al.* 2001*a*,*b*; Wenner *et al.* 2002). Very recently, using rotor-bead and optical tweezers methods, two studies uncovered that DNA surprisingly becomes overwound during the initial stages of this transition, a finding that may have implications for the functioning of DNA enzymes (Gore *et al.* 2006; Lionnet *et al.* 2006*b*).

DNA molecules are not just static duplexes. Rather, they often fluctuate between alternate structures, behaviour that can be followed using single-molecule methods. One biologically and biotechnologically important DNA motif is the hairpin loop, which can fluctuate between open and closed conformations. Equilibrium DNA hairpin opening–closing fluctuations were investigated by FCS (Bonnet *et al.* 1998). The rate associated with the opening of the hairpin was found to be essentially independent of the characteristics of the loop, whereas the rate of closing varied significantly with the loop length and sequence. Even for such a seemingly simple DNA motif, FRET-fluctuation spectroscopy has revealed sub-millisecond dynamics with a complex energy landscape between two states in a DNA hairpin loop (Wallace *et al.* 2001; Li *et al.* 2004). Deviations from Arrhenius kinetics for both opening and closing of hairpin loops were observed by FRET fluctuation spectroscopy. FCS analysis has even revealed a three-state mechanism for DNA hairpin folding that consists of a rapid equilibrium between open and intermediate forms and a closed form (Jung & Van Orden 2006). A recent study has also revealed that the rate limiting step of DNA hairpin folding is not

determined by loop dynamics, or by mismatches in the stem, but rather by interactions between stem and loop nucleotides (Kim *et al.* 2006). Another recent study of hairpin unzipping using a newly developed ultrasensitive optical tweezers passive force clamp uncovered extremely detailed information about DNA hairpin mechanical unfolding/folding energy landscapes, including the positions and heights of energy barriers (Woodside *et al.* 2006).

Structural distributions and dynamics of more complex DNA structures have also been studied using single-molecule methods. For example, by using single-molecule FRET, two subpopulations were observed for human telomeric intramolecular quadruplex and these were ascribed to coexisting parallel and anti-parallel quadruplex conformations interconverting on a minute time-scale (Ying *et al.* 2003). The free-energy barrier in the folding energy landscape of the quadruplex has been estimated to be between 3 and 15 k_BT. Interesting insights have also been obtained into the branch migration dynamics of DNA four-way Holliday junctions, intermediates in DNA strand exchange during homologous recombination (Dawid *et al.* 2004; Karymov *et al.* 2005). Using single-molecule FRET studies on immobilized Holliday junctions, it was possible to infer that the conformer transition and branch migration processes share the unstacked, open structure as the common intermediate (McKinney *et al.* 2003). Single-molecule studies also revealed that the free-energy landscape of spontaneous branch migration is highly rugged with sequence-dependent barriers and local energy minima (McKinney *et al.* 2005). It was observed that the Holliday junction fluctuation rate decreases with increasing magnesium ion concentration, while the ratio of populations of the states remains the same. A recent study using single-molecule FRET found a surprisingly large number of conformational states involved during the folding of immobilized human telomeric DNA (Lee *et al.* 2005*a*).

In the case of RNA, similar to proteins, biological function is critically dependent on three-dimensional folded structure. With many parallels to the protein case, the energy landscape of RNA folding is complicated by conformational heterogeneity due to the presence of several on- and off-pathway folding intermediates (Bokinsky & Zhuang 2005). Thus, conformational transitions in RNA are quite complicated and are best probed at the single-molecule level. The first single-molecule RNA folding experiments were carried out on a surface immobilized three-helix RNA junction that is the S15 binding fragment of the 30S ribosomal subunit using FRET (Ha *et al.* 1999). Mg^{2+}-induced folding was observed as a shift in the FRET peak position as a function of Mg^{2+} concentration, and peak broadening was attributed to foldability differences of different single RNA molecules. In a subsequent study, conformational fluctuations of the same junction were studied using FCS and FRET, as a function of Mg^{2+} and Na^{+} concentration (Kim *et al.* 2002).

Several single-molecule studies have also been performed on the relatively simple hairpin ribozyme moiety, which acts as an RNA-based self-cleaving enzyme. The folding of their branched helical junctions is essential for their catalytic role. Single-molecule FRET studies on immobilized molecules uncovered complex conformational dynamics for model two-way junction ribozymes, with four docked (active) states of distinct stabilities, explaining the heterogeneous activities of these ribozymes (figure 5*a*; Zhuang *et al.* 2002). The precise origins of this heterogeneity represent a key issue that remains to be fully resolved. Single-molecule studies on the natural four-way junction form of the hairpin ribozyme in both immobilized and freely diffusing formats demonstrated folding via an intermediate, facilitating formation of the active docked state (Tan *et al.* 2003; Pljevaljčić *et al.* 2004). A systematic single-molecule study aimed at evaluating the role of the increasing number of junctions (two-, three- and four-way junction variants) confirmed that the four-way junction strongly favour the docked (active) conformation of the two loops (Pljevaljčić *et al.* 2004). Interestingly, the four-way junction was able to fold to a compact structure even without the loop–loop docking interactions (figure 5*b*). This unique ability of the four-way junction ribozyme was ascribed to a native-like quasi-docked state which contributes to a greater propensity for formation of a tertiary structure. This work also demonstrated the importance of correct geometry of helical junction as well as loop elements for effective ribozyme folding to a functional state. Another single-molecule FRET study revealed a rarely populated docked state that was not previously detected by ensemble methods for the larger *Tetrahymena* ribozyme, and uncovered intermediate conformational states and multiple pathways during its folding (Zhuang *et al.* 2000). A subsequent in-depth smFRET study carried out a ϕ-value analysis on this ribozyme, with the results suggesting an early transition state for folding. The data suggest that kinetic traps substantially slow the folding of large structured RNAs (Bartley *et al.* 2003). Complex and heterogeneous behaviours, including multiple states and gradual FRET transitions, were also observed in the case of folding of the large RNase P RNA (Xie *et al.* 2004).

A series of mechanical unfolding studies of individual RNA molecules by the groups of Bustamante and Tinoco has also provided interesting insights into their behaviour (Liphardt *et al.* 2001). DNA handles were used in these optical tweezer experiments (as for RNase H described above). Folding and unfolding transitions of the RNA molecules could be directly studied at intermediate forces (Liphardt *et al.* 2001). Liphardt *et al.* have studied how to extract equilibrium thermodynamic information about such systems from such non-equilibrium measurements using fluctuation theorems such as the Jarzinski's equality and Crook's theorem (Liphardt *et al.* 2002; Bustamante *et al.* 2005; Collin *et al.* 2005). Intermediates were detected even for the unfolding of a simple TAR RNA hairpin in force-ramp experiments (Li *et al.* 2006). Much more complex behaviour was observed for the mechanical unfolding of the larger *T. thermophila* ribozyme (Onoa *et al.* 2003). In a systematic series of experiments on different size fragments of this molecule, a series of eight

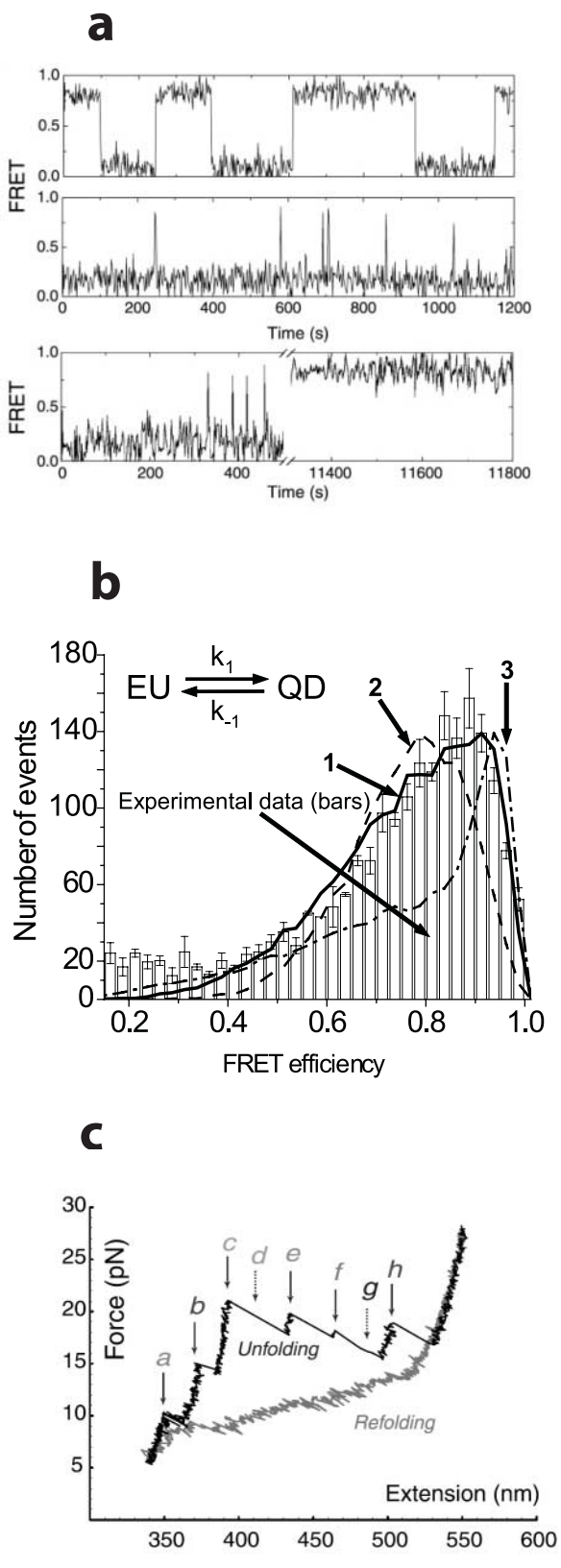

Figure 5. Single-molecule RNA dynamics. (*a*) FRET time traces for two-way junction hairpin ribozymes showing static heterogeneity in the unfolding rates for three different immobilized ribozyme molecules. From Zhuang *et al.* 2002 *Science* **296**, 1473. Reprinted with permission from AAAS. (*b*) FRET histogram for an undocked state mimic of the natural four-way junction hairpin ribozyme, showing broadening consistent with rapid fluctuations (50–100 µs) between extended undocked (EU) and quasi-docked (QD) conformations. Simulated histograms are shown for forward/reverse (k_1/k_{-1}) rate constants of $20\times10^3/6.8\times10^3\ s^{-1}$ (1), $41\times10^3/14\times10^3\ s^{-1}$ (2) and $10\times10^3/3.4\times10^3\ s^{-1}$ (3) with a corresponding equilibrium constant of 3 in favour of the quasi-docked state in all cases. Adapted from Pljevaljčić *et al.* (2004). (*c*) Optical tweezers pulling of the *Tetrahymena thermophila* ribozyme showing eight different unfolding transitions (marked with arrows from *a* to *h*). From Onoa *et al.* 2003 *Science* **299**, 1892. Reprinted with permission from AAAS.

intermediates and their structural origins were delineated for the unfolding of this ribozyme (figure 5*c*). Interestingly, some of the unfolding barriers were discovered to be 'brittle', i.e. unfolding required high forces, but occurred over a very small distance change.

Finally, single-molecule methods have monitored numerous DNA and select RNA structures during the binding or activity of associated or processing proteins; some examples are discussed in §3.3.

3.3. Biological assemblies and function

In addition to being high-sensitivity probes of structural properties of individual biological molecules, single-molecule methods are also well suited for studying the assembly of molecular complexes and their resulting function. One important area uniquely amenable to single-molecule investigations is how conformational changes are coupled with binding. An early example came from the study of a minimal RNA junction that binds a protein S15 from the 30S small ribosomal subunit (Ha *et al.* 1999). Ha *et al.* used smFRET to deduce that protein binding to the junction was accompanied by a large conformational change in the RNA junction. The magnitude of this conformational change mimicked the junction folding in the presence of magnesium alone, probably because the junction is partially preorganized for protein binding. In another study, using multi-parameter fluorescence single-molecule detection, Seidel and co-workers demonstrated the existence of three structurally distinct forms of HIV reverse transcriptase–nucleic acid complexes freely diffusing in solution (Rothwell *et al.* 2003). One of the uncovered structures had not been previously detected in X-ray crystal structures. Insights have also been gained into the chaperoned nucleic acid rearrangements and annealing of transactivation response elements (TAR: DNA and RNA) in the HIV reverse transcription process, using smFRET (Liu *et al.* 2007). Following a conformational change of TAR induced by the nucleocapsid protein from a closed conformation to a partially open Y-shaped conformation, the nucleation of annealing occurs in an encounter complex formed by two hairpins with one or both in the Y-shaped conformations. In another recent study of the folding of the bI5 intron upon binding to CBP2 cofactor, smFRET data suggested that the cofactor initially binds non-specifically to the RNA, causing large conformational fluctuations (Bokinsky *et al.* 2006). The altered conformational distribution of the RNA and the increased local concentration of the cofactor were proposed to facilitate final specific folding and complex formation.

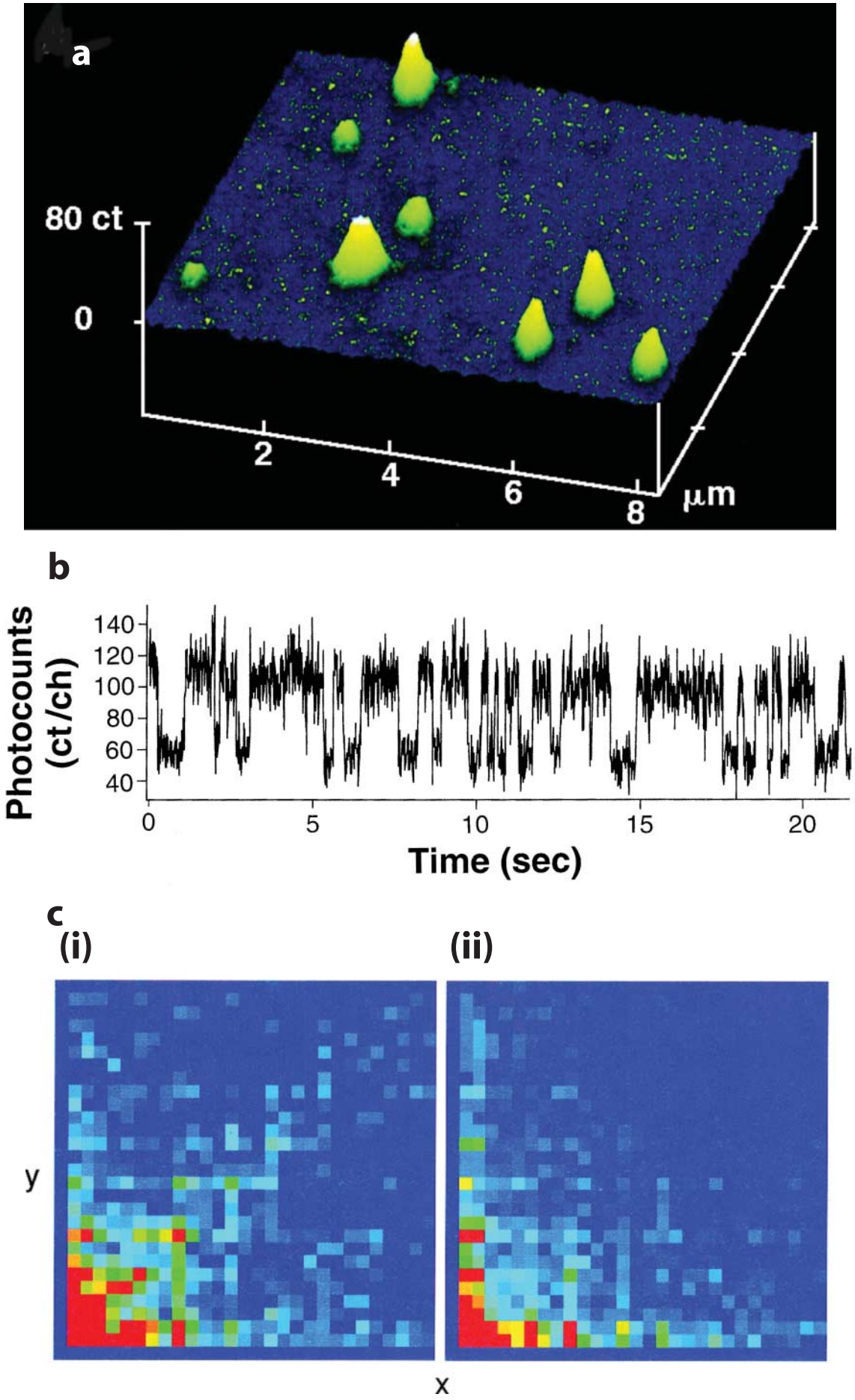

Figure 6. (*Caption overleaf.*)

From a functional point of view, recent studies have directly dissected the dynamics of tRNA on functioning ribosomes (Blanchard *et al.* 2004*a*,*b*). Using smFRET in combination with antibiotics, at least three bound tRNA states were observed, consistent with a model where fidelity of translation is controlled by both steric and kinetic effects during both initial tRNA selection and subsequent proofreading steps. Following these initial steps, the tRNA fluctuates between two states, with one of the states being favoured following peptide bond formation. These studies have provided dynamic movies of functional movements in this large and critical cellular machine.

Chromatin assembly, dynamics and remodelling are involved in virtually all chromosomal processes and have been the subjects of numerous single-molecule and single-fibre studies (Ladoux *et al.* 2000; Brower--Toland *et al.* 2002; Zlatanova & Leuba 2003). For example, two recent FRET and FCS studies used donor and acceptor probes on nucleosomal DNA to probe intrinsic conformational fluctuations in the DNA (Li *et al.* 2005; Tomschik *et al.* 2005). The FCS study detected sub-second spontaneous fluctuations in the nucleosomal DNA, while the single-molecule FRET studies observed the population of closed and open states interconverting on the 100 ms to a few second time-scales. Such fluctuations could be a key for the action of remodelling proteins that might act as Brownian ratchets by trapping the open forms of nucleosomal DNA, and may also have significant implications for gene regulation and transcription. Another study used nucleosomes assembled on heterogeneous DNA sequences, as found in native chromatin (Gemmen *et al.* 2005). A wide distribution of unravelling forces was observed here, attributed to sequence-dependent unravelling forces. Yet another recent study of remodelling enzymes by optical trapping discovered the formation of large intra-nucleosomal loops, which may be involved both in making nucleosomal DNA accessible and in nucleosomal mobility and eviction (Zhang *et al.* 2006). Finally, a recent study used AFM recognition imaging, where antibody-coated tips were used to image-specific substrates in a complex sample, to study remodelling (Bash *et al.* 2006). The results showed that histone H2A is released from nucleosomes by the action of the Swi–Snf remodeller, thereby making nucleosomal DNA accessible without complete nucleosome disruption.

The structure and function of a variety of other biological complexes have also been studied by single-molecule methods, including RecA polymerization on DNA (Leger *et al.* 1998; Bennink *et al.* 1999; Hegner *et al.* 1999; Shivashankar *et al.* 1999), synaptotagmin and complexin binding in the SNARE complex (Weninger *et al.* 2003; Bowen *et al.* 2005), chaperonin systems (Viani *et al.* 2000; Taguchi *et al.* 2001; Ueno *et al.* 2004), the assembly stoichiometry and turnover of membrane protein complexes in live cells (Leake *et al.* 2006), and direct observation of linear prion amyloid assembly via monomer addition (Collins *et al.* 2004).

3.4. Single-molecule enzymology

Single-molecule measurements have provided several novel and intriguing insights into the function of protein and RNA enzymes, which are hidden in ensemble measurements (Min *et al.* 2005; Smiley & Hammes 2006). Fluorescence studies in the mid-1990s by the Yeung and Dovichi groups used the accumulation of fluorescent product (from non-fluorescent reactant) to quantify the time-averaged activities of individual copies of protein enzymes (Xue & Yeung 1995; Craig *et al.* 1996, 1998; Tan & Yeung 1997). A surprising finding was a significant variation in the activities for different enzyme molecules, which was not a function of their local environment. Although this variation was initially attributed to individual enzyme molecules occupying different conformational substates with different catalytic efficiencies, later experiments indicated that differential post-translational modifications may have also played a major role in the observed variations (Polakowski *et al.* 2000). Thus, the molecular basis for such activity variations remains to be unequivocally determined.

Soon after, Xie and co-workers reported a pioneering study of the activity of single cholesterol oxidase enzymes that catalyse the oxidation of cholesterol by oxygen (Lu *et al.* 1998), taking advantage of the intrinsically fluorescent cofactor, flavin adenine dinucleotide (FAD). During the catalytic cycle, the fluorescent FAD oxidizes a cholesterol molecule and is itself converted to a non-fluorescent reduced form, FADH2. Following this step, it is then oxidized back to FAD by O_2. Lu *et al.* (1998) used this cycling between fluorescent and non-fluorescent states to monitor individual turnovers of this enzyme in real time (figure 6*a*,*b*), and were able to perform several detailed analyses of the time trajectories and distributions. They too observed different time-averaged activities for different individual enzymes (static heterogeneity). More interestingly, however, they also observed that individual enzyme activities appeared to fluctuate over

Figure 6. *(Overleaf.)* Memory effects in single-molecule enzyme activity. (*a*) Fluorescence image (8×8 μm) of single cholesterol oxidase (Cox) molecules immobilized in a film of agarose gel. The emission is from the fluorescent FAD, which is tightly bound to the enzyme active site. Each individual peak is attributed to a single Cox molecule. (*b*) Real-time observation of enzymatic turnovers of a single Cox molecule catalysing oxidation of cholesterol molecules (the emission intensity trajectory is recorded (shown in counts per channel (ct/ch)) with a time resolution of 13.1 ms) with a 0.2 mM cholesterol concentration and 0.25 mM saturated oxygen concentration. The time trajectory exhibits stochastic blinking behaviour as FAD interconverts between oxidized (fluorescent) and reduced (non-fluorescent) states, each on–off cycle corresponding to an individual enzymatic turnover. (*c*) Turnover waiting times (*x*- and *y*-axes are waiting times from 0 to 1 s) for (i) sequential turnovers and waiting times separated by (ii) 10 turnovers of Cox molecules. The diagonal population density in (i), which is missing from (ii), shows a memory effect that decays within 10 turnovers. From Lu *et al.* 1998 *Science* **282**, 1877. Reprinted with permission from AAAS.

time. Their analysis uncovered a molecular memory effect, with a given enzyme turnover waiting time being somewhat dependent on the previous one (figure 6*c*), interpreted as caused by slow conformational dynamics (termed dynamic heterogeneity). Such memory effects and dynamic heterogeneity have also been observed in other protein enzyme cases, and links have been made between enzyme conformational fluctuations and variations in enzymatic activity (Edman & Rigler 2000; Min *et al.* 2005; Smiley & Hammes 2006). Ribozymes and the ribosome represent another well-studied class of enzymes, where the coupling between conformational changes and the chemical turnover step have been explored in detail at single-molecule resolution (also see §§3.1–3.3; Zhuang *et al.* 2000, 2002; Blanchard *et al.* 2004*a*,*b*; Nahas *et al.* 2004). Several other enzymes have also been studied and some are discussed in §§3.5 and 3.6 (for a review, see Smiley & Hammes 2006). Overall, perhaps the most dramatic observation so far with single enzymes has been the broad distribution in catalytic rate constants even for a single enzyme molecule. Key questions that remain to be answered are whether such distributions are an intrinsic consequence of inevitable structural properties of proteins or whether biological systems actively harness and perhaps even control these distributions through structural and environmental modifications.

3.5. Motor proteins

Another extremely rich and early area of exploration by single-molecule methods has been that of motor proteins, which transform chemical potential into mechanical work and perform a variety of key functions in the cell, including transport, synthesis and packaging. Systems studied include the rotary F_1-ATPase and synthase, cytoskeletal proteins such as kinesins, dyneins and myosins, viral DNA-packaging motors, RNA and DNA polymerases, helicases and chromatin-remodelling proteins (Bustamante *et al.* 2004; Kinosita *et al.* 2004; Mallik & Gross 2004). We note that single-molecule studies of kinesin and myosin are large fields of research by themselves, and are discussed in more detail in several reviews (Block 1996; Mehta *et al.* 1999; Carter & Cross 2006; Dantzig *et al.* 2006; Block 2007; Valentine & Gilbert 2007). A few representative examples of single-molecule experiments on motor proteins are described below.

F_1-ATPase. The direct observation of rotary motion of the F_1-ATPase was a dramatic demonstration of the power of single-molecule methods to directly probe complex biological structure and mechanism in a way almost impossible in an ensemble (Kinosita *et al.* 2004). In 1997, Boyer and Walker were awarded the Nobel Prize in Chemistry for their groundbreaking biochemical and structural studies of the F_1F_0-ATP synthase motor, in part deducing that it must function as a rotary motor. In the same year, Noji *et al.* (1997) published their stunning direct visualization of precisely this rotary motion. They attached a fluorescently labelled actin filament to the rotor element, and tethered the F_1-ATPase to a coverslip. By visualizing the actin filament using fluorescence video microscopy, they were able to make movies of the rotation and estimated a near 100% efficiency for this molecular motor. In the following year, Yasuda *et al.* were able to observe 120° steps taken by the rotor, thereby directly visualizing a mechanism postulated based on the threefold structural symmetry of the motor (Kinosita *et al.* 1998). Subsequent results using single fluorophore labelling and polarization-based assay confirmed such rotary behaviour (Adachi *et al.* 2000). More recent studies using small attached beads have detected additional substeps in this rotary enzyme's mechanism of action, previously hidden due to viscous drag from the attached large actin filament (figure 7*a*; Yasuda *et al.* 2001). Additionally, Itoh *et al.* (2004) used magnetic beads attached to F_1-ATPase to reverse its rotation and were able to demonstrate synthesis of ATP from ADP, also a very dramatic demonstration of mechanically controlled reversal of chemistry in this enzyme. Finally, both ATPase and ATP synthase activities of membrane inserted F_0F_1-ATP synthase have also been studied using single-molecule polarization (Kaim *et al.* 2002) and FRET measurements (Borsch *et al.* 2002; Borsch & Graber 2005; Zimmermann *et al.* 2006).

Phage DNA-packaging motor. During the replication cycle, viruses package their replicated genome in preparation for infection of other host cells. The motors that achieve this packaging overcome large forces to package the genome to very high densities. Smith *et al.* (2001) used optical tweezers to pull on single DNA molecules as they were packaged by a portal motor of an immobilized bacteriophage. They made several intriguing observations. The motor works against very high loads (above 50 pN), making it one of the most powerful molecular motors. It is highly processive, although pauses and slips occur especially at higher packaging forces. The packaging rate decreased as a function of degree of packaging, indicating that a large internal pressure builds up during this process. Based on this result, the authors suggested that this pressure could be used as a driving force for DNA injection into the host during the viral infection process. More recent single-molecule studies (Chemla *et al.* 2005; Hugel *et al.* 2007) by the same group have resulted in several insights into the packaging mechanism, including that DNA translocation is probably triggered by phosphate release, that motor subunits act in a coordinated and processive manner, and that rotation of the motor connector complex is most probably not involved in DNA translocation.

Kinesin. Kinesin is a two-headed molecular motor that uses ATP hydrolysis to transport organelles along microtubules in cells. In early work on this system, Svoboda and co-workers used optical trapping interferometry (optical tweezers) to directly monitor the movement of individual kinesins along microtubules, discovering that it moves in 8 nm steps (Svoboda *et al.* 1993). They followed up this work with measurements of force–velocity curves (Svoboda & Block 1994). They found that kinesin can work against loads of up to 5–6 pN, and that velocity decreased linearly with increasing force. Meyhofer and Howard also observed similar results using a flexible glass fibre to measure force (Meyhofer & Howard 1995). In subsequent work, Vale, Yanagida and co-workers used low background TIR

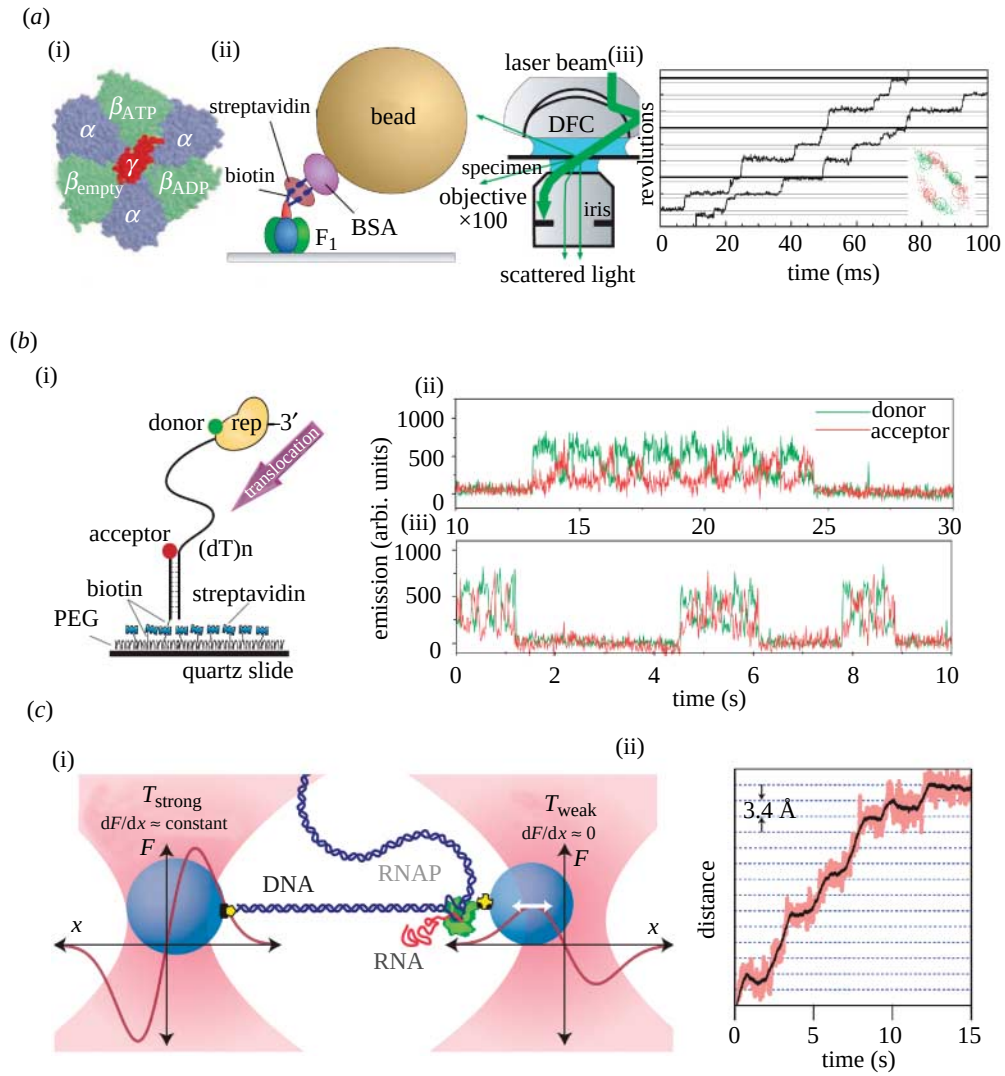

Figure 7. Single-molecule studies of molecular motors. (*a*) (i) Structure of F_1-ATPase viewed from the F_0 side. (ii) The observation system and laser dark-field microscopy set-up for observation of gold beads attached to the ATPase γ-subunit. Only light scattered by the beads was detected (DFC: dark-field condenser). (iii) Time courses of stepping rotation of 40 nm beads at 20 μM ATP concentration. Adapted by permission from Macmillan Publishers Ltd: Nature, Yasuda *et al.* 2001 *Nature* **41**, 898, copyright 2001. (*b*) (i) Donor-labelled Rep binds to a 3′ ssDNA tail and translocates towards the acceptor, and fluorescence intensity traces for a 3′ $(dT)_{80}$ tail at (ii) 22°C and (iii) 37°C. Adapted by permission from Macmillan Publishers Ltd: Nature, Myong *et al.* 2005 *Nature* **437**, 1321, copyright 2005. (*c*) Single base-pair stepping by RNA polymerase using ultrastable optical tweezers. (i) The dual optical tweezer passive force clamp. Strong trap, T_{strong}; weak trap, T_{weak}; and trap stiffness $k = dF/dx$. (ii) Base-pair stepping as a function of time under 18 pN of assisting load, median-filtered at 50 (grey) and 750 ms (black), with the distance between horizontal lines representing a 1 bp separation. Adapted by permission from Macmillan Publishers Ltd: Nature, Abbondanzieri *et al.* 2005 *Nature* **438**, 460, copyright 2005.

microscopy to study the movement of individual kinesin molecules along microtubules in the absence of a cargo (such as a bead that was used in the Svoboda work; Vale *et al.* 1996). They observed that once a kinesin molecule finds a microtubule, it moves along it very processively, with only a 1% chance of detachment per mechanical cycle. Additionally, they found that single-headed kinesins do not show processive movement, evidence for a hand-over-hand movement. In 1997, two groups used optical tweezers and light microscopy to deduce that one ATP molecule is hydrolysed per 8 nm step (Hua *et al.* 1997; Schnitzer & Block 1997). Another study using single-molecule polarization measurements detected a mobile ADP-bound state, which may be important in the kinesin stepping process (Sosa *et al.* 2001). More recently, Yildiz *et al.* (2004) have used high-resolution SPT to directly measure the load-free movement of single kinesins with nanometre-scale accuracy. They observed that kinesins singly labelled in the head region took alternate steps of approximately 17 and 0 nm, arguing strongly for a hand-over-hand model for movement and against an inchworm model. Recently, two groups have even taken such experiments to the *in vivo* level, resolving 8 nm steps (and other characteristics) of kinesin (and other motor)-directed movement of GFP-labelled peroxisomes (Kural *et al.* 2005) and quantum dot containing endocytic vesicles (Nan *et al.* 2005). Thus, numerous insights into the molecular motions and mechanisms of kinesin transport have been provided by single-molecule experiments, although much work remains to fully understand this complex process (e.g. Block 2007).

Helicases. Helicases are motor proteins that convert double-stranded DNA into single-stranded species important in numerous cellular processes, such as DNA replication, recombination, repair and viral replication. A battery of single-molecule techniques has been applied to study a variety of helicases, generating detailed mechanistic insights into their function (Joo *et al.* 2006; Lionnet *et al.* 2006*a*). For example, Lee *et al.* (2006*b*) have recently used flow forces to measure DNA unwinding in a moving replication fork. The results suggest that DNA primase acts as a brake, transiently halting replication fork progression, thus perhaps allowing for slow steps in lagging strand synthesis to keep up with progression in leading strand synthesis. In another study, the stepping rate of the HCV helicase NS3 was studied by optical tweezers, and the results led to a proposed model with dual RNA binding sites on NS3, allowing for an inchworm-like translocation and binding stepping coordinated by ATP binding (Dumont *et al.* 2006). Magnetic tweezers have also been used to provide insights into Holliday junction migration by RuvAB and DNA unwinding and strand switching by UvrD helicases (Dawid *et al.* 2004). Finally, a recent FRET study uncovered unexpected behaviour during single-stranded DNA translocation of the Rep helicase (Myong *et al.* 2005). In intramolecular FRET experiments with a short double-stranded DNA and a longer 3′ single-stranded tail, Rep demonstrated sawtooth-like FRET behaviour (figure 7*b*), apparently repeatedly shuttling from the initial binding region near the 3′ single-stranded DNA end to the double-stranded DNA blockade. Even more surprisingly, intramolecular FRET showed gradual protein conformational changes occurring on the same time-scale, presumably coordinated with the shuttling. The structural and functional significance of these intriguing observations is open to further investigation.

RNA polymerase. Several single-molecule studies have examined the activity of RNA polymerase, providing information about its binding, activity and the transcription process in general, and a couple of recent ones are mentioned here (Zlatanova *et al.* 2006). Two recent complementary studies used single-molecule FRET on diffusing molecules and magnetic tweezers to understand mechanistic features of abortive initiation by RNA polymerase (Kapanidis *et al.* 2006; Revyakin *et al.* 2006), a process where as an initial transcribing complex, it undergoes several abortive cycles of short RNA synthesis and release. Upon synthesis of a longer RNA product, the polymerase dissociates from promoter DNA and initiation factors, and proceeds to synthesize RNA in a processive manner. The results supported a mechanism where initial transcription by RNA polymerase proceeds via a DNA-scrunching mechanism involving a stressed intermediate, and are consistent with a model where the driving force for promoter escape is provided by the stress in this intermediate. High-stability optical tweezers measurements have also recently been used to resolve base-pair stepping by the polymerase along DNA (figure 7*c*; Abbondanzieri *et al.* 2005). Subsequent detailed analysis of the sequence-dependent pausing of the enzyme lend support to a two-tiered pausing mechanism, involving an enzyme inactive state and other secondary pause prolonging elements, features important in understanding the action of regulatory factors (Herbert *et al.* 2006). Finally, the base-pair resolution of the optical trap has even been used to devise a method for DNA sequencing using pauses in RNA polymerase during nucleotide-limited DNA transcription (Greenleaf & Block 2006).

3.6. Single-molecule studies in living cells

Last, we discuss how several important problems in cell biology are being addressed by single-molecule techniques, primarily fluorescence, including the structure and dynamics of lipids and proteins in cell membranes, signalling, vesicle transport, nuclear transport, and RNA dynamics and gene expression in cells. Since molecules in cells often diffuse around, these studies generally use single fluorophore tracking (SFT) that allows the dynamics of individual molecules to be tracked in real time. In addition, although not specifically at 'single-molecule' resolution, we also discuss a couple of recent applications of SPT in cells, which benefit from the same kinds of advantages as SFT experiments.

Cell membranes, lipid rafts and cell-signalling. Cell membrane structure is incredibly complex and dynamic and has been the object of several single-molecule studies. The Schindler group described early experiments of diffusion of single labelled phospholipids in model phospholipid membranes in 1996 (Schmidt *et al.* 1996), observing mean square displacements consistent with two-dimensional Brownian diffusion. In membranes of live cells, such single-molecule imaging and tracking studies have provided a significant amount of information about their inhomogeneous nature and how these physical characteristics might impact their biology.

In particular, lipid rafts are cholesterol-enriched membrane microdomains with implications for signalling, trafficking, cell polarization and also diseases such as HIV (Simons & Vaz 2004). The structure and dynamics of these rafts is a subject of continuing debate, in part due to their small size (often below the classical diffraction limit) and possibly transient nature (Edidin 2003; Shaw 2006). Several groups have used SPT of membrane-anchored proteins to deduce raft sizes between 50 and 700 nm. For example, Pralle *et al.* (2000) used optical trapping and SPT to study the diffusion of beads bound to raft and non-raft proteins in cell membranes, and deduced a raft size of approximately 25 nm. Schutz *et al.* (2000) observed approximately 700 nm membrane microdomains in muscle cell membranes. Kusumi *et al.* (2005) have carried out numerous studies in the area using high-speed SPT of attached beads or single fluorophores to study the diffusion of proteins and lipids in cell membranes, and have coined the picket-fence model for membrane diffusion. Based on observations of GPI-anchored proteins, they proposed very small and short-lived (millisecond) rafts containing only a few raft lipids. Recently, Lommerse *et al.* (2004) presented evidence for lipid microdomains even in the inner leaflet of cell membranes. Recent work using ensemble measurements

and theoretical modelling has suggested even rafts as small as 5 nm (Sharma *et al.* 2004), and other single-molecule work has shown that rafts appear not to be active driving forces in T-cell signalling (Douglass & Vale 2005), indicating that significant work remains in characterizing these dynamic structures.

Related studies of the dynamics of membrane proteins and receptors have shed new light on the dynamics of cell signalling. Using objective-type TIRF, Sako *et al.* (2000) studied the activation of EGF receptor (EGFR). In these experiments, SFT and FRET revealed binding between EGF and EGFR on membranes of semi-intact cells, with binding of a second EGF molecule occurring to complexes containing preformed EGFR dimers, followed by EGFR phosphorylation. In another study, Murakoshi *et al.* (2004) visualized Ras activation, also using FRET and SFT, demonstrating that, upon activation, Ras diffusion is significantly slowed, indicating the formation of large signalling complexes. The superior fluorescence qualities of semiconductor nanocrystals have also been used to directly monitor the diffusion of individual glycine receptors and their entry into the synapse (Dahan *et al.* 2003). Such continuing studies in combination with ensemble methods will be of great value in understanding cell membrane structure, dynamics and biology at a more molecular level (Jacobson *et al.* 2007).

Nuclear pore transport. SFT has been used to study the dynamics of translocation through nuclear pore complexes (NPCs), which enable the trafficking of biological macromolecules between the cytoplasm and the nucleus. These pores are approximately 90 nm long and approximately 50 nm wide, with additional structure extending more than 100 nm into the cytoplasm. Importins and exportins bind to and chaperone macromolecules into and out of the nucleus. Yang *et al.* (2004) used SFT to study the dynamics of GFP-labelled model protein substrate molecules as they moved through the NPC. They observed that the import–substrate complex (IC) interacts with NPCs for an average of approximately 10 ms during import, and exit from the pore appears to be the rate-limiting step. The data also indicate that transport is not sequential, and that multiple ICs are transported in a parallel or overlapping fashion. Kubitscheck *et al.* (2005) observed that the dwell times of transport receptors NTF2 and transportin 1 in the NPC were 6–7 ms long. Very interestingly, the dwell times were reduced upon binding of these receptors to substrates, indicating that loaded receptors translocate at an accelerated rate through the NPC. Their data also indicate that overlapping or parallel transport is occurring.

Vesicle trafficking and neuronal communication. Exocytocis, endocytosis and transport of vesicles from the plasma membrane are fundamental cellular processes allowing sorting of materials, uptake of nutrition and signalling molecules, as well as secretion of signalling molecules and metabolites. While many principles of vesicle transport are similar, vesicle transport within the highly specialized chemical synapses is of major biological interest, since these organelles are important building blocks in neuronal communication. Studies on large vesicles within endocrine cells have shown the existence of vesicles undergoing free diffusion, diffusion within confined volumes as well as active transport (Steyer *et al.* 1997; Steyer & Almers 1999). There are only a few studies directly reporting SPT studies of small synaptic vesicles inside living synapses since they are typically densely packed within the chemical synapse, which increases the difficulties for imaging. Interestingly, vesicle mobility in different types of synapses does not seem to be alike. While vesicles in retinal bipolar cells seem to be more mobile, vesicle mobility inside hippocampal boutons seems to be more restricted (Zenisek *et al.* 2000; Jordan *et al.* 2005; Lemke & Klingauf 2005).

Viral infection pathways. SPT has also been used during the last few years to study the pathways and mechanisms of virus entry into cells. Viral infection begins with cell contact and endocytosis of the virus, and ends with nuclear entry and gene expression. The first SPT observations of this process were made by Seisenberger *et al.* (2001) on Cy5-labelled adeno-associated virus particles. They observed initial repeated contacts of the virus particle with the cell membrane, followed by endocytosis. This was followed by free, anomalous and in a few cases, microtubule-directed diffusion in the cytoplasm for different virus particles. Although about half the viruses that entered the nucleus also showed free diffusion, a significant fraction showed unidirectional movement that was apparently microtubule directed. The authors speculated that this may be due to microtubule polymerization into tubular structures formed due to invaginations of the nuclear envelope. The group of Zhuang is studying the entry pathways of several viruses. In the case of influenza, they observed a surprising de novo formation of clathrin-coated pits, followed by endocytosis of the bound virus. They suggest that this is induced by curvature-sensing components of clathrin-coated pits that detect the membrane curvature induced by the multivalent binding of the virus particle to the membrane and may be a general and efficient way for viruses to enter host cells (Rust *et al.* 2004). Once inside the cell, the virus shows different stages of movement, beginning with actin-dependent movement in the cell periphery, followed by rapid, dynein-directed translocation on the microtubules delivering the virus from the cell periphery to the perinuclear region, and culminating with an intermittent movement involving both plus- and minus end-directed microtubule-based motilities in the perinuclear region (Lakadamyali *et al.* 2003).

Gene transcription and translation. Recently, single-molecule methods have begun to target gene expression, which is an intrinsically stochastic process, whose temporal and spatial characteristics could have a significant impact on cellular function. The Singer group used fluorescence *in situ* hybridization and digital imaging microscopy to detect individual mRNA molecules in cells (Femino *et al.* 1998). They were able to directly quantify the formation of nascent and mature RNA and observe its diffusion or transport away from the gene vicinity. Subsequent work revealed that the movement of individual mRNA transcripts in the nucleus after

release from the site of transcription was not directed, but instead occurred by simple diffusion, involving freely diffusing, corralled or constrained subpopulations (Shav-Tal *et al.* 2004). Movement of single mRNA molecules in the cytoplasm, which determine the spatial distribution of protein production, was observed to occur in a rapid, directional manner along microtubules (Fusco *et al.* 2003). Detection of transcription with single-gene sensitivity has also uncovered that transcription activity occurs in pulses (Golding *et al.* 2005; Chubb *et al.* 2006), perhaps allowing more efficient gene regulation in the cell. In order to make direct observations in real-time production of single protein molecules in individual *E. coli* cells, a fusion protein of a fast-maturing yellow fluorescent protein (YFP) and a membrane-targeting peptide were expressed under repressed conditions, in a recent study by the Xie group (Yu *et al.* 2006). Membrane-localized YFP can be detected with single-molecule sensitivity, showing that protein molecules are produced in bursts, with each burst originating from a stochastically transcribed single mRNA molecule. This method holds promise in probing low-copy number proteins in single live cells, information not accessible by current genomic and proteomic technologies.

4. PROSPECTS FOR THE FUTURE

The technologies and applications of single-molecule methods are improving and increasing by rapid leaps and bounds. The rapidly increasing number of applications in biology also highlights current limitations and catalyses progress towards novel approaches to overcome them. Improvements at every level and across several disciplines, including in instrumentation, analytical tools, dyes, sample preparation and attachment chemistries, are all of great importance in this process. Correspondingly, we will continue to see researchers from a variety of different disciplines make important contributions to the field in the coming years. Indeed, not a month passes when some current limitation of a single-molecule method is not overcome, or a new biological system is not probed.

At the molecular level, the ultimate challenge of a single-molecule experiment would be to acquire global structural time trajectories of single molecules or complexes with atomic and picosecond resolution, while simultaneously having the ability to perturb molecular energy landscapes also at atomic resolution. For example, to truly understand and test mechanistic theories of protein folding, we might like to have an atomic resolution movie of structures of an individual protein molecule as it explores the folding energy landscape. To further explore the high-dimensional energy landscape, we would also want to mechanically constrain and manipulate it in various ways, and observe the corresponding effects on the folding trajectories. While such capabilities are a far-off and perhaps lofty goal, advances in the field will probably continue to provide steady progress in this direction in the coming years. There is no doubt that some of the biggest challenges and advances in single-molecule science will be in the area of live cell measurements. However, given the current pace of research in the area, we anticipate that such measurements will soon routinely provide us with real-time structural and high-resolution localization information of multiple biological and chemical species in live cells and tissues, thus truly allowing tests of theories as well as detailed observations of a variety of *in vivo* phenomena.

Advances in instrumentation and imaging technologies will be paramount for such experiments. Current single-molecule techniques will of course continue to be used in novel and very creative ways. For example, recently described methods use the same simple PSF analysis principles as used in SMT to make dramatic improvements in the spatial resolution of biological and cellular imaging (Betzig *et al.* 2006; Rust *et al.* 2006; Sharonov & Hochstrasser 2006). In addition to fluorescence and mechanical imaging, improvements in other single-molecule optical methods such as multiphoton fluorescence and surface-enhanced Raman scattering would complement and extend currently common technologies. Less-used methods such as single-molecule conductivity measurements (He *et al.* 2006), nanopore detection and manipulation (Mathe *et al.* 2004), single-molecule applications of microfluidics (Squires & Quake 2005; Psaltis *et al.* 2006) and single-molecule or single spin magnetic resonance (Kohler *et al.* 1993; Wrachtrup *et al.* 1993; Rugar *et al.* 2004) will continue to be explored. Combination methods, such as for simultaneous single-molecule fluorescence and manipulation, will be further developed resulting in more routinely used tools (Ishijima *et al.* 1998; Lang *et al.* 2004; Sarkar *et al.* 2004; Keyser *et al.* 2006). Parallel manipulation technologies, such as dynamically controlled holographic optical tweezer arrays (Grier 2003), or optoelectronic massively parallel tweezers (Chiou *et al.* 2005) will also probably make a significant impact. Finally, technological developments in other fields might result in the experimental realization of not only currently theoretical ideas, such as the use of free-electron lasers to achieve single-molecule diffraction (Hajdu 2000; Chapman *et al.* 2006), but even of still-to-be envisioned techniques.

In addition, advances in labelling and probe technologies represent a critical challenge that needs significant input from the chemical and chemical biology communities. For example, we expect that the development of novel organic fluorophores (Willets *et al.* 2005), perhaps using combinatorial methods (Rosania *et al.* 2003), improved fluorescent nanocrystals (Michalet *et al.* 2005) or dendrimer-metal nanoclusters (Lee *et al.* 2005*c*) will provide fluorescent reporters with optimized photophysical and biological properties. New protein engineering techniques and chemical biology methods (Johnsson & Johnsson 2007) should prove very important for sample preparation and localization. For example, native and expressed chemical ligation (Muir *et al.* 1997; Dawson & Kent 2000), unnatural amino acid incorporation (Xie & Schultz 2006), labelling using targeting sequences (Chen & Ting 2005), biomimetic transamination reactions (Gilmore *et al.* 2006) and oxime ligations in general (Dirksen *et al.* 2006), the Staudinger ligation (Saxon & Bertozzi 2000; Watzke *et al.* 2006) and click chemistry (Kolb *et al.* 2001), as well as passive and

active genetically encoded probes (Zhang *et al.* 2002) will facilitate single-molecule studies *in vitro* and *in vivo* and allow for more complex sample preparation, labelling and immobilization crucial to both manipulation and fluorescence methods (Hinterdorfer & Dufrene 2006; Kienberger *et al.* 2006). Yet another critical component that will become increasingly important as the single-molecule biophysics field continues to grow is the routine and synergistic side-by-side use of theory in the planning and analysis of single-molecule experiments. On the one hand, these should result in a better understanding of the limitations of current data acquisition schemes (Ober *et al.* 2004; Watkins & Yang 2004; Talaga 2006), as well as more detailed and complex data acquisition and analysis schemes designed to extract molecular information from both single-molecule fluorescence (Yang & Xie 2002; Gopich & Szabo 2003, 2005; Lippitz *et al.* 2005; McKinney *et al.* 2006) and manipulation data in more efficient and less biased ways (Barsegov *et al.* 2006; Dudko *et al.* 2006; Moffitt *et al.* 2006; Walther *et al.* 2006). Equally important will be the side-by-side use of molecular theory and simulations that can guide and complement single-molecule experiments, together resulting in a much more enhanced understanding of the molecular system under consideration (e.g. Rueda *et al.* 2004; Wang *et al.* 2006; Merchant *et al.* 2007).

As the field matures further, additional challenges will be to use the results and methods from single-molecule experiments not only to impact biophysics at isolated molecular or cellular levels, but also to eventually integrate them into obtaining detailed insights into the complex and emergent behaviour of interacting systems of large numbers of individual molecular and cells, even at the level of whole tissues and organisms. On a more immediately societal level, we anticipate that single-molecule science will also have significant impact through novel biotechnologies, for example single-molecule DNA sequencing and drug discovery, and through developments in the overlapping field of nanotechnology, resulting, for example, in significant advances in individualized medicine. In closing, we can say that while single-molecule science and technology is accepted to have come fully of age within the last decade, our opinion is that we have still only scratched the surface, and we eagerly look forward to participating in and witnessing many exciting developments and discoveries in the field in the coming years.

A.A.D. acknowledges support by grants R01GM073104 and R01GM066833 from the National Institute of General Medical Sciences/National Institutes of Health (USA). E.A.L. acknowledges a postdoctoral scholarship from the Alexander von Humboldt Foundation. We thank the many individuals and groups that have contributed to the data and insights presented from the authors' work.

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